Tovey, Stephen C.; de Smet, Patrick; Lipp, Peter; Thomas, David; Young, Kenneth W.; Missiaen, Ludwig; De Smedt, Humbert; Parys, Jan B.; Berridge, Michael J.; Thuring, Jan; Holmes, Andrew and Bootman, Martin D. (2001). Calcium puffs are generic InsP3-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses. Journal of Cell Science, 114(Pt 22) pp. 3979–3989.

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Calcium puffs are generic InsP_3-activated elementary calcium signals and are downregulated by prolonged hormonal stimulation to inhibit cellular calcium responses

**INTRODUCTION**

Stimulation of cells with hormones that activate the enzyme phospholipase C (PLC) often evokes spatially and temporally complex intracellular Ca^{2+} signals (Berridge, 1993; Petersen et al., 1994; Berridge et al., 1998). The link between PLC and Ca^{2+} signalling is the intracellular messenger inositol 1,4,5-trisphosphate (InsP_3), which diffuses from its site of production into the cytosol and binds to specific Ca^{2+}-releasing channels (InsP_3 receptors; InsP_3Rs) (Berridge, 1993; Berridge et al., 1998). Characterization of purified receptors, recombinant receptors or cell types expressing various levels of each InsP_3R isoform have suggested that, although there is considerable functional redundancy between the InsP_3R isoforms, there might also be some isotype-specific regulation of Ca^{2+} signalling (Taylor, 1998; Taylor et al., 1999; Patel et al., 1999).

InsP_3Rs are acutely regulated by many factors, including phosphorylation, ATP, pH, accessory proteins, luminal Ca^{2+} and cytosolic Ca^{2+} (Taylor, 1998; Taylor et al., 1999; Patel et al., 1999). In addition to acute regulation of InsP_3Rs, modulation of Ca^{2+} signalling is brought about by changes in InsP_3R 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 InsP_3R content. This phenomenon has been observed with all three InsP_3R isoforms in a range of cell types (Wojcikiewicz et al., 1994a; Wojcikiewicz, 1995; Sipma et al., 1998; Youn et al., 1999). The reduction of InsP_3Rs is a specific process because the expression of other proteins involved in Ca^{2+} signalling (apart from agonist receptors themselves) is not simultaneously modulated. Such downregulation of InsP_3R isoforms...
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 (Bootman 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 (Bootman et al., 1997a; Bootman et al., 1997b). Parker and colleagues denoted these localized InsP₃ R-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 InsP₃ Rs (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; Callamars 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 InsP₃ R isoforms. 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 Typed 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. Gruener (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 acetoxymethyl 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 (Bootman 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:

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

\( f \) is the fluorescence intensity of fluo-3 recorded during the experiment. \( f_{\text{min}} \) and \( f_{\text{max}} \) are the minimal and maximal fluorescence intensities of fluo-3, reflecting the calcium-free and calcium-saturated forms of the indicator, respectively. \( f_{\text{min}} \) and \( f_{\text{max}} \) were determined by permeabilizing the cells with A23187 in the presence of 10 mM EGTA or 10 mM CaCl₂ respectively. The \( K_d \) 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 intensified camera (Photonics Science, Robertbridge, UK), and the digitized signals were stored and processed using an Imagine image processing system (Synoptics, Cambridge, UK) as described previously (Bootman et al., 1992).

**Western blotting and immunohistochemistry**

The methods used were similar to those described previously (Tovey
et al., 1997). Briefly, cellular membranes were prepared from approximately four large (150 cm²) flasks of confluent cells. For this, cells were resuspended in 10× volume of 0.32 M sucrose, 5 mM Hepes at pH 7.4 containing a protease inhibitor cocktail (0.1 mM PMSF, 0.1 mM benzamidine, 10 μM leupeptin, 10 μM pepstatin A). The cells were homogenized (8-10 strokes of a Dounce homogenizer) and then centrifuged at 500 g for 10 minutes. The resultant supernatant was then centrifuged at 100,000 g for 1 hour to pellet the microsomal fraction. The pellets were then resuspended in Hepes/sucrose buffer to a volume of ~5-10 mg ml⁻¹ (BioRad protein assay reagent) and snap frozen in liquid nitrogen.

Proteins were separated using 5% SDS-polyacrylamide gels for ~90 minutes at 20 mA per gel. 10 μg of protein well⁻¹ was used for InsP³R types 1 and 3 blots, and 20 μg was used for InsP³R type 2 blots. The gels were transferred onto nitrocellulose (Amersham hyperbond) for 3 hours at 500 mA using the BioRad semi-dry blotter. Membranes were pre-soaked in 20% v/v MeOH, 0.1% w/v SDS, 380 mM glycine, 50 mM Tris. Protein transfer was verified by staining the gels with Coomassie Blue after transfer, and the presence of the proteins on the nitrocellulose was verified with 0.1% w/v Ponceau-S. The blots were blocked for 1 hour at room temperature in 5% dried milk powder in TTBS (140 mM NaCl, 25 mM Tris, 0.05% v/v Tween 20).

They were incubated with the primary antibody for 1 hour at room temperature in 5% dried milk powder 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 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% v/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 InsP³R 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 (Vanlingen 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>Cell Type</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 ((\mu\text{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 ((\mu\text{m}))</td>
<td>1.7±5.0 (196)</td>
<td>4.2±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.
Because the cell types all expressed the three InsP₃R isoforms, it is plausible that they could be mosaics with distinct subcellular localization of each InsP₃R subtype. We therefore investigated the subcellular distribution of the InsP₃R isoforms in the six cell types using immunohistochemistry. In confocal sections (~0.6 μm in thickness), we typically observed a decreasing gradient of staining from the nuclear envelope to the plasma membrane (Fig. 1B,C), indicating that the InsP₃R 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 InsP₃R 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 InsP₃R isoforms. At the level of the light microscope, it appears that InsP₃Rs were not segregated within cells but were equally distributed.

Using real-time confocal imaging of fluo-3 fluorescence, we examined the Ca²⁺ puffs in the six cell types. Examples of the elementary Ca²⁺ puffs and global Ca²⁺ waves in some of these cells are shown in Fig. 2. For all cell types, low-frequency isolated Ca²⁺ puffs were observed by applying threshold concentrations of agonist, and global Ca²⁺ signals were evoked by higher concentrations of stimuli. The characteristics of the Ca²⁺ puffs are summarized in Table 1. No significant differences were observed in the mean amplitude or mean diameter of the Ca²⁺ puffs between any of the cell types.

![Fig. 3. Intracellular position of pacemaker Ca²⁺ puffs. (A) The histograms show the distribution of Ca²⁺ puffs relative to the nuclear envelope for each of the cell types investigated. (B) The average nuclear distances of the pacemaker Ca²⁺ 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 Ca²⁺ 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.](image)

![Fig. 4. Ca²⁺ puffs in 16HBE14o− and SH-SY5Y cells. (A) High-frequency non-regenerative Ca²⁺ puffs in a 16HBE14o− cell. The top two traces depict Ca²⁺ 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 Ca²⁺ 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 Ca²⁺ concentration (bottom trace). (B) The relatively slow elevation of the intracellular Ca²⁺ concentration observed in some SH-SY5Y cells. Although the cell shown had two active pacemaker Ca²⁺ puff sites, a regenerative Ca²⁺ rise was not observed. Instead, there was a steady shallow Ca²⁺ increase. The dashed lines in the cell images represent the positions of the nuclei.](image)
activity. The elementary events recorded here arose from InP3 R
ryanodine receptors (Bennett et al., 1996; Young et al., 2000),
indicating that, although some cells might weakly express
significant responses to 1-10 mM caffeine (data not shown),
we would provide a more reliable estimate of the global Ca2+
response, we used ratiometric imaging of Fura2 rather than single wavelength
confocal recording. The data are expressed as percentage
(mean±s.e.m.) of the amplitude of the Ca2+ fluorescence signal in
untreated cells. The number of experiments (n) is indicated in the bars.

Fig. 5. Downregulation of InsP3Rs in SH-SY5Y cells. (A) The effect of prolonged incubation with 1 mM carbachol on the expression level of (i) InsP3 R type 1 and (ii) InsP3 R type 3 in SH-SY5Y cells. Typical examples of the immunoband at 261 kDa and 248 kDa for types 1 and 3 InsP3 Rs are shown above the histograms. The immunoreactivity is expressed as a percentage of that in untreated cells. The data are expressed as mean±s.e.m. (n=3). All data were significantly different from control (P<0.05). (B) The functional loss of InsP3 Rs in cells incubated with 1 mM carbachol for 6 hours. To provide a more reliable estimate of the global Ca2+ response, we used ratiometric imaging of Fura2 rather than single wavelength confocal recording. The data are expressed as percentage (mean±s.e.m.) of the amplitude of the Ca2+ fluorescence signal in untreated cells. The number of experiments (n) is indicated in the bars.

the cell types. Furthermore, there was no striking difference in the rate of rise or decay of the Ca2+ puffs in the different cells (data not shown). None of the cell types analysed showed significant responses to 1-10 mM caffeine (data not shown), indicating that, although some cells might weakly express ryanodine receptors (Bennett et al., 1996; Young et al., 2000), the elementary events recorded here arose from InsP3Rs activity.

Our previous studies of Ca2+ puffs in HeLa cells have shown that one or a few of the Ca2+ puff sites within a cell behave as ‘pacemaker’ sites that are the first to respond during agonist stimulation, and are responsible for triggering global Ca2+ responses (Bootman et al., 1997b). In HeLa cells, such pacemaker sites are predominantly located in a perinuclear position (Lipp et al., 1997). In all of the cell types examined, we found that between one and six Ca2+ puff sites per cell were reproducibly activated by threshold agonist concentrations, and that these sites tended to be in perinuclear locations (Fig. 3; Table 1). However, the average distance of these sites from the nucleus was not the same for all cells. Instead, there was a direct correlation between average cell diameter and distance of the pacemaker Ca2+ puff sites from the nucleus for all cell types except RBL-2H3 cells. For RBL-2H3 cells, a significant proportion of the pacemaker Ca2+ puff sites were not in the cell body near the nucleus but rather in processes protruding from the cells.

The data described above suggest that, although the six cell types expressed different combinations of the three InsP3 Rs isoforms, the spatial distribution of the channels and the elementary events that they produce are not distinct. However, although the characteristics of the Ca2+ puffs were not cell-type specific, some aspects of the elementary Ca2+ signalling clearly were. In particular, the frequency at which cells could tolerate Ca2+ puffs before a global Ca2+ wave was initiated varied considerably between cells. In HeLa cells, we have previously observed that, when the Ca2+ puff frequency increases beyond 0.2 Hz, there is a transition to a steep regenerative Ca2+ increase (Bootman et al., 1997b) (Fig. 2B). Most 16HBE14o- cells were able to maintain significantly higher Ca2+ puff frequencies with only modest changes in their global Ca2+ concentration (Fig. 4A). At the other extreme, NIH-3T3 cells usually displayed only a few infrequent Ca2+ puffs before global signals were initiated (Fig. 2D). Another cell-type-specific response was seen with SH-SY5Y cells in which high-frequency Ca2+ puffs did not always trigger steeply regenerative Ca2+ responses. Instead, Ca2+ puffs were often superimposed on a shallow elevation of the global Ca2+ concentration (Fig. 4B). This pattern of response was rarely seen in the other five cell types.

**Downregulation of InsP3Rs modulates Ca2+ puff characteristics**

Because the activation and summation of Ca2+ puffs underlies the generation of global Ca2+ signals, they represent key focal points at which regulation of cellular Ca2+ responses can occur. We therefore investigated the ability of cells to regulate Ca2+ 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 InsP3 Rs 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 IP3 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 InsP3 Rs were reduced by ~90% after 6 hours incubation and became undetectable at 6-10 hours incubation. These observations are consistent with earlier investigations of agonist stimulation on InsP3 Rs expression in SH-SY5Y cells (Wojcikiewicz et al., 1992; Wojcikiewicz et al., 1994a). For both InsP3 Rs isoforms, the reduced immunoreactivity was apparent for at least 10 hours
Ca2+ puffs are generic elementary Ca2+ signals (Fig. 5A), whereas a slight recovery of both type 1 and type 3 InsP3Rs was observed after 24 hours.

To confirm that the InsP3Rs were functionally lost from the cells, we investigated the peak global Ca2+ responses that could be attained in the SH-SY5Y cells before and after InsP3R downregulation. To overcome a decreased intracellular InsP3 production owing to muscarinic receptor desensitization following the prolonged incubation with agonist (Wojcikiewicz et al., 1994a; Willars and Nahorski, 1995), Ca2+ release was evoked by a membrane-permeant InsP3 ester (InsP3BM) (Thomas et al., 2000a) at a concentration that was supramaximal for evoking global responses (100 μM) (Collins et al., 2000). A significant (46%; P<0.001) decrease in InsP3BM-evoked global Ca2+ signal was detected in Fura2-loaded SH-SY5Y cells incubated with carbachol for 6 hours (Fig. 5B). Interestingly, we observed a complete recovery of

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

Fig. 6. Changes in Ca2+ 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 Ca2+ puffs. The Gaussian curves were calculated using Microcal Origin (Northampton, USA).

![Image 5](image5)

![Image 6](image6)

![Image 7](image7)

Fig. 7. Downregulation of InsP3Rs and changes in Ca2+ 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) IP3R type 1 and (ii) IP3R type 3 in HeLa cells. The immunoreactivity is expressed as a percentage of that in untreated cells. The data are expressed as mean±s.e.m. (n=3). (B) The functional loss of InsP3Rs in cells incubated with 1 mM histamine for 6 hours assessed using ratiometric imaging of Fura2. The data are expressed as percentage (mean±s.e.m.) of the amplitude of the Ca2+ fluorescence signal in untreated cells. The number of experiments (n) is indicated in the bars. (C,D) The change in the Ca2+ puff characteristics following incubation of HeLa cells with 1 mM histamine for 4 hours.
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 Ca2+ 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 Ca2+ puffs, a 4-hour incubation in 1 mM carbachol also reduced the duration of the elementary events (Fig. 6B). The shorter lifetime of the Ca2+ puffs was not due to altered intracellular Ca2+ buffering or sequestration properties, because fitting the recovery of the elementary Ca2+ 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 Ca2+ puff amplitude and duration histograms reflect a change in the Ca2+ release capacity of InsP3R clusters.

In addition to effects on the characteristics of Ca2+ puffs, further marked effects of agonist incubation were observed in terms of the activity of pacemaking Ca2+ puff sites. The average number of pacemaking Ca2+ 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 Ca2+ release at individual pacemaking Ca2+ puff sites was reduced. In control conditions, ~75% of the sites had a Ca2+ release frequency of >5 events minute$^{-1}$ in the presence of 20 μM InsP3BM. After 4 hours of agonist incubation, this figure decreased to ~15%. Single events (a single Ca2+ 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 InsP3-generating agonist (histamine; 1 mM) with a decrease in InsP3R expression. However, in contrast to SH-SY5Y cells, the type-1 InsP3R expression did not detectably alter (Fig. 7Aii), whereas the type-3 InsP3R isoform was maximally downregulated by ~50% after 6 hours of histamine treatment (Fig. 7Aii). Incubation of HeLa cells with histamine for 6 hours caused a 32% reduction in the amplitude of global Ca2+ signals evoked by 100 μM InsP3 ester (Fig. 7B; significantly different from control; $P<0.001$), confirming that the InsP3Rs were functionally lost.

The effects of prolonged incubation of HeLa cells with 1 mM histamine on the characteristics of Ca2+ 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 InsP3 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 Ca2+ sequestration or buffering mechanisms because the first-order time constant for the exponential decay of individual Ca2+ 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 Ca2+ puff sites
were activated by 10 μM InsP_{3}BM was not significantly different in histamine-treated cells (data not shown), although the average number of pacemaking Ca^{2+} 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 Ca^{2+} 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 Ca^{2+} waves are triggered inside cells when the progressive activity of Ca^{2+} puffs reaches a threshold Ca^{2+} concentration at which CICR is activated. A likely consequence of decreased Ca^{2+} puff activity following the prolonged agonist incubation is that the cells would possess a lower propensity for initiating regenerative Ca^{2+} waves. We therefore examined the ability of HeLa cells to show regenerative Ca^{2+} waves when incubated with InsP_{3}BM. For this, we increased the stimulating InsP_{3}BM concentration to 20 μM because, at 10 μM, only non-regenerative Ca^{2+} 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 Ca^{2+} waves after a period of Ca^{2+} 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 Ca^{2+} signals in response to 20 μM InsP_{3}BM. These data indicate that, for the same InsP_{3}BM stimulus, the cells that had undergone prolonged incubation with histamine were more resistant to the activation of Ca^{2+} waves. The lack of initiation of Ca^{2+} waves in cells that had been incubated with histamine seemed to be caused mainly by the lesser activity of the pacemaking Ca^{2+} puff sites. However, a few histamine-incubated cells did manage to show reasonable levels of pacemaking Ca^{2+} puff activity in response to 20 μM InsP_{3}BM, but these also largely failed to show regenerative Ca^{2+} signals (Fig. 8B). This suggests that, in addition to the changes of the properties of the pacemaking events, the activity of the other Ca^{2+} puff sites that simply aid in the propagation of Ca^{2+} waves was also diminished.

**DISCUSSION**

Previous studies have shown that the initiation of regenerative Ca^{2+} signals in agonist-stimulated cells depends on the progressive recruitment of Ca^{2+} puffs (Bootman et al., 1997b; Marchant and Parker, 2001). With each elementary event, there is a small increase in cytosolic Ca^{2+} concentration, and the cumulative effect of successive elementary Ca^{2+} 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 Ca^{2+} puffs can be observed following hormonal stimulation of a variety of cell types (Fig. 2). Furthermore, the similarity of Ca^{2+} puffs in these cell types, which express different proportions of the three InsP_{3}R isoforms (Table 1), indicates functional redundancy of InsP_{3}Rs at the level of elementary events. In addition to the six cell types characterized here, analogous Ca^{2+} 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 (Boitinn et al., 2000) and oligodendrocytes (Haak et al., 2001). Taken together, these data indicate that diverse cell types use a generic elementary Ca^{2+} signal for constructing InsP_{3}-mediated responses.

Although the Ca^{2+} puffs might have similar characteristics in different cell types, irrespective of the InsP_{3}R 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 Ca^{2+} puffs that occur prior to Ca^{2+} wave onset. 16HBE14o− cells, in particular, seemed to be able to withstand Ca^{2+} puff frequencies that would have triggered regenerative responses in other cell types (Fig. 4A). Furthermore, although most cells showed steeply regenerative Ca^{2+} waves after a period of Ca^{2+} puff activity (e.g. Fig. 2A,B,D), SH-SY5Y cells were able to produce a cumulative increase in cytosolic with no influxion, a response that reflects the progressive summation of Ca^{2+} puffs (Fig. 4B).

It is likely that many factors (e.g. intracellular Ca^{2+} buffering, Ca^{2+} ATPase activity, intracellular InsP_{3} concentration, spacing between InsP_{3}Rs) determine how cells will respond to ongoing Ca^{2+} puff activity. In the case of 16HBE14o− cells, for example, it is plausible that the high Ca^{2+} puff frequency is tolerated owing to a weaker functional coupling between InsP_{3}Rs, 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 Ca^{2+} 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 Ca^{2+} oscillations (amplitude, duration, rise time etc) were not identical in the different cell types (data not shown). Therefore, although the Ca^{2+} puffs might be a generic InsP_{3}-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 Ca^{2+} puff sites was usually responsible for driving the cell towards the threshold for CICR. We have previously observed that the pacemaker Ca^{2+} puff sites in HeLa cells were largely distributed around the nucleus (Lipp et al., 1997). Because there are no Ca^{2+} ATPases in the inner nuclear envelope, nuclear Ca^{2+} transients can persist for significantly longer than equivalent cytosolic Ca^{2+} rises (Bootman et al., 2000). On this basis, we suggested that the activation of perinuclear Ca^{2+} puffs might be a mechanism for evoking nuclear Ca^{2+} signals with little effect on cytoplasmic Ca^{2+} 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 Ca^{2+} puff sites occurred within 3-4 μm of the nuclear envelope. Within this range, the signal from the Ca^{2+} puff can diffuse to the nuclear boundary. However, in RBL-2H3 and NIH-3T3 cells, most of the pacemaker Ca^{2+} puffs occurred further away than the diameter of the Ca^{2+} puffs, which would preclude these signals affecting nuclear Ca^{2+} levels (Fig. 3, Table 1).

It is unclear what biochemical mechanism distinguishes the pacemaking Ca^{2+} puff sites from those that simply participate in Ca^{2+} wave propagation. It is unlikely to be due to localized
such that only a fraction of the intracellular immunoreactive
insP3Rs in the six cell types only demonstrated that the density of insP3R 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 Ca2+ puff sites. Functionally, the pacemaker Ca2+ puff sites possess a greater sensitivity to insP3 than those that simply participate in Ca2+ wave propagation (Thomas et al., 2000a). A similar conclusion was reached for the ‘focal’ Ca2+ puff sites that predominantly trigger Ca2+ waves in Xenopus oocytes (Marchant and Parker, 2001).

Because Ca2+ puffs are responsible for triggering and propagating Ca2+ 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-SYS5Y cells downregulated the expression of insP3Rs in these cells (Fig. 5; Fig. 7) and concomitantly affected the activity and characteristics of Ca2+ puffs. Essentially, the consequence of prolonged agonist stimulation was to make the Ca2+ puffs less vigorous, with the amplitude, duration, frequency and number of pacemaker Ca2+ puff sites being reduced (Fig. 6; Fig. 7). Furthermore, global Ca2+ signalling was also restrained, because the number of cells displaying Ca2+ waves in response to 20 μM insP3-BM was substantially decreased (Fig. 8).

The downregulation of Ca2+ puff activity by agonist stimulation of cells can be rapid. We observed that a 2-hour incubation was sufficient to have significant effects on Ca2+ puff characteristics (data not shown). This is consistent with the observation that the half-time for loss of insP3Rs 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 insP3 sensitivity, the pacemaker Ca2+ puff sites are especially prone to regulation. Binding of insP3 to its receptor activates insP3R downregulation by stimulating ubiquitination. Once a polyubiquitinated insP3R is recognized, it is unfolded and cleaved by the proteasome (Zhu et al., 1999; Oberdorf et al., 1999). The greater insP3 sensitivity and activity of the pacemaking Ca2+ puff sites might cause them to be rapidly ubiquitinated and removed.

Although prolonged incubation with agonist had a similar effect on the Ca2+ puffs in HeLa and SH-SYS5Y cells, the extent of downregulation of insP3R isoforms was clearly different (Fig. 5; Fig. 7). A different susceptibility of insP3 R isoforms to downregulation was also reported in rat cerebellar granule cells and AR4-2J rat pancreatoma cells (Wojcikiewicz et al., 1994b). It is conceivable that, by virtue of their intrinsically higher insP3 sensitivity, the pacemaker Ca2+ puff sites are especially prone to regulation. Binding of insP3 to its receptor activates insP3R downregulation by stimulating ubiquitination. Once a polyubiquitinated insP3R is recognized, it is unfolded and cleaved by the proteasome (Zhu et al., 1999; Oberdorf et al., 1999). The greater insP3 sensitivity and activity of the pacemaking Ca2+ puff sites might cause them to be rapidly ubiquitinated and removed.

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Ca²⁺ puffs are generic element Ca²⁺ signals


