Regulation of immune receptor functional responses by G protein-coupled receptor kinases (GRKs) and arrestins

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Regulation of immune receptor functional responses by G protein-coupled receptor kinases (GRKs) and arrestins

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ABSTRACT

Biological systems consistently diminish their responses to persistent or stable stimuli, in a process termed desensitisation or adaptation. This study focuses on the process called “homologous desensitisation” that indicates the rapid and reversible loss of receptor responsiveness that occurs upon exposure to agonists (Premont, 1995; Palczewski, 1997). Key molecular determinants of this process are G protein-coupled receptor kinases (GRKs) and arrestins (Chuang, 1996a). Both GRKs and arrestins are expressed at high level in peripheral blood leukocytes (PBL) and they are potentially modulators of chemoattractant receptor-mediated immune responses (Parruti, 1993; Craft, 1995; Pitcher, 1998a). We studied human Platelet-Activating Factor receptor (hPAFR) signalling and its modulation. We showed that in a heterologous expression system recombinant hPAFR stimulated inositol phosphates production and intracellular cAMP accumulation, through the coupling with Gq/11 and Gs proteins, respectively. The Gs-coupling was a totally unpredicted finding. We also showed that PAF could stimulate intracellular cAMP accumulation in lymphocytes, suggesting that PAFR-coupling with adenylyl cyclase may be important also for endogenously expressed receptors. With the co-transfection approach we defined which GRKs were able to modulate hPAFR signalling and the mechanisms involved in this modulation. We showed that receptor pathways mediated by Gs-coupling were regulated through a strictly phosphorylation-dependent process, while Gq-mediated signalling could be modulated in a phosphorylation-independent way. We also identified in the N-terminal portion of GRK2 (N-ter) the domain involved in the down regulation of Gq-mediated signalling. This inhibition is likely through direct binding, as demonstrated by specific and selective interaction between N-ter and activated Gaq in vitro binding assays and co-immunoprecipitation.
studies. We also show that GRK2 binding to Gαq can be regulated by c-Src activity. In particular, GRK2 tyrosine-phosphorylation, mediated by c-Src, increases GRK2 affinity for Gαq, reinforcing the desensitisation pathway.
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CHAPTER 1
INTRODUCTION

1-1 G protein-coupled receptor signalling

1-1-1 Transmembrane signalling processes
A key to the successful integration of mammalian responses is the ability of the cells, tissues and organs to communicate. The communication must be timely and accurate to maintain homeostasis and to provide the host with the advantage to overcome both internal and external threats, and thus survive. One area of communication lies in the domain of extra cellular signals, which are very diverse ranging from hormones and growth factors, to neurotransmitters and primary sensory signals to physical factors. The cell transmits these stimuli internally (or chooses not to) by both specific and diverse signalling receptors that reside in the plasma membrane. These first “messages” are then transmitted, modulated and directed by signal proteins such as the G proteins, which are connected to the membrane receptor signal. These G protein-coupled membrane signalling systems are composed of fundamentally three components: a) the receptor, b) the G protein and c) the effector.

1-1-2 G protein-coupled receptors (GPCRs)
On the basis of sequences similarity, GPCRs can be divided into three major sub-families: receptors related to rhodopsin (type A); receptors related to the calcitonin receptor (type B) and receptors related to the metabotropic receptors (type C). Of these the rhodopsin sub-family is by far the largest and the most extensively investigated. Despite the structural diversity of their activating ligands, the GPCRs are predicted to share a common molecular architecture consisting of
seven transmembrane helices linked by alternating intracellular (i) and extra cellular (e) loops (Ji, 1998; Wess, 1997) (Fig 1-1). The extra cellular surface, including the extra cellular N-terminus as well as different loops, is critical for the ligand binding. The exact domains involved in ligand binding are nearly as diverse as the chemical structure of the known agonists (Strader, 1995; Schwarz, 1996). Small molecular weight ligands bind to sites within the hydrophobic core formed by the transmembrane (TM) α helices, whereas binding sites for peptides and protein agonists include the N-terminus and extra cellular hydrophilic loops joining the transmembrane domains (Wess, 1997; Schwarz, 1996).

The transmembrane portion of the receptor appears to be important in determining the state of receptor activation. Salt bridges between amino acids in the TM III and TM VII domains can maintain the receptor in an inactive state. The conserved DRY motif at the cytoplasmic side of TM III is highly conserved in members of the rhodopsin GPCR family (Probst, 1992). The invariably conserved arginine in this motif has been hypothesised to be constrained in a hydrophilic pocket formed by conserved polar residues in TM I, TM II, TM VII (Scheer, 1996; Scheer, 1997). It has been proposed that receptor activation involves protonation of the aspartate, in the DRY motif, causing arginine to shift out of the polar pocket leading to cytoplasmic exposure of buried sequences in the second and third intracellular loops (Cohen, 1993; Fahmy, 1993). Also molecular interactions between TM V and TM VI appear to be important in maintaining the receptor in an inactive state (Wess, 1997).

The intracellular receptor surface is involved with the G protein association, recognition and activation. Some consistencies with the intracellular elements of different GPCRs have been found.
**Fig 1-1. Two-dimensional model of the β2-adrenergic receptor**

This receptor model illustrates the key structural features of a GPCR belonging to the rhodopsin sub-family. The most conserved residues are indicated by one letter code. Transmembrane domains are numbered $TM I$ to $TM VII$. Both intracellular $(i)$ and extracellular $(e)$ loops are numbered and indicated. Residues that have been suggested to be actively involved in the conformational changes during receptor activation are indicated in *blue*. A critical role has been evidenced for the proline residue located in TM VI (Gether, 1997). The almost invariable disulphide bridge between extracellular loops 2 and 3 and the conserved palmitoylation site in the C-terminal tail are indicated in *white*. Residues involved in binding of epinephrine to the β2-adrenergic receptor are indicated in *red* (Strader, 1995).

Other residues are discussed in the text.
Fig 1-1

[Diagram of a protein with labeled segments and residues]
The extreme C terminus of the i3 loop as well as the membrane proximal portion of the i4 loop C terminal is likely to be alpha helical arranged (Hedin, 1993). Within these loops are conserved amino acid motifs that appear to be important in the accessibility of the loops. Conserved arginine residues in these loops are important for G protein coupling, and replacement of these amino acids will abolish any G protein coupling (Franke, 1992; Rosenthal, 1993; Zhu, 1994; Scheer, 1996). The i2 loop currently is thought to be important in the receptor-G protein recognition, while the i3 and i4 are important in G protein activation. Agonist and antagonist binding to the receptor change the conformation of these loops, in particular the i3 loop, and TM VI. The current hypothesis, based on a model proposed by Baldwin, is that with agonist activation the receptor intraloop bridges are lost opening the conformation of the i3 loop, with the result that the G protein can interlock with the receptor protein. In particular the Ga C terminus can interact within the i3 loop of the GPCR (Baldwin, 1994). This is supported by the fact that agonist binding makes the i3 loop more susceptible to enzyme cleavage (as the loop is more open and accessible) while antagonist binding decreases the susceptibility.

Also the βγ subunits have a receptor contact point that is essential for receptor recognition and G protein activation (Conklin, 1993; Bourne, 1997).

1-1-3 Heterotrimeric G proteins

G proteins are heterotrimers composed of α, β and γ subunits and are classified by virtue of their alpha subunit (Tab 1-1). To date 23 distinct α subunits encoded by 17 different genes are known and can be subdivided into four families based on the degree of primary sequence homology: Gαs, Gαi, Gαq, Gα12 (Simon, 1991). Cellular concentrations of G proteins belonging to the Gi/o family considerably exceed those of
other families (Hepler, 1992).
Thanks to the solution of the crystal structures of $G\alpha t$ and $G\alpha 1$ new understandings on G protein structure have been obtained (Noel, 1993; Lambright, 1994; Sondek, 1994; Coleman, 1994). $G\alpha$ proteins consist of two domains: a GTPase domain that is topologically identical to other guanine nucleotides binding proteins, and a $\alpha$ helical domain that is common to $\alpha$ subunits (Fig 1-2). The GTPase domain comprises five helices ($\alpha 1-\alpha 5$) and six $\beta$ sheets ($\beta 1-\beta 6$). Strong evidence supports the view that the $G\alpha$ N-terminus is the major interaction site between $\alpha$ and $\beta\gamma$ subunits (Concklin, 1993). Effector regions of $\alpha$ subunits have been mapped in three regions of the G domain $\alpha 2/\beta 4$, $\alpha 3/\beta 5$ and $\alpha 4/\beta 6$ (Berlot, 1992; Rarick, 1992; Arkinstall, 1995). The most clearly defined contact sites with receptors comprise the C terminal region of $\alpha$ subunit (Neer, 1995; Concklin, 1993, Hamm, 1988; Denker, 1995).

Table 1-1

<table>
<thead>
<tr>
<th>Classes of $G\alpha$ sub-units$^a$</th>
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<tr>
<td><strong>Class</strong></td>
</tr>
<tr>
<td>$\alpha s$</td>
</tr>
<tr>
<td>$\alpha i$</td>
</tr>
<tr>
<td>$\alpha q$</td>
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<td>$\alpha 12$</td>
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$^a$ (Neer, 1995)
**Fig 1-2. Gα protein domains**

Schematic representation of conserved sequences and interaction domains of G protein α subunits with receptors, effectors and βγ subunits. This schematic representation has been depicted in relation to three-dimensional structures obtained from G protein crystals. Sequences known to involved in GTP-binding and hydrolysis in other G proteins (as Ras, EF-Tu) and in Gα subunits are denoted G1 to G5 and are aligned for different Gα subunits. Sites for ADP-ribosylation by cholera and pertussis toxins (CTX and PTX) are indicated. Regions displaying structural differences between inactive and active conformations of Gα subunits (switch regions) are indicated. Contact sites with the γ phosphate, the guanine ring and the ribose moiety of GTP are depicted. α helices and β sheets of the G domain are numbered α1-5 and β1-6 respectively. α helices of the helical domain are denoted αA-F. Sequences connecting the helical and the G domain (linkers I and II) are indicated.
Fig 1-2

amino acid number

myristoylation

G1

G2

G3 G4 G5

PTX

CTX

α1 αA

linker 1

γ phosphate contact

switch I switch II switch III

helical domain

αB αC αD αE αF

β1

β2 β3 α2 β4

β5 αG α4

β6 α5

switch regions

regions of interaction with receptors

regions of interaction with effectors

regions of interaction with βγ

regions with highest levels of amino acid diversity

regions of interaction with Pγ

V M regions with highest levels of amino acid diversity
There is also evidence of a participation of the N-terminus of Ga in receptor interaction (Hamm, 1988; Higashijima, 1991). These conflicting observations can be reconciled by postulating a close spatial proximity between Ga N- and C-terminus. Unfortunately neither terminus is resolved in the Ga crystal structure available so far.

Five β and ten γ subunits have been described (Ray, 1995). β and γ subunits are tightly associated and can be regarded as one functional unit. The five known mammalian β subunits display a high degree of amino acid sequence identity and show a characteristic feature of a repetitive Trp-Asp (WD) motif (Simon, 1991; Watson, 1994). γ subunits are considerably more diverse.

1-1-4 Mechanisms of GPCR signalling activation

Activated receptors catalyse the exchange of GDP for GTP at the α subunit high affinity binding site for guanine nucleotides, thus governing a well-controlled dissociation-reassociation cycle of the α and βγ dimer (Birnbaumer, 1995) (Fig 1-3). Both GTP-bound Gα and βγ dimers are signalling molecules and modulate the activity of specific effector proteins. A ligand-induced change in effector activity minimally involves a cycle of five steps, wherein the reversible binding of a hormone to a receptor (1) promotes the dissociation of GDP from Gα subunit (2) and Gα activation through the binding of GTP (3), thus allowing for a change in effector activity (4) thus lasts until GTP is hydrolysed to GDP (5).

Several models for the interaction of ligands with their receptors have evolved from studies on receptors enzymes and ligand-gated ion channels (Franklin, 1983) (Fig 1-4).
Fig 1-3. Heterotrimeric G proteins activation-inactivation cycle

The cycle starts with an activated receptor enhancing displacement of GDP from an inactive heterotrimer. GTP then replaces GDP (nucleotide exchange), aided by Mg\(^{2+}\) and a GDP-GTP exchange factor (GEF). The activated (GTP-bound) G\(\alpha\) subunit dissociates from \(\beta\gamma\) subunits, and both entities activate their respective effectors. Inactivation of the G\(\alpha\) subunit takes place by GTP hydrolysis, a reaction greatly enhanced by RGS proteins, the GTPase-activating proteins (GAPs) for G\(\alpha\). The inactivated (GDP-bound) G\(\alpha\) subunit has a greater affinity for \(\beta\gamma\) subunits, allowing the heterotrimer to reassociate, thus completing the cycle.
**Fig 1-4. GPCRs activation models**

Hypothetical models of receptor activation depicting the receptor in inactive (R) or active conformations (R*, R**). Agonist (A) or inverse agonist (I) ligands can bind to the receptor. According with the ternary complex model a given conformation can determine preferential coupling to one or the other G proteins (G1 vs G2) and receptor activated conformations can be stabilised each one by a different ligand (A1, A2). All possible conformations simultaneously exist in equilibrium with each other. Other details of these models are discussed in the text.
Ligand induction model:

\[ R + A \rightarrow A\ R \rightarrow A\ R^* \]

Conformational selection model:

\[ \begin{align*}
A & \quad \uparrow \quad A \\
R & \quad \leftrightarrow \quad R^* \\
I & \quad \uparrow \quad I \\
R & \quad \leftrightarrow \quad R^* \rightarrow I\ R^* \end{align*} \]

Extended ternary complex model:

\[ \begin{align*}
A_1 & \quad A_2 \\
A_1 & \quad \leftrightarrow \quad A_2 \\
A_1 & \quad \leftrightarrow \quad R^* \\
A_1 & \quad \leftrightarrow \quad R^{**} \\
R & \quad \leftrightarrow \quad G_1 \quad G_2 \\
R^* & \quad \leftrightarrow \quad G_1 \quad G_2 \\
R^{**} & \quad \leftrightarrow \quad G_1 \quad G_2 \end{align*} \]
The "ligand induction" model predicts that transition from the receptor inactive (R) to the active state (R*), which is the only state able to interact with G proteins, is extremely rare in absence of agonist because of the energy barriers between R and R* (Bennett, 1978). Agonist (A) binding to R enables the overcome of the energy barriers and induces the transition to R*. This model is inconsistent with the high basal activity observed for many GPCRs, suggesting that the energy barrier between R and R* is surmountable also in absence of agonist. The successive "conformational selection" model can account for the basal activity of GPCRs in absence of agonists and explains the action of inverse agonists (Koshland, 1968). It postulates that transitions between R and R* can occur in absence of the agonists. Moreover, agonists bind preferentially to the R* conformation and thereby shift the equilibrium and increase the proportion of receptor in R* conformation. Inverse agonists (I) bind preferentially to R and therefore reduce the population of receptors in R* conformation. These models do not account for G protein ability to modulate receptor affinity with agonists. The newly depicted model is a ternary complex model involving the agonist, the receptor and the G protein (Lefkowitz, 1993b). Moreover, due to the fact that a single receptor can activate more than one G protein and that different receptor agonists can discriminate between multiple G protein couplings, the ternary complex has been extended (Fig 1-4). The extended ternary complex model postulates that R can exist in equilibrium with two (or more) activated states (R*, R**, and so on), each of them preferentially couples to distinct G proteins. In this way, different agonists (A1, A2, and so on) may stabilise the formation of ternary complexes with different efficacies.
1-1-5 Diversity of receptor G protein interaction

In most of the cases a GPCR agonist activates divergent signalling pathways in a cell. These results are the consequence of different mechanisms. First of all there could be considerable diversity in receptors that respond to a same agonist, as GPCR subtypes and splice variants that can be expressed in a single cell. This makes it difficult to correctly explain the multiple signalling cascades activated by an agonist. Moreover, even if a single GPCR is activated and it couples to a single G protein, it may give rise to bifurcating signalling. Following receptor activation both the \( \alpha \) subunit and the \( \beta\gamma \) dimer may convey signals to effectors (Clapham, 1993).

There are numerous examples of GPCRs that can mediate at the same time inhibition of adenylyl cyclase and activation of phospholipase C, as a consequence of a single G protein coupling. Both effectors are affected by Gi protein, in fact both signalling pathways are sensitive to pertussis toxin and are abrogated by antibodies recognising G\( \alpha \)i subunits. Activated \( \alpha i \) subunits are thought to inhibit adenylyl cyclase activity, while the activation of phospholipase C is mediated by \( \beta\gamma \) subunits released from activated Gi (Fargin, 1991; Exton, 1994). A peculiarity of G\( \beta\gamma \)-activated signalling, as opposed to the \( \alpha \) subunits, is the higher concentration of G \( \beta\gamma \) dimers required. Therefore, considerably higher concentrations of the agonists are required to produce G\( \beta\gamma \)-mediated signalling. The physiological significance of such dual activation remains unclear.

A further level of complexity is produced by receptors that can couple to multiple G proteins at the same time, both in situ and in reconstituted systems. After the first purification of receptors, G proteins and effectors, receptor-G protein interaction was studied by reconstituting purified components in phospholipid vesicles. Under these circumstances, receptors showed the ability to couple to numerous G
proteins. For example TSH receptor can couple at least to nine different G proteins (i.e. Gs, Gq, G11, Gi1, Gi2, Gi3, Go, G12 and G13) (Laugwitz, 1996). Such a high level of promiscuity is unprecedented and it is indicative of complex multifunctional signalling by the TSH receptor. However it remains to be seen whether this promiscuity among G proteins can also be observed in more physiological conditions.

1-1-6 Selectivity of receptor G protein interaction

Considering that hundreds of GPCRs transduce signals by interacting with a limited number of G proteins, the question of specificity governing the coupling of receptors to G proteins arises. Cells may differ from each other by the particular complement of receptors, α, β, and γ subunits, and effectors that they express. This complement can change substantially with developmental and metabolic state. The precise stoichiometry among the signal components can determine the predominant response pathway. Moreover there is functional and structural evidence that signal transduction components are compartmentalised in highly specialised cells. Consequently a certain subset of receptors, G proteins and effectors reside in distinct domains that may or may not have access to each other (Neubig, 1994).

Selectivity of GPCR-G protein interaction may originate also from different affinities or efficacies governing the interaction of signalling components. Even if a GPCR displays ability to couple to different G proteins at the same time, individual receptors show a rank order of interaction with closely related G proteins. There is some evidence that the α subunit is not the sole determinant of selectivity in receptor-G protein interaction. Studies on rhodopsin, conducted with biochemical and fluorescence spectroscopic approaches, demonstrated the formation of interactive complexes between transducin βγ and
rhodopsin (Phillips, 1992). In particular a specific interaction between rhodopsin and heterotrimeric G proteins depends on the γ subunit type. Both the isoprenoid moiety and the C terminal primary amino acids sequence of γ subunits are specific determinants of receptor-G protein interaction (Kelleher, 1988; Fawzi, 1991).

Expression of different G protein subunits may result in a vast number of distinct αβγ heterotrimers if all subunits can associate randomly. It is still an open question whether certain α subunits preferentially recombine with certain βγ complexes. However, there is evidence that not all possible βγ combinations can be formed (Pronin, 1992; Schmidt, 1992). Thus, in many instances, one heptahelical receptor interacts with diverse G proteins and elicits multiple intracellular signals. However, interaction of a single G protein with a given receptor in a certain cell may be governed by a high degree of selectivity imparted by specific heterotrimers.

1-2 Regulatory mechanisms of GPCRs signalling

Cellular responses activated by a GPCR are finely tuned and are generally rapidly attenuated to avoid cells over-stimulation. Numerous mechanisms are involved in the switching off of GPCR signalling and they operate at the level of the agonist, of the receptor and of the G proteins (Fig 1-5).

1-2-1 Mechanisms operating at the level of the agonist

Several mechanisms contribute to the agonist removal from receptor proximity. After receptor interaction the ligands can be diluted in the extra cellular fluids and can be excreted. A process of agonist removal that is peculiar to agonists of GPCRs and ligand-gated ion channels from the synapse is the uptake by high affinity transporters.
**Fig 1-5. Schematic model of a GPCR signalling cycle**

Schematic representation of the GPCR signalling machinery.

In the non-activated state the GPCR does not interact with G protein and GDP is bound to the α subunit of G protein (α). Upon agonist binding (A), the GPCR undergoes a conformational change to the activated state and is able to bind to the heterotrimeric G proteins and act as a guanine nucleotide exchange factor. This causes the release of GDP and the binding of GTP to the α subunit. Following the nucleotide exchange the G protein can dissociate in the Gα (α) and Gβγ (βγ) subunits. Both of them can interact with effector molecules (El, E2) and modulate their activity. Regulation of a GPCR signalling occurs with mechanisms operating at the level of the agonist, of the receptor and of the G protein. The GPCR can undergo phosphorylation (p) and this process produces uncoupling from G proteins. The deactivation of a G protein signalling is initiated by the hydrolysis of GTP by the α subunit. This process can be accelerated by proteins termed regulators of G proteins signalling (RGS).

In the GDP bound state the α subunit reassembles with the βγ dimer to form the inactive heterotrimer. Regulatory mechanisms are schematised and depicted in red. Details of each process are in the text.
Neurotransmitters are removed from the synapse by high affinity transporters. These transporters are located on neurones and glial cell, so in close proximity to the neurotransmitter site of action. This process not only prevents receptor over-stimulation but it allows for the efficient re-use of the agonist if it is taken up into the nerve of origin. The most common process of agonist removal involves its extra cellular degradation. An example of enzymatic degradation is the one involving acetylcholine released from cholinergic nerve terminals. After receptor interaction this molecule is hydrolysed by acetyl cholinesterase in the synaptic cleft and inactive acetate and choline are produced (Fernandez, 1996). Also numerous peptides undergo enzymatic degradation, principally by cell surface peptidases (Turner, 1994). Therefore, in contrast to the non-peptide neurotransmitters, which act briefly and locally, peptide signalling can be prolonged and widespread, depending on the susceptibility of a peptide to undergo degradation.

1-2-2 Mechanisms operating at the level of the receptor

Receptor loss of functional response is also indicated as desensitisation and is consequent to uncoupling of activated receptor from G proteins. Different desensitisation processes are known depending on the agents that mediate this uncoupling. It is possible to classify an agonist-dependent desensitisation, an agonist-independent desensitisation and a desensitisation due to receptor palmitoylation. Moreover, receptor endocytosis depletes the plasma membranes of high affinity receptors and contributes to desensitisation.

Receptor desensitisation occurs more rapidly than internalisation (Toews, 1984; Roth, 1991), so that it is possible to distinguish between the rapid initial uncoupling of receptor-G protein interaction and receptor sequestration. The endocytosed receptors can undergo re-sensitisation, a recovery of functional response after plasma membrane
re-exposition, or down regulation, which involves receptor degradation that produces a loss of total receptor number.

1-2-2-1 Receptor desensitisation

Desensitisation of a GPCR results in uncoupling from the transduction machinery through receptor phosphorylation. Agonist-dependent, homologous desensitisation, is mediated by G protein-coupled receptor kinases (GRKs) and their co-factors, the arrestins (Fig 1-6). GRKs produce desensitisation by phosphorylating the activated receptors, therefore GRKs need to be appropriately localised and targeted to the plasma membrane. Moreover, they have to be functionally active at physiological concentrations of agonists. The phosphorylated receptors have high affinity for the arrestin proteins. The arrestin proteins do not possess enzymatic activity but appear to function in a 1:1 stoichiometric manner, binding to the phosphorylated receptor and disrupting the interaction between the receptor and the G proteins.

Agonist-independent or heterologous desensitisation results from receptor phosphorylation by second messenger-activated protein kinases, PKA or PKC. PKA has been shown to induce phosphorylation and desensitisation of the β2-adrenergic receptor (Stadel, 1983; Benovic, 1985; Nambi, 1985). For this to take place PKA is required to translocate from the cytosol to the membrane. PKC has been implicated in the desensitisation of several receptors (m1 muscarinic receptor, vasopressin receptor, angiotensin II receptor) and upon receptor stimulation PKC undergoes translocation to the plasma membrane (Haga, 1996, Zhang, 1996a; Opperman, 1996a). This translocation requires receptors for activated C-kinases (RACKs), which target PKC isoenzymes to GPCRs causing desensitisation.
Fig 1-6. GPCR homologous desensitisation

The GPCR activated by the agonist (A) is able to couple with G proteins, promoting their activation. In this activated conformation the receptor can undergo G protein-coupled receptor kinase (GRK) phosphorylation (p). Phosphorylation in the C terminal tail or in the third intracellular loop promotes arrestin (arr.) binding to the receptor, with consequent inhibition of GPCR-G protein coupling. Details of this process are reported in the text.
As well as directly phosphorylating GPCRs, second-messenger kinases may participate in desensitisation by regulating other kinases. Recent studies showed that PKA and PKC can mediate changes in the cellular expression and activity of GRKs and arrestins thus regulating the extent of homologous desensitisation. In human peripheral blood leukocytes it was shown that T cell activation resulted in increased mRNA, protein levels and kinase activity of GRK2. Molecular analysis showed that the observed increase in GRK2 expression and kinase activity was a PKC-mediated process (Chuang, 1995; De Blasi, 1995) (Fig 1-7). On the other hand the expression of βarrestin 1, a member of the arrestin family, is regulated by intracellular cAMP (Parruti, 1993; Iacovelli, 1996). The levels of βarrestin 1 mRNA and protein were increased in different cell types when intracellular cAMP was raised by activation of receptor, Gs, or adenylyl cyclase. The mechanism of this process likely involves the activation of PKA (Fig 1-7).

All these data show a heterologous regulation of proteins involved in GPCR homologous desensitisation.

Receptor desensitisation can also result from changes in palmitoylation state. Most GPCRs have one or two cysteines located 12-15 residues C-terminal to TMD VII. Palmitoylation of these cysteines has been demonstrated for several GPCRs (Ovchinikov, 1988; O'Dowd, 1989; Kennedy, 1993). This posttranslational acylation anchors the N-terminal portion of the cytoplasmic tail to the plasma membrane, creating a fourth intracellular loop (Fig 1-1). The palmitoylation state of some receptors has been demonstrated to be a reversible process and to be influenced by agonist exposure (Moffett, 1993). Dynamic de-palmitoylation in response to agonist has been observed and this process seems to affect also GPCR desensitisation. De-palmitoylation increases the basal level of receptor phosphorylation and produces receptor uncoupling from G proteins (Moffett, 1996).
Fig 1-7. **Heterologous regulation of homologous desensitisation mechanisms**

Schematic representation of a cross talk between kinases involved in homologous and heterologous desensitisation. 

*PKC* can directly phosphorylate (*p*) and activate a member of the *GRK* family (GRK2) (Winstel, 1996) and, in T cells, can increase the expression and activity of GRK2 and GRK3, after sustained stimulation (Chuang, 1995). Increase of intracellular cAMP, likely through a *PKA*-mediated mechanism, raises mRNA and protein expression level of a member of the *arrestin* family (βarrestin1) (Iacovelli, 1996).
The precise endocytic mechanism by which GPCR internalisation is achieved remains controversial. The paradigm for the modulation of G protein-coupled receptor function in response to agonist-stimulation has been the β2-adrenoceptor (β2AR). The first evidence of agonist-induced β2AR internalisation came from early ligand binding studies with β2AR. They demonstrated a progressive loss of high affinity agonist binding during the binding assay, suggesting a loss of β2AR from the plasma membrane (Pittman, 1980; Toews, 1983). The internalised receptors were not accessible to a hydrophilic ligand (Staehelin, 1982), but were available for hydrophobic ligands, thus providing a tool to measure β2AR sequestration.

Sequestrated receptors were found associated with the light vesicle fraction and could be separated from the heavy vesicle fraction (plasma membrane) by sucrose density gradient fractionation (Harden, 1980). The translocation of β2AR, from the plasma membrane to the intracellular compartment, occurred very rapidly, with a t1/2 of about 2 min. Moreover, the sequestration was apparently independent of β2AR/Gs coupling (Clark, 1985). The only requirement for sequestration appeared to be a good level of agonist occupancy of the β2AR (Lohse, 1990a). To date the best study on the sequestration of the β2AR has been performed using immunocytochemistry to investigate sub-cellular receptor localisation. By conventional and confocal fluorescence microscopy it was demonstrated that the rapid agonist-induced translocation of β2AR into the cytosol of HEK 293 cells temporally paralleled the sequestration of β2AR, measured by radioligand binding (von Zastrow, 1992). The internalisation compartments of β2AR and other GPCRs have been identified as early endosomes. Sequestered β2AR has been shown to co-localise with endosomal markers, such as transferrin, rab 5, or clathrin (von
Zastrow, 1994). Further studies clarified that β2AR internalisation is carried out by clathrin-coated vesicles (Fig 1-8). Clathrin-coated pits are specialised regions of the plasma membrane of all eukaryotic cells (Schmid, 1997). The major structural protein of the characteristic polygonal lattice of the coated pit is clathrin. Clathrin is a trimer protein arranged in a triskelion or three-legged shape, with a globular portion at the end of each leg. Most of the protein comprises the outer shell of the lattice. Plasma membrane coated pits also contain AP-2 heterotetrameric complex, formed by α2-, β2-, σ2-, µ2-adaptin. AP-2 molecules bind to clathrin and serve as an adaptor linking receptors to the structure of clathrin cages. The arrestin- and clathrin-dependent internalisation is a dynamin-dependent event (Zhang, 1996b).

Experimental conditions, which disrupt internalisation by clathrin-coated pits (hypertonicity treatment, cytosolic acidification, intracellular potassium depletion, reduced temperature, and reduced ATP), also disrupt internalisation of β2AR (Chuang, 1980; Homburger, 1980). Moreover, the involvement of caveolae has also been reported for β2AR internalisation (Ferguson, 1996a). Caveolae represent distinct cholesterol- and glycosphingo-lipid-enriched plasma membrane and vesicular structure in the cell. The major structural component of caveolae is caveolin, a 22-24 kDa integral membrane protein (Okamoto, 1998). Receptor sequestration can likely involve both clathrin or caveolae pathways, but the preferred mechanism of endocytosis used by a particular receptor is probably dependent on both receptor-specific structural determinants and the cellular environment in which it is expressed (Fig 1-9).
Fig 1-8. β2-adrenergic receptor internalisation through clathrin-coated pits

Schematic representation of β2AR desensitisation and internalisation through clathrin coated pits. The following steps are depicted: (1) The agonist (A) bounded-GPCR can undergo GRK phosphorylation, which represents the beginning of GPCR desensitisation process. (2) The phosphorylated receptor can recruit and bind arrestin (βarr.). (3) The next step is the recruitment of clathrin, AP2, dynamin and coated pit formation, with consequent membrane invagination. (4) Coated pit constriction requires dynamin assembly at the neck. A detailed description has been reported in the text.
Fig 1-8

[Diagram of cell signaling pathways involving GRK, β arr., PIP2, and dynamin/CLATHRIN/AP2 complexes.]

- GRK
- β arr.
- PIP2
- Dynamin
- AP2
- Clathrin
Fig 1-9. Multiple mechanisms involved in GPCR endocytosis

Schematic representation of alternative mechanisms by which GPCRs are proposed to internalise. These mechanisms include caveolae, which may or may not pinch off from plasma membrane; clathrin-coated vesicles, which are excised from plasma membrane in a dynamin-dependent manner and transport membrane-bound receptors to endosomes; and non-coated vesicles, which likely endocytosed in a dynamin-independent manner to endosomes.Dynamin-dependent clathrin-coated vesicle-mediated endocytosis of β2-adrenergic receptor (β2AR) and Angiotensin II type 1A receptor (AT1AR) involves βarrestin. AT1AR endocytosis in absence of over-expressed βarrestin involves non coated vesicles and potentially caveolae. Possibility that β2ARs internalise via caveolae in some cell types is controversial and remains to be determined.
Fig 1-9

Caveolae

Clathrin-coated vesicles

Non-coated vesicles

50-80 nm

Caveolin

AT \textsubscript{1A} R, \beta \textsubscript{2} AR

\textdagger

Dynamin

AT \textsubscript{1A} R

\beta \textsubscript{1} AR, AT \textsubscript{1A} R

Clathrin

100-150 nm

ENDOSOME

\sim 100 nm
1-2-2-1 Role of GRKs and βarrestins in GPCR endocytosis

A certain number of studies indicate that GRK-mediated GPCR phosphorylation not only plays an important role in β2AR desensitisation but also serves as a signal promoting receptor internalisation (Ferguson, 1995). In particular GRKs phosphorylation of the receptor both stabilises GPCR conformation required for the internalisation and acts to promote βarrestin binding. Moreover, with sub-cellular gradient fractionation studies and with double immunofluorescence analysis using confocal microscopy it has been demonstrated that GRK2 co-localises with the internalised β2AR (Ruiz-Gómez, 1997).

Not only GRK but also βarrestin is required for β2AR sequestration. It has been shown that β2AR sequestration in different cell types perfectly correlates with the expression levels of both GRK2 and βarrestin (Menard, 1997) and sequestration of m2 muscarinic receptor is synergistically regulated by both GRK2 and arrestin (Schlador, 1997). Interestingly, co-expression of angiotensin 1a receptor, a GPCR that is not normally internalised via clathrin coated pits, with βarrestin promotes internalisation through the classic clathrin-mediated endocytic pathway (Zhang, 1996b).

Further studies demonstrate that in GPCR internalisation βarrestin seems to play a predominant role. In the absence of βarrestin binding, receptor phosphorylation is not sufficient to mediate receptor sequestration, while βarrestin over-expression can produce agonist-dependent sequestration of β2AR in the absence of GRK-mediated phosphorylation (Ferguson, 1996b). βarrestins appear to act as GPCR trafficking proteins that specifically target GPCRs for clathrin-dependent endocytosis (Zhang, 1996b). It has been demonstrated, using purified proteins, that βarrestin1 and 2 (but not visual arrestin) bind directly, stoichiometrically, and with high affinity with clathrin
(Goodman, 1996). The residues LIEF/L found in the βarrestin1 and 2 C termini have been shown to mediate this interaction (Krupnick, 1997). Moreover, immunofluorescence analysis demonstrated that in intact cells activated β2AR, βarrestin and clathrin co-localise, upon agonist addition, suggesting that the arrestin-clathrin interaction observed in vitro also occurs in vivo (Goodman, 1996). The function of βarrestin in GPCR sequestration is regulated by a phosphorylation/dephosphorylation process (Lin, 1997). Cytoplasmic βarrestin1 is constitutively phosphorylated on Ser 412. Upon GPCR stimulation βarrestin1 is recruited to the plasma membrane and becomes rapidly dephosphorylated. Recent studies showed that βarrestin1 is phosphorylated in vitro by the extracellular signal-regulated kinases (ERK1 and 2) and in intact cell studies, with HEK 293 cells, βarrestin1 phosphorylation can be modulated according to ERK activation state. These data indicate that ERK is the kinase that phosphorylates βarrestin1 in these cells (Lin, 1999) (Fig 1-10).

It is not presently clear whether βarrestin1 de-phosphorylation precedes or follows βarrestin1 receptor binding. In any case phosphorylation of Ser 412 on βarrestin1 does not seem to regulate its receptor binding. On the contrary only de-phosphorylated βarrestin1 can bind clathrin and regulate GPCR sequestration. It is interesting that Ser 412 is not present in the other members of the arrestin family. Thus they have to be regulated by different mechanisms.

Phosphorylation is not the only mechanism modulating arrestins. An agonist-dependent association of βarrestin2 with the β2-adaptin subunit of AP-2 has been demonstrated (Laporte, 1999). Moreover, βarrestin interaction with AP-2 is necessary for the clustering of β2-adrenergic receptor into clathrin-coated pits and can modulate receptor internalisation even in absence of direct βarrestin-clathrin binding.
Cytosolic β-arrestin is predominantly phosphorylated at Ser 412 as a consequence of ERK kinase activity. In the phosphorylated form β-arrestin interaction with clathrin and Src is inhibited. β-arrestin undergoes de-phosphorylation when recruited to the plasma membrane in response to agonist-stimulation. De-phosphorylated β-arrestin binds to Src and targets the agonist-occupied, GRK-phosphorylated GPCR to the clathrin coated pits for internalisation. A, agonist; GRK2, G protein-coupled receptor 2; (+) stimulatory effect; (-), inhibitory effect.
Fig 1-10

Endocytosis

- GRK2
- β-arr.
- Src
- clathrin
- ERK
In HEK 293 cells by fluorescence microscopy it has been shown that \( \beta_2 \)-adrenergic receptor, \( \beta_\text{arrestin} \), AP-2 all co-localise in clathrin-coated pits (Laporte, 2000).

**1-2-2-2 Fate of endocytosed receptors**

Once in the endosome GPCRs may either be recycled to the plasma membrane or directed to other destinations of the endocytotic pathway, such as the lysosome. Far less is known about lysosomal targeting of GPCRs for hormones and neurotransmitters. After a brief exposure to agonists, the \( \beta_2 \)AR is recycled efficiently and is rarely found in lysosomes (Koenig, 1994). It is not known if long term exposure to agonists results in the targeting of these receptors to lysosomes. Different is the behaviour of the thrombin receptor that is activated irreversibly by a unique proteolytic mechanism. Thrombin cleaves the receptor's amino terminus to reveal a new terminus, which then act as a tethered peptide ligand. Activated receptors are rapidly endocytosed and delivered to lysosomes for degradation (Hein, 1994). Receptors at the plasma membrane are eventually replenished by delivery of receptors from an intracellular pool.

**1-2-2-3 GPCR resensitisation**

Many studies have indicated that \( \beta_2 \)AR endocytosis may play a role in accelerating the recovery from desensitisation (Pippig, 1995; Pak, 1996). It was proposed that \( \beta_2 \)AR sequestration restores function by enabling a vesicle-derived phosphatase to dephosphorylate the receptor, followed by the recycling of the dephosphorylated functional \( \beta_2 \)AR receptor to the cell surface (Pippig, 1995). The importance of \( \beta_2 \)AR resensitisation, through phosphatase activity and recycling of functional receptor to the plasma membrane, was demonstrated by the
ability of calyculin A, an inhibitor of protein phosphatase and monesin, an inhibitor of intracellular trafficking, to block resensitisation of β2AR. Recently, a G protein receptor phosphatase able to dephosphorylate GRK2-phosphorylated β2AR was purified from bovine brain and shown to be a latent form of protein phosphatase 2A (Pitcher, 1995).

1-2-2-2-4 Endocytosed G protein-coupled receptor signalling
Recently, it was proposed that for some GPCRs (in particular the β2AR) events associated with receptor endocytosis are also involved in signalling to the mitogen-activated protein kinase (MAPK) cascade (Dhanasekaran, 1995; van Biensen, 1996; Gutkind, 1998). It is well known that many GPCRs regulate MAPK cascades, leading to activation of the extracellular-signal regulated kinases (ERKs), Jun-amino terminal/stress-activated kinase, and p38 MAPK, which have a number of substrates including transcription factors mediating cellular growth and differentiation. It has been shown that GPCR activation of MAPK pathway involves the small GTP-binding protein p21Ras (Koch, 1994). Depending upon the receptor and the cell type studied different signalling complexes leading to Ras activation by GPCRs have been proposed and can be grouped in three different models.

The first model involved GPCRs trans-activation of receptor tyrosine kinases and is supported by data derived from studies on the lysophosphatidic acid, α-thrombin, α2-adrenergic, m2 muscarinic receptors (van Biensen, 1995; Daub, 1997) (Fig 1-11). At least three RTKs, those for platelet-derived growth factor (PDGF), epidermal growth factor (EGF), and insulin-like growth factor-1 (IGF-1), become phosphorylated after GPCR activation (Linseman, 1995; Rao, 1995; Daub, 1996). Little is known about the mechanisms whereby GPCRs regulate RTK activity, even if Gβγ subunits, Src kinases, calcium, PKC have each been implicated in selected model systems.
Fig 1-11. A model of GPCR trans-activation of receptor tyrosine kinase.

Classical RTKs, such as the Epidermal Growth Factor receptor (EGF-R), are single transmembrane domain proteins that dimerise and trans-phosphorylate (p) (crossed arrows) upon binding of their native ligand. In this model, GPCR trans-activated EGF receptor forms the structural core of a GPCR-induced mitogenic signalling complex. (1) After hormone binding (H), GPCR activation results in dissociation of the Gα–GTP (αGTP) and Gβγ (βγ) subunits, with consequent effector (E) activation. (2) Working via undefined effectors that are probably Gβγ subunit-regulated, GPCRs induce EGF-independent activation of EGF receptor kinase. (3) EGF receptor trans-phosphorylation promotes its association with numerous phosphotyrosine-binding PTB or SH2-domain containing adaptor proteins, such as Shc and Grb2 and other signalling proteins. (4) Grb2-Sos1 recruited to the EGF receptor complex catalyses Ras-GTP exchange. Association of Ras with the MAPK kinase kinase, Raf, initiates the Raf-MEK-ERK MAP kinase cascade.
Fig 1-11

1. Myristoylation/prenylation
2. Phosphate
3. Ligand
4. Hormone

Plasma membrane

EGF-R

GTP (mek)

Ras

Raf

Shc

Grb2

Sos

ERK

MEK
The second model explains GPCR activation of MAPK cascades through GPCR signalling via focal adhesion complexes and derives from studies on bombesin, vasopressin receptors (Sinnett-Smith, 1993; Rodriguez-Fernandez, 1998) (Fig 1-12). Focal adhesions are points of cell attachment to the extracellular matrix and of cytoskeletal anchoring to the plasma membrane. Focal adhesion complexes are formed by αβ integrin heterodimers, cytoskeletal elements and catalytic signalling proteins, and their formation can lead to ERK cascade activation (Schlaepfer, 1996). It is not perfectly clear how can GPCRs activate focal adhesion-dependent MAPK pathway, but a role for a new member of the calcium- and PKC-regulated focal adhesion kinase family, Pyk2 has been proposed (Lev, 1995; Dikic, 1998).

The third model is supported by the idea that GPCR activation of the MAPK pathway requires clathrin-mediated endocytosis of GPCR (Daaka, 1998) (Fig 1-13). In HEK 293 and COS 7 cells β2AR forms complexes with activated Src kinase. This complex follows β2AR desensitisation and recruitment of βarrestin to the plasma membrane. βarrestin functions as an adaptor protein bringing activated c-Src to the agonist occupied receptor and targeting both to clathrin coated pits (Luttrell, 1999a). Co-immunoprecipitation studies with different βarrestin deleted mutants show a βarrestin/c-Src interaction region in the N-terminus of βarrestin. The amino terminal proline rich region of βarrestin can interact with the Src homology domain 3 (SH3 domain) of c-Src. Surprisingly a major interaction also has been demonstrated between βarrestin and the catalytic domain of c-Src (SH1 domain) (Miller, 2000). Moreover, the βarrestin/c-Src interaction is modulated by βarrestin phosphorylation. Dephosphorylation of βarrestin Ser 412, necessary for clathrin binding, is also required for c-Src recruitment (Luttrell, 1999a).
**Fig 1-12. A model of GPCR signalling via focal adhesion complexes.**

In this model, GPCR-induced recruitment of the Ca\(^{+2}\)-and PKC-regulated FAK family kinase (Pyk2), to integrin dimers (\(\alpha, \beta\)) initiates the assembly of a focal-adhesion-based Ras-activation complex. The signal is dependent both upon cell adhesion to the extracellular matrix and GPCR-mediated calcium signals. (1) After hormone binding (\(H\)), GPCR activation results in dissociation of the \(G\alpha-GTP (\alpha GTP)\) and \(G\beta\gamma (\beta\gamma)\) subunits, with consequent effector activation. When the activated effectors are phospholipase (\(PLC\)) \(\beta\) isoforms, it results in an increase of intracellular calcium and PKC activation. (2) Pyk2, which is predominantly cytosolic in the absence of stimulation, is recruited to integrin heterodimers (\(\alpha\beta\)), where it becomes activated, autophosphorylates and binds to PTB or SH2-domain containing adaptor proteins. (3) the resultant integrin-based complex is able to induce subsequent (4) Ras-GTP exchange that initiates the Raf-MEK-ERK cascade.
Fig 1-12

Extracellular matrix

Plasma membrane

1. Myristoylation/prenylation
2. Phosphate
3. Ligand
4. Hormone
**Fig 1-13. A model of endocytosis-dependent activation of MAPK pathway by the β2-adrenergic receptor**

In this model, β-arrestin1 (β-arr) binding to the GPCR (β2 AR) both uncouples the receptor from its cognate G proteins (αGDP βγ) and initiates the formation of a signalling complex composed of the GPCR, β-arrestin1 and c-Src. (1) Upon hormone binding (H) GPCR activates the heterotrimeric G proteins (Gα–GDP βγ), with consequent effectors stimulation. (2) One consequence of Gβγ subunits release is the enhanced GRK2-mediated phosphorylation of the agonist occupied receptor. (3) β-arrestin1 binds both to GRK-phosphorylated receptor and to c-Src, resulting in recruitment of the Src kinase to the membrane. Subsequent interaction of β-arrestin1 with clathrin, targets the receptor-β-arrestin-c-Src complex to clathrin coated pits. (4) Both β-arrestin-mediated recruitment and receptor targeting to clathrin coated pits are required for activation of the ERK pathway mediated by β2-adrenergic receptor.
Fig 1-13

Myristoylation/prenylation
Clathrin
Ligand hormone

ERK cascade

Clathrin-coated pit

\[ \beta_2 \text{ AR} \]

\[ \alpha_{GDP} \]

\[ \text{c-Src} \]

\[ \beta-\text{arr} \]

\[ \text{Clathrin} \]
The requirement of GPCR endocytosis for MAPK activation has been confirmed with receptors different from β2AR, as m1 muscarinic, μ, δ and κ opioid receptors (Vogler, 1999; Ignatova, 1999, Whistler, 1999). It's important to notice that most of these studies were obtained in over-expression systems and it is not surprising that other studies have yielded conflicting results. For example studies on α2-adrenergic, CB1 cannabinoid, m3 muscarinic, CXCR2 receptors, demonstrate that MAPK signalling is independent of GPCR endocytosis (DeGraff, 1999; Schramm, 1999; Roche, 1999; Budd, 1999; Yang 1999). Recent investigations using confocal immunofluorescence microscopy have shown that both β2AR and α2-adrenergic receptors induce ERK activation through EGFR trans-activation in COS 7 cells. Moreover these studies separate GPCR endocytosis from ERK activation, indicating that is not GPCR but the receptor kinase or other downstream effector that must engage the endocytic machinery (Pierce, 2000).

1-2-3 Mechanisms operating at the level of the G proteins

Inactive G proteins are heterotrimers formed by a GDP-bound α subunit associated with the Gβγ dimers. The activated GPCRs function as GDP/GTP exchange factors and promote the release of GDP and the binding of GTP to the α subunits. This leads to dissociation of the α subunit and the G βγ dimer. Both G α-GTP and G βγ can interact with a variety of effectors in order to modulate cellular signalling pathways. The G protein α subunit hydrolyses GTP to GDP and in turn re-associates with G βγ to form the inactive heterotrimer.

Cells have regulatory mechanisms that limit the duration or sensitivity of G-protein signalling (Koelle, 1997). For example, in order to perceive rapidly changing images our photoreceptors must confine their response to a fraction of a second after each stimulus. Thus,
photoreceptors need other mechanisms by which to limit the duration of signalling beyond the slow intrinsic GTPase activity of the visual G protein (t_1/2 for GTP hydrolysis > 10 seconds) (Chabre, 1993). GTPase activities of G-protein subunits can be activated by GTPase accelerating proteins in order to drive GTP-bound G proteins back to the inactive GDP-bound form. A recently identified class of proteins named Regulators of G Protein Signalling (RGS) accelerate the hydrolysis of Gα-bound GTP and promote the deactivation of G proteins (Fig 1-5).

**1-3 G protein-coupled receptor kinases**

Although desensitisation of a G protein-coupled signalling system can involve the receptor, the G protein, and/or the effector, impairment of the receptor's ability to activate its G protein appears to account for most desensitisation, especially within minutes of agonist-stimulation. In particular the agonist-specific inhibition of a GPCR that occurs within milliseconds to minutes of agonist challenge is called short-term homologous desensitisation. This process represents an adaptive mechanism thought to facilitate cell responsiveness to successive multiple extracellular stimuli over time. The molecular determinants of this process are G protein-coupled receptor kinases (GRKs) and arrestins.

**1-3-1 Structure of GRKs**

GRKs constitute a family of serine/threonine protein kinases. The members of this family were initially named based on the nature of their first receptor substrate identified, but it seems increasingly likely that some of these kinases do not have substrate specificity. It is more reasonable to identify these enzymes simply as GRKs and they were
numbered according to the chronological order of their discovery (Lefkowitz, 1993a). To date seven members of this family have been cloned and they are grouped in different subfamilies based on sequence and functional similarities (Tab 1-2).

These enzymes showed an overall 57-97% similarity with GRK1 and GRK2 being the most divergent (Fig 1-14). They have a central catalytic domain (of 263-266 amino acids) within is located a DLG sequence, that constitutes the ATP-binding pocket, and the Lys residue in subdomain II, which is highly conserved among protein kinases and has a role in the phospho-transfer reaction from the ATP molecule to GRK substrate. The catalytic domain is flanked by large amino- and carboxyl-terminal regulatory domains. The amino terminal domain (of about 180 amino acids) is related among all GRKs (~65% similarity); this indicates that this domain has a common function in these kinases and it has been suggested that it is involved in activated receptor recognition and binding (Inglese, 1993).

Table 1-2

<table>
<thead>
<tr>
<th>Family name</th>
<th>Tissue distribution (major sites)</th>
<th>Chromos. mapping</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRK1</td>
<td>Retina</td>
<td>13q34</td>
<td>Premont, 1995; Khani, 1996</td>
</tr>
<tr>
<td>GRK2</td>
<td>Ubiquitous (L, b, h)</td>
<td>11q13</td>
<td>Chuang, 1996a; Calabrese, 1994a</td>
</tr>
<tr>
<td>GRK3</td>
<td>Ubiquitous (L, b, o, s, h)</td>
<td>22q11</td>
<td>Premont, 1995; Calabrese, 1994b</td>
</tr>
<tr>
<td>GRK4</td>
<td>Testis (t, ce)</td>
<td>4q16.3</td>
<td>Ambrose, 1992; Sallese, 1997</td>
</tr>
<tr>
<td>GRK5</td>
<td>Ubiquitous (h, pl, lu, r)</td>
<td>10q24-qter</td>
<td>Chuang, 1996a; Bullrich, 1995</td>
</tr>
<tr>
<td>GRK6</td>
<td>Ubiquitous (b, sk, p)</td>
<td>5q35, 13qter-q21</td>
<td>Inglese, 1993, Loudon; 1994</td>
</tr>
<tr>
<td>GRK7 (gs)</td>
<td>Retina</td>
<td>-</td>
<td>Weiss, 1998</td>
</tr>
</tbody>
</table>

Reported are the major sites of expression, and human chromosome mapping. L, leukocytes; b, brain; h, heart; o, olfactory neurones; s, spleen; pl, placenta; lu, lung; r, retina; sk, skeletal muscle; p, pancreas; t, testis; ce, cerebellum. Reported GRK7 has been cloned from ground squirrell (gs)
Fig 1-14. G protein-coupled receptor kinase family and subfamilies.

The seven known GRKs can be classified in three different subfamilies according with sequence homology. All the GRKs present a centrally located catalytic domain. The C termini are involved in membrane binding and here are reported βγ binding domain of GRK2 and GRK3; GRK1, 4 and 6 lipid modifications; and also the positively charged GRK5 C terminus has been evidenced. Different splice variants of GRK4 and GRK6 have been discovered (GRK4α, β, γ, δ and GRK6 A, B, C)
Interestingly an RGS homology domain has been identified in the N-terminus of GRK2 and GRK3. RGS proteins can bind to Gα subunits accelerating the GTP hydrolysis and thereby play an important role in modulating G protein signalling. So far there is no experimental evidence to show that the RGS domain of GRK2 and GRK3 have GAP activity towards Gα subunits. However the presence of the RGS domain leads to the speculation that GRKs can play a fundamental role in modulating GPCRs signalling by binding to Gα subunits.

The carboxyl terminal domain is the most divergent region among these kinases and it presents peculiar characteristics for each GRK. This domain contains Ser and Thr residues that are sites of autophosphorylation for GRK1, GRK4, GRK5 and GRK6. This GRK region undergoes posttranslational modifications by lipid that produce membrane anchoring. Moreover the C terminus of GRK2 and GRK3 contains a pleckstrin homology domain involved in the interaction with βγ subunits and inositol phospholipids.

1-3-2 The GRK family

GRK1 or rhodopsin kinase and GRK7 constitute the first subfamily of GRKs. GRK1 is the most studied GRK and the only known member of this family with defined cellular localisation and physiological receptor substrates: rhodopsin in rods and iodopsin in cones (Lorenz, 1991) (Fig 1-14). GRK7 is the last identified member and the less characterised. It has been cloned in the ground squirrel (gs) and eastern chipmunk and it specifically localises to cones and showed ability to phosphorylate rhodopsin, suggesting that it could function as a cone opsin kinase (Weiss, 1998). GRK2 and GRK3 belong to the βARK (β-adrenergic receptor kinase) subfamily (Fig 1-15).
Fig 1-15 Domain organisation and features of GRK1 and GRK2.

Upper: Schematic diagram of GRK1.
Critical residues have been evidenced: Ser-21 represents the minor and Ser-488 and Thr-489 the major autophosphorylation sites of GRK1. GRK1 amino acid sequence terminates with a CAAX motif that directs farnesylation and carboxymethylation of this protein.

Lower: Schematic diagram of GRK2.
Critical residues have been evidenced: Ser-29 is GRK2 residue that undergoes PKCa-phosphorylation.

Critical domains have been evidenced: GRK2 RGS domain is located in the amino terminus (a.a. 51-171), while its PH domain is in the C terminus (a.a. 561-655). The catalytic domain is centrally located (a.a. 185-467) and a same feature is presented by all the other member of this family. The boxes represent domains involved in GRK2 interaction with other molecules. Gβγ binding domain (βγ) in GRK2 sequence is represented by a short 28 amino acids sequence (a.a. 643-670), including the C-terminal portion of the PH domain and 19 amino acids of the carboxyl flanking region. Mutational analysis individuates Arg-587 as the critical residue for Gβγ binding. Phospholipids bind to the PH domain of GRK2 (Phospholipids), with PIP2 binding site (PIP2) located in the amino terminus and phosphatydilserine binding site (PS) in the carboxyl terminus of the PH domain. Mutational analysis identified two GRK2 binding determinants for phospholipids binding, represented by two charged amino acids, located in the PH domain, Lys-567 and Arg-578. Two distinct CaM-binding sites (CaM) located within the N- (a.a. 1-88) and C-terminal (a.a. 593-689) regions of GRK2 were identified. GRK2 domain involved in the binding to the microsomal membranes in the N-terminal region of GRK2 (residues 88-145) (Micr. Memb.). A primary tubulin binding determinant (Tubulin) has been individuated in the catalytic domain of GRK2 (a.a. 185-467) and another binding domain has been identified also in the carboxyl terminal portion of GRK2 (a.a. 467-689).
GRK1
Catalytic domain

GRK2
Catalytic domain
PHD

CaM

RGS BOX

PIP2
PS
Phospholipids

Tubulin

S21

S488 T489

S29

K567 R578 R587

Mscr. Memb.

CaM

CaM

CaM

3-
467
561
They are ubiquitously expressed, with high expression in leukocytes, brain and heart, GRK2 protein is usually more abundant than GRK3 (Inglese, 1993; Premont 1995). GRK2 was initially identified as the kinase that phosphorylates the agonist-occupied β2-adrenergic receptor with GRK3 as a highly related isoform, but these kinases are able to phosphorylate a variety of other receptors in vitro (Benovic, 1986; Benovic, 1989; Benovic, 1991) (Tab 1-3).

The GRK4 subfamily comprises GRK4, GRK5 and GRK6 (Fig 1-14). GRK4 was cloned in the search for the Huntington’s disease locus on human chromosome 4 and was initially named IT-11, successively it was shown to be unrelated to Huntington’s disease (Ambrose, 1992). GRK4 presented the special feature to exist as four different splice variants. It undergoes alternative splicing with the alternatively spliced exons occurring in the amino and carboxyl termini (Sallese, 1994; Premont, 1996) (Fig 1-16). This kinase presents tissue specificity with expression in testis and in cerebellum (Sallese, 2000). GRK5 is one of the most extensively studied members of this family. It is distributed throughout the body with preferential expression in the heart, leukocytes, placenta and lung (Premont, 1995; Bullrich, 1995). GRK6 is the only GRK of which two different genes have been identified, one on chromosome 5 and the other on chromosome 13, the latter probably being transcriptionally inactive pseudo gene (Haribabu, 1993). The tissue distribution of GRK6 is ubiquitous, but it is highly expressed in leukocytes (Benovic, 1993; Haribabu, 1993). Recently it has been shown that GRK6 undergoes extensive alternative splicing of its extreme carboxyl terminal, yielding three different splice variants: GRK6A, B and C (Fig 1-16). This process should have functional consequences because it involves the C terminal domain with an important role in the regulation of GRK6 kinase activity and membrane binding (Premont, 1999).
**Fig 1-16. Domain organisation and features of GRK4, GRK5 and GRK6.**

**Upper:** Schematic diagram of GRK4.
The N- and C-terminal inserts produced by alternative splicing are evidenced.
The boxes represent domains involved in GRK4 interaction with other molecules. Only the GRK4 splice variants with the N-terminus insert can bind with calmodulin.
GRK4 presents a posttranslational palmitoylation in its carboxyl terminus

**Middle:** Schematic diagram of GRK5.
Lipids can non specifically stimulate GRK5 autophosphorylation on Ser-484 and Thr-485. Two CaM binding sites on GRK5 are located within residues 20-39 in the N-terminus and 540-578 in the C terminus. The N terminus is conserved in GRK6 and in two GRK4 splice variants that possess the 32 amino acid N-terminus insert. Two distinct binding sites for lipids (*lipids*) have been individuated in GRK5 sequence. One is located on the carboxyl terminal last 100 amino acids and does not present ligand selectivity among different lipidic molecules, the other binding site is in the amino terminus and it shows a high degree of selectivity toward PIP2. GRK5 domain involved in PIP2 interaction is a region of basic amino acids located in the N-terminus (a.a. 22-29). This domain is conserved in GRK6 and in two GRK4 splice variants that possess the 32 amino acid N-terminus insert. PKC phosphorylates GRK5 and the site of phosphorylation resides in the last 26 amino acids region of GRK5.

**Lower:** Schematic diagram of GRK6.
It is reported an alignment of GRK6 splice variant carboxyl-terminal sequences. Underlined letters indicate palmitoylation sites in GRK6A and the predicted protein kinase A site in GRK6B.
Fig 1-16

Catalytic domain

GRK4

S484 T485

CaM

GRK5

CaM

PKC

Lipids

GRK6

560

DCGGNCSDSEELPTRL

RIAVETATARKSSPPASSPQPEAPTSSWR

A

B

C

18 49

515 562

PIP2

CaM

PIP2

CaM
The presence of homologues in Drosophila and evidence supporting the existence of such receptor kinases in Dictyostelium and Saccaromyces cerevisiae suggests a long evolutionary history for these kinases (Pitcher, 1998a).

1-3-3 Kinase activity of GRKs

1-3-3-1 Mechanistic models of GRKs kinase activity

Studies performed on GRK-mediated phosphorylation of receptors or of peptides show several hallmark characteristics. First of all GRKs do not demonstrate clear consensus sequences in their receptor substrates. Initial studies with model peptide substrates showed some substrate specificity. GRK1 preferentially phosphorylates Ser or Thr residues followed by several acidic residues, while GRK2 phosphorylates substrates where acidic residues precede the target Ser or Thr (Onorato, 1991). GRK5 and GRK6 most actively phosphorylate peptides containing basic residues amino terminal to the serine target residues (Ohguro, 1993; Eason, 1995a; Prossnitz, 1995; Fredericks, 1996). Further investigations, based on studies with GPCRs, came to the conclusion that GRKs recognise activated (agonist-bound) conformations of GPCRs rather then linear sequences. Moreover, interaction of GRKs with activated receptors potently activates these enzymes. The ability of activated receptors to enhance the activity of GRKs toward peptide substrates suggests the hypothesis that in a cellular setting GRKs can phosphorylate non-receptor substrates following receptor activation.

GRKs recognition of the activated receptor is probably consequent to receptor conformational modifications following agonist binding. Two different mechanistic models have been proposed in order to clarify the process of GPCR-GRK interaction: the stable-complex model and the
The hysteresis model (Palczewski, 1997) (Fig 1-17). In the first model a stable ternary complex of agonist-occupied receptor and GRK is formed in a stoichiometric manner. This complex involves geometrical complementarity of the N-terminus of the GRK and different domains of the receptor. Several lines of evidence indicate that GRKs interact with receptors also at sites distinct from their sites of phosphorylation. Truncated receptors, that are not substrates of GRKs phosphorylation, retain their ability to bind the GRKs and to activate the kinases toward peptides substrates (Kim, 1993; Kameyama, 1994; Premont, 1994). In complex with the receptor the catalytic activity of the kinase is activated towards the C-terminus of the receptor with which it forms the complex, or exogenous peptides (Palczewski, 1991; Chen, 1993). The dissociation of the complex occurs when either the kinase or the receptor is phosphorylated (Pulvermüller, 1993). The hysteresis model provides that after interacting with the agonist-bound receptor the GRK remains in the active conformation for a certain time, even after dissociation from the receptor. The activated kinase is then able to phosphorylate both activated and non-activated receptors. This model is particular suitable to explain the high-gain phosphorylation observed on rhodopsin consequent to exceedingly low illumination. In particular, activated GRK1 is able to phosphorylate both photolysed and non activated rhodopsin (Aton, 1989; Binder, 1990) (Fig 1-17). While these models account for some features of GRK activation much remains to be learnt about the mechanisms of GRK regulation.

**1-3-3-2 Receptor substrates of GRKs**

It seems that the vast majority of G protein-coupled receptors represent substrates of GRKs, due to the fact that the process of homologous desensitisation is a general characteristic of this family of receptors.
Fig 1-17. Mechanistic models of GRKs kinase activity

Schematic representation of the stable-complex model and the hysteresis model. In the stable model, GPCR* forms stable complexes with GRK. Different conformations of the receptor C terminus can lead to phosphorylation of different residues of the receptor.

In the hysteresis model, the GPCR* interacts with GRK producing an activated form of the kinase (GRK*). GRK* is able to interact with and to phosphorylate both agonist-activated or inactivated receptors. Receptor agonist (A).
With the exception of GRK1 no substrate specificity has been shown for these kinases. On the other hand GRK1 is the only GRK with specificity in cellular-expression, being restricted to retinal photoreceptor cells (Lorenz, 1991). Also GRK4 is expressed in a tissue specific manner and thus it is expected to regulate a limited number of substrates. Till now no specific substrate has been identified, although olfactory-like sperm receptors and metabotropic glutamate receptor 1 have been proposed as potential targets of GRK4 (Parmentier, 1992; Sallese, 2000). All the other GRKs are widely expressed and could potentially regulate many receptors. Given the extraordinary number of GPCRs identified so far (≥ 1000) and the relatively small number of GRKs it appears that the interaction between these proteins is not based on a “one kinase for one receptor” rule (Chuang, 1996a).

Numerous attempts to determine if a particular receptor is the target of a specific GRK have been made examining both receptor phosphorylation and desensitisation.

The first biochemical approach in order to identify GRK phosphorylation of GPCRs involved in vitro kinase assays performed with purified GRKs and purified receptors reconstituted in phospholipid vesicles or with naturally occurring membranes that contain a highly enriched receptor population, such as rhodopsin in rod outer segment membranes (Lohse, 1990a; Lohse, 1992; Attramadal, 1992). A more simple and general approach is the use of purified plasma membranes from SF9 cells over-expressing a particular receptor (Pei, 1994).

Receptor phosphorylation has also been followed in intact cell studies. In order to demonstrate that a GPCR undergoes GRK phosphorylation, cells over-expressing a specific receptor were co-transfected with GRK dominant negative mutants.

The observed inhibition of agonist-stimulated receptor phosphorylation, assessed by receptor immunoprecipitation, inferred GRK action on the
receptor (Shih, 1994; Ferguson, 1995; Diviani, 1996; Loudon, 1996). While, in order to assess a specific kinase-receptor relationship, selective GRK inhibition was obtained treating permeabilised cells with GRK-specific antibodies or GRK-specific antagonist peptides (Schleicher, 1993; Diverse-Pierluissi, 1996; Oppermann, 1996b).

GRKs induced receptor desensitisation was also examined. The agonist-induced receptor inactivation was followed performing agonist-dependent GTPase assays with reconstituted phospholipid vesicles or cellular membranes (Wilden, 1986; Benovic, 1987). In other cases GPCR agonist-evoked second messenger responses were followed in intact cell studies. Part of the enormous amount of data is reported in Table 1-3.

Some general observations can be made: it seems that each receptor is a substrate of more than one GRK, even if a certain selectivity can be observed. Nevertheless, the experimental conditions that brought these results may be far from physiological, therefore there may well be considerable specificity in GRK-receptor interactions. Experimental systems need to be designed to establish the degree of specificity in vivo.
### Table 1-3

**Evidence for GRK-dependent regulation of G-protein coupled receptors**

<table>
<thead>
<tr>
<th>G protein-coupled receptors</th>
<th>Agonist-dependent phosphorylation</th>
<th>In vitro phosphorylation by GRK</th>
<th>In vivo effects of dead kinase</th>
<th>GRK2 sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A</em>₁-Purinergic</td>
<td>In vitro (Ramkumar, 1993)</td>
<td>GRK2, 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>in vivo (Ciruela, 1997)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A</em>₂/A<em>₂B</em> Purinergic</td>
<td>In vivo (Palmer, 1997)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>A</em>₃-Purinergic</td>
<td>In vitro and in vivo (Palmer, 1995)</td>
<td>GRK2</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>α</em>₂-Adrenergic</td>
<td>In vivo (α₂A-AR) (Liggett, 1992)</td>
<td>GRK2</td>
<td>P, DES</td>
<td></td>
</tr>
<tr>
<td><em>α</em>₁B-Adrenergic</td>
<td>In vivo (Lattion, 1994; Diviani, 1996)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angiotensin II 1a</td>
<td>In vivo (Oppermann, 1996)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>β</em>₁-Adrenergic</td>
<td>In vivo and in vitro (Freedman, 1995)</td>
<td>GRK2, 3, 5</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>β</em>₂-Adrenergic</td>
<td>In vivo (Sibley, 1986; Strasser, 1986; Fredericks, 1996; January, 1997).</td>
<td>GRK2, 3, 4, 5, 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**GRK**

- *GRK2 DES* (Palmer, 1997; Mundell, 1998)
- DES inhibited (Mundell, 1997)
- *P, DES* (Liggett, 1992)
- P inhibited (Diviani, 1996)
- *P, DES inhibited* (Oppermann, 1996)
- P inhibited (Freedman, 1995)
<table>
<thead>
<tr>
<th>Molecule</th>
<th>In vivo/In vitro</th>
<th>References</th>
<th>GRK Inhibitors</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR-5 chemokine</td>
<td>In vitro</td>
<td>(Aramori, 1997)</td>
<td>GRK2, 3 P, DES</td>
<td>(Aramori, 1997)</td>
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<tr>
<td>D&lt;sub&gt;1&lt;/sub&gt;A-dopamine</td>
<td>In vivo</td>
<td>(Ng, 1994; Tiberi, 1996)</td>
<td>GRK2, 3, 5 P, DES</td>
<td>(Tiberi, 1996)</td>
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<td>ET&lt;sub&gt;A,B&lt;/sub&gt;-endothelin</td>
<td>In vivo</td>
<td>(Freedman, 1997)</td>
<td>GRK2, 5, 6 P</td>
<td>P, DES inhibited (Freedman, 1997)</td>
</tr>
<tr>
<td>N-Formyl peptide</td>
<td>In vivo</td>
<td>(Heu, 1997; Prossnitz, 1997)</td>
<td>GRK2, 3</td>
<td>(Prossnitz, 1995)</td>
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<tr>
<td>In vitro</td>
<td>(Prossnitz, 1995)</td>
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<tr>
<td>Follitropin</td>
<td>In vivo</td>
<td>(Quintana, 1994; Nakamura, 1998)</td>
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<td>GnRH-R</td>
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<td>GRK2, 3, 6 DES</td>
<td>(Neill, 1998)</td>
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<td>Lutropin/choriomadotropin</td>
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<td>(Hipkin, 1993; Wang, 1997; Lazari, 1998)</td>
<td>GRK4 DES</td>
<td>(Premont, 1996)</td>
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<tr>
<td>M&lt;sub&gt;1&lt;/sub&gt; muscarinic</td>
<td>In vitro</td>
<td>(Haga, 1996)</td>
<td>GRK2 I not affected</td>
<td>(Tsuga, 1998)</td>
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<td>M&lt;sub&gt;2&lt;/sub&gt; muscarinic</td>
<td>In vivo</td>
<td>(Kwatra, 1986; Kwatra, 1987; Richardson, 1992)</td>
<td>GRK2, 3, 5, 6</td>
<td>P, DES inhibited (Pals-Rylaarsdam, 1995)</td>
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<tr>
<td>In vitro</td>
<td>(Kwatra, 1989)</td>
<td></td>
<td>(Kwatra, 1989; Kunapuli, 1994; Loudon, 1994; Richardson, 1993)</td>
<td>(Schlador, 1997; Tsuga, 1998)</td>
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<td>In vitro</td>
<td>(Debburman, 1995)</td>
<td>GRK2, 3</td>
<td>(Tsuga, 1998)</td>
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<tr>
<td>In vitro</td>
<td>(Tobin, 1995)</td>
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<tr>
<td>M&lt;sub&gt;4&lt;/sub&gt; muscarinic</td>
<td></td>
<td></td>
<td>GRK2 I</td>
<td>(Tsuga, 1998)</td>
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<tr>
<td>Neurokinin-1 (substance P)</td>
<td>In vitro</td>
<td>(Kwatra, 1993; Nishimura, 1998)</td>
<td>GRK2, 3</td>
<td>(Kwatra, 1993; Nishimura, 1998)</td>
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74
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<tr>
<th>Odorant</th>
<th>In vivo</th>
<th>Knock-out of GRK3 lacks DES (Peppel, 1997)</th>
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<tbody>
<tr>
<td>δ-Opioid</td>
<td>In vivo (Pei, 1995)</td>
<td>GRK2, 5 P (Pei, 1995; Hasbi, 1998) GRK2 DES (Pei, 1995)</td>
</tr>
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<td>Thrombin</td>
<td>In vivo (Brass, 1992; Ishii, 1994; Vouret-Craviari, 1995)</td>
<td>GRK3 DES (Ishii, 1994) GRK3 translocation (Daaka, 1997), transgenic mice overexpressing GRK3 exhibit attenuated thrombin signalling (Iaccarino, 1998)</td>
</tr>
<tr>
<td>Thyrotropin (TSH)</td>
<td></td>
<td>GRK2, 5 DES (Iacovelli, 1996; Nagayama, 1996)</td>
</tr>
<tr>
<td>V₂ vasopressin</td>
<td>In vivo (Innamorati, 1997)</td>
<td>Undefined GRKs P (Innamorati, 1997)</td>
</tr>
</tbody>
</table>

The first column provides a partial list of GPCRs that have been analysed as targets of GRK-dependent regulation. Studies that demonstrated agonist-dependent phosphorylation of G protein-coupled receptors either in cell free assays (in vitro) or in intact cells (in vivo) are listed in column two. GRKs that have been demonstrated to phosphorylate receptors in cell free assays are listed in column three, whereas effects of heterologously expressed GRKs on agonist-induced desensitisation (DES), phosphorylation (P) and internalisation (I) of individual GPCR in intact cells are shown in column four. The last column cites studies that have been analysed the effects of coexpression of GRK2 dead kinase. GnRH, gonadotropin-releasing hormone.
1-3-3-3 Non-GPCR substrates of GRKs

Growing evidence indicate that GRKs have cellular functions in addition to agonist-dependent phosphorylation and desensitisation of G protein-coupled receptors. Several laboratories have demonstrated that both GRK2 and GRK5 are able to interact with tubulin dimers and assembled microtubules (Carman, 1998a; Pitcher, 1998b). The interaction has been shown by the co-purification of GRK2 and tubulin from brain tissue and co-localisation studies in cells over-expressing GRK2. The primary tubulin binding determinants have been identified in the catalytic domain of GRK2 (a.a. 185-467) (Carman, 1998a). In addition there appear to be another binding domain in the carboxyl terminal portion of GRK2 (a.a. 467-689) (Haga, 1998). Moreover, both GRK2 and GRK5 were able to stoichiometrically phosphorylate purified tubulin dimers. Whereas the physiological functional consequences of GRK/tubulin interaction are largely unknown, a number of hypotheses have been formulated. They involve GRK regulation of tubulin assembly during processes such as mitosis and cell differentiation or tubulin-mediated regulation of the cellular localisation and/or function of GRKs.

These findings suggest the existence of signalling pathways in which GRK2 may itself act as a signal transducer. In fact in a cellular environment GPCR activation can lead to GRK2 recruitment to the plasma membrane, its allosteric activation promoting GRK2-tubulin complex formation and tubulin phosphorylation.

Synucleins have been recently identified as GRK substrate (Pronin, 2000). Synucleins are 14 kDa proteins that are highly expressed in brain that have been linked to the development of neurodegenerative disorders, such as Alzheimer and Parkinson’s diseases. Both GRK2 and GRK5 phosphorylate synucleins and this phosphorylation may inhibit synuclein function. Although the function of synucleins remains largely
unknown, it has been demonstrated that they inhibit PLD2 \textit{in vitro}. Activation of PLD2 causes rearrangement of actin cytoskeleton and vesicle formation aiding in receptor endocytosis and recycling. GRK-mediated phosphorylation of synucleins inhibits synuclein interaction with PLD2. The findings that GRKs can interact and modulate the functions of cytoskeleton proteins, such as tubulins and synucleins, suggest an important role for GRKs in the regulation of cytoskeletal dynamics.

Another substrate of GRKs is the Na\textsuperscript{+}/H\textsuperscript{+} exchanger regulatory factor (NHERF) (Hall, 1999). This factor is involved in the regulation of cellular homeostasis through regulating intracellular pH and sodium concentration. NHERF contains two PDZ domains, which are involved in protein-protein interaction and is constitutively phosphorylated on a serine residue (Ser 289). It has been demonstrated that NHERF binds to the mouse GRK6A splice variant and in \textit{in vitro} assay is phosphorylated on Ser 289. These results point to GRK6A as the kinase responsible for constitutive NHERF phosphorylation, even if the functional consequences are still undefined.

Other data identify phosducin and phosducin-like proteins as substrates of GRK2 phosphorylation. Phosducin is a soluble phosphoprotein, which plays a role in the modulation of \(\beta\gamma\) signalling. Both phosducin and phosducin-like proteins bind to \(\beta\gamma\) subunits thereby competing for effectors. It was demonstrated that phosducin and phosducin-like proteins inhibit GRK2 activity toward GPCR, probably by competing for free G \(\beta\gamma\) subunits and preventing binding of GRK2 (Hekman, 1994). On the other hand GRK2 is able to produce phosducin phosphorylation with consequent reduction of phosducin affinity for \(\beta\gamma\) subunits (Ruiz-Gómez, 2000). These observations further expand the cellular roles of GRK2 indicating that it can modulate GPCR signalling acting at the level of G protein subunits.
1-3-4 Mechanisms modulating GRK kinase activity

GRKs were initially considered as constitutively active enzymes, but many studies have shown that GRK kinase activity is finely regulated by different mechanisms. A primary route is through posttranslational modifications such as autophosphorylation and lipid modifications.

1-3-4-1 Regulatory role of GRK autophosphorylation

Autophosphorylation has been shown for GRK1, GRK5, GRK6 and GRK7, but a potential autophosphorylation site is present in all four splice variants of GRK4 in the C terminal domain (Fig 1-18). In GRK1 the residues that undergo autophosphorylation are Ser-488 and Thr-489, but also a minor autophosphorylation site has been mapped to Ser-21 (Palczewski, 1992) (Fig 1-15). Autophosphorylation does not affect GRK1 kinase activity but influences GRK1-substrate interaction (Kelleher, 1990; Buczylko, 1991). Autophosphorylated GRK1 has impaired binding with phosphorylated, light-activated rhodopsin, suggesting that this mechanism may have a role in GRK1 dissociation from its substrate following receptor phosphorylation. Moreover studies with autophosphorylation-deficient mutants indicate that this process may regulate the binding of ATP to GRK1 (Palczewski, 1995).

GRK5 can undergo autophosphorylation on different residues, suggesting a different functional consequence (Fig 1-16). Lipids can nonspecifically stimulate GRK5 autophosphorylation on Ser-484 and Thr-485. A GRK5 mutant, in which these residues are mutated into alanine, does not present affected kinase activity or impaired binding to phospholipids, however its ability to phosphorylate GPCRs is profoundly impaired. This suggest that the autophosphorylation of these residues enable GRK5 recognition and binding to activated GPCRs, probably participating in membrane localisation of this enzyme (Kunapuli, 1994a).
Fig 1-18. General domain organisation of the GRKs

Schematic diagram of a GRK, with a central kinase catalytic domain. The amino terminal region is thought to be important for recognition of activated receptors substrates, whereas the carboxyl terminal region contains the major autophosphorylation sites and GRK specific membrane targeting lipid modifications or membrane protein-binding domains. The sequences of the autophosphorylated regions of the seven known GRKs are shown, with the potential serine and threonine sites in bold and the experimentally determined autophosphorylation sites underlined. Conserved amino acids are indicated by asterisks.
On the other hand it has been demonstrated that Ca\(^{2+}\)-calmodulin can promote GRK5 autophosphorylation on residues different from Ser-484 and Thr-485 and this time the autophosphorylation process impairs rather than facilitates GRK5-GPCR interaction (Pronin, 1997a).

The GRK5 autophosphorylation sites are conserved in all three splice variants of GRK6 sequence, but it appears that GRK6 autophosphorylation is ~10 fold less efficient when compared with GRK5 and this process is not increased in presence of phospholipids. This could mean that this process is not important in the physiology of GRK6 function or that other cellular components or chemical agents are necessary to fully activate the kinase (Loudon, 1994).

GRK7 possesses a Thr residue in position 21 equivalent to the minor autophosphorylation site at Ser-21 in the GRK1 sequence. A serine is also present at position 485 that is conserved among the other members and that could be a potential autophosphorylation site. Results obtained with a histidine-tagged GRK7 construct indicate that GRK7 is also capable of autophosphorylation (Weiss, 1998).

1-3-4-2 Posttranslational lipid modifications of GRKs

Many studies have shown that GPCR phosphorylation by some members of the GRK family requires membrane localisation of GRKs. Multiple factors are likely to affect GRKs membrane association. In particular prenylation has been demonstrated to be required for translocation of GRK1 to the membrane (Lorenz, 1991). GRK1 amino acid sequence terminates with a CAAX motif that directs farnesylation and carboxymethylation of this protein. Farnesylated rhodopsin kinase has a cytosolic localisation in the absence of rhodopsin activation, but it is membrane-bound when rhodopsin is activated by light. A GRK1 non-farnesylated mutant is unable to translocate to the membrane and presents reduced ability to phosphorylate rhodopsin. However it is still
unknown how farnesyl group on GRK1 could direct membrane localisation in a light-dependent manner (Inglese, 1992). GRK4 and GRK6A are posttranslationally modified by palmitoylation at their carboxyl termini (Stoffel, 1994; Premont, 1996) (Fig 1-16). The palmitoylation reaction is a reversible process leading to the acylation of cysteine residues with a palmitic acid. Palmitoylation is found only on the membrane-bound fraction of these kinases indicating a potential role of this modification in the direction of GRKs membrane localisation. It remains to be discovered how palmitoylation, and consequent sub-cellular localisation of these GRKs, can be regulated upon GPCR activation.

1-3-4-3 Regulation of GRKs activity by lipids
Several studies have demonstrated the regulation of GRKs by charged phospholipids. Initially the data were conflicting, likely resulting from different experimental approaches. Now different laboratories agree on the regulatory role of lipids on GRKs activity. GRK2 and GRK3 are considered to be phospholipid-dependent kinases (DebBurman, 1996). PIP2 is potent activator of GRK2, even if in in vitro assays PIP2 shows biphasic regulation of the kinase, activating at low micro molar concentration and inhibiting at high micro molar concentration. However high concentrations of PIP2 are not considered as physiological. PIP2 directly interacts with GRK2 and GRK3 via their pleckstrin homology domains. A recent mutational analysis has identified specific GRK2 binding determinants for phospholipids binding in two charged amino acids located in these PH domains, Lys-567 and Arg-578 (Carman, 2000) (Fig 1-15). The functional consequence of this binding is the promotion of GRKs membrane association and thus the localisation of the GRK to its substrate (Pitcher, 1996). Other lipids have been shown to regulate GRK2 activity.
In particular phosphatydilserine binds to GRK2, on a carboxyl terminal domain distinct from the PH domain, and activates GRK2. Even if phosphatydilserine has a lower affinity for GRK2 compared with PIP2, it is able to induce a conformational change in the kinase directly activating its catalytic activity. In fact phosphatydilserine, unlike PIP2, increases GRK2-mediated phosphorylation of soluble peptides (Onorato, 1995; DebBurman, 1996).

GRK5 activity is also modulated upon lipid binding. In particular two distinct binding sites have been identified in GRK5. One is located on the carboxyl terminal 100 amino acids and does not present ligand selectivity among different lipids. Lipids binding to this domain produce increased GRK5 autophosphorylation and indirectly increase receptor kinase activity (Kunapuli, 1994a). The other binding site is in the amino terminus and it shows a high degree of selectivity toward PIP2. GRK5 domain involved in PIP2 interaction is a region of basic amino acids located in the N-terminus and mutational analysis of residues 22, 23, 24, 25, 26, 28, 29 ablates GRK5 ability to interact with PIP2 (Pitcher, 1996). This domain is conserved in GRK6 and in two GRK4 splice variants that possess the 32 amino acid N-terminus insert (GRK4α and β). The PIP2 binding does not affect GRK5 autophosphorylation activity. So in this case the functional consequence of PIP2 binding is an enhancement of GRK5 phosphorylation of receptors as a consequence of its facilitated membrane association. In fact GRK5-mediated phosphorylation of soluble peptides remains unaffected by this lipid binding, indicating that PIP2 does not increase the intrinsic kinase activity of GRK5. For all the members of the GRK4 subfamily PIP2-dependent receptor kinase activity has been demonstrated.
**1-3-4-4 Regulation of GRKs activity βγ subunits of G proteins**

A regulatory role for GRK2 and GRK3 receptor kinase activity has been attributed to the βγ subunits of G proteins. The selective modulation of these two kinases among all the known GRKs is due to the peculiarity of their C terminal tail. Both GRK2 and GRK3 possess within their carboxyl termini a pleckstrin homology domain (GRK2 residues 540-655). This protein module of approximately 100 amino acids acts as mediator of protein-protein and protein-membrane interactions. Moreover, extensive studies on Gβγ binding domain containing proteins indicate that Gβγ binding may be a common property of proteins with a PH domain flanked by appropriate sequence determinants (Touhara, 1994). Accordingly an initial Gβγ-binding site was identified in a 125 amino acid region (residues 546-670) on GRK2 sequence (Koch, 1993). Successive studies showed that a short 28 amino acid peptide (GRK2 residues 643-670), including only a C-terminal portion of GRK2 PH domain and 19 amino acids of the carboxyl flanking region, was able to bind to Gβγ (Touhara, 1994). Recently the solution structure of the GRK2 PH domain has been determined by high resolution NMR and the principal Gβγ binding determinants have been identified as Arg-587, Arg-663, Lys-665 (Fushman, 1998). The comparison of GRK2 PH domain with PH domains of other proteins showed a significant difference in topology, most notably an extension of the C terminal alpha-helix of GRK2 PH domain. The positively charged residues of this alpha-helix produce a polar surface that may interact with a highly negatively charged area on Gβγ subunits. Successive mutational analysis identified a single amino acid as a critical residue for Gβγ binding in GRK2 sequence. GRK2 mutant R587Q failed to bind Gβγ (Carman, 2000). GRK2 binding to Gβγ subunits produces the functional consequence of translocating the kinase to the membrane because Gβγ subunits are membrane associated proteins as a
consequence of γ subunit isoprenylation (Simonds, 1991). The interaction with Gβγ increases the ability of GRK2 to phosphorylate receptors (Kim, 1993; Haga, 1994; Kameyama, 1994). This activation does not directly involve a change in GRK2 catalytic activity, because GRK2-mediated peptide phosphorylation is not significantly affected by Gβγ binding, but is consequent to GRK2 membrane localisation (Pitcher, 1992). In any case Gβγ action is more specific compared with PIP2-mediated membrane translocation of these kinases, because free Gβγ subunits are produced in close proximity of the activated GPCRs and so Gβγ binding specifically targets GRK2 to its activated receptor substrates, which induce allosteric activation of the kinase. Further investigations have shown that Gβγ are unable to stimulate GRK activity in the absence of charged phospholipids, indicating that they are conditional activators (DebBurman, 1996). Ablating Gβγ binding with GRK2-R587Q mutant also abolishes agonist-dependent receptor phosphorylation. This indicates that Gβγ binding is necessary if not sufficient for GRK2-dependent receptor phosphorylation in cells (Carman, 2000). A further confirmation of this hypothesis came from studies with permeabilised olfactory cilia. When these cells were treated with a synthetic peptide, containing the carboxyl-terminal 222 amino acid residues of GRK3 and including the beta gamma binding site, both membrane translocation of GRK3 and odorant receptor desensitisation were impaired. In this olfactory cilia system the disruption of Gβγ binding is sufficient to block efficient GRK3-mediated receptor phosphorylation (Boekhoff, 1994).

Although both GRK2 and GRK3 possess determinants for Gβγ binding the presence of variable domains within the Gβγ binding site of these kinase suggest the existence of specificity for different Gβγ isoforms. Actually 6 Gβ subunits and 12 Gγ subunits are known (Ray, 1995; Watson, 1996). The search for specificity in Gβγ binding was performed
both with in vitro binding assays with purified Gβ, Gγ subunits and recombinant C terminal domains of GRK2 and GRK3 and with cellular studies in which GRK/Gβγ binding was followed with the co-immunoprecipitation approach following activation of different GPCRs. It is known that different receptors couple to specific Gβγ combinations (Diverse-Pierluissi, 1996; Daaka, 1997). A differential binding of specific Gβγ isoforms to GRK2 and GRK3 has been shown suggesting that it could be of primary importance in determining receptor specificity of these enzymes.

1-3-4-5 Regulation of GRKs by protein kinase C
Both GRK2 and GRK5 have been shown to undergo PKC phosphorylation. Purified PKC phosphorylates, albeit somewhat slowly, recombinant GRK2 with a stoichiometry of ~0.9 moles Pi/mole GRK2 and this process increased GRK2 activity toward receptors. This activation is analogous to the activation of phospholipids and βγ subunits of G proteins. In fact PKC phosphorylation does not produce increased GRK2 kinase activity toward soluble peptide substrates, suggesting that phosphorylation favours GRK2 membrane translocation and indirectly activates GRK2 activity toward membrane-bound receptors (Winstel, 1996). Recent detailed studies showed that GRK2 is preferentially phosphorylated by PKC isoforms α, γ, and δ in vitro. Two-dimensional peptide mapping of PKCα-phosphorylated GRK2 showed a single site of phosphorylation, identified as serine 29. A GRK2-S29A mutant is not phosphorylated by PKC in in vitro assays or in phorbol ester-stimulated HEK 293 cells (Krasel, 2001). This phosphorylation and its functional effects on GRK2 kinase activity have been demonstrated in intact cell studies. Direct activation of PKC in HEK 293 cells transiently expressing GRK2 produced activation of cytosolic GRK2 and translocation of GRK2 immunoreactivity from the cytosol to
the membrane fraction (Winstel, 1996). Moreover, the functional significance of PKC-dependent increase in GRK activity was demonstrated with experiments of adrenergic receptor homologous desensitisation in mononuclear leukocytes. Receptor desensitisation was significantly increased in PMA-pre-treated cells and this increase was specifically blocked by a GRK inhibitor, heparin. These results indicate that GRK2 can be preconditioned, to modulate the subsequent cellular responsiveness to receptor activation, by PKC phosphorylation (Chuang, 1995).

Regulation of GRK5 by PKC has also been demonstrated (Pronin, 1997b). Both in vitro assays and intact cell studies with PKC activators demonstrate that PKC rapidly and stoichiometrically (2 moles Pi/mole GRK) phosphorylates GRK5. The site of phosphorylation resides in the C terminal 26 amino acids. The functional consequence of this phosphorylation is reduced intrinsic kinase activity of GRK5, showed by reduced ability to phosphorylate soluble substrates and the rhodopsin receptor. The last phenomenon is in part due to reduced ability of phosphorylated GRK5 to bind to rhodopsin-containing membranes, while GRK5 affinity with phospholipids remains unaffected by PKC phosphorylation (Pronin, 1997b).

1-3-4-6 Regulation of GRKs by Ca\(^{2+}\) binding proteins

The activity of GRKs appears to be inhibited by intracellular Ca\(^{2+}\) by direct interaction of GRKs with calcium-binding proteins. The calcium-binding proteins that share a common Ca\(^{2+}\)-binding motif (the EF hand) and participate in several biochemical reactions are often called calcium sensor proteins (CSP). The EF motif consists of two perpendicularly placed \(\alpha\)-helices and one inter helical loop, which together form a single Ca\(^{2+}\)-binding site. Ca\(^{2+}\) binds to these CSP and induces conformational changes that enable these proteins to interact
with a variety of targets (Ikura, 1996).

GRK1 was the first member of the GRK family for which modulation by a CSP has been shown. Recoverin has been demonstrated to specifically inhibit GRK1 activity (Kawamura, 1993). Recoverin is a 23 kDa myristoylated CSP expressed predominantly in vertebrate photoreceptors (Korf, 1992). Direct interaction between GRK1 and recoverin was demonstrated and upon recoverin binding GRK1 catalytic activity becomes Ca\(^{2+}\) sensitive. In the presence of recoverin, high Ca\(^{2+}\) concentrations produce inhibition of GRK1 catalytic activity as tested in phosphorylation assays with urea stripped rod outer segment membranes. The inhibitory effect of recoverin is enhanced by its covalently attached myristoyl residue. Recoverin neither interacts with other members of the GRKs family nor inhibits their kinase activity. The specific and calcium-dependent recoverin-GRK1 interaction is necessary for the inhibitory effect of recoverin on rhodopsin phosphorylation and has an important role in photoreceptor light adaptation (Kawamura, 1993). The molecular mechanism was subsequently elucidated (Klenchin, 1995; Chen, 1995a).

Photoactivation of rhodopsin stimulates cGMP hydrolysis. The resulting loss of intracellular cGMP slows the influx of Ca\(^{2+}\), through cGMP-dependent cation channels, while the Ca\(^{2+}\) efflux, regulated by Na\(^{+}/K^{+}\), Ca\(^{2+}\) exchangers, remains unaffected. Under these conditions a general decrease in intracellular Ca\(^{2+}\) concentration is observed (from 550 to 50 nM). This light-induced lowering of intracellular Ca\(^{2+}\) concentration is decoded by a certain number of calcium-binding proteins that re-establish cellular homeostasis. In these low Ca\(^{2+}\) concentration conditions GRK1 is released from recoverin and phosphorylation and inactivation of photoactivated rhodopsin can be observed (Kawamura, 1993). In absence of light activation and with high intracellular Ca\(^{2+}\) concentrations GRK1 would be predicted to be complexed to recoverin,
membrane associated and inactive. In this condition rhodopsin phosphorylation was inhibited (Pitcher, 1998a). This functional model could be an oversimplification of the physiology of vision since there are a few reports that do not fit with the proposed model. Knockout mice for the recoverin gene, show no major effects in their response to photon absorption (Chen, 1995b). However, the removal of recoverin could result in an unpredictable compensation by other components of the phototransduction cascade. Since recoverin inhibits the phosphorylation of dark-adapted rhodopsin better than that of bleached rhodopsin it was proposed that recoverin-dependent regulation of GRK1 plays a role in preventing the enzyme from unwanted phosphorylation of dark-adapted rhodopsin (this phenomenon is known as "high-gain" phosphorylation) (Senin, 1997a; Senin, 1997b).

Following the evidence that GRK1 is regulated by recoverin other studies were performed to test whether this was a general phenomenon with respect to other CSP and GRK subtypes (Table 1-4). Initial studies were performed on CaM, which is an acidic protein that is considered the primary "decoder" of Ca\textsuperscript{2+} information in the cell (James, 1995; Rhoads, 1997). Different laboratories found that GRK2 and GRK3 were inhibited by CaM in a calcium-dependent manner (Chuang, 1996b; Haga, 1997a; Pronin, 1997a; Pronin, 1998). The IC\textsubscript{50} was 2 \textmu M. Since G\textbeta\gamma is a binding target of Ca\textsuperscript{2+}/CaM and the binding of GRK2 and 3 to G\textbeta\gamma is critical for the activation of these two GRK-subtypes, it was proposed that Ca\textsuperscript{2+}/CaM inhibitory action was consequent to G\textbeta\gamma sequestration. However, CaM was able to inhibit GRK2 kinase activity even in the absence of G\textbeta\gamma, indicating that the effect of CaM is not due to subtraction of G\textbeta\gamma from GRK2. The mechanism of this inhibition likely involves the direct interaction between CaM and GRK2. Two distinct CaM-binding sites located within the N- and C-terminal regions
of GRK2 were identified by performing surface plasmon resonance (SPR) analysis of GRK2-CaM interaction (Levay, 1998). The two sites are located within residues 1-88 and 593-689 of GRK2. The site of GRK2 phosphorylation by PKC was identified as serine 29, which is located in the calmodulin-binding region of this kinase. When GRK2 is phosphorylated in vitro by PKC it is not inhibited by calmodulin. These results have suggested the formulation of the following hypothesis. GRK2 is basely inhibited by calmodulin binding, but this inhibition can be abolished upon PKC phosphorylation of the kinase (Krasel, 2001).

Further studies demonstrated that among the GRK subtypes CaM interacts most potently with GRK5 (Chuang, 1996b; Haga, 1997a; Pronin, 1997a; Pronin, 1998). The IC₅₀ was 40-50 nM, indicating that CaM is approximately 50 fold more potent in inhibiting GRK5 kinase activity compared to GRK2 and 3. The other two members of this GRK subfamily, GRK4 and GRK6, were also strongly inhibited by CaM. The inhibitory effect of CaM on GRK4α (Sallese, 1997) and GRK6 (Pronin, 1997a) was ~3 fold lower than that on GRK5 and ~30 fold more potent than that on GRK2. For GRK4 the calculated IC₅₀ was 80 nM. Intriguingly, three other splice variants of GRK4 (β, γ and δ) are not inhibited by calmodulin (Sallese, 1997).

The high affinity of CaM for GRK5 suggests that this interaction can be physiologically relevant. This interaction has been extensively characterised. A direct interaction between GRK5 and Ca²⁺/CaM was demonstrated using CaM-conjugated sepharose 4B and confirmed using surface plasmon resonance (SPR) technology on a BIAcore instrument. The two CaM binding sites on GRK5 are located within residues 20-39 in the N-terminus and 540-578 in the C terminus (Levay, 1998). CaM binding does not influence the catalytic activity of GRK5, as demonstrated by the lack of inhibitory effect on GRK5 phosphorylating activity on the soluble substrate casein. Instead,
Ca\(^{2+}\)/CaM significantly reduced kinase binding to the membrane and to phospholipid vesicles. It was suggested that CaM can directly compete for both the phospholipid and receptor binding sites of GRK5. On GRK5 the PIP2-binding site coincides with the calcium/calmodulin binding site, suggesting that binding is mutually exclusive.

The following model of GRK5 regulation by CaM has been proposed. At resting Ca\(^{2+}\) concentrations GRK5 is active and able to phosphorylate agonist-occupied receptors. When a cell is stimulated and intracellular Ca\(^{2+}\) levels rise, CaM binds to GRK5 and inhibits directly receptor phosphorylation. Moreover, CaM also stimulates GRK5 autophosphorylation and so the kinase remains inhibited even when Ca\(^{2+}\) levels go down and CaM dissociates from the enzyme. Presumably, GRK5 will eventually be dephosphorylated and return to its basal level of activity. Thus, CaM-stimulated autophosphorylation may prolong the inhibitory effect of a transient increase of intracellular Ca\(^{2+}\) levels on GRK5. These studies suggest that CSP mediate the regulation of different GRK subtypes by Ca\(^{2+}\). Preliminary studies also indicate that S100 protein is able to inhibit GRK2 in a calcium-dependent manner (Haga, 1997b), suggesting that several classes of EF-hand CSP can regulate GRK activity. This mechanism is however highly selective with respect to the different CSP and GRK subtypes.

Recoverin inhibited GRK1 phosphorylating activity but in parallel experiments did not interact with GRK2 and was ineffective on ROS phosphorylation by this kinase subtype. Several other recoverin analogues, including NCS 1, VILIP 1 and hippocalcin, are also able to inhibit GRK1 but they do not inhibit GRK5 kinase activity (Tab 1-4). An analysis of VILIP in olfactory neurones indicated that in these cells VILIP does not interfere with GRK2 (Boekhoff, 1997). Taken together these data indicate that recoverin and other NCS can selectively inhibit GRK1 and do not affect other GRK subtypes. By contrast CaM strongly
inhibits GRK4 subfamily members while it has little (GRK2 and 3) or no (GRK1) effect on the other GRKs. In addition, in a recent study the group of Palczewsky identified 5 new calcium binding proteins named CABP that inhibit GRK5-mediated phosphorylation of rhodopsin with different affinities but did not affect GRK2 activity (Haeseleer, 2000).

GRK2, GRK3 and GRK5 regulate the vast majority of GPCRs and some of those, such as substance P, angiotensin II, and 5-HT2 receptors are coupled to fluctuations in intracellular Ca\(^{2+}\). For this class of GPCRs CaM can provide a feedback mechanism to modify homologous desensitisation. Moreover, due to the lack of receptor specificity different GRKs can potentially regulate the same receptor and CSP could work addressing which kinase will desensitise the activated receptor.

Table 1-4

**Selective inhibition of GRK subtypes by CSP**

<table>
<thead>
<tr>
<th>Recoverin</th>
<th>VILIP</th>
<th>NCS-1</th>
<th>CaM</th>
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<td>GRK1</td>
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CSP were ineffective (-) or able to inhibit the indicated GRK subtypes with different degree of potency (+). nd, not determined; (*), unpublished data from our laboratory.
1-3-5 GRKs sub-cellular localisation

The membrane localisation of these enzymes is a prerequisite for their kinase activity and this process is subject to complex regulatory mechanisms. Moreover GRK receptor phosphorylation requires membrane localisation. Active GPCRs can directly associate with GRKs in the presence of phospholipids. It is possible that the active GPCR directly recruits GRKs acting as an agonist-dependent membrane anchor. Inside the GRK family GRK1, GRK2 and GRK3 are present in the cytosol in resting cells, while GRK4, GRK5 and GRK6 are located in the particulate fraction that contains GPCRs.

Upon rhodopsin stimulation GRK1 translocates to the membrane and this process is dependent upon GRK1 farnesylation. While GRK7 (gs) has a high identity with human GRK1, differences in the isoprenylation sequence have been shown. In the GRK7 carboxyl terminal the sequence CVLL promotes geranylgeranylation instead of farnesylation, resulting in constitutive association with the plasma membrane. Therefore GRK7 might be more tightly associated with the membrane than GRK1 (Weiss, 1998).

GRK2 was initially described as a cytosolic enzyme, but now it is generally accepted that several GRK2 pools exist inside the cell: cytosolic, membrane-bound and microsomal membrane bound. A significant amount of GRK2 is associated with internal microsomal membranes by means of electrostatic interactions between an N-terminal region of GRK2 (residues 88-145) and an unknown protein that is an integral component of the microsomal membrane. GRK2 binding to this unidentified anchoring protein leads to kinase inactivation. The role of microsomal-associated GRK2 still remains unclear even if it has been proposed as a reservoir for GRK2 translocation to the plasma membrane upon receptor activation, or it may reflect other cellular functions of GRK2 (Garcia-Higuera, 1994;
Murga, 1996). In this context a direct association of GRK2 with both the microtubule and actin cytoskeleton has been demonstrated (Carman, 1998a; Haga, 1998). Also GRK2 binds to caveolin, which may serve as a membrane localised GRK2 inhibitor.

On the other hand GRK2 membrane translocation, necessary for GRK phosphorylation of receptor substrates, requires the simultaneous binding of two PH domain ligands, G protein βγ subunits and PIP2 (Carman, 2000).

GRK4 and GRK6A are both palmitoylated and this posttranslational modification makes possible their membrane localisation. Therefore, the GRK6B and GRK6C variants, which are predicted to lack palmitoylation, would require some other mechanism for membrane localisation. Interestingly the GRK6B variant carboxyl-terminal sequence contains a consensus PKA site that might allow phosphorylation-regulated association of the GRK6B protein with the membrane (Premont, 1999). Also GRK5 does not have lipid modifications and for this kinase autophosphorylation has been proposed to participate in membrane binding. Moreover GRK4 subfamily members possess amino and carboxyl termini regions rich in basic and polar amino acids, suggesting that electrostatic interactions with the negative charged phospholipids of the plasma membrane could help these kinases interact with the membrane. In particular PIP2 binding has a primary role. The only exception is the GRK6C variant that possesses a truncated carboxyl-terminal domain lacking the basic and proline rich domains.

1-4 GTPase activating proteins

GTP hydrolysis is a key regulator of intracellular signal transduction (Scheffzek 1998). Numerous vital processes, including protein
synthesis, visual perception, vesicular and nucleocytoplasmic transport, protein targeting, growth control and differentiation, are controlled by GTP-binding proteins. Heterotrimeric G proteins and small GTP-binding proteins function as molecular switches that cycle between GTP-bound (active) and GDP-bound (inactive) states. Upon activation exchange of the bound GDP with GTP is promoted and this induces conformational changes that allow the GTP-binding proteins to interact with effector molecules. Hydrolysis of bound GTP is the timing mechanism that returns these proteins to their GDP-bound inactive state and thereby completes the GTPase cycle (Bourne, 1991).

The intrinsic rate of GTP-hydrolysis by GTP-binding proteins is very slow but can be accelerated by orders of magnitude upon interaction with GTPase-activating proteins (GAPs) (Gideon, 1992). GAPs are primarily down regulators of GTPase signalling, but some are also effectors of signal transduction.

The importance of GTPase regulation is evident from diseases associated with mutations in either GTP-binding proteins themselves or GAPs: mutant Ras proteins that lack GTPase activity are oncoproteins (Lowy, 1996); and loss of GAP function (as a consequence of disruption or mutation of the presumed tumour-suppressor gene) is responsible for type 1 neurofibromatosis (Gutmann, 1993).

1-4-1 Discovery of the RGS family

Genetic studies of yeast, C. elegans, and Aspergillus nidulans have been used to identify proteins that function to inhibit G-protein signalling. Members of the protein family identified have been named RGS (Regulators of G protein Signalling) proteins, and the region of sequence similarity shared by these proteins is called the RGS domain (Koelle, 1996) (Tab 1-5).
### Table 1-5

**RGS proteins: their Gα target and tissue distribution**

<table>
<thead>
<tr>
<th>RGS</th>
<th>Target Gα&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Tissue expression</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGS-GAI</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;, Gα&lt;sub&gt;j&lt;/sub&gt;, Gα&lt;sub&gt;k&lt;/sub&gt; &gt; Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Ubiquitous, low in brain</td>
<td>De Vries, 1995; Berman, 1996; Wang, 1998</td>
</tr>
<tr>
<td>RGSZ1</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt; &gt; Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Brain</td>
<td>Wang, 1998</td>
</tr>
<tr>
<td>RET-RGS1</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;, Gα&lt;sub&gt;j&lt;/sub&gt;, Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Retina</td>
<td>Wang, 1998; Faurobert, 1997</td>
</tr>
<tr>
<td>RGS1</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>B-lymphocytes, lung</td>
<td>Druey, 1996; Hong, 1993</td>
</tr>
<tr>
<td>RGS2</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Ubiquitous</td>
<td>Druey, 1996; Siderovski, 1994; Hezimer, 1997</td>
</tr>
<tr>
<td>RGS3</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt; &gt; Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Ubiquitous</td>
<td>Druey, 1996; Gold, 1997</td>
</tr>
<tr>
<td>RGS4</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt; &gt; Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Brain, heart</td>
<td>Druey, 1996; Berman, 1996; Gold, 1997</td>
</tr>
<tr>
<td>RGS5</td>
<td>ND</td>
<td>Ubiquitous</td>
<td>Gold, 1997; Seki, 1998</td>
</tr>
<tr>
<td>RGS6</td>
<td>ND</td>
<td>Brain</td>
<td>Gold, 1997</td>
</tr>
<tr>
<td>RGS7</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;, Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Brain</td>
<td>Gold, 1997; Shuey, 1998</td>
</tr>
<tr>
<td>RGS8</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;, Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Brain</td>
<td>Saitoh, 1997</td>
</tr>
<tr>
<td>RGS9</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Retina (cones &gt; rods), neurones</td>
<td>He, 1998</td>
</tr>
<tr>
<td>RGS10</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Brain</td>
<td>Hunt, 1996</td>
</tr>
<tr>
<td>RGS11</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Brain</td>
<td>Gold, 1997</td>
</tr>
<tr>
<td>RGS12</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;, Gα&lt;sub&gt;12/13&lt;/sub&gt;</td>
<td>Lung, brain, spleen, testis</td>
<td>Snow, 1997</td>
</tr>
<tr>
<td>RGS13</td>
<td>ND</td>
<td>Lung</td>
<td>GB</td>
</tr>
<tr>
<td>RGS14</td>
<td>ND</td>
<td>Brain, spleen, Lung</td>
<td>Snow, 1997</td>
</tr>
<tr>
<td>RGS16&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Gα&lt;sub&gt;i&lt;/sub&gt;</td>
<td>Retina, pituitary, liver, ubiquitous?</td>
<td>Chen, 1997; Degtaryev, 1994; Buckbinder, 1997</td>
</tr>
<tr>
<td>Axin/Conductin</td>
<td>ND</td>
<td>Thymus, testis &gt; ubiquitous</td>
<td>Zeng, 1997; Behrens, 1998</td>
</tr>
<tr>
<td>D-AKAP2</td>
<td>ND</td>
<td>Testis &gt; ubiquitous</td>
<td>Huang, 1997</td>
</tr>
<tr>
<td>p115RhoGEF</td>
<td>Gα&lt;sub&gt;13&lt;/sub&gt; &gt; Gα&lt;sub&gt;12&lt;/sub&gt;</td>
<td>Ubiquitous, high in leukocytes</td>
<td>Kozasa, 1998; Hart, 1998</td>
</tr>
</tbody>
</table>

<sup>a</sup>Compiled from data published or submitted to GenBank; for some RGS proteins very little information is available. ND not determined, GB data from GenBank; * data combined from RGS-r, RGS16 and A28-RGS14, which have the same protein sequence (Snow, 1998) but show differences in distribution that still remain to be clarified.

<sup>b</sup>Note that these assignments of target Gα are mostly based on in vitro analyses that are in many cases not comprehensive. There is also no guarantee that an interaction identified in vitro is physiologically relevant. These assignments of target Gα should therefore be considered tentative pending further experimental confirmation, including intracellular localisation and tissue-specific expression.
The RGS domain was also discovered independently of the genetic experiments described above when a human RGS protein, named GAIP (G α interacting protein), was identified in a yeast two-hybrid screen using the G protein Gαi3 as probe (De Vries, 1995). GAIP was shown to bind Gαi3 in vitro, and a fragment of GAIP containing only the RGS domain proved sufficient to interact with Gαi3.

The sequence similarities among the initially identified RGS proteins have allowed many additional proteins containing RGS domains to be identified (Koelle, 1996), (Druey, 1996), searching (Druey, 1996; Koelle, 1996; Siderovski, 1996).

To date, at least 24 different RGS proteins have been identified in mammalian cells.

1-4-2 Mammalian RGS subfamilies

Given the divergence of RGS proteins and the scarcity of information on their functions, it was thought that a phylogenetic analysis might reveal the presence of subfamilies within RGS proteins and explain the relationship between RGS proteins. (Zheng, 1999).

From this analysis the mammalian RGS protein family was divided at least in six distinct subfamilies: (1) RGS-GAIP, Ret-RGS1 and RGSZ1; (2) RGS1, RGS2, RGS3, RGS4, RGS5, RGS8, RGS13 and RGS16 (also called RGS-r or A28-RGS14p); (3) RGS6, RGS7, RGS9 and RGS11; (4) RGS12 and RGS14 (also called rap1/rap2 interacting protein); (5) Axin and Conductin (also called Axin2 or Axil); and (6) p115-RhoGEF, PDZ-RhoGEF and Lsc.

Two RGS proteins, RGS10 and D-AKAP2, were not placed in any of the above subfamilies because of their distinct structural features. It is possible that each of them could group with unknown mammalian RGS proteins and form additional subfamilies.
1-4-3 Structure of RGS proteins

Some mammalian RGS proteins possess little more than the RGS domain (e.g., RGS1, RGS2, RGS4, and RGS8); however, other RGS are much larger due to the possession of lengthy N- and C-terminal extensions (e.g., RGS3, RGS9L, RGS12, and RGS14) (Tab 1-6).

The N- and C-termini of these RGS proteins share little sequence homology, suggesting that these "extensions" have unique functions. These extensions contain domains and motifs necessary for important aspects of RGS biology, such as protein translocation, membrane anchoring, and sub-cellular targeting. In other cases, these extensions are involved in selectively regulating the association of RGS proteins with protein components of specific signalling pathways and linking small GTPases to G protein activity.

1-4-3-1 RGS domain

All the RGS proteins possess a common ~125 amino acid RGS core domain. Within the RGS domain, these proteins are highly homologous (45-80%). Inside the RGS domain sequence three highly conserved GH (GAIP or GOS homology) subdomains (GH1, GH2, and GH3) can be identified (De Vries 1995; Druey 1996; Koelle 1996). Biochemical studies revealed that the RGS domain alone is sufficient for accelerating GAP activity of Go; however, modular domains and motifs within some RGS protein C- and N-termini enhance the efficacy of the GAP activity of the RGS domain (Chen, 1998; Zeng, 1998). None of the RGS proteins tested so far seems to be a GAP for Gs. Thus, in principle, RGS proteins could modulate most if not all αi-, αo- and α12/13-mediated signalling pathways.
Table 1-6

Domains/motifs within RGS proteins.

<table>
<thead>
<tr>
<th>Domain /motif</th>
<th>Biological role</th>
<th>Role in RGS biology</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGL</td>
<td>The GGL domain is present in a subfamily of RGS proteins; confers select binding to Gβ5 subunits</td>
<td>All RGS-GGL examined are specific GAPs for Go0; Gβ5 enhances the GAP activity of the RGS domain</td>
<td>Lindorfer, 1998; Snow, 1998b; Kovoor, 2000</td>
</tr>
<tr>
<td>PPr</td>
<td>Binding to proteins with SH-3, WW, or EVH-1 domains; binding induces recruitment during transcription, signalling, and cytoskeletal rearrangements</td>
<td>Unknown</td>
<td>Yu, 1994; Granneman, 1998; Zhang, 1999</td>
</tr>
<tr>
<td>PDZ</td>
<td>Assembly of signalling proteins into multimeric complexes at submembranous sites</td>
<td>Unknown</td>
<td>Snow, 1998a</td>
</tr>
<tr>
<td>PTB</td>
<td>Binds primarily to phosphorylated tyrosine residues on proteins</td>
<td>Unknown</td>
<td>Forman-Kay, 1999</td>
</tr>
<tr>
<td>RBD</td>
<td>Binding sites in Raf-1 kinases for Ras and Rap GTPases</td>
<td>Unknown</td>
<td>Ponting, 1999</td>
</tr>
<tr>
<td>GoLoco</td>
<td>Provides Go1/0 binding and GEF activity toward Go0</td>
<td>Unknown</td>
<td>Luo, 1999; Siderovski, 1999</td>
</tr>
<tr>
<td>PBT</td>
<td>Co-operates with other lipid modifications to target proteins to subcellular membranes</td>
<td>Site of cysteine palmitoylation; enhances the GAP activity of the RGS domain; receptor selectivity</td>
<td>Zeng, 1998; Xu, 1999</td>
</tr>
</tbody>
</table>

DEP, dishevelled, Egl-10, pleckstrin homology domain; Dsh, dishevelled; EVH-1, Enabled, Vasp, Homology 1 domain; GAP, GTPase-activating protein; GEF, guanine-nucleotide exchange factor; GGL, Gγ-like subunit domain; GoLoco, Go1/0 – Loco motif; PBT, polybasic track; PDZ, PSD-95, G-protein signalling; SH-3, Src homology domain-3.
1-4-3-2 DEP domain

RGS6, RGS7, RGS9 and RGS11 have an N-terminal DEP (Dishevelled homology/EGL-10/pleckstrin homology) domains. The DEP domain is approximately 80 residues in length and structural predictions indicate it to be a globular domain. The DEP domain of the Drosophila protein Dishevelled is responsible for its localisation to membranes (Axelrod, 1998). Thus, by analogy, the DEP domain of RGS9 might be responsible for its tight membranes association (Cowan, 1998). So in the absence of additional membrane anchoring motifs, the DEP domain could play a selective role in targeting the mammalian DEP domain-containing RGS proteins to specific sub-cellular membranous sites, perhaps even to specific G protein-coupled signalling pathways.

1-4-3-3 GGL domain

RGS6, RGS7, RGS9 and RGS11 subfamily of RGS proteins contain also a GGL (homology to Gγ) domain located between their DEP and RGS domains. This domain seems to have a role in determining RGS selectivity of action. The GGL domain is similar to the Gγ subunit, suggesting that the GGL domain of this RGS subfamily may interact with Gβ subunits (Snow, 1998a). Indeed, these RGS proteins associate very strongly and with high affinity to Gβ5 through their GGL domains. This association has been demonstrated when these RGS proteins were expressed in heterologous cell lines, or when they were purified from native systems (Snow, 1998a; Levay, 1999; Makino, 1999; Posner, 1999; Snow, 1999).

Moreover, it has been hypothesised that these RGS proteins can selectively inhibit Gβ5-mediated signalling by sequestering the Gβγ subunit. Both these RGS proteins and Gβ5 have the same tissue distribution. The members of this subfamily of RGS proteins are expressed predominantly or exclusively in the brain or in the retina.
(Gold, 1997; Granneman, 1998; He, 1998; Snow, 1998a). Also Gβ5 is expressed nearly exclusively in brain and retina (Watson, 1994). Gβ5 has also unusual characteristics: it dimerises predominantly with γ2 and the Gβ5γ2 selectively activates PLC-β2.

Gilman and colleagues (Posner, 1999) have demonstrated that RGS6 and RGS7 proteins can inhibit Go-mediated signalling by acting as GAPs for Go and sequestering Gβγ subunits. RGS6 and RGS7 attenuate the activation of PLC-β2 and terminate inositol trisphosphate-induced Ca²⁺ release.

1-4-3-4 DH domain

The presence of additional domains in RGS structure sometimes provides links between Ga signalling and other signalling pathways. This is the case of the DH (dbl homology) domain that acts as a GDP–GTP exchange factor (GEF) for the small G proteins.

p115RhoGEF has an RGS domain, that acts as a GAP for Ga13, and a DH domain that acts as GEF for the GTPase RhoA. GEF activity is stimulated when activated Ga13 interacts with the RGS domain of p115RhoGEF (Hart, 1998; Kozasa, 1998). The mechanism of p115RhoGEF action became clear studying the morphological changes induced in endothelial cells by thrombin (Vouret-Craviari, 1998). Thrombin receptors couple to Ga13. The GTPase reaction of Ga13 is accelerated by p115RhoGEF. This RGS protein not only regulates the signalling of Ga13 but upon Ga13 binding becomes activated. p115RhoGEF successively acts as a Ga13 effector activating RhoA and inducing downstream cytoskeletal changes (Hall, 1998). Here is a completely defined pathway, whereby an extracellular ligand induces intracellular morphological changes, and the pivotal effector molecule of the pathway belongs to the RGS family.
**1-4-3-5 PPr domain**

Other RGS modular domains are important for protein-protein interaction in multimeric complexes for example the PPr (polyproline) domain.

This motif is likely to be necessary for binding to SH-3, WW, or EVH-1 (Enabled Vasp Homology1) domain-containing proteins. SH-3 domains are found in a variety of proteins that require rapid recruitment, such as during the initiation of transcription, transmembrane signalling, and cytoskeletal rearrangements. Polyproline domains are present in the termini of RGS6, RGS7, RGS9 and RGS11.

The C-terminus of RGS9 is alternatively spliced. Alternative splicing produces two forms: RGS9S and RGS9L. RGS9S, localised in retina and pineal, has a short 18 amino acid C-terminus, while RGS9L, localised in striatum, hypothalamus, nucleus accumbens, has a 200 amino acid C-terminus rich in proline and serine residues (PPr domain) (Granneman, 1998; Zhang, 1999).

The RGS9L PPr domain contains six consensus PXXP (where P is proline and X is any amino acid), motifs that are absent from the C-terminus of RGS9S (Yu, 1994). An arginine (R) residue three residues N-terminal to the PXXP motif is often observed (RXXPXXP or class I site) (Alexandropoulos, 1995). Many PXXP-SH-3 interactions are regulated by phosphorylation. In particular, phosphorylation of serine/threonine residues has been demonstrated to inhibit the interactions. These sites of phosphorylation frequently occur immediately preceding the proline (PX[S/T]P) in the PXXP motif. The first two PXXP motifs in the PPr domain of RGS9L occur in tandem and conform to this pattern, suggesting that phosphorylation of these motifs may regulate RGS9L|PXXP]-SH-3 binding.

It has been speculated that RGS9L binding to SH-3 domain proteins could rapidly regulate the sub-cellular distribution of RGS9L, enhance...
the GTPase activity of RGS9L toward Gi/Go, and/or play an important regulatory role in a neurone-specific process, such as synaptic vesicle endocytosis (McPherson, 1999).

1-4-3-6 PDZ domain
The PDZ (PSD95/Dlg/ZO1 homology) domain is important for clustering of signalling molecules. RGS12 is a GAP for Go1 and probably Go12/13 subunits. It is the largest RGS protein described so far, with an N-terminal PDZ domain, a C-terminal serine/proline-rich coiled-coil structure that could interact with cytoskeletal proteins, a PID/PTB domain that suggests interaction with a tyrosine-phosphorylated protein, and a PDZ binding motif (Snow, 1998b; Mao, 1998).

PDZ domains play an important role in organising protein networks on membranes (Craven, 1998). This is accomplished by binding to consensus C-terminal motifs in target proteins. PDZ domains occur as single or, more frequently, as multiple tandemly repeated copies. Multiple PDZ domains are present in proteins that are involved in the clustering of proteins into multimeric receptor- or ion channel-PDZ-protein networks, particularly at the post synaptic density (Burchett, 2000). Although the in vivo targets for the PDZ domain of RGS12 are still unknown, results from yeast two-hybrid and in vitro interactions revealed that the chemokine interleukin-8 receptor B (CXCR-2) can specifically interact with the PDZ domain of RGS12 (Snow, 1998b). Thus, RGS12 might be an important scaffold molecule for components of G-protein-linked chemokine signalling pathways.

1-4-3-7 PTB domain
Due to the heterogeneity of the RGS family, other domains that can be found in RGS proteins structure, do not strictly correlate with G protein-mediated signalling
For example RGS12 contains an N-terminal phosphotyrosine-binding (PTB) domain. Although PTB binding may in some cases be independent of tyrosine phosphorylation, PTB domains bind primarily to phosphorylated tyrosine residues, such as occur on growth factor receptors (Forman-Kay, 1999). Thus, in the latter instance, RGS12 may cross-link G protein and tyrosine kinase-signalling pathways.

1-4-3-8 RBD domain
C-terminal to the RGS domain RGS12, RGS14 contain a tandemly duplicated region homologous to the Ras- and Rap-binding domain (RBD) of Raf-1 kinase. By interacting with Ras or Rap, RGS12 or RGS14 could regulate MAPK signalling in neurones. Also in this case an RGS domain can allow a cross-linking between different pathways.

1-4-3-9 GoLoco domain
RGS12 and RGS14 contain a 17 amino acid region of homology with the Drosophila Loco gene product. The last three amino acids of that region are labelled the Gi/o-Loco or "GoLoco" motif (Siderovski, 1999), since they have been identified in other non-RGS proteins that interact with Gi/o subunits. This domain has been hypothesised to confer Go binding and/or GEF activity toward Go to these RGS proteins. RGS12 and RGS14 also possess a C-terminal coiled-coil (CC) region that may be involved in the self-assembly of these proteins into multimeric complexes, or associating RGS12 with the cytoskeleton (Snow, 1997).

1-4-4 The mechanism of GTPase activation
Although RGS family members are diverse, all these proteins contain an RGS domain that should confer a GTPase activating function. The lifetime of G protein active state is determined by the combination of their slow intrinsic GTPase activity and the action of GTPase-
activating proteins (GAPs) (Gideon, 1992; Lamarche, 1994). GTP hydrolysis causes conformational changes in the G protein that are localised to two distinct regions of the molecule called: switch I and switch II (Gamblin, 1998). The structural responses of the switch regions to the loss of the γ-phosphate have been comprehensively described for the archetypal G protein, Ras, Rap2A, the heterotrimeric G protein α subunit (Coleman, 1994; Lambright, 1994; Sondek, 1994; Raw 1997), and more recently, members of the Rho subfamily of small G proteins (Rac, Cdc42 and RhoA).

Arginine and lysine residues play critical roles in phosphoryl-transfer reactions. Positively charged under physiological conditions, these residues are able to neutralise negative charges that develop on the transferred phosphoryl group (Maegley, 1996).

GAPs specific for the Rho and Ras families of small G proteins actively participate to their catalytic activity. They insert an arginine residue into the active site of the G protein and stabilise the G protein switch regions.

Ga proteins structurally differ from small GTP-binding proteins in that they contain an additional helical domain important for positioning the catalytic arginine residue (Fig 1-2).

G proteins also contain a conserved region known as the P-loop which forms a structural pocket for the β- and γ-phosphate groups of the nucleotide and additionally supplies a ligand (serine or threonine) to the octahedrally co-ordinated magnesium ion (Kjeldgaard, 1996).

A major breakthrough, in the elucidation of the nature of GTPase acceleration for Ga proteins, came from studies using fluorescently labelled guanine nucleotides and aluminium fluoride (AlFx). AlFx was originally found to activate heterotrimeric G proteins in their inactive GDP-bound state (Chabre, 1990). The crystal structure of Ga-AlFx complexes indicates that AlFx was trapped in the γ-phosphate-binding
site, thereby mimicking at least some aspects of the GTP-bound form of Ga. In addition these structures, together with biochemical data, supported the idea that GDP-AlF-4 does not simply act as a GTP analogue but, rather, mimics the transition state in the GTPase reaction (Coleman, 1994; Sondek, 1994).

The crystal structure of a complex between Ga\iri-GDP-AlF-4 and RGS4 (Tesmer, 1997) indicates that the additional 40-fold activation by RGS molecules is most likely achieved through binding to, and stabilisation of, the Ga\iri switch regions.

In crystals of RGS4 with Ga\iri only residues from 5 to 178 of RGS4 (RGS box) are visible in the electron density map. Accordingly the arms of RGS4 are disordered and play no obvious role either in binding Ga\iri or in stabilising the RGS box. There are two binding sites for RGS4 on the surface of Ga\iri. The first is remote from the active site of Ga\iri, the second and clearly functional binding site is formed by residues of switch I, switch II and the P-loop of Ga\iri (Fig 1-2). These Ga\iri residues are intimately associated with the binding and hydrolysis of GTP and interact with the most highly conserved regions of RGS4.

The RGS effects in this binding may be regarded as an allosteric contribution catalysis. Moreover, since the RGS4-Ga\iri interface involves the switch regions of Ga\iri, downstream effectors are not predicted to interact with the RGS4-Ga\iri complex, accordingly RGS4 can act at the level of Ga\iri as an effector antagonist.

Finally comparing the roles of GAPs with those of RGSs there are at least two themes in order to realise efficient GTP hydrolysis: (1) use of arginine residues for stabilising the transition state; (2) stabilisation of the switch regions in order to optimise the orientation of the catalytic machinery in the GTP-binding protein.

In heterotrimeric G proteins the arginine residue, critical for the GTPase reaction, is part of the GTP-binding protein itself and is
supplied in cis with an extra domain. The RGS protein is only required for proper alignment of the entire machinery. For Ras-related GTP-binding proteins, this arginine residue is supplied in trans by the respective GAP.

1-4-5 RGS specificity of action
It seems that RGS proteins have very little specificity in their interaction with G proteins (Tab 1-5).
Subtle differences in sequence between RGS domains, among different RGS proteins, probably dictate specificity for Ga interaction (Tesmer, 1997; Kozasa, 1998). It is also possible however that more detailed kinetic analysis will reveal an ability of RGS proteins to distinguish among members of the Ga family. On the other hand specificity of function would have to rely on different expression patterns or different regulations of the RGS proteins. Indeed, some RGS proteins do have very restricted expression patterns, in specific cells or sub-cellular compartments, and some contain conserved domains that could be targets for regulatory interactions (Druey, 1996; Koelle, 1997).
The discovery of mechanisms modulating the GAP activity of RGS proteins might be helpful in understanding how cells can integrate multiple incoming signals and fine-tune their output, thus modifying an organism behaviour (Wilkie, 2000)

1-4-5-1 RGS tissue distribution
At least one RGS protein is expressed in every organ, and many tissues express multiple RGS proteins (Tab 1-5).
Only a few RGS present specificity in tissue distribution. RGS9S is strictly localised in the retina. There is now compelling evidence that RGS9 is the physiological GAP for transducin, modulating the process of phototransduction in retinal cones (He, 1998).
RGS3, RGS5, GAIP, RGS12, RGS14 and RGSr mRNAs have the broadest expression patterns, but some show significant differences in expression levels from one tissue organ to another (De Vries, 1995; Chen, 1996; Druey, 1996; Snow, 1997). Thus, it will be very difficult to deduce a specific physiological role for a given RGS protein from the present knowledge of their tissue expression.

1.4.5.2 RGS sub-cellular localisation

All the RGS proteins, whose intracellular localisation is known, have both membrane-associated and cytosolic pools. Ga subunits are also known to be both membrane associated and cytosolic. Gas and Ga11 translocate to membranes from a cytosolic pool, with a process that is regulated by palmitoylation and myristoylation (Degtyarev, 1994; Wedegaertner, 1996).

It seems likely that RGS proteins, like Ga subunits, fluctuate between membrane-bound and cytoplasmic pools. This could facilitate their access to the membrane-bound Ga subunits if the localisation of the RGS and the Ga is on the same type of membrane.

Different mechanisms seem to be important for placing RGS proteins near their target G proteins. RET-RGS1 possesses a putative transmembrane domain and is likely an integral membrane protein (Faurobert, 1997). For other RGS proteins covalent lipid modification as palmitoylation (RGS-GAIP and RGS4) and domains essential for electrostatic interaction with membrane lipid (RGS4) have been identified. The existence of scaffolding proteins that assemble RGS protein has also been proposed.

RGS3, RGS4, and RGS16 are predominantly cytoplasmic proteins in mammalian cells. Activation of the Ga subunit by agonist or expression of a constitutively active form of Ga appears to signal translocation of these RGS proteins from the cytoplasm to the plasma membrane.
RGS1 possesses three N-myristoylation motifs within its N-terminus that could confer membrane anchoring (Denecke, 1999). However, N-myristoylation by itself is relatively weak and not sufficient to anchor a protein to the membrane. A second signal, such as palmitoylation or a series of basic amino acids within the N-terminus, is necessary for stable membrane binding.

RET-RGS1, RGSZ1, GAIP, and RGS17 possess a palmitoylation motif: the cysteine (Cys)-"rich" region, labelled cysteine string (De Vries, 1996; Faurobert, 1997; Wang, 1998; Zheng, 1999). Palmitate attachment to Cys residue is a reversible posttranslational modification used for membrane anchoring, trafficking and protein-protein interaction. In pituitary cells membrane-bound GAIP is palmitated and located on clathrin-coated vesicles (CCVs), this suggests a model whereby a GAP is separated from its target G protein, with the two coming in contact at the time of vesicle fusion (De Vries, 1996; De Vries, 1998).

RGS4, RGS5, and RGS16 all possess Cys amino acids (Cys2 and Cys12) embedded within a highly conserved 33 amino acid stretch rich in basic amino acids (PBT). An essential feature of polybasic domain-containing proteins is a positive charge contributed by the basic amino acids (Hancock, 1991). Structure analysis of the PBT of RGS4 and RGS16 indicates that the PBT has the capacity to adopt an amphipathic helix conformation (Srinivasa, 1998; Chen, 1999). Both the positively charged basic amino acids and the hydrophobic residues contribute to RGS membrane binding. The first interacts with the negative charges of the phospholipid head groups while the second intercalates into the membrane hydrophobic interior.

The PTB domain has been suggested to confer receptor selectivity. Wilkie and colleagues (Zeng, 1998) demonstrated that the RGS domain alone of RGS4 retains GTPase activity for the Gαq subunit (Popov,
1997). But the C-terminus and especially the N-terminal PBT, significantly enhance the potency of the RGS domain to attenuate Gαq-activated Ca^{2+} signalling. Removal of the PBT domain of RGS4 also eliminates RGS ability to discriminate between two different Gq-coupled receptors. Similar results were obtained with a RGS4 mutant with the N-terminal 33 a.a. deleted. This deleted mutant resulted in a non-functional protein in in vivo assays, with a loss of membrane localisation.

It was suggested that the PTB domain interacts with the receptor to position the RGS protein between Gq and the effector PI PLCβ. Thus, a particular RGS protein may desensitise specific G protein signalling pathways.

Surprising data have been produced studying protein localisation in COS 7 cells transfected with RGS-GFP constructs. It has been shown that RGS2 and RGS10 accumulate in the nucleus, RGS4 and RGS16 accumulate in the cytoplasm, while RGSz localises in the Golgi complex. None of the RGS studied showed plasma membrane localisation. The molecular determinants for nuclear localisation were identified to the conserved RGS domain, so they seem to be common features of all the members of this family. Cytosolic localisation should be allowed by sequence elements outside the RGS domain that result in either nuclear-cytoplasmic transport or cytoplasmic retention. A similar sequence has been evidenced in the N-terminus of RGS4 and RGS16.

The authors do not address whether nuclear localised RGS could have unrecognised function in nuclear processes or if their localisation, far away from membrane bound G proteins, has only a regulatory importance (Chatterjee, 2000).

1-4-5-3 Modulation of RGS level of expression

As the GTPase promoting activity of the RGS proteins is constitutive,
the regulation of expression of the RGS genes may be an important mean to control RGS activity.

Transcriptional up regulation has been shown for different RGS proteins. No data are available on the stability of the mRNA for RGS proteins and only the promoter regions of RGS4, 3 and 16 have been studied (Siderovsky, 1994; Buckbinder, 1997; Chatterjee, 1997). RGS2 and 3 seem to have importance in the regulation of neuronal signalling playing a role in the desensitisation to psycho stimulatory agents. RGS2 mRNA is induced in the rat striatum after treatment with amphetamine and RGS3 mRNA is induced after induction of neuronal plasticity (Burchett, 1998; Ingi, 1998). The gene encoding RGS16 has a p53 binding site and it is induced by the p53 tumour suppressor and by serum. Over-expression of RGS16 inhibits grow factor receptor-mediated activation of the MAPK signalling pathway (Buckbinder, 1997). RGS 16 can act as a transcriptionally inducible component of a p53-controlled negative feedback mechanism involved in cell proliferation and/or apoptosis (Snow, 1998c).

**1-4-6 RGS modulation of receptors signalling**

Many reports show that RGS proteins markedly alter hormone- and neurotransmitter-stimulated cellular responses in mammalian cells. They are able to modulate adenylyl cyclase activity, inhibit mitogen-activated protein kinases (MAPK) and inositol (1,4,5) triphosphate/Ca\(^{2+}\) signalling, attenuate K\(^+\) conductance in neurones and regulate visual signalling. Although some studies are consistent with RGS GAP activity, this property is not generalisable. A number of RGS proteins have affinity for the inactive conformation (G\(\alpha\)-GDP) and the active (GTP-bound state) of G proteins. In most cases RGS proteins stimulate desensitisation of GPCR, but they can perform broader cellular functions, also as a consequence of the fact that RGS and G\(\beta\gamma\) can not
bind simultaneously to Ga. An example is given by RGS4 regulation of GIRK (G protein-gated inwardly rectifying K⁺) channels. These channels are activated by G protein-coupled receptors and regulate the onset and offset of slow inhibitory post synaptic potentials that control neurone excitability. GIRK channels are activated by Gβγ released by Ga1 or Ga0. RGS proteins do not diminish the activation phase and peak current of GIRK channel activation, but they directly accelerate the inactivation and activation rate. The last process can be explained by RGS acceleration of Ga and Gβγ dissociation or inhibition of their reassociation, resulting in an increased amount of free Gβγ that can activate the channel.

1-4-7 RGS proteins and lymphocytes

GPCRs and their respective G proteins are expressed on lymphocytes, but do not play a major role in lymphocyte activation, which is regulated by Ig receptors and cytokine receptors. However the recognition of the critical importance of the chemokine receptors signalling is stimulating new interest in the involvement of GPCR in immunocompetent cells. Moreover numerous results indicate that G proteins may function to modulate both TCR- and IL2R-stimulated pathways. A possible mechanism whereby Ag or cytokine receptor signalling might interface with heterotrimeric G proteins could involve RGS proteins.

Recent studies demonstrate that RGSs expression is regulated upon lymphocyte activation, while other investigations indicate that chemokine receptor signalling can directly up-regulate RGS mRNA levels.

Lymphoid tissues and cells have been found to express multiple RGS molecules. Two of the first mammalian RGS genes identified, RGS1 and RGS2, were identified as mitogen-induced proteins in lymphocytes.
(Siderovsky, 1994), while RGS3 was isolated by screening a B cell cDNA library with an RGS domain probe.

Many data indicate that RGS proteins may function downstream of lymphoid cell receptors, regulating B and T cell responsiveness to lymphoid chemokines.

Using transient transfection in a mature B cell line, 2PK3, Cyster and coll. (Reif, 2000) demonstrate that RGS1 and the short isoform of RGS3 (RGS3s) are effective inhibitors of the Gαi-dependent chemotactic response toward chemokines (as stromal cell-derived factor1, SDF-1, B lymphocytes chemoattractant, BLC, and EBV-induced molecule 1 ligand chemokine, ELC), whereas RGS2 has a minimal effect on migration to these chemokines.

Functional studies in the B cell line HS Sultan transduced with a retroviral vector encoding RGS1 demonstrated that RGS1 impaired PAF-mediated increases in intracellular Ca^{2+}, SDF-1 induced cell migration and the induction of downstream signalling by a constitutively active form of Gα12.

These data, together with the observation that RGS1 expression is induced in B cells by phorbol ester, surface Ig, cAMP, PAF and TNF-α, suggest that it may modulate the inflammatory response of B cells (Moratz, 2000).

Also monocyte involvement in the inflammatory processes seems to be modulated by RGS1; in fact immunofluorescence analysis identified RGS1 in the plasma membrane of monocytes. In these cells RGS1 desensitises a variety of chemotactic receptors including receptors for N-fMLP, C5a, leukotriene B4 as shown by the determination of the GTPase activity in plasma membranes containing ligand-stimulated G-proteins with or without recombinant RGS1 (Denecke, 1999).

RGS2 was cloned as an immediate early response gene, up-regulated in T lymphocytes after activation (Heximer, 1997a). Its role in vivo was
studied by generating RGS2-deficient mice. RGS2 role in T cell activation is confirmed by the observation that T lymphocytes from these mice presented reduced TCR-stimulated cell proliferation and IL2 production. In vivo this defect translates in an impaired anti-virus response (Olivera-dos-Santos, 2000).

RGS16 has been cloned as an IL2-induced gene from human T cells. In vitro binding assays, using Jurkat cell lysates and recombinant RGS16-His tag, have demonstrated that RGS16 binds Gαq and Gαi proteins present in T cells. Moreover studies in heterologous expression system show that RGS16 is able to inhibit Gq- and Gi-mediated signalling pathways. As lymphocyte migration depends on Gαi activity, it would be predicted that IL2-triggered RGS16 expression could serve to attenuate the chemotactic signalling pathway (Beadling, 1999).

PAFR regulation by RGS16 was studied in detail in a heterologous expression system employing PAFR-transfected CHO cells. Lin and coll. (Zhang, 1999) demonstrated that RGS16 inhibits PAF-stimulated inositol triphosphates production and p38 MAPK activation. p38 MAPK is thought to play an important role in the regulation of cellular responses during infection. Perhaps RGS16 is recruited to fine tune the immune response through its effects on the expression of pro inflammatory molecules.

Studies in CEM leukaemia cells demonstrate that TNFα up regulates RGS16 mRNA levels in a calcium-sensitive manner. Calcium mobilisation can inhibit the expression of RGS16. This is unexpected because most genes are up regulated in T cells in the presence of phorbol esters and high intracellular calcium, which culminate in T cell activation. A speculative explanation for the observed inhibition of RGS16 levels is that the lower abundance of RGS16 allows the signalling of GPCR, which may be essential for proper T cell differentiation and propagation. In this way evidence was provided of a
cross talk between the cytokine receptors and GPCR through the up-regulation of an RGS protein (Fong, 2000).

1-5 Brief introduction to the thesis

The present study investigated human Platelet-Activating Factor receptor (hPAFR) signalling and its modulation, focusing on the process of homologous desensitisation. Both GRKs and arrestins are expressed at high level in peripheral blood leukocytes (PBL) and they are potentially modulators of chemoattractant receptor-mediated immune responses (Parruti, 1993; Craft, 1995; Pitcher, 1998a). We showed that in a heterologous expression system recombinant hPAFR stimulated inositol phosphates production and intracellular cAMP accumulation, through the coupling with Gq/11 and Gs proteins, respectively. The Gs-coupling was a totally unpredicted finding. We also showed that PAF could stimulate intracellular cAMP accumulation in lymphocytes, suggesting that PAFR-coupling with adenylyl cyclase may be important also for endogenously expressed receptors. With the co-transfection approach we defined which GRKs were able to modulate hPAFR signalling and the mechanisms involved in this modulation. We showed that receptor pathways mediated by Gs-coupling were regulated through a strictly phosphorylation-dependent process, while Gq-mediated signalling could be modulated in a phosphorylation-independent way. We also identified in the N terminal portion of GRK2 (N-ter), which contains an RGS homology domain, the domain involved in the inhibition of Gq-mediated signalling. We also show that GRK2 binding to Goq could be regulated by c-Src activity.
2-1 Molecular biology

The molecular biology techniques were performed according to Ausubel, 1999, and Sambrook, 1989.

2-1-1 Restriction digest

Several commercially available restriction enzymes were used to digest DNA for cloning experiments. The digestions were usually carried out as follow: about 1 µg of plasmidic DNA was incubated for 2 h at the suggested temperature with 5 U of the appropriate restriction enzyme in 30 µl of restriction buffer supplied by the enzyme manufacturer. The reaction was stopped by agarose gel electrophoresis and the DNA fragment of interest cut and processed as needed.

2-1-2 DNA purification

The phenol chloroform extraction method was used to purify the DNA fragment from the agarose gel. The DNA was run on 0.8% low melting temperature agarose gel in the presence of ethidium bromide. The band of interest was visualised on a short wave UV transilluminator and removed with a scalpel, including the smallest amount of agarose. The DNA fragment was transferred into a Microfuge tube and the volume adjusted to 300 µl with water. The agarose was melted at 65 °C for 15 min, mixed with 500 µl of Tris-HCl-buffered phenol (pH 7.5), centrifuged for 10 min at room temperature and the upper aqueous layer transferred into a new tube; the phenol extraction was repeated 2 times. Finally the aqueous layer was extracted with 500 µl of chloroform. The purified DNA was recovered by precipitation in 0.3 M...
sodium acetate, 70% ethanol. The pellet was washed with 70% ethanol, dried down, resuspended in water, and stored at -20 °C until needed.

2-1-3 Ligation of DNA fragments
The DNA ligation reactions were performed as follow: the DNA insert and the vector (molar ratio 3:1), plus 5 U of T4 DNA ligase were mixed in 30 µl of reaction buffer containing 1 mM ATP and incubated overnight at room temperature. The next day, the enzyme was heat inactivated (75 °C for 5 min) and 10 µl of the reaction were used to transform 100 µl of competent bacteria.

2-1-4 Preparation of competent cells
The competent bacteria were obtained using the rubidium chloride method (Kushner, 1978). *E. coli* XL1-blue (Stratagene) bacterial strain was streaked over night on a Luria Broth (LB) plate and the next day, one single large colony was inoculated into 10 ml of LB and grown overnight at 37 °C with shaking at 225 revolution per minute (RPM). One ml of the over night saturated culture was inoculated into 9 ml of fresh LB and grown for about 2 h, until the optical density at 550 nM was 0.3. The bacteria were subcultured 1:20 into 100 ml of pre-warmed LB, and grown until the optical density at 550 nM reached 0.48. The cells were chilled on ice and centrifuged at 1,000 x g at 4 °C for 5 min, the supernatant was removed and the pellet gently resuspended in 40 ml of 30 mM potassium acetate, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl, 15% glycerol pH 5.8. The bacteria were left on ice for 2 h, centrifuged and resuspended in 4 ml of 10 mM MOPS, 75 mM CaCl₂, 10 mM RbCl, 15% glycerol, pH 7. Cells were left on ice for 15 min and frozen in 100µl aliquots in ethanol/dry ice bath and stored at -80 °C until needed.
**2-1-5 Bacterial transformation**

Competent cells were thawed on ice and 10 μl of the ligase reaction or 10 ng of the circular plasmid were added. After gently mixing the cells, they were left on ice for 30 min and heat shocked for 1 min at 42 °C. After the addition of 1 ml of LB, the bacteria were grown at 37 °C for 1 h with constant shaking at 225 RPM. The culture was centrifuged and cells resuspended in 100 μl of fresh LB and plated on LB agar containing the appropriate selective antibiotic(s).

**2-1-6 Minipreps**

XL1-blue *E. coli* harbouring the plasmid of interest was grown overnight in LB medium and the selective antibiotic(s). The culture was centrifuged and the pellet resuspended in a solution containing 100 μl of 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl pH 8. The bacteria was left for 5 min at room temperature and then gently lysed in 200 μl of 1% sodium dodecyl sulfate (SDS), 200 mM NaOH. The solution was neutralised with 150 μl of 3 M sodium acetate pH 4.7, centrifuged for 5 min in a Microfuge, and the *E. coli* debris pellet was discarded. The supernatant was transferred into a new Microfuge tube and mixed with 1 ml of cold ethanol. The tube was centrifuged for 5 min at room temperature and the supernatant removed. The pellet was washed with 70 % ethanol, dried and resuspended in 50 μl of water. About 5 μl were used for each restriction digestion.

**2-1-7 Construction of recombinant GST-N-ter in fusion-protein expression vector and in eukaryotic expression vector**

The GRK2 N terminal region (Ala2-Thr187) cDNA was amplified using the primers 5'-CGGGATCCGCGGACCTGGAGGC-3' (forward) and 5'-TCAGGTCAGGGATGGTTGAGGC-3' (reverse) (the native sequences are underlined) in 100 μl of PCR buffer (10 mM Tris HCl, pH 8.3, 50 mM
KCl, 2 mM MgCl₂), 0.4 μg of each primer, 200 μM of dNTPs, 2.5 U of *Termus acquaticus* DNA polymerase (Amplitaq Perkin Elmer). Amplification was carried out for 30 cycles, 94 °C 1 min (denaturation), 56 °C 1 min (annealing), 72 °C 1 min (extension). The amplification ended with 10 min at 72 °C to complete the polymerisation of any incomplete chains. The polymerase chain reaction product was restriction digested with BamHI and ligated into pGEX-4T1 (Pharmacia Biotech) digested with BamHI and SmaI. The ligase product was used to transform competent cells and for minipreps and large scale plasmid purification.

Subsequently, the GRK2-Nter was subcloned in the eukaryotic expression vector pCDNA3HisC (Invitrogen, Carlsbad, CA) to be used for transfection. In order to do this the pGEX-4T1 vector encoding GRK2-Nter was digested with the BamHI and NotI restriction enzymes and the insert subcloned into the pCDNA3HisC previously digested with the same restriction enzymes. The ligase product was used to transform competent cells and for minipreps and large scale plasmid purification.

**2-1-8 Large plasmid purification on a caesium chloride gradient**

250 ml of bacteria were grown overnight in LB plus the appropriate antibiotic(s), collected in 250 ml centrifuge bottles and centrifuged 10 min at 7,000 RPM at 4 °C in JA 14 type rotor (Beckman Inc). The pellet was resuspended in 10 ml of 50 mM glucose, 10 mM EDTA and 25 mM Tris-HCl pH 8.5 mg/ml lysozyme and left on ice for 20 min. The bacterial suspension was lysed for 5 min on ice in 20 ml of 1% SDS, 200 mM NaOH and neutralised with 15 ml of 3 M potassium acetate pH 4.7. The precipitate was removed by centrifugation at 10,000 RPM for 30 min at 4 °C (JA 14). The supernatant was transferred into a new tube and the DNA precipitated for 20 min with 0.6 volumes of isopropyl
alcohol. The precipitated DNA was collected by centrifugation at 7,000 RPM (JA 14) at room temperature for 30 min, and the resultant pellet resuspended in 5 ml of 10 mM Tris-HCl, 1 mM EDTA pH 8. 5.5 g of CsCl and 500 μl of ethidium bromide were added, and the solution was transferred into quick seal tubes and centrifuged at room temperature for 18 h at 48,000 RPM in the VTI 65.2 rotor (Beckman Inc.). The plasmid was recovered from the gradient with a syringe and ethidium bromide extracted using butanol/water saturated with NaCl. The recovered DNA solution was diluted 1:3 with water, and DNA precipitated with 2 volumes of ethanol at -20 °C over night and recovered by centrifugation at 10,000 RPM for 30 min in JA 20 rotor (Beckman Inc). The DNA pellet was washed in 70% ethanol, resuspended in 500 μl of water, quantified by measuring its absorbance at 260 nM and stored at -20 °C until use.

2-1-9 RNA Isolation

Total RNA was prepared with a modified guanidine thiocyanate method (Chirgwin, 1979). Cells were washed twice with phosphate buffered saline (PBS), (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄) then, for monolayer culture, 3 ml of 4 M guanidine each 50 x 10⁶ cells were added directly to the dish, and the viscous lysate recovered by scraping with a rubber policeman. Cell suspension cultures were harvested by centrifugation and lysed by guanidinium. Cell lysates were layered on a 2 ml cushion of 5.7 M CsCl. This step gradient was centrifuged at 150,000 x g in a swinging bucket rotor (SW50.1 from Beckman Instruments, Inc.) at 18 °C for 16 h. The next day, the supernatant was removed carefully and the RNA pellet resuspended in 400 μl of water. To further purify, the RNA was precipitated with ethanol and sodium acetate. The RNA pellet was resuspended in water, quantitated by measuring its absorbance at 260
nM, and stored at -80 °C until use.

2-1-10 Northern blot analysis

The GRK6 or PAFR mRNA expression was examined by Northern blot analysis. 20 μg of total RNA were separated according to their molecular weight on a denaturing 1% agarose gel electrophoresis (containing 6% formaldehyde). The electrophoresed RNA was transferred to a nylon filter (Gene Screen Plus membrane Du Pont-New England Nuclear) through a capillary blot procedure in the presence of 10 x SSC (1.5 M NaCl, 1.5 M sodium citrate pH 7). The RNA was fixed on the membrane by baking for 2 h at 80 °C. The entire coding sequence of human PAFR was labelled by random priming, and used as a probe. The GRK6 cDNA fragment bp 1413-1822, labelled by random priming, was used as GRK6 probe. The labelled probe was purified over a Sephadex G50 spun column and used for hybridisation. About 1 million counts/ml of hybridisation mixture (50% formamide, 2% SDS, 1 M NaCl, and 10% dextran sulphate, 100 μg/ml sonicated salmon sperm DNA) was used to hybridise (16 h at 42 °C) the RNA immobilised on the nylon filters. After removal from the hybridisation solution, the membrane was washed. The washing procedure was as follows: twice with of 2 x SSC at room temperature for 5 min; twice with 2 x SSC, 1% SDS at 60 °C for 30 min; twice with 0.1 x SSC at room temperature for 30 min always with constant agitation. The washed membrane was subjected to autoradiography at -80 °C for 1-4 days. The developed film was analysed by LKB Ultroscan XL Enhanced Laser Densitometer or digitalised with image scanner Agfa-Gevaert AG and quantified by NIH Image 1.59 software. All results were confirmed at least in two separate experiments.
2-1-11 Preparation of Bacterial Recombinant Proteins

Recombinant GST-RGS4 and GST-N-ter (comprising GRK2 N-terminal region Ala2-Thr187) fusion proteins were expressed in Escherichia coli BL21 by induction for 3 h with 1 mM isopropyl-D-thiogalactoside. The fusion proteins were purified essentially according to Frangioni and Neel (Frangioni, 1993).

10 ml of bacteria were grown overnight in LB plus 100 μg/ml ampicillin. The day after 100 ml of sterile LB medium was inoculated with 2 ml of the overnight culture and the bacterial suspension bacteria was grown at 37 °C for 1 h with constant shaking at 225 RPM. At this point the peptide was induced for 3 h at 37 °C with 1 mM isopropyl-D-thiogalactoside. The bacterial suspension was then collected in 250 ml centrifuge bottles and centrifuged 10 min at 7,000 RPM at 4 °C in JA 14 type rotor (Beckman Inc). The bacteria were lysed by sonication in STE buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM 1,4-dithio-D,L-threitol (DTT), 1 mM EDTA] containing 100 μg/ml lysozyme, 1.5% N-laurylsarcosine, and protease inhibitor cocktails. After clarification by a centrifugation step (10,000 x g for 5 min), the lysate was incubated with glutathione agarose beads for 1 h at 4 °C, washed eight times with ice-cold PBS, and placed in storage buffer I (75 mM HEPES, 150 mM NaCl, 5 mM DTT, 10% glycerol) until used.

2-2 Protein biochemistry

All the protein buffers used contained the following protease inhibitors 0.1 mM phenylmethylsulphonyl fluoride, 10 μg/ml leupetin, 5 μg/ml pepstatin and 10 μg/ml benzamidine, unless otherwise indicated.
**2-2-1 Cytosol and membrane preparation**

Cytosolic and membrane preparations from cells were obtained as follows (Parruti, 1993): cells or tissue fragments were harvested by centrifugation (400 x g for 5 min), lysed in cell lysis buffer (CLB) (10 mM Tris-HCl, 5 mM EDTA, 7.5 mM MgCl₂, pH 7.4) using a polytron tissue disrupter (Janke and Kundel) at low speed for 40 sec on ice. Unbroken cells and cell nuclei were pelleted by centrifugation (800 x g for 5 min) and discarded. The supernatant was then centrifuged at 300,000 x g for 30 min at 4 °C and the protein content of the resultant supernatant (cytosol) and pellet (membranes) were measured.

**2-2-2 Protein assay**

To assess the protein concentration from cytosolic or resuspended membrane preparation a Bio-Rad protein assay was used. This assay is based on the colour change of Coomassie brilliant blue G-250 dye in response to various concentrations of proteins. Several µl of sample were diluted in 800 µl of water and mixed with 200 µl of reagent. After 5 min, a blue colouring developed whose absorbance (wave-length of 595 nM) was measured by spectrophotometer. The protein concentration is obtained by extrapolation from a standard curve using known amounts of protein.

**2-2-3 Western blot analysis**

Six different anti-GRK2 antibodies were used. An affinity-purified rabbit polyclonal anti-βARK1 antibody (LevβARK1) raised against GST-human βARK1 C-terminus (last 222-amino acids) fusion protein was kindly provided by Dr. H. LeVine III (Chuang , 1995) and it was used for Western blot analysis with a dilution of 1:1,000. An affinity-purified rabbit polyclonal anti-βARK1 antibody (BouβARK1) raised against βARK1 N-terminus was kindly provided by Dr. F. Boulay and it was
used for Western blot analysis with a dilution of 1:1,000. A monoclonal antibody anti-GRK2/3 clone C5/1.1, was available from Upstate Biotechnology, it was raised against a GST fusion to GRK3-Cter (residues 467-688), homologous to GRK2, (Oppermann, 1996b) and it was used for Western blot analysis at a concentration of 2 µg/ml. The affinity-purified rabbit polyclonal anti-SARK1 antibody (AbPF1) raised against GST-GRK2 PF1 (a.a. 50-145), the affinity-purified rabbit polyclonal anti-SARK1 antibody (AbPF2), raised against GST-GRK2 PF2 (a.a. 437-689) fusion protein were kindly provided by Prof. F. Mayor (Murga, 1996) and they were used for Western blot analysis with a dilution of 1:1,000. The affinity-purified rabbit polyclonal anti-SARK1 antibody (Ab9) raised against recombinant GRK2 was kindly provided by Dr. J.L. Benovic (Thomas Jefferson Cancer Institute of Philadelphia) and was used for Western blot analysis with a dilution of 1:1,000.

The monoclonal antibody F4C1, raised against the highly conserved epitope DGVVLVD, identical in ßarrestins and arrestin, was kindly provided by Dr. K. Palczewsky and Dr. L.A. Donoso and was used for Western blot analysis at a concentration of 0.5 µg/ml.

An affinity-purified rabbit polyclonal antibody that recognises Gαq/11 was from Santa Cruz Biotechnology, Inc., Gαq/11 (C19) and was used for Western blot analysis with a dilution of 1:1,000.

A rabbit polyclonal antibody, with epitope mapping at the amino terminus of c-Src p60 of human origin, was from was Santa Cruz Biotechnology, Inc., Src (N16) and was used for Western blot analysis with a dilution of 1:500.

The antiphosphotyrosine monoclonal antibody, horseradish peroxidase-conjugated, to detect protein tyrosine phosphorylation was from Santa Cruz Biotechnology, Inc., (PY99-HRP) and was used for Western blot analysis with a dilution of 1: 7,000.

Rabbit polyclonal antibodies that recognise RGS4 (RGS4 N-16), Gαs
(Gαs K20), Gαo (Gαi/o/t/z C20) were from Santa Cruz Biotechnology (Santa Cruz, CA) and were used for Western blot analysis with a dilution of 1: 1,000.

The goat polyclonal antibody was from and anti-GST (Pharmacia Biotechnology, Arlington Heights, IL) and was used for Western blot analysis with a dilution of 1: 500.

Usually samples containing 100 μg of postnuclear proteins were suspended in Laemmli buffer (8% SDS, 10% glycerol, 5% β-mercaptoethanol, 25 mM Tris-HCl, pH 6.5, and 0.003% bromophenol blue) by boiling for 5-10 min and electrophoresed on SDS polyacrylamide homogenous slab gel (SDS-PAGE). Proteins were electroblotted (Burnette, 1981) onto 0.22 μm Protran nitrocellulose filter (Schleicher & Schüell) with a tank transfer system. Efficiency of transference was verified by Ponceau red staining of the blots and Coomassie Blue staining of gel after transfer. The blots were saturated 1 h with 1 % bovine serum albumin (BSA) plus 1% skim milk in Tris buffered saline (TBS), (100 mM Tris-HCl pH 7.5, 0.9% NaCl and 0.05% Tween-20). The immunoblottings were performed incubating the saturated blots for 1 h at room temperature with the primary antibodies diluted in TBS at the reported concentrations. This incubation was followed by three washes with TTBS (TBS plus 0.05% Tween-20) and then the blots were incubated for 1 h at room temperature with the appropriate secondary antibodies diluted in TBS. The antibodies LevSARK1, BouSARK1, anti-RGS4, anti-Gαs, anti-Gαo, anti Gαq were developed with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5,000) and 5-bromo-4-chloro-3-indolyl-phosphate and nitro-blue tetrazolium. The immunoblotting for anti-GRK2/3 clone C5/1.1, the monoclonal antibody F4C1 antibody were developed with alkaline phosphatase-conjugated goat anti-mouse IgG (1:5,000) and 5-bromo-4-chloro-3-indolyl-phosphate and nitro-blue tetrazolium.
The polyclonal antibodies AbPF1, AbPF2, Ab9, Gq/11 (C-19), Src (N-16) were developed with the anti-rabbit horseradish peroxidase–conjugated antibodies and were revealed using a chemiluminescent method (ECL, Amersham Pharmacia Biotech).

The antiphosphotysine blots were revealed with a different method: the blots were saturated with TBS plus 2% BSA for 2 h at room temperature, then they were incubated, for 1 h at room temperature or 12 h at 4 °C, with the antiphosphotyrosine monoclonal antibody, horseradish peroxidase–conjugated, diluted 1: 7,000 in TBS plus 2% BSA, then they were washed three times with TTBS and directly revealed using the chemiluminescent method (ECL, Amersham Pharmacia Biotech).

2-2-4 Purification of Gαq Protein

The purification of Gαq was performed according to Kozasa and Gilman (Kozasa, 1995). Sf9 cells were cultured on a monolayer using TNM-FH (Life Technology) medium containing 10% foetal bovine serum and antibiotics (2.5 µg/ml fungizone, 50 µg/ml streptomycin, 50 µg/ml penicillin). To produce recombinant Gαq, SF9 cells were infected with baculoviruses encoding the Gαq subunit, the β1 subunit, and the Hist-γ2 subunit (1 plaque-forming unit per cell for each virus). Seventy-two hours later, the cells were harvested and lysed in ice-cold lysis buffer (50 mM NaHEPES pH 8, 100 mM NaCl, 0.1 mM EDTA, 3 mM MgCl₂, 10 mM β-mercaptoethanol, 50 µM GDP, plus a mixture of protease inhibitors and 0.03 mg/ml lima bean trypsin inhibitor) using a polytron tissue disrupter (Janke and Kundel) at low speed for 40 sec on ice. Unbroken cells and cell nuclei were pelleted by centrifugation (800 x g for 5 min) and discarded. The supernatant was then centrifuged at 100,000 x g for 30 min at 4 °C. The resultant pellet was suspended in wash buffer (50 mM NaHEPES pH 8, 50 mM NaCl, 3 mM MgCl₂, 10 mM
β-mercaptoethanol, 50 μM GDP, plus a mixture of protease inhibitors) and centrifuged at 100,000 x g for 30 min at 4 °C. The washed pellet (membranes) was frozen in liquid nitrogen and stored at −80 °C. Membranes were thawed and diluted to 5 mg/ml was resuspended in the ice-cold wash buffer. Sodium cholate was added to a final concentration of 1% (w/v), and the mixture was stirred on ice for 1 h prior to centrifugation at 100,000 x g at 4 °C for 40 min. Remaining procedures were carried out at 4 °C unless otherwise specified. The supernatants (membrane extract) were collected, diluted 5-fold with buffer A (20 mM NaHEPES pH 8, 100 mM NaCl, 1 mM MgCl₂, 10 mM β-mercaptoethanol, 50 μM GDP, 0.5% BRJI 58, plus a mixture of protease inhibitors) and loaded onto a Ni-NTA column (Qiagen, Chatsworth, CA), which had been equilibrated with buffer A. The column was washed with 25 column volumes of buffer B (20 mM NaHEPES pH 8, 300 mM NaCl, 1 mM MgCl₂, 10 mM β-mercaptoethanol, 50 μM GDP, 0.5% BRJI 58, 5 mM imidazole, plus a mixture of protease inhibitors). The column was then incubated at room temperature with buffer C (20 mM NaHEPES pH 8, 100 mM NaCl, 0.2 mM MgCl₂, 10 mM β-mercaptoethanol, 5 μM GTPγS, 0.2% sodium cholate, 5 mM imidazole, plus a mixture of protease inhibitors) and washed with the same buffer C. This step is utilised to elute an endogenous (Sf9 cell) αi-like protein. The Gaq was eluted by an AlF₄⁻-containing buffer (20 mM NaHEPES pH 8, 50 mM NaCl, 50 mM MgCl₂, 10 mM β-mercaptoethanol, 50 μM GDP, 1% sodium cholate, 5 mM imidazole, 50 μM AlF₄⁻, 10 mM NaF, plus a mixture of protease inhibitors. The eluted Gaq was inactivated by exchanging the elution buffer with storage buffer (20 mM HEPES pH 8.0, 100 mM NaCl, 1 mM EDTA, 3 mM MgCl₂, 3 mM DDT, 0.7% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate, 0.5 μM GDP) and used for protein-protein interaction experiments with GST-N-ter or GST-RGS4.
2-2-5 Binding of G Proteins to RGS4 and GRK2 N-terminal

Cytosolic proteins (150 μg) from HEK 293 cells transfected with the indicated Gα subunits or 100 ng of purified Gαq were mixed with 40 μl of slurry containing GST-RGS4, or GST-N-ter fusion proteins bound to glutathione agarose beads in a final volume of 400 μl of binding buffer (20 mM Tris-HCl, pH 7.5, 1 mM 1,4-dithio-D,L-threitol (DTT), 100 mM NaCl, 0.1% Lubrol, 10 μM GDP, 3 mM MgCl₂), in the presence or absence of 47 mM MgCl₂, 30 μM AlCl₃, and 20 mM NaF. After 1 h at 4 °C, the beads were washed three times with 1 ml of ice-cold binding buffer and the resins containing the eventual bound proteins were analysed by immunoblotting.

2-2-6 Binding of non phosphorylated or tyrosine-phosphorylated Gαq Proteins to GRK2 N-terminal

Cytosolic proteins (150 μg), from HEK 293 cells transfected with Gαq R183C in absence or in presence of constitutively activated c-Src (Src*), were mixed with 50 μl of slurry containing GST-NterI (a.a. 50-145 of GRK2 sequence) fusion protein bound to glutathione agarose beads. The incubation was performed in a final volume of 500 μl in the incubation buffer (20 mM Tris HCl, pH 7.5, 1mM orthovanadate, 1 mM DTT, 100 mM NaCl, 0.1% Lubrol, 10 μM GDP, 3 mM MgCl₂). After 1 h at 4 °C, the beads were washed five times with 1 ml of ice-cold incubation buffer and the resins containing the bound proteins were analysed by immunoblotting.

2-2-7 Immunoprecipitation reaction

48 h after transfection the immunoprecipitation assays were performed. Transfected cells were scraped and washed twice with ice cold phosphate-buffered saline. Cells were then lysed in 700 μl/100-mm dish of RIPA buffer (50 mM Tris, 0.3 M NaCl, 1% (w/v) sodium
deoxycholate, 1% (v/v) TX-100, 0.1% SDS, 1 mM sodium orthovanadate, pH 7.5, plus a mixture of protease inhibitors) and homogenised with a Teflon pestle. After gentle rocking for 60 min a 4 °C, the lysates were clarified by centrifugation, and an aliquot (30 μl) was used to assess protein over-expression. The immunoprecipitation reactions were performed incubating the supernatants with 1 mg/ml bovine serum albumin and specific antibodies for 4 h, followed by incubation with protein A-Sepharose for 1 h. A preimmune serum (diluted 1:125) was employed as negative control of immunoprecipitation, the specific polyclonal antibody AbPF2 (diluted 1:125) (Murga, 1996) for GRK2 immunoprecipitations and the Gαq/11 (C19) affinity-purified rabbit polyclonal antibody (4 μg/100-mm petri dishes) (Santa Cruz Biotechnology, Inc.) for Gαq immunoprecipitations. All the immunoprecipitated samples were washed four times with 1 ml of RIPA buffer and then resuspended in Laemmli buffer and boiled for 10 min before resolution by 10% SDS-PAGE and transference on nitrocellulose membranes.

2-3 Cell culture and transfection

2-3-1 Culture and transfection of COS 7 cells (chapters 3 and 4)
COS 7 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) foetal bovine serum and antibiotics (100 u/ml penicillin, 100 μg/ml streptomycin) at 37 °C in a humidified 7% CO₂ atmosphere. Transfections were performed on 70% confluent monolayers. One day before transfection, the cells were subcultured into the appropriate petri dishes, at the density of 50,000 cells/cm². COS 7 cells were transiently transfected by the diethylaminoethyl (DEAE)-dextran procedure (Kluxen, 1993). With this method a transfection medium containing plasmid DNA and DEAE-
dextran, which forms complexes that were taken up by the cells via endocytosis, was layered directly onto the cells. The transfection medium was formed by DMEM plus 10% FCS and supplemented with chloroquine diphosphate (0.1 mM) and DEAE-dextrane (0.4 mg/ml). Chloroquine was added to inhibit degradation of plasmid DNA. Plasmid DNA, diluted in distilled water to a concentration between 1 and 0.1 μg/μl, was added to the transfection medium. The cells were then incubated with pre-warmed DNA-supplemented transfection medium for five hours and then exposed transiently to a permeablising agent to increase the DNA uptake ("shock"). The shock was performed aspirating the DNA-supplemented transfection medium and exposing the cells, for 2 minutes at room temperature, to a medium (DMEM plus 10% FCS) containing 10% DMSO. The DMSO-containing medium was then aspirated, the cells were washed twice with phosphate buffer solution (PBS) and then were fed with complete medium for further 48 h. In co-transfection experiments the amount of cDNA was kept constant using the empty vector. The day after transfection, the cells were trypsinised and seeded into the appropriate dishes for functional assays or for cytosol and membrane preparation and Western blot analysis. This method was used to over-express PAFR in COS 7 cells. Moreover it was used to perform co-transfection experiments with PAFR and TSHR in COS 7 cells.

2-3-2 Culture and transfection of HEK 293 cells (chapter 4)

Cells were cultured in DMEM supplemented with 10 % foetal calf serum (FCS) and antibiotics (100 u/ml penicillin, 100 μg/ml streptomycin) and split 1:6 every 4-5 day. One day before transfection, the cells were subcultured into the appropriate petri dishes, at the density of 50,000 cells/cm². The cells were transfected using the calcium phosphate method (Graham, 1973) where an HEPES-buffered
solution was used to form a calcium phosphate precipitate that was layered directly onto the cells. The precipitate containing calcium phosphate and DNA was formed by slowly mixing an equal volume of HEPES buffer saline (HEPES 0.05 M, NaCl 0.28 M, Na₂HPO₄ 1.5 M, pH 7.05) with a solution containing calcium chloride (2.5 M) and the indicated DNA amounts. The resulting solution was directly added to the growth medium and the cells were incubated 16 h with the calcium phosphate precipitate under standard growth conditions. Then the precipitate was washed out and the cells fed with complete medium for further 48 h. This method was used to perform co-transfection experiments with 5-HT₂C receptor in HEK 293 cells. Moreover with this method different Ga-subunits were over-expressed in HEK 293 cells and their cytosolic extracts were used to perform in vitro binding assays.

2-3-3 Culture and transfection of HEK 293 cells (chapter 5)

HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) foetal bovine serum at 37 °C in a humidified 7% CO₂ atmosphere. Transfections were performed on 70% confluent monolayers in 100-mm dishes for immunoprecipitation assays or 12-well dishes for inositol phosphate assays. The cells were transfected with the lipofectamine method (Hawley-Nelson, 1993) where a solution containing DNA-liposome complexes was layered directly onto the cells. The transfection was performed using the commercially available lipofectAMINE PLUS Reagents (Life Technologies). The DNA-liposome complexes were formed mixing the indicated amount of plasmid DNA, previously pre-complexed with the plus reagent (20 µl/100-mm dishes), with the lipofectamine reagent (30 µl/100-mm dishes) in the serum-free transfection medium OPTIMEM (GIBCO) (5 ml /100-mm dishes). The lipofectamine reagent is a 3:1 (w/w) liposome
formulation of the polycationic lipid 2,3-dioleyloxy-N-2(sperminecarboxamido)ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoyl phosphatidylethanolamine (DOPE) in membrane filtered water, suitable for the transfection of DNA into cultured eukaryotic cells. Empty vector was added to keep the total amount of DNA added/dish constant. After 3 h of exposure to the transfection medium containing DNA-liposome complexes, monolayers were refed with culture medium and incubated overnight. Assays were performed 48 h after transfection. This method was used in co-transfection experiments with GRK2 to perform co-immunoprecipitation assays. Moreover this method was used in co-transfection experiments with wt Gaq to perform the inositol phosphate assays reported in chapter 5.

2-3-4 Cytofluorimetric analysis

FLAG-PAFR expression was measured by binding of monoclonal antibody, anti-FLAG M5 monoclonal antibody (Eastman Kodak Company) directed at MDYKDDDDKEF amino acid sequence present at the N-terminal Met-FLAG fusion protein of PAF receptor (Kunz, 1992). Three days after transfection, COS 7 cells were detached with phosphate buffered saline (PBS) and harvested by centrifugation. Cells were then resuspended in RPMI 1640 without phenol red at 1 x 10^6/ml, placed on ice and incubated with the primary antibodies (10 μg/ml) at 4 °C for 30 min. After washing with staining buffer (phosphate-buffered saline with 0.02% sodium azide and 0.2% bovine serum albumin), transfected cells were resuspended in foetal calf serum and incubated with a 1:40 dilution of fluorescein isothiocyanate-labelled goat anti mouse IgG (BioSource International) and analysed on a FACScan flow cytometer. Cells were incubated with propidium iodide just before acquisition, and dead cells, identified as red positive events,
were excluded from analysis. Mock-transfected cells were stained with anti-FLAG M5 antibody to identify the peak of untransfected cells. A marker including these cells was selected to exclude them from statistical analysis (negative marker). In samples transfected with the receptor, cells whose fluorescence intensity exceeded the negative marker were considered positive for transfection and their mean intensity of fluorescence (MIF) was utilised as an index of receptor expression.

2-3-5 Cyclic AMP Assay

One day after transfection, transfected COS 7 cells were seeded in 48-well plates at the density of $8 \times 10^4$ cells per well. 72 h after transfection the cells were washed twice with pre-warmed Hanks' balanced salt solution (HBSS$^+$) and incubated with a cAMP incubation buffer (HBSS$^+$ plus 0.4% BSA, 10 mM HEPES, and 0.5 mM 3-isobutyl-1-methylxanthine, pH 7.4) and the indicated agonists at 37 °C for 30 min. The reaction was stopped aspirating the cAMP incubation buffer and adding ice-cold ethanol on the cells. The ethanol extracts of each sample were collected and evaporated to dryness at ~55 °C under a stream of nitrogen. The residue was then dissolved in a cAMP assay buffer (0.05 M Tris HCl, 4 mM EDTA pH 7.5) and the intracellular cAMP content was measured using a commercial Cyclic AMP [H3] Assay System (code TRK 432, Amersham). The assay was based on the competition between unlabelled cyclic AMP, from cellular extracts, and a fixed quantity of the tritium labelled compound, [$^3$H]cAMP, for binding to a protein, which has a high specificity and affinity for cyclic AMP. Separation of the protein-bound cyclic AMP from the unbound nucleotide was achieved by adsorption of the free nucleotide on to coated charcoal, followed by centrifugation. An aliquot of the supernatant was then removed for liquid scintillation counting. The
amount of labelled protein-cyclic AMP complex formed was inversely related to the amount of unlabelled cyclic AMP present in the assay sample. Measurement of the protein-bound radioactivity enabled the amount of unlabelled cAMP in the sample to be calculated from a linear standard curve. Data are expressed as pico moles of cAMP/well.

2-3-6 Inositol Phosphate (IP) Formation Assay

One day after transfection, transfected COS 7 cells were seeded in 24-well plates at the density of 20 x 10^4 cells per well. 48 h after transfection cells were labelled for 24 h with 5 μCi/ml of myo-[2-3H]inositol (Amersham) in the inositol-free medium M199 (GIBCO). 72 h after transfection labelled cells were washed twice with pre-warmed HBSS+ and incubated at 37 °C for 15 min in an incubation buffer (HBSS+ plus 350 mg/l NaHCO3, 10 mM Hepes, 10 mM LiCl, pH 7.3) and then incubated with the indicated agonists at 37 °C for 30 min. The reaction was stopped aspirating the incubation buffer and adding ice cold 6% perchloric acid/1.5 mM EDTA. The cells were scraped, transferred into a Microfuge tube and the pH was brought to pH 7 with 10 mM KOH. The cell debris were removed by centrifugation (5 min at room temperature in a Microfuge). The supernatants, containing the intracellular inositol phosphates, were applied to ion exchange chromatography columns of dowex AG1-X8 (formate form) (200-400 mesh, 350 μl bed volume). Each column was washed with 2 column volumes of unlabelled inositol (5 mM), 2 column volumes of 5 mM sodium tetraborate/60 mM ammonium formate, and eluted by 3 ml of 0.1 M formic acid/1.5 M ammonium formate as previously described (Berridge, 1983). Total IPs were quantified by counting β emissions. The elutes were mixed with scintillation solution and subjected to scintillation counting. The results are expressed as counts/well.
2-3-7 Separation of lymphocytes

Peripheral blood leukocytes (PBL) obtained from healthy volunteers were isolated as described by DeBlasi (DeBlasi, 1986). Freshly withdrawn samples of blood were supplemented with EDTA 5 mM and sodium citrate 0.38% and were transferred in tubes containing phosphate-buffered saline (PBS). EDTA acts as an anti-coagulant. All steps thereafter were performed at 4 °C. The blood was centrifuged at 200 x g for 15 min, the supernatant was discarded and the pellet resuspended in PBS. Mononuclear leukocytes (MNL) were isolated by density gradient centrifugation (600 x g for 45 min at 4 °C) on Ficoll-hypaque. The mononuclear fraction was washed twice with PBS and then lysed in guanidine thiocyanate for Northern blot analysis. The mono-nuclear leukocyte preparation yielded a heterogeneous cell population that contained about 80% of lymphocytes, 15-20% of monocytes, and less than 2% granulocytes. MNL were further fractionated into lymphocytes and monocytes by a Percoll gradient. Granulocytes and erythrocytes were recovered at the bottom of the Ficoll gradient and granulocytes were isolated from erythrocytes by differential lysis. Incubation for 15 min at 4 °C in the hyperosmotic buffer (155 mM NH₄Cl, 10 mM NH₄HCO₃) causes disruption of erythrocytes and broken cells were removed by centrifugation (600 x g for 10 min a 4 °C) and the granulocyte pellet washed with PBS and lysed in guanidine thiocyanate for Northern blot analysis. Resting T lymphocytes were prepared from the mononuclear fraction resuspended in RPMI 1640 supplemented to 10% (v/v) FCS, 10 mM HEPES (pH 7.4) 100 U/ml penicillin, 50 μg/ml streptomycin and maintained at 37 °C in a humidified, 5% CO₂ incubator. The cells were initially stimulated with PHA at 2 μg/ml for three days. The cells were then washed twice with fresh medium and resuspended at a density of 5 x 10⁵ cells/ml. The cells were maintained resting or "blast" by adding 2 nM human IL2.
every 2 days (Rosoff, 1992).

2-3-8 cAMP accumulation assays in lymphocytes

Incubations started adding 0.1 ml ice-cold cells (20 x 10⁶/ml cells) to 0.9 ml RPMI 1630 (GIBCO) at 37 °C. The medium was supplemented with 100 µM Ro 20-1724, an inhibitor of phosphodiesterase activity, and, where necessary, PAF was added at the indicated concentrations. After 10 min the reactions were terminated by centrifugation at 10.000 x g for 30 sec at 4 °C. The supernatant was aspirated and the pelleted cells were resuspended in 100 µl of the cAMP assay buffer (0.05 M Tris HCl, 4 mM EDTA pH 7.5). The tubes were placed in a boiling water bath for 10 min and then frozen. After thawing the samples were sonicated, three pulses of 20 sec each, centrifuged at 10.000 x g for 30 sec at 4 °C. The supernatants, containing intracellular extracts, were assayed for intracellular cAMP content, using the commercial Cyclic AMP [H3] Assay System (code TRK 432, Amersham).

2-3-9 Statistical analysis

All experiments are presented as the average of duplicate or triplicate determinations repeated at least three times. Statistical analysis was carried out by Student’s t test otherwise indicated.

2-4 Miscellaneous

2-4-1 Cell sources

Cultured cells (American Tissue Culture Collection) were grown under standard conditions with the appropriate medium and subcultured as suggested from ATCC. Peripheral blood leukocytes (PBL) obtained from healthy volunteers were isolated by density gradient centrifugation (600 x g for 45 min at 4 °C) on Ficoll-hypaque followed by Percol gradient
2-4-2 Material sources

Deoxynucleotides used for PCR and AmpliTaq DNA polymerase were from Perkin Elmer; restriction endonucleases were from Gibco, Pharmacia LKB Biotechnology Inc. or Boehringer Mannheim. NuSieve 3:1 agarose was from FMC. Gene Screen Plus membranes were from New England Nuclear. 32P labelled dNTPs and the random priming kit were purchased from Amersham. Culture media, FCS, guanidine thiocyanate were from Gibco. All gel electrophoresis materials (but NuSieve agarose, FMC) were purchased from Bio-Rad. The Cyclic AMP [H3] Assay System (code TRK 432) was from Amersham. The lipofectAMINE PLUS Reagents was from Life Technologies. All chemicals were obtained from commercial sources as the highest purity material available. Protein molecular weight markers were from Pharmacia LKB Biotechnology Inc.

PAF was purchased from Bachem (Bubendorf, Switzerland), BN52021 is a gift of Dr P Braquet (Institute Henri Beaufour, Leplesis-Robensir, France), iloprost was kindly provided by Schering (Germany), forskolin, ionomycin, A 23187, cholera toxin, pertussis toxin, PHA were from Calbiochem. Human IL8 (72 a.a.) and IL2 were from Peprotech, Inc.

2-4-3 Vectors sources

The following plasmids were generous gifts: human PAFR in pCDM8-FLAG plasmid from Dr C Gerard (Harvard Medical School, Boston, Massachusetts); GRK2 C terminal domain (Gly495-Leu689, GRK2-Cter) and GRK2K220R from Dr C. Scorer (Glaxo Wellcome, Stevenage, UK); GST-RGS4 in pGEX-4T2 from Dr R. Neubig (University of Michigan, Ann Arbor); IL8-RA from Dr B. Baruch (National Institute of Health, Maryland); RGS4 in pCMV vector from Dr J. Hepler (Emory University,
Atlanta, GA); eukaryotic expression vectors bearing Gαs, Gαq, Gαo, Gαq-R183C cDNAs and baculoviruses encoding the Gαq subunit, the β1 and the His6-γ2 subunits of G protein were from Dr A. Gilman (University of Texas, Dallas); constitutively active Gαq-Q209L from Dr N. Dhanasekaran (Temple University, Philadelphia, PA); thyrotropin receptor (TSHR) cDNA from Dr L.D. Kohn (National Institute of Diabetes and Digestive and kidney diseases, Bethesda, MD); 5-hydroxytryptamine (5-HT2c) receptor cDNA from Dr A. Salzman (Rhone-Poulenc Rorer Central Research, King of Prussia, PA); GST-NterI (a.a. 50-145 of GRK2 sequence), GRK2-Y13,86,92F with all the other tyrosine mutants of GRK2, β2-adrenergic (β2 AR) receptor and m1 muscarinic receptor from Prof F. Mayor jr (Universidad Autonoma de Madrid, Spain); human p60c-SRC (wt c-Src), the constitutively active Y527F p60c-SRC (c-Src-Y527F) and the dominant negative K295R p60c-SRC (c-Src-K295R) were provided by Dr S. Gutkind (NIH, Bethesda, MD); α2 adrenergic (α2 AR) receptor cDNA and βarrestin2 from Dr R.J. Lefkowitz (Howard Hughes Medical Institute, Duke University, Durham); GRK5 and GRK6 from Dr J. Benovic (Thomas Jefferson University, Philadelphia, PA); minigene encoding the undecapeptide of Gαs C terminal (Gαs DN) from Dr H. Hamm (University of Illinois, Chicago, IL). βarrestin1, GRK2, GRK3, GRK4 were previously cloned in our laboratory.
CHAPTER 3
SIGNALLING AND DESENSITISATION OF HUMAN PLATELET-ACTIVATING FACTOR RECEPTOR

3-1 Introduction

Intercellular communication is the key process to ensure a coherent organisation of cellular, multicellular and hence whole organism functions. To respond selectively to new or increasing stimuli, biological systems consistently diminish their responses to persistent or stable stimuli, in a process termed desensitisation or adaptation. Within milliseconds to minutes after agonist challenge, cells can diminish or virtually eliminate their agonist-evoked responses, in a process that involves phosphorylation of specific receptors at one or more intracellular domains. After several hours of agonist exposure, the receptors undergo down-regulation, a process in which the cellular complement of stimulated receptors is decreased by a combination of protein degradation, transcriptional, and post-transcriptional mechanisms (Bohm, 1997). This study focuses on the process called "homologous desensitisation" that indicates the rapid and reversible loss of responsiveness to a given agonist after cellular exposure to that agonist itself (Premont, 1995; Palczewski, 1997). Key molecular determinants of this process are G protein-coupled receptor kinases (GRKs) and arrestins (Lohse, 1990b; Lefkowitz, 1993a; Chuang, 1996a). Both GRKs and arrestins are members of multigene families and, with the exception of GRK1 and GRK4 for the GRKs family and of retinal and cone arrestin for arrestin/βarrestin gene family, they do not show selective tissue distribution. Interestingly it has been shown that peripheral blood leukocytes (PBL) represent the major site of expression and function for the ubiquitous members of both families (Parruti, 1993; Craft, 1995; Pitcher, 1998a). Moreover, GRK2/3 mRNA
expression and kinase activity are regulated in the process of T-cell activation (De Blasi, 1995). These observations suggest that GRKs and arrestins may be potent modulators of receptor-mediated immune responses, raising the possibility of their relevant role in the regulation of chemoattractant receptors. The present study proposes a method to study homologous desensitisation of chemoattractant receptors. The first receptor that has been studied is the Platelet-Activating Factor receptor. Platelet-Activating Factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocoline) is a lipid mediator that exhibits diverse and potent biological effects on a variety of cells and tissues, which are activated by a specific cell surface receptor (Chao, 1993). The human Platelet-Activating Factor receptor (hPAFR) gene exists as a single copy on chromosome 1. This gene possesses two 5'-non coding exons that are alternatively spliced to a common splice acceptor site on a third exon, which contains the total open reading frame, to yield two different species of functional mRNA (transcripts 1 and 2). These two transcripts have different tissue localisation and regulation by different transcriptional factors, allowing a tissue-specific regulatory control of PAFR gene expression in different human tissues and cells (Mutoh, 1993). The existence of putative hPAFR subtypes has been proposed, based on pharmacological evidence showing different potencies of various antagonists against different cellular membranes expressing PAFR. In this way evidence has been presented for different hPAFR subtypes expressed in eosinophils, neutrophils and platelets. (Hwang, 1988; Hwang, 1990; Shukla, 1992). However, all hPAFR cDNAs that have been cloned until now contain an identical coding region sequence (Nakamura, 1991; Ye, 1991; Kunz, 1992; Sugimoto, 1992). On the other hand, by extensive homology screening of several libraries from different cell sources, under low stringency, no evidence for the existence of receptor subtypes has been found (Shimizu, 1992). These
apparent contradictions may arise from detection of different available conformational states of a single PAF receptor under different assay conditions. The cloned hPAFR is a member of the family of G protein-coupled receptors (GPCRs) and its signalling has been investigated in endogenous and over-expression systems (Hwang, 1990). Although the involvement of G proteins in PAFR responses has been clearly established, the identification of which G protein transduces the signal to the various cellular effector systems, such as adenylyl cyclase, phospholipase C, phospholipase A2, phospholipase D, mitogen-activated protein kinase and others, remains elusive. In COS 7 cells hPAFR has been shown to induce inositol phosphates (IPs) production through PLCβ1 activation via Gaq/11 (Amatruda, 1993; Murphy, 1994; Parent, 1996). PAF receptor is also classically described as coupled to Gi/o (Gutierrez-Venegas, 1991; Agrawal, 1992). Accordingly, agonist occupancy causes a decrease in adenylyl cyclase activity resulting in a decrement of intracellular cAMP. Some reports demonstrate that, under certain conditions, PAFR mediates a rise in intracellular cAMP, as in bacterial lipopolysaccharide (LPS)-primed P338D1 mouse macrophage-like cells (Asmis, 1994a; Asmis, 1994b). In these cells PAF-induced increase in cAMP levels is not due to a direct activation of adenylyl cyclase, but to an indirect stimulation via the formation and secretion of prostaglandin E2 (PGE2). PGE2 acts in an autocrine manner, interacting with its own receptors, which are coupled to G proteins of the stimulatory class, producing an increase of intracellular cAMP.

As a common feature of GPCR agonists, PAF responses are desensitised in many systems. PAFR is down regulated upon agonist exposure, as monitored by ligand binding, but also homologous desensitisation to PAF has been observed in a variety of cells and tissues with variable mechanisms among different cell types.

The aims of the study were:
1) to investigate the multiple signalling pathways stimulated by PAFR activation. This point was mainly investigated by the expression of recombinant hPAFR in heterologous expression system, which allow the analysis of molecular determinants involved in receptor signalling.

2) to characterise the G proteins and the pathways mediating PAFR signalling. This point was investigated using pharmacological and molecular tools, which allow the definition of the role of different G proteins within different pathways.

3) to investigate the molecular mechanisms of PAFR desensitisation by co-transfection of regulatory proteins and analysis of PAFR signalling.

4) to define the molecular determinants involved in the selective regulation of different PAFR signalling pathways. This point was investigated using molecular tools such as receptor kinases, RGS proteins and recombinant functional domains of regulatory proteins.

3-2 PAFR signalling in COS 7 cells

3-2-1 PAFR stimulates IPs and cAMP production in transfected COS 7 cells

Human PAFR cDNA was cloned from an undifferentiated U937 cell library and a modified hPAFR cDNA was constructed by inserting additional 30 nucleotides after the 5' ATG, encoding the amino acid sequence MDYKDDDDKEF. The Flag epitope is specifically recognised by a monoclonal antibody (Kunz, 1992). This PAFR encoded in pCDM8-FLAG plasmid was used for transient transfections in COS 7 cells. After over-expression in COS 7 cells this receptor was able to couple with various second messengers systems, including activation of phospholipase Cβ (Fig 3-1A). In parent untransfected COS 7 cells, PAF-mediated stimulation of PLC was undetectable, suggesting that an endogenous PAF receptor was not expressed in COS 7 cells.
**Fig 3-1. Dose-response curve for PAF-induced IPs production**

COS 7 cells were transiently transfected in 100 mm petri dishes with hPAFR (10 μg cDNA/2 x 10⁶ cells). 24 hours after transfection, cells were plated in 24-well for inositol phosphates (IPs) production assay.

A 48 hours after transfection, cells were loaded for 24 hours with myo-[³H] inositol. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of PAF. Inositol phosphates production, subtracted basal level (~1500 cpm/well), are expressed as cpm/well. The calculated EC50 is 45.6 nM. Data shown represent mean ± SEM.

B 48 hours after transfection, cells were loaded for 24 hours with myo-[³H] inositol, in absence or in presence of PTX 10⁻⁸ M. 72 hours after transfection cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data are mean ± SEM for three separate experiments, each performed in duplicate. PTX-treatment did not modify PAF-stimulated IPs production in PAFR-transfected cells.
Fig 3-1

A

B

IPs production (cpm/well)

IPs production (cpm/well)

PAF [nM]

PAF [μM]

- .25 .5 1

PTX 10^-8 M

- .25 .5 1
In transfected cells the production of inositol phosphates (IPs = inositol monophosphate + inositol bisphosphate + inositol triphosphate), 30 min after agonist-stimulation, increase dose-dependently on PAF concentration and was not blocked by pertussis toxin (PTX) (Fig 3-1B). This indicates that PAFR activation of PLC in COS 7 cells is mediated through the coupling with a PTX resistant G protein.

Interestingly, when cAMP accumulation studies were performed on intact PAFR-transfected COS 7 cells, PAF was able to stimulate intracellular cAMP production. This stimulation was observed employing the agonist in a range of concentrations going from 0.1 nM to 50 μM (Fig 3-2). In transfected COS 7 cells PAFR stimulation, (PAF from 10 nM to 10 μM), did not inhibit basal nor FSK-stimulated (FSK from 0.5 to 10μM) cAMP accumulation (Fig 3-3A). This indicate that PAFR expressed in COS 7 cells does not couple to Gi (at least as assessed by FSK-stimulated cAMP inhibition). This lack of coupling to Gi was only observed with PAFR. In fact another GPCR, the interleukin-8 receptor (IL8-RA), which is known to be coupled to Gi (Wu, 1993), was able to inhibit FSK-stimulated cAMP accumulation. IL8-RA did not stimulate cAMP formation, under the same conditions, as PAF receptor did (Fig 3-3B).

PAF is a lipid and at high concentrations it can alter the physical properties of cell membranes, it can enter the cell and can act independently from extracellular exposed receptors. But in this case PAF-mediated activation of adenyl cyclase depends on the presence of over-expressed hPAFR, in fact no increase in cAMP level was observed after PAF-mediated stimulation of untransfected COS 7 cells. Moreover, the Ginkgolide B (BN 520211) (Braquet, 1985), a specific PAF receptor antagonist, inhibited PAF-activation of adenyl cyclase in PAFR-transfected COS 7 cells (Fig 3-3A).
Fig 3-2. Dose-response curve for PAF-induced cAMP accumulation

COS 7 cells were transiently transfected in 100 mm petri dishes with hPAFR (10 µg cDNA/2 x 10^6 cells). 24 hours after transfection cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of PAF. cAMP accumulation, subtracted basal level (~1.6 picomoles/well), is expressed as picomoles of cAMP/well. The calculated EC50 is 44.7 nM. Data shown represent the mean ± SEM.
Fig 3-3. PAF stimulates cAMP accumulation in PAFR-transfected COS 7 cells

COS 7 cells were transiently transfected in 100 mm petri dishes with an empty vector (PBJI) or hPAFR or IL8-RA (5 μg cDNA of each/2 x 10^6 cells). After 24 hours, cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated substances. Data are expressed as picomoles of cAMP/well.

A PAF (1 μM) does not stimulate cAMP accumulation in untransfected COS 7 cells. In hPAFR-transfected cells PAF increases not only basal, but also FSK-stimulated cAMP production. cAMP accumulation induced by PAF 1 μM is reduced of ~50% by the addition of BN 520211 (20 μM). PAF is dissolved in Tris HCl 0.15 M, BSA 0.25% at pH 7.6 (Buffer). The buffer alone does not stimulate cAMP formation.

B IL8 (250 nM) was not able to decrease basal cAMP production, but it inhibited FSK-stimulated cAMP accumulation. Data are mean ± SEM values of 3 separate experiments, each performed in triplicate.
Fig 3-3

IL8-RA

---

FSK

BN (20 nM)

FSK (10 nM)

BN (20 nM)

PAP (1 nM)

Buffer

---

cAMP accumulation

(μmoles/well)

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3-2-2 PAFR stimulation of adenylyl cyclase in COS 7 cells does not involve prostaglandin synthesis and action

In order to clarify the mechanism of PAFR-activation of cAMP formation in transfected COS 7 cells, different hypotheses have been tested. In LPS-primed P388D1 cells PAF stimulates adenylyl cyclase as a consequence of PGE2 formation and secretion. The time course of this stimulation showed a great increase of PGE2 production that reached a plateau 5 minutes after PAF exposure (Asmis, 1994a; Asmis, 1994b). This time is compatible with the cAMP accumulation assays performed in the present study in transfected COS 7 cells.

Since, virtually, every cell can form eicosanoids (Campbell, 1990), it was reasonable to suspect that PAF effects on COS 7 cells might be a consequence of prostaglandin synthesis. According with this hypothesis, the blockage of prostaglandin synthesis would impair PAF-stimulated cAMP formation. The cAMP assay was performed in the presence of Flectadol (Campbell, 1990), a cyclooxygenase inhibitor that blocks prostaglandin synthesis, however PAF-stimulated cAMP production was not affected (Fig 3-4A). Moreover, neither the Ca²⁺ ionophore ionomycin, that was able to trigger PGE2 formation and consequent cAMP levels rise in P338D1 cells (Asmis, 1994a; Asmis, 1994b), nor iloprost (a synthetic analogue of prostaglandin PGI2) (Campbell, 1990) were able to stimulate cAMP formation in COS 7 cells (Fig 3-4B). All these data indicate that PAF activation of adenylyl cyclase profoundly diverges from the one described in LPS-primed P388D1 and is not dependent on prostaglandin synthesis.
Fig 3-4. PAF stimulation of adenylyl cyclase is not mediated by prostanoid synthesis in PAFR-transfected COS 7 cells

COS 7 cells were transiently transfected with of hPAFR in 100 mm petri dishes (10 µg cDNA/2 x 10^6 cells). After 24 hours cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated substances. Data are expressed as picomoles of cAMP/well.

A Transfected cells were incubated with PAF (1 µM) alone or with different concentrations of Flectadol (from 0.1 to 1 mM). Flectadol was not able to inhibit neither basal nor PAF-induced cAMP formation.

B COS 7 cells were incubated with two Ca^{2+} ionophores, A23187 (5 µM) and ionomycin (0.5 µM), or iloprost (1 µM). None of them were able to stimulate cAMP accumulation. Data are mean ± SEM values for 3 separate experiments, each performed in triplicate.
**Fig 3-4**

**A**

- **cAMP accumulation (pmoles/well)**
  - Experiment conditions:
    - A23187 5 mM
    - Ion: 0.5 mM
    - Flect: 1 mM

- **PAF (1 μM)**
  - Flect [mM]
  - 0, 1, 5, 10, 15, 20, 25
  - Bars represent the accumulation of cAMP.

**B**

- **cAMP accumulation (pmoles/well)**
  - Experiment conditions:
    - A23187 5 mM
    - Ion: 0.5 mM
    - Flect: 1 mM

- **PAF (1 μM)**
  - Flect [mM]
  - 0, 1, 5, 10, 15, 20, 25
  - Bars represent the accumulation of cAMP.
3-2-3 PAFR activation of adenylyl cyclase does not involve $\beta\gamma$-subunits of Gi

Another possible mechanism of PAFR-dependent stimulation of adenylyl cyclase could involve the direct activation of $\beta\gamma$-sensitive isozymes of adenylyl cyclase, as described for other Gi-coupled receptors (Tang, 1991; Federman, 1992; Simonds, 1999). Several lines of evidence indicated that this was not the case. First of all the effects of pertussis toxin (*Bordetella* pertussis islet-activating protein or PTX) were examined. Pertussis toxin ADP-ribosylates the $\alpha$ subunits of Gi and Go and this modification prevents receptor-mediated activation of these G proteins. PTX-treatment of PAFR-transfected COS 7 cells did not block PAF-mediated stimulation of adenylyl cyclase, as would be expected if $\beta\gamma$ released from Gi or Go were responsible (Fig 3-5A).

As positive control PTX effects, on another transfected G protein-coupled receptor, were verified in COS 7 cells. The same concentration of PTX ($10^{-8}$ M), that did not affect PAFR signalling, inhibited $\alpha_2$-adrenergic receptor ($\alpha_2$-C10 AR) stimulation of PLC in $\alpha_2$AR-transfected COS 7 cells (Fig 3-5B). This process has been largely investigated and involves $\beta\gamma$ subunits of Gi (Luttrell, 1995).

To further confirm that Gi$\beta\gamma$ subunits do not have a role in PAF-activation of adenylyl cyclase a specific Gi$\beta\gamma$-scavenger, the carboxyl terminus of GRK2 (GRK2-Cter or C-ter, hGRK2 from a.a. 495 to 689), was co-expressed to block $\beta\gamma$ signalling in COS 7 cells. This approach has been largely used to demonstrate Gi$\beta\gamma$ involvement in receptor signalling (Koch, 1993; Touhara, 1994; Luttrell, 1995). PAF-mediated cAMP activation was unaffected by GRK2-Cter over-expression indicating that this process is not mediated by $\beta\gamma$ signalling (Fig 3-6A). However, PLC activation produced by UK 14,304 ($\alpha_2$-adrenergic receptor agonist) was significantly inhibited in COS 7 cells transfected with $\alpha_2$-C10 AR and GRK2-Cter (Fig 3-6B).
Fig 3-5. PAF-mediated stimulation of adenylyl cyclase is not inhibited by PTX-treatment in PAFR-transfected COS 7 cells

**A** COS 7 cells were transiently transfected with hPAFR in 100 mm petri dishes (10 μg cDNA/2 x 10^6 cells). 24 hours after transfection, cells were plated in 48-well for cAMP accumulation assay. After other 24 hours cells were starved and incubated in the presence or absence of PTX (10^{-8} M) for 24 hours. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data are expressed as picomoles of cAMP/well. Data are mean ± SEM values for 3-5 separate experiments, each performed in triplicate. PTX-treatment did not modify PAF-stimulated cAMP accumulation, in PAFR-transfected COS 7 cells.

**B** COS 7 cells were transiently transfected with α2AR in 100 mm petri dishes (10 μg cDNA/2 x 10^6 cells). After 24 hours α2AR-transfected cells were plated in 24-well for IPs production assay. Total [^3H] inositol phosphates were measured as described under experimental procedures. Cells were loaded for 24 hours with myo[^3H] inositol in the presence or absence of PTX 10^{-8} M. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with UK 14,304 (1 μM). Data are expressed as cpm/well. Data are mean ± SEM values for three separate experiments, each performed in duplicate. (** p<0.001 versus UK-stimulated IPs production in PTX-untreated cells, as evaluated with two-side unpaired Student t test). PTX-treatment resulted in a significant inhibition UK 14,304-stimulated IPs production.
Fig 3-5

**IPs production**

(cpm/well)

<table>
<thead>
<tr>
<th>UK14,304 (1 μM)</th>
<th>0</th>
<th>500</th>
<th>1000</th>
<th>1500</th>
<th>2000</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ - PTX 10⁻³ M</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**cAMP accumulation**

(pmoles/well)

<table>
<thead>
<tr>
<th>PAF (μM)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ - PTX 10⁻³ M</td>
<td>+</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig 3-6. PAF-mediated stimulation of adenylyl cyclase is not suppressed by a fγ-scavenger co-expressed in PAFR-transfected COS 7 cells

A COS 7 cells were transiently transfected in 100 mm petri dishes with hPAFR and C-ter or an empty vector (PBJI) (8 µg cDNA of each/2 x 10^6 cells). After 24 hours cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. cAMP levels, subtracted basal activity (~1.3 picomoles/well), are expressed as picomoles of cAMP/well. Data are mean ± SEM values for 3-5 separate experiments, each performed in triplicate. C-ter co-expression did not modify PAF-stimulated cAMP accumulation at each dose of PAF.

B COS 7 cells were transiently transfected in 100 mm petri dishes with α2AR and C-ter or an empty vector (PBJI) (8 µg cDNA of each/2 x 10^6 cells). After 24 hours α2AR-transfected cells were plated in 24-well for IPs production assay. Total [³H] inositol phosphates were measured as described under experimental procedures. Cells were loaded for 24 hours with myo-[³H] inositol. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with UK 14,304 (1 µM). Data are expressed as cpm/well. Data are mean ± SEM values for three separate experiments, each performed in duplicate. (* p<0.01 versus UK 14,304-stimulated α2AR/PBJI-transfected cells, as evaluated with two-side unpaired Student t test). C-ter co-expression resulted in a significant inhibition of UK 14,304-stimulated IPs production.

C Samples containing 100 µg of postnuclear proteins, from transfected cells, were suspended in Laemmli buffer and electrophoresed on SDS polyacrylamide homogenous slab gel (15% SDS-PAGE). Proteins were electroblotted onto nitro-cellulose membranes. The membrane has been revealed using the affinity-purified rabbit polyclonal anti-GRK2 antibody raised against GST-human GRK2 C-terminus (last 222-amino acids) fusion protein, kindly provided by H. Le Vine III (Chuang, 1995). The lines contain, starting from the left: GRK2 positive control purified from SF9 (+cont.), samples from PAFR/C-ter and α2AR/C-ter-transfected COS 7 cells.
Fig 3-6

**PAFR**

![Bar graph showing cAMP accumulation](image)

**α₂AR**

![Bar graph showing IPs production](image)

**Western Blot**

[Image of Western Blot showing GRK2 and C-ter]
GRK2-Cter cDNA transfection was evaluated by Western blot analysis (Fig 3-6C). These data confirm that, while Gβγ subunits represent the mechanism mediating α2-adrenergic receptor activation of PLC (Luttrell, 1995), they are not involved in PAF-mediated cAMP stimulation.

3-2-4 PAFR couples with Gαs in PAFR-transfected COS 7 cells

Based on these results the working hypothesis was that PAFR could functionally couple to Gαs. In order to investigate PAFR coupling with Gαs, cholera toxin (CTX) was used. CTX catalyses the transfer of the ADP-ribose moiety of NAD to a specific Arg residue in Gαs subunits. In this way CTX constitutively activates these proteins by inhibiting their GTPase activity. CTX-pre-treatment of COS 7 cells would reduce the available pool of Gαs that can be activated by a Gs-coupled receptor, reducing receptor ability to stimulate adenylyl cyclase. This approach has been previously used by Eason and co-workers to demonstrate α2-adrenergic receptor coupling with Gs (Eason, 1995b). Indeed, pretreatment of COS 7 cells overnight with cholera toxin decreased PAF-mediated stimulation of adenylyl cyclase (Fig 3-7). Similar results were obtained with TSHR, which has been demonstrated to be coupled to Gs in transfected COS 7 cells (Biebermann, 1998). However, while these results suggest a PAFR coupling with Gs, they are difficulty to interpretate, because cholera toxin treatment directly and maximally activates Gs-mediated adenylyl cyclase activity.

A more successful approach involves dominant negative constructs of the alpha subunits of G-proteins, which can selectively antagonise the signal transduction of a specific G protein in vivo. In a recent paper (Gilchrist, 1999) Hamm and co-workers made minigene plasmid constructs that encode oligonucleotide sequences corresponding to the carboxyl-terminal undecapeptide of Gαi, Gαq, Gαs.
Fig 3-7. CTX effects on PAFR and TSHR in COS 7 cells

COS 7 cells were transiently transfected in 100 mm petri dishes with hPAFR (5 μg cDNA/2 x 10^6 cells) and hTSHR (5 μg cDNA/2 x 10^6 cells). After 24 hours cells were plated in 48-well for cAMP accumulation assay. 48 hours after transfection, cells were starved and incubated in the presence or absence of CTX (20μg/ml) for 24 hours. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonists. Data, subtracted basal level (~3 picomoles/well for CTX-untreated cells and ~400 picomoles/well for CTX-treated cells), are expressed as folds of increase over basal. CTX-treatment seems to block PAF- and TSH-stimulated cAMP accumulation. (** p<0.001 versus CTX-untreated control cells, as evaluated with two-side unpaired Student t test). Data are the mean ± SEM values for three separate experiments, each performed in triplicate.
cAMP accumulation
(fold over basal)

- TSH (1 nM)
- PAF (1 µM)
- CTX
- PAF/RTSHR

Fig 3.7
They demonstrated that after transient transfection the minigene constructs selectively blocked agonist-mediated response. This approach depends on competitive inhibition. When the minigene of the carboxyl-terminal undecapeptide of Gαs (GsDN) was co-transfected with hPAFR, basal IPs were significantly increased (445 ± 46 in PAFR/PBJI and 590 ± 72 in PAFR/GsDN), while basal cAMP levels remained unchanged. Interestingly specific blockage of PAF-mediated adenylyl cyclase activation was observed (~75% of inhibition), without interference with the IPs accumulation (Fig 3-8). These results clearly support a coupling of PAFR with G-proteins of the stimulatory class in PAFR-transfected COS 7 cells.

3-2-5 PAFR in lymphocytes

Although the physiological significance of Gαs activation by PAFR in transfected cells remains unclear, the stimulation of adenylyl cyclase occurs with an EC50 that is sub-micromolar (EC50 ~44 nM), which might be in the physiological range (Fig 3-2). It suggests that coupling of PAFR to adenylyl cyclase may be important in some cell types.

In order to demonstrate that this coupling can be observed in native cells, different primary cell types were analysed for their capacity to respond to PAF via adenylyl cyclase activation. PAFR is expressed on granulocytes, Kupffer cells, platelets, monocytes, endothelial cells and fibroblasts (Hwang, 1990). A positive result was obtained with lymphocytes separated from peripheral blood leukocytes. When these cells were stimulated with PAF an increase of intracellular cAMP was measured (Fig 3-9). This increase is blocked by BN 52021 (20 μM), a PAFR antagonist, supporting a receptor-mediated activity and is not inhibited by Flectadol (1 mM), a cyclooxygenase inhibitor, excluding a prostaglandin-mediated mechanism of action.
**Fig 3-8. A dominant negative G\(\alpha_s\) construct inhibits PAFR-mediated cAMP activation**

**A** COS 7 cells were transiently transfected in 100 mm petri dishes with hPAFR (8 \(\mu g\) cDNA/2 \(\times 10^6\) cells) alone or in presence of a dominant negative construct of G\(\alpha_s\) (GsDN) (30 \(\mu g\) cDNA/2 \(\times 10^6\) cells). After 24 hours, cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data, subtracted basal level (~1 picomoles/well), are expressed as picomoles of cAMP/well. Data are mean ± SEM values for 3 separate experiments, each performed in triplicate. (* \(p<0.05\) versus PAFR/PBJI-transfected cells stimulated with PAF 5 nM, ** \(p<0.001\) versus PAFR/PBJI-transfected cells stimulated with PAF 250 nM, as evaluated with repeated ANOVA and Turkey post test). G\(\alpha_s\) dominant negative co-expression resulted in a significant inhibition of agonist-stimulated cAMP accumulation.

**B** The same co-transfected cells, analysed for cAMP accumulation, were subjected to the measure of IPs production. 24 hours after transfection, cells were plated in 24-well for IPs production assay. Total \(^3\)H inositol phosphates were measured as described under experimental procedures. Cells were loaded for 24 hours with myo-\(^3\)H inositol. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentration of PAF. Data, subtracted basal level (~450 cpm/well in PAFR/PBJI and ~600 cpm/well in PAFR/GsDN), are expressed as cpm/well. Data are mean ± SEM values for three separate experiments, each performed in duplicate. G\(\alpha_s\) dominant negative co-expression did not modify PAF-stimulated IPs production at each dose of PAF.
Fig 3-9. PAF stimulates cAMP accumulation in purified lymphocytes

Lymphocytes, separated from peripheral blood leukocytes (as described under experimental procedures), were stimulated with different concentrations of PAF (10-1,000 nM), incubated at 37 °C for 10 min and subjected to cAMP accumulation analysis. 10 nM and higher concentrations of PAF were able to produce an increase of the basal level of intracellular cAMP in these cells. Data are expressed as cpm/well. Data are the mean ± SEM values for 3-5 separate experiments, each performed in duplicate.
Northern blot analysis, performed on mRNA extracted from resting T-lymphocytes, revealed that PAFR is not expressed on these cells (Fig 3-13). Other data support this observation (Calabresse, 1992). Peripheral blood leukocytes utilised in whole cell cAMP assay are a mixed population of T- and B-lymphocytes. B-lymphocytes represent about 10% of the total population and is well known that B-cells and B cell lines express a functionally active PAFR (Mazer, 1992; Muller, 1993).

**3-3 PAFR homologous desensitisation**

After having established that transfected PAFR will signal in COS 7 cells, the mechanisms modulating PAFR signalling were analysed.

**3-3-1 Members of the GRK family desensitised PAFR in COS 7 cells**

To assess this point a method to study receptor homologous desensitisation was developed. The method is based on transient expression of PAF receptor alone or co-transfected with βarrestins and GRKs in COS 7 cells. If the receptor is under the control of GRKs and arrestins, the stimulation of intracellular second messengers should be quenched in cells co-transfected with these regulatory proteins. To determine which GRKs are responsible for down modulating PAFR, five different GRKs were transiently co-transfected with the receptor.

In each transfection, cAMP and IPs levels in response to PAF were studied, in comparison with cells expressing the receptor alone. GRK2 and GRK3 inhibit PAFR signalling and in each case two of the most common biochemical modifications occurring during homologous desensitisation of G protein-coupled receptors were observed, namely a decreased sensitivity of the receptor to the agonist and agonist-reduced ability to mediate the maximal response. Moreover when GRK2 and GRK3 were expressed at the same level, GRK3 was significantly more
potent than GRK2 in blocking both IPs production and cAMP accumulation (Fig 3-10).

A control was performed on the efficiency of receptor transfection, by measuring receptor expression in each co-transfection assay. For this reason, flow cytometric analysis were performed on transfected cells, with antibodies that recognise the extracellular FLAG sequence of PAFR, showing that PAFR surface expression was not affected by GRK2 or GRK3 co-expression (Fig 3-11A). GRK2 and GRK3 expression levels were studied with Western blot analysis using a monoclonal antibody that recognises the same epitope on both kinases (Oppermann, 1996b) (Fig 3-11B).

Among the GRK4 subfamily, composed of GRK4, GRK5 and GRK6, only GRK6 was able to desensitise PAFR signalling (Fig 3-12). GRK6 is among the most recently identified members of the GRK family. In order to clarify if GRK6 tissue distribution could be related to PAFR, Northern blot analysis on different sub-populations of leukocytes were performed and both PAFR and GRK6 levels of expression were studied. The two proteins appear to have the same pattern of expression (Fig 3-13).

Mononuclear leukocytes express mRNAs of both PAFR and GRK6, but, among this population of leukocytes, monocytes have the highest expression levels for both PAFR and GRK6 mRNAs. Also granulocytes presented elevated levels of expression of both mRNAs, while resting T lymphocytes do not express PAFR, as reported in literature (Calabresse, 1992). These data suggest that monocytes and granulocytes are cells in which PAFR signalling could be regulated by GRK6.

The effects of two arrestin proteins on receptor-mediated response were also investigated. For β2AR and rhodopsin the uncoupling between receptor and G protein, occurring during desensitisation, is mediated by arrestin proteins, which specifically bind to the receptor after its phosphorylation by GRKs (Gurevich, 1995).
Fig 3-10. GRK2 and GRK3 desensitised PAFR in COS 7 transfected cells

A COS 7 cells were all transiently transfected in 100 mm petri dishes with hPAFR (8 µg cDNA/2 x 10^6 cells) and co-transfected with GRK2 (5 µg cDNA/2 x 10^6 cells) or GRK3 (8 µg cDNA/2 x 10^6 cells). All the cDNA amounts were made equal with empty vector (PBJI). After 24 hours, cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data, subtracted basal level (~1.3 picomoles/well), are expressed as picomoles of cAMP/well. Data are mean ± SEM values for 3-5 separate experiments, each performed in triplicate. GRK2 and GRK3 co-expressions were able to inhibit PAF-stimulated cAMP accumulation in PAFR-transfected COS 7 cells.

B The same co-transfected cells, analysed for cAMP accumulation, were subjected to the measure of IPs production. Cells were loaded for 24 hours with myo-[³H] inositol. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data, subtracted basal level (~1400 cpm/well), are expressed as cpm/well. Data are mean ± SEM values for three separate experiments, each performed in duplicate. (** p<0.01 and * p<0.05 versus PAFR/PBJI-transfected COS 7 cells stimulated with the same concentration of PAF, as evaluated with ordinary ANOVA). Both GRK2 and GRK3 co-expression were able to block PAF-stimulated IPs accumulation. There was a significant greater efficacy of GRK3 in inhibiting PAF-stimulated IPs production compared with GRK2 (* p<0.05 versus GRK2-transfected COS 7 cells stimulated with the same concentration of PAF, as evaluated with ordinary ANOVA).
Fig 3-10

A

**cAMP accumulation**

(units: pmoles/well)

**PAF [nM]**

- **PAFR/PBII**
- **PAFR/GRK2**
- **PAFR/GRK3**

B

**IPs production**

(units: cpm/well)

**PAF [nM]**

- **PAFR/PBII**
- **PAFR/GRK2**
- **PAFR/GRK3**
Fig 3-11. Controls of transfection efficiency

A PAFR transfection efficiency was verified with flow cytometric analysis. FLAG-PAFR expression was measured, as described under experimental procedures, by binding of monoclonal antibodies, anti-FLAG M5 monoclonal antibody (Eastman Kodak Company) directed at MDYKDDDDKEF amino acid sequence present at the N-terminal Met-FLAG fusion protein of PAF receptor (Kunz, 1992). They were analysed on a FACScan flow cytometer. Mock-transfected cells were stained with anti-FLAGM5 antibody to identify the peak of untransfected cells. In samples transfected with the receptor, cells whose fluorescence intensity exceeded the negative marker were considered positive for transfection and their mean intensity of fluorescence (MIF) was utilised as an index of receptor expression. MIF referring to PAFR/PBJI-transfected cells was considered as 100% and GRK2 and GRK3 co-transfected cells were quantified for comparison. Data are mean ± SEM values for 3-5 separate experiments, each performed in duplicate. GRK2 and GRK3 co-transfection did not alter PAFR transfection efficiency.

B Western blot analysis: Samples containing 100 µg of postnuclear proteins, from transfected cells, were suspended in Laemmli buffer and electrophoresed on SDS polyacrylamide homogenous slab gel (10% SDS-PAGE). Proteins were electroblotted onto nitro-cellulose membranes. The membrane has been revealed using anti-GRK2/3 clone C5/1.1 (monoclonal antibody available from Upstate Biotechnology). This antibody recognises with the same efficiency GRK2 and GRK3 making possible a quantitative comparison of the expression level of two kinases. The lines contain, starting from the left: samples from PAFR/PBJI, PAFR/GRK2, PAFR/GRK3-transfected COS 7 cells and GRK2 positive control purified from SF9 (+cont.).
A

Transfection efficiency (% of control)

+ PBJI  + GRK2  + GRK3

PAFR

B

Western Blot

PAFR/ PBJI  PAFR/ GRK2  PAFR/ GRK3  +cont.

79.6 KDa

GRK2

GRK3
**Fig 3-12. PAFR is desensitised by GRK6 in COS 7 cells**

**A** COS 7 cells were all transiently transfected with hPAFR (8 μg cDNA/2 x 10⁶ cells) and co-transfected with GRK4 (8 μg cDNA/2 x 10⁶ cells) or GRK5 (8 μg cDNA/2 x 10⁶ cells) or GRK6 (8 μg cDNA/2 x 10⁶ cells). All the amounts of cDNA were made equal by adding the empty vector (PBJI). After 24 hours, cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data, subtracted basal level (~1.3 picomoles/well), are expressed as picomoles of cAMP/well. Data are mean ± SEM values for 3-5 separate experiments, each performed in triplicate. (**) p<0.01 versus PAFR/PBJI-transfected COS 7 cells stimulated with the same concentration of PAF, two tailed p value as evaluated with Student t test). Only GRK6 co-expression was able to inhibit PAF-stimulated cAMP accumulation in PAFR-transfected COS 7 cells.

**B** The same co-transfected cells, analysed for cAMP accumulation, were subjected to the measure of IPs production. 24 hours after transfection, cells were plated in 24-well for IPs production assay. Total [³H] inositol phosphates were measured as described under experimental procedures. Cells were loaded for 24 hours with myo-[³H] inositol. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. In the graphic are reported data referring to PAFR/PBJI and PAFR/GRK6. Data, subtracted basal level (~1400 cpm/well), are expressed as cpm/well. Data are mean ± SEM values for three separate experiments, each performed in duplicate (* p<0.05 and **) p<0.001 versus PAFR/PBJI-transfected COS 7 cells stimulated with the same concentration of PAF, two tailed p value as evaluated with Student t test). Neither GRK4 nor GRK5 were able to inhibit PAF-stimulated IPs accumulation in PAFR-transfected COS 7 cells, while GRK6 induced a significant inhibition.
Fig 3-12

**A**

![Graph showing cAMP accumulation (pmoles/well) vs. PAF [nM].](image)

**B**

![Graph showing IPs production (cpm/well) vs. PAF [nM].](image)
Fig 3-13. Northern bolt analysis of GRK6 and PAFR mRNA expression

20 μg of total RNA from different leukocyte sub-populations were separated on a denaturing 1% agarose gel electrophoresis (containing 6% formaldehyde). The electrophoresed RNA was transferred to a nylon filter (Gene Screen Plus membrane Du Pont-New England Nuclear). The samples were loaded in the following order: 1) mononuclear leukocytes, 2) lymphocytes, 3) monocytes, 4) granulocytes, and 5) resting T-lymphocytes.

A PAFR cDNA probe (the entire coding sequence of human PAFR labelled by random priming) detected a transcript of ~4 Kb expressed in monocytes and mainly in granulocytes.

B GRK6 cDNA probe (GRK6 cDNA fragment bp 1413-1822 labelled by random priming) detected two transcripts of ~3.6 and 3 Kb mainly expressed in granulocytes. Both blots were exposed for autoradiography 24-48 hours at -80 °C.

C The panel reported ethidium bromide staining of 28S rRNA.
Fig 3-13

A

1 2 3 4 5

PAFR

~4 Kb

B

GRK6

~3 Kb

~2.4 Kb

C

rRNA

28S
Much less is known about the role of arrestins in desensitisation of other G protein-coupled receptors. Thus we attempted to assess the effect of two different arrestin proteins, βarrestin1 and βarrestin2 on PAFR signalling. The level of over-expression of both arrestin proteins was assessed with Western blot analysis, with an antibody that equally recognises both βarrestin1 and 2 (Fig 3-14C). Neither βarrestin1 nor βarrestin2 were able to block PAFR signalling (Fig 3-14A, B), suggesting that in these cells βarrestin expression is not a limiting factor in the process of desensitisation in presence of over-expressed PAFR. On the contrary, in similar experimental conditions, βarrestin1 was able to inhibit thyrotropin receptor-stimulated response (Iacovelli, 1996).

3-3-2 Phosphorylation-independent mechanisms regulating PAFR signalling

In order to verify whether PAFR desensitisation, observed in the presence of co-transfected GRKs, required the kinase activity of these regulatory proteins, experiments with a GRK2 dead kinase (GRK2-K220R or K220R) were performed. K220R mutant was generated in Benovic’s laboratory mutating a single amino acid in the GRK2 sequence. The structural features of GRK2 include a centrally localised catalytic domain, containing all the conserved amino acid residues shared by the extended family of protein kinases. A universally conserved lysine in subdomain II of the catalytic domain (Lys 220) has been shown to be directly involved in the phospho-transfer reaction from the ATP molecule to the substrate. Amino acid substitution of this lysine to arginine generated a dominant negative GRK2 (Kong, 1994) (Fig 3-15). Despite the loss of kinase activity of K220R mutant, this mutant retained the ability to inhibit PAFR signalling in co-transfected cells.
Fig 3-14. βarrestins do not desensitise PAFR in COS 7 transfected cells

A COS 7 cells were all transiently transfected with hPAFR (8 µg cDNA/2 x 10^6 cells) and co-transfected with human βarr1 (8 µg cDNA/2 x 10^6 cells) or bovine βarr2 (2 µg cDNA/2 x 10^6 cells). All the cDNA amounts were made equal by adding the empty vector. After 24 hours, cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data, subtracted basal level (~1.3 picomoles/well), are expressed as picomoles of cAMP/well. Data are mean ± SEM values for 3-5 separate experiments, each performed in triplicate. Neither βarr1 nor βarr2 were able to inhibit PAF-stimulated cAMP accumulation in PAFR-transfected COS 7 cells.

B The same co-transfected cells, analysed for cAMP accumulation, were subjected to the measure of IPs production. 24 hours after transfection, cells were plated in 24-well for IPs production assay. Total [³H] inositol phosphates were measured as described under experimental procedures. Cells were loaded for 24 hours with myo-[³H] inositol. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data, subtracted basal level (~1400 cpm/well), are expressed as cpm/well. Data are mean ± SEM values for two separate experiments, each performed in duplicate. Neither βarr1 nor βarr2 were able to inhibit PAF-stimulated IPs accumulation in PAFR-transfected COS 7 cells.

C Western blot analysis: Samples containing 100 µg of postnuclear proteins, from transfected cells, were suspended in Laemmli buffer and electrophoresed on SDS polyacrylamide homogenous slab gel (10% SDS-PAGE). Proteins were electroblotted onto nitro-cellulose membranes. The membrane has been revealed using the monoclonal antibody F4C1, anti-arrestins. This antibody recognises with the same efficiency βarr1 and βarr2 making possible a quantitative comparison of the expression level of the two arrestins. The lines contain, starting from the left: samples from PAFR/PBJI, PAFR/βarr1, PAFR/βarr2-transfected COS 7 cells.
**Fig 3-14**

A. Western Blot

- **Western Blot**
- **48 KDa**
- **BARR1**
- **BARR2**

B. CAMP accumulation (pmoles/well)

- **PAFR / PBJI**
- **PAFR / Barr1**
- **PAFR / Barr2**

<table>
<thead>
<tr>
<th>PAF [nM]</th>
<th>50</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAMP</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

C. IPs formation (cpm/well)

- **PAFR/PBJI**
- **PAFR / Barr1**
- **PAFR / Barr2**

<table>
<thead>
<tr>
<th>PAF [nM]</th>
<th>50</th>
<th>500</th>
</tr>
</thead>
<tbody>
<tr>
<td>IPs</td>
<td>4000</td>
<td>3000</td>
</tr>
</tbody>
</table>
Fig 3-15. Schematic representation of GRK2, K220R, N-ter and C-ter

a GRK2 structure: the boxed portion represents the catalytic domain that contains the ATP-binding region formed by the DLG sequence. In the catalytic domain is also located the lysine (K) involved in the phospho-transfer reaction. RGS domain (RGS dom.) (from a.a. 51 to 171) and the pleckstrin homology domain sequence (PH dom.) (from a.a. 550 to 656) are indicated. b K220R structure: GRK2 invariant lysine has been mutated to arginine (R) (Kong, 1994). c N-ter is a fusion protein with an anti-Xpress tag (Invitrogen) and GRK2 sequence from a.a. 1 to 188. d C-ter is a minigene construct that contains GRK2 sequence from a.a. 495 to 689.
In functional assays the mutant did not impair agonist-induced stimulation of adenylyl cyclase, but PAFR-induced IPs production was significantly blunted (Fig 3-16). These results confirmed the presence of two different PAFR-activated pathways, which are differently regulated. While PAF activation of adenylyl cyclase is susceptible of a classical control through a phosphorylation process, PAF activation of PLC presents a more complex regulation, not necessarily requiring receptor phosphorylation. In order to demonstrate which domain of GRK2 could play a role in the regulation of PLC activation, GRK2 structure was studied, paying attention to specific domains, which can interact with proteins involved in cellular signalling and cytoskeletal functions (Fig 3-15). GRK2 COOH-terminal region (GRK2-Cter or C-ter) contains a pleckstrin homology domain sequence (PH dom.), which has been demonstrated to bind \( \beta \gamma \) subunits, subtracting them from intracellular signalling (Koch, 1993; Inglese, 1994). The NH\(_2\)-terminal region (GRK2-Nter or N-ter) contains a putative RGS domain (a.a. 51 to 171) (Siderovski, 1996). Expression vectors containing GRK2-Nter (from a.a. 1 to 187) and GRK2-Cter (from a.a. 495 to 689) were co-transfected with PAFR. These fragments do not contain the catalytic domain of the kinase. In co-transfected cells agonist-stimulated IPs production presented a blunting, while cAMP accumulation was not affected. These results are summarised in Fig 3-16, in which GRK2, GRK2-K220R, GRK2-Cter and GRK2-Nter, that were expressed at similar levels as indicated in Fig 3-17, were compared for their capacity to modulate PAFR signalling. An additional control was performed on receptor expression. Since co-transfection might reduce the number of cell surface receptors with a consequent decrease of second messenger production, we measured PAFR expression under the co-transfection conditions used.
Fig 3-16. PAFR co-transfected with GRK2, K220R, N-ter and C-ter in COS 7 cells

A COS 7 cells were all transiently transfected with hPAFR (8 μg cDNA/2 x 10^6 cells) and co-transfected with: the empty vector PBJ1 (16 μg cDNA/2 x 10^6 cells), or GRK2 (8 μg cDNA/2 x 10^6 cells), or K220R (5 μg cDNA/2 x 10^6 cells), or N-ter (16 μg cDNA/2 x 10^6 cells), or C-ter (8 μg cDNA/2 x 10^6 cells). All the cDNA amounts were made equal by adding the empty vector (PBJ1). After 24 hours, cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection, cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data, subtracted basal level (~1.3 picomoles/well), are expressed as picomoles of cAMP/well. Data are mean ± SEM values for 3-5 separate experiments, each performed in triplicate.

B The same co-transfected cells, analysed for cAMP accumulation, were subjected to the measure of total [3H] inositol phosphates. Data, subtracted basal level (~1400 cpm/well), are expressed as cpm/well. Data are mean ± SEM values for three separate experiments each performed in duplicate. (** p<0.01 and * p<0.05 versus PAFR/PBJ1-transfected COS 7 cells stimulated with the same concentration of PAF, as evaluated with ordinary ANOVA).

C PAFR transfection efficiency was verified with flow cytometric analysis. FLAG-PAFR expression was measured, as described under experimental procedures. Transfected cells were analysed on a FACScan flow cytometer. Mock-transfected cells were stained with anti-FLAGM5 antibody to identify the peak of untransfected cells. In samples transfected with the receptor, cells whose fluorescence intensity exceeded the negative marker were considered positive for transfection and their mean intensity of fluorescence (MIF) was utilised as an index of receptor expression. MIF referring to PAFR/PBJ1-transfected cells was considered as 100% and GRK2, K220R, N-ter, C-ter co-transfected cells were quantified for comparison. Data are mean ± SEM values for 3-5 separate experiments, each performed in duplicate.
Fig 3-16

**A**

**CAMP accumulation** (pmoles/well)

- PAF 5 nM
- PAF 250 nM

<table>
<thead>
<tr>
<th>Condition</th>
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<th>250 nM</th>
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</thead>
<tbody>
<tr>
<td>+vector</td>
<td>1.0</td>
<td>±0.2</td>
</tr>
<tr>
<td>+GRK2</td>
<td>1.2</td>
<td>±0.3</td>
</tr>
<tr>
<td>+K220R</td>
<td>2.0</td>
<td>±0.5</td>
</tr>
<tr>
<td>+N-ter</td>
<td>2.5</td>
<td>±0.6</td>
</tr>
<tr>
<td>+C-ter</td>
<td>2.7</td>
<td>±0.7</td>
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</table>

**B**

**IPs production** (cpm/well)

- PAF 5 nM
- PAF 250 nM

<table>
<thead>
<tr>
<th>Condition</th>
<th>5 nM</th>
<th>250 nM</th>
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<tbody>
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<td>+K220R</td>
<td>4000</td>
<td>±700</td>
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<tr>
<td>+N-ter</td>
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<td>±800</td>
</tr>
<tr>
<td>+C-ter</td>
<td>479</td>
<td>±90</td>
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</table>

**C**

**Transfection efficiency** (% of control)

<table>
<thead>
<tr>
<th>Condition</th>
<th>PAFR/ PBJI</th>
<th>PAFR/ GRK2</th>
<th>PAFR/ K220R</th>
<th>PAFR/ N-ter</th>
<th>PAFR/ C-ter</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>100%</td>
<td>105±5%</td>
<td>103±4%</td>
<td>98±1%</td>
<td>95±3%</td>
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Fig 3-17. Western blot analysis of over-expressed GRK2, K220R, N-ter and C-ter in COS 7 cells

Samples containing 100 µg of postnuclear proteins, from transfected cells, were suspended in Laemmli buffer and electrophoresed on SDS polyacrylamide homogenous slab gel (15% SDS-PAGE). Proteins were electroblotted onto nitro-cellulose membranes.

A The membrane has been revealed using the affinity-purified rabbit polyclonal anti-GRK2 antibody raised against GRK2 N-terminus, kindly provided by F. Boulay. The lines represent: 1) GRK2 positive control purified from SF9, 2) 100 µg of postnuclear proteins from COS 7 cells transfected with PAFR and three different amounts of GRK2-Nter. In functional experiments N-ter over-expression represented in line n°3 had been taken in consideration and had been compared with GRK2 and K220R expression level. 5) 100 µg of postnuclear proteins from COS 7 cells transfected with PAFR and GRK2, 6) 100 µg of postnuclear proteins from COS 7 cells transfected with PAFR and K220R, 7) 100 µg of postnuclear proteins from COS 7 cells transfected with PAFR and the empty vector. On the left are indicated molecular weight markers.

B The membrane has been revealed using the affinity-purified rabbit polyclonal anti-GRK2 antibody raised against GST-human GRK2 C-terminus (last 222-amino acids) fusion protein, kindly provided by H. LeVine III (Chuang, 1995). The lines represent: 1) GRK2 positive control purified from SF9, 2) 100 µg of postnuclear proteins from COS 7 cells transfected with PAFR and GRK2, 3)-4) 100 µg of postnuclear proteins from COS 7 cells transfected with PAFR and two different amounts of C-ter. In functional experiments C-ter over-expression represented in line n° 3 had been taken in consideration. 5) 100 µg of postnuclear proteins from COS 7 cells transfected with PAFR and the empty vector. On the right are indicated molecular weight markers. 79.6 kDa is the molecular weight of GRK2. 26.6 kDa and 22.8 kDa are the molecular weight of GRK2-Nter and GRK2-Cter respectively.
Fig 3-17

**A**

Anti-GRK2 Blots

<table>
<thead>
<tr>
<th>Lane</th>
<th>26.6 kDa</th>
<th>43 kDa</th>
<th>67 kDa</th>
<th>94 kDa</th>
<th>79.6 kDa</th>
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**B**

C-ter

<table>
<thead>
<tr>
<th>Lane</th>
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<th>67 kDa</th>
<th>94 kDa</th>
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Binding of antibodies, produced against the FLAG sequence of PAFR, was analysed by means of flow cytometry on transfected cells. We found that PAFR surface expression was not affected in all the co-transfection conditions (Fig 3-16C) and consequently the reduced activation of PLC observed in co-transfected cells cannot be attributed to a decreased expression of the receptor. In the case of co-transfected GRK2-Cter, the blockage of inositol phosphate synthesis suggests a role of $\gamma\gamma$ subunits in PAFR activation of PLC, even if, in these cells, PAFR-induced stimulation of PLC has been demonstrated to be primarily due to $\gamma q$. On the other hand the decrease of IPs production observed after co-expression of GRK2-Nter was unexpected.

3-3-3 PAFR signalling is modulated in a similar way by GRK2 N-ter and RGS4

"Regulators of G-protein signalling" (RGS) proteins constitute a newly identified group of negative regulators of G-proteins signalling (Koelle, 1997; Hepler, 1999). They have the ability to dampen signalling via $\gamma i$, $\gamma q/11$- and $\gamma 12/13$-coupled pathways, but cannot modulate $\gamma s$-mediated signalling (De Vries, 1999). In functional assays GRK2-Nter does not affect PAF-induced cAMP accumulation, which is likely to be due to $\gamma s$-coupling, but it inhibits PAF-induced stimulation of PLC. It is reasonable to suppose that the last process is due to $\gamma q$ inhibition by an RGS-like molecule. GRK2-Nter was then compared with RGS4 concerning its ability to modulate PAFR signalling. RGS4 is one of the most extensively studied members of the RGS family (Berman, 1996). When RGS4 was co-transfected with PAFR, agonist activation of PLC remained blunted, while cAMP accumulation was unaffected (Fig 3-18). The mechanism of RGS regulation of G-proteins is not completely understood at the molecular level. RGS4 has been demonstrated to regulate Gq class signalling with a combined action (Zeng, 1998).
Fig 3-18. PAFR co-expression with RGS4 in COS 7 cells

A COS 7 cells were transiently transfected with hPAFR (8 µg cDNA/2 x 10⁶ cells) alone or co-transfected with RGS4 (8 µg cDNA/2 x 10⁶ cells). After 24 hours, cells were plated in 48-well for cAMP accumulation assay. 72 hours after transfection cells were stimulated for 30 min at 37 °C with the indicated concentrations of the agonist. Data are expressed as picomoles of cAMP/well. Data are mean ± SEM values for three separate experiments, each performed in triplicate. RGS4 over-expression was not able to inhibit cAMP accumulation stimulated by PAF in PAFR-transfected COS 7 cells.

B The same co-transfected cells, analysed for cAMP accumulation, were subjected to the measure of IPs production, as described under experimental procedures. Data are expressed as cpm/well. Data are mean ± SEM values for three separate experiments each performed in duplicate. (* p<0.05 versus PAFR/PBJI cells stimulated with the same agonist concentration, as evaluated with ordinary ANOVA). RGS4 co-expression was able to block PAF-stimulated IPs production.
It acts by accelerating the rate of the GTPase reaction, catalysed by the alpha subunits of Gaαi or Gaαq, working as an allosteric catalyst. On the other hand it has high affinity for the Gaαq and its binding conveys competitive inhibition of effector activation, acting as an effector antagonist at the level of the Gaα subunit.

3-4-1 Discussion

3-4-2 PAFR coupling with Gaαs

A large number of G protein-coupled receptors have been found to exert multiple intracellular effects, either by coupling to multiple G proteins and subsequent activation of multiple effectors, by coupling to an individual G protein, and via the secondary influences of one signalling cascade on others. Numerous reports indicate that under certain circumstances, receptors coupled to Gi proteins elicit stimulation of cAMP production. More interestingly, several lines of evidence strongly suggest that these receptors are coupled directly to Gs as well as to Gi (Eason, 1992; Cruciani, 1993; Van Sande, 1993). Such receptors show a complex biphasic response. For instance the α2-adrenergic receptor, after over-expression in heterologous systems, inhibit adenylyl cyclase at low agonist concentrations, while at high agonist concentrations a stimulation of adenylyl cyclase was seen (Eason, 1992). Further investigations showed that in COS 7 cells α2AR stimulates cAMP synthesis through adenylyl cyclase type-II (AC-II) by releasing βγ from Gi and simultaneously interacting with Gs, to provide the activated Gaαs-GTP, required for stimulation of AC-II activity by βγ (Federman, 1992). In fact βγ subunits can activate two isoforms of adenylyl cyclase but only in the presence of activated Gs (Simonds, 1999). Even if coupling to multiple signal transduction pathways is common among G protein-coupled receptors, the ability to couple to Gi as well as to Gs is
particularly intriguing, because the receptor is able to simultaneously evoke both stimulatory and inhibitory regulation of a single effector. PAF-mediated stimulation of COS 7 cells, transfected with hPAFR, produced activation of adenylyl cyclase in a receptor-mediated manner. In fact, untransfected cells did not respond to the agonist and a PAF receptor antagonist could block PAF-promoted cAMP accumulation in transfected cells. This stimulation is not mediated by prostaglandin synthesis because an inhibitor of eicosanoid synthesis was not able to block this signalling. Moreover, this stimulation cannot be explained with Gi/o coupling and release of βγ subunits, as indicated by the lack of effect of PTX and of βγ-scavenger (GRK2-Cter or C-ter). In any case no Gi/o coupling emerges from cAMP accumulation assays on PAFR-transfected cells, in fact, in a range of concentrations going from 10 pM to 50 μM, PAF was neither able to inhibit basal nor forskolin-stimulated cAMP production. Some indications in favour of PAFR coupling with Gαs were obtained from experiments performed with CTX. pre-treatment with CTX produces ADP-ribosylation of Gαs subunits and is used to ablate Gs coupling. In CTX-treated cells PAF was not able to further stimulate adenylyl cyclase and the same behaviour was showed by a Gs-coupled receptor, the TSHR (Fig 3-7). The limit of this approach is that CTX-treatment fully activates Gαs and downstream effectors, making it difficult to interpret these data. More clear evidence was obtained from experiments based on a dominant negative approach. This method is based on the ability of the carboxyl terminus of Gα subunits to act as a dominant negative construct of Gα protein signalling in vivo. The carboxyl terminal undecapeptide of the Gα subunits is not only an essential region for receptor contact but is also important for determining G protein-receptor specificity. When the minigene plasmid construct encoding the last eleven amino acids of Gαs was co-transfected with PAFR, the receptor-mediated activation of
PLC was unaffected, while, in the same transfection, the stimulation of cAMP production was significantly blunted (Fig 3-8). These results confirm that PAF-mediated activation of adenylyl cyclase requires receptor-Gαs coupling.

It is worth considering whether the PAFR coupling in our studies had physiological significance. PAFR coupling to Gs may be cell type-dependent, as it was observed in COS 7 cells over-expressing PAFR. Moreover, as it can be noted in intact cell cAMP studies, the stimulation response occurs with an EC50 of ~44nM, which may be considered in the physiological range. There is growing evidence that some classes of Gi-coupled receptors can produce stimulation of cAMP as shown by α2AR upon over-expression in CHO cells (Eason, 1995b), δ opioid receptor endogenously expressed in a neuroblastoma cell line (Cruciani, 1993) and cloned human and dog 5-HT1D receptors expressed in CHO cells and Y1 kin-8 cells respectively (Van Sande, 1993). All these receptors exert stimulatory effects on cAMP accumulation, which have been attributed to Gs-coupling. On the other hand, functional coupling to cAMP stimulation appears to be specific of certain Gi-coupled receptors, so that some inhibitory receptors display only inhibition of adenylyl cyclase activity, as IL8 receptor upon over-expression in COS 7 cells (Fig 3-3B). However, PAFR coupling with Gs was demonstrated in an over-expression system. In order to assess whether this apparently paradoxical ability of PAFR to mediate stimulation of cAMP production could be confirmed in cells endogenously expressing the receptor, intact cell cAMP accumulation assays were performed in a few cells. Interestingly, in human lymphocytes, PAF was able to increase basal cAMP levels in a receptor-mediated manner and in a prostaglandin-independent manner (Fig 3-9). Patke et al. (Patke, 1994) reported that PAF modulates cAMP generation in normal human B cells in a time- and dose-dependent manner. Optimal levels of enhanced cAMP
production occurred 30 min after agonist exposure and with a range of PAF concentrations going from $10^{-5}$ to $10^{-7}$ M. In our hands cAMP accumulation assay was performed on lymphocytes after 30 min of agonist exposure with a maximal stimulation with PAF 1 µM. These lymphocytes were a mixed population of T- and B-lymphocytes purified from peripheral blood leukocytes. However, due to the lack of PAFR expression on resting T-lymphocytes, as assessed by Northern blot analysis (Fig 3-13), and by analogous results reported by Patke’s study on human B cells, the observed increased of cAMP production could derive only from PAF-stimulated B-lymphocytes.

What role does PAFR-induced stimulation of adenylyl cyclase plays in cellular physiology? It has been observed that cAMP inhibits PAF synthesis in neutrophils (Fonteh, 1993) and causes the down-regulation of human PAFR mRNA at transcriptional and post-transcriptional levels in monocytes (Thivierge, 1993). The present observation that PAFR can stimulate cAMP could provide the basis for a negative feedback. PAF-stimulated increase in intracellular cAMP can induce PAFR down regulation by acting at the transcriptional level (Fonteh, 1993; Thivierge, 1993).

**3-4-3 Phosphorylation-dependent and -independent mechanisms modulate PAFR signalling in COS 7 cells**

It was suggested that agonist-induced kinase activation and consequent phosphorylation of the receptor cytoplasmic tail might be essential for PAFR desensitisation. Numerous evidences indicate that both GRKs and second messenger-activated protein kinases play a role in the modulation of PAFR signalling.

Previous studies of our laboratory demonstrated that in human mononuclear leukocytes Platelet-Activating Factor is able to induce translocation of GRK (SARK) activity from cytosol to membrane
Translocation of cytosolic kinases is the first step in the process of receptor desensitisation. As a consequence of receptor activation the kinase becomes co-localised with the receptor that can be phosphorylated and uncoupled from the transduction system. Other studies in rabbit platelets suggested that PAFR is modulated in vivo by GRKs, because it undergoes homologous desensitisation in PKC-independent way (Morrison, 1989). Phosphorylation of the carboxyl-terminal cytoplasmic tail of hPAFR, especially at distal Ser/Thr residues, seems to be critical for signal attenuation induced by the agonist (Ali, 1994). In *in vitro* kinase assay a synthetic peptide of the guinea pig PAFR (gpPAFR) carboxyl-terminal tail was strongly phosphorylated by recombinant GRK2, suggesting that this kinase or its relatives might be involved in PAFR homologous desensitisation (Takano, 1994). The most extensive study on hPAFR was conducted in heterologous expression system (RBL2H3, rat basophilic leukaemia, transfected cells) by Snyderman and colleagues, who showed a regulation of this receptor through homologous, heterologous and also chemoattractant receptor cross-desensitisation. They demonstrated that agonist-stimulated PAFR phosphorylation in the cytoplasmic tail is associated with homologous desensitisation and that this ligand-stimulated receptor phosphorylation is mediated via the activation of both GRKs and PKC. The hPAFR possesses 11 serines and threonines in the predicted cytoplasmic tail, two of these sites conforming to the consensus sequence for potential phosphorylation by protein kinase C. hPAFR has also one consensus sequence for phosphorylation by PKA in its cytoplasmic tail and is a target for PKA phosphorylation, but this process did not correlate with receptor desensitisation. More detailed analysis revealed that the PAF-mediated response is regulated via phosphorylation at two levels of the signalling pathway, i.e. at the receptor level and at PLCβ3. PAF-stimulated phosphorylation of PLCβ3
by PKC resulted in the inhibition of new responses stimulated by PAF (Ali, 1997).

Our study performed with the co-transfection approach showed that, in the βARK subfamily of GRKs, GRK3 is more potent in producing PAFR homologous desensitisation, while, in the GRK4 subfamily, only GRK6 is able to inhibit PAFR signalling. Although these results were obtained in heterologous expression systems, using over-expression conditions, we obtained evidence that PAFR is a potential substrate for GRK3 and GRK6. With Northern blot analysis both PAFR and GRK6 levels of expression were followed on subpopulations of leukocytes. These two proteins appear to have the same pattern of expression, supporting the idea that GRK6 could have a role in PAFR desensitisation also in native cells.

The co-transfection approach showed the ability of GRK family members to modulate PAFR signalling not only in a phosphorylation-dependent manner, but also with phosphorylation-independent mechanisms. The transfection model used allows a comparative analysis of two PAFR-activated pathways in the same transfection. Each pathway can act as an internal control for the effects measured on the other. This provides evidence that the two pathways are differently regulated. While they are both inhibited upon GRK2 over-expression, the level of desensitisation was different with a stronger blockage of Gq-mediated signalling. Moreover, Gq-mediated signalling can be selectively inhibited upon K220R, C-ter and N-ter co-expression. The ability of K220R to produce inhibition of receptor signalling cannot be attributed to kinase activity of GRK2, because the Lys→Arg mutation ablates GRK2 kinase activity. Moreover, in intact cell studies, the over-expression of this dead kinase impaired agonist-induced phosphorylation of the receptor, as it has been shown for β1-adrenergic receptor transfected in HEK 293 cells (Freedman, 1995), α1B-
adrenergic receptor expressed in COS 7 cells (Diviani, 1996), and endothelin receptors transfected in HEK 293 cells (Freedman, 1997). Phosphorylation-independent control of PAFR-induced stimulation of PLC, mediated by GRK2, was further confirmed by the absence of any effect on PAFR Gs-mediated pathway (Fig 3-16). On the other hand, GRK2-K220R over-expression generally produce reduction of agonist-induced receptor desensitisation. In fact, desensitisation of m2 muscarinic acetylcholine receptor (Pals-Rylaarsdam, 1995), delta-opioid receptor (Pei, 1995), CCR2B receptor (Aragay, 1998a) all coupled to Gi proteins, or β1-adrenergic receptor coupled to Gs protein, were reduced upon K220R over-expression. Interestingly none of these receptors are coupled to Gq/11 proteins. More interestingly when desensitisation processes of a Gq-coupled receptor were examined, different results were obtained. Parathyroid hormone (PTH) receptor is desensitised in the presence of over-expressed K220R. This reduced PLC stimulation produced by PTH was not mimicked by C-ter transfection, indicating that other GRK2 domains have a role in the blockage of the inositol phosphates pathway (Dicker, 1999).

Our experiments on PAFR not only indicate a specific activity of K220R on Gq-mediated signalling, but show that the N-ter domain is involved in the inhibition of PAF-stimulated IPs production. This domain comprises a putative RGS box and, in functional assays, N-ter has a similar behaviour to RGS4 (Fig 3-18). Although these findings do not clarify if N-ter action is due to its modulation of Goq GTPase activity or to its physical binding with Goq, with consequent impairment of effector activation, they represent the first evidence that N-ter in intact cells can interfere in a specific way with PLC signalling pathway. This suggests a growing complexity in the biochemical mechanisms involved in the regulation of PLC-linked G protein-coupled receptors as well as in the potential roles played by GRK in different cell systems.
A diagram summarising the mechanisms of down regulation of PAFR signalling pathways is reported in Fig 3-19. In this picture the ability of GRK2, GRK3, GRK6 to inhibit PAFR activation of both adenyl cyclase and phospholipase C stimulation is reported, while K220R, C-ter and N-ter can only block PAF-mediated activation of phospholipase C.
Fig 3-19. A model for PAFR signalling down regulation

Detailed description of the model is reported in the discussion.
4-1 Introduction

In chapter 3 GRK2 regulation of different GPCR signalling pathways was demonstrated. In this chapter the results are confirmed and extended, to examine whether GRK2 can desensitise other receptors. The thyrotropin receptor (TSHR) was chosen for analysis because TSHR couples to different G proteins and GRK2 has been shown desensitise TSHR signalling (Allgeier, 1994; Iacovelli, 1996).

Thyrotropin (thyroid stimulating hormone, TSH) regulates thyroid growth and differentiated function, interacting with a membrane receptor belonging to the large superfamily of heptahelical G protein-coupled receptors (Nagayama, 1989). In the human thyroid, the ligand bound to TSHR leads to stimulation of adenylyl cyclase and phospholipase C, by interacting with Gαs and Gαq/11 (Allgeier, 1994).

The relevance of these two signalling pathways for thyroid physiology is only poorly understood. The cAMP regulatory cascade is thought to control growth and differentiated functions, whereas Ca\(^{2+}\) and diacylglycerol have been suggested to stimulate iodination and thyroid hormone synthesis (Vassart, 1992).

TSHR signalling is also characterised by endogenously constitutive activity toward Gs-dependent adenylyl cyclase activation, a feature that discriminate TSHR from other glycoprotein hormone receptors (Cetani, 1996). Phospholipase C activation by primarily Gs-coupled receptors requires greater receptor density and greater agonist concentrations, as opposed to stimulation of adenylyl cyclase by the same agonist (Gudermann, 1996). A three to fourfold increase of agonist concentration required for TSH-induced IP production was observed in
human thyroid slices and in CHO and COS 7 cells transfected with recombinant TSHR (Van Sande, 1990; Biebermann, 1998).

TSHR is well known to undergo homologous, but not heterologous, desensitisation (Rapoport, 1976; Kaneko, 1976; Shuman, 1976). It has been reported that only the hormone-bound TSH receptor is likely to be involved in homologous desensitisation (Nagayama, 1994), but the molecular mechanisms of TSH receptor desensitisation remain largely unclear. Some studies reported GRK5 and βarrestin1 as playing a pivotal role in TSHR homologous desensitisation. GRK5 and βarrestin1 appear to be the predominant GRK and arrestin isoforms in the thyroid cells, as determined by reverse transcription and polymerase chain reaction (RT-PCR) on human and rat thyroid mRNA (Nagayama, 1996a; Nagayama, 1996b). Moreover, in functional studies, using rat thyroid FRTL5 cells, enhancement or suppression of GRK5 expression, by stable transfection with sense or antisense cDNA, produced an increased or attenuated homologous desensitisation of the TSHR respectively (Nagayama, 1996a). Other studies on TSHR desensitisation have been performed in non-thyroidal eukaryotic cells with the recombinant receptor expressed in CHO (Haraguchi, 1993; Shy, 1993), HEK 293 (Nagayama, 1994), COS 7 cells (Iacovelli, 1996), with results sometimes diverging from those previously reported.

Since Northern and Western blot analysis revealed a substantial expression of GRK2 in rat thyroid cell line FRTL5, the involvement of GRK2/βarrestin1 machinery in TSHR homologous desensitisation was investigated. Studies in COS 7 cells showed that TSH-induced cAMP accumulation in cells transfected with the TSH receptor was reduced by 35-45% when co-transfected with GRK2 and/or βarrestin1 (Iacovelli, 1996). This observation strongly indicates that GRK2/βarrestin1 can regulate TSHR signalling.

The aims of the study were:
1) to investigate, at the molecular level, the regulation of Gq- and Gs-mediated signalling by GRK2 and its functional domains. This point was addressed by expression of recombinant TSHR, which is able to stimulate both IPs production and cAMP accumulation and investigating the regulatory effects of GRK2 and GRK2 recombinant functional domains.

2) to identify which is the domain of GRK2 that modulates Gq-mediated signalling and the mechanisms involved.

4-2 GRK2-Nter modulates Gq-coupled GPCR signalling

4-2-1 TSHR signalling

In order to investigate the regulatory role of GRK2 on different GPCR-stimulated second messengers we used TSHR, which is able to couple to different G proteins and to activate different signalling pathways (Tornquist, 1993). The first set of experiments was performed in COS 7 cells, where the heterologous expression of TSHR had already been well established (Biebermann, 1998). TSHR, transfected into COS 7 cells, couples with Gs and Gq and, upon stimulation, activates PLCβ and adenylyl cyclase, resulting in the production of IP, diacylglycerol and cAMP. The basal cAMP production in TSHR-expressing cells was about 5 fold greater than in mock-transfected cells (Fig 4-1A). This enhanced production, even in the absence of agonist-stimulation, confirms that TSH receptor possesses a marked constitutive activity (Biebermann, 1998). According to Biebermann, the majority of TSHR are assumed to be in a pre-activated state; agonist binding shifts the isomerisation equilibrium either toward the preferred Gs coupling state or to the Gq/11-interacting active conformation. Mutational analysis indicate that a tyrosine residue, located in the fifth transmembrane domain (TM5), can be regarded as the crucial molecular switch, allowing the
unliganded, inactive receptor to adopt a pre-activated conformation. This preferential, pre-activated conformation constitutively stimulates cAMP accumulation without activating phospholipase C. The amount of second messengers produced upon stimulation depends on the activity of the transfected TSH receptor, since untransfected COS 7 cells do not express endogenous receptor and do not respond to the thyrotropic hormone. In TSHR-transfected cells, TSH (1 x 10^{−8} M) stimulation increased intracellular cAMP concentration more than 8 fold over the basal level (Fig 4-1B,C). As previously reported (Tornquist, 1993) the amount of TSH that stimulates IP production is about one order of magnitude greater than that activating cAMP accumulation. The IPs production in TSHR-transfected cells upon stimulation with TSH (1 x 10^{−6} M) was about 3 fold over the basal level (Fig 4-1C).

### 4-2-2 GRK2 regulates TSHR signalling

Binding of the agonist to TSH receptor reduces the second messenger response to subsequent stimuli. This rapid and agonist-dependent regulation is similar to the homologous desensitisation observed for several G protein-coupled receptors (Bunemann, 1999; Freedman, 1996). The molecular mechanisms of TSH receptor desensitisation, which involves a decreased coupling of the receptor to Gs and Gq, are only partially understood. To test if TSHR could undergo GRK2-mediated homologous desensitisation, we measured the effects of thyrotropin hormone-stimulation on the adenylyl cyclase and PLC response of COS 7 cells transfected with TSHR receptor, alone or in co-transfection with GRK2. In heterologous expression systems if a receptor is sensitive to the co-expressed GRK, any agonist-stimulated second messenger production should be significantly reduced.
Fig 4-1. Dose-response curves of TSH-induced cAMP accumulation and IPs production

COS 7 cells were transiently transfected with 3 μg cDNA/1 x 10^6 cells. After 24 hours, cells were plated in 48-well for cAMP accumulation assay and in 24-well for inositol phosphates (IPs) production assay. For total [³H] IPs measurement cells were loaded for 24 hours with myo-[³H] inositol.

A 72 h after transfection, intracellular cAMP levels were measured in unstimulated COS 7, cells transfected with an empty vector or with TSHR. THSR transfection produced a significant increase in cAMP levels, about 5 fold, according with TSHR constitutive ability to stimulate adenylyl cyclase.

B 72 hours after transfection, cells transfected with the TSHR were stimulated for 30 min at 37 °C with the indicated concentrations of TSH. Data are expressed as picomoles of cAMP/well and are mean ± SEM of three independent experiments, each performed in triplicate. The calculated EC50 is ~6 nM.

C 72 hours after transfection, cells transfected with the TSHR were stimulated for 30 min at 37 °C with the indicated concentrations of TSH. For inositol phosphates production assay (described under experimental procedures) data are expressed as cpm/well and are mean ± SEM of three independent experiments, each performed in duplicate. The calculated EC50 is ~140 nM.
Fig 4.1
This approach has been extensively used to demonstrate the regulation of a variety of GPCRs by different GRK subtypes (Aragay, 1998; Freedman, 1996; Freedman, 1997).

In comparison with TSHR-transfected cells, TSHR/GRK2-co-transfected cells showed reduced basal levels of cAMP (30% reduction), moreover an inhibition of agonist-stimulated cAMP accumulation (30% and 40% of inhibition upon stimulation with TSH $1 \times 10^{-8}$ and $1 \times 10^{-7}$ M respectively) was observed (Fig 4-2A). In contrast basal IP levels were not modified upon GRK2-co-transfection, while agonist-stimulated IP accumulation (in the presence of LiCl) was significantly reduced when compared to receptor-transfected cells alone (60% and 70% of inhibition upon stimulation with TSH $1 \times 10^{-7}$ and $1 \times 10^{-6}$ M respectively) (Fig 4-2B).

The decreased TSH-induced responses, observed in the presence of GRK2-co-transfection, are consistent with desensitisation of TSHR previously reported by other groups (Iacovelli, 1996; Iacovelli, 1999). The effects of GRK2 on $\mathrm{G}_{\alpha_s}$- and $\mathrm{G}_{\alpha_q}$-mediated responses (cAMP accumulation and IP production respectively) were somehow different, since the TSH-stimulated cAMP response was desensitised to a lower extent than the receptor-stimulated IP production (Fig 4-2). This may indicate that TSHR coupling to $\mathrm{G}_{\alpha_s}$ is more efficient than the coupling to $\mathrm{G}_{\alpha_q}$, thus resulting in a lower sensitivity of $\mathrm{G}_{\alpha_s}$ to desensitising mechanisms. Alternatively, we can hypothesise additional mechanisms, possibly phosphorylation-independent, as suggested by a recent report (Dicker, 1999), showing that K220R could desensitise the parathyroid hormone receptor-stimulated $\mathrm{G}_{\alpha_q}$ response. To test the latter possibility, we used the dead kinase GRK2 mutant (K220R), in which the catalytic activity is disrupted (Kong, 1994).
Fig 4-2. Regulation of TSHR-mediated signalling by GRK2

COS 7 cells were transiently transfected with TSHR (3 μg/1 x 10^6 cells) plus vector control, GRK2 (4 μg/1 x 10^6 cells) (empty circles) or K220R (2 μg/1 x 10^6 cells).

A 72 hours after transfection cells were stimulated for 30 min at 37 °C with the indicated concentrations of TSH and cAMP accumulation assay was performed. Data are expressed as percentage of maximal stimulation (TSH 100 nM) and show mean ± SEM of three experiments.

B 72 hours after transfection cells were stimulated for 30 min at 37 °C with the indicated concentrations of TSH and inositol phosphates (IPs) production assay was performed. Data are expressed as percentage of maximal stimulation (TSH 1000 nM) and are mean ± SEM of three experiments.
Fig 4-2

**A**

- cAMP accumulation (% of control)
- TSHR/PBJI
- TSHR/K220R
- TSHR/GRK2

**B**

- IP production (% of control)
- TSHR/PBJI
- TSHR/K220R
- TSHR/GRK2
Over-expression of K220R did not affect TSH-stimulated cAMP response, indicating that in COS 7 cells endogenous GRK2 was not sufficient to desensitise over-expressed TSHR, in agreement with other GPCRs (Ito, 1999). On the other hand, TSH-stimulated IP production was substantially blunted by K220R (~70% of inhibition for both TSH 1 x 10^{-7} and 1 x 10^{-6} M stimulation) (Fig 4-2B), consistent with the idea that a phosphorylation-independent mechanism is selectively involved in the regulation of TSHR-Gαq-mediated signalling. In order to determine which domain of GRK2 was involved in this effect, N-ter and C-ter of GRK2 were co-expressed with TSHR and TSH-stimulated cAMP and IP responses were measured (Fig 4-3). Neither basal nor TSH-stimulated cAMP accumulation were affected by the expression of these domains, further indicating that only a phosphorylation-dependent mechanism is involved in the desensitisation of this pathway. Gαq-mediated response was not affected by C-ter, but was substantially blunted by N-ter, indicating that this is the domain involved in the phosphorylation-independent regulation of TSHR-Gαq-mediated response. Over-expression of GRK2, its dead kinase, N-ter and C-ter kinases were confirmed by Western blot analysis.

The results obtained with the TSH receptor are similar to those obtained with the PAFR. They indicate that GRK2 is able to produce uncoupling of the receptors from both Gs and Gq proteins, while a selective blockage of Gq-mediated signalling is observed in presence of GRK2 dead kinase co-expression and the GRK2 domain involved in this inhibition is N-ter domain, thus suggesting a phosphorylation-independent mechanism.
COS 7 cells were transiently transfected with TSHR (3 μg/1 x 10^6 cells) plus vector control, GRK2 (4 μg/1 x 10^6 cells), K220R (2 μg/1 x 10^6 cells), N-ter (8 μg/1 x 10^6 cells), C-ter (4 μg/1 x 10^6 cells) or RGS4 (4 μg/1 x 10^6 cells) cDNA. All the cDNA amounts were made equal by adding the empty vector.

A 72 hours after transfection, cells were stimulated for 30 min at 37 °C with TSH 10 nM (empty bars) or 100 nM (filled bars) and cAMP accumulation assay was performed. Data are expressed as percentage of maximal stimulation (TSH 100 nM) and are mean ± SEM of three or four experiments, each performed in triplicate.

B 72 hours after transfection, cells were stimulated for 30 min at 37 °C with TSH 100 nM (filled bars) or 1000 nM (striped bars) and inositol phosphates (IPs) production assay was performed. Data are expressed as percentage of maximal stimulation (TSH 1000 nM) and are mean ± SEM of three or four experiments, each performed in duplicate. Statistically significant differences versus control cells (TSHR/PBJI-transfected cells, stimulated with the same concentration of agonist) are indicated (* p<0.05 and ** p<0.01; Dunnett's test).
IP production (% of control)

- Vector
- +GRK2
- +K220R
- +N-ter
- +C-ter
- +RGS4

CAMP accumulation (% of control)

- Vector
- +GRK2
- +K220R
- +N-ter
- +C-ter
- +RGS4
**4-2-3 RGS4 regulates TSHR signalling**

The ~190 residue N terminus of GRK2 has been suggested to contain receptor binding determinants, however its overall structure and function remain largely uncharacterised. Siderovski and colleagues identified a sequence homology between RGS domains and a ~120 residue region in the N terminus of GRK2 (Siderovski, 1996). We studied the effects of RGS4 (co-expressed in parallel assays) on the different pathways stimulated by TSHR. RGS4 is known to bind to G\(\alpha_q\) (but not to G\(\alpha_s\)) and to desensitise the G\(\alpha_q\)-stimulated intracellular response (Berman, 1998; De Vries, 1999; Hepler, 1999). In our model RGS4 significantly reduced TSH-stimulated IP production (40% and 60% of inhibition upon stimulation with TSH 1 \(\times 10^{-7}\) and 1 \(\times 10^{-6}\) M respectively), without any effect on the basal IP values, while it was ineffective on cAMP response stimulated by TSH (Fig 4-3). This is in agreement with other reports for different receptors (Hepler, 1997).

**4-2-4 5-HT\(_{2c}\) receptor signalling**

In order to assess whether the effect of N-ter on GPCR-stimulated-G\(\alpha_q\) signalling could be observed with other receptors and other cell lines, we studied the serotonin receptor (5-HT\(_{2c}\)R) transfected in HEK 293 cells. This receptor is primarily coupled to G\(\alpha_q\) and its stimulation results in PLC-mediated phosphoinositide hydrolysis, to liberate the second messengers diacylglycerol and inositol triphosphate (Berg, 1994). The 5-HT\(_{2c}\) receptor has been shown to undergo desensitisation both *in vivo* and *in vitro* at a functional level through mechanisms that include phosphorylation and down regulation, which are analogous to those described for the \(\beta_2\)-adrenergic receptor (Westphal, 1995). We transiently transfected HEK 293 cells to express 5-HT\(_{2c}\) receptor and measured the effects of GRK2-co-expression on 5-HT-stimulated IP production.
production. Untransfected HEK 293 cells did not respond to 5-HT, while in transfected cells 5-HT (10 μM) stimulated IP production by ~5 fold. 5-HT$_{2C}$ receptor-mediated signalling was desensitised by over-expression of wild type GRK2 (~70% of inhibition) (Fig 4-4). Co-transfection of N-ter also significantly reduced the 5-HT-induced IP accumulation, further indicating that the interaction of this domain of GRK2 with Gαq results in a negative regulation of Gαq-mediated signalling. Consistently, the co-expression of the dead kinase mutant K220R also inhibited 5-HT-induced IP production (~60% of inhibition), further indicating that GRK2 can desensitise 5-HT$_{2C}$-mediated signalling in a phosphorylation-independent mechanism. The co-expression of C-ter did not affect the 5-HT-stimulated IP production, suggesting that this domain is not involved in Gq signalling regulation. As expected, the over-expression of RGS4 significantly reduced 5-HT-stimulated IP accumulation. The over-expression of different proteins was confirmed by Western blot analysis. These results further suggest or confirm that the N-ter is able to desensitise GPCR-stimulated Gαq signalling.

4-2-5 N-ter directly modulates Gq signalling
To assess whether N-ter regulates directly Gαq, rather than the receptor, Gαq was over-expressed in HEK 293 cells and its signalling was induced by aluminium fluoride (AlF$_{4}^{-}$). AlF$_{4}^{-}$ is an activator of GDP-bound G protein α subunits. In fact aluminium fluoride complexes can activate the heterotrimeric G proteins, by binding next to GDP in the nucleotide site of their Gα subunit and acting as analogues of γ-phosphate of the GTP molecule (Antonny, 1992). In vitro AlF$_{4}^{-}$ is able to activate purified Gαq, which consequently stimulates partially purified PLC (Smrcka, 1992).
HEK 293 cells were transiently transfected with 5-HT2C receptor (1.5 μg/1 x 10^6 cells) plus vector control, GRK2 (2 μg/1 x 10^6 cells), GRK2-K220R (1 μg/1 x 10^6 cells), N-ter (4 μg/1 x 10^6 cells), C-ter (2 μg/1 x 10^6 cells) or RGS4 (2 μg/1 x 10^6 cells) cDNA. All the cDNA amounts were made equal by adding the empty vector. After 24 hours cells were plated in 6-well for inositol phosphates (IPs) production assay and cells were loaded for 24 hours with myo-[^3H] inositol. 48 hours after transfection cells were stimulated for 30 min at 37 °C with 5-HT 10μM. Data are expressed as percentage of control cells (transfected only with 5-HT2C receptor) and represent mean ± SEM of three or four experiments. Statistically significant differences versus control (+vector) are indicated (** p<0.01; Dunnett’s test).
Fig 4-4

Graph showing IP production (% of control) for various conditions: Vector, GRK2, K220R, N-187R, C-187R + RGS4.
Accordingly AlF4− can elevate cellular IP levels in cells over-expressing recombinant Gαq. By itself the transfection of Gαq produces a small but reproducible elevation of cellular IP content. The addition of AlF4− causes a ~5 fold increase of basal IP levels. These results suggest that increased amount of Gαq caused a modest elevation in the cellular level of its GTP-bound form, and addition of AlF4− activates Gαq, making it able to further activate the effector enzyme.

The co-expression of N-ter decreased Gαq-dependent IP production stimulated by AlF4− (30 μM AlCl3 and 10 mM NaF) (Fig 4-5), thus indicating that N-ter directly inhibits Gαq signalling. In a parallel set of experiments, HT2C receptor was additionally co-transfected (Fig 4-5). These cells were stimulated at the same time with AlF4−, to activate Gαq, and with 10 μM 5-HT, to bring the receptor to the activated conformation. The stimulation of the receptor with 5-HT did not further increase AlF4−-stimulated IP production, confirming that Gαq was already maximally stimulated by AlF4− (IP production was 21,700 and 22,800 cpm/well in cells with Gαq alone or Gαq plus 5-HT2C respectively).

However, N-ter-co-transfection produced a greater inhibition in presence of the activated receptor, indicating that the receptor enhances the ability of N-ter to interact with Gαq, reinforcing its ability to inhibit Gαq signalling. The observed effect of the N-ter is proportional to the amount of transfected cDNA with the highest effect observed transfecting 4 fold more N-ter than Gαq cDNA. The increased Gαq inhibition, observed in the presence of activated receptor, can be explained considering receptor ability to recruit N-ter to the membrane, promoting its localisation near the target Gαq. In fact, N-ter has always been considered the domain involved in activated receptor recognition and binding (Pitcher, 1998a).
Fig 4-5. Regulation of Gaq signalling by N-ter and RGS4

A HEK 293 cells were transiently transfected with Gaq (0.5 μg/1.5 x 10^6 cells) with indicated amounts of N-ter (referred to 1.5 x 10^6 cells) (filled squares). In a parallel set of experiments cells were additionally co-transfected with 5HT2C receptor (0.5 μg/1 x 10^6 cells) (empty squares) cDNA.

B HEK 293 cells were transiently transfected with Gaq (0.5 μg/1.5 x 10^6 cells) with indicated amounts of RGS4 (referred to 1.5 x 10^6 cells) (filled squares). In a parallel set of experiments cells were additionally co-transfected with 5HT2C receptor (0.5 μg/1 x 10^6 cells) (empty squares) cDNA. After 24 hours, cells were plated in 60 mm petri dishes for inositol phosphates (IPs) production assay. 48 hours after transfection cells were stimulated for 30 min at 37 °C with AlF4⁻ (30 μM AlCl₃ and 10 mM NaF) and 10 μM 5-HT was added where the receptor had been transfected, to make the receptor in the active (agonist-bound) conformation. Data, subtracted basal level, are expressed as percentage of maximal stimulation of cells transfected with Gaq plus 5HT2C receptor and are representative of two similar experiments.
Fig 4-5
Co-transfection of RGS4 affected Gaq signalling in a similar manner, consistent with previous findings, showing that the direct interaction of RGS4 with the receptor contributes to the ability of RGS4 to regulate G protein signalling (Zeng, 1998). Also in this case RGS4-induced inhibition is completely dependent on the expression level, with the highest effect measured transfecting 3 fold more RGS4 cDNA than Gaq.

**4-3 GRK2-Gαq binding**

**4-3-1 GRK2 binding to HEK 293 cell membranes**

N-ter showed a similar behaviour to RGS4 in the selective regulation of Gaq-mediated response. RGS4 is known to bind to Gaq (Heximer, 1997), it was so hypothesised that the N terminal region of GRK2, that contains an RGS homology domain, could directly interact with the Gaq subunit to regulate its signalling. Both Gaq and Gα11 are palmitoylated and show a prevalent membrane localisation.

HEK 293 cells have a relevant endogenous expression of these G proteins, as assessed by Western blot analysis. For this reason, plasma membranes obtained from HEK 293 cells were employed to show a possible interaction between GRK2-Nter and Gaq. Recombinant GST-N-ter (GRK2 Ala²-Thr¹⁸⁷) fusion protein was incubated with membranes from untransfected HEK 293 cells, in the absence or presence of AlF₄⁻ to activate Gα. Incubation was stopped by centrifugation and pelleted membranes were subjected to SDS-PAGE electrophoresis, transferred to nitrocellulose membrane and immunoblotted with anti-GST antibody to reveal GST-N-ter binding. Western blot analysis showed that GST-N-ter binds to plasma membranes even at basal conditions but the binding was substantially increased in the presence of AlF₄⁻, which activates endogenous Gα (Fig 4-6).
Fig 4-6. Specific binding of GRK2-Nter to activated Gaq

A Recombinant purified GST-N-ter fusion protein was incubated with untransfected HEK 293 cells membranes in the absence (-) or presence (+) of AlF4⁻ (30 μM AlCl₃ plus 20 mM NaF) to activate the Ga. Incubation (1 h at 4 °C) was stopped by centrifugation (300,000 x g) and pelleted proteins were resolved by 8% SDS-PAGE, blotted onto nitrocellulose membrane, and GST-N-ter bound to HEK 293 cells membranes was revealed by immunoblot with anti-GST antibody.

B Cytosolic proteins (150 μg) from Gaq-transfected HEK 293 cells were incubated (1 h a 4 °C) with recombinant purified GST, GST-N-ter or GST-RGS4 fusion proteins conjugated to glutathione agarose beads, in the absence (-) or presence (+) of AlF4⁻ (30 μM AlCl₃ plus 20 mM NaF) to activate Gaq. After three extensive washings glutathione agarose beads, with bound proteins, were electrophoresed on 8% SDS-PAGE, and after blotting onto nitrocellulose membrane, Gaq bound to the columns was revealed by immunoblot with anti-Gaq antibody. Also starting material (S) (30 μg of cytosolic preparation) is included in the immunoblot.

C Purified Gaq (100 ng) was incubated (1 h a 4 °C) with recombinant purified GST-N-ter fusion protein conjugated to glutathione agarose beads, in the absence (-) or presence (+) of AlF4⁻ (30 μM AlCl₃ plus 20 mM NaF) to activate Gaq. After three extensive washings glutathione agarose beads, with bound proteins, were electrophoresed on 8% SDS-PAGE, and after blotting onto nitrocellulose membrane, Gaq bound to the columns was revealed by immunoblot with anti-Gaq antibody. Also starting material (S) (80 ng) is included in the immunoblot. All the experiments shown were repeated at least three times with similar results.
**Fig 4-6**

**A**

HEK293 membranes

anti-GST BLOT

\[ \text{AlF}_4^- \quad - \quad + \quad \rightarrow \text{N-ter} \]

**B**

anti-G\(\alpha_q\) BLOTs

<table>
<thead>
<tr>
<th>GST resin</th>
<th>GST-N-ter resin</th>
<th>GST-RGS4 resin</th>
</tr>
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<tbody>
<tr>
<td>G(\alpha_q)</td>
<td>-</td>
<td>+</td>
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</tbody>
</table>

**C**

anti-G\(\alpha_q\) BLOTs

From purified G\(\alpha_q\)
This indicates a possible interaction of GST-N-ter with the activated Go. Mayor and co-workers (Murga, 1996) showed that GRK2 associates with intracellular microsomal membranes both in vitro and in situ, moreover with [35S] GTPγS binding and immunoblot analysis they confirmed the presence of heterotrimeric G proteins in their preparations. They also demonstrated that a fusion protein derived from the carboxyl terminus of GRK2 does not affect this association, excluding a PH domain-mediated interaction, whereas fusion proteins corresponding to the amino terminal region of the kinase markedly inhibit it.

These observations further confirm that GRK2 may bind to plasma membranes, by interacting with Go proteins through its RGS domain.

4-3-2 Go proteins binding

To further assess the possibility that GRK2 binds to Go subunits in an AlF4⁻-dependent manner, in vitro binding assays were performed. GRK2 affinity resin was prepared by conjugating recombinant GST-N-ter, the same GRK2 construct used in membrane binding experiments, to glutathione agarose beads. Cytosolic proteins from HEK 293 cells, transiently transfected with different Go subtypes, were employed as source of Go subunits. Cytosolic extracts from Gaq-, Gao-, Gas-transfected cells were incubated with the affinity resin. Unbound proteins were removed by extensive washing, while bound proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Immunoblot analysis with specific polyclonal antibodies, that recognise different Go subunits, were used to reveal Gobinding. Control experiments were performed with a GST-RGS4 affinity resin to check the experimental conditions. In GST-N-ter assay only activated Gaoq showed binding ability to the resin. When the incubation was done in the absence of AlF4⁻ (i.e. Go is in the inactive state), only a negligible
interaction between $\alpha_q$ and GST-N-ter was detected, while in the presence of AlF$_4^-$, to activate the $\alpha_q$, a substantial fraction of $\alpha_q$ (estimated ~25% of starting material) was bound to GST-N-ter (Fig 4-7). The ability to bind to GST-N-ter appeared to be selective for $\alpha_q$, since $\alpha_s$ and $\alpha_o$ failed to bind to GST-N-ter, even when activated by AlF$_4^-$. Despite their presence in the starting material. In parallel experiments binding of GST-RGS4 fusion protein to different $\alpha$ proteins was also measured (Fig 4-7). According to previous reports (Tesmer, 1997), in the presence of AlF$_4^-$ we observed a significant binding of RGS4 to $\alpha_o$ and $\alpha_q$, but not to $\alpha_s$ (Heximer, 1997). Due to the cell origin of $\alpha$ source, the involvement of other proteins in GRK2-$\alpha_q$ interaction could not be ruled out. In order to rule this hypothesis, experiments with purified $\alpha_q$ were performed. Recombinant $\alpha_q$, obtained from infected SF9 cells and affinity purified according to Kozasa (Kozasa, 1995), was incubated with GST-N-ter or GST (negative control) affinity columns, in the absence or presence of AlF$_4^-$. $\alpha_q$ showed a strictly AlF$_4^-$-dependent binding only with GST-N-ter (Fig 4-6). Addition of AlF$_4^-$ to inactive $\alpha$(GDP) produces a stable conformation that mimics the transition state produced during hydrolysis of $\alpha$(GTP) to $\alpha$(GDP). RGS proteins are able to discriminate between the different activation states of the $\alpha$ subunits, exhibiting preferential binding to $\alpha$(GDP/AlF$_4^-$). This preference is possibly due to the ability of RGS proteins to bind and stabilise the transition state of the GTP hydrolysis, acting as catalysts of the reaction. To determine if GRK2 binding to $\alpha_q$ was also selective for the transition state, the ability of GST-N-ter to bind to AlF$_4^-$ activated $\alpha_q$ or to $\alpha_q$ constitutively activated mutant ($\alpha_q$-Q209L) were compared. Q209L mutation abolishes $\alpha_q$ intrinsic GTPase activity (Dhanasekaran, 1994); consequently a fraction of this mutant should be in the GTP-bound form when over-expressed in a heterologous system (Berman, 1996).
Cytosolic proteins (150 µg) from different Gα subunits-transfected in HEK 293 cells (Gαs, Gαo, Gαq, Gαq-Q209L) were incubated (1 h at 4 °C) with recombinant purified GST-N-ter or GST-RGS4 fusion proteins conjugated to glutathione agarose beads, in the absence (-) or presence (+) of AlF4⁻ (30 µM AlCl₃ plus 20 mM NaF). After three extensive washings glutathione agarose beads with bound proteins were electrophoresed on 8% SDS-PAGE and, after blotting onto nitrocellulose membrane, Gα proteins bound to the columns were revealed by immunoblot with specific antibodies for different Gα proteins. Also starting material (S) (30 µg of cytosolic preparation) is included in the immunoblot. The experiments were repeated at least three times with similar results.

Fig 4-7. Selective binding of GRK2-Nter to activated Gαq
Fig 4-7

Figures 4-7 show the results of a Western blot analysis using anti-Gαs, anti-Gαo, anti-Gαq, and anti-Gαq - Q209L antibodies on GST-N-ter and GST-RGS4 proteins. The blots demonstrate the detection of Gαs, Gαo, Gαq, and Gαq - Q209L proteins.
In fact, over-expression of Gaq-Q209L in HEK 293 cells produced a 6 fold increase in the basal levels of IP (7,000 and 44,000 cpm for vector and Gaq-Q209L-transfected cells respectively), confirming that this mutant is blocked in an active conformation. When cytosolic extracts of cells transfected with this mutant were employed in GST-N-ter interaction experiments, a substantial binding of Gaq-Q209L was observed even in the absence of AlF4-. This binding was not further enhanced by the activating solution, confirming that this mutant is already in the active conformation (Fig 4-7). In parallel experiments, Gaq-Q209L failed to bind to GST-RGS4, both in the presence or absence of AlF4-, consistent with the evidence that RGS4 did not bind to constitutively activated Gaq (Berman, 1996). The interaction of GRK2-Nter with Gaq is likely to be associated to the RGS box, which is present in this region of GRK2. Although the domain is the same involved in RGS4-Gaq interaction, substantial differences between GRK2-Nter and RGS4 are seen. N-ter was more selective than RGS4 in interacting with different Ga, since among all G proteins tested, it binds only to activated Gaq. Additionally N-ter is able to bind to Gaq both in the AlF4- and in the GTP-bound form (i.e. Gaq-Q209L mutant), while RGS4 only interacted with the AlF4--activated form of Gaq and Gaq. On the basis of these observed differences we speculated that the molecular determinants of RGS domain/Gaq binding are different to some extent between GRK2-Nter and RGS4.

4-3-3 GRK2/Gaq interaction in intact cells

In order to determine if GRK/Gaq interactions occur in intact cells, GRK2/Gaq co-immunoprecipitation experiments were performed. HEK 293 cells were transfected with GRK2 and either Gaq or Gaq-R183C. This particular mutation (R183C) of Gaq nearly abolishes GTPase activity, by trapping the GTP-bound active state of Gaq but does not
directly confer the active state. Thus, a portion of the expressed Gαq will accumulate in the active state due to basal stimulation of GPCRs during cell culture. For immunoprecipitation, cell lysates were incubated with either GRK2-polyclonal antibodies or with a preimmune serum.

Subsequent blotting of this immunoprecipitation material with a anti-GRK2 specific antibody revealed that the GRK2 polyclonal antibodies effectively immunoprecipitated GRK2 in both cells co-expressing Gαq and those expressing Gαq-R183C, while preimmune serum did not precipitate GRK2. Immunoblotting of the immunoprecipitates, with anti-Gαq polyclonal antibody to detect Gq, revealed that only small amounts of wild type Gq co-precipitated with GRK2, whereas a significant amount of Gαq-R183C co-immunoprecipitated with GRK2 (Fig 4-8, Fig 4-9). This experiment is in full agreement with our in vitro data (Fig 4-6) and strongly suggest the ability of GRK2 to bind tightly to the active state of Gαq as opposed to other RGS.

4-4 Discussion

4-4-1 The GRK2 N-terminus selectively regulates Gq-mediated signalling

The experiments reported in this chapter demonstrate that GRK2 regulates GPCR signalling at multiple levels.

TSH receptor represents a key tool because it allows, due to its ability to couple with two different G proteins, to investigate two different signalling pathways, activated by the same receptor, in the same system. As for PAFR it was found that GRK2 desensitises the TSH receptor in an agonist-dependent manner, inhibiting all receptor-activated intracellular pathways.
HEK 293 cells were transfected as indicated in the figure and 5 μg/1 x 10^6 cells of each plasmid were used. All the cDNA amounts were made equal adding the empty vector. Whole cell lysates from transfected cells were subjected to immunoprecipitation with anti-GRK2 antibodies (I) or with a preimmune antiserum (P) followed by incubation with protein A-agarose. Initial cell extracts (lysates) (10% of total used in immunoprecipitation) and samples from the immunoprecipitation reaction (IPP) were resolved by 10% SDS-PAGE, blotted, and analysed with anti-GRK2 (upper panel) and anti-Gαq antibodies (lower panel). A clear co-immunoprecipitation of Gαq-R183C (Gαq*) was observed only in presence of co-transfected GRK2 and only when the immunoprecipitation reaction was performed with anti-GRK2 antibodies. A representative Western blot is shown.

IP: immunoprecipitation; IB: immunoblotting

Fig 4-8. Co-immunoprecipitation of activated Gαq and GRK2 from HEK 293 cells

HEK 293 cells were transfected as indicated in the figure and 5 μg/1 x 10^6 cells of each plasmid were used. All the cDNA amounts were made equal adding the empty vector. Whole cell lysates from transfected cells were subjected to immunoprecipitation with anti-GRK2 antibodies (I) or with a preimmune antiserum (P) followed by incubation with protein A-agarose. Initial cell extracts (lysates) (10% of total used in immunoprecipitation) and samples from the immunoprecipitation reaction (IPP) were resolved by 10% SDS-PAGE, blotted, and analysed with anti-GRK2 (upper panel) and anti-Gαq antibodies (lower panel). A clear co-immunoprecipitation of Gαq-R183C (Gαq*) was observed only in presence of co-transfected GRK2 and only when the immunoprecipitation reaction was performed with anti-GRK2 antibodies. A representative Western blot is shown.

IP: immunoprecipitation; IB: immunoblotting
IP: anti-GRK2 Ab (I), preimmune (P)
IB: anti-Goαq Ab

Fig 4-8

IP: anti-GRK2 Ab (I), preimmune (P)
IB: anti-Goαq Ab

anti-GRK2 BLOT

anti-Goαq BLOT

GRK2
Goαq*

lysates

Anti-GRK2 IPPs

P I P I P I

GRK2

IgG

Gαq*

- - + - - - - + +
- - - - - - - + + + + + + + + + + +
HEK 293 cells were transfected as indicated in the figure (5 µg/1 x 10^6 cells of each plasmid were used). All the cDNA amounts were kept equal adding the empty vector. Whole cell lysates from transfected cells were subjected to immunoprecipitation with anti-GRK2 antibodies or with a preimmune antiserum followed by incubation with protein A-agarose. Initial cell extracts (10% of total used in immunoprecipitation) and samples from the immunoprecipitation reaction (IPPs) were resolved by 10% SDS-PAGE, blotted, and analysed with GRK2 (upper panel) and Gaq antibodies (lower panel). A small amount of co-immunoprecipitated Gaq was observed. A representative Western blot is shown.

IP: immunoprecipitation; IB: immunoblotting
Fig 4-9

IP: anti-GRK2 Ab (I), preimmune (P)
IB: anti-Gαq Ab

anti-GRK2 BLOT

anti-Gαq BLOT

GRK2  -  +  +  -  -  +  +  +  +
Gαq    -  wt  *  -  -  wt  wt  *  *

lysates  ←  Anti-GRK2 IPPs  →
P  I  P  I  P  I
However the dead kinase K220R is able to modulate only Gq-mediated signalling, showing that regulation of the Gs-mediated signalling requires phosphorylation. The GRK2 domain involved in the down regulation of Gaq signalling is the N-ter portion.

The experiments with the 5HT2c receptor confirmed that the effects produced by N-ter can be applied to other Gq-coupled receptors in other cellular systems. Due to the presence of RGS domain in N-ter, it was hypothesised that its action could be due to a direct interaction with the G protein.

In cells transfected with Gaq, direct activation of Gaq signalling is inhibited by N-ter. This indicates that N-ter acts at the level or downstream of Gaq and not at the level of the receptor. However, the simultaneous expression of an activated receptor can potentiate N-ter inhibitory activity. Parallel experiments were performed with RGS4, another regulator of G protein signalling, whose behaviour is well described and a similar regulatory profile was found.

4-4-2 GRK2 N-terminus specifically and selectively binds to Gaq

Direct binding experiments demonstrate the ability of N-ter to bind in a specific and selective way to both AlF4- activated and GTP-bound (Gaql-Q209L) Gaq. These experiments reveal differences between RGS4 and N-ter binding, that can be explained through the low similarity between the two RGS domains.

Mutagenesis analysis of the RGS homology domain of RGS4 shows several amino acids important for G protein binding and RGS GAP activity, which are not conserved in N-ter. The crystal structure of Gaq1-GDP-AlF4–RGS4 complex revealed that one amino acid (N128) of RGS4 protrudes in the nucleotide binding pocket of Gaq. This residue, which seems to play a catalytic role, is not conserved in N-ter (Tesmer 1997). These observations suggest that N-ter may not modulate GTPase
activity of Gaq.

Another substantial difference is given by the ability of N-ter and RGS4 to bind to different Gaq conformational states. RGS4 binds selectively to AlF4^-activated Gaq, thus causing the stabilisation of the transition state of the GTP hydrolysis, accelerating the reaction and the switching off G protein signalling. On the contrary GRK2 is able to bind to both AlF4^-activated and constitutively activated (GTP-bound) Gaq, supporting the idea that its inhibitory action on G protein signalling is due to its being an effector antagonist rather than a GTPase activating protein.

These results show that GRK2 is a multidomain kinase that regulates GPCR signalling by interacting with different proteins of the signalling cascade. The catalytic domain phosphorylates the receptor (important for homologous desensitisation), the PH domain interacts with dissociated Gβγ (important for kinase targeting to membranes) and the N-terminus, likely through the RGS box, interacts with the activated Gaq for regulation of Gaq signalling.

While many subtypes of GPCR and Gβγ interact with GRK2, the interaction of N-ter with Gaq appears to be selective.

Therefore GRK2 appears to rule GPCR signalling in a complex manner and multiple interacting mechanisms are likely to be important for the fine-tuning of this process in the living cells. The results reported in the present and previous chapters allow us to hypothesise a model of GRK2 modulation of GPCR signalling, depending on GPCR ability to couple to different transduction systems (Fig 4-10).
Phosphorylation-dependent receptor modulation

Phosphorylation-independent receptor modulation

Fig 4-10. A model for GRK2 phosphorylation-dependent and -independent modulation of GPCRs signalling

Detailed description of the model is reported in the discussion.
A seven transmembrane receptor can couple, at the same time, with G\(_\alpha q\) and G\(\alpha s\) proteins, so that upon agonist (A) binding it undergoes conformational modifications that enable its interactions with both alpha subunits (\(\alpha s\) and \(\alpha q\)). In this agonist-bounded form it is also a substrate of GRK2 activity. GRK2 phosphorylation (p) can occur in one or multiple residues of the receptor in the C-terminal tail or third intracellular loop (Pitcher, 1998a). Upon GRK2 phosphorylation, the interaction of the receptor with both G proteins is impaired. In fact, in functional assays the over-expression of GRK2 caused GPCR signalling inhibition, independently of the nature of receptor-G protein coupling. GRK2 can further modulate the receptor-activated Gq-mediated signalling through its N-terminal domain. As evidenced by in vitro binding assays and co-immunoprecipitation reactions, GRK2 can bind to G\(\alpha q\), in the activated conformation or in the conformation of the GTPase reaction transition state. The functional consequence of this binding, as shown with functional assays, is the selective inhibition of G\(\alpha q\) signalling.
5-1 Introduction

The specific action of different GRKs toward a GPCR has been difficult to identify until now, with the exception of rhodopsin kinase, whose physiological target been discovered. For GRK2, which is ubiquitously expressed, the investigation is further complicated by the recently discovered ability to phosphorylate also non-receptor substrates. In agreement with the important physiological role of GRK2, its specificity of action may be a consequence of modulation of its expression levels, subcellular localisation and phosphorylation activity. GRK2 activity is in fact regulated by interactions with βγ subunits, several lipids, anchoring proteins and agonist-bound receptors (Aragay, 1998; Carman, 1998; Pitcher, 1998a). Posttranslational modifications, such as phosphorylation on serine or tyrosine residues by other kinases, can also modulate GRK2 activity. It has been reported that phosphorylation by PKC induced increased GRK2 activity toward receptors, probably due to enhanced kinase association to the plasma membrane (Chuang, 1995; Winstel, 1996).

The most intriguing regulation involves kinases of the Ras/MAPK pathway, indicating a strict intercommunication between different signalling transduction systems inside the cell. Emerging evidence indicates that stimulation of Gq- or Gi-coupled GPCRs also activate the mitogen-activated protein kinase (MAPK) cascade in a Ras-dependent way (Gutkind, 1998; Lefkowitz, 1998; Luttrell, 1999a). Interestingly it has been suggested that GRK2 and βarrestin directly participate to the process of MAPK cascade activation, either by facilitating receptor internalisation and/or the interaction with additional proteins (Lefkowitz, 1998; Luttrell, 1997; Daaka, 1998).
In fact, binding of βarrestin1 or βarrestin2 as well as preventing receptor-mediated G protein activation, recruits other molecules such as c-Src to the receptor, and helps to target the phosphorylated receptor for internalisation via clathrin-coated pits (Ferguson, 1996). The recruitment of c-Src and the engagement of clathrin-coated pits have been shown to be essential for β2-adrenergic receptor-mediated ERK activation (Daaka, 1998). Recent investigations demonstrated that a fraction of GRK2 cellular pool exists in a phosphorylated form. Mass spectrometry and mutational analysis localise the site of phosphorylation to a carboxyl terminal serine residue, Ser 670, which is located in a peptide sequence, conforming to an ERK consensus phosphorylation sequence, i.e. PX(S/T)P (Pitcher, 1999). In confirmation, in vitro experiments show that ERK1 phosphorylates GRK2 and β2-adrenergic receptor stimulation in HEK 293 cells promotes the presence of activated MAPK and GRK2 in the same multimolecular complex (Elorza, 2000). As a functional consequence of this association, ERK-phosphorylated GRK2 displays a reduced activity toward activated GPCRs and presents a dramatically impaired Gβγ-mediated activation (Pitcher, 1999). It is interesting to note that ERK phosphorylation at Ser 670 is specific to GRK2, because this residue is not conserved in GRK3. This observation together with the inhibitory effect of ERK on βarrestin1, suggesting the existence of an autoregulatory loop in the GPCR/MAPK pathway (Lin, 1999). A decreased activity of GRK2 and βarrestin1 upon MAPK phosphorylation would attenuate the coupling of GPCR to the ERK/MAPK pathways decreasing c-Src recruitment and/or receptor internalisation (Luttrell, 1999a; Luttrell, 1999b). Other experiments demonstrated a direct role of Src-like kinases in the modulation of G protein-coupled receptor signalling and desensitisation. In vitro experiments with purified proteins showed that c-Src can directly phosphorylate GRK2 on
tyrosine residues. More important is that, in COS 7 cells, agonist-stimulation of β2-adrenergic receptors leads to a rapid and transient tyrosine phosphorylation of GRK2, which results in the enhancement of GRK2 intrinsic activity (Sarnago, 1999). In this case Src-dependent phosphorylation of GRK2 provides a positive feedback loop. Increased GRK2 activity produces a more efficient blockage of GPCR signalling, while reinforcing the arrestin/Src/Ras/MAPK pathways. This hypothesis is in agreement with the proposed role of GRK2 as a “signal switching” molecule.

The aims of the study were:

1) to investigate the existence of novel mechanisms able to modulate the physical and functional interaction between GRK2 and Gαq.

The study focused on c-Src kinase, based on the evidence that the GRK2 tyrosine residues that are phosphorylated by c-Src kinase are within the RGS homology domain of GRK2.

2) to investigate the mechanisms of c-Src modulation of GRK2 interaction with Gαq.

This point was investigated using a co-immunoprecipitation approach, to follow GRK2/Gαq* interaction, and looking at the modulation of this interaction produced by recombinant mutants of c-Src kinase with enhanced or ablated kinase activity.

3) to investigate the relative role of GRK2 and Gαq tyrosine-phosphorylation in the modulation of their interaction.

This point was investigated using the co-immunoprecipitation approach with the aid of a mutated GRK2 with impaired Src-mediated tyrosine-phosphorylation, to assess the role of GRK2 tyrosine-phosphorylation, and with in vitro binding assay, to assess the role of Gαq tyrosine-phosphorylation.

4) to investigate the functional consequences of the modulation of GRK2/Gαq interaction by c-Src.
This point was investigated following AlF4⁻-stimulated Gαq signalling and its modulation by GRK2, in presence or in absence of its tyrosine-phosphorylation.

5-2 Modulation of GRK2/Gαq interaction by c-Src

5-2-1 Src-phosphorylation site in GRK2 sequence

The work of Mayor and colleagues (Sarnago, 1999) reported the kinetic parameters of Src-mediated GRK2 phosphorylation, without identifying which tyrosine residue is the target of c-Src activity. Subsequent investigations were conducted in order to shed new light on this subject. A fusion protein of GRK2 N terminus (a.a. 50-145) undergoes tyrosine phosphorylation in in vitro assay, in the presence of a constitutively activated mutant of c-Src (c-Src-Y527F or Src*), suggesting that the GRK2 tyrosine residue phosphorylated by c-Src is located in the N terminus. These studies, were performed with protein fragments that may not retain the correct tertiary structure and thereby residues may be exposed which are not the sites of in vivo phosphorylation. Further studies with a C-terminal deleted mutant of GRK2 (a.a. 1-546) confirm that GRK2 tyrosine residues, phosphorylated by c-Src, could be located in GRK2 N-terminus. In fact, this construct was still tyrosine-phosphorylated by a constitutively activated mutant of c-Src (c-Src-Y527F). The analysis of GRK2 amino acidic sequence identified four tyrosine residues in GRK2 domain (residues 51-145) that could be potential sites of c-Src-phosphorylation (Fig 5-1). Interestingly tyrosine in position 86 (Y86) is located in a consensus sequence for kinases of Src family, the sequence YEEI. It is well known that this sequence, upon tyrosine-phosphorylation (pYEEI) is an interaction site for the SH2 domain of c-Src.
Fig 5-1. Potential Src-phosphorylation sites in GRK2 sequence

Top: the overall structure of GRK2 is shown. The N-terminal RGS domain of ~120 residues is boxed in *light grey*. The central catalytic domain of ~270 residues is shown in an *empty box*, whereas the C-terminal pleckstrin homology domain of ~100 residues is shown in a *green box*.

Bottom: Amino acid sequence of GRK2 RGS domain (residues 51-173) is shown. Tyrosine residues Y65, Y86, Y92 and Y112 that were mutated to phenylalanine are reported in *magnified red* characters. The other tyrosine residue, included in GRK2 RGS domain but outside the potential c-Src-phosphorylation domain, is reported in *magnified black character*. The *arrow* underlies GRK2 domain (a.a. 51-145) that is substrate of Src-phosphorylation.
Potential Src-phosphorylation sites in GRK2 sequence.
Tyrosine in position 86 has been mutated, together with tyrosine in position 92 to obtain the GRK2-Y86,92F mutant (Fig 5-1). The double mutant choice was suggested by the possibility that sometimes replacement of a crucial determinant at the canonical phosphorylation site is tolerated if the same residue is present at subsidiary close positions (Pinna, 1996). This observation is particularly true for tyrosine kinases that do not have well-defined site specificity. Other GRK2 mutants were produced with single mutations of other two tyrosine residues present in GRK2 sequence (residues 51-145) and the GRK2-Y65F and GRK2-Y112F constructs were obtained. Two other tyrosines (Y13, Y46) upstream of the potential Src-phosphorylation domain were also mutated, to produce GRK2-Y13F, GRK2-Y46F mutants. Moreover, double, triple and quadruple mutants were obtained with different combinations of mutated tyrosine residues (Fig 5-2). All these mutants were tested for their ability to undergo c-Src-phosphorylation. The most interesting results were obtained with GRK2-Y86,92F and GRK2-Y13,86,92F mutants. Adequate control experiments underlined that these two GRK2 mutants were correctly over-expressed, in HEK 293 and COS 7 cells.

Moreover GRK2 mutant kinase activity, on a receptor substrate, rhodopsin, and a soluble substrate, casein, were similar to wt GRK2. It is noteworthy that, in \textit{in vitro} phosphorylation assays with the constitutive active mutant of c-Src (Src*), the double mutant GRK2-Y86,92F and the triple mutant GRK2-Y13,86,92F showed decreased Src-phosphorylation. To verify whether the phosphorylation was impaired also \textit{in vivo} under more physiological conditions, these two mutants were transfected together with $\beta_2$-adrenergic receptor and wild type c-Src in COS 7 cells; their ability to be tyrosine-phosphorylated upon $\beta_2$-adrenergic receptor stimulation was subsequently tested.
Fig 5-2. GRK2 tyrosine mutants

The first column reports the list of GRK2 mutants, tested for their ability to undergo Src phosphorylation. The second column indicates the experimental conditions: Src* indicates that GRK2 mutants were co-transfected in COS 7 cells with the constitutively activated mutant of c-Src (Src*). After immunoprecipitation with anti-GRK2 antibody, tyrosine phosphorylation of these mutant was analysed with Western blot, using an anti-phosphotyrosine antibody. c-Src + iso indicates that GRK2 mutants were co-transfected with c-Src and β2-adrenergic receptor. Transfected cells were stimulated with isoproterenol (10 µM) for 5 min and then GRK2 mutants were immunoprecipitated with an anti-GRK2 antibody. Their tyrosine phosphorylation was analysed, in comparison with wt GRK2, with Western blot, with anti-phosphotyrosine antibody. The third column reports an estimate of Src kinase activity on the different mutants.
<table>
<thead>
<tr>
<th>GRK2 mutants</th>
<th>experimental assay</th>
<th>Src phosphorylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRK2 -Y13F</td>
<td>Src*</td>
<td>unaffected</td>
</tr>
<tr>
<td>GRK2 -Y46F</td>
<td>Src*</td>
<td>unaffected</td>
</tr>
<tr>
<td>GRK2 -Y65F</td>
<td>Src*</td>
<td>unaffected</td>
</tr>
<tr>
<td>GRK2 -Y112F</td>
<td>Src*</td>
<td>unaffected</td>
</tr>
<tr>
<td>GRK2 -Y86,92F</td>
<td>c-Src + iso</td>
<td>impaired</td>
</tr>
<tr>
<td>GRK2 -Y13,86,92F</td>
<td>Src*</td>
<td>impaired</td>
</tr>
<tr>
<td>GRK2 -Y13,86,92F</td>
<td>c-Src + iso</td>
<td>impaired</td>
</tr>
<tr>
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<td>c-Src + iso</td>
<td>impaired</td>
</tr>
<tr>
<td>GRK2 -Y13,65,86,92F</td>
<td>c-Src + iso</td>
<td>impaired</td>
</tr>
<tr>
<td>GRK2 -Y46,65,86,92F</td>
<td>c-Src + iso</td>
<td>impaired</td>
</tr>
</tbody>
</table>
Transfected cells were stimulated with a β2-adrenergic receptor agonist (isoproterenol 10 µM) at different time intervals, then GRK2 was immunoprecipitated with a specific polyclonal antibody and tyrosine-phosphorylation was analysed using anti-phosphotyrosine antibodies. This analysis revealed that in contrast with wt GRK2, showing a maximal tyrosine-phosphorylation within 5 min of agonist exposure, both mutants presented impaired agonist-stimulated tyrosine-phosphorylation (Fig 5-3). Fig 5-3 reports results obtained with the triple mutant GRK2-Y13,86,92F, but similar results were obtained with GRK2-Y86,92F. These data are in agreement with the results obtained with GRK2 N terminus fusion protein (a.a. 50-145) and GRK2 C terminal deleted mutant (a.a. 1-546) and they further indicate that the tyrosine residue, target of Src-phosphorylation, is located in the GRK2 N terminus. It is important to notice that Src-phosphorylation is conditioned by other factors than the presence of specific determinants in the sequence of the phospho-acceptor. In this regard GRK2 not only contains a putative binding site for the SH2 domain of c-Src but also a proline sequence in its C terminus (similar to a Type II proline rich motif) that could associate to the SH3 domain of c-Src, constituting another interaction site.

Proline sequence in GRK2 C terminus is a putative additional interaction site. It is not necessary for c-Src interaction, as indicated by the experiment with the C-terminus deleted mutant, but could increase the affinity of GRK2 for c-Src.

5-2-2 Src* increases the interaction between GRK2 and activated Gαq

The previous data support the hypothesis that a Src-phosphorylation site is located in the N-terminus of GRK2.
Fig 5-3. Isoproterenol-induced tyrosine phosphorylation of GRK2 and GRK2-Y13,86,92F mutant

A Time course of isoproterenol-stimulated wt GRK2 and GRK2-Y13,86,92F mutant. COS 7 cells, transiently transfected with wt GRK2 or GRK2-Y13,86,92F, plus c-Src and β2-adrenergic receptor (1 μg cDNA of each/1 x 10^6 cells), were stimulated (or not) for the indicated times with isoproterenol (ISO 10 μM). Immunoprecipitates (anti-GRK2 IPP) of GRK2 from RIPA buffer lysates were resolved by 8% SDS-PAGE, blotted, and analysed with anti-phosphotyrosine (anti-pyr) antibodies (upper panel); after stripping, the presence of GRK2 was analysed in the same gel using a specific anti-GRK2 antibody (Ab9) (lower panel). The arrows indicate the migration of GRK2.

B Whole cell lysates were used for immunoblotting with anti-phosphotyrosine (anti-pyr) antibodies (upper panel); after stripping the levels of expression of GRK2 and GRK2-Y13,86,92F mutant were analysed using a specific anti-GRK2 antibody (Ab9) (middle panel) while c-Src expression was verified with a commercial anti-Src antibody (lower panel). In this way it was checked that the same amount of each protein was expressed in every transfection condition. The arrows indicate the migration of GRK2 and c-Src.

C Quantification of isoproterenol-stimulated tyrosine-phosphorylation of GRK2 and GRK2-Y13,86,92F mutant. Tyrosine-phosphorylation of wt GRK2 and mutated was measured by scanner laser densitometry, and the data were normalised to the amount of GRK2 protein present in the immunoprecipitates, as assessed with the anti-GRK2 antibodies. In each transfection unstimulated controls were taken as the basal condition. Stimulated data points are mean ± S.E. from three independent experiments.
Isoproterenol-induced tyrosine phosphorylation of GRK2 (fold over basal)

Fig 5-3
Phosphorylation of a tyrosine residue changes the polarity environment introducing a negative charge that could significantly modify the ability of GRK2 N terminus to interact with other signalling proteins. The putative Src-phosphorylation targets, Y86 and Y92, are located in the GRK2 RGS domain, which is involved in G protein alpha subunit interaction. All these observations raised the question whether c-Src activity might regulate GRK2/Gαq interaction.

In order to investigate the possible regulatory role of c-Src on GRK2/Gαq binding a constitutively activated mutant of c-Src (Src*) was used. This mutant has the tyrosine residue Y527, critical for kinase activity, mutated to phenylalanine. Y527 is located in the C-terminal of the c-Src SH1 domain (Src homology domain 1 or kinase domain), called regulatory region, and, under basal conditions, is phosphorylated by another kinase (C-terminal Src kinase or Csk) and bound to the c-Src SH2 domain. Formation of this intracellular bridge favours the interaction of two other c-Src domains, the SH3 domain and the polyproline helix. These intramolecular interactions result in inhibition of c-Src kinase activity. Activation of c-Src is achieved by dephosphorylation of Y527 by a phosphatase. Mutation of Y527 to phenylalanine destabilises the two intramolecular interactions producing a constitutively activated mutant.

GRK2/Gαq interaction was studied with the co-immunoprecipitation approach. GRK2 and the constitutively activated mutant of Gαq (Gαq-R183C or Gαq*) were co-expressed in HEK 293 cells in the presence or in the absence of the constitutively activated mutant of c-Src (Src*). Immunoblotting of transfected cells lysates, with anti-GRK2 polyclonal antibodies, revealed that GRK2 expression levels were unmodified by Gαq* and Src* co-expression. Moreover, Gαq* expression levels were similar in the absence or in the presence of co-expressed GRK2 or Src* (Fig 5-4A).
Fig 5-4. Co-immunoprecipitation analysis of GRK2 and Gaq* in the absence or the presence of Src*

A Whole cell lysates, from HEK 293 cells transfected as indicated in the figure, were immunoprecipitated with anti-GRK2 antibodies. Cell lysates and samples from anti-GRK2 immunoprecipitates (anti-GRK2 IPP) were resolved by 10% SDS-PAGE, blotted and analysed with anti-GRK2 (upper panel) and anti-Gaq antibodies (lower panel). There is an increase in the amount of co-immunoprecipitated Gaq-R183C (Gaq*) when a constitutively active mutant of c-Src (Src*)(3 μg/3 x 10^6 cells) was co-expressed together with GRK2 (3 μg/3 x 10^6 cells) and Gaq-R183C (3 μg/3 x 10^6 cells).

IP: immunoprecipitation.

B Quantification of Gaq*-GRK2 co-immunoprecipitated. The amount of co-immunoprecipitated Gaq* was measured by scanner laser densitometry and the data were normalised to the amount of GRK2 protein present in the immunoprecipitates, as assessed with the anti-GRK2 antibodies. In each experiment the transfection in absence of Src* was taken as the basal condition. Data are presented as mean ± S.E. of five experiments. * p<0.05. (the two-tailed p value is 0.0185 analysed with paired Student t test).
Fig 5-4

A  IP: anti-GRK2 Ab (I), preimmune (P)

Anti-GRK2 IPPs

Cell lysates

anti-GRK2 BLOT

Anti-Gaq Ab

GRK2

Gαq

Src

anti-Gαq BLOT

B  IP: anti-GRK2 Ab
IB: anti-Gαq Ab

Fold of Gαq*-GRK2 co-precipitated

GRK2

Gαq

Src

244
For immunoprecipitation cell lysates were incubated with either anti-GRK2 polyclonal antibody or a preimmune serum (Fig 5-4A). Subsequent blotting of the immunoprecipitated material, with anti-Gαq specific antibody, revealed that anti-GRK2 polyclonal antibody did not precipitate Gαq* in the absence of over-expressed GRK2, but co-immunoprecipitated Gαq* when GRK2 was co-transfected. Moreover, the amount of co-immunoprecipitated Gαq* was greater when Src* was co-expressed. On the other hand the preimmune serum didn’t precipitate GRK2 nor Gαq*. Quantification of five different experiments indicates that Src* co-expression produces ~2 fold increase of co-immunoprecipitated Gαq* (Fig 5-4B). Src* ability to increase GRK2/Gαq* interaction was verified under different transfection conditions as shown in Fig 5-5A. Increasing amounts of Gαq* (1.5, 1, 3, 5 μg/3 x 10^6 cells) were transfected with the same quantity of GRK2 (5 μg/3 x 10^6 cells) in the absence or presence of Src* and then GRK2 immunoprecipitation was performed. The amount of Src* was kept constant, (3 μg/3 x 10^6 cells), in order to obtain a maximal tyrosine-phosphorylation of its substrates. With the exception of the transfection condition in which 1.5 μg of Gαq* was transfected without showing any detectable GRK2-Gαq* co-immunoprecipitation, in all other conditions Src* co-transfection always increased GRK2/Gαq* interaction. Similar results were obtained with Gαq wild type. wt Gαq has a lower affinity for GRK2 than Gαq*, and only a small amount can be co-immunoprecipitated with anti-GRK2 antibody. In any case Src* co-expression increased also GRK2/wt Gαq interaction (Fig 5-5B).
Fig 5-5. Immunoblot analysis of GRK2 and wt Gαq and a constitutively activated mutant Gαq-R183C in anti-GRK2 immunoprecipitates

A Whole cell lysates, from HEK 293 cells transfected with the same amount of GRK2 (3 μg/3 x 10^6 cells) and increasing amount of Gαq-R183C construct (0.5, 1, 3, 5 μg/3 x 10^6 cells), were subjected to immunoprecipitation with anti-GRK2 antibodies. Samples from anti-GRK2 immunoprecipitates (anti-GRK2 IPP) were resolved by 10% SDS-PAGE, blotted, and analysed with anti-GRK2 antibodies (upper panel) and anti-Gαq antibody (lower panel). The co-expression of a constitutively active mutant of c-Src (Src*) (3 μg/3 x 10^6 cells) produces an increase in the amount of co-immunoprecipitated Gαq-R183C.

B The same experiment was performed with wt Gαq. Also in this case an increase in the amount of co-immunoprecipitated Gαq was observed in presence of Src*. Immunoblot analysis of cell lysates was reported to check for the expression level of the different transfected proteins.
5-2-3 c-Src kinase activity is necessary to increase GRK2/Goq interaction

To determine whether c-Src kinase activity was necessary to increase GRK2/Goq interaction, we compared the effect of wt c-Src, a dominant negative c-Src (c-Src-K295R or SrcDN) and Src* (Fig 5-6). Both GRK2 and Goq* were over-expressed in HEK 293 cells alone or in the presence of wt c-Src or of its mutants. GRK2 was then immunoprecipitated and the amount of co-immunoprecipitated Goq* was quantified. The interaction was significantly increased only in presence of Src* (Fig 5-6).

This interaction was also studied in parallel experiments, performed by immunoprecipitating Goq* and measuring the amount of co-immunoprecipitated GRK2 (Fig 5-7). Also in this case a significant effect was observed only in the presence of Src*. The immunoprecipitation of Goq appears to be more sensitive to evaluate Src*-induced GRK2/Goq* increased interaction.

Phosphotyrosine immunoblotting of cell lysates revealed a generally increased tyrosine-phosphorylation of cellular proteins upon over-expression of Src* in HEK 293 cells. Further analysis of the same immunoprecipitated materials showed that in the presence of Src* both GRK2, in Goq* immunoprecipitates, and Goq*, in GRK2 immunoprecipitates, are tyrosine phosphorylated (Fig 5-8).

Upon Src* over-expression both proteins undergo tyrosine-phosphorylation and both proteins are tyrosine-phosphorylated in the immune complex. Interestingly several investigators have reported that the alpha subunit of Gi, Gt, Go, Gs, Gq/11 are substrates of tyrosine kinases in vitro (Moyers, 1995). In particular in Rat-1 fibroblasts transformed with the v-src oncogene, Gαq/11 becomes phosphorylated at tyrosine residues (Liu, 1996).
**Fig 5-6. Immunoblot analysis of GRK2 and Gαq-R183C in anti-GRK2 immunoprecipitates, in presence of wt c-Src, Src* and SrcDN**

**A** HEK 293 cells were transfected with GRK2 (3 μg/3 x 10^6 cells), Gαq* (3 μg/3 x 10^6 cells) and different c-Src mutants (3 μg/3 x 10^6 cells) as indicated in the figure. Cells were subjected to immunoprecipitation with anti-GRK2 antibodies. Samples from anti-GRK2 immunoprecipitates (anti-GRK2 IPP) were resolved by 10% SDS-PAGE, blotted, and analysed with anti-GRK2 antibodies (upper panel) and anti-Gαq antibody (lower panel).

**B** Quantification of Gαq*/GRK2 co-immunoprecipitation. Data are the mean ± S.E. of five experiments. (* p<0.05 as analysed with paired Student t test).
Fig 5-7. Immunoblot analysis of GRK2 and Goq-R183C in anti-GRK2 and anti-Goq immunoprecipitates, in absence or in presence of wt c-Src or Src*

A HEK 293 cells transfected with GRK2, Goq-R183C (Goq*) and wt c-Src or Src* (3 μg cDNA of each/3 x 10⁶ cells) were subjected to immunoprecipitation with anti-GRK2 antibodies. Cell lysates and samples from anti-GRK2 immunoprecipitates (anti-GRK2 IPP) were resolved by 10% SDS-PAGE, blotted, and analysed with anti-GRK2 antibodies (upper panel), anti-Goq antibody (middle panel) and anti-Src antibody (lower panel). Only the co-expression of Src* produces a significant increase in GRK2-Goq* interaction. For the quantification of GRK2-Goq* interaction the amount of co-immunoprecipitated Goq* was measured by scanner laser densitometry and the data were normalised to the amount of GRK2 protein present in the immunoprecipitates, as assessed with the GRK2 antibodies. In each experiment the transfection in absence of Src* was taken as the basal condition. Data are mean ± S.E. of five experiments. * p<0.05. (the two-tailed p value is 0.0185 analysed with paired Student t test).

B HEK 293 cells transfected with GRK2, Goq* and wt c-Src or Src* (3 μg cDNA of each/3 x 10⁶ cells) were subjected to immunoprecipitation with anti-Goq polyclonal antibody. Cell lysates and samples from anti-Goq immunoprecipitates (anti-Goq IPP) were resolved by 10% SDS-PAGE, blotted, and analysed with anti-GRK2 antibodies (upper panel), anti-Goq antibody (middle panel) and anti-Src antibody (lower panel). Only the co-expression of Src* produces an increase in GRK2-Goq* interaction. For the quantification of GRK2-Goq* interaction the amount of co-immunoprecipitated GRK2 was measured by scanner laser densitometry and the data were normalised to the amount of Goq* protein present in the immunoprecipitates, as assessed with the Goq antibodies. In each experiment the transfection in absence of Src* was taken as the basal condition. Data are mean ± S.E. of 3 experiments. * p< 0,05. (the two-tailed p value is 0,028 analysed with unpaired Student t test).
A

<table>
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<tr>
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<tr>
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<td><strong>anti-Src BLOT</strong></td>
<td><strong>c-Src</strong></td>
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Fold-stimulation of Gαq-Gαq co-p.:

- Src Src*

IP: anti-GRK2 Ab
IB: anti-Gαq Ab

Fold-stimulation of Gαq-Gαq co-p.:

- Src Src*

IP: anti-Gαq Ab
IB: anti-GRK2 Ab
Fig 5-8. In GRK2-Gαq* immune complex both proteins are phosphorylated on tyrosine residues

Whole cell lysates, from HEK 293 cells transfected with the same amount of GRK2 (3 μg/3 x 10^6 cells) and two different amounts of Gαq-R183C (3-5 μg), were subjected to immunoprecipitation with anti-GRK2 (A) and with anti-Gαq polyclonal antibody (B). Immunoblot analysis of cell lysates and of the two different kinds of immunoprecipitations (IPP) was performed with anti-phosphotyrosine antibody. When Src* (3 μg/3 x 10^6 cells) was co-expressed, both GRK2 and Gαq-R183C resulted phosphorylated in tyrosine in the immune complex.

IP: immunoprecipitation; IB: immunoblotting.
**Fig 5-8**

**A**

anti-ptyr BLOT

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Lysates  

Anti-GRK2 IPPs

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**B**

anti-ptyr BLOT

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Lysates  

Anti-Gαq IPPs

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<td>Src</td>
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IP: anti-GRK2 Ab  
IB: anti-Gαq Ab  
IP: anti-Gαq Ab  
IB: anti-GRK2 Ab
Moreover, Yamamoto and colleagues (Umeme, 1997) demonstrated that Gaq/11 can be activated upon tyrosine phosphorylation presumably produced through c-Src kinase activity. Gaq residue involved in this reaction was identified as Tyr 356.

5-2-4 GRK2 tyrosine-phosphorylation has a primary role in the modulation of Gaq* interaction

Src-induced covalent modification of both proteins could be at the basis of the increased GRK2/Gaq* binding. In order to investigate if GRK2 tyrosine-phosphorylation could have a role, we used a mutated kinase (GRK2-Y13,86,92F) with the proposed three tyrosines, targets of Src-phosphorylation, mutated into phenylalanine residues. When co-transfected with Src*, GRK2 mutant showed a 50% reduction of phosphorylation, as compared to the wild type kinase (Fig 5-9). In both co-immunoprecipitation assays this mutant did not show any significant increase in Gaq* interaction in the presence of co-expressed Src* (Fig 5-10). These results suggest that GRK2 tyrosine-phosphorylation plays a primary role in the modulation of GRK2-Gaq* binding. In fact, in conditions in which c-Src ability to phosphorylate GRK2 was impaired, no increased interactions with Gaq* were promoted. To further investigate this hypothesis we performed an in vitro binding assay in order to demonstrate that Gaq* tyrosine-phosphorylation, induced by Src* kinase activity, fails to produce increased GRK2 interaction. HEK 293 cells were transfected with Gaq* alone or in the presence of co-transfected Src*. Upon Src* overexpression Gaq* undergoes tyrosine-phosphorylation, as shown by Western blot analysis of cell lysates performed with anti-phosphotyrosine antibody (Fig 5-11A).
Fig 5-9. A mutated GRK2-Y13,86,92F presented impaired tyrosine-phosphorylation.

A HEK 293 cells were transfected with wt GRK2 (wt) or GRK2-Y13,86,92F (YF), in the absence or the presence of Src* (3 µg cDNA of each/3 x 10⁶ cells). Cells were then subjected to immunoprecipitation with anti-GRK2 antibodies. Samples from anti-GRK2 immunoprecipitates (anti-GRK2 IPPs) were resolved by 10% SDS-PAGE, blotted and analysed with anti-ptyr antibody (lower panel), and after stripping with anti-GRK2 antibodies (upper panel). Src* co-expression produces a significant increase in wt GRK2 tyrosine-phosphorylation, while GRK2-Y13,86,92F mutant presented impaired tyrosine-phosphorylation.
**Fig 5-10. A mutated GRK2-Y13,86,92F that presented impaired tyrosine-phosphorylation failed in increasing Gαq* interaction in presence of Src* co-expression**

**A** HEK 293 cells transfected with GRK2-Y13,86,92F, Gαq-R183C in the absence or the presence of Src* (3 μg cDNA of each/3 x 10^6 cells) were subjected to immunoprecipitation with anti-GRK2 antibodies. Samples from anti-GRK2 immunoprecipitates *(anti-GRK2 IPP)* were resolved by 10% SDS-PAGE, blotted, and analysed with anti-GRK2 antibodies *(upper panel)*, anti-Gαq antibody *(lower panel)*. The co-expression of Src* does not produces a significant increase in GRK2-Gαq* interaction. For the quantification of Gαq*-GRK2 interaction the amount of co-immunoprecipitated Gαq* was measured by scanner laser densitometry and the data were normalised to the amount of GRK2 protein present in the immunoprecipitates, as assessed with the GRK2 antibodies. The transfection in absence of Src* was taken as the basal condition. Data are mean ± S.E. of five experiments. * p<0.05. (the two-tailed p value is 0.0185 analysed with paired Student t test).

**B** The same transfected cells were subjected to immunoprecipitation with anti-Gαq polyclonal antibody. Samples of anti-Gαq immunoprecipitates *(anti-Gαq IPP)* were resolved by 10% SDS-PAGE, blotted, and analysed with anti-GRK2 antibodies *(upper panel)*, anti-Gαq antibody *(lower panel)*. Neither in this case the co-expression of Src* produced an increase in GRK2-Gαq* interaction. For the quantification of GRK2-Gαq* interaction the amount of co-immunoprecipitated GRK2 was measured by scanner laser densitometry and the data were normalised to the amount of Gαq* protein present in the immunoprecipitates, as assessed with the Gαq antibodies. The transfection in absence of Src* was taken as the basal condition. Data are mean ± S.E. of 3 experiments. * p< 0.05. (the two-tailed p value is 0.028 analysed with unpaired Student t test).
A

anti-GRK2 IPPs
anti-GRK2 BLOT
- GRK2-Y13,86,92F
anti-Gαq BLOT
- Gαq*
- Src*

B

anti-Gαq IPPs
anti-GRK2 BLOT
- GRK2-Y13,86,92F
anti-Gαq BLOT
- Gαq*
- Src*

Fig 5-10

IP: anti-GRK2 Ab
IB: anti-Gαq Ab

Fold stimulation of
Gαq*-GRK2 cop.

- Src*
GRK2

- Src*
GRK2-Y13,86,92F

IP: anti-Gαq Ab
IB: anti-GRK2 Ab

Fold stimulation of
Gαq*-GRK2 cop.

- Src*
GRK2

- Src*
GRK2-Y13,86,92F
**Fig 5-11. Gaq* tyrosine phosphorylation failed to increase GRK2-Gaq* interaction**

HEK 293 cells were transfected with Gaq-R183C in the absence or in the presence of Src* (3 µg cDNA of each/3 x 10^6 cells). Cytosolic proteins (150 µg) from both transfections were incubated (1 h at 4 °C) with recombinant purified GST, GST-N-terI fusion proteins conjugated to glutathione agarose beads. After extensive washings, as described under “Experimental Procedure”, glutathione agarose beads with bound proteins and cells lysates (30 µg of starting cytosolic preparation) were electrophoresed on SDS-PAGE and transferred onto nitrocellulose membrane. Western blot analysis was then performed with anti-Gaq antibody (*upper panels*) and anti-phosphotyrosine antibody (*lower panels*). For the quantification of Gaq*/N-terI interaction the amount Gaq* bound to the beads was measured by scanner laser densitometry. The transfection in absence of Src* was taken as the basal condition. Data are mean ± S.E. of four different experiments. No significant difference was observed in Gaq*/N-terI binding between the two cytosolic extracts.
A

Lysates

anti-Goq
BLOT

anti-ptyr
BLOT

GST

GST-NterI

- Src*

- Gαq*

- Gαq*

B

GRK2/αq interaction (arbitrary units)

- Src*
Cytosolic extracts from Gaq* and Gaq*/Src*-co-transfected cells were incubated with glutathione agarose beads conjugated with GST or with GST-NterI (a.a. 50-145 of GRK2 sequence). Unbound proteins were removed by extensive washing, while bound proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. Immunoblot analysis with anti-Gaq polyclonal antibodies was used to reveal Gaq binding. Both cytosolic extracts presented a specific binding only with GST-NterI-conjugated glutathione agarose beads, but they also bound with the same efficiency. These results indicate that tyrosine phosphorylation of Gaq* does not produce the increased interaction with the N-terminal portion of GRK2.

5-2-5 Increased GRK2/Gaq* interaction induced by GPCR stimulation

In order to verify if GPCR activation could be the physiological trigger of the increased GRK2/Gaq* interaction, the m1 muscarinic receptor (mAChR) was transfected together with GRK2 and constitutively activated Gaq in HEK 293 cells. Several reports have recently established that stimulation of M1 mAChR and other GPCR leads to c-Src-mediated phosphorylation of several cellular proteins (Gutkind, 1998). Transfected cells were stimulated with carbachol (10 μM) for different periods of time (0-10 min). GRK2 was then immunoprecipitated and the amount of co-immunoprecipitated Gaq* was measured. Carbachol-stimulation resulted in a marked increase in tyrosine-phosphorylation of immunoprecipitated GRK2, which was maximal within 5-10 min of agonist exposure. These results demonstrate that M1 receptor activation triggers the rapid agonist-mediated tyrosine-phosphorylation of GRK2. A parallel increase in the amount of co-immunoprecipitated Gaq* was observed during the time course of carbachol-stimulation (Fig 5-12).
Fig 5-12. Increased GRK2/Gαq* interaction produced upon GPCR activation

HEK 293 cells transfected with m1 muscarinic receptor (6 µg/1.5 x 10^6 cells), GRK2 (1.5 µg/1.5 x 10^6 cells) and Gαq-R183C (1.5µg/1.5 x 10^6 cells), at 48 h from transfection, were stimulated for 2, 5, 10 min and then subjected to immunoprecipitation with anti-GRK2 antibody. Samples from anti-GRK2 immunoprecipitates were resolved by 10% SDS-PAGE, blotted, and analysed with anti-phosphotyrosine antibodies (anti ptyr) (upper panel), after stripping the expression levels of GRK2 and Gαq* were checked with anti-GRK2 (middle panel) and anti-Gαq antibodies (lower panel). Quantification of Gαq*/GRK2 interaction was done. The amount of co-immunoprecipitated Gαq* was measured by scanner laser densitometry and the data were normalised to the amount of GRK2 protein present in the immunoprecipitates, as assessed with the GRK2 antibodies. In each experiment the unstimulated sample was taken as the basal condition. Data are presented as mean ± S.E. of three experiments.
Fig 5-12

anti-ptyr BLOT

anti-GRK2 BLOT

anti-Gαq BLOT

Carbachol 10 μM

Fold stimulation of Gαq•-GRK2 co-ip.

0 2 5 10 min

Carbachol 10 μM

0 1 2 3 4 5 6

0 2 5 10 min
These results further support the idea that GRK2 tyrosine-phosphorylation, produced under physiological conditions, reinforces GRK2/Gαq* interaction.

5-3 Functional consequences of Src*-modulated GRK2/Gαq interaction

5-3-1 Gαq-mediated signalling is inhibited by GRK2 and GRK2-Y13,86,92F over-expression

Finally we verified if Src action on GRK2/Gαq binding could have any functional consequence.

In the previous chapters we reported that GRK2 inhibits Gαq activation of PLCβ through direct binding of its RGS domain to Gαq. We subsequently investigated if the Src-promoted increase in GRK2/Gαq interaction could produce an increased inhibition of Gαq signalling.

wt Gαq was over-expressed in HEK 293 cells and its signalling was stimulated with A1F4-+. An increase of basal inositol phosphates level was measured (~5 fold). When wt GRK2 or GRK2-Y13,86,92F were co-transfected together with wt Gαq no variation in basal IPs levels was observed but the A1F4+-stimulated signalling was significantly inhibited, as compared to cells expressing only the G protein (Fig 5-13). These data indicate that the three tyrosine residues mutated in GRK2 N-terminus are not critical determinants in Gαq interaction and even after tyrosine to phenylalanine mutation GRK2 retains its ability to bind and to inhibit Gαq signalling.

Moreover, GRK2-Y13,86,92F is an important tool in functional assays, because, due to impaired Src-mediated tyrosine-phosphorylation, it can be used to discriminate between Src-dependent and -independent mechanisms.
Fig 5-13. Both GRK2 and GRK2-Y13,86,92F inhibit AlF4- stimulated Gαq signalling

HEK 293 cells transfected with Gαq wt together with vector (control) or the indicated GRK2 constructs were subjected to total-[³H]inositol phosphates assay. 72 h after transfection cells were stimulated at 37 °C for 30 min with AlF4⁻ (30 μM AlCl₃ and 20 mM NaF) and the total-[³H]inositol phosphates produced were measured. Data, subtracted of basal values and expressed as cpm/well, are mean ± SE from 3 to 5 separate experiments. Both GRK2 and GRK2-Y13,86,92F constructs produced a significant decrease of AlF4⁻-stimulated Gαq signalling. (** p<0.01, *** p<0.001 ANOVA test with Tukey post-test were used for statistical analysis.
5-3-2 Src* further increases GRK2-mediated inhibition of Gαq signalling

Co-transfection of Src* with Gαq produced an increase in the basal levels of total IPs (about 2 fold), as compared to cells transfected only with Gαq, but also a significant inhibition of AlF₄⁻-stimulated PLCβ activity (35% of inhibition) (Fig 5-14). Moreover, when Src* was co-expressed with GRK2, in the same conditions in which increased GRK2/Gαq interaction was produced, the inhibition of Gαq signalling was more pronounced (more than 50% of inhibition compared with cells transfected only with GRK2 and Gαq) (Fig 5-14). This observation seems to confirm the hypothesis that Src* activity on GRK2/Gαq interaction has the functional consequence to inhibit Gαq signalling.

On the other hand, co-transfection of Src* with GRK2-Y13,86,92F, that presented impaired tyrosine kinase phosphorylation, does not produce any effect when compared with cells that express only GRK2-Y13,86,92F and Gαq. These data further indicate that GRK2 tyrosine-phosphorylation, necessary for increased GRK2/Gαq interaction, reinforces GRK2 ability to inhibit AlF₄⁻-stimulated Gαq signalling.

5-4 Discussion

5-4-1 GRK2 tyrosine-phosphorylation induced by c-Src increases GRK2/Gαq interaction

The aim of this chapter was to investigate whether Src-phosphorylation of GRK2 could produce any effect on GRK2 ability to bind to the alpha subunit of Gq. In vitro phosphorylation assays, performed with a constitutively activated c-Src mutant and the recombinant GRK2 N-terminus (a.a. 50-145), suggested that the tyrosine target of Src-phosphorylation resides within this domain of GRK2.
**Fig 5-14. Src* over-expression is able to inhibit Gαq-mediated signalling, upon AlF₄⁻ activation**

HEK 293 cells, transfected with Gαq wt alone or with the indicated GRK2 and c-Src constructs, were subjected to total-[³H]inositol phosphates assay. 72 h after transfection cells were stimulated at 37 °C for 30 min with AlF₄⁻ (30 µM AlCl₃ and 20 mM NaF) and the total-[³H]inositol phosphates produced were measured. Data, subtracted of basal values and expressed as percent of control cells (cells of the same type of transfection without co-transfected c-Src), are mean ± SE from 3 to 5 separate experiments. (* p< 0.05, ** p<0.01 as analysed with paired Student t test (p value between the two columns: pcDNA and pcDNA+Src* is 0.004 and the one between the two columns GRK2 and GRK2+Src* is 0.03).
In subsequent mutagenesis analyses all tyrosine residues that lay in this domain were mutated to phenylalanine; two tyrosine residues located in the flanking N-terminal region were also mutated. All these mutants were tested for their ability to undergo Src-phosphorylation. No mutant presented a completely blocked Src-phosphorylation in the presence of the constitutively activated c-Src. This is probably due to the fact that under forced experimental conditions other sites can be phosphorylated by c-Src, in addition to those that have been identified.

In more physiological conditions, in intact cell studies, by stimulating c-Src with a β2-adrenergic receptor agonist, two mutants (GRK2-Y86,92F and GRK2-Y13,86,92F) presented profoundly impaired Src-phosphorylation (80% inhibition as compared to GRK2 wild type). Both Y86 and Y92 could be potential targets of Src-phosphorylation. These residues are located in the GRK2 RGS domain that is directly involved in Gαq interaction. A more detailed analysis indicated that the two tyrosines are located in the fourth alpha helix of the RGS box and crystallographic analysis revealed that in this RGS4 region are located amino acids directly involved in Gα interaction (Fig 5-15). This observation raised the hypothesis that GRK2 phosphorylation promoted by c-Src could directly affect GRK2/Gαq binding. Co-immunoprecipitation assays clearly indicated that GRK2/Gαq interaction was increased about 2 fold by c-Src activity. The immunoblot analysis of the immunoprecipitation reactions, performed with anti-phosphotyrosine antibody, revealed that both GRK2 and Gαq are tyrosine-phosphorylated in the immune complex formed in the presence of constitutively activated c-Src. However GRK2 tyrosine-phosphorylation plays a primary role in reinforcing Gαq binding, as shown from experiments performed with the GRK2 mutant with impaired Src-mediated phosphorylation.
Fig 5-15. Alignment of GRK2 and GRK3 N termini with RGS domain

*Top:* overall topology of GRK2 and GRK3.

*Bottom:* GRK2 and GRK3 (residues 51-173) were aligned with RGS domains of different proteins. In particular RGS4 (residues 59-176). The predicted secondary structure is represented by the *black bars* labelled α1-α9 for each of the α-helices in this structure. Hydrophobic residues thought to be largely involved in forming the hydrophobic core of this structure are shown in *grey*. Residues in RGS4 shown to contact Ga (Tesmer, 1997) are designated α.
An extensive investigation on residues that represent important contact sites between RGS4 and Goi1, as shown by the crystallographic analysis of the complex, has been performed (Tesmer, 1997).

The mutagenesis analysis allowed to discriminate between RGS4 amino acids important for GAP activity or for G protein binding (Wieland, 2000). In RGS4 sequence the interaction residues are Glu-87, Asn-88, Asn-128.

Neither of them are conserved in GRK2 structure (Fig 5-15). Mutation of Asn-88, Asn-128 in RGS4, or of the corresponding residues in RGS16, seriously impaired GAP activity, while Glu-87 mutation did not affect the efficacy but highly reduced the potency of GAP activity toward Goi1, thus indicating a reduced affinity for the G protein.

It seems that this highly conserved glutamate in RGS proteins interacts with the equally well conserved lysine in Goi and Goq family members and it is important for high affinity binding but is apparently not directly involved in the acceleration of the GTPase hydrolysis. The binding is a consequence of electrostatic attraction between polar amino acids with opposite charges. The glutamate-87 lies in the fourth-alpha helix of the RGS domain (α4). In GRK2 sequence the glutamate residue is replaced by an alanine and this residue is separated by six amino acids by the tyrosine 86, hypothetical target of Src-phosphorylation. Upon Src-phosphorylation a negative charge is introduced on GRK2 in the same alpha helix side where the corresponding RGS4 glutamate is located, even if on the subsequent wheel. Due to the importance of the negative charged residue in RGS4/Goi/q interaction, we could hypothesise that the increased interaction between GRK2 and Goq, observed upon Src-phosphorylation, could be consequent to the introduction of a new negative charge on the fourth alpha helix of GRK2 RGS domain.
5-4-2 Increased GRK2/Gαq interaction induced by c-Src further inhibits Gαq signalling

The functional consequences of the observed GRK2/Gαq increased interaction were then investigated. We previously reported that GRK2 N-terminus binding to Gαq produces a partial inhibition of Gαq signalling. Experiments of total inositol phosphate production were performed in HEK 293 cells over-expressing wt Gαq and Gαq signalling was stimulated with AlF₄⁻. When GRK2 was co-expressed with Gαq more than 40% inhibition of second messenger production was measured, but the contemporaneous co-expression of Src⁺ produced an additional 50% inhibition, which was completely reversed upon expression of the dominant negative construct of c-Src. This further inhibition requires Src activity, in particular Src-mediated phosphorylation of GRK2 is necessary. In fact, the GRK2 mutant that does not undergo Src-phosphorylation is not able to produce a further blockage of Gαq signalling, in the presence of co-expressed Src⁺. It seems that in conditions in which GRK2/Gαq binding is reinforced a further higher blockage of Gαq signalling can be observed.

In Fig 5-16 we reported a schematic model of GRK2 modulation of Gq-coupled receptors signalling. Upon agonist (A) binding the receptor is in the active conformation and can undergo GRK2 phosphorylation with consequent uncoupling from the transduction machinery. In addition, GRK2 contains an RGS domain, which leads to fairly selective inhibition of Gq-mediated signal transduction pathways. Receptor phosphorylation increases its affinity for βarrestin (β-arr.) proteins that translocate to the membrane and bind to the receptor. βarrestin is actually accepted as a multidomain protein and can recruit c-Src kinase through the binding with its SH3 domain (Luttrell, 1999).
Fig 5-16. A model for GRK2 involvement in Gαq-mediated signalling termination

Detailed description of the model is in the discussion.
β-arrestin binds to c-Src and also targets the agonist-occupied, GRK2-phosphorylated GPCR to the clathrin-coated pits for internalisation (Lin, 1999). In this way c-Src is positioned in the close proximity of both GRK2 and Ga\textsubscript{q} and can phosphorylate both proteins. We propose that recruited c-Src can phosphorylate GRK2, modulating its kinase activity and its ability to couple to Ga\textsubscript{q}. Upon GRK2 tyrosine-phosphorylation GRK2-Ga\textsubscript{q} binding is reinforced and Ga\textsubscript{q}-mediated signalling further inhibited.
CHAPTER 6
FINAL DISCUSSION

Four subtypes of GRKs are expressed in leukocytes, GRK2, GRK3, GRK5, GRK6, and two different arrestins, βarrestin1 and βarrestin2 (Lefkowitz, 1993a; Chuang, 1996). Moreover, GRKs levels of expression and kinase activity are modulated according with the state of cell maturation and activation (Chuang, 1992; Chuang, 1995; Loudon, 1996). The high level of expression of these kinases observed in mature leukocytes and the modulation of the enzyme activities suggest that GRKs and arrestins may be potent modulators of receptor-mediated immune responses. Potential targets of GRK/arrestin action in leukocytes are GPCRs that mediate chemotaxis. It has been reported that, in phagocytic leukocytes, chemoattractant receptors can desensitise in the presence of agonists, suggesting the involvement of a GRK/arrestin mechanism in this process (Morrison, 1989). In vitro experiments showing agonist-dependent phosphorylation of N-formyl peptide (fMLP), C5a, PAF receptors, further support this possibility (Ali, 1994; Prossnitz, 1995; Langkabel, 1999). The present study focuses on the process of homologous desensitisation of the human Platelet-Activating Factor receptor. This process was mainly investigated with a cellular model, in which the expression of recombinant PAFR in heterologous expression system allows the analysis of the molecular determinants involved in a signalling pathway. First of all the pathways activated by PAFR, upon agonist-stimulation, were defined. In COS 7 cells PAFR stimulates IPs production and intracellular cAMP accumulation through the coupling with Gq/11 and Gs proteins, respectively. The Gs-coupling was a totally unpredicted finding, because the common view of PAFR is of a Gi-coupled receptor. Numerous GPCRs that are classified as Gi-coupled receptors have been
described to be able to stimulate adenylyl cyclase through Gs coupling, as the 5-HT\textsubscript{1D} receptor, the δ opioid receptor and the α\textsubscript{2}AR (Cruciani, 1993; Van Sande, 1993, Eason, 1995). Moreover, our observation that PAF can stimulate cAMP accumulation in human peripheral blood lymphocytes, in a receptor-mediated manner, suggests that PAFR coupling with adenylyl cyclase may be important also for endogenously expressed receptors. No obvious physiological role for this coupling can be envisaged in lymphocytes, even if it is well documented that cAMP causes negative modulation of PAFR at transcriptional and posttranscriptional level (Font, 1993; Thivierge, 1993). The process of PAFR homologous desensitisation was then investigated using the co-transfection approach. My results showed that GRKs that are expressed at high levels in leukocytes as well as GRK2, 3 and 6 are able to induce PAFR desensitisation, while GRK4, which has a restricted expression in spermatozoa (Sallese, 1997), is not able to desensitise PAFR. In co-transfection experiments GRK2, 3 and 6 were able to inhibit both pathways activated by PAFR, but the mechanisms involved in these inhibitions seem to be multiple. I investigated at the molecular level the mechanisms involved in GRK2 inhibition of PAFR signalling and showed that GRK2 blockage of the Gs-mediated signalling was through a strictly phosphorylation-dependent process, while the Gq-mediated signalling could be modulated in a phosphorylation-independent way. I also identified the GRK2 domain involved in the down regulation of Gq-mediated signalling. This domain is the N terminal portion of GRK2, a domain that contains an RGS box (Siderovski, 1996). The blockage of a Gq-mediated signalling by GRK2-Nter domain is likely through direct binding to Gαq. I demonstrated that GRK2-Nter is able to bind in a selective and specific way to activated Gαq, \textit{in vitro} binding assays. Moreover, a direct interaction, between GRK2 and activated Gαq, was also observed in intact cell studies, using the co-immunoprecipitation
approach. These results expand the general view of GRK2 as a multidomain kinase able to regulate a GPCR signalling by interacting with different proteins of the signalling cascade, through its catalytic, PH and RGS domains, and they furnish evidence of the fundamental contribution of GRK2 in the modulation of Gq-mediated signalling. This kinase was initially studied in relation to the β2-adrenergic receptor that is a Gs-coupled GPCR and GRK2 was initially named β2-adrenergic receptor kinase (βARK). But now GRK2 seems to possess a special versatility for regulating the activity of Gq-coupled receptors. GRK2 is inhibited by Ca\(^{2+}\)-calmodulin and thereby regulated by Gq-mediated alterations in intracellular free Ca\(^{2+}\) levels (Iacovelli, 1999). This inhibition is released through PKC activity, which can also be controlled via Gq (Chuang, 1995; Krasel, 2001). In addition GRK2 contains an RGS domain, which leads to fairly selective inhibition of Gq-mediated signal transduction pathways.

The present study provides further evidence that GRK2 is a finely regulated enzyme. Not only GRK2 level of expression, subcellular localisation, kinase activity (Pitcher, 1998a), but also GRK2 ability to bind to Gαq can be modulated. Previous studies showed that GRK2 undergoes phosphorylation by other kinases, as well as PKC, ERK1, c-Src and each covalent modification influenced GRK2 kinase activity. In particular the GRK2 residues that undergo Src-phosphorylation are likely located in the RGS domain indicating a potential role for Src-phosphorylation in the modulation of GRK2/Gαq interaction. GRK2 tyrosine-phosphorylation, mediated by c-Src, has the effect of increasing GRK2/Gαq affinity. This increased interaction has the functional consequences of further increase the inhibition of Gαq-mediated signalling by tyrosine-phosphorylated GRK2.

Since activation of GPCRs has been shown to increase Src-mediated protein phosphorylation (Gutkind, 1998) and it has been reported that
GRK2 is a good substrate for c-Src \textit{in vitro} (Sarnago, 1999), our results strongly suggest that GRK2 tyrosine-phosphorylation is directly involved in the modulation of GPCR signalling. Src-phosphorylation increased GRK2 activity (Sarnago, 1999) and would help to block G protein-mediated signalling. Moreover, we show that Src-phosphorylation increase GRK2/Gαq binding, reinforcing the GRK2 desensitisation pathway. This suggested modulation of GRK2 by c-Src might also have physiological implications. In this regard, GRK2 appears to be important in the function and dysfunction of the cardiovascular system (Pitcher, 1998a). Recent data indicate that c-Src activation plays a critical role in hypertrophic growth regulation of cardiac myocytes (Fuller, 1998). Therefore, the interaction between c-Src and GRK2 in such a pathway may help to understand the consequences of some altered physiological conditions. The lack of GRK2 activity during embryonic development results in a lethal phenotype with marked myocardial hypoplasia (Jaber, 1996) while increased levels of GRK2 are associated with congestive heart failure and cardiac hypertrophy (Ungerer, 1993).
ABBREVIATIONS

(d)ATP  (deoxy)adenosine triphosphate
(d)CTP  (deoxy)cytidine triphosphate
(d)GTP  (deoxy)guanosine triphosphate
(d)TTP  (deoxy)thymidine triphosphate
AC    adenylyl cyclase
ATP   adenosine triphosphate
α2AR α2-adrenergic receptor
β2AR β2 adrenergic receptor
βarr  βarrerstin
bp    base-pair
BSA   bovine serum albumin
C-ter carboxy terminal
CTX   cholera toxin
CaM   calmodulin
cAMP  adenosine 3' 5'-cyclic-monophosphate
cGMP  guanosine 3' 5'-cyclic-monophosphate
CHO   Chinese hamster ovary
CLB   cell lysis buffer
CSP   calcium sensor protein
DMEM  Dulbecco’s modified minimal essential medium
dNTP  deoxynucleotides triphosphate
DTT   dithiothreitol
EDTA  ethylenediamine tetraacetic acid
ERK   extracellular signal regulated protein kinase
F     forward primer
FCS   foetal calf serum
FSH   follicle stimulating hormone
FSK   forskolin

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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>Gα</td>
<td>G protein alpha subunit</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor(s)</td>
</tr>
<tr>
<td>GRK</td>
<td>G protein-coupled receptor kinase(s)</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione S-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>HEK 293</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>HEPES</td>
<td>(N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>5-HT</td>
<td>serotonin</td>
</tr>
<tr>
<td>hPAFR</td>
<td>human Platelet-Activating Factor receptor</td>
</tr>
<tr>
<td>i1</td>
<td>intracellular loop</td>
</tr>
<tr>
<td>IL8-R</td>
<td>interleukin-8 receptor</td>
</tr>
<tr>
<td>IP</td>
<td>inositol phosphate</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Broth</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated kinase</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)-propanesulfonic acid</td>
</tr>
<tr>
<td>N-ter</td>
<td>amino terminal</td>
</tr>
<tr>
<td>NCS</td>
<td>neuronal calcium sensor</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBL</td>
<td>peripheral blood leukocytes</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PGE</td>
<td>prostaglandin E</td>
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<tr>
<td>Abbreviation</td>
<td>Full Name</td>
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<tr>
<td>PH</td>
<td>pleckstrin homology</td>
</tr>
<tr>
<td>PIP2</td>
<td>phosphatidylinositol 4 5-bisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PS</td>
<td>phosphatidylserine</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>PTX</td>
<td>pertussis toxin</td>
</tr>
<tr>
<td>R</td>
<td>reverse primer</td>
</tr>
<tr>
<td>RGS</td>
<td>regulatory of the G protein signalling</td>
</tr>
<tr>
<td>RK</td>
<td>rhodopsin kinase</td>
</tr>
<tr>
<td>RPM</td>
<td>revolution per minute</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDSL</td>
<td>site-direct spin labelling</td>
</tr>
<tr>
<td>Sf9</td>
<td><em>spodoptera frugiperda</em></td>
</tr>
<tr>
<td>βARK</td>
<td>β-adrenergic receptor kinase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TM</td>
<td>transmembrane helices</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>TSH</td>
<td>thyroid stimulating hormone</td>
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