Agonist-evoked inositol trisphosphate receptor (IP₃R) clustering is not dependent on changes in the structure of the endoplasmic reticulum

Mark CHALMERS*, Michael J. SCHELL† and Peter THORN¹

*Department of Pharmacology, University of Cambridge, Cambridge, CB2 1PD, U.K., and †Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd, Bethesda, MD 20814-4799, U.S.A.

The size and number of IP₃R (inositol 1,4,5-trisphosphate receptor) clusters located on the surface of the ER (endoplasmic reticulum) is hypothesized to regulate the propagation of Ca²⁺ waves in cells, but the mechanisms by which the receptors cluster are not understood. Using immunocytochemistry, live-cell imaging and heterologous expression of ER membrane proteins we have investigated IP₃R clustering in the basophilic cell line RBL-2H3 following the activation of native cell-surface antigen receptors. IP₃R clusters are present in resting cells, and upon receptor stimulation, form larger aggregates. Cluster formation and maintenance required the presence of extracellular Ca²⁺ in both resting and stimulated cells. Using transfection with a marker of the ER, we found that the ER itself also showed structural changes, leading to an increased number of ‘hotspots’, following antigen stimulation. Surprisingly, however, when we compared the ER hotspots and IP₃R clusters, we found them to be

distinct. Imaging of YFP (yellow fluorescent protein)–IP₃R transfected in to living cells confirmed that IP₃R clustering increased upon stimulation. Photobleaching experiments showed that the IP₃R occupied a single contiguous ER compartment both before and after stimulation, suggesting a dynamic exchange of IP₃R molecules between the clusters and the surrounding ER membrane. It also showed a decrease in the mobile fraction after cell activation, consistent with receptor anchoring within clusters. We conclude that IP₃R clustering in RBL-2H3 cells is not simply a reflection of bulk-changes in ER structure, but rather is due to the receptor undergoing homotypic or heterotypic protein–protein interactions in response to agonist stimulation.

Key words: calcium, endoplasmic reticulum (ER), inositol trisphosphate receptor (IP₃R), RBL-2H3 cell line, yellow fluorescent protein (YFP), agonist.

INTRODUCTION

A rise in cytosolic Ca²⁺ is a key trigger for the regulation of a huge variety of cell processes [1]. One source of cytosolic Ca²⁺ in many cells is the release of Ca²⁺ from intracellular stores, such as the ER (endoplasmic reticulum) in response to the generation of the soluble messenger IP₃ (inositol 1,4,5-trisphosphate). IP₃ opens the IP₃R (IP₃ receptor), present on the ER membrane, and Ca²⁺ flows into the cytosol. It follows that the positioning of IP₃R can be an important parameter for the local delivery of Ca²⁺ to specific sites within the cell. A good example of this is in pancreatic acinar cells where IP₃R are specifically enriched in the apical domain and locally regulate Ca²⁺-dependent secretion [2–4].

Polarized epithelial cells like acinar cells, are probably an exceptional case in that their IP₃R are relatively stably positioned within the cell [5] with essentially no mobility, as shown in confluent Madin–Darby canine kidney cells [6]. In most other cell-types IP₃R are diffusible within the ER membrane and can even be dynamically positioned during cell stimulation. For example, activation of neutrophils leads to a cell-shape change and repositioning of the whole Ca²⁺ store [7]. More recently, long-term agonist stimulation in the smooth muscle cell line A7r5 was shown to lead to global repositioning of type 1 IP₃R (IP₃RI), but not type 3 IP₃R (IP₃RIII) [8].

In addition to these global movements of IP₃R, it has recently been recognized that a more local clustering of IP₃R can also occur. For example, the maturation of oocytes before fertilization

leads to IP₃R clustering [9–11], and in other cell types, such as the basophilic cell line RBL-2H3, IP₃R clustering can be rapidly triggered by activation of the Ca²⁺ signalling cascade [12,13]. The consequences of receptor clustering are not clear but since cytosolic Ca²⁺ can both positively and negatively regulate the IP₃R, the close apposition of IP₃R following clustering may influence the Ca²⁺ signal produced [14].

The mechanism(s) of IP₃R clustering is not known. In principle it could be a process intrinsic to the IP₃R (or an accessory protein) or it could be due to changes in the ER itself. Wilson et al. [12] demonstrated that this process in RBL-2H3 cells is rapid, reversible and regulated by cytosolic Ca²⁺. They showed that although the structure of the ER does change, that change is too small to account for IP₃R clustering. Recently, Tateishi et al. [15] indicated that it is likely that a conformational change induced by the occupancy of IP₃R by IP₃ is the critical trigger for clustering in COS cells; in that study the ER structure apparently did not change at all. A limitation of both these studies [12,15] is that any dynamic change of the ER was not quantified and therefore not directly compared with IP₃R clustering.

In the present study, we have compared and quantified the localization and dynamics of transfected-ER-markers with that of the IP₃R following the stimulation of cell surface receptors in RBL-2H3 cells. Despite similar percentage areas, numbers and time-courses of clustering, to our surprise the ER hotspots and the IP₃R clusters did not colocalize. We conclude that the processes that regulate the clustering of the ER and IP₃R in these cells are

Abbreviations used: BAPTA/AM, [bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)]; DMEM, Dulbecco's modified Eagle's medium; DNP, 2,4-dinitrophenyl; ER, endoplasmic reticulum; EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; FCS, foetal calf serum; FRAP, fluorescence recovery after photobleaching; IP₃, inositol 1,4,5-trisphosphate; IP₃R, IP₃ receptor; MEM, minimum essential medium; Mf, mobile fraction; PFA, paraformaldehyde; ROI, region of interest; YFP, yellow fluorescent protein.

¹ To whom correspondence should be addressed (email pt207@cam.ac.uk).

distinct, suggesting that IP₃R clustering is due chiefly to protein–protein interactions.

MATERIALS AND METHODS

Solutions

Experiments were performed in Hanks BSA buffer (in mM: 125 NaCl, 5 KCl, 0.7 Na₂HPO₄, 0.7 NaH₂PO₄, 15 NaHCO₃, 5.5 glucose, 0.75 MgCl₂, 1.8 CaCl₂ and 0.5 % BSA). Ca²⁺ free Hank's BSA buffer contained 500 μ M EGTA and no CaCl₂. High Ca²⁺ Hank's BSA buffer contained 5 mM CaCl₂. Confocal-imaging time-lapse microscopy was performed in Phenol Red free DMEM (Dulbecco's modified Eagle's medium), (Invitrogen), with 25 mM Hepes, 4 mM glutamine and 5 % FCS (foetal calf serum) (Invitrogen).

Antibodies

Rabbit polyclonal anti-IP₃RII receptor antibody (Chemicon International) was used at a dilution of 1:20. Rabbit polyclonal anti-type 2 IP₃R (IP₃RII) antibody raised against keyhole-limpet haemocyanin conjugated to a peptide corresponding to residues 62–75 (PMNRYSAQKQFWKAC) of rat IP₃RI which is common to all IP₃R subtypes (S17; a gift from Professor C. W. Taylor, Gurdon Institute, Cambridge, U.K.) was used at a dilution of 1:100. Western blot analysis showed that in RBL-2H3 lysates both antibodies exclusively recognized a band at 250 kDa (see Figure 1D). Rabbit anti-calreticulin polyclonal antibody (Calbiochem) was used at a dilution of 1:100. The secondary antibodies, Cy3 and fluorescein isothiocyanate-conjugated donkey anti-(rabbit IgG) (Strattech Scientific Ltd) and an Alexa Fluor[®] 488-conjugated goat anti-(rabbit IgG) (Molecular Probes) were all used at a dilution of 1:200. Applied alone these secondary antibodies showed no staining.

Cell culture

RBL-2H3 cells (DSMZ German Collection of Microorganisms and Cell Cultures) were maintained in monolayer cultures in 75 cm³ plastic tissue-culture flasks containing Eagle's MEM (minimal essential medium) containing L-glutamine, NaHCO₃ and Earl's salts supplemented with 10 % FCS and 100 units · ml⁻¹ penicillin-streptomycin in a humidified atmosphere of 5 % CO₂ at 37 °C. When the RBL-2H3 cells reached confluency they were detached from culture plates by washing with Dulbecco's PBS without Ca²⁺ and Mg²⁺, followed by 0.25 % trypsin solution. The RBL-2H3 cells were seeded at a density of approx. 1 × 10⁶ cells/75 cm³.

Antigen stimulation of primed RBL-2H3 cells

RBL-2H3 cell surface FCεR1 IgE receptors were primed by the addition of anti-DNP-IgE (2,4-dinitrophenylated IgE) (1 μ g · ml⁻¹ in Eagle's MEM with 10 % FCS and 100 units · ml⁻¹ penicillin-streptomycin) for 12–24 h. Cells were washed 3 times with Eagle's MEM to remove excess IgE and activated by the addition of the polyvalent antigen, DNP-BSA (37 °C).

Immunofluorescence

Monolayers of RBL-2H3 cells were grown on sterilized glass coverslips (25 mm diameter, No. 1 thickness; BDH, Cincinnati, OH, U.S.A.) for at least 24 h before drug treatment. Cells were washed in Dulbecco's PBS with Ca²⁺ and Mg²⁺ then in Pipes (dipotassium salt) buffer (80 mM K-Pipes, 5 mM EGTA,

2 mM MgCl₂, pH 6.5) and then fixed with 4 % (w/v) PFA (paraformaldehyde) in K-Pipes buffer for 30 min. The remaining steps were carried out in PBS without Ca²⁺ and Mg²⁺. Cells were washed and then permeabilized with PBS/0.1 % Triton X-100 for 5 min, washed again, and then blocked for 1 h with 2 % (v/v) donkey serum and 2 % (v/v) gelatin. The coverslips were then inverted on to a 50 μ l droplet of primary antibody and placed on to parafilm in a humidified chamber for 1–12 h. The coverslips were then placed in clean dishes and washed 4 times over a period of 15 min. The coverslips were soaked in 2 % (v/v) donkey serum for 15 min before placing the coverslips in a clean dish. The coverslips were inverted on to a 50 μ l droplet of secondary antibody for 30 min. Coverslips were then washed for 15 min before mounting on to glass slides in 20 μ l of Prolong antifade (Molecular Probes, Eugene, OR, U.S.A.).

Plasmid construction and transient transfection

The expression plasmid coding for EYFP (enhanced yellow fluorescent protein)–IP₃RI was constructed from the full-length (aa 1–2749) IP₃RI cDNA lacking the S1 splice site [16] as described by Parker et al. [17]. DsRed2-ER (Becton Dickinson Biosciences, Clontech) was used in this study for fluorescent labelling of the ER in living cells. DsRed2-ER has the ER targeting sequence of calreticulin, fused to the 5' end of DsRed2; and the ER-retention sequence, KDEL, fused to the 3' end of DsRed2. The expression plasmid coding for the P450–EGFP (enhanced green fluorescent protein) was constructed using the full-length (aa 1–490) image clone 4162143 mouse cytochrome P450 (family 2; subfamily C, polypeptide 29) as a template. The cDNA was sub-cloned in-frame into the p-EGFP-N1 vector (Becton Dickinson Biosciences, Clontech), using the restriction enzymes BamHI and EcoRI (Promega, U.S.A.) which places the GFP at the C-terminus. The cloning was confirmed by both restriction digest and sequencing. Transfection was carried out using FuGENE[™] 6 Transfection reagent (Roche Molecular Biochemicals). The transfected cells were processed for immunofluorescence studies 12–48 h post-transfection.

Confocal-imaging

Images of cells were captured using a Zeiss confocal laser-scanning microscope, model 510 fitted with an $\times 63$, 1.4 numerical aperture, oil immersion objective (Zeiss) and a 37 °C heated stage-mount (Harvard Apparatus). Images were captured in an optical slice of approx. 1 μ m with the appropriate filters: fluorescein isothiocyanate, Alexa Fluor[®] 488, EYFP and EGFP were excited using the 488 nm line of a krypton/argon laser and viewed with a 505–530 band-pass filter. Cy3 and DsRed2 were excited with the 543 nm line of a helium/neon laser and collected with a 560 nm long-pass filter. All images were captured using the multi-track mode of the microscope to decrease crosstalk of fluorescent signals.

FRAP (fluorescence recovery after photobleaching)

Typically, a square perinuclear ROI (region of interest) (400 pixel² = 14.5 μ m²) was defined and photobleaching conducted using the argon 488 nm laser with an increased power and approx. 150 scan iterations. At least twenty pre-bleach images were acquired. Following ROI bleaching, a series of post-bleach images were acquired until the bleached ROI fluorescence-intensity recovered to a plateau, which was approx. 300 sec for the ER membrane proteins. To confirm that FRAP was due to diffusion, the recovery of fluorescence following bleaching was examined in PFA-fixed RBL-2H3 cells. As expected, following

the bleach-protocol there was insignificant recovery (results not shown).

Mf (mobile fraction)

The Mf (mobile fraction) refers to the percentage of the starting fluorescence that diffuses in to a bleached ROI during the time-course of the experiment. The Mf was calculated according to the following equation:

$$\text{Mf} = 100 \left[\frac{(F_{\text{precell}} - F_{\text{background}})}{(F_{\infty} - F_{\text{background}}) - (F_0 - F_{\text{background}})} \right] \times \left[\frac{(F_{\infty\text{cell}} - F_{\text{background}})}{(F_{\text{pre}} - F_{\text{background}}) - (F_0 - F_{\text{background}})} \right]$$

where: F_{precell} is whole cell pre-bleach intensity; F_{pre} is bleach ROI pre-bleach intensity; $F_{\infty\text{cell}}$ = asymptote of fluorescence recovery in the whole cell; $F_{\text{background}}$ is mean background intensity; F_{∞} = bleach ROI asymptote; F_0 is bleach ROI immediate post bleach intensity.

We corrected for the loss of total cellular fluorescence due to bleaching. Pre-bleach fluorescence intensity of the whole cell ROI (F_{precell}), was divided by the whole-cell ROI intensity at time 't' and the fraction was then converted into a percentage.

Diffusion coefficient

Proteins in membranes move by diffusion (D , $\mu\text{m}^2 \cdot \text{sec}^{-1}$) reflecting the mean-squared displacement that a protein explores through time. To actually model this behaviour in the ER, formulas have evolved to account for diffusion in complex three-dimensional membrane networks, which we have used to calculate the effective diffusion coefficient, D_{eff} [22].

IP₃R cluster and ER hotspot image-analysis

Confocal images were processed with MetaMorph software (Universal Imaging Corporation) in conjunction with ImageJ (version 1.33a, National Institutes of Health) to analyse the number, size and area of IP₃R clusters or labelled ER hotspots. Individual confocal z-sections (optical slice 0.8–1.2 μm) were analysed separately. To minimize fluorescence interference from neighbouring confocal slices within a cell, alternate confocal z-slices were analysed. The mean fluorescence intensity of each slice was measured by manually defining the cell boundary excluding the nuclear region. IP₃R clusters and labelled ER hotspots were defined as having a fluorescence intensity greater than twice the mean fluorescence intensity of the cell and an area of greater than 0.056 μm^2 . A binary image was created of the IP₃R clusters or labelled ER hotspots and the ImageJ particle analysis function was then used to count the number and measure the size of each. The total area occupied by the IP₃R clusters or labelled ER hotspots could then be defined as a percentage of each confocal z-slice.

Statistical analyses

All numerical data are presented as means $\pm$ S.E.M. Statistical analyses was performed using Microsoft Excel and GraphPad Prism. Data sets with just two groups were subjected to a two-tailed, un-paired Student's t test. Most data sets with at least three groups were subjected to one-way ANOVA, and a Newman-Keuls

test was used for post-hoc comparisons. Denotation by asterisks *, **, *** represent significance of $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

RESULTS

Native IP₃RII cluster-formation following antigen stimulation

In primed RBL-2H3 cells the IP₃RII was distributed diffusely across the cell, with occasional bright IP₃RII clusters (Figure 1A shows data using Chemicon anti-IP₃RII antibody, AB3000). IP₃RI and IP₃RIII were not detected using isoform-specific antibodies by immunofluorescence, presumably due to low levels of expression (results not shown; see [18]). Following stimulation with the antigen DNP-BSA, the IP₃RII distribution changed dramatically; large IP₃RII clusters appeared, often concentrated in the perinuclear region (Figure 1A; Figure 1C shows S17 antibody staining). Over the time-course of DNP-BSA stimulation the mean immunofluorescence intensity did not change, which at rest was 48.7 ± 4.43 a.u. (absolute units) (mean $\pm$ S.E.M., $n = 20$ cells) at 10 min was 51.67 ± 1.51 a.u. ($n = 9$ cells) and at 1 h was 52.22 ± 2.5 a.u. ($n = 9$ cells). This supports the idea that it is IP₃RII reorganization, rather than addition or loss of receptors that underlies the observed clustering.

The antigen-induced clustering of the IP₃RII was significantly inhibited if RBL-2H3 cells were incubated in Ca^{2+} -free buffer, or by prior incubation in BAPTA/AM [bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester)] (2 μM , 45 min), indicating a dependence on Ca^{2+} (Figure 1B). Finally, 1 μM ionomycin treatment alone induced the clustering of IP₃RII (Figure 1B). Taken together our data confirm previous results showing that an elevation in intracellular cytosolic Ca^{2+} is necessary to induce IP₃R clustering [12].

IP₃RII clustering following antigen stimulation appears visually as an increase in the number and size of IP₃RII clusters. To test this observation we performed quantitative image analysis. Analysis of the S17 antibody staining (Figures 1E–1G) and AB3000 antibody staining (Figures 1H–1J) showed similar changes but did reveal one difference. The percentage area (relative to the whole cell area) occupied by IP₃RII clusters, recorded using the S17 antibody staining, was consistently larger than that obtained for the AB3000 antibody staining (Figure 1H). This was not due to differences in the size of the clusters, which were similar for both antibodies, but was due to the identification of fewer numbers of clusters by the AB3000 antibody. These differences in cluster numbers were consistently observed. They could reflect specific differences in the effective affinities of the antibodies for their epitopes or they could be due to different levels of background staining; either of which would affect our analysis of receptor clustering. Notwithstanding these differences, both antibodies detected very significant increases (< 0.001 , $n = 31$ –36 cells for the S17 antibody, $n = 9$ –20 cells for the AB3000 antibody) in the percentage area occupied by clusters (Figures 1E and 1H) and in the cluster area after DNP-BSA stimulation for 10 and 30 min (Figures 1F and 1I). As in the case of DNP-BSA-induced changes in the number of clusters, data measuring the change in cluster number for the two antibodies was not consistent. Cluster number apparently decreased when using the S17 antibody (Figure 1G) but did not significantly change after 10 min and only showed a small increase after 30 min when staining with the AB3000 antibody (Figure 1J).

Focussing on the 10 min time-point, it is clear that for both antibodies the dramatic increase in the percentage area occupied by clusters is due to an increase in cluster size and not cluster number. This suggests a mechanism whereby individual clusters

Figure 1 Clustering of native IP₃RIIs

(A) Primed RBL-2H3 cells show a diffuse homogenous pattern of IP₃RII immunostaining (AB3000 antibody) throughout the cell. DNP-BSA ($1 \mu\text{g} \cdot \text{ml}^{-1}$) stimulation caused the IP₃RIIs to cluster. (B) Ca²⁺ is necessary for IP₃R clustering. Either EGTA 500 μM (left, pretreated for 1 h) or BAPTA/AM pre-treatment (for 45 min) inhibited DNP-BSA ($1 \mu\text{g} \cdot \text{ml}^{-1}$, 1 h) evoked IP₃R clustering. Ionomycin ($1 \mu\text{M}$ for 30 min, 1 mM extracellular Ca²⁺) enhanced IP₃R clustering. Images are maximum projections from stacks of z-sections. Scale bars represent 10 μm . (C) IP₃R clustering is not an artefact of a particular antibody as the use of a different IP₃R antibody, S17, also showed DNP-BSA dependent receptor clustering. (D) The RBL-2H3 cell lysate ($10 \mu\text{g} \cdot \text{lane}^{-1}$) was separated by SDS/PAGE and probed with anti-IP₃RII antibodies AB3000 and S17. For both antibodies, staining was observed exclusively at a single band of approx. 250 kDa consistent with the expected mass of IP₃RII. (E–G) Analysis of the percentage area occupied by clusters (S17 antibody), the mean cluster-size and the number of clusters shows changes in the response to DNP-BSA ($1 \mu\text{g} \cdot \text{ml}^{-1}$, 31–36 cells). All significance tests are with reference to primed cells. (H–J) Analysis of the percentage area occupied by clusters (AB3000 antibody), the mean cluster-size and the number of clusters shows changes in response to DNP-BSA ($1 \mu\text{g} \cdot \text{ml}^{-1}$, 9–20 cells). In the absence of extracellular Ca²⁺, the percentage area occupied by IP₃RII clusters and the mean cluster-size transiently but significantly increased after 10 min DNP-BSA ($1 \mu\text{g} \cdot \text{ml}^{-1}$) treatment (10–14 cells) but decreased to control levels after 1 h (not shown). All significance tests are with reference to primed cells.

act to seed IP₃RII aggregation. This is contrary to the impression gained from simply looking at the images, that cluster numbers increase, and reinforces the importance of our quantitative image analysis.

In both control and Ca²⁺-free media (cells pre-incubated for 1 h in Hanks media containing 500 μM EGTA) DNP-BSA stimulation for 10 min led to a significant ($P < 0.001$) increase in the percentage area occupied by clusters, which was due to a large increase in cluster size (Figure 1I; $P < 0.001$, $n = 10$ –14). While this increase was sustained for the 30 min period of stimulation in control cells, it was not sustained in the cells bathed in Ca²⁺-free media (Figures 1H and 1I) where IP₃R clustering returned to pre-stimulus levels. This transient IP₃R clustering most likely reflects the Ca²⁺ signal which, in the absence of extracellular Ca²⁺, would be a transient release of Ca²⁺ from intracellular stores. In support of this idea, Wilson et al. [12] have previously shown IP₃R clustering elicited by thapsigargin-induced depletion of intracellular stores.

We next used the heterologous expression of DsRed2-ER to study the general morphology and integrity of the ER in RBL-2H3 cells. DsRed2-ER expression in DNP-BSA-stimulated RBL-2H3 cells appeared as a fine meshwork of interconnecting tubules in the ER and nuclear envelope (Figure 2A). The gross distribution of DsRed2-ER was very similar to the ER marker proteins calreticulin (Figure 2A) and Bip (immunoglobulin heavy-chain binding protein) labelled by immunostaining; consistent with previous reports [11,15]. It was also similar to the pattern of heterologously expressed GFP-P450, another ER marker protein (see Figure 3B and [19]).

Examination of the structural changes in the ER, by high-resolution imaging of serial z-sections, was difficult in live RBL-2H3 cells due to movement of the highly dynamic ER during image acquisition. Therefore, to determine if changes might take place, we used PFA-fixed cells (Figure 2B). DsRed2-ER in primed PFA-fixed RBL-2H3 cells shows the typical interconnected tubular ER network. DNP-BSA stimulation results in subtle changes giving a coarser looking structure to the ER [12] with the appearance of regions of higher fluorescence intensity which we term hotspots. The addition of ionomycin led to ER vesicularization and apparent break-up. These data indicate that DNP-BSA stimulation does affect the structure of the ER. This is consistent with the data obtained from *Xenopus* oocytes [9] and raises the possibility that changes in ER structure might lead to the restricted diffusion of IP₃RII and in so doing promote IP₃RII cluster formation.

IP₃RII clusters do not co-localize with DsRed2-ER hotspots

To test this hypothesis we investigated the spatial relationship between IP₃RII clusters and DsRed2-ER hotspots. In DNP-BSA stimulated cells IP₃RII clusters are dispersed along the tubules of the ER network (Figure 3A) but do not appear to overlap with the DsRed2-ER hotspots. These data suggest that, despite their similar sizes, IP₃RII clusters and ER hotspots are different. To further test this we used the GFP-P450 construct as an independent ER marker. Once again, DNP-BSA stimulation led to IP₃RII clustering with receptor clusters appearing on the GFP-P450 stained ER but not colocalizing with GFP-P450 hotspots (Figure 3B).

We also applied ionomycin, which is known to promote IP₃R clustering (see Figure 1B) and changes in ER structure [20]. Application of 1 μM ionomycin caused an increase in IP₃RII clustering (Figure 3C) but only modestly changed the DsRed2-ER structure. The application of 5 μM ionomycin, however, not only caused a dramatic increase in IP₃RII clustering but also led

Figure 2 Changes in the structure of the ER following antigen stimulation

(A) DsRed2-ER overexpression (red) in DNP-BSA ($1 \mu\text{g} \cdot \text{ml}^{-1}$, 30 min)-stimulated RBL-2H3 cells showed a similar distribution to the immunolocalization of the ER marker, calreticulin (green). (B) DNP-BSA ($1 \mu\text{g} \cdot \text{ml}^{-1}$, 30 min) stimulation, resulted in a subtle increased coarseness in the ER and the formation of ER hotspots. Ionomycin ($5 \mu\text{M}$ in 5 mM CaCl_2 , 20 min) caused ER vesicularization and apparent loss of ER connectivity. The images are maximum projections of a stack of z-sections. Scale bars represent $10 \mu\text{m}$. The higher magnification ROIs (bottom row) are the regions bounded by the red squares in the low magnification images (top row).

to prominent vesicularisation of the ER (Figure 3C) consistent with previous findings [20].

The qualitative observations of a lack of colocalization of IP₃R clusters with ER hotspots were reinforced when we performed image analysis. We applied a similar threshold analysis to the images, as in Figure 1, and determined the extent of changes in IP₃RII clustering and formation of ER hotspots. These comparisons indicated that the general trend of changes were similar for IP₃RII and DsRed2-ER: both showed an increase in the area of clusters (as a percentage of total cell area), an increase in cluster size and an increase in the number of clusters after DNP or ionomycin treatment (Figures 4A–4C). However, this quantification did show distinct differences; in particular DsRed2-ER immunofluorescence revealed almost no clustering before stimulation. To quantify the degree of co-localization between the two ER proteins in the same cell we binarized images of the IP₃RII and labelled ER, using the previously applied threshold to the images, and then measured the percentage area occupied by the IP₃RII clusters that did not overlay with the area occupied by DsRed2-ER (Figure 4D). The results indicate that, under all conditions, most regions (>75 %) containing the IP₃RII clusters do not colocalize with the regions of DsRed2-ER hotspots. Furthermore, we conducted similar experiments using hetero-

logously expressed GFP-P450, a known ER marker [19], and obtained similar results indicating that our findings reflect real changes in the ER and are not dependent on the possible specific behaviour of the DsRed2-ER construct. We conclude that IP₃R clustering evoked by either DNP-BSA or ionomycin does not correlate directly with changes in the ER structure.

The IP₃R remains within the ER during clustering

The lack of overlay between the IP₃RII clusters and ER hotspots suggested that the structural reorganization of the ER into hotspots was not responsible for the clustering of IP₃RII. However, the images could also be interpreted as an indication that the IP₃RII had actually left the ER. To test this we counterstained IP₃RII in stimulated cells with markers of other cell compartments such as the Golgi apparatus, where IP₃RII might well be expected to traffic through the secretory pathway and also through endosomes and lysosomes. We also tested for possible ubiquitination of IP₃RII using an anti-ubiquitin antibody. In none of these experiments did we find evidence for IP₃RII export from the ER to other compartments or evidence for ubiquitination (results not shown). This indicates that IP₃RII is retained in the ER during cell stimulation-induced receptor clustering.

Figure 3 IP₃R clusters are distinct from ER hotspots

The ER was labelled with either DsRed2-ER or the GFP-P450 construct. In either case, after DNP-BSA ($1 \mu\text{g} \cdot \text{ml}^{-1}$, 30 min)-stimulation clusters of IP₃Rs, as observed by immunostaining, are seen to localize in the ER but not in ER clusters (**A**, **B**). (**C**) Ionomycin ($1 \mu\text{M}$ in 5 mM extracellular Ca^{2+} , 20 min) induced large immunostained IP₃RII clusters (green) that colocalize approximately with the tubular DsRed2-ER network (red) but not with the DsRed2-ER hotspots. Ionomycin ($5 \mu\text{M}$ in 5 mM extracellular Ca^{2+}) induced fragmentation of the DsRed2-ER and clustering of IP₃RII, again with little colocalization in the clusters. Images are maximum projections from a stack of z-sections. Higher magnification images are single confocal images. Scale bar represents $10 \mu\text{m}$.

To further examine IP₃R clustering, we heterologously expressed a construct composed of IP₃RI fused with YFP to determine changes in IP₃R distribution in live cells. Similar to the situation for endogenous IP₃RII, live primed-RBL-2H3 cells expressing YFP-IP₃RI showed a diffuse, homogeneous pattern of fluorescence with few clusters (Figure 5A). DNP-BSA stimulation caused the formation of YFP-IP₃RI clusters (Figure 5B). Image analysis confirmed that DNP-BSA stimulation significantly increased the total area occupied by YFP-IP₃RI clusters relative to primed cells (Figure 5C). This was due to significant increases in both the mean size of YFP-IP₃RI clusters and the number of YFP-IP₃RI clusters per unit cell area. These data are similar to the data for the native IP₃RII and suggest that YFP-IP₃RI is a good marker for IP₃R clustering in RBL-2H3 cells.

We next performed FRAP experiments to determine if YFP-IP₃RI was present in a contiguous ER compartment. In primed

RBL-2H3 cells, YFP-IP₃RI clusters were seen throughout the cell (Figure 6A). Following local photobleaching, (Figures 6A and 6B) a dramatic fall in fluorescence was seen in the bleached region followed by a slow recovery of fluorescence in the photobleached region. This recovery was paralleled by a fall in fluorescence in the unbleached region; suggesting that YFP-IP₃RI was present in a single continuous ER compartment (Figure 6B [21]). Similar effects of bleaching and recovery were seen in DNP-BSA-treated cells (images not shown) supporting the idea that IP₃Rs are retained within the ER even after stimulation.

Quantification of IP₃R diffusion

To quantify any possible changes in YFP-IP₃RI expression after cell stimulation we fitted the FRAP curves to the mathematical model put forward by Siggia et al. [22]. The fitted data (Figure 6C,

Figure 4 Quantification of IP₃R clustering and ER hotspots

(A) The percentage area occupied by DsRed2-ER or IP₃R clusters increased with DNP-BSA (1 μg · ml⁻¹, 30 min), 1 μM ionomycin (5 mM CaCl₂, 10 min) and 5 μM ionomycin (5 mM extracellular Ca²⁺, 10 min) treatment. This increase was a reflection of changes in cluster size (B) and cluster number (C). In all cases the changes in DsRed2-ER clustering induced by DNP-BSA or ionomycin were significant ($P < 0.001$) compared with the relevant primed-cell parameters. The levels of significance for IP₃R clusters, again all with reference to the primed data, are shown on the graphs. All data were obtained from 2–5 cells. (D) A binary image of the IP₃R clusters and ER hotspots was generated and the percentage area of colocalization quantified. In DNP-BSA (1 μg · ml⁻¹, 30 min) stimulated cells, and with 1 μM or 5 μM ionomycin (5 mM extracellular Ca²⁺, 20 min), IP₃R clusters did not colocalize with DsRed2-ER hotspots.

dotted lines) approximated well with the raw data (Figure 6C, dark lines) and was then used to calculate the Mf of YFP-IP₃R in the ER and the diffusion coefficient of YFP-IP₃R (Figures 6D and 6E). For comparison we carried out the same analysis using GFP-P450. The Mf for the two heterologously expressed proteins was similar but only YFP-IP₃R showed a significant decrease after DNP-BSA treatment ($n = 12$ cells) consistent with the idea that cluster formation might anchor YFP-IP₃R.

Comparison of the diffusion coefficients of YFP-IP₃R showed that it was a lot slower than GFP-P450 but neither were affected by DNP-BSA treatment (Figure 6E). This indicates that mobile YFP-IP₃R is capable of diffusing freely and that DNP-BSA-induced changes in ER morphology do not hinder movement of this receptor pool.

IP₃R clustering occurs over a time-course relevant to the generation of the cytosolic Ca²⁺ signal

Our quantification of agonist-induced IP₃R clustering and the demonstration that it is likely to be independent of changes in

Figure 5 YFP-IP₃R cluster formation following DNP stimulation

(A, B) Live primed RBL-2H3 cells show a diffuse homogeneous pattern of YFP-IP₃R expression throughout the cell with evidence of a few clusters. DNP-BSA (1 μg · ml⁻¹, 30 min) stimulation caused the formation of larger YFP-IP₃R clusters. Images are maximum projections from a stack of z-sections. Scale bars represent 10 μm. (C) In primed YFP-IP₃R cells the percentage area occupied by clusters increased significantly after DNP-BSA (1 μg · ml⁻¹, 30 min) stimulation ($P < 0.01$, 9 cells), as did YFP-IP₃R cluster-size and cluster-number ($P < 0.05$, 9 cells for both parameters).

ER structure led to the question of the possible role of receptor clustering in shaping the Ca²⁺ response. For example, at one extreme it might be imagined that DNP-BSA stimulation leads to a single Ca²⁺ spike that is then terminated by a mechanism involving IP₃R clustering. To test this we used a ratiometric fura 2 measurement of cytosolic Ca²⁺ from a large number of single cells on a cover dish. DNP-BSA was applied in a concentration range 0.001–10 μg · ml⁻¹. All concentrations of DNP-BSA induced an initial peak Ca²⁺ response followed by trains of Ca²⁺ oscillations that lasted as long as our recording (up to 30 min, Figure 7). These ongoing oscillations are most likely due to periodic Ca²⁺ release from stores [23] and our records therefore support the idea that IP₃R clustering does occur over the time-course of active Ca²⁺ signalling and is therefore likely to be of relevance in the generation of the Ca²⁺ signals.

DISCUSSION

By quantifying the dynamic changes in IP₃R distribution and ER structure in RBL-2H3 cells upon cell stimulation, we show that, although the changes are similar, the clustering of IP₃Rs occurs independently of changes in the bulk of the ER. Furthermore, we show that the IP₃R does not leave the ER and is not ubiquitinated during cell stimulation. Instead our data demonstrate that the IP₃R is diffusible within the ER compartment even after cell stimulation. Thus the mechanisms controlling IP₃R redistribution are independent of ER changes and are likely to depend on protein–protein interactions.

IP₃R clusters are present in resting cells

Upon first inspection, it appeared to us that the increased number of IP₃R clusters after stimulation was consistent with previous

Figure 6 Diffusion of ER proteins is affected by cell stimulation

(A) Live RBL-2H3 cells heterologously expressing YFP-IP₃RI showed typically diffuse receptor localization. A local application of high-intensity light photobleached a small region within the cell (box). This transiently decreased the local fluorescence following the time-course shown in (B). (C) Similar photobleaching experiments were performed for GFP-P450 and the fluorescence recovery compared with that of YFP-IP₃RI. In both cases the recovery curve, fitted according to Siggia et al. [22] (dotted lines) approximated well with the data and was used to calculate the Mf and diffusion coefficients for the two ER membrane proteins (D, E).

accounts [12]. However, our analysis now shows that, at least at the earliest time point of 10 min, this is not the case and the most significant change is an increase in the size of the clusters (see Figures 1E–1J).

This conclusion, of course, depends on how clusters are identified. By our criteria, a cluster is a region of fluorescence that is twice the average fluorescence and occupies a contiguous area of more than 0.056 μm². By contrast others have used edge-detection [12] or visual identification [15] both of which are methods suited to detecting larger objects but are likely to miss smaller aggregations of IP₃Rs.

Possible mechanisms of IP₃R clustering

Our data argue against IP₃R clustering occurring as a secondary consequence of ER changes and instead indicate that the mechanism must reside within the receptor or within an associated protein. Wilson et al. [12] also observed ER changes upon cell-stimulation but suggested that the ER changes were too small to explain changes in IP₃RII distribution. We now show that this is

not the case; the structural changes in the ER are of a similar scale to those of IP₃RII, however, our data unequivocally show that the two are not colocalized. In contrast with this work on RBL-2H3 cells, Tateishi et al. [15] state that in COS cells the ER doesn't change upon cell-stimulation. This maybe a cell-specific phenomena or it may be a reflection of the quantitative approach we have taken versus the more qualitative analysis of Tateishi et al. [15]. Whatever the differences, all three studies (ours, [12] and [15]) conclude that IP₃RII clustering is not ER-dependent.

Wilson et al. [12] showed that the agonist-evoked clustering of IP₃Rs in RBL-2H3 cells could be mimicked by raising cytosolic Ca²⁺ by treatment with either ionomycin or thapsigargin and could be inhibited by removal of extracellular Ca²⁺. This led to the conclusion that cytosolic Ca²⁺ was the trigger for clustering. By contrast, Tateishi et al. [15] have provided recent evidence that the critical trigger is occupancy of the IP₃R with IP₃ and a subsequent conformational change of the IP₃R. Their evidence suggests that agonist-evoked clustering of IP₃Rs occurs after the Ca²⁺ signal has reached a maximum but coincides with a peak in IP₃ production [15]. Furthermore, they show that ionomycin-induced

Figure 7 DNP-BSA-induced calcium oscillations observed over long periods of time

In fura 2 loaded cells the ratio of light emitted at 510 nm from the cells alternately excited at 340 and 380 nm showed a rapid peak-response followed by trains of spikes from cell to cell that were heterogeneous in terms of size and frequency. Lower panels: magnified traces pre-stimulation and at the times indicated after DNP-BSA stimulation.

IP₃R clustering is inhibited in the presence of the phospholipase C inhibitor, U-73122 [15]. Finally, they show that mutant IP₃Rs, unable to change conformation upon IP₃ binding, are not cluster competent [15].

RBL-2H3 cells predominantly express IP₃RII and it is this native expressed isoform that we have immunolocalized and shown to be capable of clustering. In our study we also employed YFP-IP3RI and showed that even this overexpressed receptor clusters upon antigen stimulation. The possible formation of heterotetrameric receptors means that we cannot determine whether clustering is isoform-specific. It is perhaps surprising that clustering can occur at all in cells overexpressing IP₃Rs since it might be expected that the expressed construct would flood the ER with receptors and mask any clustering. Perhaps even more surprising is that the absolute numbers in our analysis of clustering of native IP₃RII, as measured with AB3000 antibody staining and YFP-IP3RI expression are similar (compares Figures 1H, 1I and 1J with Figure 5C).

Functional consequences

We now show that over the time-course of agonist-induced IP₃R clustering the RBL-2H3 cells show an ongoing oscillatory Ca²⁺ response. These oscillations have previously been shown to depend on Ca²⁺ release from intracellular stores [23] and are therefore likely to be influenced by IP₃RII clustering. We have tried simultaneous measurement of IP₃RII movement and the Ca²⁺ signal but, at present, do not have the temporal resolution to determine if receptor clustering is transiently associated with each Ca²⁺ spike. Tateishi et al. [15] present evidence to suggest that this is the case and the obvious question remains as to whether clustering represents a mechanism to enhance the Ca²⁺ response or is it inhibitory, forming part of the mechanisms to terminate the Ca²⁺ spike?

Theoretical modelling predicts that IP₃R clustering affects both elementary and global Ca²⁺ signalling events, and also suggests that there is an optimum extent to IP₃R clustering for effective elevation of the intracellular Ca²⁺ concentration. Shuai and

Jung [14] predicted that IP₃R clustering serves to amplify the cells' Ca²⁺ signalling capability, enabling a large Ca²⁺ response to low-levels of IP₃ produced by physiological stimulation. However, when IP₃R clusters become very large, the global Ca²⁺ signals elicited by low IP₃ levels would be significantly decreased due to increased distance between IP₃R clusters, and thus decrease the probability of saltatoric propagation among them [24]. As large IP₃R clusters do not allow room for differential responses to stimuli of different intensity [14], the distribution of IP₃Rs in clusters of variable size would optimize both the sensitivity and increase the response range to stimuli [25]. IP₃R clusters consisting of 20–50 IP₃Rs separated by 2–3 μ m are predicted to favour the production of Ca²⁺ waves and repetitive global oscillations with multiple initiation sites [14,26,27]. These theoretical values for IP₃R cluster-size and -separation agree with experimentally demonstrated values for Ca²⁺ release sites in the *Xenopus* oocyte [24].

YFP-IP3RI diffusion characteristics

Our data measured the effect of a physiologically relevant stimulus (DNP-BSA) on the diffusion characteristics of heterologously expressed IP₃Rs. Caution is needed in the interpretation of these experiments, since overexpression probably compromises the endogenous mechanisms of IP₃R clustering. However, the significant and selective (compared with GFP-P450) decrease in the YFP-IP3RI Mf is interesting. It is also interesting to compare our Figures showing the diffusion coefficients of YFP-IP3RI. Our mean value of $0.056 \pm 0.003 \mu\text{m}^2 \cdot \text{s}^{-1}$ ($n = 12$) is much slower than the $0.26 \mu\text{m}^2 \cdot \text{s}^{-1}$ described for IP₃RI in hippocampal neurons [28] but does fall within the broad range described for ER-localized GFP-fusion proteins, of 0.02 – $0.5 \mu\text{m}^2 \cdot \text{s}^{-1}$ [29,20].

Conclusions

The observed changes in the ER, and IP₃R distribution upon cell-stimulation in RBL-2H3 cells were shown to be quantifiably different, indicating that the two processes are probably independent. Furthermore, our quantification now shows that, at least at early time-points, it is cluster size rather than increasing cluster numbers that dominates receptor aggregation. How these changes in ER and IP₃R distribution are related to cell function is not clear, although we have shown that they do occur over a time-course relevant to agonist-evoked Ca²⁺ spiking. Since it is the Ca²⁺ signal in RBL-2H3 cells that regulates secretion [30], the reorganization of the ER and IP₃Rs is therefore likely to be relevant to the control of cell secretion.

This work was supported by Medical Research Council grants (G0000214 and G0400669) to P.T. and a Royal Society Fellowship to M.J.S.

REFERENCES

- Berridge, M. J. (2002) The endoplasmic reticulum: a multifunctional signaling organelle. *Cell Calcium* **32**, 235–249
- Lee, M. G., Xu, X., Zeng, W., Diaz, J., Wojcikiewicz, R. J. H., Kuo, T. H., Wuytack, F., Racymaekers, L. and Muallem, S. (1997) Polarized expression of Ca²⁺ channels in pancreatic and salivary gland cells. Correlation with initiation propagation of [Ca²⁺]_i waves. *J. Biol. Chem.* **272**, 15765–15770
- Ito, K., Miyashita, Y. and Kasai, H. (1996) Micromolar and submicromolar Ca²⁺ spikes regulating distinct cellular functions in pancreatic acinar cells. *EMBO J.* **16**, 242–251
- Thorn, P., Fogarty, K. E. and Parker, I. (2004) Zymogen granule exocytosis is characterized by long fusion pore openings and preservation of vesicle lipid identity. *Proc. Natl. Acad. Sci.* **101**, 6774–6779
- Turvey, M. R., Fogarty, K. E. and Thorn, P. (2005) Inositol (1,4,5)-trisphosphate receptor links to filamentous actin are important for generating local Ca²⁺ signals in pancreatic acinar cells. *J. Cell Sci.* **118**, 971–980

- 6 Crutwell, C., Bernard, J., Hilly, M., Nicolas, V., Tunwell, R. E. A. and Mauger, J.-P. (2005) Dynamics of the Ins(1,4,5) P_3 receptor during polarisation of MDCK cells. *Biol. Cell* **97**, 699–707
- 7 Stendahl, O., Krause, K. H., Krischer, J., Jerstrom, P., Theler, J. M., Clark, R. A., Carpentier, J. L. and Lew, D. P. (1994) Redistribution of intracellular Ca^{2+} stores during phagocytosis in human neutrophils. *Science* **265**, 1439–1441
- 8 Vermassen, E., Van Acker, K., Annaert, W. G., Himpen, B., Callewaert, G., Missiaen, L., De Smedt, H. and Parys, J. B. (2003) Microtubule-dependent redistribution of the type-1 inositol 1,4,5-trisphosphate receptor in A7r5 smooth muscle cells. *J. Cell Sci.* **116**, 1269–1277
- 9 Terasaki, M., Runft, L. L. and Hand, A. R. (2001) Changes in organization of the endoplasmic reticulum during *Xenopus* oocyte maturation and activation. *Mol. Biol. Cell* **12**, 1103–1116
- 10 Goud, P. T., Goud, A. P., Van Oostveldt, P. and Dhont, M. (1999) Presence and dynamic redistribution of type I inositol 1,4,5-trisphosphate receptors in human oocytes and embryos during in-vitro maturation, fertilization and early cleavage divisions. *Mol. Human Reprod.* **5**, 441–451
- 11 Boulware, M. J. and Marchant, J. S. (2005) IP $_3$ receptor activity is differentially regulated in endoplasmic reticulum subdomains during oocyte maturation. *Curr. Biol.* **15**, 765–770
- 12 Wilson, B. S., Pfeiffer, J. R., Smith, A. J., Oliver, J. M., Oberdorf, J. A. and Wojcikiewicz, R. J. H. (1998) Calcium-dependent clustering of inositol 1,4,5-trisphosphate receptors. *Mol. Biol. Cell* **9**, 1465–1478
- 13 Hong-Geller, E. and Cerione, R. A. (2000) Cdc42 and Rac stimulate exocytosis of secretory granules by activating the IP(3)/calcium pathway in RBL-2H3 mast cells. *J. Cell Biol.* **148**, 481–493
- 14 Shuai, J. W. and Jung, P. (2003) Optimal ion channel clustering for intracellular calcium signaling. *Proc. Natl. Acad. Sci.* **100**, 506–510
- 15 Tateishi, Y., Hattori, M., Nakayama, T., Iwai, M., Bannai, H., Nakamura, T., Michikawa, T., Inoue, T. and Mikoshiba, K. (2005) Cluster formation of inositol 1,4,5-trisphosphate receptor requires its transition to open state. *J. Biol. Chem.* **280**, 6816–6822
- 16 Mignery, G. A., Newton, C. L., Archer, B. T. and Sudhof, T. C. (1990) Structure and expression of the rat inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* **265**, 12679–12685
- 17 Parker, A. K., Gergely, F. V. and Taylor, C. W. (2004) Targeting of inositol 1,4,5-trisphosphate receptors to the endoplasmic reticulum by multiple signals within their transmembrane domains. *J. Biol. Chem.* **279**, 23797–23805
- 18 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 Ca^{2+} binding proteins in RBL-2H3 rat basophilic leukemia cells. *Cell Calcium* **22**, 475–486
- 19 Szczesna-Skorupa, E., Chen, C. D., Rogers, S. and Kemper, B. (1998) Mobility of cytochrome P450 in the endoplasmic reticulum membrane. *Proc. Natl. Acad. Sci.* **95**, 14793–14798
- 20 Subramanian, K. and Meyer, T. (1997) Calcium-induced restructuring of nuclear envelope and endoplasmic reticulum calcium stores. *Cell* **89**, 963–971
- 21 Bannai, H., Inoue, T., Nakayama, T., Hattori, M. and Mikoshiba, K. (2004) Kinesin dependent, rapid, bi-directional transport of ER sub-compartment in dendrites of hippocampal neurons. *J. Cell Sci.* **117**, 163–175
- 22 Siggia, E. D., Lippincott-Schwartz, J. and Bekiranov, S. (2000) Diffusion in inhomogeneous media: theory and simulations applied to whole cell photobleach recovery. *Biophys. J.* **79**, 1761–1770
- 23 Narenjkar, J., Marsh, S. J. and Assem, E. S. (1999) The characterization and quantification of antigen-induced Ca^{2+} oscillations in a rat basophilic leukaemia cell line (RBL-2H3). *Cell Calcium* **26**, 261–269
- 24 Callamaras, N., Marchant, J. S., Sun, X. P. and Parker, I. (1998) A continuum of InsP $_3$ -mediated elementary Ca^{2+} signalling events in *Xenopus* oocytes. *J. Physiol.* **509**, 81–91
- 25 Bray, D., Levin, M. D. and Morton-Firth, C. J. (1998) Receptor clustering as a cellular mechanism to control sensitivity. *Nature* **393**, 85–88
- 26 Swillens, S., Dupont, G., Combetter, L. and Champeil, P. (1999) From calcium blips to calcium puffs: theoretical analysis of the requirements for interchannel communication. *Proc. Natl. Acad. Sci.* **96**, 13750–13755
- 27 Thul, R. and Falcke, M. (2004) Release currents of IP(3) receptor channel clusters and concentration profiles. *Biophys. J.* **86**, 2660–2673
- 28 Fukatsu, K., Bannai, H., Zhang, S., Nakamura, H., Inoue, T. and Mikoshiba, K. (2004) Lateral diffusion of inositol 1,4,5-trisphosphate receptor type 1 is regulated by actin filaments and 4.1n in neuronal dendrites. *J. Biol. Chem.* **279**, 48976–48982
- 29 Levin, M. H., Haggie, P. M., Vetrivel, L. and Verkman, A. S. (2001) Diffusion in the endoplasmic reticulum of an aquaporin-2 mutant causing human nephrogenic diabetes insipidus. *J. Biol. Chem.* **276**, 21331–21336
- 30 Mahmoud, S. F. and Fewtrell, C. (2001) Microdomains of high calcium are not required for exocytosis in RBL-2H3 mucosal mast cells. *J. Cell Biol.* **153**, 339–350

Received 14 July 2005/11 October 2005; accepted 8 November 2005

Published as BJ Immediate Publication 8 November 2005, doi:10.1042/BJ20051130