Analysis of molecular structure, cellular localisation and functional role of G protein-coupled receptor kinase GRK4 and related kinases

Thesis

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Analysis of Molecular Structure, Cellular Localisation and Functional Role of G Protein-Coupled Receptor Kinase GRK4 and Related Kinases

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ABSTRACT

For a number of G protein-coupled receptors (GPCRs) a rapid, and reversible loss of responsiveness occurs upon exposure to agonists (homologous desensitisation). Two types of proteins play a major role in determining homologous desensitisation: G protein-coupled receptor kinases (GRK), (Chuang, 1996a; Krupnik, 1998), which phosphorylate agonist-occupied GPCRs, and the cofactor, (S)-arrestin (Krupnik 1998). The GRK family consists, so far, of six members named GRK 1 to 6 (Bunemann, 1999). GRK4, unlike the other GRKs exists in two splice variants (Sallese 1994) and was thought to be expressed only in testis (Sallese 1994; Ambrose 1992). In the present study one human and two rat splice variants of GRK4 have been identified. The four human isoforms of GRK4 were named GRK4α, GRK4β, GRK4γ, and GRK4δ. GRK4 expression in the testis is restricted to spermatozoa and germinal cells, where it associates with mitochondria and acrosomal membranes. Using an antibody common to all the GRK4 isoforms, only GRK4γ was detectable in human sperm. Moreover, GRK4α can phosphorylate rhodopsin and this is inhibited by calmodulin (CaM) in a calcium dependent manner, whereas the other three isoforms do not phosphorylate rhodopsin and do not interact with CaM. In-situ hybridisation and immunohistochemical analysis showed that GRK4 is also selectively expressed in the somatodendritic part of Purkinje cells. The metabotropic glutamate receptor 1 (mGluR1) has been found to be expressed in sperm and Purkinje cells and to colocalise with GRK4. Studies using an heterologous expression system (HEK293) have shown that mGluR1 signalling is regulated by GRKs including GRK4.
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CHAPTER 1

INTRODUCTION

1.1 The G protein-coupled receptor transduction machinery

1.1.1 General introduction

Single cells need to continuously communicate with other cells in order to maintain the coherent function and specialisation of the system to which they belong. Cell surface receptors perform this important task by receiving information coming from outside and transducing signals inside the cell. Approximately one thousand of the genome's hundred thousand genes (Clapham, 1996) encode for G protein-coupled receptors (GPCR), all member of this family have a characteristic structure and span the cell membrane seven times. GPCRs recognise a wide array of molecules, ranging from neurotransmitters, hormones, chemokines, and autacoid substances to light and odorants. Ligand binding causes as yet poorly defined conformational changes in the receptor followed by specific interactions with distinct classes of G protein heterotrimers (α and βγ subunits), which are associated with the cytoplasmic surface of the plasma membrane (Fig.1.1a).

The interaction between G protein and GPCR triggers the exchange of GTP for GDP on the α subunit, leading to dissociation of the G protein from the receptor and of the α subunit from the βγ complex (Fig.1.1b). The released G protein subunits, α-GTP and free βγ, are then able to interact with distinct effector molecules, such as adenylyl cyclases (AC), phospholipase C (PLC) isoforms, ion-channels
**Figure 1.1 Representation of signal transduction, mechanism in the β2AR system**

The receptor activated by agonist (A) (a) is able to couple with several Ga subunits. These Ga subunits exchange GDP for bound GTP and dissociate from the activated receptors (R*) and the Gβγ complexes (b). The Ga subunits subsequently activate effectors, such as adenylyl cyclase (AC). GRK2 translocates to the plasma membrane (b), through binding to R*-Gβγ complexes, and PIP2. R* is phosphorylated by its GRK, and binds an arrestin molecule (c) to terminate activation (desensitisation).
and tyrosine kinases, resulting in a variety of cellular functions, leading to the desired physiological response (Rens-Domiano, 1995; Langhans-Rajasekaran, 1995). The process of signal transduction mediated by GPCRs must be regulated in order to prevent overstimulation, to achieve signal termination and to allow the receptor to be responsive to subsequent stimuli. The mechanisms of signal attenuation include removal of agonist from the extracellular fluid, desensitisation, endocytosis, and down regulation of the receptors. Several mechanisms contribute to the removal of hormones and neurotransmitters from extracellular fluid, but their relative importance depends on the nature of the agonists.

One fundamental mechanism of regulation is homologous desensitisation, which rapidly occurs after receptor occupancy by the agonist. Desensitisation is an adaptive mechanism of receptor regulation that prevents overstimulation and allows receptors to respond to repeated stimuli (Freedman, 1996; Chuang, 1996a).

The desensitisation process for GPCRs is a multistep phenomenon. Receptor phosphorylation and subsequent uncoupling from the G protein occur first (Lohse, 1993) (Fig. 1.1b). Immediately thereafter, the receptor is engaged by the arrestin protein (Fig. 1.1c) which promotes sequestration in an intracellular compartment (endocytosis), where the dephosphorylation by specific phosphatases allows the resensitisation of the receptor, which is recycled to the plasma membrane (Lohse, 1993; Pitcher, 1995a). When the stimulation is persistent, then the receptor is downregulated (Lohse, 1993) through degradation of the protein and/or reduction of the steady-state mRNA levels.
1.1.2 Receptor structure

Despite the remarkable structural diversity of their activating ligands, all GPCRs, share a common molecular architecture, consisting of seven transmembrane helices (TM I-VII) linked by alternating intracellular (i1-i3) and extracellular loops (Fig. 1.2). The extracellular domain of the receptor (including the N-terminal domain, and the different extracellular loops) has been shown to be critical for ligand binding, whereas the intracellular region is known to be involved in G protein recognition and activation (Strader, 1994; Schwartz, 1994). A high-resolution structure of a GPCR is not currently available, primarily due to the difficulty of generating crystals of integral membrane proteins suitable for x-ray crystallography. However, low-resolution structures (Schertler, 1993; Unger, 1995) have been deduced for the transmembrane core of the photoreceptor rhodopsin. Electron cryomicroscopic analysis of two dimensional rhodopsin crystals confirmed the presence of seven transmembrane helices that are arranged in a ring-like fashion, thus forming a tightly packed helical bundle (Schertler, 1995). It was estimated that the extracellular and intracellular surface of the rhodopsin transmembrane core covers an area of 42 x 20 Å² and 31 x 22 Å² respectively. Based on the low resolution structure of bovine rhodopsin Baldwin proposed a model of a GPCR core in an attempt to correlate the various peaks of the rhodopsin projection structure to individual TM helices of the rhodopsin sequence (Baldwin, 1993). This model contained a number of molecular constraints that were postulated for the individual transmembrane helices, based primarily on a rigorous sequence comparison of more than 200 different GPCRs. According to the Baldwin model, TM IV, VI, and VII are nearly perpendicular to the plane of the membrane, whereas TM I-III are
Figure 1.2 Topology of β2AR inserted in the plasma membrane

The model is based on hydropathicity analysis of the human β2AR according to the method of Kyte and Doolittle (Kyte, 1982). The amino acids are indicated by one letter code. Transmembrane domains are numbered TMI to TMVII. Intracellular loops are indicated as i1, i2, i3. The 11 potential sites of GRK-mediated phosphorylation on the C-terminal of the receptor are indicated with square boxes. The residues phosphorylated by both GRK3 and GRK5 are shown in black while GRK5 specific phosphorylation sites are shown in grey (Fredericks, 1996).
tilted, thus producing an arc-shaped feature in the projection structure. Several recent molecular genetics and biochemical studies confirm that the basic features of the Baldwin model are probably correct. Studies involving the functional rescue of a misfolded mutant GPCR by complementary (secondary) mutations demonstrated the proximity of individual TM helices as predicted by Baldwin (Liu, 1995b; Mizobe, 1996). Similar results were obtained in biochemical studies by investigating the ability of histidine-substituted GPCR mutants to bind metal ions (Sheikh, 1996) or of cysteine substituted rhodopsin molecules to form intramolecular disulphide bonds (Yang, 1996). Two recent mutagenesis studies also provided experimental evidence that TM I- VII are arranged in a clockwise fashion (Fig. 1.3) as viewed from the cell interior (Mizobe, 1996). Although the structural features of the transmembrane receptor core are now beginning to be known in detail, the molecular architecture of the extracellular receptor surface is presently unknown. On the other hand, recent biophysical studies have shed some light on the structure of the intracellular receptor surface, that is predicted to be involved in G protein coupling. Nuclear magnetic resonance and circular dichroism with peptides derived from distinct intracellular GPCR domains have shown that the extreme C-terminal of the i3 loop as well as the membrane proximal portion of the C-terminal domain (i4) are likely to be alpha helical (Jung, 1995; Jung, 1996). Since many of the investigated peptides still retain biological activity (as demonstrated by their ability to inhibit receptor/G protein interaction or to activate G proteins), it is likely that the isolated peptides fold in a fashion similar to the native receptor. Another biophysical approach which has been applied to study GPCR structure is the method of site-direct spin labelling (SDSL), which offers the advantage of obtaining structural information from intact receptor protein (Altenbach, 1996;
Figure 1.3 Scheme of the M3 muscarinic receptor as viewed from the intracellular side

The seven transmembranes hydrophobic stretches of amino acids (I-VII) are arranged in a clockwise model (Baldwin, 1993). Numbers refer to amino acid positions of the rat M3 muscarinic receptor sequence (Bonner, 1987). The amino acids highlighted in black are predicted to selectively interact with the Gq/11 proteins (Blin, 1995). Intracellular loops are indicated as i1, i2, i3.
Farahbakhsh, 1995). In this method, one or more reactive cysteine residues are introduced into the receptor molecule and modified with a small nitroxide containing spin marker. The electron paramagnetic properties of such spin labelled receptor protein and the accessibilities of the attached spin markers to collision with polar and non polar paramagnetic agents are then analysed to elucidate: 1) the localisation of labelled residue with respect to the membrane/cytoplasm interface; 2) the identity and orientation of the secondary structure of the target receptor region. SDSL studies of bovine rhodopsin suggest that the i3 loop is largely alpha helix, forming regular extension of TM V and TM VI by 1,5 and 3 turns, respectively (Altenbach, 1996). In addition SDSL studies indicate that the TM III/i2 loop junction, including the Glu/Asp-Arg motif which is conserved among most GPCR, is also alpha helically arranged. However, no clear conclusion was reached regarding the possible secondary structure of most i2 loop.

1.1.3 Receptor conformation and signalling

Mutation within a short stretch of amino acids in the C-terminal end of i3 loop of alpha 1B and β2-adrenergic receptor (β2AR) result in elevated basal (agonist-independent) signalling activity (Lefkowitz, 1993b). The constitutively active receptor displays greater affinity for agonists and there is a close positive correlation between intrinsic agonist activity and affinity for the constitutively active receptor mutant. These observations led (Lefkowitz, 1993b) to the proposal of an allosteric ternary complex model, introducing a further step that regulates the transition of an inactive state of the receptor (R) to an active state (R*) (Fig. 1.4). Receptors are assumed to exist in equilibrium between R and R*, and only R* effectively interacts with
Figure 1.4 Allosteric ternary complex model of receptor activation

This hypothetical model of receptor activation shows the receptor in the inactive (R) or active conformation (R*). K indicates the equilibrium constant for receptor shuttling between R and R* without ligand. When the ligand is an agonist (AG) the equilibrium is shifted towards the active complex AG-R*-G. When the ligand is an inverse agonist (Inv-AG) the equilibrium is shifted towards the inactive (R) conformation.
G proteins. Agonist binding shifts the equilibrium from R to R* and stabilises the ternary complex consisting of agonists (H)-bound R* and G protein (HR*G), finally resulting in G protein activation. An equilibrium between R and R*, however, implies that at any time a certain proportion of the receptor resides in the active state R*, even in the absence of agonists. Increased receptor numbers are predicted to increase agonist independent activity, because stochastically more R* will be present at any given time. This naturally occurring constitutive activity is the prerequisite for the phenomenon of inverse agonism induced by certain antagonistic drugs (Bond, 1995; Milligan, 1995). Agonists are presumed to have a high affinity for R* and to increase the ratio R*/R (Fig.1.4), whereas inverse agonists have high affinity for R and decrease the ratio R*/R (Fig.1.4). Classical competitive antagonist have equal affinity for R and R* and do not shift the equilibrium (Fig.1.4).

Naturally occurring mutations of several G protein-coupled receptors have been identified as principle causes of human disease (Parma, 1994; Birnbaumer, 1995). It has been suggested that any mutation capable of disrupting a labile inactive receptor conformation will shift the equilibrium from R to R*, by releasing a structural constraint in R, thereby exposing a critical activator receptor domain for interaction with a G protein. Numerous point mutations of the TSH receptor have been described in patients suffering from toxic hyperthyroid adenomas or non-autoimmune autosomal dominant hyperthyroidism (Duprez, 1994; Kopp, 1995). A scenario of multiple activated states of GPCRs was developed by Hofmann, 1995, which allows activation of multiple G proteins by one activated receptor. This clearly occurs in the disc membrane of retinal rods. Light induced isomerization of the 11 cis retinal chromophor of rhodopsin, which is bound to a cavity formed by different TM helices, leads to
several intermediate conformational species up to the functionally most important form, characterised by maximal absorbance of 380 nm referred to as metarhodopsin II (Hofmann, 1995) (Meta II). This represents the active form of rhodopsin R*, that is able to interact with the G protein transducin.

1.1.4 Conformational changes involved in receptor activation

To understand the molecular mechanisms of receptor/G protein coupling, better structural information about the conformational differences between the resting state (R) and the ligand-activated state (R*) of GPCRs must be obtained. Most of our current knowledge about the structural changes accompanying GPCR activation is derived from studies on the photoreceptor rhodopsin. A variety of biochemical and biophysical studies (including proteolysis, Fourier transform, infrared spectroscopy, circular dichroism) revealed structural differences between native rhodopsin and Meta II. These studies suggest, that GPCR activation is not accompanied by a major conformational rearrangement of the receptor protein. The current feeling is that ligand induced changes in the relative orientation of individual TM helices affect the conformation of the intracellular receptor surface, thus allowing productive coupling to G proteins. This hypothesis has received strong experimental support by elegant SDSL studies (Farahbakhsh, 1993). One study suggests that rhodopsin activation involves a relatively small outward movement of the cytoplasmic portion of TM III (relative to the other helices in the transmembrane receptor core) as well as yet undefined structural change in the i2 loop (Farahbakhsh, 1993). Analysis of the spectral properties of a series of rhodopsin double cysteine mutants, in which both cysteines were modified by nitrogen spin markers, indicated that
rhodopsin activation also involves an outward movement of the cytoplasmic end of TM VI, accompanied by a clockwise rotation, as viewed from the cell interior, of approximately 30° (Farrens, 1996). Farrens, 1996, also showed that disulphide cross-linking of the cytoplasmic ends of TM III and VI prevented rhodopsin activation (as determined by the inability of the cross linked proteins to activate transducin), supporting the view that relative movements between these two TM helices are essential for converting GPCRs into their activated state. Essentially similar results were obtained when the cytoplasmic ends of TM III and VI (in rhodopsin) were cross-linked by metal ions following systematic histidine substitution mutagenesis (Farrens, 1996). Taken together these results are consistent with the notion that GPCR activation involves an opening of the intracellular receptor surface, thus enabling the G protein to interact with previously inaccessible regions of the receptor protein. Since GPCRs are thought to share a conserved molecular architecture, it is likely that conformational changes similar to those found with rhodopsin also occur in other GPCR. In agreement with this hypothesis a recent study analysing the conformational changes associated with activation of the yeast α-factor pheromone receptor showed that agonist binding leads to increased accessibility of the i3 loop, as demonstrated by an increased trypsin cleavage rate (Bukusoglu, 1996). Since this phenomenon was also observed in G protein deficient yeast mutants, the conformational rearrangement of the i3 loop after ligand activation is intrinsic to the receptor protein. The interaction of antagonist with the alpha factor receptor led to a reduction of proteolytic cleavage within the i3 loop, clearly indicating that agonists and antagonists induce distinct conformational states of the receptor. A similar conclusion was reached by Gether, 1995, who examined ligand-dependent structural changes of the purified
1.2AR modified by a cysteine specific fluorescence marker molecule. Although addition of the full agonist isoproterenol led to decreased fluorescence of the modified receptor protein, incubation with classical β-adrenergic antagonist resulted in a small but significant increase in baseline fluorescence (Gether, 1995).

1.1.5 Receptor-mediated G protein activation

In most cases, a single GPCR, when activated by appropriate ligand, can recognise and activate only a limited set of structurally related G proteins (Dohlman, 1991). To understand how this selectivity is achieved at a molecular level is the focus of an increasing number of laboratories. Besides the structural information encoded by the receptor and G protein primary sequence, other factors, including receptor density and restricted localisation of specific G protein heterotrimers and the receptor on the plasma membrane, may also contribute to the observed degree of coupling selectivity (Gudermann, 1996). Although the structures of a few different G protein heterotrimers have recently been resolved by x-ray crystallography (Wall, 1995), the molecular architecture of the receptor G protein interface still remains poorly defined. The development of accurate molecular models of the receptor/G protein interface requires the identification of specific regions on the G protein that are in contact with functionally critical receptor sites.

The available structural information, together with several biochemical and molecular genetic studies (Rens-Domiano, 1995), defines a surface on the G protein heterotrimer that is likely to face the plasma membrane. This surface contains all the major G protein sites predicted to be in contact either with the plasma membrane (such as the fatty acid modified N-terminus of Ga and the prenylated
C-terminus of Gγ) or with the receptor protein (including for example, the C-terminal, segments of the α5 helix and the α4β6 loop of Ga) (Fig. 1.5). Based on these constraints, complemented by evolutionary trace analysis (Onrust, 1997), a low-resolution model of the receptor/G protein complex has been proposed recently. In this model, the guanine nucleotide (GDP) which is buried in a cleft between the α-helical and the RAS like domain of Ga, is predicted to be located approximately 30 Å away from the plasma membrane. Since it is unlikely that the nucleotide can be contacted directly by the intracellular loops of the receptor proteins (which are rather short in many cases), GPCRs are thought to trigger GDP release by an allosteric mechanism. It is possible that binding of the C-terminal region of Ga to the receptor protein leads to changes in the conformation and/or orientation of the adjacent α5 helix, which are then propagated to the preceding β6/α5 loop, which is in direct contact with guanine nucleotide. The loss of binding interactions in this loop may trigger GDP release, ultimately leading to G protein activation (Onrust, 1997).

To identify the receptor site that can interact with the C-terminus of Ga a widely used approach, has been to analyse hybrid receptors constructed between functionally distinct members of the GPCR subfamily. A study from Liu, 1995a, took advantage of the observation that the M2 muscarinic receptor, a prototypical Ga1/o-coupled receptor, cannot interact with wild type Gaq to a significant extent, while the M2 receptor can efficiently couple to mutant Gaq subunits in which the last 5 amino acids are replaced with the corresponding Ga1 or Ga0 sequence (Liu, 1995a). Analysing a large number of mutant muscarinic receptors (Liu, 1995a) revealed that the ability of the M2 muscarinic receptor to interact with the hybrid G protein...
Figure 1.5 Representation of interaction domains of Gα proteins with receptor, effector, and βγ subunits

Interaction domains are depicted in agreement with the known three dimensional structures of G protein crystals. Sequences shown to be involved in GTP-binding and hydrolysis in RAS and Gα subunits are denoted G1 to G5. ADP-ribosylation sites for cholera (CTX) and pertussis (PTX) toxins are indicated. Switch regions, that undergo structural changes between active and inactive conformations, contact sites with the gamma phosphate, the guanine ring, and the ribose moiety of GTP are shown. Alpha helices and β sheets of the G domain are numbered α1-5 and β1-6. Alpha helices of the helical domain are denoted αA-F. Sequences connecting the helical and G domain (linkers I and II) are indicated.
specifically depends on the presence of a four amino acid motif (V385, T386, I389, and L396) located at the i3 loop/TM VI junction.

These four amino acids directly correspond to the residues forming the AALS motif in the M3 receptor which play an important role in the specific recognition of the Gq/11 proteins (Burstein, 1995). Replacement of the VTIL motif in M2 muscarinic receptor with the AALS sequence virtually abolished the ability of the resulting mutant receptor to functionally interact with mutant Gaq subunits, containing an Ga1 or Gao sequence at their C-terminus (Liu, 1995). On the other hand, substitution of the VTIL motif into the wild type M3 muscarinic receptor resulted in a hybrid receptor that was able to activate a hybrid G protein with increased efficacy and efficiency (as compared with wild type Gaq). The most straightforward explanation of these findings is that the VTIL motif becomes accessible to the C-terminus of Ga1/o-subunits upon receptor activation, and that this interaction is critical for determining coupling selectivity and triggering G protein activation. It should be of considerable interest to examine whether this putative receptor/G protein contact is functionally conserved in other classes of GPCR. In apparent contrast to the findings reported by Liu et al. (Liu, 1995a), more recent studies have shown that a photo-affinity derivative of a receptor-mimetic peptide corresponding to the C-terminal part of the i3 loop of the α2 adrenergic receptor (which shares considerable sequence homology with the same region of the M2 receptor) can be cross linked to the N-terminus of Gao and to a site within the last 60 amino acids of βγ (Taylor, 1996). Since the N- and C-terminal sequences of Gαs are predicted to be located in close proximity to each other on the same side of the G protein heterotrimer (Wall, 1995), it is possible that the receptor-mimetic peptide, that was derivatised with a rather extended photo-affinity reagent, labelled G
protein sites adjacent to the actual peptide binding site. It is also possible that the C-terminal part of the i3 loop can simultaneously bind to both the N- and C-termini of Gα or that the effects observed in the genetic complementation experiments (Liu, 1995) could be due to indirect conformational changes induced by different mutations. In any case cross-linking and genetic complementation approaches should eventually lead to the identification of the major receptor Gα contact site. Considerable evidence suggests that the G protein βγ complex is also directly in contact with the receptor protein and that receptor/G protein coupling selectivity may depend on the molecular identity of all three components of the G protein heterotrimer (Gudermann, 1996). In the absence of high-resolution structure of a receptor/G protein complex, the identification of all functionally relevant receptor/G protein contact sites represents a big challenge.

The majority of studies indicate that the selectivity of G protein recognition is primarily determined by amino acids located in the i2 loop and the N- and C-terminal portion of the i3 domain. The membrane proximal portion of the C-terminal i4 region may also contribute to the receptor/G protein interface; however the gonadotropin releasing hormone receptor appears to be completely devoid of a C-terminal tail, suggesting that this region is not essential for G protein coupling. The conclusions drawn from studies using the chimeric receptor approach generally agree well with biochemical experiments using short synthetic peptides derived from distinct intracellular receptor segments. Several laboratories have shown that peptides corresponding to the N- and C-termini of the i2 loop can mimic or inhibit receptor interaction with G protein (Konig, 1989). In agreement with several mutagenesis studies (Wong, 1990; Blin, 1995) investigations with short receptor peptides (Konig, 1989) also indicate that these regions act in a co-operative fashion to
dictate proper G protein recognition. The fact that several peptides can directly activate G proteins suggest that these sequences are not accessible to the G protein heterotrimer in the inactive state of the receptor. This view is consistent with the concept outlined above that receptor activation is likely to involve an opening of the intracellular receptor surface, thus enabling the G protein to interact with previously buried key amino acids on the receptor.

### 1.1.6 Signal processing via alternatively spliced receptors

The generation of several second messengers by one extracellular stimulus is often mediated by ligands acting on related receptor subtypes that are encoded by distinct genes and display distinct G protein coupling specificities.

A remarkable versatility of receptor subtypes characterizes the GPCR family; for example 12 mammalian serotonin, 10 adrenergic, 5 muscarinic acetyl choline, and 5 dopamine receptors have been identified by molecular cloning (Peroutka, 1994; Wang, 1994; Watson, 1994). The subtypes show differences in their tissue expression pattern as well as in their signal transduction properties, even among very closely related subspecies (Watson, 1994; Eason, 1992; Green, 1992; Levy, 1993; Eason, 1994). The first cloned gene for a GPCR lacked introns in the coding region. The assumption that this feature was characteristic for the entire receptor family was abandoned after isoforms of various receptors were found to arise through alternative splicing of the primary transcript of a single copy gene. In the case of glycoprotein hormone receptor, the extracellular N-terminal domain is composed of nine exons in the thyroid stimulating hormone (TSH) (Gross, 1991) and the follicle stimulating hormone (FSH) (Heckert, 1992) receptor, and of ten exons for luteinizing-chorionic...
gonadotropin (LH-CG) (Koo, 1991) receptor, whereas the region comprising the seven transmembrane domain is encoded by one large exon. Alternative splicing gives rise to isoforms lacking the entire membrane-anchoring domain or distinct exons in its N-terminus (Aatsinki, 1992; Graves, 1992).

Alternatively spliced receptor isoforms that differ in their seven transmembrane domain or their intracellular C-terminal regions have been implicated in altered receptor function (Table 1.1). In some cases altered receptor function elicited by C-terminal receptor splice variants can be related to changes in agonist induced desensitisation and down regulation (e.g. turkey β1 adrenergic receptor, μ-opioid and SSTR2 somatostatin receptor). In other cases, divergent C-terminal regions of the receptor have been reported to deeply affect ligand binding and G protein-coupling characteristics. Similar binding and G protein-coupling characteristics (Gudermann, 1996) was reported for four C-terminal splice variants of the bovine prostaglandin E receptor EP3 (Gudermann, 1996). All receptor isoforms appear to be coupled to Gαi and Gαs proteins. Overexpression (> 10^6 receptor per cell) increases Gαs stimulation and leads to an additional Gαq interaction. While the efficacy of the receptor/G protein interaction varies between different isoforms, the basic coupling pattern appears to be invariant. The phenomenon of pronounced additional coupling to Gαs of a normally Gαi coupled receptor, depending on receptor density, has also been reported for α2 adrenergic receptor and M4 muscarinic receptor (Eason, 1992; Dittman, 1994). Conversely, alternative splicing of α1c adrenergic receptor produces three isoforms (α1C1, α1C2 α1C3) that differ in length and sequence of the C-terminal domain, but show no difference in functional parameters when expressed in chinese hamster ovary (CHO) cells (Hirasawa, 1995).
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<tr>
<td>turkey b1-</td>
<td>Intronic sequences between exons I and II: 59-aa C-terminal extension</td>
<td>C-terminal extension inhibits endocytosis and down-regulation in response to agonist</td>
<td>Hertel, 1990</td>
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<td>adrenergic</td>
<td></td>
<td></td>
<td>Wang, 1995</td>
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<td>Calcitonin</td>
<td>(a) 16-aa insertion in i1, (b) 37-aa insertion in e2 leading to type 1 (no insert), type 2 (e2), and type 3 (i1) isoforms</td>
<td>(a) Abolishes PLC activation, (b) alters ligand recognition and binding kinetics</td>
<td>Houssami, 1994, Nussenzweig, 1994, Zolnierowicz, 1994, Nakamura, 1995</td>
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<td>D2 and D2S</td>
<td>29-aa insertion in i3 leading to D2L and D2S isoforms</td>
<td>Preferential coupling of isoforms to Gi2 vs Gi3</td>
<td>Monsma, 1989</td>
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<td>D2</td>
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<td>Hayes, 1992, Guiramand, 1993</td>
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<td>mGluR1 glutamate</td>
<td>C-terminal truncation of full-length receptor: (mGluR1a) 85-bp insertion (mGluR1b), novel 3' end (mGluR1c)</td>
<td>Extent and kinetics of Ca2⁺-induced Cl-currents are altered (mGluR1a vs mGluR1c)</td>
<td>Pin, 1992</td>
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<td>Neurokinin-1</td>
<td>Deletion of 96 aa from C-terminus resulting in NK1Rlong and NK1Rshort</td>
<td>Reduced ligand affinity and coupling efficacy of deleted mutant</td>
<td>Fong, 1991</td>
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<td>μ opioid</td>
<td>Variations in length and aa composition of C-terminal define rMOR1A and rMOR1B</td>
<td>Increased resistance of rMOR1B to agonist-induced desensitisation</td>
<td>Zimprich, 1995</td>
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<td>PACAP type 1</td>
<td>Absence or presence of either one or two 84-bp cassettes inserted into i3 leading to PACAP-R, PACAP-R-hip, PACAP-R-hop1, PACAP-R-hop2, and PACAP-R-hip-hop1 isoforms</td>
<td>Presence of hip cassette impairs coupling to AC and abolishes PLC stimulation; presence of hop cassettes is without functional consequences</td>
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<td>Glandin E</td>
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<td>SSTR2</td>
<td>Divergent C-terminus define SSTR2A (unspliced) and SSTR2B</td>
<td>Spliced isoform (SSTR2B) is more efficient in inhibiting cAMP accumulation</td>
<td>Vanetti, 1993, Reisine, 1993</td>
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1.2 The GRK family

1.2.1 Introduction to the GRKs

GRKs are a family of serine/threonine protein kinases that appear to regulate a large number of GPCRs (Table 1.2). The multigene family of GRKs consists so far of six members named GRK 1 to 6, according to the time of their discovery (Premont, 1995) (Fig. 1.6). Among these six subtypes, rhodopsin kinase (RK) corresponds to GRK1, β-adrenergic receptor kinase 1 (βARK1) to GRK2, and βARK2 to GRK3. Based on sequence homology (Fig. 1.7) these six GRK subtypes are classified into three subfamilies: GRK1 is alone in the first (rhodopsin kinase subfamily), GRK2 and GRK3 form the second (βARK subfamily), while GRK4, GRK5 and GRK6 constitute the third (GRK4 subfamily). The identification of four human and four rat splice variants of GRK4, and two human splice variants of GRK6, expands the level of diversity within this gene family. The presence of known homologues in Drosophila and the evidence of such receptor kinases in Dictostelium and Saccharomyces cerevisiae suggest a long evolutionary history, that may be parallel to that of the G protein-coupled receptors themselves (Lefkowitz, 1993a).

The six-mammalian GRK proteins have 34-84% overall sequence identity (Fig. 1.7). They share a similar structural organisation with a central kinase catalytic domain, flanked by an approximately 185-amino acid N-terminal region thought to be important for substrate recognition and a C-terminal region responsible for the intracellular localisation (Lefkowitz, 1993a) (Fig. 1.6). The N- and C-terminal domains are not well conserved among the family. The N-terminus is less well characterised and until now was thought to be involved in
<table>
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<td>a1B-adrenergic</td>
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<td>Freedman, 1995a</td>
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<td>Inglese, 1993; Premont, 1996</td>
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<td>Odorant</td>
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<td>Opioid k</td>
<td>GRK2, 3</td>
<td>Raynor, 1994</td>
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Figure 1.6 Overall features of GRK structure

Schematic representation of the six GRK variants showing the common central catalytic domain (open box), the Gβγ binding domain in GRK2 and GRK3 (βγ), the CaM binding domain (CaM), and the putative "RGS box". Dotted CaM in GRK4 and GRK6 means that this domain is deduced by homology with GRK5. The principal mechanisms of membrane anchoring are indicated: Farnesylation for GRK1, βγ binding for GRK2 and GRK3, palmitoylation for GRK4 and 6, and charge for GRK5 (+++). The catalytic domain of all GRKs differs from other protein kinases for the presence of three invariant amino acids DLG.
Figure 1.7 Sequence identity among the GRK family

Values close to the labels indicate the range of similarity among the orthologous GRKs cloned so far. The values on the lines connecting two different GRKs referred to their relative identity. The numbers are expressed as percentage of identity. (INV GRKs = all the invertebrate GRK).
the recognition of activated receptor (Palczewski, 1991). Recently it was shown to contain a gelsolin homology region which may be important for phosphatidyl-inositol-4,5-bisphosphate (PIP2) binding (Pitcher, 1996). Moreover it contains a putative RGS (Regulator of G protein signalling) domain (Siderovski, 1996).

The C-terminal domain is the most variable region in GRKs, with the exception of a conserved region adjacent to the catalytic domain that includes proline rich motifs and autophosphorylation sites. GRK1 contains two autophosphorylation sites at serine 488 and threonine 489, that are both conserved in GRK5 and 6 (Premont, 1994; Loudon, 1994). Different GRK subtypes are anchored to the plasma membrane, through distinct mechanisms, all involving the C-terminal domain, and this is essential for their function. Phosphorylation of the substrate (activated receptor) occurs on multiple residues in the C-terminal tail of the receptor (e.g. in the case of rhodopsin and β2AR) or at the third intracellular loop (e.g. in the case of α2 adrenergic). Structurally the GRKs show significant amino acid similarity to other kinases only in the centrally located catalytic domain spanning about 240 amino acid residues. This domain shows about 45-95% sequence identity within the GRK family, but only 33-36% identity with the closest other relatives, the second messenger kinases protein kinase A (PKA) and protein kinase C (PKC), (Lefkowitz, 1993a).

The catalytic domains of all GRKs share certain invariant amino acids in addition to those present throughout the protein kinase superfamily. Of particular interest is the DLG signature in the core of the catalytic domain, instead of DFG present in all other protein kinases, except casein kinases (Inglese, 1993).
1.2.2 Membrane targeting mechanisms for GRKs

GRKs utilise distinct mechanisms to achieve membrane association. GRK1, GRK2, and GRK3 are primarily cytosolic proteins (Freedman, 1996), which upon receptor stimulation, translocate from the cytosol to the plasma membrane (Inglese, 1992a; Inglese, 1992b). In contrast, GRK4, GRK5 and GRK6, do not exhibit agonist-dependent membrane association. GRK5 appears to be constitutively associated with the membrane (Premont, 1994) and this observation could potentially explain the higher basal (agonist-independent) activity of this kinase as compared to GRK2, (Premont, 1994). Despite the differences between the three GRK subfamilies, membrane association of all kinases appears to be mediated, at least in part, by determinants in their C-terminal domains.

1.2.2.1 Isoprenylation

The amino acid sequence of GRK1 terminates in a CAAX motif, which directs its isoprenylation and carboxyl-methylation (Inglese, 1992a). Isoprenylation is a post translational modification in which the amino acid sequence of the CAAX motif specifies which isoprenoid homologue is attached to the protein, either farnesyl (C15) or geranyl-geranyl (C20). For GRK1 the sequence is CVLS, which directs farnesylation of the kinase. The farnesylated kinase is predominantly a cytosolic protein that translocates in a light dependent manner to the rod outer segment (ROS) membrane (Inglese, 1992a). Removal of the farnesyl group by mutation of the CVLS motif (cysteine (C) is mutated to serine (S)) results in the inability to translocate in a light-dependent fashion and in reduced ability to phosphorylate rhodopsin (Inglese, 1992a). In contrast,
mutation of serine to leucine yields a kinase that is modified by geranyl-geranylation and is constitutively associated with the membrane (Inglese, 1992a). The geranyl-geranylated kinase, although impaired in its ability to translocate to ROS membranes in a light-dependent fashion, phosphorylates rhodopsin at a comparable rate to wild type (farnesylated) GRK1. Isoprenylation of GRK1 is required for membrane association and for activity. Furthermore the specific modification found in vivo, farnesylation, confers to GRK1 the ability to associate with membranes in a stimulus (light) dependent fashion.

Similar studies with isoprenylated mutants of GRK2 have shown that both kinase activity and degree of membrane association increase with isoprenylation status (Inglese, 1992b). Non isoprenylated kinase does not bind to ROS membranes. In contrast, a mutant farnesylated form of GRK2 is partially associated with the membrane and a mutant geranyl-geranylated form of GRK2 is constitutively associated with the membrane (Inglese, 1992b). Notably, the GRK2 isoprenylation mutants, unlike GRK1, fail to display light-dependent translocation indicating that determinants other than isoprenylation mediate the agonist-dependent translocation of this enzyme. As compared to the native enzyme, the isoprenylated mutants of GRK2 display an enhanced ability to phosphorylate GPCR substrates, demonstrating the importance of membrane localisation for GRK function.

GRK2 and GRK3 lack a CAAX motif but still rely, interestingly, on isoprenylation for membrane association since their C-terminal contains an approximately 100 amino acid region of sequence homology termed the pleckstrin homology (PH) domain (residues 553-651 in GRK2) (Touhara, 1994; Touhara, 1995) (Fig. 1.8). PH domains are found in numerous proteins where they form a distinct structural module (Cohen, 1995). The PH domain of these enzymes binds Gβγ
Figure 1.8 Carboxy terminal of GRK2 mapping the $\beta\gamma$ binding domain

The complete $\beta\gamma$ binding domain is represented by the open box. Pleckstrin homology domain (PH). The black box indicates the localisation of the minimal $\beta\gamma$ binding peptide (18 residue) on GRK2 and GRK3 (Chuang, 1997), which are amplified to illustrate the amino acid compositions. Circle indicates the location of PIP2 binding on the PH domain.
and through this protein-protein interaction, associates with the membrane (Pitcher, 1992). Notably Gβγ binding sites on GRK2 and GRK3 include and extend slightly beyond the C-terminus of its constituent PH domain (residues 546-670) (Pitcher, 1992) (Fig. 1.8). The gamma subunit of Gβγ is isoprenylated with the isoprenoid geranyl-geranyl, a modification that anchors the Gβγ complex to the plasma membrane. The Gβγ binding region of GRK2 has been mapped to residues 546-670 and a glutathione S-transferase (GST) fusion protein containing this sequence as well as a 18 amino acid peptide composed of residues 648-665 inhibits the interaction of Gβγ with GRK2 in vitro (Koch, 1993; Chuang, 1997).

Originally, Gβγ was shown to enhance the activity of a partially purified preparation of a kinase with similar properties to GRK2 against purified reconstituted muscarinic cholinergic receptors (Haga, 1992). In subsequent experiments Gβγ was also shown to enhance GRK2-mediated phosphorylation of the purified reconstituted β2AR (Pitcher, 1992), but did not enhance its activity towards a soluble peptide substrate (Pitcher, 1992; Kim, 1993). These results suggest that the principal mechanism by which Gβγ enhances GRK2-mediated GPCR phosphorylation is by promoting the membrane association of GRK2, thus placing the kinase and its receptor substrate in close proximity (Pitcher, 1992) In addition, Gβγ-mediated translocation of GRK3 has also been demonstrated in permeabilised preparations of rat olfactory cilia, a system expressing endogenous levels of kinase (Haga, 1994a). Agonist-stimulated translocation of GRK3 was blocked by Gβγ sequestrators, a GST fusion protein encompassing the C-terminus of GRK3 (residues 467-688) and a peptide derived from this region (residues 664-670). For GRK2 and GRK3, Gβγ plays an integral role mediating the translocation and membrane association of these kinases. Gβγ may however play an additional role, by facilitating the
interaction of GRK2 and GRK3 with their receptor substrate. Activated GPCRs or synthetic receptor peptides are able to enhance GRK2-mediated phosphorylation of soluble peptide substrate. The interaction of GRK2 with an activated receptor substrate analogue results in allosteric activation of the kinase (Kim, 1993; Haga, 1994a). Gβγ synergistically enhances GPCR-mediated activation of GRK2, but Gβγ binding alone does not lead to direct activation of GRK2. Gβγ appears to facilitate the interaction between GPCR and GRK, thus promoting the GPCR-mediated allosteric activation of this enzyme.

GRK2 is a predominantly soluble protein, that has been shown to translocate in an agonist-dependent manner (Daaka, 1997). The Gβγ-mediated membrane association of GRK2 observed in vitro provides a model to explain this agonist-dependent translocation. In this model agonist-occupancy of a GPCR leads to the activation of heterotrimeric G proteins and the release of free Gβγ dimer. The Gβγ subsequently interacts with GRK2 and/or GRK3 and helps to target these enzymes to their membrane incorporated receptor substrates. This model has been demonstrated in intact cells using COS7 cells transiently overexpressing GRK2 (Daaka, 1997). Activation of β2AR promotes membrane association of GRK2, a process that is inhibited by overexpressing a C-terminal GRK2 peptide encompassing the βγ-binding domain. Furthermore, agonist-stimulated formation of a GRK2/Gβγ complex can be directly demonstrated by co-immunoprecipitation (Daaka, 1997). Although agonist occupancy of β2AR and lysophosphatidic acid receptor promotes GRK/Gβγ complex formation for both GRK2 and GRK3, thrombin receptor stimulation specifically induces GRK3/Gβγ complex formation (Daaka, 1997). These results suggest differences in Gβγ binding specificity between GRK2 and GRK3. Isoprenylation plays a central role in mediating the membrane association of GRK1, 2 and 3 either through direct covalent
modification of the kinase (GRK1) or through a protein-protein interaction between the kinase and isoprenylated Gβγ (GRK2 and GRK3). Notably, the isoprenyl moiety plays a critical role in conferring agonist-dependence on the membrane association of all three GRK.

### 1.2.2.2 Membrane anchoring of the GRK4 subfamily

GRK4 and GRK6 are not isoprenylated and do not bind Gβγ (Premont, 1995). These kinases are however palmitoylated (Premont, 1996; Stoffel, 1994) (Fig. 1.6). Palmitoylation is the acylation of a protein with palmitic acid through a thioester bond. Palmitic acid is a 16-carbon saturated fatty acid that confers to modified proteins a higher avidity for membranes. The most prevalent form of palmitoylation is the modification of a cysteine residue through a thioester bond. GRK6 contains a cluster of cysteines in its C-terminus at positions 561, 562 and 565; mutation of these cysteines to serines abolishes the palmitoylation of the kinase (Stoffel, 1994). By analogy with GRK6, the probable sites of palmitoylation on GRK4 are the cysteines at residues 561 and 578 (the C-terminal cysteine residue) (Premont, 1996). Palmitoylated GRK4 and GRK6 are found exclusively associated with membranes in cellular systems (Stoffel, 1994). These results are similar to those previously reported for other palmitoylated proteins (Linder, 1993; Robinson, 1995). The importance of membrane localisation for GRK6 function is demonstrated by observation that palmitoylated GRK6 is approximately 10-fold more active at phosphorylating the β2AR in vitro than its non-palmitoylated counterpart (Stoffel, 1998).

The thioester bond of palmitoylated proteins is extremely labile and this characteristic allows the removal of palmitic acid from the modified protein. Several proteins of the GPCR transduction
machinery have been shown to undergo cycles of palmitoylation and depalmitoylation following agonist treatment of GPCR. These include the \( \beta_2 \)AR (Mouillac, 1995), the \( \alpha_2 \)-adrenergic receptor (Kennedy, 1994), nitric oxide synthetase (Robinson, 1995) and the \( \alpha \) subunits of heterotrimeric G proteins (Wedegaertner, 1994). In the case of \( \text{Gas} \), the agonist-dependent loss of palmitic acid parallels the translocation of this protein from the membrane to the cytosol (Levis, 1992). The palmitoylation of GRK4 and GRK6 may potentially provide a mechanism for the dynamic regulation of the membrane association of these enzymes.

GRK5 has a C-terminal domain rich in basic and polar amino acids that may promote membrane association through interaction with the negatively charged phospholipid head groups (Premont, 1995) (Fig. 1.6).

### 1.2.2.3 Regulation of GRK activity via non covalent interactions with lipid ligands

GRKs are lipid-dependent enzymes. Thus agonist-occupied GPCRs serve as GRK substrates only when present in physiological membranes or alternatively when incorporated into lipid or detergent/lipid micelles (Pitcher, 1995b; Onorato, 1995). A number of lipid ligands for the GRKs have been identified, which functionally belong to two categories. The binding of any lipid ligand potently enhances GRK-mediated GPCR phosphorylation by targeting the GRK to the membrane and placing the enzyme and its receptor substrate in close proximity. Subsets of lipid ligands directly enhance GRK catalytic activity. As previously described GRK2/G\( \beta \gamma \) complex formation promotes GRK2-mediated GPCR phosphorylation. The G\( \beta \gamma \)-mediated translocation of GRK2 is observed in cellular systems or \textit{in vitro} using receptor substrates reconstituted in heterogeneous
("crude") lipid environments. However, purified GPCR reconstituted into pure phosphatidylcholine (PC) vesicles fail to serve as GRK substrates even in the presence of Gβγ (Pitcher, 1995b; DebBurman, 1996). Furthermore, Gβγ fails to promote the association of GRK2 with pure PC vesicles (Pitcher, 1995b). These results suggest that GRK2/Gβγ complex formation requires the presence of a lipid cofactor. Indeed, low concentrations (<10µM) of the lipid phosphatidylinositol-4,5-bisphosphate (PIP2) have been shown to specifically promote GRK2/Gβγ complex formation and thereby GRK2-mediates GPCR phosphorylation (Pitcher, 1995b; DebBurman, 1996). PIP2 binds to the N-terminus of the GRK2 PH domain in vitro (Harlan, 1994) (Fig. 1.8). Thus the coordinated binding of the two ligands (PIP2 and Gβγ) to the N- and C-termini of the PH domain of GRK2, is required for the effective membrane localisation and function of this enzyme. The concentrations of PIP2 that promotes GRK2/Gβγ-mediated GPCR phosphorylation are comparable to those believed to be physiologically relevant and have no direct effect on GRK2 catalytic activity. However, high concentrations of PIP2 (> 200µM) directly inhibit the catalytic activity of GRK2. This inhibitory effect of PIP2 is however, unlikely to be significant in vivo, since the required concentrations of PIP2 are approximately 20-fold higher than those physiologically relevant. Additionally, the inhibition of GRK2 activity observed at these high concentrations is mediated, at least partially, via binding to the ATP binding site of GRK2 and not to the PH domain (DebBurman, 1996).

Lipids other than PIP2 also play a role in regulating GRK2 activity. Thus negatively charged phospholipids such as phosphatidylserine (PS) enhance GRK2-mediated GPCR phosphorylation (Onorato, 1995). Several characteristics distinguish the enhanced GRK2 activity observed in the presence of these negatively charged phospholipids
from that observed in the presence of PIP2. Firstly, PS-enhanced GRK2 activity requires the presence of 10-20 fold higher concentrations. Since the physiological mol fraction percent for PS is estimated 10% as compared to 1-3% for PIP2 however, both regulators would be predicted to be of physiological relevance. A second feature distinguishing regulation by these two lipids concerns their mechanism of action. Thus in contrast to PIP2, PS directly increases the catalytic activity of GRK2 (Onorato, 1995; DebBurman, 1996). PS has been shown to bind to the C-terminus of GRK2. Unlike PIP2, the PH domain of this enzyme has not been directly implicated as the site of interaction (DebBurman, 1996). In the light of the differences between the binding characteristics of PS and PIP2 it is interesting to speculate that they bind to distinct C-terminal sites. It should be noted, however, that as for PIP2 (although at approximately 10-20-fold higher lipid concentrations) a synergistic enhancement of GRK2 mediated GPCR phosphorylation has been reported in the presence of PS and Gβγ (DebBurman, 1996).

GRK5 does not contain a sequence for isoprenylation (Harlan, 1994) and does not bind Gβγ (Premont, 1994). GRK5 is however a membrane-associated enzyme. When transiently overexpressed in COS7 cells GRK5 is found exclusively associated with the plasma membrane (Premont, 1994) Additionally, purified GRK5 binds to ROS membranes, an association that is not affected by light activation of rhodopsin (Premont, 1994). Thus GRK5 is constitutively associated with the membrane in an agonist-independent fashion. In vitro, GRK5 associates with liposomes composed of crude lipid mixture (Kunapuli, 1994a), but not with purified phosphatidylcholine (PC) (Pitcher, 1996). Thus, the association of GRK5 with the membrane is not dependent upon receptor activation or the presence of receptor, but rather upon specific lipid binding determinants present on the kinase.
Two distinct lipid-binding domains have been identified on GRK5, one in the amino and one in the C-terminus of the enzyme. The N-terminal lipid-binding domain exhibits a high degree of specificity for PIP2. GPCR reconstituted in pure PC vesicles fail to serve as substrates for GRK5 (Pitcher, 1996). However, the incorporation of physiological concentrations of PIP2 into vesicles restores the ability of these kinases to associate with membranes and phosphorylate GPCRs. As is the case of PIP2-mediated activation of GRK2, PIP2 has no direct effect on the catalytic activity of GRK5 (Pitcher, 1996). Binding of PIP2 to GRK5 thus enhances GPCR phosphorylation by promoting the membrane localisation of this kinase. The PIP2 binding site is localised at the N-terminus of GRK5 (Fig. 1.9), and specifically at a region homologous to the PIP2 binding site of gelsolin. GRK4, GRK5, and GRK6 are highly homologous across this region; indeed all these enzymes display PIP2-mediated membrane association and enhanced GPCR phosphorylation (Pitcher, 1996) (Fig. 1.9). PIP2 can, therefore play a role in mediating the membrane association of GRK2, GRK3, GRK4, GRK5, and GRK6. However, the structure, location and regulation of the PIP2 binding site distinguishes GRK2 and GRK3 from GRK4, GRK5, and GRK6.

GRK5 exhibits phospholipid stimulated autophosphorylation to a stoichiometric ratio of 2 mole of phosphate per mole enzyme (Kunapuli, 1994a). A GST fusion protein encompassing the C-terminus of GRK5 (residues 489-590) blocks this lipid effect, indicating the presence of a lipid binding site in the region of the kinase (Kunapuli, 1994a). Although lipid binding promotes autophosphorylation of GRK5, autophosphorylation of GRK5 does not affect lipid binding. Thus an autophosphorylation mutant inhibits the phospholipid-stimulated autophosphorylation of the native enzyme (Kunapuli, 1994a). In the absence of additional lipid ligands,
**Figure 1.9 Amino terminal of GRK4 subfamily mapping the PIP2 binding domain**

The location of the PIP2 binding domain on GRK5 is represented by the open box. The alignment of the N-terminal basic domain of GRK4, GRK5, GRK6 are shown. The residues postulated to represent the PIP2 binding site of GRK5 are boxed. The sequence involved in PIP2 binding to gelsolin is also shown.
using as substrates GPCR reconstituted in vesicles composed of only PC and PS, PS promotes membrane association of GRK5 and GRK5-mediated GPCR phosphorylation. PS binding requires the presence of a number of basic residues in the C-terminus of GRK5 (residues 547, 548, 553, 556 and 557), localising the site of PS interaction and distinguishing it from that of PIP2 (Kunapuli, 1994a). Notably, PS-mediated activation of GRK5 requires approximately 10-fold higher lipid concentrations than that required for PIP2-mediated activation of the enzyme. Furthermore, unlike PIP2, PS binding directly increases the catalytic activity of GRK5. Interestingly, the PS-dependent increase in the catalytic activity of the enzyme is dependent on the presence of intact autophosphorylation sites (Kunapuli, 1994a). Thus an autophosphorylation mutant of GRK5 although capable of binding PS is not directly activated by the lipid. The observation that the catalytic activity of the autophosphorylation deficient mutant of GRK5 is not enhanced by PS may provide an explanation for the observations of impaired ability of the autophosphorylation mutant of GRK5 to phosphorylate receptor substrates (Kunapuli, 1994a). In the absence of lipid activators native GRK5 and the autophosphorylation deficient mutant exhibit equivalent abilities to phosphorylate a soluble peptide substrate (Kunapuli, 1994a). However, the inability of PS to directly activate the autophosphorylation deficient mutant of GRK5 would specifically impair the ability of this kinase to phosphorylate lipid-incorporated substrates (such as receptor). Interestingly, synergistic enhancement of GRK5 activity is observed in the presence of PIP2 and PS (Kunapuli, 1994a). Thus binding of PS to a C-terminal region of GRK5 facilitates binding of PIP2 to the N-terminus (Kunapuli, 1994a). GRK5 membrane association is clearly distinct from that of GRK1, GRK2 and GRK3. The constitutive association of GRK5 with membranes
creates a population of kinase in close proximity to its receptor substrates, a proximity that potentially allows for a more rapid kinase response following agonist-occupancy of G protein-coupled receptors.

1.2.3 N-terminal interacting proteins

Recently, a new feature of the GRKs has come to light. Significant amounts of GRK2 have been shown to be associated with several membrane fractions after differential centrifugation (Garcia-Higuera, 1994a). The binding of GRK2 to these fractions is reversible. Membranes treated with proteinase K or boiled were unable to bind GRK2, indicating that the association was through a protein-protein interaction (Garcia-Higuera, 1994a). GRK2 was found to bind to this protein with nanomolar affinity. The highest specific activity for this interacting protein was in the microsomal membrane fraction and binding was blocked by the addition of a GST fusion protein containing residues 50-145 of the N-terminus of the kinase but not by a fusion protein containing the C-terminus (Murga, 1996). This excludes the possibility that the microsomal membrane binding is Gβγ. Addition of microsomal membranes, containing this protein, inhibited the activity of GRK2 when rhodopsin was used as a substrate; the inhibition was relieved by addition of aluminium fluoride or GTPγS plus mastoparan. This finding allows for the potential regulation of GRK2 activity through a GTP dependent mechanism; either through the release of G protein βγ subunits after activation of Gα or potentially through GTP binding of the inhibitory anchoring protein. These findings raise the possibility that additional as yet unidentified proteins direct GRKs to distinct subcellular compartments and potentially to different receptor substrates.
1.2.4 Tissue distribution of GRKs

Most of the GRKs discovered to date are expressed in a diverse array of mammalian tissues (Table 1.3), with the exception of GRK1 and GRK4, which are specifically localised in the retina and pineal gland (GRK1), and in the testis (GRK4), (Sallese, 1997; Pitcher, 1998a) which suggests limited substrate specificity. Indeed, is likely that retinal opsin constitutes the physiologic substrate for GRK1. GRK4, which is expressed principally in the testis, might be expected to desensitise an olfactory like sperm receptor. The distribution of the remaining GRKs has been established primarily by multiple tissue RNA blots, which distinguish the pattern of tissue expression only to a limited degree. Only GRK2 and GRK3 have been compared quantitatively for tissue expression, and GRK3 levels are generally 10-20% the level of GRK2. Exceptions to this generalisation includes the testis, neurones of the nigrostriatal pathways and the olfactory tubercle, where GRK3 predominates or is exclusively expressed (Arriza, 1992; Pei, 1995). Specific probes for either GRK2 or GRK3, used for in situ hybridisation and immunohistochemistry of rat brain, have clearly demonstrated the expression of GRK2 and GRK3 outside areas known to contain adrenergic neurones (Arriza, 1992; Walensky, 1995). These studies suggest that a broad range of receptors may be regulated by GRK2 and GRK3 intervening in the regulation of immune receptors or neurotransmitters as well.

1.2.5 Substrate specificity

Phosphorylation-dependent homologous desensitisation is a general mechanism that regulates GPCR-mediated signalling. Given the extraordinarily large number of GPCRs identified so far (≥1,000)
Table 1.3 GRK gene family

<table>
<thead>
<tr>
<th>Family name</th>
<th>Tissue distribution (major sites)</th>
<th>Chromosome 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>
</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.
and the relatively small number of GRKs (6 genes) (Chuang, 1996a; Premont, 1995; Pitcher, 1998a) it appears evident that the interaction between these proteins is not based on a one-kinase-for-one-receptor rule. It seems reasonable to extrapolate from the available data and speculate that most GRKs initiate desensitisation of multiple receptors. For a specific receptor, however it is conceivable that one GRK is primarily, if not exclusively, responsible for initiating homologous desensitisation. Alternatively, a receptor potentially phosphorylated by several GRK may demonstrate cell type-specific desensitisation properties that are determined by the GRK expressed in the cells under study. Most of the data on GRK substrate specificity derive from experiments with purified receptor and enzymes combined under non-physiological conditions, which were optimised primarily for the prototype of GRK, RK, and ARK1. More recently GRK phosphorylation assays have been performed with purified kinases and purified plasma membrane fragments containing high levels of receptor protein (≥ 80 pmol/mg) (Pei, 1995). By examining the stoichiometry and kinetics of receptor phosphorylation, these studies suggest GRK substrate specificity in several cases. GRK1 and GRK2, for example, can each phosphorylate both rhodopsin and S2AR, but with significantly different activity. While GRK1 shows ~2-fold greater activity on rhodopsin that on S2AR; ARK1 shows ~20-fold greater activity on S2AR than on rhodopsin (Benovic, 1986). A similar analysis suggests that the M2 muscarinic acetylcholine receptor and S2AR are comparable ARK1 substrates (Kunapuli, 1994b), while rhodopsin, the M2 muscarinic (Kunapuli, 1994b) and the α2C2 adrenergic receptors (Pei, 1994) are significantly poorer GRK5 substrates than S2AR. Likewise, the M3 muscarinic acetylcholine receptor appears to be a good substrate for
GRK2 and GRK3, but a comparably poor substrate for GRK5 and GRK6 (DebBurman, 1995).

GRK substrate specificity has also been demonstrated with synthetic peptides. Whereas GRK1 and GRK2 are active on peptides containing acidic residues flanking serine or threonine on the N- and C-terminal sides, (Onorato, 1991) GRK5 and GRK6 most actively phosphorylate peptides containing basic residue N-terminal to serine (Kunapuli, 1994b; Loudon, 1994). Mammalian cells transfected with epitope tagged receptors and individual GRKs facilitate evaluation of both agonist induced receptor phosphorylation and desensitisation in a model system, which preserves the integrity of the intracellular environment. Receptor phosphorylation is assessed by immunoprecipitation of receptor from cells labelled with $^{32}$P and challenged with agonist, while desensitisation is assessed by intact cell assays for second messenger production. This approach has been used to show that the $\beta_1$-adrenergic (Freedman, 1995) and $\delta$-opioid receptors (Pei, 1995; Kieffer, 1995) can undergo agonist-promoted phosphorylation by several GRKs, and that receptor phosphorylation is associated with diminished signalling through the receptor. For the $\beta_1$-adrenergic receptor, phosphorylation data obtained in the intact cell system were similar to those obtained using purified GRK and Sf9 cell plasma membranes containing high levels of $\beta_1$-adrenergic receptors (Freedman, 1995). With intact cells, it is also possible to estimate the relative contribution of second messenger-dependent kinases and GRKs to the process of agonist induced receptor phosphorylation. Addition of cell permeant activators or inhibitors of cAMP dependent protein kinase (PKA), to human embryonic kidney (HEK293) cells reveals that PKA is responsible for roughly half of the agonist induced phosphorylation of $\beta_1$-adrenergic receptor (Freedman, 1995). Agonist-induced phosphorylation of $\delta$ opioid, in contrast,
appears to depend on GRKs almost exclusively, in HEK293 cells. Although PKC isoforms can phosphorylate the δ opioid receptor in HEK293 cells, downregulation of PKC isoforms appears to have no effect on agonist induced phosphorylation of this receptor (Pei, 1995). The above mentioned body of evidence addresses the question whether or not a given GRK can phosphorylate a given receptor. Whether or not a given GRK does phosphorylate and initiate desensitisation of a given receptor in vivo or in the intact cells is a more difficult question to answer. Heparin has been used to inhibit GRK mediated phosphorylation and desensitisation of the β2AR, α2A-adrenergic, δ1-adrenergic receptors in permeabilised cells, (Lohse, 1989; Roth, 1991; Liggett, 1992; Zhou, 1995). More recently a dominant negative mutant of GRK2 has been used to antagonise GRK action in intact cells (Kong, 1994). The dominant negative GRK2 binds to, but cannot phosphorylate agonist occupied receptors. The dominant negative GRK2 mutant when overexpressed in cells known to express βARK1 and GRK5, is able to reduce the agonist induced phosphorylation of the δ1-adrenergic (Freedman, 1995), δ-opioid (Pei, 1995) and M2 muscarinic acetylcholine receptors (Tsuga, 1994); it also appears to reduce agonist induced desensitisation of the β2AR. Furthermore, the dominant negative βARK1 inhibits receptor phosphorylation promoted only by agonist, and not by second messengers (Pei, 1995; Freedman, 1995). Neither heparin nor the dominant negative βARK1 can be considered an inhibitor of a single GRK. Although the dominant negative should bind only those activated receptors recognised by βARK1, this binding should competitively inhibit the activity of any protein which might otherwise bind to the activated receptor. More precise determination of the role of a single GRK in receptor regulation may be made with specific antibodies introduced into permeabilised cell preparations. In rat
nasal cilia where GRK3 expression is predominant, odorant induced second messenger signals terminate within 100 milliseconds. Pretreatment of these cilia with anti GRK3 antibodies attenuates odorant receptor phosphorylation (Schleicher, 1993). Similar specificity of GRK neutralisation may be achieved in intact cells with antisense oligonucleotides, which have recently been shown to decrease β2AR desensitisation specifically by βARK1 in several cell line (Shih, 1994).

1.2.6 Unconventional physiological GRK substrates

GRK mediated GPCR phosphorylation plays a critical role in the rapid agonist induced desensitisation and targets the receptor for internalisation. However these enzymes may have additional targets. It has been demonstrated that GRK2 is associated with microtubules and soluble tubulin and that tubulin is an excellent substrate for this enzyme in vitro (Haga, 1998; Pitcher, 1998b). Notably the kinetic parameters of GRK2 mediated tubulin phosphorylation resemble those of GRK2 mediated β2ARs phosphorylation (the supposed physiological substrate of this enzyme) and far exceed those of a peptide substrate. Agonist occupancy of GPCRs promotes GRK2-tubulin complex formation and tubulin phosphorylation. These observations suggest a potential role for GRK2 in modulating the phosphorylation status of tubulin in intact cells. The interaction of GRK2 with activated receptor substrate in vitro leads to an allosteric activation of this enzyme. The physiological relevance of this activation remains somewhat obscure, since to date the only substrates identified for this enzyme are the activated receptors themselves. The identification of a potential non-GPCR GRK2 substrate raises the possibility that GRK2 may itself act as a signal
transducer. Agonist occupancy of GPCRs promotes the membrane localisation of GRK2 and the specific targeting of the enzyme to its activated receptor substrates. Allosteric activation of GRK2 by activated receptors would thus be predicted to promote GRK2 mediated phosphorylation of tubulin in an agonist stimulated manner. In such a model, GRK2 would act to link GPCR activation with one component of the cellular cytoskeleton. Moreover other cytoskeletal proteins such as actin have been reported to bind and inhibit GRK5 activity. Actin inhibits the kinase activity by directly reducing GRK5 mediated phosphorylation of both membrane bound GPCRs and soluble substrates.

1.2.7 Regulation of GRK expression

Several studies point to the potential importance of changes in cellular GRK activity/protein levels, in the regulation of cellular functions mediated by a variety of GPCRs. In cells overexpressing GRKs, the second messenger production stimulated by a number of GPCRs including $\beta_2$-(Pippig, 1993), $\beta_1$-(Freedman, 1995), $\alpha_1$B-(Diviani, 1996)-adrenergic receptors, D1A-dopamine (Tiberi, 1996) receptors and thyroid stimulating hormone (TSH) receptors (Nagayama, 1996; Iacovelli, 1996) are substantially blunted. Conversely, the treatment of cells with an antisense oligonucleotide to the mRNA for GRK2 and GRK3 has been shown to cause reduction in the $\beta_2$AR desensitisation levels (Shih, 1994). Finally, in transgenic mice with cardiac-specific overexpression of GRK2, isoproterenol-stimulated left ventricular contractility in vivo was reduced and functional coupling of $\beta$-adrenergic receptors was attenuated (Koch, 1995). A number of cellular mechanisms by which gene expression/activity of GRKs can be regulated have recently been
characterised. Using human peripheral blood leukocytes, a marked
increase in GRK2 mRNA, immunoreactivity and kinase activity was
demonstrated during the process of T-cell activation (De Blasi, 1995).
GRK3 levels were also increased, although to a lower extent, while
GRK5 and GRK6, also present in these cells, were not affected (De
Blasi, 1995). The stimulation of the T-cell antigen receptor by
phytohemagglutinin for 2-4 days was the protocol for T-cell activation.
A maximal increase in GRK2 expression could be induced by
application of PMA, thus demonstrating that GRK2 is regulated
through a PKC-mediated intracellular pathway (De Blasi, 1995) (Fig.
1.10). Functional studies on the same T-cell activation model showed
that this increase in GRK2 expression could result in a significant
blunting of the β-adrenergic receptor-mediated cAMP production (De
Blasi, 1995).

Most studies on the regulation of GRK/arrestin in vivo have
focused on the heart, particularly in relation to heart failure. A
decreased responsiveness of β-adrenergic receptors have been
demonstrated in chronic heart failure and this is thought to
exacerbate the loss of cardiac contractility (Hammond, 1993). This
impaired function is paralleled by a pronounced downregulation of β1-
adrenergic receptor subtype, while the β2AR subtype is unaffected.
Samples from the left ventricles of patients with dilated or ischemic
cardiomyopathy showed that the mRNA levels of GRK2 and the
kinase activity were increased almost threefold as compared to non-
failing control ventricles (Ungerer, 1993). This change was selective,
as GRK3 and arrestin levels were similar to the controls (Ungerer,
1994). GRK5 and GRK6 were not studied since they were not yet
identified when these studies were performed. Increased GRK activity
was also observed in the left ventricle in a porcine model of heart
failure. These findings indicate that GRK2 is increased in heart
Figure 1.10 Heterologous regulation of homologous desensitisation mechanisms

In homologous desensitisation only the agonist-occupied receptor is desensitised by a GRK/arrestin mechanism. In heterologous desensitisation receptor-stimulated PKA or PKC phosphorylates and desensitises different types of receptor substrates. These two mechanisms can crosstalk. PKC can directly phosphorylate and activate GRK2 and, in T-cells, can increase the expression and activity of GRK2 and GRK3 after sustained stimulation. An increase in intracellular cAMP raises β-arrestin1 mRNA and protein expression, probably through a PKA-mediated mechanism. Abbreviations are: CT, cholera toxin; Fsk, forskolin.
failure and this change may heighten the desensitisation of β-adrenergic receptors, thus contributing to the altered catecholamine response which worsens the myocardial contractility. This may also have important implications in the development of future strategies to fight myocardial β-adrenoceptor desensitisation. Indeed chronic treatment of pigs with β-blocker bisoprolol induced a significant decrease in GRK activity in heart ventricles (Ping, 1995). β-blockers are used to treat heart failure and their ability to decrease GRK in the heart, if proved to be general, might contribute to their therapeutic effect. An even more attractive strategy was recently suggested by the observation that isoproterenol-stimulated left ventricular contractility was enhanced in mice overexpressing a GRK2 inhibitor (Koch, 1995). The GRK2 inhibitor was a transgenic construct, coding for the C-terminal 194 amino acids (amino acid 495 to 689) of bovine GRK2, which includes the PH domain. This domain, acts as a GRK inhibitor by preventing the kinase binding to Gβγ and impairing its membrane anchoring, a process critical for receptor phosphorylation. The possibility to decrease GRK activity, either by β-blockers or by overexpression of a GRK2 inhibitor by gene therapy indicates attractive future strategies to rescue receptor functionality. This should finally result in improved cardiac functionality. However, the contribution of altered G protein gene expression to heart failure must also be taken into consideration (Ping, 1995).

Whether changes in GRK activity are specific for heart failure or a general phenomenon associated with sustained sympathetic nervous system activation is an important and unanswered question (Hammond, 1993). Another study supports the possibility of GRK activity being related to the degree of sympathetic stimulation in vivo (Garcia-Higuera, 1994a). In the liver of neonatal rats immediately after birth, there is a dramatic transient increase of catecholamines
and the GRK phosphorylating activity is increased. β-adrenoceptors were desensitised within the same time frame, suggesting that desensitisation mechanisms are important in modulating catecholamine activities at birth. The same study also reported that in 1 day-old rats exposed to hypoxia for two minutes to induce a huge increase in catecholamines, the GRK activity was markedly increased.

Based on studies on rat brain, it was also proposed that the GRK/arrestin desensitising mechanism could be involved in the development of tolerance to chronic opioid treatment. In rats treated chronically with morphine, the immunoreactivity of GRK2, but not GRK3, was increased by ~35% and β-arrestin was increased by 18% in the locus coeruleus, which is a brain area involved in opiate actions (Terwilliger, 1994). In many other brain regions these proteins were unaffected. That GRK2 might be involved in the opioid tolerance process is supported by the observation that GRK can regulate or phosphorylate δ (Pei, 1995) and κ (Raynor, 1994) opioid receptor subtypes. However the ability of GRK to regulate the μ opioid receptor, which is the relevant subtype in the locus coeruleus, remains to be demonstrated. Considering the weak effect of morphine on GRK and β-arrestin, confirmatory studies are needed to define the possible role of these regulatory proteins in opioid tolerance.

1.2.8 Regulation of GRK activity

Different GPCRs can be phosphorylated by the same GRK subtype, indicating that receptor specificity is not the key determinant for kinase-receptor interaction. Therefore it is conceivable that intracellular mechanisms exist to regulate the activity of GRKs and to provide some degree of selectivity for their interaction with GPCRs (Fig. 1.11).
Figure 1.11 Regulated interaction between GPCRs and GRKs
Several mechanisms that may modulate the activities of GRKs have been identified. First the stimulated receptor (i.e. agonist-occupied) is able to activate GRK (Chen, 1993; Haga, 1994a). Conformational changes of the receptors induced by agonist binding causes the exposure of two physically and functionally distinct domains. One contains the sequence that is phosphorylated by GRK and the second acts as an activator of these kinases. For example in the M$_2$ muscarinic receptor, the sites that are phosphorylated by GRK2 and the domains able to activate this kinase were found to be located in different intracellular regions of the receptor (Haga, 1994). PIP$_2$ and G$\beta$$\gamma$, which are major determinants for GRK membrane targeting, are also potent activators of these kinases (Pitcher, 1995b; DebBurman, 1996; Pitcher, 1996).

Intracellular calcium can modulate GRK activity by different mechanisms; it activates PKC which can phosphorylate different GRKs and modulate their activity (DebBurman, 1996; Winstel, 1996; Pronin, 1997) and it can bind to and activate calcium sensor proteins (CSP). Different CSPs can selectively regulate the activity of GRK subtypes.

Actin can inhibit GRK5 activity by directly reducing GRK5-mediated phosphorylation of both membrane-bound GPCRs and soluble substrates. The actin binding is displaced by calmodulin (CaM) that inhibits GRK5-mediated phosphorylation of membrane bound GPCRs but not soluble substrates such as casein. Thus in the presence of actin, CaM determines the substrate specificity of GRK5 by promoting the phosphorylation of soluble rather than membrane-bound substrates. These conclusions have been derived so far only from in vitro studies.
1.2.8.1 Effects of PKC phosphorylation

GRK2 modulates receptor-mediated production of second messengers, which in a feed-back loop can act on GRK2 to modulate its activity. In fact it has been shown that GRK2 activity is increased by PKC-mediated phosphorylation (Chuang, 1995), indicating that GRK2 can be pre-conditioned to regulate the subsequent cellular responsiveness to receptor activation, providing the cell with a mechanism by which specific homologous desensitisation can be regulated heterologously (Fig. 1.10). More recently it has been found that PKC phosphorylation may act as a general mechanism for regulating GRK function. PKC rapidly and stoichiometrically phosphorylates GRK5, resulting in a significant reduction in its activity (Pronin, 1997a).

This is in striking contrast with the ability of PKC to phosphorylate and activate GRK2. The differential regulation of GRK2 and GRK5 by PKC suggest that GRK diversity may play an important role in the differential desensitisation of various receptors.

GRK expression levels and subtypes may also be critical in determining how rapidly receptor signalling is attenuated, in particular for those receptors coupled to PLC stimulation. For example myocardial GRK2 and GRK5 appear to play an important role in regulating β2AR function which is coupled to stimulation of cAMP production. In contrast the myocardial type 1A of angiotensin II receptor, which stimulate PLC and consequently PKC, appears to be regulated by GRK2 and not by GRK5. Perhaps PKC activation by angiotensin receptor stimulation may turn off GRK5 activity.
1.2.8.2 Effect of CSP

Interaction of Ca\(^{2+}\) with a large number of Ca\(^{2+}\)-binding proteins represents one of the mechanism by which this second messenger controls many biological processes (Schafer, 1996; Ikura, 1996). Calcium binds with high affinity to Ca\(^{2+}\)-binding proteins and induces conformational changes that enable the proteins to interact and regulate a variety of targets. One class of protein shares a common Ca\(^{2+}\)-binding motif, the EF-hand (Ikura, 1996). This structural motif, first identified in the crystal structure of parvalbumin, consists of two perpendicularly placed \(\alpha\)-helices (helices E and F in parvalbumin) and one interhelical loop, which together form a single Ca\(^{2+}\)-binding site. The mechanism of this molecular switch lies in the conformational change induced by Ca\(^{2+}\) binding. EF-hand proteins with regulatory roles are often termed CSPs, whereas those involved in Ca\(^{2+}\) buffering and transport are termed Ca\(^{2+}\)-buffer proteins (Ikura, 1996). CSPs represents a heterogeneous class of proteins which includes CaM, neurone-specific calcium sensor proteins, named neuronal calcium sensors (NCS), such as recoverin, visin-like-protein (VILIP), neurocalcin, hippocalcin and the recently identified S100 family members.

1.2.8.3 Interactions between recoverin and GRK1

The first evidence of interaction and regulation of GRKs by a CSP were obtained in studies on retinal phototransduction. Confirming earlier observations by Kawamura, 1993 several studies suggested that the Ca\(^{2+}\) effect on cGMP phosphodiesterase (PDE) is likely to be mediated by recoverin, which was able to decrease light-dependent rhodopsin phosphorylation (Nikonov, 1993; Gorodovikova, 1994). This
results in a prolonged lifetime of catalytically active rhodopsin and therefore in a larger number of transducin molecules activated by rhodopsin. Recoverin (also called S-modulin in the frog) is a myristoylated CSP expressed predominantly in vertebrate photoreceptor cells.

The molecular mechanisms of recoverin inhibition of rhodopsin phosphorylation have been elucidated (Klenchin, 1995; Chen, 1995). Recoverin acts directly on RK to decrease its catalytic activity; no components other than rhodopsin, RK and recoverin are required. Inhibition of rhodopsin phosphorylation by recoverin was Ca\(^{2+}\)-dependent. The covalently attached myristoyl residue enhanced the inhibitory effect of recoverin as determined in an assay of phosphorylation of urea-stripped rod outer segments (ROS) membranes by purified recombinant RK. The IC\(_{50}\) for myristoylated recoverin was 0.8 \(\mu\)M and the IC\(_{50}\) for nonacylated-recoverin was 8 \(\mu\)M at saturating Ca\(^{2+}\) concentrations.

Since myristoylated recoverin was a better inhibitor than nonacylated-recoverin, it was suggested that the interaction of recoverin with ROS membranes enhances the inhibitory effect. However, this point is still controversial and requires further investigation (Klenchin, 1995; De Castro, 1995). In another study, by Klenchin, 1995, the inhibitory effect of recoverin was not affected by changing the concentration of bleached rhodopsin or the amount of membranes, leading to the conclusion that recoverin does not require binding to rhodopsin or ROS membranes.

Physical interactions between recoverin and RK likely represent the mechanism of inhibition of the kinase. Recoverin binds directly to RK in a Ca\(^{2+}\) dependent manner. This is a high affinity interaction and immobilised recoverin can be used as affinity matrix to purify GRK1 (Chen, 1995).
1.2.8.4 Interactions between CaM and fARK subfamily

While investigating whether the interaction and regulation of GRKs by CSP is a general mechanism, three different laboratories have found that GRK2 and GRK3 are inhibited by CaM in a Ca^{2+}-dependent manner (Chuang, 1996b; Haga, 1997b; Pronin, 1997b) with an IC\textsubscript{50} of 2 µM. CaM is an acidic protein, that is considered the primary "decoder" of Ca\textsuperscript{2+} information in the cell (James, 1995; Rhoads, 1997), exerting many of its functions when bound to Ca\textsuperscript{2+} (4 Ca\textsuperscript{2+} ions per CaM molecule). Numerous proteins are regulated by Ca\textsuperscript{2+}/CaM, e.g. kinases, phosphodiesterases, calcium pumps, adenylyl cyclase. G\beta\gamma is also a binding target of Ca\textsuperscript{2+}/CaM while the binding of GRK2 and GRK3 to G\beta\gamma is critical to mediate the activation of these two GRK-subtypes. Therefore, G\beta\gamma was proposed as a possible site of indirect interaction between GRK2 and GRK3 with Ca\textsuperscript{2+}/CaM. However CaM was able to inhibit GRK2 kinase activity even in the absence of G\beta\gamma, indicating that the effect of CaM is not due to sequestration of G\beta\gamma from GRK2 (Haga, 1997b; Pronin, 1997b). Preliminary results indicate that the inhibition is likely the consequence of a direct interaction between CaM and GRK2.

1.2.8.5 Interactions between CaM and GRK4 subfamily

The analysis of different GRK isoforms led to the unexpected observation that CaM is a potent inhibitor of GRK5 (Chuang, 1996b; Pronin, 1997b). The IC\textsubscript{50} was 40-50 nM, indicating that CaM is approx. 50 fold more potent in inhibiting GRK5 kinase activity as 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 GRK4 (Sallese, 1997) and GRK6 (Pronin, 1997b) was ~3 fold lower than that on GRK5 and ~30 fold more potent than that on GRK2. The calculated IC\textsubscript{50} for GRK4 was 80 nM (Sallese, 1997).

The high affinity interaction between CaM and GRK5 has been extensively characterised. CaM impaired the ability of GRK5 to phosphorylate bovine retinal ROS in a calcium-dependent manner and this effect was prevented by the CaM inhibitor peptide CaMBd. A direct interaction between GRK5 and Ca\textsuperscript{2+}/CaM was demonstrated using CaM-conjugated Sepharose 4B and confirmed using surface plasmon resonance (SPR) technology on a BIAcore instrument (Levay, 1998). The calculated K\textsubscript{d} for the interaction is ~10 nM, a value in good agreement with the IC\textsubscript{50} for CaM-dependent inhibition of ROS phosphorylation. This binding does not influence the catalytic activity of GRK5, as demonstrated by the lack of inhibitory effect on its phosphorylating activity towards the soluble GRK substrate casein. Instead, Ca\textsuperscript{2+}/CaM significantly reduced kinase binding to the membrane and to phospholipid vesicles. Kunapuli, 1994a demonstrated that binding of GRK5 to phospholipids is critical in the activation of the kinase. Thus, inhibition of GRK5 association with the membrane by Ca\textsuperscript{2+}/CaM would hinder kinase activation, with a consequent inhibitory effect on receptor phosphorylation.

Direct binding of CaM to GRK4 has also been documented (Sallese, 1997). However, the inhibitory effect of CaM on membrane binding was more evident on GRK5 than on GRK4 and is likely to account for the ~3 fold more potent inhibitory effect of CaM on rhodopsin phosphorylation by GRK5 than by GRK4 or GRK6. Unlike GRK4 and GRK6, that utilise covalent lipid modification to enhance binding to phospholipid membranes, GRK5 appears to interact directly with phospholipids via regions rich in basic amino acids (Palczewski, 1997;
Krupnick, 1998). This different mechanism of membrane targeting provides a likely explanation for the different kinase sensitivity to CaM. However, additional mechanisms may be important (Pronin, 1997b), for example, it was observed that the IC$_{50}$ of CaM to inhibit GRK5 binding to ROS membranes (~300-400 nM) was 6-8 fold higher that the IC$_{50}$ for rhodopsin phosphorylation. Moreover, at high CaM concentrations (2 μM) ~20% of the kinase remained bound to ROS membranes even though rhodopsin phosphorylation was reduced by >99%. In addition CaM inhibited GRK5-mediated phosphorylation of the soluble substrate phosvitin (IC$_{50}$ ~600 nM) suggesting that CaM interacts with regions of GRK5 which are likely to be involved in substrate binding. Taken together these results indicate that CaM can directly compete for both the lipid and receptor binding sites of GRK5.

Unexpectedly it was found that CaM activates GRK5 autophosphorylation (Pronin, 1997b). It was already known that phospholipids can activate GRK5 autophosphorylation, resulting in the activation of this kinase. However, GRK5 autophosphorylation activated by CaM was found to occur on S and T residues different from the residues S 484 and T 485, substrates for phospholipid-induced autophosphorylation. Indeed CaM-activated GRK5 autophosphorylation was observed even after mutation of S 484 an T 485 into aspartic acid (D). Autophosphorylation is not the major determinant in inhibiting GRK5 activity, but it may enhance the sensitivity of GRK5 to CaM (Pronin, 1997b).

The following model of GRK5 regulation by CaM has been proposed (Pronin, 1997b). 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 directly inhibits receptor phosphorylation. However, since CaM-
stimulated autophosphorylation also inhibits GRK5 activity, the kinase should remain inhibited even when Ca\(^{2+}\) levels decrease 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. A similar regulatory cycle has been demonstrated for CaM-kinase II (Gnegy, 1993).

The CaM binding site in GRK5 was identified (Pronin, 1997b) and found to be located near the N-terminus of the kinase (amino acids 20-39). GRK5-GST fusion proteins lacking this domain were unable to inhibit CaM-induced GRK5 autophosphorylation. In addition using SPR technology on a BIAcore instrument (Levay, 1998) GRK1, GRK2, GRK5 (each kinase is representative of a different subfamily) were found to possess two distinct CaM binding domains. Most of the CaM binding sites identified contain a basic amphipathic α-helical structure with a large number of positively charged residues as well as hydrophobic residues that repeat with a three to four period (James, 1995; Rhoads, 1997). Consistent with this model, α-helical wheel projection of the region of GRK5 which binds CaM shows the segregation of basic and hydrophobic residues to opposite sides of the helix, thereby making them available for interaction with acidic and hydrophobic patches of CaM.

The sequence corresponding to the CaM binding site was conserved within the GRK4 subfamily (GRK4, GRK5 and GRK6), which all interact with CaM at high affinity. By contrast, the putative binding site is poorly conserved in GRK1, GRK2 and GRK3, which interact with CaM at much lower affinity. Residues 22-39 of GRK5 were suggested to be involved in binding to PIP2, which leads to the activation of these kinases (Pitcher, 1996). Since the putative PIP2
and CaM binding domains are substantially overlapping, CaM could compete for binding of PIP2 to GRK5. In agreement with this, it has been shown that CaM inhibits direct binding of the kinase to phospholipid vesicles.

In GRK4, the region homologous to the CaM binding site of GRK5 lies entirely within exon 2, which is alternatively spliced (Sallese, 1997). GRK4β and GRK4δ subtypes, which do not contain this sequence, were unable to bind to CaM, further supporting the role of this sequence as a CaM interacting site. However additional sites are apparently important for the CaM-GRK4 interactions, since GRK4γ, which contains the sequence encoded by exon 2, but lacks exon 15 located near the C-terminus, was unable to bind to CaM (Sallese, 1997).

1.2.8.6 Selectivity in the regulation of GRK by CSP

It has been suggested that CSPs act as functional analogues in mediating 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, 1997a) suggesting that several classes of EF-hand CSPs can regulate GRK activity. This mechanism is however highly selective with respect to the different CSPs and GRK subtypes (see Table 1.4), (Iacovelli, 1999).

Chen showed that recoverin, which inhibited GRK1 phosphorylating activity by direct binding to this kinase, did not interact with GRK2 in parallel experiments and was ineffective on ROS phosphorylation by this kinase subtype (Chen, 1995). Recoverin is a member of the NCS family; several other members of this family including NCS 1, VILIP 1 and hippocalcin, also inhibit GRK1 in a calcium-dependent manner (De Castro, 1995). However these NCS
Table 1.4 Selective inhibition of GRK subtypes by CSP

<table>
<thead>
<tr>
<th></th>
<th>Recoverin</th>
<th>VILIP</th>
<th>NCS-1</th>
<th>CaM</th>
<th>References</th>
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<tr>
<td>GRK1</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>Klenchin, 1995; De Castro, 1995</td>
</tr>
<tr>
<td>GRK2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>Chuang, 1996b; Haga, 1997b; *</td>
</tr>
<tr>
<td>GRK3</td>
<td>nd</td>
<td>-</td>
<td>nd</td>
<td>+</td>
<td>Chuang, 1996b; Boekhoff, 1997</td>
</tr>
<tr>
<td>GRK4</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>+++</td>
<td>Sallese, 1997</td>
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<tr>
<td>GRK5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>Chuang, 1996b; Iacovelli; 1999</td>
</tr>
<tr>
<td>GRK6</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>+++</td>
<td>Pronin, 1997b</td>
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</table>

CSP were uneffective (-) or able to inhibit the indicated GRK subtypes with different degree of potency (+). nd, not determined; (*), unpublished data from our laboratory.
are not able to inhibit GRK5 kinase activity (Iacovelli, 1999). An analysis of VILIP in olfactory neurones indicated that in these cells, VILIP does not interfere with GRK (Boekhoff, 1997). Since GRK2 is the relevant GRK subtype in olfactory neurones, these results further confirm that VILIP selectively inhibits GRK1 (De Castro, 1995) and does not affect either GRK3 (Boekhoff, 1997) or GRK5 (Table 1.4). Taken together these data indicate that recoverin and other NCS can selectively inhibit GRK1 but do not affect the other GRK subtypes. By contrast CaM strongly inhibits GRK4 subfamily members while it has little (for GRK2 and GRK3) or no (for GRK1) effect on the other GRKs. This profile of selectivity is consistent with the tissue distribution of these proteins. Recoverin, which is a retinal protein, preferentially regulates RK, while the ubiquitously expressed CaM preferentially interacts with GRK5 which was found in a large variety of tissues and cells.

1.2.8.7 Functional implications

The functional consequences of the recoverin-GRK1 interaction were intensively investigated. Calcium ions regulate several steps of the phototransduction process, by modifying the activity of different Ca$^{2+}$-binding proteins, which in turn interact with key enzymes in the cascades (Polans, 1996; Koutalos, 1996). Several pathways are activated when the concentration of Ca$^{2+}$ decreases after photon absorption and they all lead to negative regulation of the effect of light. These feedback mechanisms speed up the recovery of the response to light and diminish the effect of illumination, thus enabling the cell to adapt to background light. Recoverin is involved in one such mechanism. Reduction of Ca$^{2+}$ levels by photon absorption uncouples recoverin from RK, thus allowing the inhibitory
effect of rhodopsin phosphorylation to proceed. This results in a shorter lifetime of rhodopsin in the photolysed state, which is one mechanism of light adaptation. There are however observations suggesting that the above model might represent an oversimplification and perhaps may even be incorrect (Polans, 1996). For example knockout mice for the recoverin gene, show no major effects in their response to light flashes. However these data must be interpreted with caution, since the removal of the recoverin gene could result in an unexpected compensation by other components of the phototransduction cascade. Recent studies (Senin, 1997a; Senin, 1997b) showed that 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).

Some functional consequences of regulating GRK subtypes by CaM can be predicted. A number of GPCRs are substrates of GRK2, GRK3 and GRK5 and are coupled to fluctuations in intracellular Ca\(^{2+}\) concentrations, e.g. substance P and angiotensin II receptors. CaM can therefore provide a feedback mechanism for modifying homologous desensitisation of these receptors. Furthermore, in any cell, a number of receptor substrates for GRK2, GRK3 and GRK5 may be present; calcium sensors may therefore mediate a novel route of crosstalk in which one Ca\(^{2+}\)-regulating receptor agonist can regulate the phosphorylating activity of these GRK subtypes, thus modulating the efficiency of a different receptor. Few functional data are available, Haga, found that in transfected CHO cells the sequestration of the M\(_2\) muscarinic receptor is attenuated by treatment with Ca\(^{2+}\) ionophore A23187 (Haga, 1997). Since M\(_2\)
muscarinic receptor sequestration is a specific and sensitive sensor of GRK2 activity (Tsuga, 1994), these results indicate that the activity of GRK2 in intact cells is suppressed by increased Ca\textsuperscript{2+} concentrations.

1.2.9 New levels of transduction regulators

A novel mechanism for crosstalk in signal transduction is revealed by the identification of intracellular pathways that can modify the GRK/arrestin homologous desensitisation machinery (Fig. 1.10). The mechanisms so far identified include PKC and Ca\textsuperscript{2+}-binding proteins, involved in the regulation of GRKs and cAMP-PKA involved in the regulation of arrestins. The existence of other pathways can not be excluded. Since PKC, PKA and Ca\textsuperscript{2+}-binding proteins can be activated by a large variety of receptor agonists, it can be envisaged that the functional state of the GRK/arrestin machinery is under continuous regulation in response to the fluctuating extracellular environment, thus providing the cell with another homeostatic mechanism. A challenging point is to define how these different effectors work in integrated systems.

The role of intracellular calcium in the modulation of regulatory proteins is becoming more and more relevant. For example, other CSPs could be involved in the regulation of GPCR-mediated signalling. Additionally a new class of phosphatases named G protein-coupled receptor phosphatases (GRP), has recently been identified. These GRP dephosphorylate desensitised receptors, thus favouring resensitisation (Pitcher, 1995a; Vinós, 1997; Montini, 1997). Interestingly GRPs possess two EF-hands and their activity is calcium dependent.
All these observations support the idea of a complex integrated network, working towards efficient signalling. The identification of the players and the definition of their relative function represents a major goal in this field.

The existence and the functional importance of these regulatory pathways may not be universal, but it would be expected to be dependent on factors, such as receptor selectivity and cell type specificity. For example GRK/arrestin regulation of different receptors, and the regulation of the same receptor in different cell types may have different levels of significance (Shih, 1994).

The GRK/arrestin regulatory pathway can also be modulated in vivo. Their regulation may have important implications, as in the case of heart failure. There are a number of hypotheses that need to be confirmed or substantiated. For example the interesting possibility that the activity of GRK in vivo follows the changes in the sympathetic tone to provide a protective mechanism, needs to be demonstrated by direct approaches. Whether the inhibition of GRK activity in heart failure, either by drug treatment or gene therapy, represents an additional strategy to treat this pathological state is at the moment an attractive but unanswered question.

The possible role of GRK/arrestin in the phenomenon of tolerance and withdrawal after chronic agonist or antagonist treatment also deserves further studies. The development of specific inhibitors of these kinases would provide an invaluable tool to further our understand this area. The GRK/arrestin regulatory pathway can now be viewed as an intracellular mechanism which is regulated and which may represent a suitable target for therapeutic strategy in the future.
1.3 Arrestin gene family

1.3.1 Introduction to arrestins

Retinal arrestin was the first member of the arrestin family to be cloned (Shinohara, 1987) and shown to be identical to the highly autoantigenic 48 kDa protein (S-antigen), already described in the photoreceptor cells. Further studies have shown immunoreactivity in tissues other than retina, suggesting that members of this gene family might also be implicated in the regulation of signals induced by different agonists. The cloning of the first arrestin homologue from bovine brain, termed S-arrestin, (Lohse, 1990b) finally provided evidence of the existence of arrestin like proteins in extra-retinal tissues. So far many homologues have been cloned and the arrestin gene family consists of four recognised families: (1) rhodopsin arrestin or S-antigen (arr), (2) β-arrestin 1 (βarr1), (3) β-arrestin 2 (βarr2) and (4) cone arrestin (Carr) (Table 1.5, Fig. 1.12, Wilson, 1993; Palczewski, 1994). The insect retinal arrestins could be considered a fifth family. Gene duplication leading to the generation of a new gene family whose members have distinct but related functions is at the basis of the concept of adaptive evolution (Li, 1983). Further diversity in the mammalian forms has been generated by alternative splicing. Subtypes 1A and 1B of β-arrestin 1 are distinguished by the inclusion or exclusion of a 24 base/8 amino acid cassette (exon 13) in the C-terminal region (Parruti, 1993). Subtypes L and S of arrestin 3 (equivalent to β-arrestin 2) are generated by alternative splicing of a 33 base/11 amino acids insert in a region yet closer to the C-terminus (Sterne-Marr, 1993). Even arrestin, which is a single product that includes exon 13 when expressed in retinal or pineal tissue, was found with or without exon 13 when expressed in
<table>
<thead>
<tr>
<th>Name</th>
<th>T. distribution (major sites)</th>
<th>Receptor targets</th>
<th>Splice variants</th>
<th>Chrom. mapping</th>
<th>References</th>
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<tr>
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<td>Rhodopsin</td>
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<td></td>
<td>Calabrese, 1994a</td>
</tr>
<tr>
<td>Cone arrestin</td>
<td>Retina</td>
<td>nd</td>
<td>--</td>
<td>Xcen-q21</td>
<td>Palczewski, 1994</td>
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<tr>
<td>ß-arrestin1</td>
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<td>2</td>
<td>11q13</td>
<td>Wilson, 1993</td>
</tr>
<tr>
<td></td>
<td></td>
<td>adrenergic and rhodopsin</td>
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<td>2</td>
<td>17p13</td>
<td>Wilson, 1993</td>
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<tr>
<td></td>
<td></td>
<td>Odor and</td>
<td></td>
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<td>Calabrese, 1994a</td>
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Receptor targets indicate GPCRs which interact with different arrestin subtypes as assessed with pure protein or coexpression experiments. Only few receptors are indicated and this reflects the relatively low number of studies carried out. ß-arrestin1 and 2 can bind to rhodopsin, but with low affinity. Abbreviations are: AR, adrenoceptors, Odor, odorant receptors, L, human peripheral blood leukocytes, O, olfactory neurons, nd, not determined.
Figure 1.12 Percentage of identity among the arrestin gene family

In this scheme crossing of two different arrestins gives the percentage amino acid identity. Crossing of the same arrestins gives the amino acids identity throughout the species. arr = S-antigen, βarr1 = βarrestin 1, βarr2 = βarrestin 2, Carr = cone arrestin.
peripheral blood leukocytes (Parruti, 1993). Very recently a new splice variant from human retina, produced by splicing out exon 12, has been cloned (Smith, 1996). The bovine variant, arrestin p44 is generated by splicing out the exon 16 from the transcript, corresponding to the last 35 amino acids (Smith, 1994). Sequence analysis of the arrestin superfamily often shows regions that are conserved within the members of one subfamily but highly divergent between the different subfamilies. This combination of characteristics is often considered as indicative of of a multigene family, that has diverged but also conserved regions specific to the function of each family. Obviously some domains are better conserved across the superfamily. One of these at the N-terminus (bp 79-97), is cited in PCGENEs subprogram PROSITE as an arrestin signature (Craft, 1995). Some arrestin features revealed by sequence analysis have been confirmed by mutagenesis studies, which have identified several key functional regions within the arrestin molecule (Gurevich, 1995a). The N-terminal half of arrestin (residue 1-191) was found to contain domains involved in both activation and recognition (interacting with the portions of rhodopsin that changes conformation upon light activation, indicated as A-R in Fig. 1.13) and phosphorylation-recognition (interacting with the phosphorylated C-terminus of rhodopsin, indicated as P-R in Fig. 1.13). When these primary binding sites are simultaneously engaged by binding to phosphorylated light activated rhodopsin, arrestin undergoes a rearrangement into a high affinity binding conformation (Gurevich, 1994), that results in the mobilisation of a secondary hydrophobic binding site (residue 191-355, indicated as S-Bs in Fig. 1.13). The conformational change that exposes the secondary binding site appears to be partially controlled by intramolecular interaction of the regulatory N- and C-terminal regions (indicated as R-R1 and R-R2 respectively in Fig. 1.13).
Figure 1.13 Domain organisation of the arrestin family

The functional regions of arrestins: from left to right; the regulatory region R-R1 (amino acid 1-29), the region sensitive to the receptor conformational change upon activation A-R (amino acid 29-163), the region capable of recognising the phosphorylated receptor P-R (amino acid 163-191), the hydrophobic region working as a secondary binding site S-Bs (amino acid 191-355) for the receptor, and the acidic regulatory region R-R2.
The contributions of the primary and secondary binding sites to arrestin-receptor interaction appear to be comparable. The ionic nature of the interaction involved in the phosphorylation-recognition suggests that the negatively charged phosphorylated C-terminal of the receptors interacts with the positively charged residues of arrestins. The residues 163-191 of the arrestin play a significant role in phosphorylation-recognition (Gurevich, 1995a).

### 1.3.2 Regulation of arrestins

Arrestin proteins do not possess enzymatic activity and appear to function in a 1:1 stoichiometric manner in uncoupling the GRK-phosphorylated receptor from G proteins. Though relatively less attention has been paid to the role of these proteins in homologous desensitisation, it has been demonstrated that, overexpression of β-arrestins could result in enhanced desensitisation of β2-(Pippig, 1993), β1-(Freedman, 1995), α1B-(Diviani, 1996)-adrenergic receptors, as for GRK. For TSH receptors, the agonist-stimulated cAMP production and cell growth has been shown to be attenuated by overexpression of the two β-arrestin 1 isoforms in FRTL5 cells (Iacovelli, 1996)

The expression of β-arrestin1 is regulated by intracellular levels of cAMP (Iacovelli, 1996; Parruti, 1993). The levels of β-arrestin1 mRNA and immunoreactivity were increased in different cell types when intracellular cAMP was raised after receptor activation (by iloprost or TSH), Gs (by cholera toxin) or adenylyl cyclase (by forskolin) (Fig. 1.10). The peak of the effect occurred after 6-8 hours of treatment. Cycloheximide completely prevented this effect, strongly indicating that a newly synthesised protein(s) plays a major role in the cAMP-induced increase of β-arrestin1 mRNA. The need for newly
synthesised protein(s) may also explain the reason why several hours are needed to induce this effect. The mechanism by which cAMP increases β-arrestin1 expression likely involves the activation of PKA, as indicated by the evidence that okadaic acid, a potent and specific inhibitor of phosphatases 1 and 2A, dramatically potentiated the effect of forskolin and iloprost. The mechanism suggested by these data is a PKA-mediated induction of gene expression, which is attenuated by intracellular phosphatases (Parruti, 1993).

1.4 Physiological mechanisms modulating the GPCR responses

1.4.1 Introduction to GPCR regulation

Usually the response to GPCR agonists are rapidly attenuated by different mechanisms devoted to prevent overstimulation and to restore the sensitivity of the receptor to subsequent stimuli.

1.4.2 Removal of the agonist from extracellular fluid

Dilution of the agonist in the extracellular fluid and subsequent excretion reduces its concentration so that it can no longer induce a detectable response in target cells. Uptake by high affinity carriers is among the most widely used mechanisms for removal of monoamines (i.e. dopamine, nor-epinephrine, serotonin) and amino acid neurotransmitter (i.e. gamma aminobutyric acid, glycine) from the synapse. Although many peptides are taken up by target cells during agonist-induced endocytosis of their receptors, this process occurs after receptor stimulation and desensitisation, so it can not regulate the concentration of peptides in extracellular fluids. The major mechanism for removing peptides from extracellular fluid is
extracellular degradation. In a manner similar to the degradation of acetylcholine in the synaptic cleft by acetyl cholinesterase, many peptides are degraded by cell surface peptidases (Sterne-Marr, 1993; Smith, 1996). The active site of these peptidases is usually projecting into the extracellular fluid: peptidases are ideally placed to degrade neuropeptides at the cell surface and to restrict the number of intact peptide molecules available to interact with the receptor and to initiate signalling.

1.4.3 GPCR desensitisation

Two major patterns of rapid desensitisation have been characterised, namely agonist-specific or homologous desensitisation and agonist-non-specific or heterologous desensitisation (Lohse, 1993). The term homologous indicates that desensitisation occurs only when one receptor is activated by a specific agonist, and involves only the subsequent response of the same receptor, without effects on other receptors present in the same cell. Conversely, heterologous desensitisation indicates that stimulation by one agonist attenuates the response to multiple and distinct agonists acting through distinct receptors. Phosphorylation is an essential step for both homologous and heterologous desensitisation, but the molecular mechanisms are quite distinct.

The process of homologous desensitisation is intrinsically related to the function of the GRK serine/threonine (S/T) kinases (Freedman, 1996; Chuang, 1996a; Krupnick, 1998; Palczewski, 1997) and their associated inhibitory proteins, arrestins (Smith, 1994; Craft, 1995). The essential steps of homologous desensitisation are as follows (Fig. 1.14). First, the agonist binds to the GPCR, thus
Figure 1.14 Pathways of signalling and regulatory mechanisms of GPCR

Agonist (A) binding of the β2AR is followed by activation of Gαs and adenylyl cyclase (signal 1). PKA phosphorylation of the receptor initiate its uncoupling from Gs and facilitate its coupling to Gai, which inhibits adenylyl cyclase (signal 2) GRK phosphorylation of the β2AR, and subsequent β-arrestin binding, completely desensitises the receptor. In addition β-arrestin is involved in the internalisation of the receptor via clathrin coated pit-derived vesicles. Internalisation of the receptor is required for activation of mitogen activated kinase (MAPK) (signal 3) as well as for dephosphorylation and resensitisation of the receptor.
activating the G protein. Then, the GRK is translocated to the membrane, to be physically colocalised with the receptor substrate. For GRK2 and GRK3 the presence of free βγ made available by receptor activation plays an essential role in their targeting to the membrane and may determine GRK-receptor selectivity (Pitcher, 1995b). Once the kinase is membrane-associated, it will be in the vicinity of a number of different GPCR, which are potential substrates. However, only the receptor that is stimulated by the agonist is in the appropriate conformational state to be phosphorylated and desensitised by these kinases. The strict agonist-binding requirement for GRK phosphorylation makes this mechanism strictly homologous. Phosphorylation can occur at the C-terminus, as for rhodopsin and β2AR, or at the third intracellular loop, as for M2 muscarinic and α2-adrenergic receptor (Gurevich, 1995b). GRK-phosphorylated receptors are only partially desensitised, but the phosphorylation process increases the affinity for arrestin. Binding of arrestin to the receptor subsequently induces the maximal homologous desensitisation; this means a reduction by 70-80% of the ability of fully activated rhodopsin or β2AR to stimulate the respective G proteins. A recent report suggests that binding of arrestin to the receptor may be a step towards receptor sequestration (Gurevich, 1994) (Fig. 1.14). GRK-initiated homologous desensitisation disappears very rapidly with a t1/2 from milliseconds to 15 seconds. Since GRKs act only on agonist-occupied receptors, it is not surprising that GRK-initiated desensitisation is most important under conditions of high receptor occupancy (i.e. at neural synapses, where agonist concentration is greater). In this regard, it has been observed by immunohistochemistry in the rat brain that GRK2 and GRK3 are localised at postsynaptic densities and presynaptic axon terminals. The relationship between heterologous desensitisation,
mediated by second messenger-dependent kinases (PKA and PKC) and homologous desensitisation, mediated by GRKs, is not completely understood and could be specific for receptor or cell type. In the β2AR model, established on cultured fibroblasts and carcinoma cells, desensitisation by one type of kinase appears to be independent of the other. For desensitisation of odorant receptors in rat nasal cilia, however, second messenger-dependent kinases and GRKs appear to be interdependent, and inhibition of either kinase completely abrogates desensitisation.

Moreover recent findings suggest that signal switching rather than signal desensitisation could be a more appropriate description for the second messenger-dependent signal fading. In the case of β-adrenergic receptor, for example, most of its action is mediated by coupling to Gs and activation of adenylyl cyclase. However in some cases such as heart it has been shown to interact with Gi as well (Xiao, 1995). What had not been appreciated until recently was the ability of PKA to phosphorylate the β2 adrenoceptor favouring its uncoupling from Gs and facilitating Gi coupling (Lefkowitz, 1998), (Fig. 1.14).

The information so far accumulated depicts a convincing picture of a molecular mechanism involving agonist binding to the receptor and subsequent sequestration. The biochemical details of receptor regeneration are much less clear and still a matter of investigation. For instance in the case of rhodopsin, removal of the agonist induces arrestin dissociation and dephosphorylation by protein phosphatase 2A. For other GPCRs, a low pH in endosomal compartments is probably responsible for phosphatase activation and a receptor conformational change that promotes arrestin disassembling (Palczewski, 1997).
1.4.4 Internalisation of GPCRs as a possible mechanism for desensitisation

Sequestration of β2AR was originally proposed as a mechanism of desensitisation, based on the observation that the light membrane fraction containing sequestrated β2AR did not contain Gs (Gurevich, 1995b). By applying specific inhibitors to digitonin-permeabilised A431 cells, Lohse demonstrated that sequestration of β2AR alone contributed to about 20-30% desensitisation of this GPCR (Lohse, 1990a). While other studies confirmed that β2AR sequestration may be linked to its rapid agonist-induced desensitisation, it is clear that sequestration alone cannot fully explain desensitisation. Moreover it has been shown that β2AR desensitisation occurs more rapidly than its internalisation (Toews, 1984; Roth, 1991), so that it is possible to distinguish between the rapid initial uncoupling of β2AR/Gs interaction and β2AR sequestration.

1.4.5 Agonist-induced internalisation of GPCRs

Another level of regulation of GPCRs is their internalisation or sequestration, following agonist exposure (Fig. 1.14). Numerous studies demonstrated that agonist exposure promoted translocation of GPCRs from the cell surface to intracellular compartments. Sequestration was thus originally defined as agonist-induced internalisation of a GPCR from the plasma membrane to a poorly defined cytosolic compartment where the GPCR is physically isolated or sequestered from membrane-bound G protein.

The rapid redistribution of cell surface β2AR was initially discovered by the observation that agonist treatment of bullfrog erythrocytes resulted in a decreased number of cell surface β2AR,
with concomitant increase in an intracellular compartment (Chuang, 1979). Moreover, early ligand binding studies with β2AR demonstrated a progressive loss of high affinity agonist binding during the binding assay, suggesting a loss or 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). Sequestered β2AR displayed reduced binding affinity and guanine nucleotides had no effect on agonist binding, both hallmarks for the absence of GPCR/G protein coupling. The translocation of β2AR from the plasma membrane to the intracellular compartment occurred very rapidly, with a t1/2 of about 2 min (Gurevich, 1995b). Sequestration was apparently independent on 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, utilised immunocytochemistry to investigate subcellular receptor localisation. By conventional and confocal fluorescence microscopy it was demonstrated that the rapid agonist-induced translocation of β2AR into the cytosol of HEK293 cells temporally paralleled the sequestration of β2AR, measured by radioligand binding (von Zastrow, 1992). There is much evidence to suggest that the agonist induced translocation of β2AR from plasma membrane to intracellular vesicles occurs via the clathrin coated pit/endosome pathway employed by constitutively recycling receptors (Fig. 1.14), (i.e. low-density lipoprotein and transferrin receptor) and growth factor receptors (i.e. EGF receptor). Incubation of astrocytoma cells with
EGF and isoproterenol resulted in comigration of EGF receptor and β2AR to a low density region of a sucrose gradient, suggesting that both receptors were processed in parallel (Wakshull, 1985). Techniques that disrupt internalisation by clathrin-coated pits (hypertonicity treatment, cytosolic acidification, intracellular potassium depletion, reduced temperature, and reduced ATP) also disrupt internalisation of β2AR (Homburger, 1980; Chuang, 1980). Finally, immunocytochemical subcellular localisation of β2AR, following agonist treatment, demonstrated colocalisation with the transferrin receptor, suggesting a processing similar to that of constitutively recycling transferrin receptor (von Zastrow, 1992). Many receptors, such as muscarinic (Koenig, 1996), 5-HT2A (Berry, 1996) delta and μ opioid (Keith, 1996), alpha1B (Fonseca, 1995), internalise via clathrin coated pits (Tan, 1993). A temperature-sensitive clathrin heavy chain mutant was used to demonstrate that the yeast pheromone receptor internalise, via clathrin coated pits (Tan, 1993). To date there is no evidence that the rhodopsin undergoes light-induced internalisation, consistent with the localisation of rhodopsin on an internal membrane.

1.4.6 Role of GRKs and arrestins in internalisation of GPCRs

Truncation of the C-terminus of βAR (from residue 364), removing all the putative βARK phosphorylation sites, did not affect its sequestration (Strader, 1987). In contrast, truncation proximal to residue 354 including the phosphorylation site, significantly impaired βAR sequestration (Cheung, 1989). Moreover, a β2AR mutant construct with the four putative βARK phosphorylation sites in the C-terminus mutated, was defective not only in agonist-induced
phosphorylation and desensitisation, but also in sequestration (Hausdorff, 1991).

Evidence is now accumulating that agonist-induced phosphorylation is likely to be important for the internalisation of many GPCRs. A ser/thr rich sequence has been postulated to play an important role in sequestration of M1, M2, M3 muscarinic receptors (Moro, 1993). Mutation or truncation of the ser/thr residue in the C-terminus of the thrombin receptor inhibits both agonist induced phosphorylation and sequestration (Shapiro, 1996).

Recent studies directly demonstrated a role for GRKs in agonist-induced sequestration of GPCRs. $\beta$ARK phosphorylation of muscarinic receptors enhance their sequestration, and coexpression of the M2 muscarinic receptor with a dominant negative $\beta$ARK decreases sequestration (Tsuga, 1994). The direct role of $\beta$2AR phosphorylation on its sequestration was demonstrated using a phosphorylation and sequestration defective mutant ($\beta$2AR-Y326A) (Barak, 1994). Overexpression of $\beta$ARK with this mutant enhanced its phosphorylation and sequestration (Ferguson, 1995). In addition to $\beta$ARK, GRK3-6 also phosphorylate the $\beta$2AR-Y326A mutant and rescue its sequestration (Menard, 1996). Interestingly, coexpression with GRK4 and GRK6 resulted in agonist-independent phosphorylation of $\beta$2AR-Y326A, accompanied by an increase in basal receptor sequestration. Further evidence that $\beta$ARK directly plays a role in $\beta$2AR receptor sequestration was provided by the observation that dominant negative $\beta$ARK attenuates both phosphorylation and sequestration of coexpressed wild type $\beta$2AR. It is now becoming evident that the ability of GRKs to promote sequestration of GPCRs depends on arrestins (Fig. 1.14). Overexpression of $\beta$-arrestin alone with $\beta$2AR-Y326A promoted its sequestration, and this effect was enhanced by $\beta$ARK coexpression (Ferguson, 1996). Moreover the $\beta$-
arrestin mutant V53D inhibited the ability of ßARK to rescue sequestration of ß2AR-Y326A and the sequestration of coexpressed wild type ß2AR. These arrestin mutants have been referred to as dominant negative proteins. The role of both GRKs and arrestin in regulating sequestration of GPCRs was recently highlighted by the observation that ß2AR sequestration in different cell types correlates better with the expression levels of both ßARK and ß-arrestin (Menard, 1997) and sequestration of M2 muscarinic receptor is synergistically regulated by both ßARK1 and arrestin (Schlador, 1997). Interestingly, coexpression of angiotensin 1a receptor, a GPCR which is not normally internalised via clathrin coated pits, with ß-arrestin promotes internalisation through the classic clathrin mediated endocytic pathway (Zhang, 1996). Recent studies demonstrated that arrestins can both desensitise agonist-activated GPCRs and promote their sequestration, by interacting not only with GPCRs but also with clathrin, the major protein component of the clathrin-based endocytic machinery (Goodman, 1996). ß-arrestin, but not visual arrestin, interacts specifically and stoichiometrically, with clathrin. Moreover, immunofluorescence analysis demonstrated that activated ß2AR, ß-arrestin and clathrin colocalise in intact cells, upon agonist addition, suggesting that the arrestin-clathrin interaction observed in vitro also occurs in vivo in the presence of activated cell receptors. Thus ß-arrestin may act as a signal for internalisation of agonist-activated GPCR, by virtue of its ability to target the desensitised receptor to clathrin-coated pits. Through high affinity interactions with both receptor and clathrin, the visual arrestin fulfils the functional properties of an adapter protein. In view of the many studies demonstrating that internalisation is strictly involved in the regulation of GPCR activity, as well as studies demonstrating that arrestins interact with multiple GPCRs, it is tempting to speculate
that various other GPCR signalling systems may use a similar mechanism of arrestin-promoted internalisation.

The predominant clathrin binding domain in non-visual arrestin has been localised to hydrophobic and acid residues between AA 367-385 in arrestin 3 (Goodman, 1997). Interestingly, the two visual arrestins lack a significant portion of this domain and are not very homologous in the remaining residues. A small, highly conserved region in the clathrin heavy chain N-terminal domain and containing critical basic and hydrophobic residues, was found to be important for arrestin binding (Goodman, 1997). Other GPCR signalling system are likely to utilise a similar mechanism, whereby binding of non visual arrestins to the agonist-activated and GRK-phosphorylated GPCR results in concomitant desensitisation and targeting of GPCRs to the clathrin-coated pits for internalisation. By virtue of their ability to promote internalisation of GPCR, a process now believed to be critical for recovery of responsiveness following agonist-induced desensitisation, GRK and arrestins have now been linked to both desensitisation and resensitisation of GPCRs following agonist stimulation. This hypothesis raises the intriguing possibility that GPCRs may have other functions.

1.4.7 Resensitisation

Although GPCR sequestration may not be essential for rapid agonist-induced desensitisation, recent studies have indicated that it may play a role in accelerating the recovery from desensitisation (Pippig, 1995; Pak, 1996) (Fig. 1.14). Several studies have indirectly demonstrated that sequestration is involved in resensitisation of the β2AR. Sequestration of the receptor directly promotes its resensitisation, since the inhibition of internalisation prevents
resensitisation. It was subsequently proposed that β2AR sequestration restores function by enabling a vesicles-derived phosphatase (Fig. 1.14) to dephosphorylate the receptor, followed by the recycling of the dephosphorylated functional β2AR receptor to the cell surface (Pippig, 1995). The importance of β2AR resensitisation by both 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 GRP able to dephosphorylate βARK phosphorylated β2AR was purified from bovine brain, and shown to be a latent form of protein phosphatase 2A (Pitcher, 1995a). Internalisation of the thrombin receptor also appears to be central in resensitisation, but in a unique manner involving an already internalised-Golgi localised intracellular pool of thrombin receptors. Stimulation of the thrombin receptor induces internalisation and subsequent targeting to lysosomes, while the intracellular pool of thrombin receptor is translocated to the cell surface. (Hein, 1994). Recent data on the β2AR show that internalisation is required for activation of ERK1 (extracellular signal regulated protein kinase) and ERK2 by the Goiβγ mediated pathway (Daaka, 1997), (Fig. 1.14).

1.5 Physiological role of GRK4

1.5.1 GPCR transduction system and sperm

Sperm cells can be subdivided in three main parts, the long tail used for forward progressive motion towards the oocyte, the midpiece that includes the most anterior part of the sperm tail characterised by the presence of tightly packed mitochondria that contribute to the
production of energy required for motility (Ford, 1990), and the sperm head containing the nucleus with the genetic material to be delivered to the oocyte. The acrosome is found in the most anterior part of the sperm head (Kopf, 1991). The acrosome is a flattened, membrane bound vesicle that surrounds the anterior part of the sperm nucleus; it has similarities with a secretory vesicle that contains digestive enzymes which are released through the process of exocytosis when the cells receive the appropriate signal (Kopf, 1991). The entire spermatozoon, including the acrosome is surrounded by the plasma membrane. Fertilisation involves cell-cell interaction at many levels between gametes of different mating type. Following their deposition on the female reproductive tract the mammalian sperm undergoes a series of biochemical modifications (capacitation) required for the acrosome reaction to occur in response to the appropriate stimulus (Zaneveld, 1991). Reaching the proximity of the oocytes the spermatozoa pass through the various oocyte coatings, namely the cumulus oophorus, the corona radiata, the zona pellucida and the vitelline membrane. The cumulus oophorus and the corona radiata are layers of follicle cells (Zaneveld, 1991). The cumulus cell matrix consists mostly of hyaluronic acid. The zona pellucida (ZP) is a thick extracellular matrix comprising the outer layer of the egg (Wassarman, 1995) and consists principally of glycoproteins, while the vitelline membrane is the plasma membrane that surrounds the oocyte. After penetrating the mass of cumulus cells surrounding the egg, by the use of its forward progressive motion, and gaining access to the egg surface; in mammals the sperm egg interaction can be subdivided into four steps (Fig.1.15). Sperm bind to the zona pellucida (step 1); sperm is induced to undergo acrosome reaction (step 2), a regulated exocytotic event that is essential for subsequent steps; sperm penetrate the ZP (Step 3) and can bind and fuse (step 4) to the
**Figure 1.15 Mammalian fertilisation steps**

**a:** Sperm bind to the zona pellucida (ZP). **b:** Sperm undergo the acrosome reaction and penetrate the ZP. **c:** Sperm bind to the egg plasma membrane. **d:** Sperm fuse to the egg plasma membrane and activate zygotic development.
egg plasma membrane (Snell, 1996); this latter event somehow activates the egg to initiate zygotic developments. All these processes are highly regulated and involve the activation of membrane receptor and G proteins (Ward, 1992) ultimately leading to protein phosphorylation and/or an increase in intracellular calcium (Snell, 1990; Breitbart, 1997). Three main signal transduction pathways have been identified that can result in protein phosphorylation, increased sperm motility and acrosome reaction (Zaneveld, 1993). The adenylyl cyclase, cAMP, PKA pathway; the PLC diacylglycerol, PKC pathway; and the guanylate cyclase, cGMP, protein kinase G pathways. These pathways are activated by several GPCRs identified on spermatozoa. Several GPCR agonists have been shown to act on sperm cells such as endothelin (Kamada, 1994), cannabinoid (Chang, 1993), platelet activating factor (PAF) (Breinardt, 1989), and odorant receptors (Vanderhaeghen, 1993). In the present study I documented the expression of GRK4 in spermatozoa and germinal cells, where it is associated with mitochondrial and acrosomal membranes.

1.5.2 Metabotropic glutamate receptor 1 and Purkinje cells

Based on structural homology and mode of ligand-receptor interaction, GPCRs can be subdivided into 3 main subfamilies. The first includes rhodopsin, and most of the receptors for small molecule neurotransmitters and for glycoprotein hormones; subfamily 2 includes receptors for peptide hormones, such as calcitonin and vasoactive intestinal peptide; subfamily 3 includes metabotropic glutamate receptors (mGluR), GABAB receptors, pherohormone receptors and the Ca2+-sensing receptor; these receptors bind the ligand in the extracellular N-terminal domain (Fig. 1.16). mGluR constitute a family of large G protein-coupled receptors with little
Figure 1.16 Phylogenetic tree, illustrating the putative evolutionary relationship between three GPCR families

Sequences were aligned across the seven transmembrane domain regions and loop only. CASR calcium sensing receptor; CCK1 chemokine receptor; D1, dopamine; ET, endothelin receptor; GABA-BR1b, GABA receptor; GRF, growth releasing hormone receptor; NPY, neuropeptide Y receptor; VIP, vasointestinal peptide receptor.
sequence homology to the super family of smaller G protein linked receptors (Pin, 1995). Eight members of the mGluR family have been identified, several of which generate subtypes by alternative mRNA splicing (Pin, 1995). The eight mGluR receptors are generally classified in three subgroups on the basis of their sequence homology, agonists selectivity and signal transduction pathways (Fig. 1.17), (Conn, 1997; Ozawa, 1998). The first group includes mGluR1 and mGluR5, which share 62% homology at the amino acid level are coupled to PLC in transfected cells and are sensitive to quisqualic acid as the most potent agonist. Five splice variants of mGluR1 have been described, namely mGluR1a, mGluR1b, mGluR1c mGluR1d, mGluR1e (Conn, 1997; Pin, 1995). In mGluR1f, the insertion of 85 bases in the C-terminal tail introduces a stop codon that results in a 318 amino acid deletion and also changes the reading frame, leading to a different C-terminal amino acid sequence from mGluR1a. The functional significance of the different splice variants has not yet been fully explored. It has been suggested that the C-terminus of mGluR might play a role in the sub cellular targeting of the receptor (Brakeman, 1997) and residues of the C-terminal close to the inner surface of the plasma membrane have been shown to be important for G protein coupling (Mary, 1998; Pin, 1995). mGluR1 transcripts are widely distributed in the adult rat brain and are specifically localised to neurones. Most intensely labelled neurones are Purkinje cells of the cerebellar cortex and neurones in the hippocampus (Masu, 1991). mGluR1 knock out mice do not show any gross abnormalities in the hippocampus or cerebellum (Conquet, 1994). Moreover the hippocampus of these mutant mice appears to be electrophysiologically normal; even long-term potentiation (LTP) is induced normally (Conquet, 1994).
Figure 1.17 Dendrogram and pharmacological classification of the mGluR family

The homology scheme includes the bovine parathyroid Ca$^{2+}$-sensing receptor (CASR). The numbers on the top indicate the percentage amino acid sequence identity between mGluR, at the junction of the horizontal lines. The most potent group 2 agonist is (2S,1'S,2'S)-2-(carboxycyclopropyl)glycine (L-CCG-I). The most potent group 3 agonist is L-2-Amino-4-phosphonobutyrate (L-AP4). The most potent calcium receptor agonist is gadolinium (Gd$^{3+}$).
In contrast, these mice show an impaired motor co-ordination, lack of cerebellar long-term depression (LTD) and persistent multiple climbing fibre innervation of cerebellar Purkinje cells (Kano, 1997; Conquet, 1994; Aiba, 1994). This severe motor deficit, which results from cerebellar dysfunction, resembles the cerebellar-ataxic spontaneous mutant mice such as Purkinje-cell-degeneration (PCD) mice (Ryo, 1993), whose phenotypes lead to postnatal degeneration of cerebellar Purkinje cells and male sterility due to morphological sperm abnormalities. From these studies emerge a fundamental role of mGluR1 in the Purkinje cell function, while the function of mGluR1 in other brain areas could be carried out by other mGlu receptor subtypes. This study shows, (i) that GRK4 is selectively expressed in sperm and Purkinje cells where it colocalises with mGluR1 and regulates its signalling when coexpressed in heterologous system and (ii) that mGluR1 is expressed in spermatozoa.
CHAPTER 2

MATERIALS AND METHODS

2.1 Molecular biology

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

2.1.1 DNA cloning

2.1.1.1 Restriction digest

Several commercially available restriction enzymes were used to digest DNA for cloning experiments. The digestions were usually carried out as follows: 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.

2.1.1.2 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.

2.1.1.3 Klenow treatment

In a few cases it was necessary to fill in the ends of DNA produced by restriction enzymes, in order to sub-clone them into a vector. One µg of the digested DNA was purified from the low melting point agarose by phenol/chloroform extraction and resuspended in 40 µl of water plus 5 µl of klenow buffer, (1 x final concentration), 200µM of dNTPs and 2 U of Klenow polymerase The reaction was carried out for 20 min at 37°C then the enzyme was inactivated at 75°C and the fragments ligated as described in section 2.1.1.5.

2.1.1.4 Phosphatase and Kinase treatment

To sub-clone DNA fragments containing the same restriction enzyme at both sides, it is useful to treat the vector with the calf intestinal alkaline phosphatase (CIP) to remove the phosphates and avoid religation of the plasmid. Polymerase chain reaction (PCR) products must be phosphorylated before they can be ligated to a dephosphorylated vector. Usually 1 µg of vector was treated for 1 h at 37°C with 5 U of CIP in the same reaction solution of the restriction
enzyme. About 1 μg of pure PCR product was treated for 1 h at 37°C in 50 μl of reaction buffer supplied by the enzyme manufacturer, containing 1 mM ATP and 5 U of T4 polynucleotide kinase. The DNAs were electrophoresed on an agarose gel, phenol/chloroform purified and used for the ligation reaction.

2.1.1.5 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.

2.1.1.6 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° 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 CaCl$_2$, 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.

2.1.1.7 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).

2.1.2 Plasmidic DNA preparations

2.1.2.1 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 lefted 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.

**2.1.2.2 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 lisozyme 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 isopropl 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 a 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.
2.1.3 DNA sequencing

DNA was sequenced using the dideoxy sequencing method according to Sanger (Sanger, 1992, Sanger, 1977) using a sequencing kit from Pharmacia Biotech. Three μg of plasmid DNA purified as in section 2.1.2.2, were denatured with 200 mM NaOH and precipitated. After neutralization with 3 volumes of ethanol and 0.3 M sodium acetate pH 4.5, DNA was resuspended in annealing buffer (supplied from the kit) and 10 pmol of a specific primer were annealed at 65°C for 10 min and 37°C for 10 min. The reaction was divided into four separate tubes, each one containing the primer, the template, the labelling nucleotide ($^{35}$S-dATP), the T7 DNA polymerase, four deoxynucleotides, and one dideoxynucleotide. The products of the four reactions were electrophoresed on 6% acrylamide gel, dried and subjected to autoradiography. The DNA sequence was deduced from the autoradiographyc film.

2.1.4 Amplifications

2.1.4.1 Cloning of GRK4 and identification of mGluR1 by PCR

To obtain first cDNA strand, 1μg of total RNA was reverse transcribed in 50 μl of 50 mM Tris-HCl pH 8.3, 75 mM KCl, 3 mM MgCl$_2$, 10 mM dithiothreitol (DTT), 0.5 mM of each of the dNTPs, 1 μM random hexamers, 40 U of cloned Moloney murine leukaemia virus (M-MLV) reverse transcriptase (Bethesda Reseach Laboratories) for 1h at 37°C, and the reaction stopped by chilling to 0°C. The cDNA was amplified using the primers 5'-ACC AAG AAT CCA ACC AAG CG-3' (forward, F1 in Fig. 3.2) and 5'-GTG GGA CGA GAC GCT AAC A-3'
(reverse, R1 in Fig. 3.2) 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 aquaticus* 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. All the nested PCRs were performed using the above conditions and 0.1 μl of the original PCR reactions as template. The analysis of mGluR1 expression in rat testis was performed by amplifying the cDNA prepared as above, with the primers 5'-GGC CAT TCA AGT CCA AGA ATC T-3' (bp 859-879) and 5'-GTG TGG ACT GCT GAG ACA TA-3' (bp 1053-1035 of the rat sequence: GeneBank accession number X57569). The PCR was carried out for 30 cycles as follow: 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min. The rat GRK4 cDNA was amplified using the primers 5'-TGG ACC CAG AAA AGT TCG AC-3' (forward F2 in Fig. 3.7) and 5'-CTG TAG CTC GCA CTT GAC AG-3' (reverse R2 in Fig. 3.7), or 5'-CCC ACC TTT CTG TCC TGA T-3' (forward F3 in Fig. 3.7) and 5'-GGG TCT TGC TTT GTG GTC TG-3' (reverse R3 in Fig. 3.7), or F2-R3 to obtain the full length cDNA. The amplifications were performed with the EZ rTth RNA PCR kit (Perkin Elmer) which exploits the properties of rTth DNA polymerase to act as both a reverse transcriptase and as a DNA polymerase in the same buffer system. Moreover the use of the thermoactive and thermostable enzyme, rTth DNA polymerase, for both the reverse transcription and PCR steps, allows for increased specificity in primer binding as well as avoiding secondary structure in the RNA template. The following thermal profile was used: 1 h at 60°C to retrotranscript the RNA, 1.5 min to denature the cDNA and 40 cycles 95°C for 15 seconds (sec), 60°C for 1 min, The amplification ended with 7 min at 60°C to complete the polymerisation of any
incomplete chains. PCR products were separated by gel electrophoresis using 1 or 2% agarose as needed and visualised with ethidium bromide. The nature of the amplification products was confirmed by restriction analysis and sequencing after subcloning in a modified pBluescript II SK+.

2.1.4.2 Relative expression of different GRK4 isoforms by PCR

Undegraded total RNA was reverse-transcribed as described in section 2.1.2.3 and amplified for 30 cycles. Primers F0 (5'-GCG GCG GCG CCA GGA CAT G-3')-R0 (5'-GCA ACT TCA TAT TCT GCC ACT G-3'), F1-R1, F2-R2, F3-R3 encompassing respectively the human N-terminal, C-terminal, and the rat N-terminal, C-terminal splice variants were used to quantify the relative amount of the different GRK4 isoforms. The components of PCR reactions were tested for contaminants by 40 cycle reactions without added DNA template. PCR products were separated and visualised by gel electrophoresis using 3:1 NuSieve agarose at 2%.

The nature of the amplification products was confirmed by restriction analysis and/or sequencing. Two or more GRK4 isoforms differing only in the presence or absence of a DNA insertion (the specific spliced exon) when amplified with a pair of oligonucleotides flanking the spliced region (such as one of the couple of oligos listed above), all the isoforms represent indifferent templates for the PCR reaction. As a consequence, the relative abundance of these PCR products directly measures the ratio of the two transcripts at any cycle of amplification.

The relative density of bands visualized on a Polaroid film was measured by NIH Image 1.59 software after digitalisation with image scanner Agfa-Gevaert AG.
2.1.4.3 Preliminary genetic analysis of GRK4

In collaboration with the “Università degli Studi di Siena, Centro delle Encefalo-Neuro-Miopatie Genetiche”, three members of two unrelated families presenting as clinical symptoms were selected: moderate ataxia, marked cerebellar atrophy at magnetic resonance imaging (MRI), low level of intelligence associated with marked morphological abnormalities of the majority of spermatozoa (> 80%). Genomic DNA was extracted (for procedure see section 2.1.5.3) from white blood cells separated as in 2.5.1, and used for PCR analysis. Ten ng of genomic DNA were amplified using the primers 5'-GCC GCC GCG GTC GGG CTG-3' and 5'-GCT TTC AGC AGC AGC GAG TTG-3' encompassing the 5' untraslated sequence of GRK4, which contain several direct CGG (Ambrose, 1992) repeats. Several attempts to amplify this GC rich region failed. In contrast a robust amplification was obtained when taq polymerase was added after a hot start of 5 min at 95°C and the reaction was cycled 48 times with the following atypical thermal profile: 98°C, 5 sec; 66°C, 30 sec; 72°C, 30 sec. PCR products were analysed by 3 % NuSieve 3:1 agarose gel electrophoresis.

2.1.5 Preparation and analysis of mammalian RNA and DNA

2.1.5.1 RNA Isolation

Total RNA was prepared with a modified guanidine thiocynate method (Chirgwin 1979). Tissues or 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 guanidine. 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.

### 2.1.5.2 Northern blot analysis

GRK4 or mGluR1 mRNA expression, was examined by Northern blot analysis (Alwine, 1977). Twenty µg of total RNA were separated according to 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) using a capillary blot procedure in the presence of 10 x SSC (1.5 M NaCl, 1.5 M Na₃citrate pH 7). The RNA was fixed on the membrane by baking for 2 h at 80°C. A rat mGluR1 plasmid construct kindly provided by Dr. Nakanishi, (Masu, 1991) was digested with Nco I and Xba I. The excised cDNA fragment (1614-4194bp), was labelled by random priming and used as a probe. The cDNA fragment (1278-1806bp) (see Fig. 3.2) was used as a GRK4 probe (P1). When this was labelled by random priming, the brain hybridisation pattern revealed by autoradiography was barely detectable even after long term exposures (≥15 days). A substantial improvement of the results was obtained when a larger cDNA
fragment (P2) (619-1806bp) generated by a modified PCR reaction, was used. Two to 5 ng of P2 were used as a template after gel purification. The reaction mixture (10 µl final volume) contained 15 pmol of dATP, dGTP and dTTP, 7.5 pmol of dCTP, 25 µCi (8.3 pmol) of $^{32}$P-dCTP (3,000 Ci/mmol), 200 ng of primers (forward 5'-CGA GCC ACA GGA AAA ATG TA-3', and reverse R1 described in section 2.1.4.1), 1 µl of 10 x PCR buffer and 2 U of taq polymerase. 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 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 in at least two separate experiments.

2.1.5.3 Southern blot analysis of genomic DNA

High molecular weight genomic DNA was prepared from human peripheral blood leukocytes. Cells were treated with 100 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 % SDS and 100 µg/ml proteinase K for 16 h at 37°C, followed by phenol/chloroform extraction. RNA was removed from the preparation by treatment with DNAs-free RNAs A (50 µg/ml) for 2 h at 37°C, followed by a second phenol/chloroform
High molecular weight DNA (10 µg) was digested with different restriction endonucleases, fractionated by a 0.8% agarose gel, and transferred to a nylon filter (Gene Screen Plus membrane Du Pont-New England Nuclear). Filters were hybridised as in the Northern blot analysis using the GRK4 P1 probe (bp 1278-1806 in Fig. 3.2) but labelled by the modified PCR reaction described in section 2.1.2.6. The blot was hybridised overnight, washed to a final stringency of 2 x SSC, 1 % SDS at 65°C and subjected to autoradiography at -80°C for 1-7 days. The hybridisation signal was evident after 1 day exposure, but a long-term exposure (7 days) was performed to reveal possible additional hybridisation bands.

2.1.6 Construction of recombinant GRK4α in baculovirus vector

The baculovirus construct expressing recombinant GRK4α was obtained using the BAC-to-BAC™ baculovirus expression system (Luckow, 1993), (GIBCO). pCMV-GRK4α was digested with Sal I and partially digested with Hind III; the ~1800 bp full length cDNA was recovered from agarose gel electrophoresis and subcloned in the pFastBAC1 vector digested with the same enzymes. This pFastBAC1-GRK4α was transformed into DH10BAC competent cells that contain the bacmid (consisting of a low copy number mini F replicon, a kanamycin resistance marker, the lacZα gene that contains the attachment site for bacterial transposon, and essential bacuviral genes), and a helper plasmid with transposition functions. After 2 days of growth on LB Agar plates containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 300 µg/ml X-gal and 40 µg/ml isopropyl-ß-D-thiogalactoside (IPTG), large white colonies were harvested and recombinant bacmid DNA isolated by purification over a caesium chloride gradient. Five µg of recombinant bacmid DNA were
used to transfect 1 x 10^6 Sf9 cells with the calcium phosphate method described in section 2.3.1. Five days post transfection when the cells showed clear sign of viral infection the virus (Baculo4) was harvested, amplified a few times to get a high titre, and stored at -80°C until use. Virus was titred by the end point dilution method (Summers, 1987). The production of recombinant GRK4α protein was tested by Western blot analysis of Sf9 infected Baculo4.

2.1.7 Preparation of digoxigenin-labelled riboprobes

Two different probes were used for in-situ hybridisation analysis. RP1 probe was generated by full length GRK4γ in pBluescript II SK+ linearised with EcoRI and transcribed with T7 RNA polymerase. The N-terminal region of human GRK4α (bp -16-480) was amplified by PCR and subcloned into the plasmid PCRII topo (Invitrogen). This construct was linearised with EcoRV and transcribed with SP6 RNA polymerase (sense control probe, RP2) or was linearised with BamHI and transcribed with T7 RNA polymerase (antisense probe, RP3) according to the instruction of the transcription kit manufacturer (Promega Corporation). Briefly 1 μg of linearised cDNA was incubated for 2 h at 37°C with 1 x transcription buffer (40 mM Tris-HCl, 60 mM MgCl₂, 100 mM DTT, 20 mM Spermidine pH 8), nucleotide mix (10 mM dATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-11-UTP, pH 7.5) 1 U/μl RNAsin, 1 U/μl of appropriate RNA polymerase into a final volume of 20 μl. Following the transcription reaction, template DNA was removed by digestion with 1 U/μl of DNase I RNAse free (15 min at 37°C) while RNA was precipitated in 0.4 M LiCl, 75 % of ethanol and resuspended in RNAse-free water. The digoxigenin labelled riboprobes were stored at -80°C until use.
2.1.8 Two-Hybrid screening

The two hybrid screening (Fields, 1989; Chien, 1991; Luban, 1995) was performed using the MATCHMAKER Two-hybrid system 2 kit from CLONTECH Laboratories Inc. The following experiments were performed exactly according to the manufacturer instructions. All the control experiments suggested from the kit instructions were carried out with positive results. The GRK4γ was subcloned in the Sal I restriction site of the pAS2-1 vector, in frame with the GAL4DNA binding domain (GAL4-GRK4γ). The construct was checked by sequencing, while the expression of the fusion protein was analysed by western blot, using the anti GAL4 (0.1 µg/ml) monoclonal antibody (S. Cruz Biotechnology). Y190 yeast strain was transformed with GAL4-GRK4γ and in turn, 500 ml of this yeast culture (OD600nm = 0.25) was cotransformed with 100µg of human testis cDNA library fused to the GAL4 activator domain (in pGAD10 vector). The cotransformed yeast were plated on selective media and incubated for up to 10 days at 30°C. Control plates were transformed with GAL4DN-BD/murine p53 in pAS2-1 and GAL4AD/SV40 large T-antigen in pACT2. Unfortunately after β-galactosidase assay, the colonies turned blue only in the control plates.

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 tissues and cells were obtained as follows (Parruti, 1993b): 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 as in section 2.2.3.

2.2.2 Preparation of subcellular fractions

Subcellular fractionation was performed according to Garcia-Higuera, 1994a with minor modifications. Frozen bovine sperm were thawed and rapidly pelleted at 800 x g for 20 min. The pellet was resuspended in 4 volumes of CLB plus 250 mM sucrose and homogenised, on ice, with 10 strokes of a motorised Teflon pestle and all the subsequent steps were carried out at 4°C. After a low speed centrifugation at 800 x g for 10 min the pellet was resuspended in CLB, sonicated with 4 pulses of 15 sec and treated with 40 μg/ml of DNAse I for 1 h at 37°C (nuclear enriched fraction). The supernatant (post-nuclear preparation) was centrifuged at 3,000 x g for 10 min to obtain a plasma membrane pellet. Centrifugation of the supernatant at 10,000 x g for 20 min provided the crude mitochondrial pellet. The supernatant was centrifuged at 300,000 x g for 30 min to obtain the microsomal membranes (pellet) and the cytosol (supernatant).
2.2.3 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.

2.2.4 Western blot analysis

Three different anti-GRK4 antibodies were used. I-20 and K-20, available from Santa Cruz Biotechnology, were raised respectively against the epitope corresponding to amino acids 478-496 (GRK4δ) and 527-545 (GRK4α). They were reported to be specific for GRK4 and to recognise GRK4 murine, rat, bovine and human. Nab was generated (see section 2.2.9) from a GRK4α epitope including amino acids 84-146. Anti GRK5 and 6 were polyclonal antibodies (Santa Cruz Biotechnology) raised against epitopes in the C-terminus of the respective protein. They are able to recognise antigens from, rat, bovine and human origins. Anti-rat mGluR1 polyclonal antibody was generated by Upstate biotechnology with a 21 residue immunogenic peptide (KPNVTVASVILRDYKQSSSTL) corresponding to the C-terminus of mGluR1 plus an additional lysine at the N-terminus. This antibody cross-reacts with mGluR1 from human, rat, mouse, monkey, and chicken origin. Usually samples containing 100 μg of
protein prepared as in section 2.2.1 were suspended in Laemmli buffer (8 % SDS, 10 % glycerol, 5 % β-mercaptoethanol, 25 mM Tris-HCl, pH 6.5 and 0.003 % bromophenol blue), boiled for 5-10 min and electrophoresed on SDS polyacrylamide homogenous slab gel (PAGE). Proteins were electroblotted (Burnette, 1981) onto nitro-cellulose membranes with a tank transfer system. Efficiency of transfer was verified by Ponceau red staining of the blots and Coomassie Blue staining of the gel after transfer. The blot was saturated for 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 immunoblotting was performed using 0.1 μg/ml of the Santa Cruz antibodies, 2 μg/ml of NAb and 1 μg/ml of anti-mGluR1 and developed with alkaline phosphatase-conjugated goat anti-rabbit IgG (1:5,000) and 5-bromo-4-chloro-3-indolyl-phosphate and nitro-blue tetrazolium. Western blot analysis for the mGluR1 was performed as above except for protein preparation.

HEK293 cells were disrupted in 20 mM Tris-HCl pH 7.5, 0.32 M sucrose at 4°C with a dounce homogeniser equipped with a Teflon pestle (15 strokes). Intact or partially disrupted cells were eliminated by a low speed centrifugation (800 x g for 20 min at 4°C). The supernatant was centrifuged at 12,500 RPM (JA 20 rotor) for 30 min at 4°C and pellet was resuspended in the same buffer. After centrifugation the final pellet was solubilised in Laemmli sample buffer II (2.5 % SDS, 25 mM Tris-HCl pH 6.8, 25 mM DTT, 6.25 % glycerol) heated at 85°C for 10 min and 40 μg of proteins were used.

The resulting blots were scanned four times with an LKB Ultroscan XL Enhanced Laser Densitometer. All results were confirmed at least in two separate experiments.
2.2.5 Translocation assay

Crude cytosolic proteins (100 μg) from HEK293 transfected with the four GRK4 splice variants were incubated on ice in phosphorylation buffer (section 2.2.11) with 6 μM ROS and 50 μM of cold ATP and then incubated for 30 min under room light at 30°C. The samples were centrifuged (48,000 x g, 30 min, 4°C) to separate the ROS membranes from the soluble fraction (Chuang, 1996b). SDS sample buffer was added to the ROS pellets and supernatants, and Western blot analysis was performed, to detect which fraction contains GRK4. To test if CaM could regulate the GRK4 membrane binding in the reaction mix, 5 μM of CaM was included in the absence or presence of 1 mM Ca\(^{2+}\). The same procedure was followed to study the GRK5 binding to ROS membrane.

2.2.6 CaM Sepharose 4B binding assay

CaM Sepharose 4B or unconjugated Sepharose 4B gel was washed twice in 500 μl of water and equilibrated in binding buffer (20 mM Tris-HCl pH 7.5, 1 mM DTT, 100 mM NaCl, 0.1 % Lubrol PX). The gel was pelleted each time by centrifugation for a few seconds in a bench top microfuge. Each equilibrated gel pellet (60 μl) was resuspended in binding buffer containing various components in a total volume of 850 μl (Chuang, 1996b). In appropriate samples, GRK5 (100 nM) or crude cytosolic proteins (150 μg) from HEK293 transfected with the four GRK4 splice variants were used. Samples with calcium contained 1 mM Ca\(^{2+}\), as suggested by the manufacturers of CaM Sepharose 4B, while in samples without calcium, 6 mM EGTA was added to chelate any trace of calcium present. Samples were incubated for 1 h on a
rotor at 4°C, and unbound materials removed by two rounds of centrifugation and washes with 1 ml of ice-cold binding buffer. SDS sample buffer was added to the final gel pellet, which was heated to 95°C to detach gel-bound materials, and solubilized proteins electrophorised on a 10% polyacrylamide gel. Proteins were transferred to nitrocellulose paper and identified with appropriate antibodies as indicated in the figure legends.

2.2.7 Partial purification of recombinant GRK4 isoforms

To obtain a partially pure preparation of the different GRK4 isoforms, HEK293 cells were harvested 72 h post transfection in buffer H (20 mM HEPES, pH 7.2, 5 mM EDTA, and 20 mM NaCl). The cells were broken by polytron (4 stokes of 10 sec on ice) and a post nuclear fraction isolated by centrifugation at 800 x g at 4°C. Triton X-100 (1%) was added to the post nuclear preparation in buffer H and incubated for 1 h at 4°C. After centrifugation 1 h at 100,000 x g at 4°C, the supernatant was filtered through a 0.2 µm filter and loaded on a S-Sepharose ionic exchange column (Pharmacia Biotech. Inc.). Proteins were eluted with a NaCl gradient (ranging from 100 to 1,000 mM NaCl) in buffer H; kinase activity was recovered at ~ 500 mM NaCl and assayed as in section 2.2.11.

2.2.8 Homogeneity purification of GRK4α

About 200 μg of membranes from GRK4α transfected HEK293 or Baculo4 infected Sf9 were incubated for 1 h at 4°C in buffer 1 (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 1.5% N-Laurylsarkosine, 2% Triton X-100), buffer 2 (10 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 5 mM DTT, 2% digitonin), buffer 3 (10 mM Tris-
HCl, 150 mM NaCl, 1 % nonidet NP-40, 1 % sodium deoxycholate, 0.1% SDS), buffer 4 (10 mM Tris-HCl, 250 mM KCl, 5 mM EDTA, 2 % Triton X-100). The soluble fractions were separated from the residual membranes by centrifugation, and both fractions were assayed for GRK4 content and activity using Western blot analysis or a phosphorylation assay. 300 x 10^6 Sf9 cells infected with 1 x 10^9 Baculo4, or 100 x 10^6 of HEK293 cells transfected with GRK4α were harvested by centrifugation 72 hours later, resuspended in CLB containing 250 mM sucrose, and broken with a motorised glass/Teflon potter (10 stokes of 10 sec). A post nuclear supernatant was prepared and centrifuged at 50,000 x g for 30 min at 4°C. The membrane fraction was solubilised in buffer 4, diluted four times in buffer A (20 mM HEPES pH 7.2, 5 mM EDTA, 1 mM DTT) filtered through a 0.2 μm membrane, and loaded over a 5 ml S-Sepharose ionic exchange column equilibrated in the same buffer. The column was washed with 25 volumes of the same buffer and proteins eluted with a gradient ranging from 1 to 100 % of buffer B (20 mM HEPES pH 7.2, 1 mM DTT, 5 mM EDTA, 0.1 % Lubrol PX, 1 M NaCl). Fifty 3.5 ml fractions were collected and 100 μl of each were assayed by Western blot for the presence of GRK4. The positive fractions (~ 400 mM NaCl) were pooled, diluted 5 times in buffer B without NaCl and loaded on a 5 ml affigel heparin (Bio-rad) column (In few purifications this step was omitted without compromising the quality of the preparation). After washing the column with 25 column volumes of buffer B containing 100 mM NaCl, GRK4 was eluted using a linear gradient as for S-Sepharose column. The positive fractions (~ 600 mM NaCl) were pooled and diluted 6 times with buffer C (20 mM HEPES pH 7.2, 1 mM DTT, 0.1 % Lubrol PX, 1 mM CaCl₂), loaded on a 2 ml CaM Sepharose 4B column pre-equilibrated in the same buffer. The column was washed 6 times with buffer C containing 100 mM NaCl and eluted
with 6 column volumes of EB (20 mM HEPES pH 7.2, 1 mM DTT, 0.1 % Lubrol PX, 200 mM NaCl 1 mM EGTA). The fractions containing GRK4α were divided into small aliquots and stored at -80°C.

2.2.9 Generation of GRK4 antiserum

A fusion protein between 6-His and the N-terminal (amino acids 84 to 146) region of GRK4α was constructed by inserting the EcoR I-Rsa I GRK4 cDNA fragment into pBluescript II SK+ and subsequently was subcloned into pQE9 (Ab-pQE) using the restriction endonucleases Bam HI and Hind III. *E. coli* strain M15[pREP4] bearing the Ab-pQE was grown in LB plus ampicillin and kanamycin until the optical density (600 nm) was about 0.7-0.9. At this point the peptide was induced for 4 h at 37°C with 1 mM IPTG. The cells were harvested by centrifugation and the pellet lysed in 5 ml per gram of wet weight buffer G, (6 M Guanidinium-HCl, 0.1 M sodium phosphate, 10 mM Tris-HCl pH 8) for 1 h with stirring. The insoluble materials were discarded after centrifugation and the supernatant loaded onto a Ni-NTA-agarose (QUIAGEN INC.) column. The column was washed 10 times with buffer G, and 5 times with buffer U (8 M urea, 100 mM sodium phosphate, and 10 mM Tris-HCl pH 8). The column was also washed with buffer w (8 M urea 100 mM sodium phosphate, 10 mM Tris-HCl pH 6.3) until the optical density at 280 nm was less than 0.01. The protein was eluted with 10 ml of buffer E1 (8 M urea, 100 mM sodium phosphate, and 10 mM Tris-HCl pH 5.9) and 10 ml of buffer E2 (8 M urea, 100 mM sodium phosphate, and 10 mM Tris-HCl pH 4.5) collecting 2 ml fractions.

Ten μl of each fraction were analysed on SDS-PAGE and fractions containing the protein of interest were quantified as in section 2.2.3. One mg of the fusion protein, without further purification, was mixed
with an equal volume of complete Freund's adjuvant and used to immunise two New Zealand rabbits. They were boosted after 15 and 30 days with 500 μg of antigen containing the same volume of incomplete Freund's adjuvant. The rabbits were bled, and the presence and specificity of anti GRK4 antibodies was tested by western blot analysis. The antiserum reacted specifically with bacterial recombinant GRK4; in contrast, no reactivity of the rabbit antiserum was obtained before immunisation.

The polyclonal antibody was affinity purified using the antigenic peptide coupled to CNBr-activated Sepharose 4B (Fluka Chemie AG). The final concentration of the antibody was obtained by determining the absorbance of the eluate at 280 nm. Western blot and immunoelectron-microscopy analysis were performed using the affinity-purified antibody (NAb) at the concentrations indicated in section 2.2.4 and 2.4.1 respectively.

2.2.10 Preparation of urea treated ROS

ROS were prepared from about 50 retinas suspended in 50 ml of Tris-acetate buffer (65 mM NaCl, 2 mM MgCl₂, 10 mM Tris-acetate pH 7), plus 34 % w/w sucrose, shaken vigorously and centrifuged at 2,000 x g for 5 min. The supernatant, containing the ROS, was diluted with 2 volumes of Tris-acetate buffer (TAB), and centrifuged as above. The crude ROS pellet was resuspended in 30 ml of 0.77 M sucrose, 1 mM MgCl₂, in TAB, and further purified on a stepwise sucrose gradient. The interface between 0.84 and 1 M sucrose was collected, diluted 1:1 with TAB, and ROS membrane sedimented at 48,000 x g for 20 min. RK free ROS membranes were prepared by suspending the purified ROS in 50 mM Tris-HCl pH 8, 5 mM EDTA, 5 M urea (1 ml/retina), sonicating on ice (4 min maximum power), diluting with 2
volumes of 50 mM Tris-HCl pH 7.4, and centrifuging at 100,000 x g for 45 min. The pellet was washed four times with TAB, and resuspended at a protein concentration of 1-2 mg/ml in 50 mM Tris-HCl. All operations were carried out in the dark or under red dim light. Urea-treated ROS preparations consisted of 95% rhodopsin as assessed by Coomassie brilliant blue staining of PAGE and showed negligible endogenous RK activity.

2.2.11 Phosphorylation assays

Rhodopsin was used in the present study to evaluate the GRK4 enzymatic activity. One μg of partially pure or 0.1 μg of pure GRK4 were added to a reaction mixture containing 300 pmol urea-treated ROS, 100 μM [γ-32P]-ATP (1-2 cpm/fmol), 20 mM Tris-HCl, 8 mM MgCl2, 2 mM EDTA, 5 mM NaF, at pH 7.4 and a total volume of 60-150 μl. In appropriate samples, heparin, polylysine, calcium, CaM or CaM inhibitor were added, as indicated in the figure legends. The reaction was carried out at 30°C in the presence (or absence) of light for 30 min. The reaction was stopped by the addition of the same volume of SDS sample buffer and proteins electrophoresed on 10% SDS-PAGE. The gel was stained with Coomassie brilliant Blue, dried, and subjected to autoradiography.

For a quantitative measurement of GRK4 activity, two methods were used: (A) rhodopsin bands (Mr ~35 Kd) identified by Coomassie blue staining, were isolated and counted for 32P radioactivity; (B) measurement of relative density of rhodopsin bands imprinted on the autoradiographic films by LKB Ultroscan XL Enhanced Laser Densitometer.
2.3 Cell culture and transfection

2.3.1 Culture and transfection of HEK293 cells

Cells were cultured in DMEM (GIBCO) 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 dish, at the density of 50,000 cells/cm². The cells were transfected using the calcium phosphate method (Graham, 1973) where a HEPES-buffered solution is used to form a calcium phosphate precipitate that is layered directly onto the cells. The precipitate containing calcium phosphate and DNA is 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 DNA (5µg/10⁶ cells). The cells were incubated 16 h with the precipitate under standard growth conditions. Then the precipitate was washed out and the cells fed with complete medium for further 48 h. The cDNAs used for transfections were from the sources listed below. The four human GRK4s were reconstructed by standard molecular biology techniques described in sections 2.1, subcloned into the eukaryotic expression vector pCMV and used for transfection. GRK5 and GRK6 already in eukaryotic expression vectors were provided by Dr. Lefkowitz (Duke university). The mGluR1a and mGluR1b in the pcDNA1 vector were kindly provided by Dr. Corsi (Glaxo-Wellcome).

2.3.2 Measurements of inositol phosphate hydrolysis

Inositol phosphate (IP) hydrolysis was measured in HEK293 cells that had been plated in 6 well petri dishes and transfected with
mGluR1 alone or in combination with the testing kinase. One day post-transfection, the cells were washed with PBS and incubated for 18 h with DMEM/Glutamax-1 (GIBCO), then washed and incubated over night with MEM/Glutamax-1 containing 3 μCi/well of myo-[3H]inositol (Amersham). On the third day, cells were washed twice and incubated for 1-2 h at 37°C in 1 ml of HEPES buffered saline (146 mM NaCl, 4.2 mM KCl, 0.5 mM MgCl₂, 0.1 % glucose, 20 mM HEPES pH 7.4); washed again twice with HEPES buffered saline and pre-incubated for 15' in the same buffer containing 10 mM LiCl, 1.8 U/ml glutamic pyruvic transaminase (GPT), 2 mM Na-pyruvate, to avoid the possible action of glutamate released from the cells (Carruthers, 1997). The agonist at the concentrations indicated in the figure legends, was added for 30 minutes. The reaction was stopped by replacing the incubation medium with 1 ml of ice cold perchloric acid (5%). The cells were scraped, transferred into a microfuge tube and the pH was adjusted to 7.5 with KOH. The cell debris was removed by centrifugation (5 min at room temperature in a microfuge). The supernatant containing the inositol phosphates was applied to an ion exchange chromatography column of dowex AG1-X8 (formiate form) (200-400 mesh, 350 μl bed volume). The Column was washed with 10 column volumes of water, 2 column volumes of cold 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) The elute was mixed with scintillation solution, subjected to scintillation counting and the results expressed as counts/well. Total radioactivity remaining in the membrane fraction was counted after solubilisation in 10 % Triton X-100, 0.1 M NaOH and used to standardise the results where necessary (Carruthers, 1997). Data are expressed as percentage of the mock transfected cells or percentage of the maximal stimulation
as indicated. All experiments are presented as the average of
duplicate or triplicate determinations repeated at least three times.

2.3.3 Functional expression of human β2AR in Sf9 cells

In these experiments the baculoviral constructs bearing the cDNA
for human β2AR, GRK2, and GRK5 were kindly provided by Dr.
Lefkowitz (Pei, 1994; Premont, 1994), while GRK4 was obtained as in
section 2.1.6. Sf9 cells (1 x 10^6/well) seeded in 24 well plates were
infected with the β2AR alone or in combination with one of the
GRKs, keeping the multiplicity of infection to 1. Two days post
infection, control and infected cells were washed twice with pre­
warmed cAMP incubation buffer (Hanks’ balanced salt solution
containing 0.4 % BSA, 10 mM HEPES, and 0.5 mM 3-isobutyl-1-
methylxanthine, pH 7.3) and treated for 30 min at 37°C with 1 μM
isoproterenol in the same buffer. The reaction was stopped and the
incubation buffer replaced with 300 µl of cold ethanol. The
intracellular cAMP content was measured using a commercially
available RIA kit (Amersham). Data are expressed as picomoles of
cAMP/well.

Experiments were performed at least three times in duplicate.
Possible variation of β2AR expression, between the receptor alone
and coinfection with the kinase was ruled out by ligand binding
experiments. Briefly Sf9 membranes were resuspended in binding
buffer (75 mM Tris-HCl, 12.5 mM MgCl2, 1 mM EGTA, 2 nM ³H-CGP
pH 7.4) with or without 1µM cold CGP as competing ligand and
incubated 1 h at 37°C. Membranes were isolated by vacuum filtration,
through Whatman glass fibre filters, and subjected to scintillation
counting. Usually β2AR expression levels were about 300-600
fmol/mg of protein (~2,000-4,000 sites per cell).
2.3.4 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.

2.3.5 Purkinje cells primary culture

Cerebellar neurones were prepared from Sprague-Dawley rats as described previously by Yuzaki, 1992, with minor modifications to obtain a Purkinje cell rich culture. Seven days old pups were sacrificed by cervical dislocation and the cerebella excised and minced with a scalpel. The cerebellar cells were disgregated with 0.025 % trypsin and 0.01 % DNaseI in Krebs Ringer plus 0.03 % MgSO$_4$, 0.3 % BSA for 15 min at 37°C. The cells were washed with the same buffer containing 40 μg/ml of trypsin inhibitor and 0.01 % DNaseI and dissociated by repeated passage through a fine-tipped pipette. The cell suspension was centrifuged at 400 x g for 2 min, and cells resuspended carefully in 2 ml of the same buffer. After 30-45 min, the Purkinje cells are enriched from granules by gravity. The upper part of the suspension (granules) was removed very carefully and the sediment (Purkinje cells) was rinsed with culture medium. Recovered cells were plated at a density of 20-25 x 10$^4$ cell/cm$^2$ onto poly-L-lysine coated chamber slides in serum free defined medium (Fisher 1982): Eagle's medium supplemented with 1 mg/ml BSA, 10 μg/ml insulin, 0.1 nM L-thyroxin, 0.1 mg/ml transferrin 1 μg/ml aprotinin, 30 nM selenium, 100 μg/ml streptomycin and 100 U/ml penicillin. The cultures were maintained in a humidified atmosphere of 5 % CO2 in air at 37°C. This neuronal culture consisted of about 2-
3% of Purkinje cells; they survive for 3-4 weeks and maintain constant characteristics.

2.4 Morphometrical analysis

2.4.1 Immuno-electron-microscopy

For electron microscopy analysis, biopsies from human testis containing healthy spermatozoa were processed as in Baccetti, 1994. Tissue or cells were fixed for 1 h at 4°C in 3% paraformaldehyde, 0.1% glutaraldehyde in PBS, rinsed overnight in PBS plus 5% sucrose and incubated for 30 min in PBS containing 50 mM NH$_4$Cl. The pellet obtained by centrifugation of spermatozoa samples was embedded in 3% agar in PBS. All specimens were dehydrated and embedded in Lowicryl K4M (Balzers, Furstentum, Liechtenstein) at -35°C according to Carlemalm, 1990. Ultra thin sections were cut from the embedded block using a Supernova Reichert-Jung (Wien, Austria) ultramicrotome and collected on formvar-coated nickel grids. After rehydration, the grids were treated for 30 min with 5% normal goat serum (NGS) in TBS containing 1% BSA and then incubated overnight at 4°C in the polyclonal antibody I-20 anti-GRK4 diluted 1:50 in TBS, 1% NGS, 0.1% BSA. The grids were washed by floating twice in TBS, 0.1% BSA, 0.05% Tween-20 and once in TBS, 0.1% BSA and then incubated, for 1 h at room temperature, respectively in goat anti-rabbit IgG, 10 nm colloidal gold conjugated (Biocell, Cardiff, UK), diluted in TBS, 1% NGS, 0.1% BSA. After incubation, the samples were washed consecutively in TBS, 0.1% BSA, 0.05% Tween-20; TBS, 0.05% Tween-20; TBS; distilled water, and then stained with uranyl acetate. The observation was carried out at the transmission electron
2.4.2 Whole-mount in situ hybridisation

Thirty day old rats were anaesthetised with Nembutal (40 mg/Kg body weight, intraperitoneally), perfused transcardiacaclly first with PBS and then 4 % paraformaldehyde in PBS. The brain was excised quickly from the skull and immersed overnight in the same fixative. After 3 washes in PBS plus 0.1 % tween-20 (PTW) the brain was manually sectioned to obtain slices of about 1 mm in thickness The slices were washed again and digested for 30 min at room temperature in 10 μg/ml of Proteinase K. Digestion was blocked by incubation with 2 mg/ml glycine in PTW. The slices were rinsed in PTW, and acetylated by incubation in 0.25 % acetic anhydride in 100 mM triethanolamine, 150 mM NaCl pH 8 and then washed again in PTW. After a new fixation with paraformaldehyde, the sections were prehybridised overnight at 60 °C, in 50 % formamide, 5 X SSC pH 4.5, 1 mg/ml of total yeast RNA, 100 μg/ml heparin, 1 X Denhardt (0.02 % Ficoll 400, 0.02 % poly-vinyl-pyrrolidone, 0.02% BSA), 0.1 % Tween-20, 0.1 % CHAPS, 5 mM EDTA). The hybridisation was carried out 18 h at 60°C in prehybridisation solution containing the specific ribobrobe (RP1, RP2 or RP3), 1μg/ml concentrated. The sections were then washed at high stringency (0.2 X SSC 60°C), saturated with 2 % Boehringer Mannheim Blocking Reagent, 20 % sheep serum in Maleic Acid Buffer (MAB), (100 mM Maleic acid, 150 mM NaCl pH 7.5) and incubated overnight at 4°C with alkaline phosphatase-conjugated goat anti-digoxigenin IgG 1:2,000 in the same solution. The day after the slices were washed abundantly with MAB and developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitro-blue tetrazolium. The
staining was fixed with 100 mM MOPS pH 7.4, 2 mM EGTA, 1 mM MgSO4, 3.7 % formaldehyde, dehydrated with ethanol and photographed using a stereo microscope equipped with a Yashica camera.

2.4.3 Immunohistochemistry

Under deep anaesthesia with Nembutal (40 mg/Kg body weight, intraperitoneally), rats were perfused transcardiacally with PBS and then with formaline in PBS. The brain was immersed overnight in the same fixative, embedded in paraffin, and serial sections (5µm) cut and used for immunohistochemistry. According to Salvatore, 1999, sections were pre-treated with 0.3 % H2O2 in ethanol at 4°C for 15 min to inhibit endogenous peroxidase activity and washed in PBS containing 0.05 % Tween-20. Sections were incubated with 50 % NGS for 10 min at 37°C to reduce non specific immunostaining and then with the specific antibody (anti-GRK4, K-20 or I-20, or anti GRK5 or anti GRK6 all were used at 1µg/ml) in PBS 1 % NGS over night at 4°C. Detection of immunoreactivity was accomplished using a vectastain elite ABC kit (Vector Laboratories) Sections were incubated with anti rabbit IgG biotin-conjugated (1:100) in 50 % NGS for 30 min at room temperature and finally incubated with the ABC reagent for 30 min at room temperature. After each incubation step, sections were carefully washed with PBS. The immunolocalisation was visualised using 0.04 % of 3,5'-diaminobenzidine (DAB), 0.33 % H2O2 in PBS; tissues were counterstained with methylene blue. Negative control sections were processed in the same way using the primary antibody preadsorbed with an excess of antigenic peptide or 1 % NGS instead of the primary antibody. Photomicrographs were taken using Zeiss Axiophot microscope (Carl Zeiss Inc.).
2.4.4 Immunofluorescence

Fertile human spermatozoa were washed twice in PBS. After centrifugation at 1,000 g for 20 min, the pellets were resuspended in PBS and smeared on glass slides. The samples were air-dried, fixed for 10 min in methanol at -20°C, extracted for 5 sec in acetone at -20°C, redried, washed three times in PBS and saturated with PBS, 5% NGS, 1% BSA for 20 min. Spermatozoa were incubated overnight at 4°C in polyclonal antibody I-20, diluted at 2.5 μg/ml in PBS, 1% NGS, 0.1% BSA. All the slides were washed three times in PBS, 0.1% BSA and incubated in FITC goat anti-rabbit IgG antibody (Calbiochem, La Jolla, CA), 1:100 in PBS, 1% NGS, 0.1% BSA. Finally, the glass slides were washed three times in PBS, mounted in PBS, glycerol, 1:10, containing 5% propyl-gallate and observed in a light microscope Leitz Aristoplan (E. Leitz, Rockleigh, NJ) equipped with fluorescence optics.

Twenty day old Purkinje cell primary cultures were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature. The autofluorescence was quenched by incubation for 30 min in 50 mM NH₄Cl, 50 mM glycerol in PBS and non specific interactions were blocked by treatment with blocking solution (0.005% saponin, 0.2% BSA in PBS) for 1 h at room temperature. Cells were incubated (Salvatore 1999), overnight at 4°C with K-20 (1μg/ml) polyclonal antibody in blocking solution. The chamber slides were carefully washed with PBS and incubated with blocking solution containing Alexa-488 anti rabbit IgG (Molecular Probes) (1:400) for 1 h in the dark. The cells were washed in PBS and incubated for 1 h in blocking solution containing 3 μg/ml of Alexa-594 labelled anti mGluR1 (anti mGluR1 was conjugated by alexa-594 protein labelling kit (Molecular
probes) exactly according the manufacturer instruction). When cells were labelled with monoclonal anti calbindin-D$_{28k}$ (Sigma), (1:200); the alexa-488 anti mouse IgG was used as secondary antibody. After each incubation step, cells were carefully washed with PBS (6 times/3 min each). After immunostaining the coverslips were mounted on slides with Mowiol 4-88, and observed with a Zeiss Axiophot (Carl Zeiss Inc.). Colocalisation of the mGluR1 and GRK4 antigens was assessed by INSIGHT PLUS laser scanning confocal microscope system (Meridian, Oketos) equipped with an Olympus IMT-2 inverted microscope. Ten groups of optical Z-section serial slices from each experiment were taken with 0.5 µM Z-steps from the top to the bottom of the specimen. Fluorescent images were recorded using a Dage CCD camera, and stored directly on computer. Merging of both immunofluorescence generated the colocalisation maps by colour.

### 2.5 Miscellaneous

#### 2.5.1 Human, bovine, rat tissues and cell sources

Macroscopically normal human tissues, obtained from surgically excised samples, were rapidly frozen in liquid nitrogen. Human testis from biopsies and spermatozoa from volunteers were fixed as described in sections 2.4.1 and 2.4.4. Heart samples were myocardial specimens from hearts explanted for idiopathic congestive cardiomyopathy; lung and liver and breast samples were taken 5-8 cm away from the periphery of tumour tissue. Skeletal muscle and adipose tissue specimens were from rectum abdomens and subcutaneous soft tissues of laparotomized patients. Bovine tissues were collected in the local slaughterhouse. Bovine spermatozoa were
purchased from SemenItaly S.r.l. Montesilvano (PE, Italy). Rat tissues were collected from the internal animal care unit. 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. The mononuclear fraction was washed twice with PBS and the pellet lysed respectively in guanidine thiocyanate or CLB in the case of Northern blot or Western blot analysis. The mono-nuclear leukocyte (MNL) preparation yielded a heterogeneous cell population that contained about 80 % of lymphocytes, 15-20 % of monocytes, and less that 2 % granulocytes. In some cases the leukocytes 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 (1600 RPM x 10 min a 4°C) and the granulocyte pellet washed with PBS and used as needed.

2.5.2 Material sources

Deoxynucleotides used for PCR and Amplitaq DNA polymerase were from Perkin Elmer; M-MLV reverse transcriptase, T7 DNA polymerase, other modifying enzymes, and 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. ³⁵S and ³²P labelled dNTPs and the random priming kit were purchased from Amersham.
Culture media, FCS, guanidine thiocyanate were from GIBCO; heparin (from porcine intestinal mucous), proteinase K, and isoproterenol were from Sigma. All gel electrophoresis materials (but NuSieve agarose, FMC) were purchased from Bio-Rad.
RESULTS

CHAPTER 3

CLONING AND FUNCTIONAL PROPERTIES OF NEW GRK4 SPLICE VARIANTS

3.1 Introduction

GRKs are responsible for homologous desensitisation of GPCRs (Pitcher, 1998a; Haga, 1994b). The mechanism of action of these kinases has been extensively investigated for GRK1, GRK2 and GRK3. Receptor activation triggers translocation of GRKs from cytosol to plasma membrane (Strasser, 1986), where they phosphorylate agonist-occupied receptors. GRK5 and GRK6 likely regulate receptor substrates in a similar manner. GRK4 presents some peculiar characteristics with respect to other GRKs. GRK4 was the only GRK for which splice variants had been identified (Sallese, 1994). Unlike GRK2, GRK3, GRK5 and GRK6 which are widely distributed in different tissues and cells (Chuang, 1992; Parruti, 1993; Kunapuli, 1993; Benovic, 1993; Benovic, 1991), the expression of GRK4 is extremely restricted since substantial amounts of GRK4 mRNA have been found only in the testis (Ambrose, 1992; Sallese, 1994). This unique site of expression makes GRK4 similar to GRK1, which is expressed only in the retina where it regulates phototransduction. Based on these observations it was suggested that GRK4 may have strong substrate selectivity and that the receptor substrate, which is as yet unknown, may be located in the testis. Despite these properties which make GRK4 a unique member within the GRK family, this receptor kinase has been poorly investigated. All the
studies reported so far have been performed in heterologous expression systems. Premont, 1996, showed that recombinant GRK4, expressed in COS7 cells, was palmitoylated, had desensitising activity when LH/CG receptor was cotransfected, and was able to phosphorylate in vitro purified $\beta_2$AR when partially purified. Moreover the complete genomic structure of human GRK4 was reported.

The aims of this project were to identify new splice variants of GRK4, to define the exact testicular cell in which GRK4 is expressed, to explore the intracellular distribution and membrane targeting mechanism, to identify the physiological substrate for GRK4 and to elucidate the role of the GRK4 subfamily members in receptor desensitisation.

3.2 Identification of new GRK4 splice variants

3.2.1 Cloning of human isoforms

Preliminary analysis of the sequence alignment of GRK4 with the closest members of this kinase subfamily (GRK5 and GRK6), revealed that the original GRK4 sequence (Ambrose, 1992) possessed two major gaps one located near the N-terminus and one located near the C-terminus (Fig. 3.1). In previous work, I documented the existence of two isoforms of GRK4, which differed by a 96 bp/32 amino acid fragment filling the apparent gap in the N-terminal region (Sallese, 1994). These two isoforms have been named GRK4A and GRK4B according to the presence or the absence of the 32 amino acid fragment, respectively (Sallese, 1994). On the basis of previous experience, it seemed possible that the gap near the C-terminus could be due to the existence of additional splice variants. A PCR
Figure 3.1 Alignment of the human GRK4δ (IT11) protein with GRK5 and GRK6

The ClustalW program from the computational service of EMBL was used to align the human GRK4δ (Ambrose, 1992) with human GRK5 (Kunapuli, 1993) and GRK6 (Benovic, 1993). Amino acid residues that are identical in all the aligned GRKs are denoted by an asterisk (*), conserved substitutions as a colon (:), and semi-conserved substitutions as a point (.). Dashes indicate gaps introduced to obtain optimum alignment.
Figure 3.2 Deduced amino acid sequence of two human GRK4 isoforms

**a:** DNA sequence of the PCR product obtained from human testis RNA. The arrows indicate the forward (F1) and reverse (R1) primers used for the amplification. The alternatively spliced sequence is boxed. **b:** Schematic representation of GRK4 showing the position where the insert is located within the protein. The catalytic domain is indicated as an open box. The new insert (amino acids 515 to 562), and the N-terminal spliced region, are shown as filled boxes.
Figure 3.3 PCR analysis of human testis GRK4

One µg of total RNA from human testis was reverse transcribed with random hexamers. PCR using 0.8 µg of each primer F1-R1 (a), or F0-R0 (b) were carried out, and 20 µl of PCR analysed by electrophoresis in 3% NuSieve 3:1 agarose gel. The two amplification products (528 and 393 Bp. long in a, whereas 294 and 198 Bp. long in b) are indicated.
the shorter form (Fig. 3.3a). The sequences encompassing the site of splicing in the N-terminal region of GRK4 was also amplified from human testis RNA using the oligos F0-R0 (for details see section 2.1.4.2). Two PCR bands were generated, corresponding to the two splice variants identified earlier, and the longer product was \(~2\) fold more abundant (Fig. 3.3b) than the shorter one. This is different from my previous finding on human brain RNA, where the shorter isoform GRK4B was much more abundant. The identification of two alternatively spliced sequences at the C-terminus of GRK4 indicates the existence of four splice variants of this kinase that, according to Premont, 1996, have been named GRK4a, GRK4b, GRK4γ and GRK4δ (Fig. 3.4). GRK4δ was previously known as GRK4B (Sallese, 1994) or IT11 (Ambrose, 1992) while GRK4γ corresponds to GRK4A (Sallese, 1994).

### 3.2.2 Genomic Southern blot analysis of human GRK4

Whether these isoforms were generated by alternative mRNA splicing of a single gene or represented transcripts of different genes was investigated. The F1-R1 cDNA fragment of GRK4δ, which is able to recognise all the isoforms, was used to probe blots of human genomic DNA digested with different restriction endonucleases. This analysis revealed a single restriction fragment (Fig. 3.5), even after long term (7 days) exposure of filters, in agreement with the existence of a single gene for GRK4 in the human genome and suggesting that the four isoforms were generated by alternative mRNA splicings. During the preparation of this work, Premont, 1996, reported the identification of the same splice variants and demonstrated that they are generated by the alternative splicing of
Figure 3.4 Schematic representation of four splice variants of human GRK4

The sequences that are alternatively spliced in the N-terminal (amino acids 18-49) and in the C-terminal (amino acids 515-562) regions are in filled boxes. The open box (cd) represents the catalytic domain. The dashed box represents the putative RGS domain. The circle (CaM) indicates the CaM binding domains. The catalytic domain of all GRKs differs from other protein kinases for the presence of three invariant amino acids DLG. The spliced sequences have been deposited in the Gene Bank/EMBL Data Bank (accession numbers X98118; X97879; X97880; X97881).
Figure 3.5 Southern blot analysis of human genomic DNA

Large molecular size DNA (10 µg) was treated with the indicated restriction endonucleases, fractionated by a 0.8% agarose gel, transferred to a nylon membrane and probed as described in the section 2.1.5.3. Washed filters were exposed at -80°C for 4 days. Molecular size standards in Kb are shown.
exon 15 in the C-terminal region and exon 2 in the N-terminal region since he cloned the human gene for GRK4.

3.2.3 Cloning of different isoforms of rat GRK4

The work from Premont, 1995, did not answer the question of whether all four putative mRNA isoforms were synthesised in vivo. Amplification of a single portion of the GRK4 cDNA does not reveal the relationship between the N- and the C-terminal splice variants. Amplification with two oligos encompassing all the possible splice variants would be representative of the natural ratio of the different transcripts (Siebert, 1992). However the amplification of human testis GRK4 with two oligos that encompass all the coding region showed that the expression level among the various isoforms is very different, therefore it is hard to detect the low abundant isoforms. To overcome this problem the amplification reactions were carried out for many cycles. In these conditions the reactions generated several bands and only few of them were at the expected size. Taking into account the presence of GRK4 plasmid in the laboratory (which represent a potential source of contamination) it was extremely hard to decide which bands reflected isoforms really expressed in cells and which were generated by potential contamination (Scherczinger, 1999), or non specific amplifications.

To address this problem, the rat GRK4 cDNA was studied. Four different oligos shown in the Fig. 3.5 (the rat GRK4 sequence was obtained from gene bank, accession number X97568) were synthesised and used in a single step PCR (Myers, 1991) (see section 2.1.4.1). Either the region that encompasses the N-terminal (oligo F2-R2 Fig. 3.6 and 3.7c) human splice variants, or the region
Figure 3.6 Deduced amino acid sequence of rat GRK4 isoforms

Deduced amino acid sequence of rat GRK4 (RAT GRK4) was aligned with the human GRK4α (Hum GRK4). Asterisks indicate conserved residues. The alternatively spliced sequences are boxed with solid line in the full length clone. The dashed boxes indicate the sequence found to be alternatively spliced in the partial clones N2 or N3. Small arrows numbered (2-16) indicates the exons junctions in the human GRK4. The full length cDNA of rat GRK4 (RAT GRK4) was obtained from the PCR product F2-R3, whereas partial clones (N2, N3) were obtained from the PCR product F2-R2. The horizontal arrows indicate the positions of the primers on the rat GRK4 sequence.
Figure 3.7 PCR analysis of rat GRK4 mRNA

a. One µg of total testis RNA was reverse-transcribed with primers R2 (N-ter), or R3 (F-length, and C-ter), and amplified using 0.8 µg of primers F2-R2 (N-ter), F2-R3 (F-length), and F3-R3 (C-ter). Ten µl of the PCR reactions analysed by electrophoresis in 3 % NuSieve 3:1 agarose gels are shown. Predicted sizes of the PCR products are indicated as Bp. 

b: One µg of total cerebellar RNA was amplified as in a (N-ter) and 10 µl of the PCR product is shown. Predicted sizes of the PCR products are indicated as Bp. 

c: Diagram showing the rat GRK4 splice variants. Arrows denote the positions and directions of primers along the rat GRK4 cDNA; the denominations are indicated. The sequences that are alternatively spliced are in dashed boxes. The open box (cd) represents the catalytic domain.
encompassing the C-terminal region (oligo F3-R3 Fig. 3.6 and 3.7c) and the entire cDNA (oligo F2-R3 Fig. 3.6 and 3.7c) was amplified from rat testis. Gel electrophoresis of the PCR products showed i) three main bands in the N-terminal region (Fig. 3.7a), with the longer product being 10 times more abundant than the shorter ones, ii) two main bands in the C-terminal amplification (Fig. 3.7a), with the longer product being 5 times more abundant, and iii) a smear at the expected size for the entire cDNA amplification (Fig. 3.7a). Two nested PCR (with the N- oligos (F2-R2) and the C- oligos (F3-R3) were performed on the full length amplification to assess if this PCR product contained GRK4. Electrophoretic analysis of the nested PCR showed three bands in the N- amplification and two bands in the C- amplifications, corresponding to the bands previously shown for the original N- and C-terminal amplification products (Fig. 3.7a).

The smear present in the full length amplification product was purified from agarose gel electrophoresis and cloned in the TOPO vector using the TOPO TA cloning kit (Invitrogen). Two clones were rescued and fully sequenced (C1 and C2). The C1 clone was colinear with the human GRK4α isoform (Fig. 3.6) while the clone C2 had two gaps that correspond exactly to the lack of the exon number 6 and 14 of the human genomic structure (Fig. 3.6). These data are consistent with the C-terminal PCR that showed two bands differing of about 138 bp, though it does not explain the three bands obtained from the N-terminal PCR. To assess the nature of the N-terminal amplification, these products were purified from agarose gel electrophoresis, cloned in the TOPO vector and three different clones (N1, N2, N3) were analysed. The N1 sequence was collinear with the human GRK4α, the N2 was identical to the N-terminal region of the clone C2 (lacks the exons 6) while the clone N3 was new and presented a large gap.
corresponding to the lack of the exon 6 and 7 (human genomic structure) Fig.3.6 and 3.7c.

To analyse if the rat GRK4 shows different patterns isoform in various tissues, the cerebellar RNA was amplified. PCR of the N-terminal region using the oligos F2-R2 generated 5 amplification products (indicated with 1, 2, 3, 4, 5 in Fig. 3.7b), three of them corresponding to those previously amplified from the testis (1, 2, 3) plus two (4, 5). Imaging analysis of the DNA bands visualised by UV stimulated ethidium bromide, showed that: the product 1 was the most abundant, about three times of products 3, 4 or 5 that were similar among them. The product 2 was the less abundant, representing about half of band 3. These results indicates that GRK4 could be present even more splice variants depending on the species and the expression site. A complete structure-function correlation of these kinases is a matter for further investigation.

3.3 Expression and characterisation of GRK4 kinase activity

To study the GRK4 structure-function relationship, the four putative GRK4 cDNAs were reconstructed, since the PCR cloning of the different splice variants generated only small pieces of the entire cDNAs. GRK4δ also called IT11A a kind gift of Gusella (Ambrose, 1992) was digested with the Nar I restriction enzyme and cloned in the compatible Cla I restriction sites of the eukaryotic expression vector pCMV5. GRK4γ was obtained by inserting a fragment from the long N-terminal splice variant in the GRK4δ through several cut and paste steps. The GRK4α was generated by subcloning the Nco I-EcoRV fragment from the long C-terminal splice variant into the GRK4γ. The GRK4β variant was obtained by subcloning a fragment from the short N-terminal variant into the GRK4α construct. Finally all the
isoforms were subcloned in the pCMV5 vector. All the splice variants were transformed in XL1 blue bacterial strain and plasmid DNA was prepared by the caesium chloride gradient.

To assess if the four cDNAs can produce recombinant GRK4 they were transfected (10 µg/100 mm dish), using the calcium phosphate method (Graham, 1973), into HEK293 cells and the protein expression levels were determined by immunoblot (Fig. 3.8). Despite the lipid modification (Premont, 1996; Premont, 1995), significant amount (~50% of total) of the immunoreactivity was recovered in the cytosolic fraction (data not shown).

Several studies have demonstrated that rhodopsin contained in the ROS of bovine retina can be phosphorylated by GRKs in a light-dependent manner, and that it can be used to investigate GRK activity quantitatively (Sohlemann, 1993; Freedman, 1996; Kunapuli, 1994b; Benovic, 1993). Thus, Rhodopsin was used in the present study to evaluate the GRK4 enzymatic activity. Different amounts (from 1 to 50 µg) of a crude cytosolic preparation from GRK4-transfected HEK293 were used to phosphorylate ROS, but the effect was negligible as compared to untransfected cells. Even when GRK4 transfected cells were treated for 24 h with 10 mM of sodium butyrate (Russo, 1997; Virlon, 1998), (a compound which is able to lower the GRK independent ROS phosphorylation) the ROS phosphorylation was unsatisfactory (Fig. 3.9). To obtain an higher specific activity at least 20 petri dishes (150 mm) were transfected with each one of the GRK4 isoforms. 72 hours later, the transfected cells were harvested, and GRK4 was extracted with Triton X-100 and partially purified by one step ionic exchange chromatography with a S-Sepharose column, eluting the phosphorylating activity with increasing NaCl concentration (Loudon, 1994). This purification step enriched the kinase activity by about tenfold in the fraction eluted between 450 to
Figure 3.8 Expression of GRK4 splice variants (α, β, γ and δ) in HEK293 cells

Membrane proteins (100 μg) from HEK293 transfected with the four GRK4 isoforms were blotted, and immunoreactivity was determined using the K-20 (isoforms α and β) and I-20 (isoforms γ and δ) polyclonal antibodies. Mr standards in kilodaltons are indicated.
Figure 3.9 ROS phosphorylation by cytosolic GRK4

Cytosolic proteins from GRK4α transfected HEK293 were used to phosphorylate 300 pmol urea-treated ROS. Auto, indicate the residual rhodopsin kinase activity as a background. The phosphorylation activity of 1 and 5 μg of GRK4α or vector (vec) transfected HEK293 is shown. The arrow indicates bands of phosphorylated rhodopsin (Opsin) as revealed by autoradiography after PAGE. Each experiment was repeated two times.
600 mM of NaCl. Using this partially purified preparation a robust phosphorylation of rhodopsin was obtained by GRK4α while GRK4β, GRK4γ and GRK4δ did not phosphorylate ROS, as compared to a similar preparation from HEK293 cells transfected with the vector alone (Fig. 3.10). Phosphorylation of rhodopsin by GRK4α was agonist (light)-dependent with an effect similar to that of GRK2 and GRK5 taken as positive controls (Fig. 3.11). The phosphorylation of rhodopsin by GRK4α was inhibited by heparin (Fig. 3.11), a known selective inhibitor of GRKs (Benovic, 1989), with an IC₅₀ of 770 ng/ml. In addition GRK4 activity was enhanced by polyanions such as poly-L-lysine (Fig. 3.11) as shown previously for other members of the GRK4 subfamily (Loudon, 1994; kunapuli, 1994b).

3.4 In vitro regulation of GRK4 activity

3.4.1 Homogeneity purification of GRK4α

To further characterise the functional properties of GRK4, proteins were expressed using the baculovirus expression system. The baculovirus expression system was chosen because it usually gives higher amounts of heterologous gene expression, as compared to other eukaryotic expression systems. In many cases the recombinant proteins are soluble and easily recovered from infected cells, at a late stage of infection, when the host protein synthesis is diminished (Carstens, 1979). In addition, baculoviruses have a restricted host range, which is limited to specific invertebrate species (Groner, 1986); hence they are safe to use, since they are non-infectious for vertebrates (Carbonell, 1987). To generate recombinant baculovirus, (Luckow, 1993) site specific transposition of an expression cassette into a baculovirus shuttle vector (bacmid), was carried out in E. coli.
Figure 3.10 Phosphorylation of rhodopsin by partially purified GRK4

The four GRK4 isoforms (α, β, γ and δ) prepared as in section 2.2.7 and a similar preparation of HEK293 transfected with the vector alone (Vec) were assayed for their ability to phosphorylate ROS. The arrow indicates bands of phosphorylated rhodopsin (Opsin) as revealed by autoradiography after PAGE. Each experiment was repeated at least three times. The amount of the four isoforms used in the experiment was similar as confirmed by immunoblot in b. b: Cytosolic proteins (100 μg) from HEK293 transfected with the four GRK4 isoforms were blotted and immunoreactivity was determined using the common antibody NAb. Mr standards in kilodaltons are indicated.
Figure 3.11 Characterisation of GRK4α kinase activity

**a:** rhodopsin phosphorylation by partially purified GRK4α, and purified GRK5 and GRK2 was light dependent and inhibited by heparin. Heparin concentrations were 10 µg/ml for GRK4α, GRK2 and 100 µg/ml for GRK5.  

**b:** inhibition of GRK4α kinase activity by different doses of heparin. 

**c:** activation of GRK4α kinase activity by coincubation with 2 µM poly-L-lysine. The arrows indicate bands of phosphorylated rhodopsin (Opsin) as revealed by autoradiography after PAGE. Each experiment was repeated two-three times.
The bacmid contained the "low-copy number mini-F replicon", a kanamycin resistance marker, and a segment of DNA encoding the LacZα peptide from pUC-based cloning vector. A short segment containing the attachment sites for the bacterial transposon Tn7 was inserted into the N-terminus of the LacZα gene so that it did not disrupt the reading frame of the LacZ peptide. Recombinant bacmids are constructed by transposing a mini-Tn7, element from a donor plasmid (pFastBac) to the mini-Tn7 attachment site on the bacmid, when the Tn7 transposition functions are provided in trans by a helper plasmid (a schematic representation of the process is shown in Fig. 3.12).

First the GRK4α cDNA was subcloned in the pFastBac vector downstream of the polyhedrin promoter. This construct was transformed into DH10Bac E. coli, which contains the bacmid and the helper plasmid expressing a transposon. The colonies containing recombinant Bacmid (Baculovirus-GRK4) were identified by disruption of the LacZ gene and by the ability to grow in antibiotic-selective media. A positive colony was isolated and viral DNA was prepared and purified over a caesium chloride gradient. This DNA was used to transfect the *spodoptera frugiperda* (Sf9) cells, using the calcium phosphate method (Graham, 1973). The Sf9 cells transfected with the recombinant baculovirus produce complete viral particles (Baculo4) that express the GRK4 protein.

Data from the literature suggested that GRK4 could have a higher specific phosphorylation activity in the palmitoylated form (Loudon, 1997), which is present in the membrane fraction (Stoffel, 1994). The best conditions to extract the membrane bound GRK4 were identified. Four different extraction buffers were tested (see section 2.2.8) to evaluate their ability to extract active GRK4. Cell membrane preparations were lysed and the residual particulate fractions were
Recombinant GRK4 Expression

Figure 3.12 Generation of recombinant baculoviruses-GRK4α using the Bac-to-Bac expression system

GRK4α was cloned into pFASTBAC1, and the recombinant plasmid was transformed into DH10BAC E. coli cells. The mini-Tn7 element on the pFASTBAC1 plasmid transposed to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids were identified since the disruption of the lacZα gene. High molecular weight DNA was prepared from selected E. coli clones containing the recombinant bacmid, and this DNA is then used to transfect Sf9 cells, which produced the recombinant baculovirus particles (Baculo4).
separated from the solubilised fractions by centrifugation. In both fractions the amount of GRK4 was measured by Western blotting (Fig. 3.13a). The buffer 3 and buffer 4 (for detailed description see section 2.2.8) solubilised most of the membrane-bound GRK4, (Fig. 3.13a). The phosphorylation assay showed that, among all extraction buffers tested the number 4 was the best to preserve the GRK4 activity; hence it was adopted as extraction buffer in the purification step (Fig. 3.13b).

Recombinant GRKs are generally purified using two chromatographic steps; ionic exchange chromatography on S-Sepharose column, followed by affinity chromatography on heparin-Sepharose column (Loudon, 1994). About 500 x 10⁶ cells were infected with approximately 1 x 10⁸ Baculo4. 72 hours later, the cells were harvested by centrifugation, broken with a Dounce homogeniser and the membrane fraction lysed in buffer 4. The membrane lysate was separated on a S-Sepharose followed by the heparin-Sepharose. However the purity of protein obtained was unsatisfactory. To further purify and concentrate the GRK4, the ability of GRK4 to bind Ca⁺²/CaM was exploited. The material eluted from the heparin-Sepharose column was loaded onto a CaM-Sepharose column and eluted with EGTA.

This GRK4 preparation was more than 98 % pure as assessed by Coomassie blue staining on SDS PAGE (Fig. 3.14) and mass spectrometry. A phosphorylation assay of this purified preparation of GRK4 on the ROS displayed a high specific activity and similar results were obtained from transiently transfected HEK293 (Fig. 3.15).
Figure 3.13 Solubilisation of membrane bound GRK4

**a:** Two hundred µg of membranes from GRK4α transfected HEK293 were solubilised with four different extraction buffers (see section 2.2.8). Insoluble (I) and soluble (S) fractions were assayed for GRK4 immunoreactivity by western blot analysis. **b** Phosphorylation assay of each of the soluble fractions (10 µg) tested in **a**. Phosphorylated rhodopsin (Opsin) is indicated. CLB was used as a control. Similar results were obtained when membranes from GRK4α infected Sf9 were used.
Figure 3.14 Purified GRK4α from Sf9 cells

One hundred ng of recombinant GRK4α purified from Sf9 cells were electrophoresed on a 10 % PAGE and stained with Coomassie blue (GRK4α). Pharmacia Mr standards (Ladder) are: phosphorylase B (94,000), albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100).
GRK4α transfected HEK293 cells were purified as in section 2.2.8. Phosphorylating activity was assayed in different fractions (F1 to F5), eluted from CaM-Sepharose 4B. Ten μl of purified GRK4α containing about 40 ng of proteins was assayed using 300 pmol of rhodopsin, for 30 min at 30°C. Reactions were separated by PAGE on 10% gel and dried for autoradiography. Phosphorylated rhodopsin (Opsin) is indicated.
3.4.2 Inhibition of GRK4α by Ca\textsuperscript{2+}/CaM

In our laboratory the calcium-binding protein CaM was recently shown to inhibit GRK5 phosphorylation activity through direct binding (Chuang, 1996b). Since GRKs share conserved residues in their primary sequence, the possible regulation of GRK4 by CaM was also investigated. Phosphorylation of rhodopsin by GRK4α was potently inhibited by 1 μM CaM in a calcium-dependent manner (Ca\textsuperscript{2+} was 1 mM) (Fig. 3.16a). This inhibition was CaM-dependent, since it was completely reverted by addition of 0.5 μM CaM inhibiting peptide (CaMBd) (Fig. 3.16a), whose sequence is derived from the CaM-dependent protein kinase II. Computer analysis of the dose-response inhibition of GRK4 activity by CaM (Fig. 3.16b) showed an IC\textsubscript{50} = 80 nM, similar to GRK5 (IC\textsubscript{50} = 40 nM) and ~25 fold more potent than that on GRK2 (IC\textsubscript{50} ~2 μM) (Chuang, 1996b).

3.4.3 Direct binding of GRK4 to CaM

A possible mechanism through which Ca\textsuperscript{2+}/CaM could inhibit GRK4α activity is by direct binding, as previously shown for GRK5 (Chuang, 1996b). To address this possibility, an \textit{in vitro} binding assay using CaM-conjugated Sepharose 4B gel was performed, using an unconjugated Sepharose 4B as negative control (Fig. 3.17). GRK5 was used as a positive control for this assay since it was previously shown to bind CaM-Sepharose (Chuang, 1996b). Both GRK4α and GRK5 bound to CaM-Sepharose 4B in a Ca\textsuperscript{2+}-dependent manner (Fig. 3.17a and b). Based on internal standard, >80% of the total amount of GRK4 and GRK5 added to the binding assay was estimated to bound by CaM-conjugated gel, in the presence of Ca\textsuperscript{2+}. In the absence of Ca\textsuperscript{2+}, no detectable binding of GRK4 and GRK5 to CaM was found.
Figure 3.16 Inhibition of GRK4α by Ca2+/CaM

a: Rhodopsin phosphorylation by GRK4α in the presence of 1 µM CaM ± 1 mM Ca2+. The effect of Ca2+/CaM was prevented by CaM inhibitor CaMBd (0.5 µM). b: Rhodopsin phosphorylation by GRK4α, in the presence of the indicated concentration of CaM (plus 1 mM Ca2+). Each experiment was repeated two-three times.
Figure 3.17 Direct binding of GRK4 splice variants to CaM

a: CaM-conjugated Sepharose 4B (CaM-Sg) was incubated (at 4°C for 1 h) with 150 µg cytosolic proteins from HEK293 cells expressing GRK4α or in b with 100 nM purified GRK5. Experiments were in the presence or absence of Ca2+. Unconjugated Sepharose 4B (Sg) was used as negative control. Bound GRK4α or GRK5, separated by centrifugation followed by extensive washing, was incubated in SDS sample buffer at 95°C for 5 min, ran on 10% PAGE, blotted onto nitrocellulose paper and revealed by specific antibodies. c: The four splice variants of GRK4 (α, β, γ and δ) were assayed in parallel binding experiments to CaM-Sg performed as described above. The starting material contained the same amount of each isoform as documented by immunoblot (d). The blot c was developed for much longer to allow the trace amount of bound GRK4β, γ and δ to be detected. The experiments shown are representative of three similar.
Neither bound to unconjugated Sepharose 4B gel (Fig. 3.17a and Chuang, 1996b).

The effect of Ca\textsuperscript{2+}/CaM on GRK4\textgreek{S}, GRK4\textgreek{y} and GRK4\textgreek{d} could not be tested in the phosphorylation assay, since these isoforms did not phosphorylate ROS (Fig. 3.10). The binding of the four isoforms to CaM-conjugated gel was measured (Fig. 3.17c). Experiments were done in parallel; 150 µg of cytosolic proteins from HEK293 cells transfected with the four isoforms were incubated with CaM-conjugated Sepharose 4B gel, in the presence or the absence of Ca\textsuperscript{2+}. The level of expression of each isoform was the same, as documented by immunoblot analysis (Fig. 3.17d). A substantial and Ca\textsuperscript{2+}-dependent binding of GRK4\textgreek{a} to CaM was observed, while only trace amount of GRK4\textgreek{S}, GRK4\textgreek{y} and GRK4\textgreek{d} were bound (Fig. 3.17c).

3.4.4 In vitro modulation of GRK4 membrane binding by CaM

To further elucidate the mechanism of CaM inhibition on GRK4 phosphorylation, the effect of CaM on the interaction between GRK4 and ROS membrane was investigated. Cytosolic proteins extracted from GRK4-transfected HEK293 cells were incubated under phosphorylating conditions with ROS membranes (Chuang, 1996b); the mixture was centrifuged to separate the membrane fraction from the soluble proteins, and both fractions were separated by PAGE and immunoblotted. Most of the GRK4 immunoreactivity is translocated from the soluble fraction to the membrane fraction (Fig. 3.18); in addition all four isoforms can bind ROS membrane (Fig. 3.18). The amount of GRK4 membrane binding was independent of the activation state of rhodopsin, since the binding was still present even when the translocation experiment was carried out in the dark (Fig. 3.18). The presence of Ca\textsuperscript{2+}/CaM (at a dose able to inhibit ROS
Figure 3.18 GRK4 binding to ROS membranes

Urea-treated ROS was incubated (at 30°C for 30 min) with 100 μg of cytosolic proteins from HEK293 cells expressing one of the four GRK4 isoforms (α, β, γ, δ) under phosphorylating conditions. ROS membranes were separated by centrifugation (48,000 x g, 30 min, 4°C). The pellet (Pe) and supernatant (S) were resuspended in SDS sample buffer, run on 10 % PAGE, blotted onto nitrocellulose paper, and GRK4 was revealed using the common antibody Nab (central panel). The starting material contained similar amount of each isoforms as documented by immunoblot in the left panel. Right panel shows the same experiment using only GRK4α, carried out in the dark. The experiment shown is representative of two similar.
phosphorylation by GRK4) in the incubation mixture did not alter the translocation of GRK4, which was still recovered in the membrane fraction, presumably because it is bound to ROS membranes (Fig. 3.19a). These results suggest that CaM inhibits GRK4 phosphorylation, without preventing its membrane association. In contrast binding of GRK5 to ROS membranes is inhibited by Ca$^{2+}$/CaM (Chuang, 1996b; and Fig. 3.19b). This discrepancy could be explained by the presence of a palmitoyl moiety on the GRK4 molecule, which is absent in GRK5. The lipid modification on GRK4 produces a tight binding to lipid membranes dampening the displacement by CaM. GRK5 interacts loosely to the ROS membrane, since binding is mediated only by positively charged amino acids at the C-terminal region of the protein (Premont, 1995), consequently CaM can prevent membrane association.
Figure 3.19 Binding of GRK5 to ROS membranes is inhibited by Ca2+/CaM in contrast to GRK4α

Urea-treated ROS was incubated (at 30°C for 30 min) with 100 µg of cytosolic recombinant GRK4α or GRK5 under phosphorylating conditions, in the presence or absence of Ca2+ and/or CaM (5 µM). At the end of the incubation the samples were chilled and centrifuged (48,000 x g, 30 min, 4°C) to pellet membranes. The pellet (Pe) and supernatant (S) were resuspended in SDS sample buffer, run on 10 % PAGE, blotted onto nitrocellulose paper, GRK4α and GRK5 were revealed using respectively K-20 (panel a) and anti-GRK5 (panel b) specific antibodies. The experiment shown is representative.
3.5 Discussion

3.5.1 Identification of new GRK4 splice variants

GRK4 was identified by molecular cloning while searching for expressed genes from the human chromosome 4p16.3 region as candidates for Huntington's disease (Ambrose, 1992), which has been mapped at the D4S127 locus. On the basis of sequence homology, this kinase was recognised as a new member of the GRK family. The six members of GRK gene family share high structural homology. The catalytic domain is the most conserved region, indicating a similar mechanism of action. GRK4, however, presents some specific characteristics (Ambrose, 1992; Sallese, 1994) which make it a distinct member of this family. The present study substantially extends previous findings and provides a number of new insights that might indicate novel mechanisms of action for this kinase family.

A new human splice variant of GRK4 has been identified, which was not seen in the original work by Ambrose (Ambrose, 1992). This variant includes an additional stretch of 47 amino acids, located near the C-terminus of the kinase. An additional splice site, previously identified in our laboratory (Sallese, 1994), is located in the N-terminal region of the protein and can generate two new isoforms. Thus four isoforms of this kinase are possible: the short form, lacking both splice sequences, named GRK4δ; the isoform containing the 32 amino acid sequence near the N-terminus (GRK4γ); the isoform that contains the 47 amino acid sequence near the C-terminus (GRK4β); and finally the longest form, which contains both splice sequences (GRK4α). The GRK4 nomenclature is taken from Premont where the genomic structure of GRK4 and the identification of the four possible
isoforms was first reported (Premont, 1996). The sequence of the splice variants described by Premont is identical to that reported in the present study.

In addition, the sequence of two full lengths and three partial clones of rat GRK4 are reported. The rat GRK4 was cloned by a single step PCR using primers encompassing the open reading frame derived from a rat sequence found in the EMBL database. The deduced amino acid sequence of one clone (C1) was colinear (only 3 amino acids shorter) with the longest human sequence (GRK4α), and with the sequence obtained from the database and subsequently published (Virion, 1998). Virion describes a full length GRK4 clone which lacks 31 amino acids in the N-terminus, and two partial clones, one lacking 51 amino acids in the N-terminus (generated by the previous 31 amino acids gap plus the 22 adjacent amino acids) and one presenting a gap of 47 amino acids in the C-terminal region. The clone C2 of the present work also included the entire open reading frame but presented two gaps; one in the N-terminus of 31 amino acids identical to that published by Virion, 1998, and one of 47 amino acids corresponding to a partial clone of Virion, 1998.

In conclusion, the findings of the present report are similar to those reported by others (Virion, 1998). Moreover, the present work represent the first demonstration that the two spliced regions can occur in one GRK4 molecule.

Our results also establish that the longer isoforms are the predominant mRNA variants in the testis, and that GRK4 is spliced differently in different species and/or tissues. This suggests that the various GRK4 variants, may have different physiological functions, however protein expression data are clearly needed to substantiate such an hypothesis.
Sequence analysis of the rat splice variant showed that the lack of 31 amino acids in the C2 clone matches exactly the human GRK4 exon VI, the lack of 52 amino acids of the N3 clone matches the human GRK4 exon VI and VII, and the lack of 47 amino acids of the C2 clone matches the human GRK4 exon XIV (Premont, 1996). These data along with Southern blot analysis performed by Virion, 1998, indicate that the cloned sequence described here is the rat counterpart of human GRK4. Surprisingly the overall amino acid identity between the two proteins is only 76% whereas identity between other rat and human GRKs is in the range of 86-98% (Sallese, 1997). The explanation for this unusual level of diversity, for a GRK protein among different species, could reside in the specific function exerted by GRK4.

In most marine invertebrates with external fertilisation, sperm egg interaction exhibits some degree of species selectivity (sperm fertilises conspecific eggs more efficiently than heterospecific eggs), (Lopez, 1993; Wassarman, 1995). The species selectivity could occur at one or more specific recognition steps in the fertilisation cascade; including attachment of the sperm to the egg envelope, induction of the acrosome reaction, interaction of the acrosomal protein with the egg envelope, (Wassarman, 1995; Ohlendieck, 1995; Breitbart, 1997; Bigler, 1997). This ancestral protection mechanism against interspecies fertilisation is conserved in mammals and is exerted at the molecular level by a faster evolution (likely through positive Darwinian selection) of the proteins involved in gamete recognition (Lin, 1993; Swanson, 1995; Lee, 1992), than proteins with other functions. Since the identity between human and rat GRK4 is lower than in other GRKs (Fig. 3.20), this might indicate that GRK4 is one of the genes participating in fertilisation selectivity. A further level of diversity is created by the alternative splicing of different exons.
3.20 Comparison of amino acids sequences of human and rat GRK by phylogram analysis

The ClustalW program from the computational service of EMBL was used to align amino acid sequences of the human and rat GRK1 (U63973, U63971), GRK2 (M80776, M87854), GRK3 (X69117, M87855), GRK4α (X97881, present study) GRK5 (L15388, U34841), GRK6 (L16862, X97439). The phylogram illustrates the relative evolutionary relationship among the GRK family members.
3.5.2 Molecular determinants of GRK4 activity

In this study a simple method to assess the kinase activity of GRK4 was developed which is based on transient expression in HEK293 cells, followed by partial purification and phosphorylation of ROS. We consistently found that GRK4α phosphorylates rhodopsin. This is different from previous results from our group (Sallese, 1994) and from others (Premont, 1996), which indicated that GRK4 was not able to phosphorylate this receptor. The apparent discrepancy is likely due to the different expression systems and kinase isoforms used in these studies. Similar to other GRKs, phosphorylation of rhodopsin by GRK4α was strictly agonist (light)-dependent, inhibited by heparin and stimulated by poly-L-lysine (Chuang, 1992; Benovic, 1993; Kunapuli, 1993; Kunapuli, 1994b). An agonist-dependent phosphorylating activity of GRK4α was previously shown using as a substrate the purified β2AR reconstituted on vesicles containing 5% PIP2 (Premont, 1996).

GRK4α kinase activity was potently inhibited by CaM and this effect was strictly dependent on Ca²⁺, and was prevented by the CaM inhibitor, CaMBd. A direct interaction between GRK4α and Ca²⁺/CaM was revealed using CaM-conjugated Sepharose 4B. In a recent study a similar inhibition of GRK5 by Ca²⁺/CaM (Chuang, 1996b) was reported. Two other groups have shown that in the presence of Ca²⁺, GRK1 is inhibited by the photoreceptor-specific recoverin by direct binding (Chen, 1995; Klenchin, 1995). Recoverin is a member of the family of neurone-specific CSP named neuronal calcium sensors (NCS) and several other members of this family were also able to inhibit GRK1 (De Castro, 1995). Since CaM and NCS are both Ca²⁺ sensors, these studies suggest that they may act as functional
analogues in mediating the regulation of different GRK subtypes by Ca$^{2+}$. This mechanism is however highly selective, with respect to the GRK subtypes. While GRK1, but not GRK2, is regulated by recoverin and other NCS, GRK4 and GRK5, which belong to the GRK4 subfamily, are potently inhibited by CaM (Iacovelli, 1999). CaM had little or no effect on members of other GRK subfamilies (Chunag, 1996b; Iacovelli, 1999). The effect of Ca$^{2+}$-sensor proteins appears to be rather general, and the GRK subtype selectivity indicates that different kinases can be specifically regulated in different target tissues.

The properties of the four GRK4 splice variants were investigated and some functional differences were found and in particular only GRK4α, but not GRK4β, GRK4γ and GRK4δ, was able to phosphorylate rhodopsin. Since all four isoforms are able to desensitise some G protein-coupled receptors, as suggested by the inhibition of LH/CG-induced cAMP accumulation in cotransfection experiments (Premont, 1996), these results indicate that the four GRK4 isoforms may have different receptor substrate specificities.

The interaction of the four GRK4 isoforms with CaM was also investigated and it was found that only GRK4α, but not GRK4β, GRK4γ and GRK4δ was able to bind to CaM in a calcium dependent manner. We speculate that the long kinase isoform GRK4α, which contains the two inserts at the N- and C-termini, closely resembles GRK5 with which it shares some common properties, including the ability to phosphorylate rhodopsin and sensitivity to CaM inhibition by direct binding (Chuang, 1996b). By contrast the other GRK4 isoforms may have distinct properties such as substrate specificity or regulation by phospholipids, but this remains to be demonstrated. The insert located in the N-terminal domain has been reported to contain some sequence homology to gelsolin and has been postulated
to be the binding site for PIP2, which is critical for kinase activity (Pitcher, 1996). The lack of phosphorylating activity of GRK4β and GRK4δ could be explained by the lack of an N-terminal insert and hence the putative binding site for PIP2. However we also observed that GRK4γ, which contains the N-terminal insert but not the one located at the C-terminus, was also unable to phosphorylate rhodopsin, strongly indicating that both inserts are important for a functional kinase.
CHAPTER 4

GRK4 TISSUE DISTRIBUTION

4.1 Cellular localisation of GRK4

4.1.1 Identification of testicular cells expressing GRK4

When GRK4 was identified, it was evident that unlike GRK2, GRK3, GRK5 and GRK6, it is not widely distributed in tissues and cells. Substantial expression of GRK4 was found only in testis (Ambrose, 1992; Sallese, 1994). However a limited number of tissues were examined at that time and the possibility of GRK4 being expressed in tissues not previously considered had to be tested. Therefore a systematic Northern blot examination was undertaken, in order to evaluate the expression of GRK4 on a large number of human and rat tissues and cell lines. As expected, a single mRNA band of about 2.5 kb (Ambrose, 1992) was detected on RNA from human, bovine, rat, mouse testis (a representative blot of rat and human tissues is shown in Fig. 4.1). Rat testis RNA was used as a positive control in all the experiments. Samples analysed were: i) human testis, liver, heart, lung, adipose tissue, brain cortex and skeletal muscle; ii) cells isolated from human peripheral blood, monocytes, monocytes activated by adherence, lymphocytes, polymorphonuclear leukocytes; iii) human cell lines, Jurkat (lymphoid leukaemia cells), HL60 (promyelocitic leukaemia cells), K562 (myeloid leukaemia cells), MRC5 (embryonic lung), Hep G2 (hepatoma cells), IMR32 (neuroblastoma cells), MCF7 (breast adenocarcinoma cells), SW626 (ovarian adenocarcinoma cells), O143 (osteosarcoma cells) MDA-MB
Figure 4.1 Representative Northern blot of human and rat GRK4

Total cellular RNA (20µg) from rat testis (T), ovary (OV), olfactory bulb (OB), adenohypophysis (A), and human embryonic lung fibroblast (MRC5), breast adenocarcinoma (MCF7) cell lines, testis (T), cerebellum (C), was hybridised as in section 2.1.5.2. Ribosomal RNAs are indicated as Mr standard. Data represent at least three separate experiments.
(mammary carcinoma cells); iv) rat brain regions, such as cortex, cerebellum, n. accumbes, striatum, hypothalamus, hippocampus, brainstem, olfactory bulbs; v) rat tissues and cells, PBL, stomach, intestine, prostate, seminal vesicles, uterine tubes, ovary, adrenal gland, adenohypophysis, C6 (glioma cell line); bovine epididimus. No expression of GRK4 could be documented in all these tissues and cells, strongly supporting the idea that GRK4 mRNA is expressed exclusively in testis.

Different testicular cell types were next analysed by Northern blot (Fig. 4.2). GRK4 mRNA was not expressed in the mouse Sertoli cells TM4 and in the Leydig cells TM3 (Mather, 1980), while it was abundantly expressed in the spermatogonia GC-1 spg (Hofman, 1992), (Fig. 4.2), suggesting that sperms are the cell site of expression of GRK4. In agreement with this, mRNA expression was lower in testis from immature rats (20 days-old), compared to testis from adult mature animals (60 days-old) (Fig. 4.2). As previously observed, in some tissues an additional hybridisation band at ~4 Kb (Premont, 1996) on GC-1 spg was also present (Fig. 4.2). The significance of this additional band remains to be defined.

4.1.2 Biochemical study of GRK4 expression in spermatozoa

To investigate the cellular and ultrastructural localisation of GRK4 within testis anti-GRK4 polyclonal serum (I-20) obtained from Santa Cruz Biotechnology was first tested on recombinat GRK4 protein. This antiserum should recognise GRK4γ and GRK4δ. On a blot containing: GRK4γ, GRK1 partially purified from bovine retina and recombinants GRK2, GRK3, GRK5 and GRK6 (Fig. 4.3), overexpressed in Sf9, only GRK4 was recognised by this antibody, thus confirming the selectivity reported by the manufacturer (Fig. 4.3). The expression
Figure 4.2 Northern blot analysis of GRK4 mRNA

Total RNA (20 µg) from mouse Leydig cells TM3 (L), Sertoli cells TM4 (Se), spermatogonia GC-1 spg (Sp) and testis (Te), (a), and from 20 or 60 days-old rat testis (b), was hybridised with a GRK4 human cDNA probe common to the four isoforms. Washed filters were exposed at -80°C for 24-48 h. Data represent two separate experiments.
Figure 4.3 Selective I-20 immunoreactivity for GRK4

Western blot analysis of total protein (100μg) extracted from Sf9 cells, infected with GRK2, GRK3, GRK5, and GRK6, and bovine retina (GRK1) or GRK4γ-transfected HEK293 cells (GRK4γ). The I-20 antiserum was used at 0.1 μg/ml. The duration of the development step was much longer than usual to allow possible faint cross-reactivity to be visualised.
of GRK4 was then studied on cytosolic and membrane preparations obtained from several tissues, including rat testis, ovary, and adenohypophysis, human testis and bovine spermatozoa. A strong immunoreactive band at Mr ~61 Kd was detected only on bovine sperm membrane (Fig. 4.4). This band was totally abolished by preincubation of antiserum with the antigen peptide (Santa Cruz Biotechnology, CA), thus confirming its specificity (Fig. 4.4). Since GRK4 is expressed at higher level in mature spermatozoa, which are mainly present in the deferent duct, GRK4 can not be easily detected in testis by Western blot.

These results also confirm that GRK4 is selective localised on membranes in native tissue such as spermatozoa. When sperm membranes, containing GRK4, were washed with high salt concentrations (1 M NaCl) only ~30 % of GRK4 was detached (Fig. 4.5). This indicates that the majority of native protein may undergo post-translational modification, which could tightly anchor GRK4 to the membranes, and GRK4 is known to be palmitoylated (Premont, 1996). Subcellular fractions of sperm, separated by differential centrifugation, as described, in the section 2.2.2 (Garcia-Higuera I, 1994b), were examined to evaluate whether GRK4 was associated with any specific intracellular organelles. GRK4 immunoreactivity was substantially associated with enriched mitochondrial and microsomal fractions, with no immunoreactivity observed on enriched nuclear fraction, cytosolic proteins or plasma membrane (Fig. 4.6).

In more recent experiments, GRK4 immunoblots have revealed two specific bands of Mr ~58 and ~62 Kd. Immunoblots performed using as standard the short form (GRK4δ) and the isoform containing the insert at the N-terminus (GRK4γ) showed that the doublet recognised by the specific antibody I-20 identifies GRK4γ and GRK4δ as the lower and higher band respectively (Fig. 4.7).
Figure 4.4 Expression of GRK4 in sperm membrane

**a:** GRK4 expression on bovine spermatozoa (lanes 1 and 2), rat adenohypophysis (3 and 4) and rat testis (5 and 6). Immunoreactivity of I-20 antibody was determined using 100 μg of proteins from cytosol (lanes 1, 3 and 5) or membranes (lanes 2, 4, and 6) from each tissue. **b:** Blot identical to **a** revealed with I-20 antibody preincubated with a tenfold excess of antigen peptide for 2 h at 4°C. Molecular mass standards (77, 61, 41 and 36 kilodaltons) are indicated on the left. Data represent two separate experiments.
Figure 4.5 Characterisation of GRK4 in bovine sperm membrane

The total membrane pellet (70 μg) was resuspended in CLB containing 1 M NaCl and incubated for 1 h at 4°C. The extracted proteins and membranes were separated by centrifugation at 300,000 x g for 30 min at 4°C, and the entire pellet (lane 1) and supernatant (lane 2) were analysed by immunoblot using I-20 antiserum. Molecular mass standards (94, 67, and 43 kilodaltons) are indicated on the left.
Figure 4.6 Analysis of GRK4 protein, by 1-20 in subcellular fractions of bovine sperm

Cytosol (lane 1), enriched nuclear fraction (lane 2), plasma membranes (lane 3), mitochondria (lane 4) and microsomes (lane 5) were fractionated as described in section 2.2.2. Fifty µg of proteins were loaded in lanes 1-4 while 20 µg of proteins were loaded for the microsome fraction (lane 5). Molecular mass standards (77, 61, 41 and 36 kilodaltons) are indicated on the left. Data represent two separate experiments.
Figure 4.7 Immunoblot of bovine sperm GRK4 isoforms

Bovine sperm membranes (Sperm), and HEK293 (100 µg) expressing recombinant GRK4γ and GRK4δ were separated on 10 % PAGE, blotted, and their immunoreactivity determined using the I-20 antiserum. Molecular mass standards (94, 67, and 43 kilodaltons) are indicated on the right.
4.1.3 Ultrastructural localisation of GRK4 in spermatozoa

To further elucidate the subcellular localisation of GRK4 on sperm cells the ultrastructural localisation of the kinase was performed in collaboration with Prof. Baccio Baccetti (University of Siena Italy). Electron microscopy and immunocytochemistry on Lowicryl embedded free spermatozoa or on testicular tissue, revealed that GRK4 was present in spermatozoa, spermatids and in previous stages of spermatogenesis. In mature spermatozoa (Fig. 4.8), most gold particles are localised on acrosomes and mitochondria. In the acrosome GRK4 localisation seems to be concentrated on the outer and inner membranes, mostly on the cytoplasmic surface and only few gold particles are sometimes extended to the acrosomal content (Fig. 4.8). After the acrosomal reaction (Zaneveld, 1993), when the anterior nuclear region is covered only by the inner acrosomal membrane, the particles show a monolayered distribution, localised between membrane and nucleus, in the thin subacrosomal layer (Fig. 4.8b). In the mitochondria of the ejaculated spermatozoa, gold granules appear to be more concentrated on the outer mitochondria membrane (Fig. 4.8c), and sometimes are present also on the cristae (Fig. 4.8c, d). Fluorescent microscopy confirmed these observations, showing positive labels in the acrosomal region (Fig. 4.8e-h), including equatorial segment (Fig. 4.8e and f), as well as in the midpiece of the tail, mainly close to the centriole (Fig. 4.8e, f, i, l). In the testicular tissues (Fig. 4.9), immature germinal cells show gold granules concentrated in the outer mitochondrial membrane during the whole spermatogenesis (Fig. 4.9d). Clusters of particles are also present on the membranes of cytoplasmic vesicles (endoplasmic reticulum) (Fig. 4.9b) and in the Golgi complex (Fig. 4.9a). At the
Figure 4.8 Ultrastructural localisation of GRK4 on human sperm

a-d: Submicroscopical immunogold localisation in human spermatozoa treated with I-20 antibody. In a the presence of colloidal gold granules is evident in the acrosome, specifically on the inner (small arrows) and on the outer (large arrows) membranes. In b the particles are present only in the inner membrane after the acrosomal reaction. The localisation of GRK4 clearly appears into the mitochondria of the midpiece of the tail (b, c, d) (arrows). a: X 26,000; b, c: X 21,000; d: X 54,000. e-n: UV and phase contrast micrographs of human spermatozoa treated with the I-20 antibody: an intense fluorescence is visible on the acrosomal (e-h) and on the midpiece (e-l) regions. The fluorescent staining is completely absent in the control (m, n). X 900. A, acrosome; ES, equatorial segment; N, nucleus; M, mitochondria; MP, midpiece; AX, axoneme.
Figure 4.9 Submicroscopic immunogold localisation of GRK4 in human testis

At the spermatocyte stage, the reaction is positive (arrows) into the Golgi complex (a). At the spermatid stage, the gold granules are localised (arrows) on Golgi derived cytoplasmic vesicles (b), in the mitochondria (d) and in the acrosome (e). In the young spermatids, the nucleus and the acrosome are devoid of granules (c). a, b: X 21,000; c, d: X 27,000; e: X 52,000. GC, Golgi complex.
beginning of its formation, the acrosome appears almost devoid of particles (Fig. 4.9c), when it starts to be moulded and condensed in older spermatids, groups of particles migrate from the cytoplasmic membrane to small areas of the outer acrosomal surface (Fig. 4.9e), possibly carried out by small cytoplasmic vesicles. The axoneme and the nuclei are consistently not labelled.

4.1.4 Analysis of GRK4 isoforms using a common antibody

The two commercially available anti GRK4 antibodies, I-20 and K-20, were generated against peptides derived from the C-terminal alternatively spliced region of human GRK4α and GRK4δ respectively; neither is able to recognise all four isoforms. The antibody I-20 recognises GRK4γ and GRK4δ but not GRK4α or GRK4β (Premont, 1996), while K-20 recognises GRK4α and GRK4δ but not GRK4γ or GRK4δ (present work). A third antibody (NAb), raised against a sequence (amino acids 84-146) of GRK4α common to all four isoforms was generated. This antibody NAb, which is able to recognise all four recombinant GRK isoforms (Fig. 4.10a), revealed only one major band of immunoreactivity on human sperm membranes. Based on gel migration, this corresponded to GRK4γ (Fig. 4.10a), while no immunoreactive band was evident at the level of GRK4α, GRK4δ or GRK4δ (Fig. 4.10a). These data indicate that the GRK4α isoform is expressed at lower levels or not expressed at all, as compared to the GRK4γ. Electron microscopy immunocytochemistry on sperm cells using NAb confirmed the results obtained with I-20 (Fig. 4.10b). GRK4 was specifically recognised on the inner and outer acrosomal membranes, on the outer mitochondrial membranes and sometimes on the cristae.
Figure 4.10 Analysis of GRK4 with NAb

**a:** Immunoblot of GRK4 isoforms. HEK293 membranes (100 μg) expressing recombinant GRK4α (lane 1), GRK4β (lane 2), GRK4δ (lane 3) and GRK4γ (lane 4) were blotted and immunoreactivity was determined usings NAb. Immunoblot of human sperm membranes (100 μg) with NAb is in lane 5. **b:** Ultrastructural localisation of GRK4 on human sperm with the polyclonal antibody NAb. Gold granules are localised on the inner and outer acrosomal membranes (arrows; X 23,000). The localisation of GRK4 is evident on the outer mitochondrial membrane (long arrows) and sometimes on the cristae (short arrows; X 57,000). Data represent two separate experiments.
4.2 Identification of GRK4 expression in cerebellar Purkinje cells

4.2.1 Brain distribution of GRK4 by in-situ hybridisation

The expression of GRK4 was considered to be restricted to the testis (Ambrose, 1992). However with the aid of the extremely sensitive RT-PCR technique trace amount of GRK4 mRNA in the brain were detected (Sallese, 1994). Whether these very low mRNA levels, detectable only by PCR, were generated by very few copies, widely distributed without any functional meaning, or by a significant amount, localised in selected cells or regions, was not clear. The present study was aimed in part to define the possible expression of GRK4 in brain areas and in neurones using whole mount in situ hybridisation and immunohistochemistry. The use of these techniques for the analysis of GRK4 expression were validated using rat and bovine testis, where this kinase is highly expressed (Ambrose, 1992; and present work).

A nearly full length GRK4 antisense RNA probe RP1 (which recognises all GRK4 splice variants) labelled with digoxigenin, was hybridised on a whole mount specimen of bovine testis (Fig. 4.11a). An intense staining of the testis tubule was generated, while the intertubular area which includes the Leydig cells, was completely unlabelled (Fig. 4.11a). Similar results were obtained when the probe RP2, which includes only the N-terminal region of GRK4 was used. The specificity of the hybridisation signal was examined by preventive digestion of the specimens with RNAse h (Fig. 4.11b). This treatment completely prevented any hybridisation, (Fig. 4.11 is representative of different experiments). In situ hybridisation of the rat brain using the probe RP1 or RP2 showed that GRK4 mRNA is mainly expressed in the cerebral cortex, hippocampus and cerebellum (Fig. 4.12a and b).
**Figure 4.11 GRK4 in situ hybridisation on bovine testis**

a: The human GRK4 cRNA probe (RP1) which recognises all the GRK4 splice variants, was labelled with digoxigenin and used to detect GRK4 mRNA in bovine testis. An intense staining was observed inside the testis tubuli, while the intertubular spaces that include the Leydig and myoid cells were completely unlabelled. The hybridisation was completely prevented in parallel experiments carried out using specimens pretreated with RNAse H (b).
**Figure 4.12 GRK4 in situ hybridisation in rat brain**

*a*: Brain in situ hybridisation showing that GRK4 mRNA is mainly expressed in the cerebral cortex, and hippocampus. In the hippocampus, the hybridisation signals were seen throughout CA1-CA4 regions (closed arrow) as well as in the dentate gyrus (open arrow). Moderate expression was detected in the posterior part of the cingulate cortex (open arrowheads), in the region of the pyriform cortex, (closed arrowheads).

*b*: Rat cerebellar GRK4 hybridised in situ using the RP1 probe. An intense staining, indicated with the arrow, was observed in the Purkinje cells layer.

*c*: Bovine cerebellum obtained from a local slaughterhouse, was fixed in 3% paraformaldehyde and hybridised with the RP1 probe. The arrow indicates the selective expression of GRK4 mRNA in the Purkinje cell bodies.

*d*: Negative control of rat cerebellar specimen hybridised with the RP3 sense probe.
The use of the sense probe did not show any specific labelling (Fig. 4.12d). The hippocampal staining was extended from CA1 to CA4 and included the dentate gyrus (Fig. 4.12a). Moderate expression was detected in the brain neocortex in the region of the pyriform cortex, the insular (rhinal) cortex and all the parietal cortexes (motor area and somatosensory area) up to the posterior part of the cingulate cortex. The cerebellar cortex represents the preferential site of expression of GRK4 in the brain. Microscopic examination of bovine cerebella in situ hybridisation, revealed that GRK4 mRNA was selectively expressed in the Purkinje cell bodies (Fig. 4.12), while the labelling in the molecular and granular layer was negligible.

**4.2.2 Northern Blot analysis**

The in-situ hybridisation results were inconsistent with the Northern blot data so far accumulated. To clarify this problem I set up a more sensitive Northern blot analysis differing from earlier ones by using a different cDNA region as a probe (P2, 619-1806 bp) and labelled to a higher specific activity by a modified PCR (Parruti, 1993b), (for details see section 2.1.2.6). Using this method a specific hybridisation band from rat cerebellar RNA at the expected Mr of 2.5 Kb was obtained (Fig. 4.13). The amount of GRK4 mRNA found in the cerebellum represents about 3 % of the testis expression level, when quantified by NIH image software. This observation could have two different explanations: i) the amount of RNA in the spermatogonia is much higher than in the Purkinje cells because spermatogonia undergo active mRNA and protein synthesis and need to accumulate proteins necessary at later stages of differentiation, when transcriptional and translational quiescence will take place; or ii) the Purkinje cells represent a smaller percentage of the cerebellar cells,
Figure 4.13 Northern blot analysis of cerebellar GRK4

20μg of total RNAs from rat testis (T), brain cortex (B) and cerebellum (C) were separated on 1 % agarose-formaldehyde gel. The RNA was blotted on a nylon filter, and probed with a PCR-labeled 1187-Bp. cDNA fragment (bases 619-1806, which includes the catalytic and the C-terminal domains) at 42°C in 50 % formamide. After high stringency washing, the blot was exposed for autoradiography, six days at -80°C.
compared to the percentage of spermatogonia in the whole testis.

4.2.3 Immunohistochemical localisation of GRK4 on rat brain

To confirm the in-situ hybridisation results, GRK4 expression was analysed by immunohistochemistry, using the antibodies 1-20 and K-20. The expression of GRK4 was first characterised in rat testis (Fig. 4.14a and b), where high level of immunoreactivity was evident in the inner part of the seminiferous tubuli, confirming and extending previous results. The labelling intensity varied according to the stage of spermatogenesis. Weak immunoreactivity levels were observed in stages IX-XI tubules containing many leptotene to late pachytene primary spermatocytes, while no signal was seen in the myoid interstitial cells. Microscopic observations (of the slides) revealed that the highest signal was systematically observed over stage XIII tubule sections, where primary spermatocytes, elongated spermatids and spermatozoa are present.

Further investigations were focused on the cerebellum. Rat brain fixation with formalin, which displayed a satisfactory level of tissue preservation was used in this study. Immunohistochemical labelling with the I-20 polyclonal antibody revealed a greater positivity at the molecular layer of the cerebellar cortex where Purkinje cells project their dendrites and a weaker positivity at the level of Purkinje cell bodies (Fig. 4.14c). Immunolabelling with K-20 antibody detected a very strong immunoreactivity exclusively in the somatodendritic region of the Purkinje cells (Fig. 4.14d). Both antibodies stained the granular cell layer at negligible levels. Immunoreactivity was completely abolished by pre-absorption of GRK4 antibodies with the antigenic peptide for 2 h before incubation with the brain sections (Fig. 4.14f). Figure 4.14e show an enlargement of the impressive punctuate
Figure 4.14 GRK4 immunohistochemical analysis

a: Rat testis showing an intense immunoreactivity of the I-20 antibody inside the seminiferous tubule. b: Higher magnification of one labelled tubule. The immunostaining on the spermatogonia and sperm head are indicated by open and closed arrows respectively. c: The staining of the cerebellar cortex with I-20 revealed a preferential labelling of the molecular layer, where the Purkinje cells project their dendrites, and weaker labelling of the Purkinje cell bodies. d: The cerebellar labelling with K-20 detected a very strong immunoreactivity throughout the molecular and Purkinje cell layer with a negligible staining in the granular cell layer. e: Higher magnification of Purkinje cells labelled with K-20 showing a granular pattern of GRK4 immunoreactivity inside the neurone except the nucleus (N). f: As a negative control, cerebellar sections were incubated with the GRK4 antibody pre-absorbed with an excess of its antigenic peptide for 2 h, all immunoreactivity was completely abolished by this treatment.
staining of GRK4 inside a Purkinje cell, while the nucleus (N), which is clearly visible is unlabelled.

To evaluate the amount of GRK4 expressed in Purkinje cells, the immunoreactivity of other GRK members was determined in parallel in different brain areas. Immunostaining of the cerebral cortex with anti-GRK4, or anti-GRK6 showed a moderate labelling throughout all the cortical layers including the few neurones present in the molecular layer I (Fig. 4.15a and c). The staining was localised in the perikaryon of small neurones and absent in dendrites and in other cellular processes. Anti-GRK5 polyclonal antibody was not able to stain any cells in rat brain cortical sections (Fig. 4.15b). The same antibodies showed a completely different behaviour when incubated with cerebellar sections. In the cerebellum the labelling of anti-GRK5 and anti GRK6 were faint on the Purkinje cells body and absent on the molecular and granular layer (Fig. 4.15e and f), while the labelling of anti GRK4 was prominent in the somatodendritic region of the Purkinje cells and irrelevant in all the other cerebellar regions (Fig. 4.15d). This analysis shows that GRK4 is expressed in several brain areas at low level, but the only relevant expression site is in Purkinje cells; in contrast GRK6 expression level is similar in different brain areas.

Extended histological examinations were performed on rat cerebellum at different developmental stages (from P10 to three months). No positive staining was observed in the cerebellum of 10 day old rats in the molecular layer or in the granular cell layer (Fig. 4.16a). Even Purkinje cells were unlabelled at this stage, indicating that GRK4 expression appears at later stages of development. At developmental stage P20-30 GRK4 immunological staining was at maximum level in Purkinje cells (Fig. 4.16b and c), while in older rats (P60 and P90), the immunoreactivity plateaued see Fig. 4.16d and e).
Figure 4.15 Selective expression of GRK4 in the Purkinje cells

Immunostaining with anti-GRK4, -GRK5, -GRK6 antibodies in cerebral cortex and cerebellum. In the cerebral cortex moderate expression of GRK4 and GRK6 was detected, while GRK5 labelling was absent. In the cerebellum a low expression level of GRK5 and GRK6 was found, while GRK4 was expressed at higher level.
Figure 4.16 Developmental expression of GRK4 on rat cerebellar cortex

GRK4 immunostaining of rat cerebellum at different postnatal developmental stages. The expression of GRK4 was negligible at P10 (a) while the maximal expression was observed around P20 (b), and P30 (c). In adult rats, P60 (d), and P90 (e), the immunoreactivity was slightly reduced in comparison to P30 animals.
4.3 Discussion

4.3.1 Identification of GRK4 expressing cells

Northern blot analysis of mRNA expression on a large variety of tissues and cells showed that GRK4 is substantially expressed in testis only. In the present study, expression was localised to human and bovine sperm and germinal cells, where it is associated with membranes of intracellular organelles. Polyclonal antibodies selective for GRK4, showed on a variety of tissues and cells, that the kinase is expressed only on membranes prepared from spermatozoa. Using an antibody that recognises all GRK4 splice variants, the relative expression of native GRK4 isoforms was also investigated. We found that only one isoform, GRK4γ, is detectable in human sperm membranes. This finding is unexpected since, based on the analysis of mRNA expression in testis, a higher expression of the longest form was postulated (Premont, 1996; and present paper). The lack of the other three isoforms may indicate that: i) GRK4α, GRK4β and GRK4δ are expressed at low levels, not detectable by our immunoblot protocol; ii) they are not expressed in mature sperm, but rather in germ cells; iii) they are highly unstable proteins; iii) they are not expressed at all.

4.3.2 Subcellular localisation of GRK4

Western blot and immunocytochemical analysis consistently show that GRK4 is essentially a membrane associated kinase. A fraction of GRK4 is associated very tightly with sperm membranes, indicating a post-translational modification of the native protein (acylation?), as
already shown for GRK1 and GRK6 (Inglese, 1992a; Cha, 1997; Loudon, 1997). Palmitoylation of the four GRK4 isoforms, transiently expressed in COS7 cells, has been recently reported (Premont, 1996). About 30% of the membrane-associated proteins were however detached after high salt treatment, indicating that palmitoylation cannot account for all membrane affinity and other mechanisms, perhaps based on charge, may play a role. For GRK5, amino acids at the C-terminus are positively charged and it has been proposed that they may interact with negatively charged phospholipid head groups to promote its membrane association (Premont, 1995). For GRK4, the new 47-amino acid sequence identified in the present investigation, confers to the isoform GRK4S a positive charge similar to GRK5 suggesting a similar interaction with phospholipids. It is also possible that different mechanisms may target GRK4 to different organelles. For other members of this family, such as GRK2 and GRK3, a mechanism of transient translocation to the plasma membrane, involving binding to βγ subunits has been extensively characterised (Daaka, 1997; Pitcher, 1995a). Constitutive association with membranes seems to be a characteristic common to the GRK4/GRK5/GRK6 subfamily of GRKs (Premont, 1995; Premont, 1996).

Premont, 1996, failed to identify GRK4 in sperm while we consistently found GRK4 immunoreactivity in membranes from human and bovine sperm. This was possible only after the method has been optimised in the early experiments a number of non-specific bands were observed. We believe that the differences in Western blot protocols may account for their negative results. Some features of the western blot protocols for GRK4 analysis are hard to explain. For example the Western blot of bovine sperm generates an immunoreactive band only if incubated with the I-20 antibody while
no immunoreactivity is observed if K-20 or Nab (Nab reacts with all the recombinant human isoforms) antibodies are used. However Nab gave a stronger signal than I-20 when tested in parallel on the same amount of recombinant GRK4. In addition none of the antibodies recognise any specific bands in testis from human, rat, mouse or bovine. The analysis of three mice testicular cell lines was negative even on the GC-1 (spermatogonia cell line) that gave a strong hybridisation signal in Northern blot analysis. Moreover, none of the commercially available antibodies showed immunoreactivity on a human sperm extract (these antibodies were raised in rabbits injected with human GRK4 antigen peptides) while as stated above, I-20 does react with bovine sperm. The possibility that immunoreactive bands considered non specific so far, (since their Mr was different from the theoretical Mr), are post translational modified GRK4 can not be ruled out.

The present study was also aimed at investigating the cellular and ultrastructural localisation of GRK4. Immunochemical and ultrastructural analysis were performed to define the subcellular localisation of this kinase. It was found that GRK4 is present in germinal cells throughout their whole life. In the spermatozoa it is associated with the two acrosomal membranes (where it migrates from cytoplasmic membranes after the maturation of the organelle) and to the outer mitochondria membrane. Analysis of the immature germinal cells showed that GRK4 was present in the membranes of cytoplasmic vesicles (endoplasmic reticulum) and in the Golgi complex. The acrosome, at the beginning of its formation appears almost devoid of gold particles but when it starts to be moulded and condensed in older spermatids, groups of gold particles are seen to migrate from the cytoplasm to small areas of the outer acrosomal surface. Possibly they are carried by small cytoplasmic vesicles. It
was hoped that the ultrastructural localisation of GRK4 might help understand its function. The association of GRK4 with highly specialised sperm organelles, which are essential for fertilisation strongly indicates that this kinase is involved in this process. Whether this is to phosphorylate an as yet unidentified G protein-coupled receptor or an unusual substrate remains a matter for further investigation.

4.3.3 Localisation of GRK4 in the cerebellum

Using the extremely sensitive RT-PCR method, trace amount of specific GRK4 mRNA could be found on tissues other than testis (i.e. brain cortex), (Sallese, 1994). These very low levels of mRNA, detectable only by PCR, could be a consequence of very few copies, widely distributed without any functional meaning, or of a significant amount localised in selected cells or regions.

Using whole mount in situ hybridisation and immunohistochemistry we have now found that GRK4 is expressed in selected brain regions including cerebral cortex, hippocampus, dentate gyrus and cerebellar cortex. In the cerebellar cortex GRK4 is localised in the somato-dendritic region of the Purkinje cells, while virtually no mRNA hybridisation or immunoreactivity was detected on other cell types within the cerebellar cortex. Selective expression in Purkinje cells was only observed for GRK4. By contrast GRK5 and GRK6, which are structurally closely related to GRK4, are either not expressed (GRK5) or expressed at relatively low levels in the cerebellar cortex. Additionally, previous studies showed that GRK2 and GRK3, which are abundantly expressed in many brain regions, are found in different cell types within the cerebellar cortex (Arriza, 1992).
Substantial changes in GRK4 levels in Purkinje cells were identified during postnatal development. This kinase, which was barely detectable at P10, reached a maximal peak of expression at P20-P30 and then declined to moderate levels in adult animals (60 to 90 days-old). The first two-three weeks after birth represent a critical stage for the development of the architecture of cerebellar cortex (Altman, 1972; Shatz, 1990). During this time a developmentally regulated regression of the innervation of the Purkinje cells by climbing fibres (CFs) occurs in this region (Offermanns, 1997; Crepel, 1982); likely the key step for this process is a strong metabotropic activity that reduces the multiple CF innervation (Kano, 1997). This process finally results in a one-to-one relationship between a Purkinje cell and a CF that is found in the adult animal. Studies with mouse knock outs for the mGluR1 receptor, strongly indicate that mGluR1 receptors play a critical role in this mechanism (Kano, 1997). In mice lacking mGluR1, a persistent multiple climbing fibre innervation of cerebellar Purkinje cell was observed, which could account for the motor discoordination observed in these animals (Aiba, 1994; Conquet, 1994). The peak expression of GRK4 in Purkinje cells, around the third week, may therefore, be important for regulating the critical role of mGluR1 signalling at this stage of development.
CHAPTER 5

IDENTIFICATION AND REGULATION OF GRK4 SUBSTRATES

5.1 Two-hybrid screening to identify GRK4 interacting proteins.

The strategy to identify GRK4 physiological substrates was based on the two hybrid screening (Fields, 1989; Luban, 1995). The yeast two-hybrid assay is based on the observation that many eukaryotic transcriptional activators are composed of two separable, functionally independent domains: a DNA binding domain (DNA-BD) and a transcriptional activator domain (AD). Both domains are required to activate a gene and usually the two domains are part of the same protein. As an example for the GAL4 transcription factor the DNA-BD recognises and binds to a sequence in the upstream region of GAL4 responsive genes, while the AD interacts with other components of the transcription machinery (Ma, 1987). If physically separated and expressed in the same host cells, the GAL4-BD and AD do not interact with each other and cannot activate the responsive genes (Ma, 1988). When GAL4 DNA-BD and AD are fused independently with two genes encoding interacting proteins and expressed in the same host cell, the DNA-BD will be associated with the transcriptional AD. As a result of this interaction, a functional GAL4 transcription factor is reconstituted thus activate reporter genes (LacZα and HIS3), which are phenotypically detectable.

Little was known about the domains of GRK that might interact with substrates, consequently it was decided to use the entire GRK4γ (GRK4γ is the isoform present in human sperm cells) as a bait for the two hybrid assays and to screen this with a human testis cDNA
library. First the GRK4γ was subcloned into the pASII vector, to obtain a fusion protein between the activator domain of the GAL4 promoter and the GRK4γ. This construct was transformed into the yeast strain Y190 and grown on selective media. The expression of the fusion protein was tested by western blot analysis of proteins extracted from the transformed yeast using an antibody that recognises the GAL4 activator domain. Fig. 5.1 shows an immunoreactivity band at 78 Kd corresponding to the Mr of GRK4 plus GAL4 activator domain, that is absent in the wild type yeast.

To confirm that the two-hybrid assay was working properly, I performed a series of controls with several constructs supplied by the Matchmaker two-hybrid screening kit (Clontech); all of them worked properly. Unfortunately screening of the human testis cDNA library did not rescue any transformed yeast with the expected phenotype, such as the ability to grow in minimal medium without leucine, tryptophan and histidine and presence of β-galactosidase activity.

5.2 GRK induced desensitisation of human β2AR expressed in Sf9 cells

Recently an increasing number of proteins have been expressed and purified from heterologous expression systems, such as bacteria or eukaryotic cells: CHO, BHK, COS, HEK293, Sf9. G protein-coupled receptors, that contains seven hydrophobic regions, spanning the plasma membrane, can be expressed at considerable levels only in eukaryotic cells and in particular the baculovirus expression system has been extensively used (Kuhn, 1999; Moriya, 1999; Ross, 1994). I set up a method to study homologous desensitisation of GPCRs in Sf9 cells. Endogenous β2ARs are absent in Sf9 cells, and
Figure 5.1 Fusion protein between GRK4γ and GAL4-AD

One hundred μg of total yeast extract was electrophoresed on 10 % PAGE and blotted onto nitrocellulose membrane. Western blot analysis performed with the monoclonal antibody anti GAL4-AD is shown. WT, refers to wild type yeast; GAL4-GRK4γ, refers to the fusion protein between GRK4γ and GAL4-AD. Mr standards in kilodaltons are also indicated.
isoproterenol treatment does not change intracellular cAMP levels (Fig. 5.2). Cells infected for 48 hours with a β2AR-containing baculoviral vector show a functional coupling with stimulatory G proteins similar to the native system (Kleymann, 1993). The infection of Sf9 cells with the receptor (MOI=1) increased the basal cAMP content from 1 to 6 pmol/well, while 30 min stimulus with 1 μM of isoproterenol raises the cAMP levels to 23 pmol/well (Fig. 5.2). The desensitisation of β2AR by GRK2, (positive control), GRK4 and GRK5 was studied. When the cells were co-infected with β2AR and GRK2, the isoproterenol induced cAMP production were reduced by 25 %, compared to the receptor alone (Fig. 5.2). Co-infection with GRK4 or GRK5 reduced the cAMP production by 50 % (Fig. 5.2). The GRKs lowered the basal cAMP levels in β2AR infected Sf9 cells, but they did not alter the intracellular cAMP levels when infected alone (not shown). However, it is difficult to assess which kinase has the strongest desensitisation activity on β2AR, because their expression levels could not be directly compared.

These results indicate that functional β2ARs can be expressed in the baculovirus system and that the Sf9 cells can be used for studying the functional properties of receptors and their regulatory mechanisms. Moreover, it was shown for the first time that GRK4 can cause homologous desensitisation of β2AR.

5.3 mGluR1 receptor as candidate GRK4 substrate

Part of this study was aimed at identifying which GPCR might be regulated by GRK4. mGluR1 is a receptor mainly expressed in Purkinje cells (Takacs, 1997; Grandes, 1994; Shigemoto, 1992) and in addition, Northern blot analysis of rat testis, brain cortex and cerebellum showed (Fig. 5.3) that mGluR1 was undetectable at P10,
Figure 5.2 cAMP accumulation in Sf9 cells infected with β2AR and individual GRK

β2AR infected Sf9 were stimulated with the presence (+) or absence (-) of 1 μM isoproterenol and cAMP accumulation was evaluated after 30 min using a RIA kit. Results are a mean ± SE (pmol/well) for three individual experiments performed in triplicate. Mock, indicates the cAMP level measured in wild type Sf9. Cells infected with the receptor alone are marked Vec, while cells coinfected with the receptor and GRK are indicated by GRK2, GRK4α, or GRK5.
**Figure 5.3 Northern blot analysis of mGluR1**

Developmental profile of mGluR1 mRNA from rat testis (Te), brain cortex (B) and cerebellum (C) at P10, P20, P30, P60. Each lane contains 20 μg of total RNAs from the indicated tissues. After hybridisation with the radiolabelled rat mGluR1 probe, blot was washed at 60°C in 2 x SSC, 1 % SDS and exposed for autoradiography 2 days at -80°C. Positions of 28S (4.7 Kb) and 18S (1.9 KB) ribosomal RNAs, used as Mr markers, are indicated.
at P20 the expression increased in the cerebellum while it remained undetectable in the testis and brain cortex and at P30 it was present in the cortex and cerebellum. At P60 the mGluR1 expression in the cortex and cerebellum reached the adult level, while it was still undetectable in the testis. This mGluR1 expression profile is coincident with the expression profile described in section 4.2.3 for GRK4. Moreover an analysis of the topological distribution of GRK4 in rat brain paralleled well the distribution of mGluR1 receptor.

To determine whether mGluR1 is expressed in the testis, the major expression site for GRK4, PCR analysis was performed on several tissues outside the central nervous system and cerebellum (included as a positive control). An mGluR1 amplification band at the expected Mr was evident in cerebellum and testis only (Fig. 5.4a). To corroborate the PCR results and to detect which testicular cells express mGluR1, an immunohistochemical analysis of rat testis was performed. A positive mGluR1 immunoreactivity was observed inside the seminiferous tubules, and particularly, in spermatogonia, spermatocytes, spermatides and in sperm head (Fig. 5.4b). Neither Sertoli nor interstitial Leydig cells were labelled by the anti-mGluR1 antibody. This impressive colocalisation of mGluR1 and GRK4 suggests that they may be functionally linked. Based on the pattern of expression, as well as on the developmental profile of these two proteins, we hypothesised that mGluR1 could represent a reasonable candidate for being regulated by GRK4. In order to provide experimental evidence for this, we measured IP production in HEK293 cells transiently transfected to overexpress mGluR1 alone or along with GRK4.
Figure 5.4 Analysis of testis mGluR1 by PCR and immunohistochemistry

a: One μg of total RNA from rat testis was reverse transcribed and amplified with two oligos encompassing 859-1053 bp of rat mGluR1 (Nakanishi, 1991). Ten μl of the PCR reaction separated on a 2 % agarose gel showed a strong mGluR1 amplification in testis (Te) and cerebellum (C) used as a positive control; while no amplification arose from lung (L), thyroid (Th), and adrenal gland (A). M, indicates the Mr ladder. b, c: Immunohistochemical analysis of testis mGluR1 at low (b) and high (c) magnification. The staining can be observed inside the seminiferous tubules, in spermatogonia, spermatocytes spermatids and on the sperm head (arrow). Neither Sertoli nor interstitial Leydig cells are labelled by the anti-mGluR1 antibody.
5.3.1 IP production in HEK293 cells transiently transfected with mGluR1 receptor

In a heterologous expression system if a receptor is sensitive to a co-expressed GRK, then any agonist-stimulated second messenger production should be significantly reduced. This approach has been extensively used to demonstrate the regulation of a variety of GPCRs by different GRK subtypes (Aragay, 1998; Freedman, 1997; Freedman, 1996). This set of experiments has been performed in HEK293 cells instead of Sf9, as the baculoviral vectors for mGluR1 were not available. Moreover in HEK293 cells the heterologous expression of mGluR1 was already well established. mGluR1, transfected into HEK293, couples with the PLCβ and if stimulated results in the production of IP and diacylglycerol (Prezeau, 1996). The amount of IP produced upon stimulation is a measure of the activity of the transfected receptor, since HEK293 cells do not express endogenous mGluR1 receptor and do not respond to the agonist quisqualate (Prezeau, 1996). mGluR1a and mGluR1b differ only by 20 amino acids at their C-termini (Fig. 5.5) (Conn, 1997). mGluR1a transfected HEK293 cells treated with 100 μM quisqualate showed an increase of IP formation over the basal level. A similar effect was observed in mGluR1b-expressing cells (Fig. 5.6). No effect of quisqualate was observed in mock transfected-cells (Fig. 5.6). As previously reported (Prezeau, 1996; Carruthers, 1997; Mary, 1998) the basal IP production in mGluR1a expressing cells was about two fold greater than in mock-transfected cells (Fig. 5.6)

The basal IP level in mGluR1b transfected HEK293 cells was comparable to mock transfected cells (Prezeau, 1996); 100 μM quisqualate increased the IP production by about 280 %. In mGluR1a transfected cells the quisqualate increased the IP production by only
Figure 5.5 Schematic representation of mGluR1

The first 20 amino acids depicted in black correspond to the signal peptide. The putative glutamate (Glu) binding domain is indicated as the LIVBP homologous domain. In this region the hydrophobic segment proposed to form the binding pocket is boxed, and the amino acids involved in Glu binding are indicated with asterisks. The cysteine residues conserved among the mGluRs are indicated by the big black filled circles. The putative intracellular phosphorylation sites are indicated by the small black ball attached to the amino acid chain. The putative glycosylation sites are indicated by Y. The domains involved in the specificity of G protein activation are represented with thicker black circles. The position where alternative splicing between mGluR1a and mGluR1b occurs is indicated by the vertical black bar. Specific mGluR1b residues are in grey.
Figure 5.6 Transfection of HEK293 cells with mGluR1a but not mGluR1b leads to an increase in the basal PLC activity

HEK293 cells transfected with the carrier DNA alone (Mock) or expressing the mGluR1a or mGluR1b were labelled with [3H]inositol. After being washed with the HEPES buffered saline, cells were incubated for 30 min in the absence (-) or presence (+) of 100μM of quisqualate. Results are mean ± SE of 8 duplicate determinations. Data are expressed as percentage of the basal IP production measured in mock transfected cells. *, p<0.05 (paired t test) compared with mock transfected cells.
160 % however since the mGluR1a basal was already about 220 % of the mock transfected cells, the IP production in mGluR1a transfected cells upon stimulation was more than 350 % of the basal level. These data are the result of four separate experiments performed in parallel with mGluR1a and mGluR1b transfected cells where it is possible to compare directly the amount of IP formation. The expression level of mGluR1a towards mGluR1b was not evaluated because anti mGluR1b antibodies were not available; it was assumed that their expression levels were comparable since both receptors were cloned in the same vector. In a different set of experiments the mGluR1a and mGluR1b transfected cells were used separately so that the biological variability of the cells can impair a direct comparison.

Binding of the agonist to mGluR1 receptor reduces the IP response to subsequent stimuli. This rapid and agonist-dependent regulation is similar to the homologous desensitisation observed for several G-coupled receptors (Bunemann, 1999; Freedman, 1996). The molecular mechanisms of mGluR1 receptor desensitisation, which involves a decreased coupling of the receptor to Gq, are only partially understood. mGluR receptor is thought to be desensitised by PKC, but this is based only on pharmacological evidence; in addition different authors (Balazs, 1997; Yuzaki, 1992; Aronica 1993; Catania, 1990) reported the presence of PKC independent desensitisation.

To test if mGluR1 could undergo GRK-mediated homologous desensitisation, we measured the effect of quisqualate stimulation on the PLC response in HEK293 cells transfected with mGluR1 receptors, alone or in combination (cotransfection) with GRK4.

For HEK293 cells transfected with the human mGluR1a receptor cDNA, the IP accumulation was biphasic resembling the constitutive and agonist stimulated activity respectively, when the inositol phosphatases are blocked by LiCl. In cells cotransfected with cDNA
for mGluR1α and GRK4α the IP accumulation (in the presence of LiCl) was significantly reduced by 25 % (Fig. 5.7) after 30 min as compared to mGluR1α transfected cells. This reduction was consistent and displayed the same magnitude at the basal or quisqualic-stimulated IP production (Fig. 5.7a and 5.7b). These reductions in the IP accumulation will be referred as desensitisation. Moreover the concentration dependencies of quisqualate stimulated accumulation of IP, differed considerably between mGluR1 alone or in cotransfection with GRK4α (Fig. 5.7b); however the two curves were parallel, indicating that the GRK4 effect was primarily on the basal, i.e. agonist independent, activity. Likely the amount of mGluR1 present in the active conformation by chance (without external agonist added) is sufficient to engage all the transfected GRK4 and therefore no further effect could be seen after increasing the number of activated receptors by the agonist.

Augmented basal level of IP could also be a consequence of glutamate released from the cells in the culture medium. To examine this, dose-response curves of agonist induced IP accumulation were determined in HEK293 cells transfected with the glutamate transporter (Kanai, 1992). In this case the apparent agonist independent activity of GRK4 on mGluR1α was prevented and only agonist dependent effects were now observed (Fig. 5.8). Overexpression of different proteins were confirmed by Western blot analysis (not shown).

Cotransfection of HEK293 cells with mGluR1b and GRK4α cDNAs did not change the basal IP levels, when compared to receptor-transfected cells alone, but the kinase was able to reduce agonist-stimulated inositol phosphate production by 40 % (Fig. 5.9a). The dose-response curves of quisqualate-stimulated IP hydrolysis showed that the desensitisation effect of GRK4α on mGluR1b was completely
Figure 5.7 Homologous desensitisation of mGluR1α

**a:** HEK293 cells transfected with the carrier DNA alone (Mock), mGluR1α or mGluR1α plus GRK4α. IP accumulation was measured after 30 min in the absence (-) or presence (+) of 100μM of quisqualate. Data are expressed as percentage of the basal IP production measured in mock transfected cells. Results are mean ± SE of 10 duplicate determinations. *, p<0.05, (paired t test) compared with the mGluR1 transfected cells.

**b:** HEK293 cells were treated as in **a,** and IP accumulation was stimulated with growing doses of quisqualate. The open squares refer to receptor transfected alone, while the filled triangles refer to the cotransfection between mGluR1 and GRK4α.
Figure 5.8 Dose response desensitisation of mGluR1a transfected HEK293 cells

HEK293 cells were transfected with expression plasmid encoding the glutamate transporter (EAAC1), (Kanay, 1992), and mGluR1a along with the plasmid encoding either no proteins (filled diamonds), or GRK4α (open squares). Cells were processed for the agonist stimulated IP hydrolysis as described in section 2.3.2. Cells were stimulated with the indicated concentrations of quisqualate for 30 min. The signal above basal was obtained by subtracting from each data point the inositol phosphate value obtained from unstimulated control cells. Each value was then divided by the signal above basal and multiplied by 100 to obtain the percentage of control. The plot is representative of two separate experiments performed in duplicate.
Figure 5.9 Homologous desensitisation of mGluR1b

a: HEK293 cells transfected with the carrier DNA alone (Mock), mGluR1b or mGluR1b plus GRK4α. IP accumulation was measured after 30 min in the absence (-) or presence (+) of 100μM of quisqualate. Data are expressed as percentage of the basal IP production measured in mock transfected cells. Results are mean ± SE of 6 duplicate determinations. *, p<0.05, (paired t test) compared with the mGluR1b transfected cells. 

b: HEK293 cells were treated as in a, and IP accumulation was stimulated with increasing doses of quisqualate. The open squares refer to receptor transfected alone, while the filled triangles refer to the cotransfection between mGluR1b and GRK4α.
agonist dependent (Fig. 5.9b). Similar experiments were performed to assess if other GRK4 isoforms could regulate the mGluR1 receptors. Cotransfection of mGluR1 with GRK4α, GRK4δ, GRK4γ, or GRK4δ showed that only GRK4α and to a lesser extend GRK4δ significantly regulated the mGluR1a (Fig. 5.10a) signalling in this heterologous system; whereas, only GRK4α could regulate mGluR1b (Fig. 5.10b).

The GRK2 and GRK3 which are ubiquitously expressed, have been shown to desensitise a large variety of GPCRs (Pitcher, 1998a). GRK5 and GRK6 are members of the GRK4 subfamily which are selectively expressed in defined tissues. Their expression in Purkinje cells is negligible, but GRK6 seems to be coexpressed, with mGluR1 in different brain areas. Consequently, the ability of other GRKs to regulate mGluR1 was examined.

Cotransfection of the mGluR1 receptor with other GRKs (GRK2, GRK3, GRK5 or GRK6) were performed and the IP levels measured and compared to the receptor transfected alone. All the GRKs showed desensitisation activity on mGluR1a (Fig. 5.11a) and mGluR1b (Fig. 5.11b). Overexpression of different kinases were confirmed by Western blot analysis (not shown). To test if the experimental conditions were appropriate and selective, the ability of GRK2 and GRK4 to modulate the PAF, and serotonin (5-HT2A) receptors (two Gq linked GPCR like mGluR1) signalling was investigated. GRK2 showed a marked reduction of serotonin or PAF induced IP accumulation (Fig. 5.12a and b), GRK4 decreased the serotonin induced IP to a lesser extend compared to GRK2 (Fig. 5.12a), while GRK4 was ineffective on the PAF stimulated IP (Fig. 5.12b) accumulation.

These results indicated that the assay conditions and the GRK expression levels are adequate to obtain a specific regulation of the
Figure 5.10 Inhibition of mGluR1a and mGluR1b receptor signalling by GRK4 isoforms

**a**: HEK293 cells were cotransfected with mGluR1a and either the empty vector (Vec), or GRK4α, GRK4β, GRK4γ, GRK4δ. Transfected cells were metabolically labelled with [3H]inositol and stimulated for 30 min with 100 μM of quisqualate. Data were calculated as in Figure 5.8. The means ± SE from two separated experiments performed in duplicate are plotted. *, p<0.05, (paired t test) compared with the Vec transfected cells. **b**: Same as in a except that mGluR1b replaced mGluR1a cDNA.
Figure 5.11 Desensitisation of mGluR1a and mGluR1b by overexpressed GRKs

**a:** HEK293 cells transfected with mGluR1a cDNA and individuals GRK cDNAs, GRK4α, GRK5, GRK6, GRK2, or empty vector (Vec), were metabolically labelled with [3H]inositol and stimulated for 30 min with 100 μM of quisqualate. Data were calculated as in Figure 5.10. *, p<0.05, (paired t test) compared with the Vec transfected cells. **b:** Same as in **a** except that mGluR1b replaced the mGluR1a cDNA.
Figure 5.12 Receptor specific signal suppression by overexpressed GRKs

HEK293 cells were transfected with plasmids encoding the 5-HT$_{2a}$ receptor (a) or PAF receptor (b), along with plasmids encoding either no proteins (Vec), GRK2, or GRK4a. Cells were processed as described in section 2.3.2, using 10 μM serotonin or 10 nM PAF for 30 min to activate the receptors (+). Data are expressed as the percentage of the basal (-) receptor transfected alone. *, p<0.05, (paired t test) compared with the Vec +, transfected cells.
assayed receptors but possible artefacts due to the variation of receptor levels cannot be ruled out.

It is difficult to determine the exact density of mGluR1 receptors through binding experiments, because the $^3$H glutamate (the only ligand of mGluR1) has a low affinity for its receptor (Prezeau, 1996). Possible variations in the expression of mGluR1a were therefore examined by western blot analysis using a commercially available polyclonal antiserum (see section 2.2.4). A strong immunoreactive band was evident at Mr 150,000 (Fig. 5.13) in mGluR1a and mGluR1a plus GRK(n) transfected cells. The intensities were similar; when assessed by NIH image analyser the amount of mGluR1a in the presence of the kinase resulted to be slightly greater than the receptor transfected alone.

5.3.2 Analysis of mGluR1 internalisation in primary cultures of Purkinje cells

We have established the procedure for Purkinje cell primary culture, starting from 7 day-old rat cerebellum (Yuzaki, 1992). Immunohistochemical analysis of these cultured cells confirmed that GRK4 is expressed in the Purkinje cells, (identified by immunolabelling with calbindin), and showed that it colocalised with mGluR1 (Fig. 5.14a). Moreover, 30 min quisqualate-treatment induced a substantial translocation of the receptor from the plasma membrane to an intracellular compartment (usually referred to as receptor internalisation) and the GRK4 associated immunofluorescence was colocalised with the receptor at the subcellular level during this internalisation (Fig. 5.14b).
Figure 5.13 Western blot analysis of mGluR1 transfected HEK293

For comparative purposes the mGluR1 immunoreactivity was analysed in HEK293 transfected alone and in combination with, GRK4α, GRK5, GRK6, or GRK2. The blot was revealed with anti rat-mGluR1 antibody, the immunoreactive bands are indicated (mGluR1a). The molecular weight standards in Kd are indicated on the left.
Figure 5.14 Confocal analysis of GRK4 and mGluR1 in Purkinje cells primary culture

Confocal images of Purkinje cells, treated for 5 min with 100 μM of quisqualate (5 min) or untreated (0 min). GRK4 was localised using K-20 antiserum and revealed with Alexa-488-conjugated anti rabbit IgG (red colour). The mGluR1 was detected with Alexa-594-conjugated anti rat-mGluR1 (green colour). Electronic merge of GRK4 and mGluR1 immunofluorescence is shown in the lower panels (Merge). The arrows indicate one of the vesicles where GRK4 and mGluR1 are colocalised.
5.4 GRK4 as candidate gene involved in cerebellar ataxia associated with sperm abnormalities

Evidence is now accumulating that altered G protein mediated signal transduction plays a major role in the pathogenesis of several human diseases (Rockman 1997; Pitcher, 1998a; Themmen, 1996). Although GRK are key regulators of a variety of G-coupled receptors, the possible involvement of these kinases in human pathological conditions has been poorly investigated so far. Limiting factors for extensive analysis of GRK structure and function in relation to human diseases can be summarised as follows: I) despite the evidence that many different G-coupled receptors are regulated by GRKs, the functional role of such regulation in vivo remains poorly defined; II) the majority of GRK isoforms are ubiquitously expressed. This makes it difficult to relate a specific GRK to a specific disease.

GRK4, unlike the majority of GRKs, is expressed selectively in only a few cell types, in particular in specialised organelles (i.e. the acrosome) of sperm cells which are essential for fertilisation, and in the somato-dendritic part of Purkinje cells, where it might play a role in the regulation of Purkinje cells, including the long term depression (LTD) (Levenes, 1998; Daniel, 1998). It has been documented that the a GPCR complex (including mGluR1) is responsible for LTD, a phenomenon which eventually leads to neuronal degeneration (cerebellar cortex atrophy) possibly resulting in ataxia (Aiba, 1994). Based on its unique profile of tissue distribution and ability to modulate mGluR1 signalling, it was speculated that a genetic alteration of GRK4 could result in cerebellar alterations (such as cerebellar atrophy and ataxia), associated with sperm abnormalities. In collaboration with "Centro delle Encefalo-Neuro-Miopatie
Genetiche, Università degli Studi di Siena”, we selected three members of two unrelated families presenting both clinical symptoms (Malandrini, 1993). These patients presented moderate ataxia, marked cerebellar atrophy at magnetic resonance imaging (MRI), low level of intelligence associated with marked morphological abnormalities of the majority of spermatozoa (> 80%). By contrast testis size and endocrine parameters were normal (Malandrini, 1993).

5.4.1 Preliminary genetic analysis of GRK4 gene

There are not many studies on the genetic variability of GRK4. Preliminary data indicate that GRK4 may be high polymorphic as compared to other members of the GRK family, since both non synonymous and synonymous mutations have been observed (Ambrose, 1992; Premont, 1996). An Ala-Val and a Val-Ala substitution in codons 454 and 142, were reported (Ambrose, 1992; Premont, 1996). However, this change in the amino acid sequence is a common polymorphism with allele frequencies on normal chromosomes of 54 % and 46 % for Ala and Val respectively, usually without effect on protein functionality. Neither population or functional studies have been made for the other amino acid polymorphisms (Premont, 1996). The analysis of the GRK4 gene, using published sequences, was done to investigate the possible presence of di-tri nucleotide repeat regions, which are possible sources of length polymorphisms. In the 5' untranslated region, two tri-nucleotide (GGC/CCG) repeat regions were found; they are constituted by five and six units (Ambrose, 1992). These potential length polymorphisms are reminiscent of the sequence at the fragile X mental retardation 1 (FMR1) locus that undergoes expansion in fragile X-associated mental retardation (Verkerk, 1991). However, in
both regions, the units are not regular, showing the deletion of a guanine in the fourth unit of the first region and the transversion from the second guanine to a cytosine in the first and in the last unit repeat of the second region. Strong expansions in the number of triplet units, i.e. CAG, CTG, CGG, were found in many hereditary ataxias in both coding and non-coding regions (Fu, 1991; Mitas 1997; Reddy, 1997). These data suggest that the 5' region might be a hot spot for gene alterations. Further sequence analysis identified other repeat areas in the intronic regions of GRK4: they are constituted by repetition of TG units, and are placed 5' and 3' flanking introns of exon 14; they are constituted by 34 and 18 repeat units, respectively.

In attempt to verify some size differences in tri-nucleotide repeat regions among affected and non-affected individuals, an amplification of about 300 bp was carried out in the 5' untranslated region over the two affected brothers (cerebellar ataxia associated with sperm abnormalities), and the healthy sister. A unique, clean product was obtained, (Fig. 5.15) but no size differences were detected. The study is continuing in two parallel directions: I) amplifying all the exons and intron/exon junctions and analysing any mutations by direct sequencing of the PCR products II) amplifying the repeat regions and analysing the PCR products by PAGE.
Figure 5.15 PCR analysis of length polymorphism

Ten ng of genomic DNA were amplified by PCR as described in section 2.1.4.3, and separated on 3 % NuSieve 3:1 agarose. The two affected brothers (B1, B2) and the healthy sister (Si), are shown. ST, indicate the \( \Phi X \) 174 DNA, HaellIII digests, as a standard.
5.5 discussion

5.5.1 Novel GRK4 substrates

This part of the study was aimed at identifying a physiological substrate for GRK4. One possible approach was by screening a cDNA library to identify GRK4 interacting proteins. Since it is unclear which GRK domains interact with the substrate or if the expression of a single domain is able to assume the correct three dimensional structure to interact with the substrate, the entire protein was used as a bait. Two-hybrid screening of a testis cDNA library using GRK4γ as a bait did not rescue any clone with the expected phenotype. One possible explanation for this, is that the majority of GRK4 is not free to interact with the prey because palmitoylation localises the protein to the membrane.

One problem in identifying physiological GRK substrates is their wide tissue distribution. The physiological substrate of GRK1 was likely to be rhodopsin since it is expressed only in the retina (Pitcher, 1998a; Chuang, 1996a). The highly selective localisation of GRK4, together with the knowledge accumulated so far about other GRKs (Freedman, 1996) should provide some insight into which receptor substrate interacts with this kinase. The present study localised the GRK4 to the sperm acrosome and to Purkinje cells. The GRK4 expression in the cerebellum at different developmental stages paralleled mGluR1 expression. Frungieri, 1996, reported that glutamic acid is present in the testis and epididymis but so far, in the literature, mGluR1 was reported to be expressed exclusively in the central nervous system (Conn, 1997; Ozawa, 1998). To strengthen the hypothesis that mGluR1 could be the physiological substrate for
GRK4, testis was analysed for mGluR1 expression by PCR. Amplification of mGluR1 from different tissues including cerebellum and testis revealed that mGluR1 transcript was present in testis mRNA. Immunohistochemical analysis also demonstrated that mGluR1 is expressed on the spermatogenic line including the head of mature sperm. These data suggest that mGluR1 could be a good candidate to be regulated by GRK4. In addition several studies reported (Aronica, 1993; Yuzaki, 1992) that a component in the desensitisation of mGluR1 involves mechanisms other than phosphorylation by PKC or ADP-ribosylation but is homologous (GRK induced?) and reversible (Balazs, 1997).

5.5.2 mGluR1 regulation by GRK

To test whether mGluR1 is regulated by GRK4 the proteins were co-expressed in a heterologous system (HEK293 cells) to see whether the presence of GRK4 could result in reduced mGluR1-mediated second messenger production. This approach has been used extensively to demonstrate the role of different GRKs in the regulation of GPCRs (Freedman, 1996; Pitcher, 1998a). We found that, in the presence of GRK4, IP production stimulated by mGluR1a and mGluR1b is significantly reduced, indicating that these receptor subtypes can be regulated by GRK4. Western blot analysis performed on human mature sperm cells showed that GRK4γ is the only detectable GRK4 isoform, while GRK4γ and GRK4δ are the isoforms revealed on bovine sperm. PCR analysis performed on whole testis RNA showed that the longer isoforms are most abundantly expressed. The likely explanation for this apparent discrepancy is that the longer isoforms could be expressed at earlier stages of spermatogenesis, to regulate a classical GPCR, since only GRK4α is
able to phosphorylate the rhodopsin and desensitise the mGluR1. The shorter isoforms are likely expressed in mature sperm cells, where they could phosphorylate a novel substrate, since they are inactive on the classical GRK substrate. According to previous results, the overexpression of the mGluR1a subtype resulted in enhanced second messenger production even in the absence of the agonist, suggesting that this receptor, unlike the mGluR1b subtype, possesses a marked constitutive activity (Prezeau, 1996). According to Mary, 1998, the strong agonist independent activity of mGluR1a is due to the particularly long intracellular C-terminus that suppress the inhibitory action of a cluster of basic residues present in the other mGluR receptor which confer specific PLC-coupling properties including the absence of constitutive activity. However the high mGluR1a basal activity could also be accounted for by the release of glutamate from the cells in the culture medium (Desai, 1995). In native systems such as neurones, the glutamate transporter mediates uptake of released glutamate, to keep its extracellular concentration below the active concentration.

However, in our experimental conditions mGluR1a basal activity is mainly due to glutamate released from the cells.

In fact when the EAAC1 glutamate transporter was co-transfected, it was able to abolish the apparent mGluR1 basal activity likely re-uptaking glutamate present in the medium.
CHAPTER 6

Final discussion

GRK4 is not the only GRK identified in sperm, but a previous study showed that in the mid piece of rat sperm, GRK3 and \( \beta \)-arrestin2 are colocalised with odorant-like receptors that are likely substrates for these desensitisation proteins (Walensky, 1995). GRK3 and \( \beta \)-arrestin2 however are widely distributed in different organs and cells, with the highest expression in brain and leukocytes (Arriza, 1992; Parruti, 1993a), while for GRK4, sperm represents one out of two relevant sites of expression. We therefore hypothesised that one physiological role of GRK4 could be highly specific for and strictly related to the function of spermatozoa.

The present study documents that GRK4 is not associated with plasma membranes but rather with membranes of specialised intracellular structures such as mitochondria, the acrosome and vesicles. This is a totally unpredicted finding that substantially expands the general view that GRKs are kinases transiently or stably associated with plasma membranes where they phosphorylate and regulate surface GPCR. One previous study on rat liver (Garcia-Higuera, 1994b) reported the association of a substantial fraction (39\%) of GRK2 with endoplasmic reticulum, while the majority of this kinase in the same cells was found in the cytosol (43\%) or associated with the plasma membranes (18\%).

Our results also raise the question of the possible function of GRK4 on these intracellular structures. The acrosome is a highly specialised vesicle, localised on top of the sperm head, involved in fundamental steps of fertilisation, including capacitation, acrosome reaction and interaction with mature eggs (Cohen-Dayag, 1994,
Zaneveld, 1993; Zaneveld, 1991). The capacitated spermatozoa have the potential to undergo the acrosome reaction as soon as they bind to the zona pellucida (ZP) of the egg. Binding to ZP proteins leads to signal transduction and, in turn, to an exocytotic event (Snell, 1996). It involves the fusion and vesiculation of the outer acrosomal membrane with the surrounding plasma membrane and culminates in the dispersal and release of the acrosome contents (mostly proteolytic enzymes), (Wassarman, 1995). As a consequence the inner acrosomal membrane on which GRK4 is found on the cytoplasmic side is exposed, resembling the classical localisation for a GRK. Once sperm has undergone the acrosome reaction, penetrated the ZP, and reached the perivitelline space between egg plasma membrane and ZP, it can bind to and fuse with egg plasma membrane (Wassarman, 1995). Finally the entire sperm cell penetrates the oocyte (Wassarman, 1995; Raz, 1998). The acrosome is therefore a highly specialised structure involved in a variety of serial events fundamental to fecundation.

No obvious roles for GRK4 can be envisaged in these processes although surface recognition between oocyte and spermatozoon may be affected by this kinase. It is noteworthy that, after the acrosomal reaction, GRK4 appears to be found on the cytoplasmic side of the inner acrosomal membrane which, at that stage, works as a plasma membrane and is the site where the surface recognition processes of the spermatozoon occurs. A large family of proteins, called disintegrins, are involved in mammalian gamete fusion, and binding to the egg integrins (Wassarman, 1995). Additionally there is evidence indicating a role of GPCRs including PAF (Reinhardt, 1999), muscarinic (Baccetti, 1995), odorant-like (Vanderhaeghen, 1997), atrial natriuretic peptide (Rotem, 1998), and prostaglandin receptors (Shimizu, 1998), in sperm motility and acrosome reaction. The
presence of orphan heptahelical receptors or mGluR1 in sperm has also been reported (Meyerhof, 1991 and present study). All these receptors that are mainly coupled to G proteins identified on the plasma and/or outer acrosomal membranes, can be potentially regulated by phosphorylation from kinases of the GRK family.

The mechanisms that regulate mGluR glutamate receptors have been, at least in part, elucidated by previous studies. It was demonstrated that mGluR1 can be desensitised and that PKC is involved in this process, although a PKC-independent component of mGluR1 desensitisation was also documented (Aronica, 1993). An increased intracellular calcium, either induced by NMDA receptor activation or released by intracellular stores, seems to play an important role in the activation of PKC or more generally in the regulation of group I mGluR-mediated responses (Kawabata, 1996). RGS proteins are a recently discovered family of proteins (~20 members identified so far) which can bind to the α subunit of heterotrimeric G proteins and stimulate their GTPase activity thus reducing G protein signalling. RGS proteins selectively interact with Ga1 and Gaq, but not with Gas (De Vries, 1999, Hepler, 1999; Zerangue, 1998; Berman, 1998). RGS proteins are also involved in the regulation of glutamate receptors. It was documented that RGS4 markedly attenuated the mGluR1a and mGluR5a-mediated response (Saugstad, 1998).

The present study provides the first evidence that a GRK, and in particular the GRK4 subtype, is involved in the regulation of mGluR receptors. This is the first report that GRKs can regulate receptors of the third GPCR subfamily (Barnes, 1998 and section 1.5.2). The present findings add further support to the idea that GPCR-mediated signalling is strictly controlled by multiple and redundant molecular mechanisms that allow the cell to be exposed to the optimal level of

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stimulation. In the case of the group I mGluR, different regulators are likely involved which can act at the receptor level (such as GRK4 and PKC) (Nakanishi, 1998) and at the level of the Gq (such as RGS4), (Saugstad, 1998). Intracellular messengers such as calcium can, in turn modulate these regulatory processes (Kawabata, 1996). In this regard it is interesting that GRKs are modulated by calcium sensor proteins, in particular it was demonstrated in this study that GRK4 kinase activity is inhibited by CaM in a calcium dependent manner through a physical protein-protein interaction. A rise of intracellular calcium, which can activate PKC, can, in turn result in inhibition of GRK4 through CaM. The possible roles of other calcium sensor proteins present in Purkinje cells (such as hippocalcin) in the regulation of GRK4 is presently being investigated in our laboratory.

What are the relative contributions of these regulatory mechanisms to mGluR1-mediated signalling and is there a link to the process of LTD (De Zeeuw, 1998) and motor learning? These are important question still to be addressed. A physiological role for GRK4 can be envisaged; when glutamate is released at the synapse of the climbing fibre on the Purkinje neurone it stimulates the mGluR1 that is coupled to Gαq and stimulates PLC: this results in a depolarisation and an influx of Ca2+. Repeated increases in the concentration of Ca2+ will induce LTD of the active parallel fibre synapses, and potentiation of all inhibitory synapses. A defect in one of the proteins of the mGluR1 complex, could lead to the impairment seen in LTD (Aiba, 1994; Levenes, 1998; Inoue, 1998). The mGluR1 is likely to be regulated by homologous desensitisation through a GRK, probably GRK4 and this means that a functionally defective GRK4 could lead to the over stimulation of Purkinje cells and their degeneration. Genetic analysis of candidate patient families for GRK4 alteration has been initiated in the present study and is ongoing.
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>(d)ATP</td>
<td>(deoxy)adenosine triphosphate</td>
</tr>
<tr>
<td>(d)CTP</td>
<td>(deoxy)cytidine triphosphate</td>
</tr>
<tr>
<td>(d)GTP</td>
<td>(deoxy)guanosine triphosphate</td>
</tr>
<tr>
<td>(d)TTP</td>
<td>(deoxy)thymidine triphosphate</td>
</tr>
<tr>
<td>AD</td>
<td>activator domain</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>β2AR</td>
<td>β2 adrenergic receptor</td>
</tr>
<tr>
<td>bp</td>
<td>base-pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C-terminal</td>
<td>carboxy terminal</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
</tr>
<tr>
<td>CaMBd</td>
<td>calmodulin binding domain</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3' 5'-cyclic-monophosphate</td>
</tr>
<tr>
<td>CFs</td>
<td>climbing fibres</td>
</tr>
<tr>
<td>cGMP</td>
<td>guanosine 3' 5'-cyclic-monophosphate</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CIP</td>
<td>calf intestinal alkaline phosphatase</td>
</tr>
<tr>
<td>CLB</td>
<td>cell lysis buffer</td>
</tr>
<tr>
<td>CSP</td>
<td>calcium sensor protein</td>
</tr>
<tr>
<td>DAB</td>
<td>3 5'-diaminobenzidine</td>
</tr>
<tr>
<td>DIG-11-UTP</td>
<td>digoxigenin-11-uridine triphosphate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified minimal essential medium</td>
</tr>
<tr>
<td>DNA-BD</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotides triphosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EP3</td>
<td>prostaglandin E receptor 3</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated protein kinase</td>
</tr>
</tbody>
</table>
F forward primer
FCS foetal calf serum
FSH follicle stimulating hormone
g gravity
Gα G protein alpha subunit
GPCR G protein-coupled receptor(s)
GRK G protein-coupled receptor kinase(s)
GST glutathione S-transferase
HEK293 human embryonic kidney
HEPES (N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid)
i1 intracellular loop
IP inositol phosphate
IPTG isopropyl-β-D-thiogalactoside
Kb kilobases
LB Luria Broth
LH-CG luteinizing-chorionic gonadotropin
LTD long-term depression
LTP long-term potentiation
M-MLV Moloney murine leukaemia virus
MAB maleic acid buffer
MAPK mitogen activated kinase
Meta II metarhodopsin II
mGluR1 metabotropic glutamate receptor 1
MOI multiplicity of infection
MOPS 3-(N-morpholino)-propanesulfonic acid
MRI magnetic resonance imaging
N-terminal amino terminal
NCS neuronal calcium sensor
NGS normal goat serum
P postnatal day
PAF platelet activating factor
PAGE polyacrylamide gel electrophoresis
PBS phosphate buffered saline
PC phosphatidylcholine
PCR polymerase chain reaction
PDE phosphodiesterase
PH pleckstrin homology
PIP2 phosphatidylinositol 4,5-bisphosphate
PKA protein kinase A
PKC protein kinase C
PLC phospholipase C
PS phosphatidylserine
R reverse primer
RGS regulatory of the G protein signalling
RK rhodopsin kinase
RPM revolution per minute
SDS sodium dodecyl sulfate
SDSL site-direct spin labelling
Sf9 *spodoptera frugiperda*
SPR surface plasmon resonance
ßARK ß-adrenergic receptor kinase
SSC sodium chloride/sodium citrate
SSTR2 somatostatin receptor 2
TAB Tris-acetate buffer
TBS Tris buffered saline
TM transmembrane helices
Tris tris(hydroxymethyl)aminomethane
TSH thyroid stimulating hormone
X-gal 5-bromo-4-chloro-3-indolyl-beta-D-galactoside
ZP zona pellucida
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