

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

Stephen C. Tovey^{1,*}, Patrick de Smet^{2,*}, Peter Lipp^{1,†}, David Thomas¹, Kenneth W. Young³, Ludwig Missiaen², Humbert De Smedt², Jan B. Parys², Michael J. Berridge¹, Jan Thuring⁴, Andrew Holmes⁴ and Martin D. Bootman^{1,5}

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

Journal of Cell Science 114, 3979-3989 (2001) © The Company of Biologists Ltd

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 evokes 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 InsP₃R

results from a profound acceleration of InsP₃R 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 InsP₃R 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 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 InsP₃R 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-excitabile 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 InsP₃R 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 glutamine (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 Tissue Culture Collection (HeLa cells, CCL-2; SH-SY5Y, CRL2266; NIH-3T3, CRL1658; RBL-2H3, CRL2256). The 16HBE140– cells were a kind gift of Dieter C. Gruenert (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:

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

f is the fluorescence intensity of fluo-3 recorded during the experiment. f_{\min} and f_{\max} are the minimal and maximal fluorescence intensities of fluo-3, reflecting the calcium-free and calcium-saturated forms of the indicator, respectively. f_{\min} and f_{\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 intensifying camera (Photronics Science, Robertsbridge, 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 cm³) 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 the above blocking solution. The InsP₃R-type-1-specific polyclonal antibody (Parys et al., 1995) was used at a 1:1000 dilution. The InsP₃R-type-2-specific KM1083 monoclonal antibody was a kind gift of K. Mikoshiba and was used at 1 μg ml⁻¹. The InsP₃R-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 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. (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;

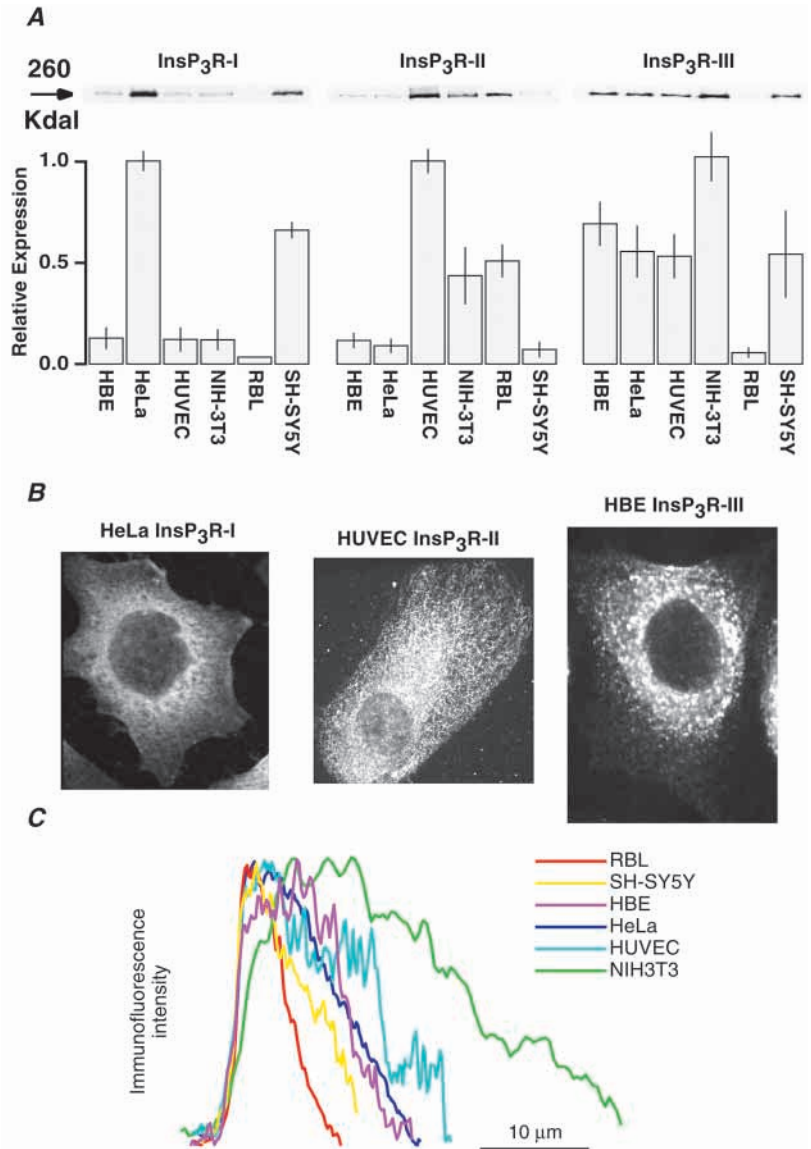


Fig. 1. InsP₃R expression and localization. (A) The histograms illustrate the relative abundances of the three InsP₃R isoforms in the cell types listed. The data were obtained by analysing the density of the immunoreactive bands at ~260 kDa. The data show means ± s.e.m. of three blots. (B) Typical confocal images (*z* depth < 1 μm) of (left) HeLa cells stained for type 1 InsP₃R, (middle) HUVEC cells stained for type 2 InsP₃R and (right) 16HBE140– cells stained for type 3 InsP₃R. In most of the cell types, a reticular distribution of staining could be observed but, in 16HBE140– cells in particular, the ER seemed to be considerably fragmented after fixation. (C) Intensity profiles of immunofluorescence from the nuclear envelope to the plasma membrane. These profiles were obtained by sampling the immunofluorescence intensity of the major InsP₃R isoform in each cell type across a line one pixel wide running from the nuclear envelope to the plasma membrane.

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).

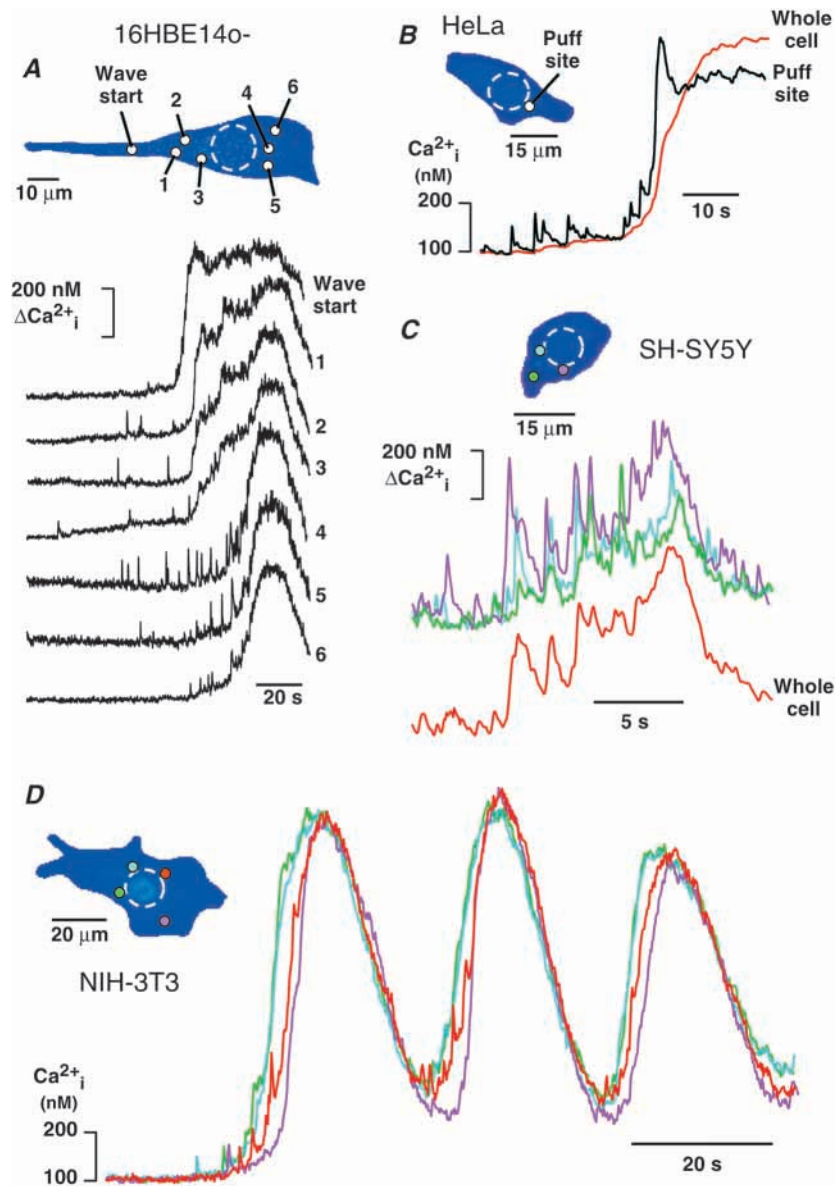


Fig. 2. Ca^{2+} puff activity during local and global signalling. All of the cell types analysed in the present study displayed Ca^{2+} puffs when treated with appropriate agonist concentrations. The aim of this figure is to show some of the different modes in which Ca^{2+} puff activity can be observed in these cells. (A) Rapid Ca^{2+} puff activity in a 16HBE140- cell stimulated with $5 \mu\text{M}$ ATP. Several Ca^{2+} puff sites were active before and during the onset of the Ca^{2+} wave. White circles on the inset cell image show the locations of the active puff sites, and the Ca^{2+} signals recorded at these regions are depicted by the correspondingly numbered traces. (B) A HeLa cell stimulated with $1 \mu\text{M}$ histamine and in which a single Ca^{2+} puff sites was responsible for triggering a regenerative Ca^{2+} wave. The black trace represents the Ca^{2+} signal observed at the puff site and the red trace depicts the global Ca^{2+} signal observed by averaging fluo-3 fluorescence across the whole cell. (C) Ca^{2+} puffs firing at three different sites within a single carbachol-stimulated SH-SY5Y cell ($1 \mu\text{M}$ carbachol). Coloured circles on the inset cell image show the locations of the active puff sites, and the Ca^{2+} signals recorded at these regions are depicted by the correspondingly coloured traces. This example illustrates the observation that, sometimes, the Ca^{2+} puff sites appear to fire in synchrony, whereas, at other times, they do not. (D) Ca^{2+} puffs in a NIH-3T3 cell stimulated with $1 \mu\text{M}$ ATP. Ca^{2+} puffs were observed at the regions marked by the blue, green and red circles on the inset cell image, and the correspondingly coloured traces indicate the Ca^{2+} changes observed at these sites. Ca^{2+} puffs were observed during the initial latency before the first Ca^{2+} oscillation and also on the rising phases of subsequent Ca^{2+} oscillations.

RESULTS

Characteristics of Ca^{2+} puffs are independent of the InsP_3R isoform

For analysis of Ca^{2+} puff characteristics, we chose six cell types that have been previously shown to express different combinations of the three InsP_3R isoforms. Using isoform-specific antibodies, we examined the differential expression of InsP_3R s 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_3R isoform, but the ratio of expression differed significantly (Fig. 1A). Some of the cell types seemed to be reasonable models for a single InsP_3R isoform (e.g. RBL-2H3 and 16HBE140- cells, which expressed high levels of types 2 and 3 InsP_3R s, respectively), whereas others demonstrated a predominant expression of pairs of InsP_3R isoforms (e.g. HeLa/SH-SY5Y expressed types 1 and 3 InsP_3R s; HUVEC/NIH-3T3 cells expressed types 2 and 3 InsP_3R s).

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

	16HBE140-	HeLa	HUVEC	NIH-3T3	RBL-2H3	SH-SY5Y
Amplitude (nM)	178±108 (602)	127±143 (521)	49±26 (529)	73±49 (376)	42±28 (232)	123±105 (664)
Ca^{2+} puff diameter (μm)	3.2±0.8 (30)	3.7±0.6 (40)	3.1±0.5 (30)	4.1±0.8 (20)	2.5±0.5 (32)	3.0±0.6 (40)
Distance from nucleus (μm)	1.7±5.0 (196)	4.4±6.2 (238)	6.2±6.1 (180)	17.5±14.9 (149)	12.3±11.3 (54)	3.3±4.2 (544)

The characters in parentheses indicate the number of Ca^{2+} puffs analysed.

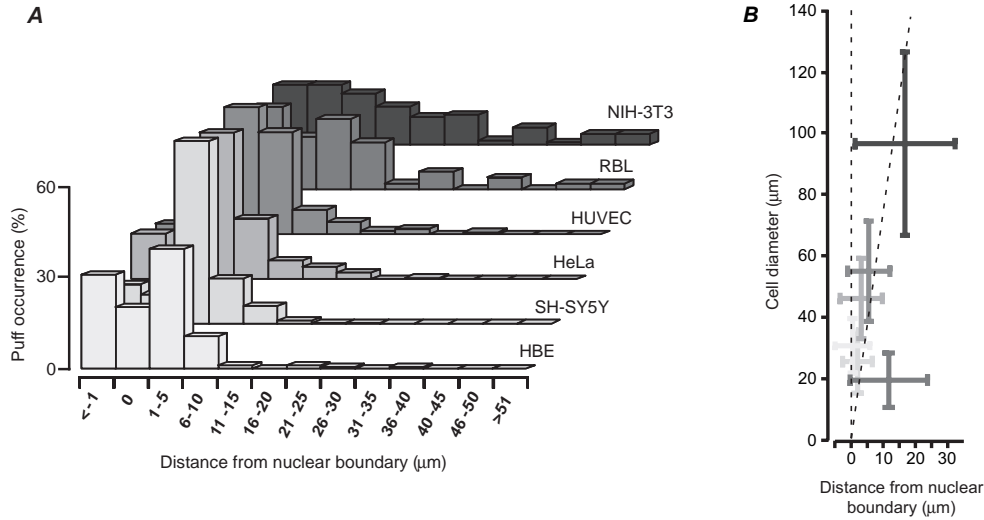


Fig. 3. Intracellular position of pacemaker Ca²⁺ puffs. (A) The histograms show the distribution of Ca²⁺ puffs relative to the nuclear envelope for each of the cell types investigated. (B) The average nuclear distances of the pacemaker Ca²⁺ puff sites are plotted against cell diameter, showing a roughly linear correlation for all the cell types except RBL-2H3 cells. The data are shown as mean \pm 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.

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 μ 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 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 InsP₃R 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

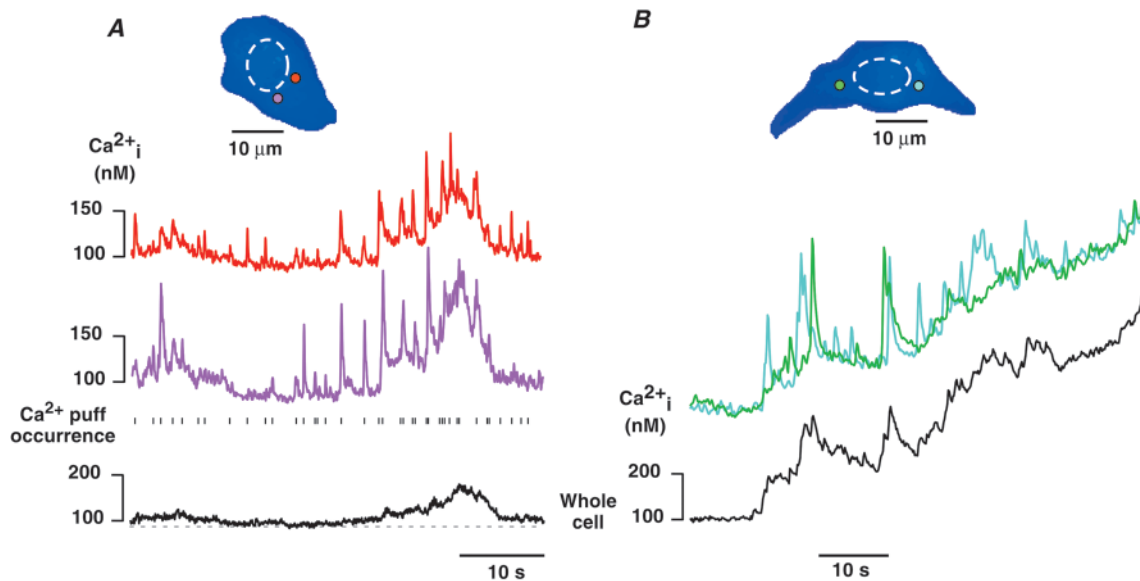


Fig. 4. Ca²⁺ puffs in 16HBE14o- and SH-SY5Y cells. (A) High-frequency non-regenerative Ca²⁺ puffs in a 16HBE14o- cell. The top two traces depict Ca²⁺ puffs firing at the sites marked by the correspondingly coloured circles in the inset cell image. The ticks beneath the second trace denote the occurrence 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.

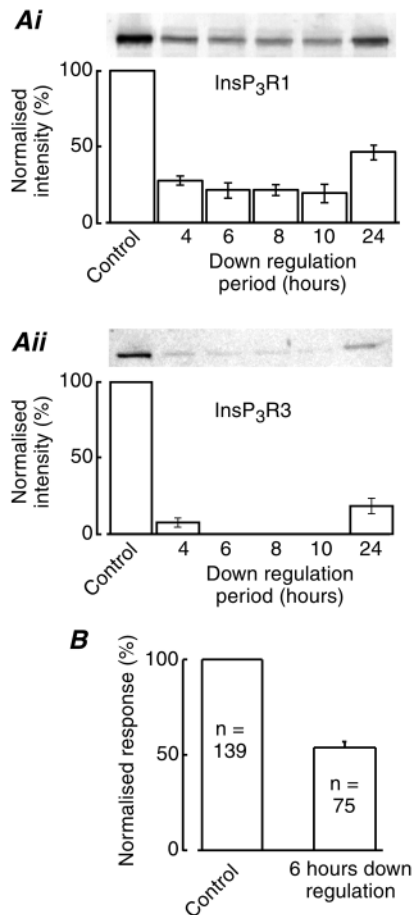


Fig. 5. Downregulation of InsP₃R_s in SH-SY5Y cells. (A) The effect of prolonged incubation with 1 mM carbachol on the expression level of (i) InsP₃R type 1 and (ii) InsP₃R type 3 in SH-SY5Y cells. Typical examples of the immunoband at 261 kDa and 248 kDa for types 1 and 3 InsP₃R_s are shown above the 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 InsP₃R_s 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 InsP₃R 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 InsP₃R 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 InsP₃R_s 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 InsP₃R 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 IP₃R 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 InsP₃R_s were reduced by ~90% after 4 hours of incubation and became undetectable at 6–10 hours incubation. These observations are consistent with earlier investigations of agonist stimulation on InsP₃R expression in SH-SY5Y cells (Wojcikiewicz et al., 1992; Wojcikiewicz et al., 1994a). For both InsP₃R isoforms, the reduced immunoreactivity was apparent for at least 10 hours

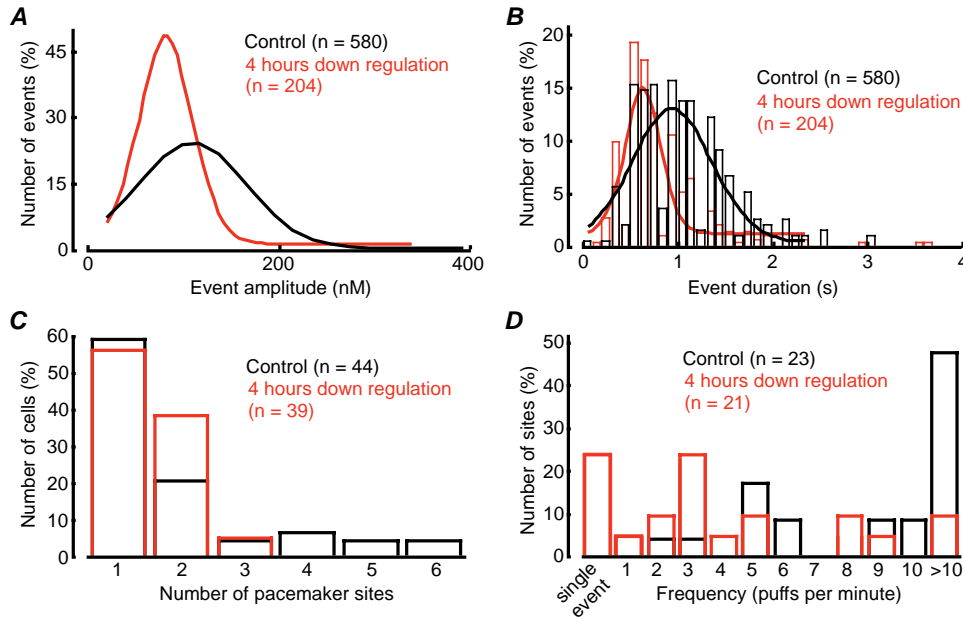


Fig. 6. Changes in Ca²⁺ puff characteristics in SH-SY5Y cells following prolonged agonist stimulation. (A–D) The effects of incubating SH-SY5Y cells with 1 mM carbachol for 4 hours on the characteristics of Ca²⁺ puffs. The Gaussian curves were calculated using Microcal Origin (Northampton, USA).

(Fig. 5A), whereas a slight recovery of both type 1 and type 3 InsP₃R_s was observed after 24 hours.

To confirm that the InsP₃R_s 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

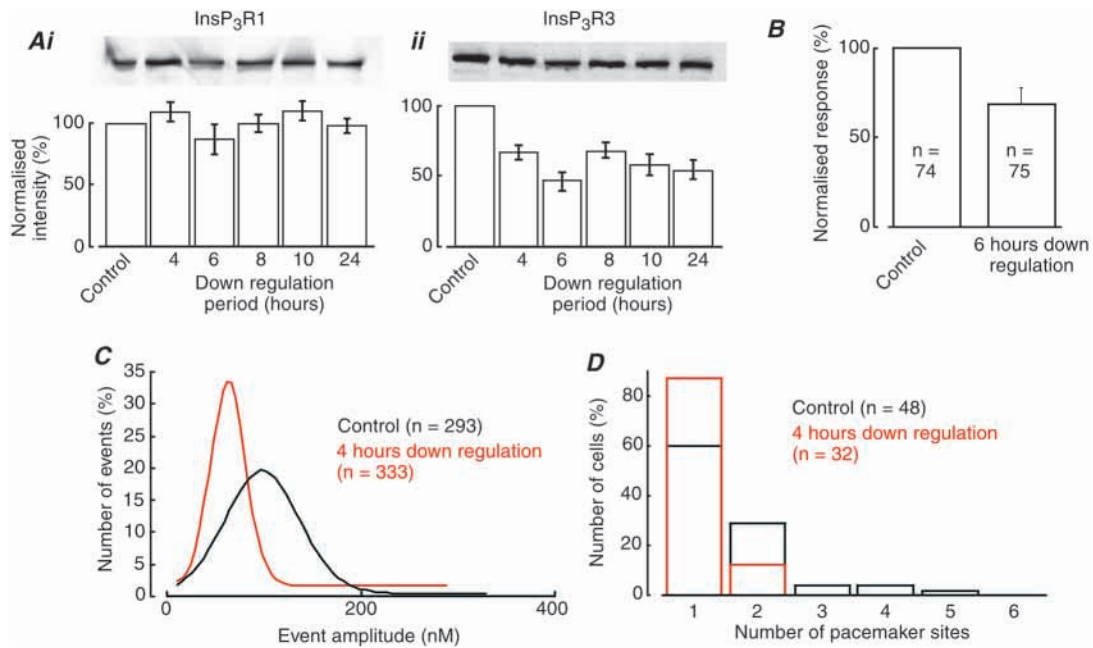


Fig. 7. Downregulation of InsP₃R_s and changes in Ca²⁺ puff characteristics in HeLa cells following prolonged agonist stimulation. (A) The effect of prolonged incubation with 1 mM histamine on the expression level of (i) IP₃R type 1 and (ii) IP₃R type 3 in HeLa cells. The immunoreactivity is expressed as a percentage of that in untreated cells. The data are expressed as mean ± s.e.m. ($n=3$). (B) The functional loss of InsP₃R_s 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.

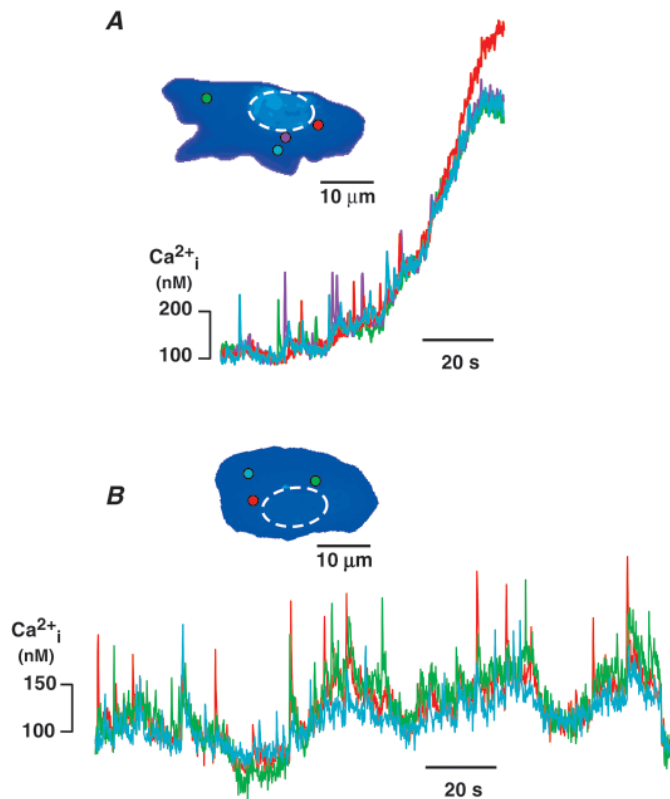


Fig. 8. Inhibition of Ca^{2+} wave initiation following prolonged agonist stimulation of HeLa cells. Typical responses of single HeLa cells to $20 \mu\text{M}$ InsP_3BM either before (A) or after (B) a 4-hour incubation with 1 mM histamine. (A) An example in which the pacemaking Ca^{2+} puff activity 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 amplitude of global responses after 24 hours of incubation with carbachol (data not shown), even though the InsP_3R expression had recovered only modestly (Fig. 5A). The latency for generation of global Ca^{2+} signals using the supramaximal InsP_3BM concentration was not different between control and carbachol-treated cells, suggesting that there was no change in their ability to hydrolyse the ester into its active product.

We next sought to examine the effect of InsP_3R downregulation on the characteristics of Ca^{2+} puffs in the SH-SY5Y cells. Because the amplitude of Ca^{2+} puffs is proportional to intracellular InsP_3 concentration (Thomas et al., 1998), we used InsP_3BM to evoke elementary Ca^{2+} release. In this way, we could match the InsP_3 concentration in control and carbachol-stimulated cells. Application of $20 \mu\text{M}$ InsP_3BM evoked Ca^{2+} puffs in 67% of the SH-SY5Y cells after a latency period of typically ~ 1 –5 minutes. In control cells, the amplitude of the Ca^{2+} puffs ranged from $\sim 15 \text{ nM}$ to $>300 \text{ nM}$.

The distribution of event amplitudes could be described by a single Gaussian curve with a mean of $115 \pm 15 \text{ nM}$ (mean \pm 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 ($80 \pm 5 \text{ nM}$; mean \pm 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 $\tau = 0.31 \pm 0.02 \text{ second}^{-1}$ and $0.25 \pm 0.02 \text{ second}^{-1}$, 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_3R 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, $\sim 75\%$ of the sites had a Ca^{2+} release frequency of $>5 \text{ events minute}^{-1}$ in the presence of $20 \mu\text{M}$ InsP_3BM . After 4 hours of agonist incubation, this figure decreased to $\sim 15\%$. 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_3R expression. However, in contrast to SH-SY5Y cells, the type-1 InsP_3R expression did not detectably alter (Fig. 7Ai), whereas the type-3 InsP_3R isoform was maximally downregulated by $\sim 50\%$ 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 Ca^{2+} signals evoked by $100 \mu\text{M}$ InsP_3 ester (Fig. 7B; significantly different from control; $P < 0.001$), confirming that the InsP_3Rs 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 $10 \mu\text{M}$ InsP_3 ester was reduced from $103 \pm 23 \text{ nM}$ to $62 \pm 18 \text{ nM}$ (mean \pm 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 ($\tau = 0.30 \pm 0.02 \text{ second}^{-1}$ and $0.24 \pm 0.02 \text{ second}^{-1}$, respectively; $P > 0.05$). The frequency with which pacemaking Ca^{2+} puff sites

were activated by 10 μM 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 μM because, at 10 μM , 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 μM 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 μM 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 (Hüser 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. 16HBE140– 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 16HBE140– 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 16HBE140–) 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

InsP₃ production (Thomas et al., 2000a). Furthermore, immunostaining 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-SY5Y 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 InsP₃BM 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-SY5Y cells, the extent of downregulation of InsP₃R isoforms was clearly different (Fig. 5; Fig. 7). A different susceptibility of InsP₃R 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 InsP₃Rs, type 1 InsP₃Rs were found to be selectively downregulated. It is currently unclear why certain InsP₃R 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 InsP₃Rs (~50% loss of type 3 InsP₃Rs, which are roughly half of all receptors) correlated roughly with the reduction in the Ca²⁺ response triggered by a supramaximal InsP₃BM concentration (Fig. 7). In SH-SY5Y cells, the loss of InsP₃R expression was much more marked but the cells still managed a significant response to supramaximal InsP₃BM (Fig. 5). This might indicate that, in SH-SY5Y, there is a large reserve of InsP₃Rs, such that only a fraction of the intracellular immunoreactive

InsP₃Rs are required to generate a maximal Ca²⁺ liberation. HeLa cells, by contrast, do not have a receptor reserve.

In summary, our data show that Ca²⁺ puffs are a generic elementary Ca²⁺ signal used by different cell types for constructing InsP₃-mediated responses. The different InsP₃Rs appear to be functionally redundant at the level of elementary Ca²⁺ signalling. In addition, Ca²⁺ puff sites are susceptible to regulation during prolonged cellular stimulation and, as a consequence, global Ca²⁺ signalling is inhibited.

This work was funded by the BBSRC 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

- Bennett, D. L., Cheek, T. R., Berridge, M. J., De Smedt, H., Parys, J. B., Missiaen, L. and Bootman, M. D. (1996). Expression and function of ryanodine receptors in non-excitabile cells. *J. Biol. Chem.* **271**, 6356-6362.
- Berridge, M. J. (1993). Inositol trisphosphate and calcium signalling. *Nature* **361**, 315-325.
- Berridge, M. J. (1997). Elementary and global aspects of calcium signalling. *J. Physiol.* **499**, 291-306.
- Berridge, M. J. and Galione, A. (1988). Cytosolic calcium oscillators. *FASEB J.* **2**, 3074-3082.
- Berridge, M. J., Bootman, M. D. and Lipp, P. (1998). Calcium – a life and death signal. *Nature* **395**, 645-648.
- Berridge, M. J., Lipp, P. and Bootman, M. D. (2000). The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* **1**, 11-21.
- Bobanovic, F., Bootman, M. D., Berridge, M. J., Parkinson, N. A. and Lipp, P. (1999). Elementary [Ca²⁺]_i signals generated by electroporation functionally mimic those evoked by hormonal stimulation. *FASEB J.* **13**, 365-376.
- Boittin, F.-X., Coussin, F., Morel, J.-L., Halet, G., Macrez, N. and Mironneau, J. (2000). Ca²⁺ signals mediated by Ins(1,4,5)P₃-gated channels in rat uterine myocytes. *Biochem. J.* **349**, 323-332.
- Bootman, M. D. and Berridge, M. J. (1995). The elemental principles of calcium signalling. *Cell* **83**, 675-678.
- Bootman, M. D. and Berridge, M. J. (1996). Subcellular Ca²⁺ signals underlying waves and graded responses in HeLa cells. *Curr. Biol.* **6**, 855-865.
- Bootman, M. D., Taylor, C. W. and Berridge, M. J. (1992). The thiol reagent, thimerosal, evokes Ca²⁺ spikes by sensitizing the inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* **267**, 25113-25119.
- Bootman, M. D., Niggli, E., Berridge, M. J. and Lipp, P. (1997a). Imaging the hierarchical calcium signalling system in HeLa cells. *J. Physiol.* **499**, 307-314.
- Bootman, M., Berridge, M. J. and Lipp, P. (1997b). Cooking with calcium; the recipes for composing global signals from elementary events. *Cell* **91**, 367-373.
- Bootman, M. D., Thomas, D., Tovey, S. C., Berridge, M. J. and Lipp, P. (2000). Nuclear calcium signalling. *Cell Mol. Life Sci.* **57**, 371-378.
- Brind, S., Swann, K. and Carroll, J. (2000). Inositol 1,4,5-trisphosphate receptors are downregulated in mouse oocytes in response to sperm or adenophostin A but not to increases in intracellular Ca²⁺ or egg activation. *Dev. Biol.* **223**, 251-265.
- Callamaras, N., Marchant, J. S., Sun, X. P. and Parker, I. (1998). Activation and co-ordination of InsP₃-mediated elementary Ca²⁺ events during global Ca²⁺ signals in *Xenopus* oocytes. *J. Physiol.* **509**, 81-91.
- Collins, T. J., Lipp, P., Berridge, M. J., Li, W. and Bootman, M. D. (2000). Inositol 1,4,5-trisphosphate-induced Ca²⁺ release is inhibited by mitochondrial depolarization. *Biochem. J.* **347**, 593-600.
- De Smedt, H., Missiaen, L., Parys, J. B., Bootman, M. D., Mertens, L., Van Den Bosch, L. and Casteels, R. (1994). Determination of the relative amounts of inositol trisphosphate receptor mRNA isoforms by polymerase chain reaction. *J. Biol. Chem.* **269**, 21691-21698.

- DeSmedt, H., Missiaen, L., Parys, J. B., Henning, R. H., Sienaert, I., Vanlingen, S., Gijssens, A., Himpens, B. and Casteels, R. (1997). Isoform diversity of the inositol trisphosphate receptor in cell types of mouse origin. *Biochem. J.* **322**, 575-583.
- Haak, L. L., Song, L. S., Molinski, T. F., Pessah, I. N., Cheng, H. P. and Russell, J. T. (2001). Sparks and puffs in oligodendrocyte progenitors: cross talk between ryanodine receptors and inositol trisphosphate receptors. *J. Neurosci.* **21**, 3860-3870.
- Hüser, J. and Blatter, L. A. (1997). Elementary events of agonist-induced Ca²⁺ release in vascular endothelial cells. *Am. J. Physiol.* **42**, C1775-C1782.
- Jacob, R., Merritt, J. E., Hallam, J. E. and Rink, T. J. (1988). Repetitive spikes in cytoplasmic calcium evoked by histamine in human endothelial cells. *Nature* **335**, 40-45.
- Jellerette, T., He, C. L., Wu, H., Parys, J. B. and Fissore, R. A. (2000). Down-regulation of the inositol 1,4,5-trisphosphate receptor in mouse eggs following fertilization or parthenogenetic activation. *Dev. Biol.* **223**, 238-250.
- Koizumi, S., Bootman, M. D., Bobanovic, L. K., Schell, M. J., Berridge, M. J. and Lipp, P. (1999). Characterization of elementary Ca²⁺ release signals in NGF-differentiated PC12 cells and hippocampal neurones. *Neuron* **22**, 125-137.
- Lipp, P., Thomas, D., Berridge, M. J. and Bootman, M. D. (1997). Nuclear calcium signalling by individual cytoplasmic calcium puffs. *EMBO J.* **16**, 7166-7173.
- Lipp, P., Laine, M., Tovey, S. C., Burrell, K. M., Berridge, M. J., Li, W. and Bootman, M. D. (2000). Functional InsP₃ receptors that may modulate excitation-contraction coupling in the heart. *Curr. Biol.* **10**, 939-942.
- Marchant, J., Callamaras, N. and Parker, I. (1999). Initiation of IP₃-mediated Ca²⁺ waves in *Xenopus* oocytes. *EMBO J.* **18**, 5285-5299.
- Marchant, J. S. and Parker, I. (2001). Role of elementary Ca²⁺ puffs in generating repetitive Ca²⁺ oscillations. *EMBO J.* **20**, 65-76.
- Mountian, I., Manolopoulos, V. G., De Smedt, H., Parys, J. B., Missiaen, L. and Wuytack, F. (1999). Expression patterns of sarco/endoplasmic reticulum Ca²⁺-ATPase and inositol 1,4,5-trisphosphate receptor isoforms in vascular endothelial cells. *Cell Calcium* **25**, 371-380.
- Oberdorf, J., Webster, J. M., Zhu, C. C., Luo, S. G. and Wojcikiewicz, R. J. (1999). Down-regulation of types I, II and III inositol 1,4,5-trisphosphate receptors is mediated by the ubiquitin/proteasome pathway. *Biochem. J.* **339**, 453-461.
- Parker, I. and Yao, Y. (1991). Regenerative release of calcium from functionally discrete subcellular stores by inositol trisphosphate. *Proc. R. Soc. Lond. B Biol. Sci.* **246**, 269-274.
- Parrington, J., Brind, S., De Smedt, H., Gangeswaran, R., Lai, F. A., Wojcikiewicz, R. and Carroll, J. (1998). Expression of inositol 1,4,5-trisphosphate receptors in mouse oocytes and early embryos: the type I isoform is upregulated in oocytes and downregulated after fertilization. *Dev. Biol.* **203**, 451-461.
- Parys, J. B., De Smedt, H., Missiaen, L., Bootman, M. D., Sienaert, I. and Casteels, R. (1995). Rat basophilic leukemia cells as model system for inositol 1,4,5-trisphosphate receptor IV, a receptor of the type II family: functional comparison and immunological detection. *Cell Calcium* **17**, 239-249.
- Patel, S., Joseph, S. K. and Thomas, A. P. (1999). Molecular properties of inositol 1,4,5-trisphosphate receptors. *Cell Calcium* **25**, 247-264.
- Petersen, O. H., Petersen, C. C. H. and Kasai, H. (1994). Calcium and hormone action. *Annu. Rev. Physiol.* **56**, 297-319.
- Rooney, T. A., Sass, E. J. and Thomas, A. P. (1990). Agonist-induced cytosolic calcium oscillations originate from a specific locus in single hepatocytes. *J. Biol. Chem.* **265**, 10792-10796.
- Sienaert, I., Huyghe, S., Parys, J. B., Malfait, M., Kunzelmann, K., De Smedt, H., Verleden, G. M. and Missiaen, L. (1998). ATP-induced Ca²⁺ signals in bronchial epithelial cells. *Pflügers Arch.* **436**, 40-48.
- Simpson, P. B., Mehotra, S., Lange, G. D. and Russell, J. T. (1997). High density distribution of endoplasmic reticulum proteins and mitochondria at specialized Ca²⁺ release sites in oligodendrocyte processes. *J. Biol. Chem.* **272**, 22654-22661.
- Sipma, H., Deelman, L., De Smedt, H., Missiaen, L., Parys, J. B., Vanlingen, S., Henning, R. H. and Casteels, R. (1998). Agonist-induced down regulation of type 1 and type 3 inositol 1,4,5-tris-phosphate receptors in A7r5 and DDT1 MF-2 smooth muscle cells. *Cell Calcium* **23**, 11-21.
- Sun, X. P., Callamaras, N., Marchant, J. S. and Parker, I. (1998). A continuum of InsP₃-mediated elementary Ca²⁺ signalling events in *Xenopus* oocytes. *J. Physiol.* **509**, 67-80.
- Taylor, C. W. (1998). Inositol trisphosphate receptors: Ca²⁺-modulated intracellular Ca²⁺ channels *Biochim. Biophys. Acta* **1436**, 19-33.
- Taylor, C. W., Genezzani, A. A. and Morris, S. A. (1999). Expression of inositol trisphosphate receptors. *Cell Calcium* **26**, 237-251.
- Thomas, A. P., Renard, D. C. and Rooney, T. A. (1991). Spatial and temporal organization of calcium signalling in hepatocytes. *Cell Calcium* **12**, 111-126.
- Thomas, A. P., Bird, G. S. J., Hajnoczky, G., Robb-Gaspers, L. D. and Putney, J. W. (1996). Spatial and temporal aspects of cellular calcium signalling. *FASEB J.* **10**, 1505-1517.
- Thomas, D., Lipp, P., Berridge, M. J. and Bootman, M. D. (1998). Hormone-stimulated calcium puffs in non-excitabile cells are not stereotypic, but reflect activation of different size channel clusters and variable recruitment of channels within a cluster. *J. Biol. Chem.* **273**, 27130-27136.
- Thomas, D., Lipp, P., Tovey, S. C., Berridge, M. J., Li, W. H., Tsien, R. Y. and Bootman, M. D. (2000a). Microscopic properties of elementary Ca²⁺ release sites in non-excitabile cells. *Curr. Biol.* **10**, 8-15.
- Thomas, D., Tovey, S. C., Collins, T. J., Bootman, M. D., Berridge, M. J. and Lipp, P. (2000b). A comparison of fluorescent indicators and their use in measuring elementary and global Ca²⁺ signals. *Cell Calcium* **28**, 213-223.
- Tovey, S. C., Godfrey, R. E., Hughes, P. J., Mezna, M., Minchin, S. D., Mikoshiba, K. and Michelangeli, F. (1997). Identification and characterization of inositol 1,4,5-trisphosphate receptors in rat testis. *Cell Calcium* **21**, 311-319.
- Van Acker, K., Bautmans, B., Bultynck, G., Maes, K., Weidema, A. F., De Smet, P., Parys, J. B., De Smedt, H., Missiaen, L. and Callewaert, G. (2000). Mapping of IP₃-mediated Ca²⁺ signals in single human neuroblastoma SH-SY5Y cells: cell volume shaping the Ca²⁺ signal. *J. Neurophysiol.* **83**, 1052-1057.
- Vanlingen, S., Parys, J. B., Missiaen, L., De Smedt, H., Wuytack, F. and Casteels, R. (1997). Distribution of inositol 1,4,5-trisphosphate receptor isoforms, SERCA isoforms and Ca²⁺ binding proteins in RBL-2H3 rat basophilic leukemia cells. *Cell Calcium* **22**, 475-486.
- Willars, G. B. and Nahorski, S. R. (1995). Quantitative comparisons of muscarinic and bradykinin receptor-mediated Ins(1,4,5)P₃ accumulation and Ca²⁺ signalling in human neuroblastoma cells. *Br. J. Pharmacol.* **114**, 1133-1142.
- Wilson, B. S., Pfeiffer, J. R., Smith, A. J., Oliver, J. M., Oberdorf, J. A. and Wojcikiewicz, R. J. (1998). Calcium-dependent clustering of inositol 1,4,5-trisphosphate receptors. *Mol. Biol. Cell* **9**, 1465-1478.
- 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.
- Wojcikiewicz, R. J., Nakade, S., Mikoshiba, K. and Nahorski, S. R. (1992). Inositol 1,4,5-trisphosphate receptor immunoreactivity in SH-SY5Y human neuroblastoma cells is reduced by chronic muscarinic receptor activity. *J. Neurochem.* **59**, 383-386.
- Wojcikiewicz, R. J., Furuichi, T., Nakade, S., Mikoshiba, K. and Nahorski, S. R. (1994a). Muscarinic receptor activation down-regulates the type-I inositol 1,4,5-trisphosphate receptor by accelerating its degradation. *J. Biol. Chem.* **269**, 7963-7969.
- Wojcikiewicz, R. J., Tobin, A. B. and Nahorski, S. R. (1994b). Muscarinic receptor-mediated inositol 1,4,5-trisphosphate formation in SH-SY5Y neuroblastoma cells is regulated acutely by cytosolic Ca²⁺ and rapid desensitization. *J. Neurochem.* **63**, 177-185.
- Yao, Y., Choi, J. and Parker, I. (1995). Quantal puffs of intracellular Ca²⁺ evoked by inositol trisphosphate in *Xenopus* oocytes. *J. Physiol.* **482**, 533-553.
- Young, K. W., Challiss, R. A. J., Nahorski, S. R. and Mackrill, J. J. (1999). Lysophosphatidic acid-mediated Ca²⁺ mobilization in human SH-SY5Y neuroblastoma cells is independent of phosphoinositide signalling, but dependent on sphingosine kinase activation. *Biochem. J.* **343**, 45-52.
- Young, K. W., Bootman, M. D., Channing, D. R., Lipp, P., Maycox, P. R., Meakin, J., Challiss, R. A. J. and Nahorski, S. R. (2000). Lysophosphatidic acid-induced Ca²⁺ mobilization requires intracellular sphingosine 1-phosphate production: potential involvement of endogenous Edg-4 receptors. *J. Biol. Chem.* **275**, 38532-38539.
- Zhu, C. C., Furuichi, T., Mikoshiba, K. and Wojcikiewicz, R. J. (1999). Inositol 1,4,5-trisphosphate receptor down-regulation is activated directly by inositol 1,4,5-trisphosphate binding - studies with binding-defective mutant receptors. *J. Biol. Chem.* **274**, 3476-3484.
- Zhu, C. C. and Wojcikiewicz, R. J. (2000). Ligand binding directly stimulates ubiquitination of the inositol 1,4,5-trisphosphate receptor. *Biochem. J.* **348**, 551-556.