Calcium puffs are generic InsP$_3$-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses.
Calcium puffs are generic InsP₃-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses

Stephen C. Tovey¹, Patrick de Smet²,*, Peter Lipp¹,†, David Thomas¹, Kenneth W. Young³, Ludwig Missiaen², Humbert De Smedt², Jan B. Parys², Michael J. Berridge¹, Jan Thuring⁴, Andrew Holmes⁴ and Martin D. Bootman¹,⁵

¹Laboratory of Molecular Signalling, The Babraham Institute, Babraham, Cambridge, CB2 4AT, UK
²Laboratory of Physiology, K.U. Leuven Campus Gasthuisberg O/N, Herestraat 49, B-3000 Leuven, Belgium
³Department of Cell Physiology and Pharmacology, Medical Sciences Building, University of Leicester, University Road, Leicester, LE1 9HN, UK
⁴Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge, CB2 1EW, UK
⁵Department of Zoology, University of Cambridge, Downing Street, Cambridge, CB2 3EJ, UK

*These authors contributed equally to this study
†Author for correspondence (e-mail: peter.lipp@bbsrc.ac.uk)

Accepted 7 August 2001

SUMMARY

Elementary Ca²⁺ signals, such as ‘Ca²⁺ puffs’, which arise from the activation of inositol 1,4,5-trisphosphate receptors, are building blocks for local and global Ca²⁺ signalling. We characterized Ca²⁺ puffs in six cell types that expressed differing ratios of the three inositol 1,4,5-trisphosphate receptor isoforms. The amplitudes, spatial spreads and kinetics of the events were similar in each of the cell types. The resemblance of Ca²⁺ puffs in these cell types suggests that they are a generic elementary Ca²⁺ signal and, furthermore, that the different inositol 1,4,5-trisphosphate isoforms are functionally redundant at the level of subcellular Ca²⁺ signalling. Hormonal stimulation of SH-SY5Y neuroblastoma cells and HeLa cells for several hours downregulated inositol 1,4,5-trisphosphate expression and concomitantly altered the properties of the Ca²⁺ puffs. The amplitude and duration of Ca²⁺ puffs were substantially reduced. In addition, the number of Ca²⁺ puff sites active during the onset of a Ca²⁺ wave declined. The consequence of the changes in Ca²⁺ puff properties was that cells displayed a lower propensity to trigger regenerative Ca²⁺ waves. Therefore, Ca²⁺ puffs underlie inositol 1,4,5-trisphosphate signalling in diverse cell types and are focal points for regulation of cellular responses.

Key words: Calcium, Signalling, Inositol 1,4,5-trisphosphate

INTRODUCTION

Stimulation of cells with hormones that activate the enzyme phospholipase C (PLC) often evoke spatially and temporally complex intracellular Ca²⁺ signals (Berridge, 1993; Petersen et al., 1994; Berridge et al., 1998). The link between PLC and Ca²⁺ signalling is the intracellular messenger inositol 1,4,5-trisphosphate (InsP₃), which diffuses from its site of production into the cytosol and binds to specific Ca²⁺-releasing channels (InsP₃ receptors; InsP₃Rs) (Berridge, 1993; Berridge et al., 2000).

Three isoforms of InsP₃Rs have been defined, each encoded by a different gene. Most, if not all, individual cells express multiple isoforms, which can combine in either homo- or heterotetramers (De Smedt et al., 1994; Wojcikiewicz, 1995; Taylor et al., 1999). Characterization of purified receptors, recombinant receptors or cell types expressing various levels of each InsP₃R isoform have suggested that, although there is considerable functional redundancy between the InsP₃R isoforms, there might also be some isotype-specific regulation of Ca²⁺ signalling (Taylor, 1998; Taylor et al., 1999; Patel et al., 1999).

InsP₃Rs are acutely regulated by many factors, including phosphorylation, ATP, pH, accessory proteins, luminal Ca²⁺ and cytosolic Ca²⁺ (Taylor, 1998; Taylor et al., 1999; Patel et al., 1999). In addition to acute regulation of InsP₃Rs, modulation of Ca²⁺ signalling is brought about by changes in InsP₃R expression during long-term hormonal stimulation (Wojcikiewicz et al., 1994a) and cellular development and differentiation (Parrington et al., 1998; Brind et al., 2000; Jellerette et al., 2000).

It has been demonstrated that persistent activation of cell surface hormone receptors coupled to PLC leads to a decrease of InsP₃R content. This phenomenon has been observed with all three InsP₃R isoforms in a range of cell types (Wojcikiewicz et al., 1994a; Wojcikiewicz, 1995; Sipma et al., 1998; Young et al., 1999). The reduction of InsP₃Rs is a specific process because the expression of other proteins involved in Ca²⁺ signalling (apart from agonist receptors themselves) is not simultaneously modulated. Such downregulation of InsP₃Rs...
results from a profound acceleration of InsP3 R protein degradation (Wojcikiewicz et al., 1994a) initiated by InsP3 binding to its receptor (Zhu et al., 1999), and involves the ubiquitin-proteasome pathway (Oberdorf et al., 1999; Zhu and Wojcikiewicz, 2000). Although biochemical aspects of downregulation are well documented, the functional consequences of the decrease in cellular InsP3 R content on the characteristics and generation of intracellular Ca2+ signals have not been extensively characterized.

Hormone-evoked Ca2+ signals are commonly observed as Ca2+ waves in which an initial Ca2+ increase in a subcellular region triggers a regenerative propagation of the Ca2+ signal throughout the cell; a ‘global’ response (Boorman and Berridge, 1995; Berridge et al., 1998). Such Ca2+ waves can occur repetitively, giving rise to a series of Ca2+ spikes or oscillations (Jacob et al., 1988; Thomas et al., 1991; Thomas et al., 1996; Berridge, 1997).

We have previously found that the initiation and propagation of global Ca2+ signals in HeLa cells relies on the spatiotemporal recruitment of ‘elementary’ Ca2+ release events (Boorman et al., 1997a; Bootman et al., 1997b). Parker and colleagues denoted these localized InsP3R-dependent events as ‘Ca2+ puffs’ (Parker and Yao, 1991; Yao et al., 1995). The non-stereotypical nature of Ca2+ puffs indicates that they arise from sites containing variable numbers of InsP3Rs (Sun et al., 1998; Thomas et al., 1998).

When a cell is stimulated with a Ca2+-mobilizing hormone, there is usually a period of several seconds (‘latency’) before a global Ca2+ wave is observed. The recruitment of Ca2+ puffs occurs during this latency, and the cumulative activity of Ca2+ puffs provides the pacemaker Ca2+ rise necessary to trigger an ensuing regenerative response via the process of Ca2+-induced Ca2+ release (CICR) (Bootman et al., 1997b; Bobanovic et al., 1999; Marchant et al., 1999). Once triggered, the Ca2+ wave spreads throughout the cell in a saltatory manner, reflecting the sequential activation of elementary Ca2+ release sites spaced ~1-6 μm apart (Bootman et al., 1997a; Callamaras et al., 1998).

Surprisingly, in most HeLa cells, only one or a few ‘pacemaker’ Ca2+ puff sites are active during the latency, and the activity of these few individual sites determined whether a global Ca2+ wave or an abortive response was evoked. Repetitive stimulation of a cell consistently recruited the same pacemaker Ca2+ puff site (Bootman et al., 1997b). The consistent recruitment of pacemaker puff sites by repetitive stimulation is in accordance with earlier video imaging studies of Ca2+ signals in several cell types, which indicated that InsP3-dependent Ca2+ waves usually arise from a conserved cellular region (Rooney et al., 1990; Bootman and Berridge, 1996; Simpson et al., 1997).

In the present study, we examined the characteristics of Ca2+ puffs in various cell types that expressed different levels of the three InsP3Rs. Our data indicate that the characteristics of the Ca2+ puffs did not significantly differ between the cell types, consistent with such signals being a generic elementary building block for Ca2+ signals in non-excitable cells. Although there were no discernable differences in the elementary Ca2+ signals themselves, some cell types varied in the ways in which they recruited Ca2+ puffs. In addition, we examined the dynamic regulation of Ca2+ puffs during prolonged cell stimulation. These data suggest that reduction of InsP3 R expression has a profound effect on the activity of Ca2+ puffs, with the consequence that cells show a lower propensity to trigger regenerative global Ca2+ signals.

**MATERIALS AND METHODS**

**Cell culture**

All cells, except HUVECs, were cultured in Eagle’s minimum essential medium supplemented with glucose (2 mM), penicillin (55 units ml−1), streptomycin (55 mg ml−1), and serum (5%) of a 1:1 foetal: newborn bovine mixture. Cells were incubated in a humidified atmosphere (5% O2: 95% CO2; 37°C), with the culture media replaced every other day, and cells passaged when they reached ~80% confluence. In preparation for experiments, cells were plated onto glass coverslips and returned to the incubator for 24–48 hours to ensure adequate adhesion. Some of the cell lines were obtained from the American Type Culture Collection (HeLa cells, CCL-2; SH-SY5Y, CRL2266; NIH-3T3, CRL1658; RBL-2H3, CRL2256). The 16HBE14o− cells were a kind gift of Dieter C. Gruneret (University of Vermont, Colchester, USA). The primary HUVECs were obtained from Clonetics (BioWhittaker, Walkersville, MD, USA) and were grown, as specified, in the vendor’s Endothelial Cell Growth Medium.

**Calcium imaging**

Prior to imaging the culture medium was replaced with an extracellular medium (EM) containing: NaCl, 121 mM; KCl, 5.4 mM; MgCl2, 0.8 mM; CaCl2, 1.8 mM; NaHCO3, 6 mM; glucose, 5.5 mM; HEPES, 25 mM; pH 7.3. Cells were loaded with fluo-3 by incubation with 2 μM fluo-3 acetoxyethyl ester (Molecular Probes) for 30 minutes, followed by a 30 minute de-esterification period. All incubations and experiments were carried out at room temperature (20-22°C). Confocal cell imaging was performed as described elsewhere (Boorman et al., 1997b). Briefly, a single glass coverslip was mounted on the stage of a Nikon Diaphot inverted microscope attached to a Noran Oz laser-scanning confocal microscope, equipped with a standard argon-ion laser for illumination. Fluo-3 was excited using the 488 nm laser line and the emitted fluorescence was collected at wavelengths >505 nm. Images were acquired using the confocal microscopes in image mode at 7.5 Hz. Off-line analysis of the confocal data was performed using a modified version of NIH Image. Absolute values for Ca2+ were calculated according to the equation:

\[
[Ca^{2+}] = K_d (f - f_{\text{min}}) / (f_{\text{max}} - f)
\]

f is the fluorescence intensity of fluo-3 recorded during the experiment. fmin and fmax are the minimal and maximal fluorescence intensities of fluo-3, reflecting the calcium-free and calcium-saturated forms of the indicator, respectively. fmin and fmax were determined by permeabilizing the cells with A23187 in the presence of 10 mM EGTA or 10 mM CaCl2 respectively. The Kd of fluo-3 for Ca2+ inside cells was determined empirically to be 810 nM (Thomas et al., 2000b). The cells were typically monitored for 1–5 minutes, during which time a sufficient number of elementary events could be recorded without serious bleaching of the indicator. For video imaging using Fura2, a coverslip bearing Fura2-loaded adherent cells was mounted on the stage of a Nikon Diaphot inverted epifluorescence microscope. Fluorescent images were obtained by alternate excitation at 340 nm and 380 nm (40 milliseconds at each wavelength) using twin xenon arc lamps (Spex Industries, Edison, NJ, USA). The emission signal at 510 nm was collected by a charge-coupled device intensifying camera (Photonics Science, Robertsbridge, UK), and the digitized signals were stored and processed using an Imagine image processing system (Synoptics, Cambridge, UK) as described previously (Boorman et al., 1992).

**Western blotting and immunohistochemistry**

The methods used were similar to those described previously (Tovey
The InsP3 R-type-1-specific polyclonal antibody (Parys et al., 1995) was used at a 1:1000 dilution. The InsP3 R-type-2-specific KM1083 monoclonal antibody was a kind gift of K. Mikoshiba and was used at 1 µg ml⁻¹. The InsP3 R-type-3-specific antibody was obtained from Transduction Laboratories (Lexington, KY, USA) and was used at 0.25 µg ml⁻¹ (i.e. the recommended 1:1000 dilution). After incubation with the primary antibodies, the blots were washed three times for 10 minutes each in TTBS, and incubated with secondary antibody (HRP Conjugates, 1:3000, Sigma) for 1 hour at room temperature. The blots were then washed three times for 10 minutes each. The blots were developed using Amersham ECL reagents and the intensities of the bands were measured using NIH Image.

The immunostaining was performed using methods described previously (Lipp et al., 2000). Briefly, the cells were fixed in 4% w/v paraformaldehyde in PBS for 30 minutes at room temperature and permeabilized with Triton (0.2% v/v in PBS). The cells were then blocked for 30 minutes using 3% w/v bovine serum albumin, 0.1% v/v Triton in PBS. The protocol was then the same as for the western blotting described above, except that PBS was used in place of TTBS, and the cells were incubated overnight at 4°C in the blocking solution. The primary antibodies were all at the same concentrations given above and the secondary antibodies were all fluorescein labelled. The confocal immunofluorescent images were obtained using an UltraView microscope (PerkinElmer Life Sciences, Cambridge, UK).

For the InsP3 R downregulation experiments, western blotting was performed as previously described (Sipma et al., 1998). Briefly, microsomes from SH-SY5Y and HeLa cells were prepared according to Parys et al. (Parys et al., 1995). Microsomal proteins were analysed by SDS-PAGE on a 3-12% linear gradient polyacrylamide gel and transferred to Immobilon-P (Millipore corporation, USA). Blots were blocked for 1 hour in buffer (KH₂PO₄, 10 mM; NaH₂PO₄, 32 mM; NaCl, 154 mM; Tween-20, 0.1%; milk powder 5%; pH 7.5) and incubated with the primary antibodies described above in the same buffer without milk powder for 1 hour. Alkaline-phosphatase-coupled anti-mouse or anti-rabbit antibodies were used as secondary antibodies. The immunoreactivity, visualized as fluorescent light (Vistra, ECF western blotting kit, USA), was detected and quantified with a Storm 840 Fluorimager and the ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA, USA) exactly as described previously (Vanliingen et al., 1997).
RESULTS

Characteristics of Ca\textsuperscript{2+} puffs are independent of the InsP\textsubscript{3} R isoform

For analysis of Ca\textsuperscript{2+} puff characteristics, we chose six cell types that have been previously shown to express different combinations of the three InsP\textsubscript{3} R isoforms. Using isoform-specific antibodies, we examined the differential expression of InsP\textsubscript{3} Rs in these cells. In general agreement with previous observations at both mRNA and protein levels (Wilson et al., 1998; Sipma et al., 1998; Sienaert et al., 1998; Mountian et al., 1999; De Smedt et al., 1997), we found that all of the cell types expressed detectable amounts of each InsP\textsubscript{3} R isoform, but the ratio of expression differed significantly (Fig. 1A). Some of the cell types seemed to be reasonable models for a single InsP\textsubscript{3} R isoform (e.g. RBL-2H3 and 16HBE14o-cells, which expressed high levels of types 2 and 3 InsP\textsubscript{3} Rs, respectively), whereas others demonstrated a predominant expression of pairs of InsP\textsubscript{3} R isoforms (e.g. HeLa/SH-SY5Y expressed types 1 and 3 InsP\textsubscript{3} Rs; HUVEC/NIH-3T3 cells expressed types 2 and 3 InsP\textsubscript{3} Rs).

Table 1. Characteristics of Ca\textsuperscript{2+} puffs in the different cell types

<table>
<thead>
<tr>
<th></th>
<th>16HBE14o-</th>
<th>HeLa</th>
<th>HUVEC</th>
<th>NIH-3T3</th>
<th>RBL-2H3</th>
<th>SH-SY5Y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amplitude (nM)</td>
<td>178±108 (602)</td>
<td>127±143 (521)</td>
<td>49±26 (529)</td>
<td>73±49 (376)</td>
<td>42±28 (232)</td>
<td>123±105 (664)</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} puff diameter (\textmu m)</td>
<td>3.2±0.8 (30)</td>
<td>3.7±0.6 (40)</td>
<td>3.1±0.5 (30)</td>
<td>4.1±0.8 (20)</td>
<td>2.5±0.5 (32)</td>
<td>3.0±0.6 (40)</td>
</tr>
<tr>
<td>Distance from nucleus (\textmu m)</td>
<td>1.7±5.0 (196)</td>
<td>4.4±6.2 (238)</td>
<td>6.2±6.1 (180)</td>
<td>17.5±14.9 (149)</td>
<td>12.3±11.3 (54)</td>
<td>3.3±4.2 (544)</td>
</tr>
</tbody>
</table>

The characters in parentheses indicate the number of Ca\textsuperscript{2+} puffs analysed.
Ca\textsuperscript{2+} puffs are generic elementary Ca\textsuperscript{2+} signals

Because the cell types all expressed the three InsP\textsubscript{3}R isoforms, it is plausible that they could be mosaics with distinct subcellular localization of each InsP\textsubscript{3}R subtype. We therefore investigated the subcellular distribution of the InsP\textsubscript{3}R isoforms in the six cell types using immunohistochemistry. In confocal sections (~0.6 \textmu m in thickness), we typically observed a decreasing gradient of staining from the nuclear envelope to the plasma membrane (Fig. 1B,C), indicating that the InsP\textsubscript{3}R density was highest in the perinuclear regions, and least at the periphery of the cells. Such a gradient of staining was true for the most highly expressed or least abundant InsP\textsubscript{3}R isoform in each cell type; only the intensity of staining varied. No distinctive patches of staining were observed within the cells for any of the InsP\textsubscript{3}R isoforms. At the level of the light microscope, it appears that InsP\textsubscript{3}Rs were not segregated within cells but were equally distributed.

Using real-time confocal imaging of fluo-3 fluorescence, we examined the Ca\textsuperscript{2+} puffs in the six cell types. Examples of the elementary Ca\textsuperscript{2+} puffs and global Ca\textsuperscript{2+} waves in some of these cells are shown in Fig. 2. For all cell types, low-frequency isolated Ca\textsuperscript{2+} puffs were observed by applying threshold concentrations of agonist, and global Ca\textsuperscript{2+} signals were evoked by higher concentrations of stimuli. The characteristics of the Ca\textsuperscript{2+} puffs are summarized in Table 1. No significant differences were observed in the mean amplitude or mean diameter of the Ca\textsuperscript{2+} puffs between any of

Fig. 3. Intracellular position of pacemaker Ca\textsuperscript{2+} puffs. (A) The histograms show the distribution of Ca\textsuperscript{2+} puffs relative to the nuclear envelope for each of the cell types investigated. (B) The average nuclear distances of the pacemaker Ca\textsuperscript{2+} puff sites are plotted against cell diameter, showing a roughly linear correlation for all the cell types except RBL-2H3 cells. The data are shown as mean±s.e.m. The dashed line (drawn by eye) indicates the trend for the pacemaker Ca\textsuperscript{2+} puff sites to be more distant from the nuclear boundary as the cell size increases. The cell size was estimated by measuring the diameter of cells along randomly chosen lines that crossed the cell! body and nucleus.

Fig. 4. Ca\textsuperscript{2+} puffs in 16HBE14o– and SH-SY5Y cells. (A) High-frequency non-regenerative Ca\textsuperscript{2+} puffs in a 16HBE14o– cell. The top two traces depict Ca\textsuperscript{2+} puffs firing at the sites marked by the correspondingly coloured circles in the inset cell image. The ticks beneath the second trace denote the occurrence of a Ca\textsuperscript{2+} puff at either of the sites. The average frequency of events in this recording was 0.8 Hz. Despite this high frequency, there was only a modest effect on the global Ca\textsuperscript{2+} concentration (bottom trace). (B) The relatively slow elevation of the intracellular Ca\textsuperscript{2+} concentration observed in some SH-SY5Y cells. Although the cell shown had two active pacemaker Ca\textsuperscript{2+} puff sites, a regenerative Ca\textsuperscript{2+} rise was not observed. Instead, there was a steady shallow Ca\textsuperscript{2+} increase. The dashed lines in the cell images represent the positions of the nuclei.
the elementary events that they produce are not distinct. However, although the characteristics of the Ca\(^{2+}\) puffs were not cell-type specific, some aspects of the elementary Ca\(^{2+}\) signalling clearly were. In particular, the frequency at which cells could tolerate Ca\(^{2+}\) puffs before a global Ca\(^{2+}\) wave was initiated varied considerably between cells. In HeLa cells, we have previously observed that, when the Ca\(^{2+}\) puff frequency increases beyond 0.2 Hz, there is a transition to a steep regenerative Ca\(^{2+}\) increase (Bootman et al., 1997b) (Fig. 2B). Most 16HBE14o–cells were able to maintain significantly higher Ca\(^{2+}\) puff frequencies with only modest changes in their global Ca\(^{2+}\) concentration (Fig. 4A). At the other extreme, NIH-3T3 cells usually displayed only a few infrequent Ca\(^{2+}\) puffs before global signals were initiated (Fig. 2D). Another cell-type-specific response was seen with SH-SY5Y cells in which high-frequency Ca\(^{2+}\) puffs did not always trigger steeply regenerative Ca\(^{2+}\) responses. Instead, Ca\(^{2+}\) puffs were often superimposed on a shallow elevation of the global Ca\(^{2+}\) concentration (Fig. 4B). This pattern of response was rarely seen in the other five cell types.

**Downregulation of InsP\(_3\)Rs modulates Ca\(^{2+}\) puff characteristics**

Because the activation and summation of Ca\(^{2+}\) puffs underlies the generation of global Ca\(^{2+}\) signals, they represent key focal points at which regulation of cellular Ca\(^{2+}\) responses can occur. We therefore investigated the ability of cells to regulate Ca\(^{2+}\) puff activity dynamically during prolonged agonist stimulation. For this analysis, SH-SY5Y cells and HeLa cells were used because we have previously characterized their elementary and global responses (Bootman et al., 1997b; Van Acker et al., 2000; Young et al., 2000).

Types 1 and 3 InsP\(_3\)R isoforms were the most readily detectable in SH-SY5Y cells (Fig. 1), and so we concentrated on these two proteins. Incubation of SH-SY5Y cells in culture with 1 mM carbachol (a muscarinic receptor agonist) resulted in a marked loss of IP\(_3\)R types 1 and 3 immunoreactivity (Fig. 5Ai,ii). The intensity of the band detected by the type-1-specific antibody was reduced by ~80% after 6 hours of incubation (Fig. 5Ai). Type 3 InsP\(_3\)Rs were reduced by ~90% after 6 hours of incubation (Fig. 5Ai,ii). The intensity of the band detected by the type-3-specific antibody was reduced by ~80% after 6 hours of incubation (Fig. 5Ai). Type 3 InsP\(_3\)Rs were reduced by ~90% after 6 hours of incubation (Fig. 5Ai,ii). The intensity of the band detected by the type-3-specific antibody was reduced by ~80% after 6 hours of incubation (Fig. 5Ai). Type 3 InsP\(_3\)Rs were reduced by ~90% after 6 hours of incubation (Fig. 5Ai,ii). The intensity of the band detected by the type-3-specific antibody was reduced by ~80% after 6 hours of incubation (Fig. 5Ai). Type 3 InsP\(_3\)Rs were reduced by ~90% after 6 hours of incubation (Fig. 5Ai,ii). The intensity of the band detected by the type-3-specific antibody was reduced by ~80% after 6 hours of incubation (Fig. 5Ai). Type 3 InsP\(_3\)Rs were reduced by ~90% after 6 hours of incubation (Fig. 5Ai,ii). The intensity of the band detected by the type-3-specific antibody was reduced by ~80% after 6 hours of incubation (Fig. 5Ai).
Ca\textsuperscript{2+} puffs are generic elementary Ca\textsuperscript{2+} signals

(Fig. 5A), whereas a slight recovery of both type 1 and type 3 InsP\textsubscript{3}Rs was observed after 24 hours.

To confirm that the InsP\textsubscript{3}Rs were functionally lost from the cells, we investigated the peak global Ca\textsuperscript{2+} responses that could be attained in the SH-SY5Y cells before and after InsP\textsubscript{3}R downregulation. To overcome a decreased intracellular InsP\textsubscript{3} production owing to muscarinic receptor desensitization following the prolonged incubation with agonist (Wojcikiewicz et al., 1994a; Willars and Nahorski, 1995), Ca\textsuperscript{2+} release was evoked by a membrane-permeant InsP\textsubscript{3} ester (InsP\textsubscript{3}BM) (Thomas et al., 2000a) at a concentration that was supramaximal for evoking global responses (100 \textmu M) (Collins et al., 2000). A significant (46%; \(P<0.001\)) decrease in InsP\textsubscript{3}BM-evoked global Ca\textsuperscript{2+} signal was detected in Fura2-loaded SH-SY5Y cells incubated with carbachol for 6 hours (Fig. 5B). Interestingly, we observed a complete recovery of

![Fig. 6. Changes in Ca\textsuperscript{2+} puff characteristics in SH-SY5Y cells following prolonged agonist stimulation. (A-D) The effects of incubating SH-SY5Y cells with 1 mM carbachol for 4 hours on the characteristics of Ca\textsuperscript{2+} puffs. The Gaussian curves were calculated using Microcal Origin (Northampton, USA).](image)

![Fig. 7. Downregulation of InsP\textsubscript{3}Rs and changes in Ca\textsuperscript{2+} puff characteristics in HeLa cells following prolonged agonist stimulation. (A) The effect of prolonged incubation with 1 mM histamine on the expression level of (i) IP\textsubscript{3}R type 1 and (ii) IP\textsubscript{3}R type 3 in HeLa cells. The immunoreactivity is expressed as a percentage of that in untreated cells. The data are expressed as mean\pm s.e.m. (\(n=3\)). (B) The functional loss of InsP\textsubscript{3}Rs in cells incubated with 1 mM histamine for 6 hours assessed using ratiometric imaging of Fura2. The data are expressed as percentage (mean\pm s.e.m.) of the amplitude of the Ca\textsuperscript{2+} fluorescence signal in untreated cells. The number of experiments (\(n\)) is indicated in the bars. (C,D) The change in the Ca\textsuperscript{2+} puff characteristics following incubation of HeLa cells with 1 mM histamine for 4 hours.](image)
In this way, we could match the InsP$_3$ concentration in control and carbachol-stimulated cells, and the amplitude of global responses after 24 hours of incubation with carbachol (data not shown), even though the InsP$_3$R expression had recovered only modestly (Fig. 5A). The latency for generation of global Ca$^{2+}$ signals using the supramaximal InsP$_3$ ester had recovered only modestly (Fig. 5A). The latency for generation of global Ca$^{2+}$ signals using the supramaximal InsP$_3$ ester (Fig. 7B; significantly different $P>0.05$). These data suggest that the reduction in Ca$^{2+}$ puff amplitude and duration histograms reflect a change in the Ca$^{2+}$ release capacity of InsP$_3$R clusters.

Fig. 8. Inhibition of Ca$^{2+}$ wave initiation following prolonged agonist stimulation of HeLa cells. Typical responses of single HeLa cells to 20 μM InsP$_3$BM either before (A) or after (B) a 4-hour incubation with 1 mM histamine. (A) An example in which the pacemaking Ca$^{2+}$ puff activity was able to drive the cell towards the threshold for triggering a regenerative global Ca$^{2+}$ wave. (B) The failure of the Ca$^{2+}$ puffs to trigger a regenerative response in a cell pre-incubated with 1 mM histamine. The cell shown in (B) was chosen because it displayed three active pacemaking Ca$^{2+}$ puff sites and was one of the most active cells following the histamine preincubation. However, despite the considerable activity of the pacemaking Ca$^{2+}$ puff sites, they caused only modest changes in the global Ca$^{2+}$ concentration. The cells shown in this figure were from the same passage and are typical of the responses from three independent experiments. Coloured circles on the inset cell images depict the positions of the pacemaking Ca$^{2+}$ puffs.

The distribution of event amplitudes could be described by a single Gaussian curve with a mean of 115±15 nM (mean±s.e.m.) (Fig. 6A). Incubation of the SH-SY5Y cells with 1 mM carbachol for 4 hours significantly narrowed the spread of Ca$^{2+}$ puff amplitudes, resulting in a sharper Gaussian curve in the lower amplitude range (80±5 nM; mean±s.e.m.; Fig. 6A).

In addition to reducing the average amplitude of Ca$^{2+}$ puffs, a 4-hour incubation in 1 mM carbachol also reduced the duration of the elementary events (Fig. 6B). The shorter lifetime of the Ca$^{2+}$ puffs was not due to altered intracellular Ca$^{2+}$ buffering or sequestration properties, because fitting the recovery of the elementary Ca$^{2+}$ signals to a first order exponential function revealed no significant difference between control and carbachol-treated cells (the exponential decay constant $\tau$=0.31±0.02 second$^{-1}$ and 0.25±0.02 second$^{-1}$, respectively; $P>0.05$). These data suggest that the reduction in Ca$^{2+}$ puff amplitude and duration histograms reflect a change in the Ca$^{2+}$ release capacity of InsP$_3$R clusters.

In addition to effects on the characteristics of Ca$^{2+}$ puffs, further marked effects of agonist incubation were observed in terms of the activity of pacemaking Ca$^{2+}$ puff sites. The average number of pacemaking Ca$^{2+}$ puff sites decreased from 1.9 to ~1.0 after a 4-hour incubation period with 1 mM carbachol (Fig. 6C). After incubation with the agonist, no individual cells were observed to display more than three such sites. Furthermore, the frequency of Ca$^{2+}$ release at individual pacemaking Ca$^{2+}$ puff sites was reduced. In control conditions, ~75% of the sites had a Ca$^{2+}$ release frequency of >5 events minute$^{-1}$ in the presence of 20 μM InsP$_3$BM. After 4 hours of agonist incubation, this figure decreased to ~15%. Single events (a single Ca$^{2+}$ puff detected during an average 3-minute confocal recording period) were not observed in control cells. After 4 hours of carbachol treatment, single events were observed in 24% of the records (Fig. 6D).

HeLa cells also responded to prolonged incubation with an InsP$_3$-generating agonist (histamine; 1 mM) with a decrease in InsP$_3$R expression. However, in contrast to SH-SY5Y cells, the type-1 InsP$_3$R expression did not detectably alter (Fig. 7A), whereas the type-3 InsP$_3$R isoform was maximally downregulated by ~50% after 6 hours of histamine treatment (Fig. 7A). Incubation of HeLa cells with histamine for 6 hours caused a 32% reduction in the amplitude of global Ca$^{2+}$ signals evoked by 100 μM InsP$_3$ ester (Fig. 7B; significantly different from control; $P<0.001$), confirming that the InsP$_3$Rs were functionally lost.

The effects of prolonged incubation of HeLa cells with 1 mM histamine on the characteristics of Ca$^{2+}$ puffs showed a similar general tendency but were not quite as prominent as those described above for SH-SY5Y cells. The mean amplitudes of elementary events evoked by 10 μM InsP$_3$ ester was reduced from 103±23 nM to 62±18 nM (mean±s.e.m.) (Fig. 7C). Furthermore, the duration of the elementary events was reduced in a similar way to that shown for SH-SY5Y cells (data not shown). As with the SH-SY5Y cells, this effect was not due to changes in the Ca$^{2+}$ sequestration or buffering mechanisms because the first-order time constant for the exponential decay of individual Ca$^{2+}$ puffs was not significantly different in control or histamine-treated cells ($\tau$=0.30±0.02 second$^{-1}$ and 0.24±0.02 second$^{-1}$, respectively; $P>0.05$). The frequency with which pacemaking Ca$^{2+}$ puff sites...
were activated by 10 mM InsP3BM was not significantly different in histamine-treated cells (data not shown), although the average number of pacemaking Ca2+ puff sites per cell was reduced from 1.8 to 1.1 (Fig. 7D). The changes in amplitude, kinetics and frequency of the elementary events were not due to reduction of the Ca2+ loading within the lumen of the endoplasmic reticulum (ER), because the response to thapsigargin-induced discharge of the ER stores was unaffected by agonist preincubation (data not shown).

Our previous studies (Bootman et al., 1997b) and those of Parker and colleagues (Marchant et al., 1999) have demonstrated that Ca2+ waves are triggered inside cells when the progressive activity of Ca2+ puffs reaches a threshold Ca2+ concentration at which CICR is activated. A likely consequence of decreased Ca2+ puff activity following the prolonged agonist incubation is that the cells would possess a lower propensity for initiating regenerative Ca2+ waves. We therefore examined the ability of HeLa cells to show regenerative Ca2+ waves when incubated with InsP3BM. For this, we increased the stimulating InsP3BM concentration to 20 mM because, at 10 mM, only non-regenerative Ca2+ puffs are usually observed in HeLa cells. In a typical experiment using matched cells from the same passage number, we found that 71% (n=17) of the control cells showed Ca2+ waves after a period of Ca2+ puff activity (Fig. 8A). By contrast, 86% of the cells (n=29) incubated with 1 mM histamine for 4 hours failed to show global regenerative Ca2+ signals in response to 20 mM InsP3BM. These data indicate that, for the same InsP3BM stimulus, the cells that had undergone prolonged incubation with histamine were more resistant to the activation of Ca2+ waves. The lack of initiation of Ca2+ waves in cells that had been incubated with histamine seemed to be caused mainly by the lesser activity of the pacemaking Ca2+ puff sites. However, a few histamine-incubated cells did manage to show reasonable levels of pacemaking Ca2+ puff activity in response to 20 mM InsP3BM, but these also largely failed to show regenerative Ca2+ signals (Fig. 8B). This suggests that, in addition to the changes of the properties of the pacemaking events, the activity of the other Ca2+ puff sites that simply aid in the propagation of Ca2+ waves was also diminished.

**DISCUSSION**

Previous studies have shown that the initiation of regenerative Ca2+ signals in agonist-stimulated cells depends on the progressive recruitment of Ca2+ puffs (Bootman et al., 1997b; Marchant and Parker, 2001). With each elementary event, there is a small increase in cytosolic Ca2+ concentration, and the cumulative effect of successive elementary Ca2+ release events drives the cell towards the threshold for a regenerative CICR response (Bootman et al., 1997b; Marchant and Parker, 2001).

The present study demonstrates that analogous Ca2+ puffs can be observed following hormonal stimulation of a variety of cell types (Fig. 2). Furthermore, the similarity of Ca2+ puffs in these cell types, which express different proportions of the three InsP3R isoforms (Table 1), indicates functional redundancy of InsP3Rs at the level of elementary events. In addition to the six cell types characterized here, analogous Ca2+ puffs have been visualized in *Xenopus* oocytes (Yao et al., 1995), endothelial cells (Häuser and Blatter, 1997), PC12 cells (Koizumi et al., 1999), smooth muscle cells (Boittin et al., 2000) and oligodendrocytes (Haak et al., 2001). Taken together, these data indicate that diverse cell types use a generic elementary Ca2+ signal for constructing InsP3-mediated responses.

Although the Ca2+ puffs might have similar characteristics in different cell types, irrespective of the InsP3R isoforms present, there are subtle differences in the ways in which cells use these events. The most obvious difference observed in the present study was in the frequencies of Ca2+ puffs that occur prior to Ca2+ wave onset. 16HBE14o– cells, in particular, seemed to be able to withstand Ca2+ puff frequencies that would have triggered regenerative responses in other cell types (Fig. 4A). Furthermore, although most cells showed steeply regenerative Ca2+ waves after a period of Ca2+ puff activity (e.g. Fig. 2A,B,D), SH-SY5Y cells were able to produce a cumulative increase in cytosolic with no inflexion, a response that reflects the progressive summation of Ca2+ puffs (Fig. 4B).

It is likely that many factors (e.g. intracellular Ca2+ buffering, Ca2+ ATPase activity, intracellular InsP3 concentration, spacing between InsP3Rs) determine how cells will respond to ongoing Ca2+ puff activity. In the case of 16HBE14o– cells, for example, it is plausible that the high Ca2+ puff frequency is tolerated owing to a weaker functional coupling between InsP3Rs, so that higher levels of activity are required to provoke a regenerative response. With the exception of RBL-2H3 cells, all of the cell types analysed here displayed regular baseline Ca2+ oscillations when stimulated with appropriate concentrations of agonist (Fig. 2D). However, as has been noted previously (Berridge and Galione, 1988; Thomas et al., 1996), the characteristics of global Ca2+ oscillations (amplitude, duration, rise time etc) were not identical in the different cell types (data not shown). Therefore, although the Ca2+ puffs might be a generic InsP3-mediated elementary event, differential recruitment or modulation of these signals might lead to cell-specific elementary and global response patterns.

Another similarity between the cell types analysed here was that the activity of one or a few pacemaking Ca2+ puff sites was usually responsible for driving the cell towards the threshold for CICR. We have previously observed that the pacemaker Ca2+ puff sites in HeLa cells were largely distributed around the nucleus (Lipp et al., 1997). Because there are no Ca2+ ATPases in the inner nuclear envelope, nuclear Ca2+ transients can persist for significantly longer than equivalent cytosolic Ca2+ rises (Bootman et al., 2000). On this basis, we suggested that the activation of perinuclear Ca2+ puffs might be a mechanism for evoking nuclear Ca2+ signals with little effect on cytoplasmic Ca2+ levels (Lipp et al., 1997).

This scheme might apply to four of the six cell types (HeLa, HUVEC, SH-SY5Y and 16HBE14o–) tested here because >70% of their pacemaker Ca2+ puff sites occurred within 3-4 μm of the nuclear envelope. Within this range, the signal from the Ca2+ puff can diffuse to the nuclear boundary. However, in RBL-2H3 and NIH-3T3 cells, most of the pacemaker Ca2+ puffs occurred further away than the diameter of the Ca2+ puffs, which would preclude these signals affecting nuclear Ca2+ levels (Fig. 3, Table 1).

It is unclear what biochemical mechanism distinguishes the pacemaking Ca2+ puff sites from those that simply participate in Ca2+ wave propagation. It is unlikely to be due to localized
InsP$_3$ production (Thomas et al., 2000a). Furthermore, immunostaining InsP$_3$Rs in the six cell types only demonstrated that the density of InsP$_3$R expression decreased with distance from the nucleus to the cell periphery (Fig. 1B) and did not reveal any prominent regions that could underlie the pacemaking Ca$^{2+}$ puff sites. Functionally, the pacemaker Ca$^{2+}$ puff sites possess a greater sensitivity to InsP$_3$ than those that simply participate in Ca$^{2+}$ wave propagation (Thomas et al., 2000a). A similar conclusion was reached for the ‘focal’ Ca$^{2+}$ puff sites that predominantly trigger Ca$^{2+}$ waves in Xenopus oocytes (Marchant and Parker, 2001).

Because Ca$^{2+}$ puffs are responsible for triggering and propagating Ca$^{2+}$ waves in cells, it is conceivable that they are key points at which a cell could regulate its response to hormonal stimulation. Prolonged stimulation of HeLa and SH-SY5Y cells downregulated the expression of InsP$_3$Rs in these cells (Fig. 5; Fig. 7) and concomitantly affected the activity and characteristics of Ca$^{2+}$ puffs. Essentially, the consequence of prolonged agonist stimulation was to make the Ca$^{2+}$ puffs less vigorous, with the amplitude, duration, frequency and number of pacemaker Ca$^{2+}$ puff sites being reduced (Fig. 6; Fig. 7). Furthermore, global Ca$^{2+}$ signalling was also restrained, because the number of cells displaying Ca$^{2+}$ waves in response to 20 µM InsP$_3$BM was substantially decreased (Fig. 8).

The downregulation of Ca$^{2+}$ puff activity by agonist stimulation of cells can be rapid. We observed that a 2-hour incubation was sufficient to have significant effects on Ca$^{2+}$ puff characteristics (data not shown). This is consistent with the observation that the half-time for loss of InsP$_3$Rs in agonist-stimulated cells is <2 hours, compared with >8 hours in unstimulated conditions (Wojcikiewicz et al., 1994a; Wojcikiewicz et al., 1994b). It is conceivable that, by virtue of their intrinsically higher InsP$_3$ sensitivity, the pacemaker Ca$^{2+}$ puff sites are especially prone to regulation. Binding of InsP$_3$ to its receptor activates InsP$_3$R downregulation by stimulating ubiquitination. Once a polyubiquitinated InsP$_3$R is recognized, it is unfolded and cleaved by the proteasome (Zhu et al., 1999; Oberdorf et al., 1999). The greater InsP$_3$ sensitivity and activity of the pacemaking Ca$^{2+}$ puff sites might cause them to be rapidly ubiquitinated and removed.

Although prolonged incubation with agonist had a similar effect on the Ca$^{2+}$ puffs in HeLa and SH-SY5Y cells, the extent of downregulation of InsP$_3$R isoforms was clearly different (Fig. 5; Fig. 7). A different susceptibility of InsP$_3$R isoforms to downregulation was also reported in rat cerebellar granule cells and AR4-2J rat pancreatoma cells (Wojcikiewicz, 1995). In these cell types, which express almost exclusively types 1 and 2 InsP$_3$Rs, type 1 InsP$_3$Rs were found to be selectively downregulated. It is currently unclear why certain InsP$_3$R isoforms are more sensitive to downregulation than others, although it has been suggested that homotetramers are more resistant to ubiquitination (Oberdorf et al., 1999).

In HeLa cells, the extent of downregulation of InsP$_3$Rs (~50% loss of type 3 InsP$_3$Rs, which are roughly half of all receptors) correlated roughly with the reduction in the Ca$^{2+}$ response triggered by a supramaximal InsP$_3$BM concentration (Fig. 7). In SH-SY5Y cells, the loss of InsP$_3$R expression was much more marked but the cells still managed a significant response to supramaximal InsP$_3$BM (Fig. 5). This might indicate that, in SH-SY5Y, there is a large reserve of InsP$_3$Rs, such that only a fraction of the intracellular immunoreactive InsP$_3$Rs are required to generate a maximal Ca$^{2+}$ liberation.

HeLa cells, by contrast, do not have a receptor reserve.

In summary, our data show that Ca$^{2+}$ puffs are a generic elementary Ca$^{2+}$ signal used by different cell types for constructing InsP$_3$-mediated responses. The different InsP$_3$Rs appear to be functionally redundant at the level of elementary Ca$^{2+}$ signalling. In addition, Ca$^{2+}$ puff sites are susceptible to regulation during prolonged cellular stimulation and, as a consequence, global Ca$^{2+}$ signalling is inhibited.

This work was funded by the BBSC Intracellular Response Initiative (grant ICR07498, to M.D.B. and M.J.B.), the MRC (grant G9808140, to P.L., M.D.B. and M.J.B.) and the Concerted Actions of the K.U. Leuven (grant 99/08, to L.M., H.D.S. and J.B.P.). P.D.S. is a Senior Research Assistant of the Fund for Scientific Research-Flanders. M.D.B. gratefully acknowledges support from the Royal Society.

REFERENCES


Brind, S., Swann, K. and Carroll, J. (2000). Inositol 1,4,5-trisphosphate receptors are downregulated in mouse oocytes in response to sperm or adenosine 5' monophosphate but not to increases in intracellular Ca$^{2+}$ or egg activation. Dev. Biol. 223, 251-265.


Ca\textsuperscript{2+} puffs are generic elementary Ca\textsuperscript{2+} signals


Wojcikiewicz, R. J. H. (1995). Type-I, type-II and type-III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *J. Biol. Chem.* 270, 11678-11683.


