A steady-state mechanism can account for the properties of inositol 2,4,5-trisphosphate-stimulated Ca^{2+} release from permeabilized L1210 cells

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We have investigated the effects of sub-maximal $\text{Ins}(2,4,5)\text{P}_3$ concentrations on the Ca^{2+} permeability of the residual undischarged Ca^{2+} stores in electroporated or digitonin-permeabilized L1210 cells by measuring Ca^{2+} -efflux rate after addition of the ATPase inhibitor thapsigargin. Low concentrations of $\text{Ins}(2,4,5)\text{P}_3$, causing rapid discharge of a small proportion of the releasable Ca^{2+} , result in a substantial stimulation of Ca^{2+} efflux after thapsigargin addition. This indicates firstly that in the absence of thapsigargin there must have been a substantial, counterbalancing, increase in rate of Ca^{2+} pumping, and secondly that the increased Ca^{2+}

permeability is more consistent with a steady state than with a quantal model of Ca^{2+} release. Similar increases in passive Ca^{2+} permeability are produced by addition of concentrations of ionomycin which produce equivalent changes in Ca^{2+} loading to those produced by $\text{Ins}(2,4,5)\text{P}_3$, although the time course and initial rate of Ca^{2+} release are very much slower. In the presence of a Ca^{2+} -buffering system, the time course of Ca^{2+} release by $\text{Ins}(2,4,5)\text{P}_3$ becomes superimposable on that of ionomycin, indicating that the initial rapid phase of $\text{Ins}(2,4,5)\text{P}_3$ -stimulated Ca^{2+} is at least partially due to positive feedback from extravesicular Ca^{2+} .

INTRODUCTION

It is now generally agreed that $\text{Ins}(1,4,5)\text{P}_3$, the water-soluble product of receptor-mediated $\text{PtdIns}(4,5)\text{P}_2$ hydrolysis, is responsible for the release of Ca^{2+} from intracellular stores [1]. Endoplasmic reticulum, or a specialized part of it, has been shown to contain high-affinity $\text{Ins}(1,4,5)\text{P}_3$ -binding sites which are associated with a tetrameric Ca^{2+} channel that opens on binding of $\text{Ins}(1,4,5)\text{P}_3$ [2–5]. However, some aspects of this mechanism remain controversial. High on the list of these is the phenomenon of quantal Ca^{2+} release, originally described by Muallem et al. [6], where sub-maximal concentrations of $\text{Ins}(1,4,5)\text{P}_3$ cause the rapid release of a small fraction of stored Ca^{2+} from permeabilized cells or microsomal preparations. The cessation of Ca^{2+} release after a short time is not a result of a conventional channel-inactivation phenomenon, since successive equal small doses of $\text{Ins}(1,4,5)\text{P}_3$ release successive equal quantities of Ca^{2+} . This behaviour was described as increment detection by Meyer and Stryer [7]. The explanation of this effect remains in doubt. Muallem et al. [6] used the term 'quantal release' to describe their hypothesis whereby at a particular $\text{Ins}(1,4,5)\text{P}_3$ concentration a fraction of the stores unloads completely, leaving the remainder unaffected, i.e. the stores are divided into a series of subsets of varying sensitivity to $\text{Ins}(1,4,5)\text{P}_3$. Behaviour of this sort has been observed by Parker and Ivorra [8] and by Parker and Yao [9], using *Xenopus* oocytes. Taylor and Potter [10] and Oldershaw et al. [11] have shown that with permeabilized hepatocytes sub-maximal doses of $\text{Ins}(1,4,5)\text{P}_3$ do not, after the initial Ca^{2+} efflux, increase the unidirectional efflux of ^{45}Ca from the remaining stores. However, Irvine [12] has proposed a steady-state model that can explain many of the features of quantal release or increment detection, based on co-operativity between intravesicular Ca^{2+} and $\text{Ins}(1,4,5)\text{P}_3$ in determining channel opening. Evidence that intravesicular Ca^{2+} can control the responsiveness of the stores has been found by Missiaen et al. [13], although other studies have not supported Irvine's hypothesis [14]. Recently, Missiaen

et al. [15], using A7r5 smooth-muscle cells, have published ^{45}Ca -efflux data which strongly suggest that a steady-state rather than a truly quantal mechanism is operating under their experimental conditions. We have adopted a rather different approach, but in this paper we report data which also support the idea of a steady state rather than a quantal mechanism. Our experimental system uses L1210 cells, permeabilized by electroporation or by digitonin. By using a high cell concentration (approx. $10^7/\text{ml}$), rather large changes in medium Ca^{2+} concentration are caused by InsP_3 addition, which perhaps reflect the condition *in vivo* more closely than would the use of a low cell number or highly buffered media. To overcome the problem of $\text{Ins}(1,4,5)\text{P}_3$ metabolism, we have used the very slowly metabolized analogue $\text{Ins}(2,4,5)\text{P}_3$ to cause long-lasting steady-state changes in Ca^{2+} loading. Addition of the Ca -ATPase inhibitor thapsigargin [16] during the steady state results in a Ca^{2+} -efflux rate which is a measure of the Ca^{2+} permeability of the store and is also a measure of the Ca^{2+} -pumping rate before thapsigargin addition. A similar technique, but using hexokinase and glucose to remove ATP, has been used by others (e.g. [7]). Using this system we show that the effects of $\text{Ins}(2,4,5)\text{P}_3$ can best be explained by a steady-state mechanism, that even a very small fractional Ca^{2+} release results in a large increase in pump rate, and that the dynamics of store unloading causing the transition into the new steady state are dependent on the Ca^{2+} -buffering characteristics of the medium.

EXPERIMENTAL

Materials

Fluo-3 pentammonium salt, a membrane-impermeant Ca^{2+} indicator, was purchased from Molecular Probes, Eugene, OR, U.S.A. Ca^{2+} ionophore ETH1001 was purchased from Fluka Chemicals, Glossop, Derbyshire, U.K. Phosphocreatine, creatine kinase, EGTA, ionomycin and thapsigargin were purchased from Sigma Chemical Co., Poole, Dorset, U.K.; ATP and dithiothreitol were from B.C.L., Lewes, Sussex, U.K.; silver

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Figure 1 Conversion of fluorescence changes into changes in $[Ca]_{total}$

Assay conditions were as described under 'Methods' section. (a) shows a typical fluorescence trace of Ca²⁺ release from electroporated L1210 cells loaded to steady state with Ca²⁺. Ca²⁺ efflux was started by the addition of thapsigargin (TG; 300 nM). Ins(2,4,5)P₃ was added to a final concentration of 8 μ M. Calibration pulses of Ca²⁺ were added as marked at the arrows. (b) shows the data from (a) after linearization of the response to changes in $\Delta[Ca]_{total}$ by using a polynomial derived from the Ca-buffering curve shown in Figure 2.

Figure 2 Ca-buffering curve for the assay conditions described for Figure 1

The composition of the assay medium was as described under 'Methods' except that the total volume used was 8 ml. Cells (2.5×10^7 /ml) were allowed to accumulate Ca²⁺ until a steady state of loading was obtained. Individual 1 ml batches of the incubation mixture were then transferred to the fluorimeter, and for each 1 ml sample a different size of calibration pulse of Ca²⁺ was added. The size of the Ca²⁺ addition was then plotted against the resulting change in fluorescence, and a polynomial was fitted to the points by using 'Cricket Graph'. In the case shown, the polynomial for the curve drawn through the data points was:

$$\Delta[Ca]_{total} = 2.61 \times 10^{-3} + 1.01 \times 10^{-2} \Delta F + 2.48 \times 10^{-5} (\Delta F)^2 + 5.77 \times 10^{-8} (\Delta F)^3$$

This equation was then used to convert the ΔF data of Figure 1(a) into the $\Delta[Ca]_{total}$ data of Figure 1(b). The polynomial was found to vary somewhat from day to day, probably due to different batches of cells, so that a new polynomial was derived for each experiment.

foil, used as electrode plates for cell permeabilization by electroporation, was obtained from Aldrich, Gillingham, Dorset, U.K.; digitonin was from B.D.H., Poole, Dorset, U.K.; and tissue-culture materials were from GIBCO, Uxbridge, U.K. Ins(2,4,5)P₃ was a gift from Dr. R. F. Irvine, AFRC Institute of Animal Physiology, Babraham, Cambridge, U.K.

Methods

Cell growth

Mouse lymphoma L1210 cells were grown essentially as described previously [17] in a system of gas-permeable batch culture. Cells were grown over a 4-day period to a density of 6.0×10^5 cells/ml, as measured on a Coulter electronic cell counter. A 42 ml portion of cells (6.0×10^5 cells/ml) was centrifuged at 2000 g for 5 min in a bench centrifuge at 25 $^{\circ}$ C. The pellet was resuspended in 25 ml of wash buffer, consisting of 0.9% (w/v) NaCl and 20 mM-Hepes/KOH, pH 7.0 at 30 $^{\circ}$ C, and centrifuged as above. This wash was repeated before finally resuspending the final pellet of cells in 1 ml of assay buffer to give a cell density of 2.5×10^7 cells/ml.

Cell permeabilization

Permeabilization by electroporation was carried out with a Bio-Rad Gene Pulser with a custom-designed electroporation cuvette. Cells were subjected to five pulses of 0.8 kV (2 kV/cm) with a capacitance of 25 μ F and a time constant of 0.3 ms. Unlike commercially available electroporation cuvettes, the custom-designed cuvettes had electrode plates made from silver foil. This avoided possible artefacts shown to be due to solubilization of cations from the electrode plates [18].

Permeabilization by digitonin was performed by adding 10 μ g of digitonin (in 1 μ l of dimethyl sulphoxide) to cells suspended in the assay medium.

Figure 3 Effect of $\text{Ins}(2,4,5)\text{P}_3$ concentration on thapsigargin-induced passive Ca^{2+} -efflux rate

(a) shows Ca^{2+} -efflux data, processed by the method described in Figures 1 and 2, under the assay conditions described under 'Methods' without additional Ca buffering. (b) shows similar data, but in the presence of additional Ca buffering, provided by addition of $40\ \mu\text{M}$ EGTA and $20\ \mu\text{M}$ Ca^{2+} . Additions at the arrows marked (1) were the following $\text{Ins}(2,4,5)\text{P}_3$ concentrations: in (a): (a) $8.0\ \mu\text{M}$, (b) $2.0\ \mu\text{M}$, (c) $1.5\ \mu\text{M}$, (d) $1.0\ \mu\text{M}$, (e) $0.75\ \mu\text{M}$, (f) $0.25\ \mu\text{M}$ and (g) $0\ \mu\text{M}$; in (b): (a) $8.0\ \mu\text{M}$, (b) $4.0\ \mu\text{M}$, (c) $2.0\ \mu\text{M}$, (d) $1.5\ \mu\text{M}$, (e) $1.0\ \mu\text{M}$, (f) $0.75\ \mu\text{M}$ and (g) $0\ \mu\text{M}$; additions at the arrows marked (2) were $300\ \text{nM}$ thapsigargin. The data shown are from one typical experiment on a single batch of cells in each case. Similar data was obtained for between 3 and 10 replicates of each InsP_3 concentration on different batches of cells under buffered and non-buffered conditions.

Ca^{2+} uptake

The assay medium consisted of $100\ \text{mM}$ -KCl, $20\ \text{mM}$ -Hepes/KOH, pH 7.0, $1\ \text{mM}$ -dithiothreitol, $5\ \text{mM}$ -succinate (K^+ salt), $50\ \mu\text{g/ml}$ creatine kinase, $10\ \text{mM}$ -phosphocreatine, $2\ \text{mM}$ - MgCl_2 , $0.7\ \text{mM}$ -ATP and $1\ \mu\text{M}$ -Fluo 3 (pentammonium salt) in a $1\ \text{ml}$ final volume containing 2.5×10^7 permeabilized cells. Experiments were carried out at 30°C . Ca^{2+} uptake was routinely measured with a Ca^{2+} -sensitive electrode. Ca^{2+} -sensitive membranes contained the neutral ionophore ETH1001 and were prepared by the method described by Clapper and Lee [19]. After the cells had loaded to steady state, the incubation mixture was transferred to a Shimadzu RF-5000 fluorimeter coupled to an IBM-compatible computer. Ca^{2+} release was measured by Fluo 3 fluorimetry. pCa values were determined, and traces of $[\text{Ca}]_{\text{total}}$ were generated by mathematical manipulation of fluorescence data by using Microsoft 'Excel' and Computer Associates 'Cricket Graph' software (see the Results section).

For some experiments, additional Ca^{2+} buffering was provided by addition of $40\ \mu\text{M}$ EGTA plus $20\ \mu\text{M}$ Ca^{2+} to the uptake medium. Under these conditions, fluorescence response was linear to changes in $[\text{Ca}]_{\text{total}}$ over the required $[\text{Ca}]$ range.

Under both the experimental conditions described above, the InsP_3 -sensitive pool was $76 \pm 3\%$ ($n = 10$) of the total ionomycin-releasable Ca^{2+} -pool.

RESULTS

To be comparable with the situation inside cells *in vivo*, we wished in the first instance to use experimental conditions where the free Ca^{2+} concentration in the medium could change quite substantially. This raised an immediate problem, in that the response of Fluo 3 is markedly non-linear over the Ca^{2+}

concentration range of interest. The situation is illustrated in Figure 1(a). Permeabilized L1210 cells were incubated in the medium until the free Ca^{2+} concentration was lowered to a steady value, in this case equivalent to a pCa of 7.1. The accumulated Ca^{2+} was then released by a combination of thapsigargin and a maximal dose of $\text{Ins}(2,4,5)\text{P}_3$, decreasing the pCa to about 6.5. However, the calibration pulse of $0.5\ \text{nmol}$ of Ca^{2+} added at pCa 7.1 gives a very similar fluorescence change to the addition of $2\ \text{nmol}$ of Ca^{2+} at pCa 6.5. There is some Ca^{2+} re-uptake after the additions of $\text{Ins}(2,4,5)\text{P}_3$ and the second Ca^{2+} pulse. This uptake is insensitive to thapsigargin (which is added at a maximally effective dose), but is sensitive to protonophore uncouplers (results not shown). However, the uptake is slow compared with the mixing time and does not distort the calibration procedure described below. The non-linearity of response to Ca^{2+} concentration changes makes analysis of rates and extents of Ca^{2+} release very difficult. To overcome the problem, we assayed the Ca^{2+} -buffering capacity of the medium by measuring the fluorescence change caused by adding known quantities of Ca^{2+} to experimental incubations identical with that of Figure 1(a). The fluorescence change was then plotted as a function of $\Delta[\text{Ca}]_{\text{total}}$ (Figure 2), and a curve was fitted to the points by using a third-order polynomial. This equation was then used to translate the raw fluorescence data into amount of Ca^{2+} released (or accumulated) from a set starting point. This latter point was taken as the steady-state fluorescence value obtained after the cells had loaded with Ca^{2+} from the medium. The result of the transformation is given in Figure 1(b), showing that the Ca^{2+} calibration pulses are now of the right size relative to one another and to the left-hand ordinate. The transformed data therefore allow an accurate estimate of the rate and extent of Ca^{2+} movements in terms of amount as distinct from free concentration.

Figure 4 Time course of $\text{Ins}(2,4,5)\text{P}_3$ - and ionomycin-stimulated Ca^{2+} release under different Ca-buffering conditions

Experimental conditions were as described in Figures 3(a) and 3(b) for panels (a) and (b) respectively, with (b) containing 40 μM EGTA and 20 μM added Ca^{2+} . For the traces marked (a) the arrow (1) shows addition of 0.75 μM $\text{Ins}(2,4,5)\text{P}_3$, and for the traces marked (b) the arrow (1) shows addition of 0.75 nM ionomycin. For all traces the arrow marked TG shows addition of 300 nM thapsigargin. Fluorescence change was converted into nmol of Ca^{2+} released as described in Figures 1 and 3.

The quantal model of Ca^{2+} release predicts that sub-maximal concentrations of InsP_3 will empty some Ca^{2+} pools (which will then make no further contribution to Ca^{2+} movements) and leave others unaffected. Thus it would be expected that the passive Ca^{2+} leak rate would be unaltered by addition of low InsP_3 concentrations. Figure 3(a) shows an experiment to test this prediction. Electroporated cells, loaded to steady state, were treated with sub-optimal concentrations of $\text{Ins}(2,4,5)\text{P}_3$. After the rapid phase of Ca^{2+} release there is a new steady state established (e.g. Fig. 3a, traces e, f), reflecting a balance between Ca^{2+} efflux and Ca^{2+} uptake. Addition of thapsigargin to inhibit the Ca-ATPase then allows measurement of the Ca^{2+} -efflux rate (and therefore, by difference, the Ca^{2+} -pumping rate) immediately before thapsigargin addition. Figure 3(a) clearly shows that even very low concentrations of $\text{Ins}(2,4,5)\text{P}_3$, giving very little Ca^{2+} release (e.g. Figure 3a, traces e, f) result in a greatly increased Ca^{2+} -efflux rate and therefore, by implication, greatly increased turnover through the Ca-ATPase. The Ca^{2+} leakage rate is even higher at higher $\text{Ins}(2,4,5)\text{P}_3$ concentrations. In the particular experiment shown in Figure 3(a), the situation is complicated by the presence of a slow and limited re-uptake of Ca^{2+} after $\text{Ins}(2,4,5)\text{P}_3$ addition, presumably into an InsP_3 -insensitive pool. Although it is likely that the re-uptake will lead to a small underestimate of the initial rate of thapsigargin-induced Ca^{2+} leak from the InsP_3 -sensitive pool, it is unlikely that such a limited transfer of Ca^{2+} into the insensitive pool would increase the total Ca^{2+} leakage rate, particularly since the total vesicular

Ca^{2+} pool has been substantially decreased. However, to eliminate this possibility, we carried out the experiment shown in Figure 3(b), where a low capacity Ca-EGTA buffer was added to the incubation medium. The Ca^{2+} and EGTA concentrations were chosen so that the final steady-state pCa obtained was the same as in Figure 3(a) (pCa 7.3) and that the ΔpCa on addition of maximal $\text{Ins}(2,4,5)\text{P}_3$ was decreased to about 30 % of that in the absence of the buffer. Under these conditions, the fluorescence response to Ca^{2+} addition was linear, so that data was processed into $\Delta\text{Ca}_{\text{total}}$ movements by using a linear function derived for the buffering conditions. It is clear that, even under these circumstances where there is no Ca^{2+} re-uptake, the presence of $\text{Ins}(2,4,5)\text{P}_3$ leads to a substantially faster Ca^{2+} leak rate after thapsigargin addition. Once again, the leak rate increases progressively with $\text{Ins}(2,4,5)\text{P}_3$ concentration. Thus, when store loading is changed by the addition of sub-optimal $\text{Ins}(2,4,5)\text{P}_3$ concentrations, there appears to be an enhanced Ca^{2+} permeability and a compensating increase in pump rate during the new steady state.

A feature of the traces in Figure 3(b) as compared with those in Figure 3(a) is that the time course of Ca^{2+} release appears to be significantly slower in the buffered system (Figure 3b). Since the holes made by electroporation are rather small, it was possible that slow Ca^{2+} release could be a result of change in accessibility of the stores to externally added $\text{Ins}(2,4,5)\text{P}_3$. The effect was therefore investigated in more detail by using digitonin-permeabilized cells (Figure 4). Once again, $\text{Ins}(2,4,5)\text{P}_3$ -stimulated Ca^{2+} release is very much slower in the buffered system (Figure 4b, trace a) than in the unbuffered system (Figure 4a, trace a). The time course of Ca^{2+} release in the unbuffered system, showing a fast phase followed by a much slower phase, is qualitatively similar to time courses previously reported for $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release [7,20]. However, in the presence of a Ca/EGTA buffer, the same concentration of $\text{Ins}(2,4,5)\text{P}_3$ releases Ca^{2+} in a slow, apparently monophasic, manner. It has been observed in various previous studies [6,14] that ionomycin releases Ca^{2+} very much more slowly than InsP_3 , even at concentrations which produce a similar change in store loading. This is also shown in Figure 4(a). Although the final level of Ca^{2+} release produced by addition of 0.75 μM $\text{Ins}(2,4,5)\text{P}_3$ and 7.5 nM ionomycin are the same, the time courses of release are very different. Significantly, however, the rate of Ca^{2+} leakage after thapsigargin addition is the same in both cases. A different result is obtained in the presence of the Ca-buffer (Figure 4b). Here, as well as there being similar rates after thapsigargin addition, the time course of Ca^{2+} release caused by $\text{Ins}(2,4,5)\text{P}_3$ is the same as that caused by ionomycin. It should also be noted that the initial rate of Ca^{2+} release caused by ionomycin in the unbuffered condition and by both ionomycin and $\text{Ins}(2,4,5)\text{P}_3$ in the buffered condition is very similar to that caused by addition of thapsigargin to the new steady state. This is not the case for $\text{Ins}(2,4,5)\text{P}_3$ in unbuffered conditions.

The data in Figure 4 show that, for a particular change in store loading, the change in steady-state Ca^{2+} permeabilities caused by $\text{Ins}(2,4,5)\text{P}_3$ and ionomycin are the same, both in the buffered and in the unbuffered systems. Figure 5 shows that this is true across a broad range of $\text{Ins}(2,4,5)\text{P}_3$ and ionomycin concentrations. The slope of the line relating change in Ca^{2+} loading to change in Ca^{2+} permeability is, however, different for the buffered and unbuffered condition, and this may reflect the fact that the changes in $[\text{Ca}^{2+}]_{\text{free}}$ are less in the former condition, giving rise to smaller changes in the velocity of the Ca-pump.

A possible explanation for the above observations would be if the changes in Ca^{2+} -pumping rate and leakage rate were caused by the changes in the extra-vesicular $[\text{Ca}^{2+}]_{\text{free}}$, rather than by a

Figure 5 Stimulation of the thapsigargin-induced passive Ca^{2+} -efflux rate after Ca^{2+} release by $\text{Ins}(2,4,5)\text{P}_3$ or ionomycin

In (a) and (b) assay conditions and addition times were as for Figures 4(a) and 4(b), except that various concentrations of $\text{Ins}(2,4,5)\text{P}_3$ (○) or ionomycin (●) were used to perturb the steady-state Ca^{2+} loading to different extents. In each case, the change in steady-state Ca^{2+} loading [i.e. the amount of Ca^{2+} released by $\text{Ins}(2,4,5)\text{P}_3$ or ionomycin at steady state] is plotted on the abscissa, and the subsequent thapsigargin-induced leak rate is plotted on the ordinate. Ionomycin concentrations used were in the range 0–40 nM and $\text{Ins}(2,4,5)\text{P}_3$ concentrations were up to 2 μM . Data are combined results from two separate experiments in each case.

Figure 6 Lack of effect of increased extravesicular free Ca^{2+} on the thapsigargin-induced passive Ca^{2+} -efflux rate

Assay conditions were as for Figure 3(b), except that for the points (○) a pulse of Ca^{2+} was added instead of $\text{Ins}(2,4,5)\text{P}_3$ to perturb the steady-state Ca^{2+} concentration by the number of nmol shown. The pulse was immediately followed by thapsigargin (300 nM) to start passive Ca^{2+} efflux. Each point represents the mean of three determinations on separate lots of cells. For comparison, the points marked (●) are data where the steady-state extravesicular Ca^{2+} concentration has been perturbed with various $\text{Ins}(2,4,5)\text{P}_3$ concentrations as described in Figure 5(b).

direct effect of $\text{Ins}(2,4,5)\text{P}_3$ on Ca^{2+} permeability. To test this, we tried adding a pulse of Ca^{2+} to perturb the steady-state $[\text{Ca}^{2+}]_{\text{free}}$, followed immediately by thapsigargin to allow measurement of the Ca^{2+} -leakage rate at the new value of $[\text{Ca}^{2+}]_{\text{free}}$. The results are shown in Figure 6 in comparison with data for $\text{Ins}(2,4,5)\text{P}_3$. It is clear that a change in $[\text{Ca}^{2+}]_{\text{free}}$ by itself does not change the

Figure 7 Effect of changing $[\text{Ca}^{2+}]_{\text{free}}$ on Ca^{2+} -efflux rate in the presence of $\text{Ins}(2,4,5)\text{P}_3$ and thapsigargin

Conditions were as described for Figure 3(b). At the arrow marked (1), 1 μM $\text{Ins}(2,4,5)\text{P}_3$ was added to traces (b) and (c) and 2 μM $\text{Ins}(2,4,5)\text{P}_3$ was added to trace (a). At the arrow marked TG, 300 nM thapsigargin was added to all three traces. Ca^{2+} (1.2 nmol) was added as indicated to trace (b) to change the Ca^{2+} concentration. Traces are typical of three similar experiments.

Ca^{2+} -leakage rate. However, a further possibility is that a change in $[\text{Ca}^{2+}]_{\text{free}}$ could alter the activity of $\text{Ins}(2,4,5)\text{P}_3$ in causing release from others pools, i.e. that the increased $[\text{Ca}^{2+}]_{\text{free}}$ after thapsigargin addition gives increased quantal release. This is addressed in the experiment shown in Figure 7, where (trace c) a low concentration of $\text{Ins}(2,4,5)\text{P}_3$ is followed by thapsigargin in the normal protocol. Addition of Ca^{2+} during this efflux does not change the efflux rate at all (trace b, efflux rate after Ca^{2+} addition 0.6 ± 0.03 nmol/min compared with c, 0.6 ± 0.03 nmol/min; $n = 3$) and the rate remains substantially lower than that in trace (a), 1.26 ± 0.04 nmol/min ($n = 3$), where a higher concentration of $\text{Ins}(2,4,5)\text{P}_3$ has been used to give a similar change in $[\text{Ca}^{2+}]_{\text{free}}$.

DISCUSSION

The results described above strongly suggest that, in our experimental system, Ca^{2+} release by $\text{Ins}(2,4,5)\text{P}_3$ takes place by a steady-state, rather than by an all-or-none quantal mechanism. In the latter case, no enhanced Ca^{2+} efflux would be seen under partial unloading conditions, since the empty stores would not contribute to the process, as has been reported [10,11,21].

A striking feature of our findings is that even rather limited Ca^{2+} release leads to a substantial increase in Ca^{2+} leakage and a counterbalancing Ca^{2+} -pumping activity. Inhibition of the microsomal Ca-ATPase by intravesicular Ca^{2+} is a well-established phenomenon [22,23], so that any decrease in intravesicular Ca^{2+} load would be expected to lead to an increase in pump activity. However, we seem to be faced with several problems. Firstly, why do some experimental techniques give rise to results compatible with a steady-state mechanism ([15]; the present paper), whereas others indicate a purely quantal mech-

anism [8–11,21]? Secondly, under our conditions, how are the dynamics of Ca^{2+} release compatible with a steady-state mechanism? Thirdly, what sort of model can resolve these difficulties?

In addressing the first problem, two different types of experiments have given results consistent with quantal release: those on whole single cells, studied with imaging techniques [8,9], and those using large populations of permeabilized cells. Considering the second type first, since they are more comparable with our system, a common feature between our system and that of Missiaen et al. [15] is that the InsP_3 -releasable pool is a very high percentage (approx. 80%) of the total exchangeable or ionomycin-sensitive pool. This means that changes in permeability of the InsP_3 -sensitive pool are not masked by the unaltered permeability of the insensitive pool. Such masking might be possible in the results of Taylor and Potter [10] and Oldershaw et al. [11], using hepatocytes, where the sensitive pool is only about 40–50% of the total. However, this is not true of the experiments of Bootman et al. [21], who report data very similar to that of Oldershaw et al. [11], but on HeLa cells showing an 80% InsP_3 -sensitive Ca^{2+} pool. A more fundamental experimental difference is that both we and Missiaen et al. (Figures 1 and 2 of [15]) have used thapsigargin to trigger Ca^{2+} efflux, whereas the experiments reported on hepatocytes and HeLa cells [10,11,21] have used a glucose–hexokinase trap to remove ATP. ATP has been shown to modulate the InsP_3 -sensitive Ca^{2+} channel [24], so that removal of ATP could possibly alter channel activity and contribute to the observed differences in behaviour. Also, both we and Missiaen et al. [15] have measured Ca^{2+} coming out, rather than Ca^{2+} remaining in the vesicles, and this may give a more sensitive measure of efflux rate.

Moving to the second problem, of the dynamics of Ca^{2+} release, our observations show that under rather unbuffered conditions, where $[\text{Ca}^{2+}]_{\text{free}}$ is allowed to change, there is a very rapid initial Ca^{2+} release which rapidly attenuates into a much slower process. This is in agreement with the data from rapid kinetic measurements of InsP_3 -stimulated Ca^{2+} release [7,20], and also with the finding that the initial rate of Ca^{2+} release caused by InsP_3 is substantially faster than that caused by ionomycin for a comparable change in Ca^{2+} loading [14]. The origin of this may be in positive feedback from changes in extravesicular $[\text{Ca}^{2+}]_{\text{free}}$. This is suggested by our observation that, in the presence of a Ca/EGTA buffer, the time course of Ca^{2+} release by $\text{Ins}(2,4,5)\text{P}_3$ becomes very much slower and superimposable on that for ionomycin. Positive Ca^{2+} feedback on the InsP_3 -sensitive channel has been observed by Bezprozvanny et al. [25] and Finch et al. [26], and the possible contribution of such a process to Ca^{2+} spiking has been discussed by Parker & Yao [9]. At first sight, it is rather surprising that such a pronounced effect of EGTA on rate of Ca^{2+} release has not (to our knowledge) been reported previously. Indeed, the data of Oldershaw et al. [11] and Bootman et al. [21] show that there is very rapid $\text{Ins}(1,4,5)\text{P}_3$ -stimulated Ca^{2+} release in the presence of more concentrated Ca/EGTA buffers than those used here. It seems likely, on the basis of preliminary experiments in this laboratory (J. W. Loomis-Husselbee, unpublished work), that the effect is more pronounced when $\text{Ins}(2,4,5)\text{P}_3$ is used to release Ca^{2+} than when $\text{Ins}(1,4,5)\text{P}_3$ is used. It is thus possible that the degree of co-operativity for channel opening between

extravesicular Ca^{2+} , intravesicular Ca^{2+} [12] and InsP_3 may be very different for $\text{Ins}(1,4,5)\text{P}_3$ and $\text{Ins}(2,4,5)\text{P}_3$.

Applying these sorts of considerations to experiments where quantal release is seen in single cells [8,9], it seems possible that such apparent quantal release can be generated by mechanisms which are compatible with the steady-state mechanism indicated by our results. In intact cells, local $[\text{Ca}^{2+}]_{\text{free}}$ changes are likely to be very much more pronounced than those observed in permeabilized systems. Particular stores, which may be sensitized by high Ca^{2+} loading [12,13], or may possess a higher density of InsP_3 receptors, may respond preferentially to a change in InsP_3 concentration. Positive feedback by released Ca^{2+} in the vicinity of the store would then lead to rapid Ca^{2+} release from that particular store, while others might remain completely unaffected. Thus heterogeneity of store emptying (or quantal release) could arise from a combination of heterogeneity of store loading and heterogeneity of extravesicular $[\text{Ca}^{2+}]_{\text{free}}$, features that are likely to persist only in an attenuated form in bulk permeabilized cell suspensions.

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REFERENCES

- Berridge, M. J. and Irvine, R. F. (1989) *Nature* (London) **341**, 197–205
- Ferris, C. D., Haganir, R. L., Supattapone, S. and Snyder, S. H. (1989) *Nature* (London) **342**, 87–89
- Ross, C. A., Meldolesi, J., Milner, T. A., Satoh, T., Supattapone, S. and Snyder, S. H. (1989) *Nature* (London) **339**, 468–470
- Mignery, G. A. and Südhof, T. C. (1990) *EMBO J.* **9**, 3893–3898
- Maeda, N., Kawasaki, T., Nakade, S., Yokota, N., Taguchi, T., Kasai, M. and Mikoshiba, K. (1991) *J. Biol. Chem.* **266**, 1109–1116
- Muallem, S., Pandol, S. J. and Beeker, T. G. (1989) *J. Biol. Chem.* **264**, 205–212
- Meyer, T. and Stryer, L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3841–3845
- Parker, I. and Ivorra, I. (1990) *Science* **250**, 977–979
- Parker, I. and Yao, Y. (1991) *Proc. R. Soc. London Ser. B* **246**, 269–274
- Taylor, C. W. and Potter, B. V. L. (1990) *Biochem. J.* **266**, 189–194
- Oldershaw, K. A., Nunn, D. L. and Taylor, C. W. (1991) *Biochem. J.* **278**, 705–708
- Irvine, R. F. (1990) *FEBS Lett.* **263**, 5–9
- Missiaen, L., Taylor, C. W. and Berridge, M. J. (1991) *Nature* (London) **352**, 241–244
- Combettes, L., Claret, M. and Champeil, P. (1992) *FEBS Lett.* **301**, 287–290
- Missiaen, L., De Smedt, H., Droogmans, G. and Casteels, R. (1992) *Nature* (London) **357**, 599–602
- Thastrup, O., Cullen, P. J., Drøbak, B. K., Hanley, M. R. and Dawson, A. P. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2466–2470
- Cullen, P. J., Irvine, R. F., Drøbak, B. K. and Dawson, A. P. (1989) *Biochem. J.* **259**, 931–933
- Loomis-Husselbee, J. W., Cullen, P. J., Irvine, R. F. and Dawson, A. P. (1991) *Biochem. J.* **277**, 883–885
- Clapper, D. L. and Lee, H. C. (1985) *J. Biol. Chem.* **260**, 13947–13954
- Champeil, P., Combettes, L., Berthon, B., Doucet, E., Orlowski, S. and Claret, M. (1989) *J. Biol. Chem.* **264**, 17665–17673
- Bootman, M. D., Berridge, M. J. and Taylor, C. W. (1992) *J. Physiol. (London)* **450**, 163–178
- Dawson, A. P. and Fulton, D. V. (1983) *Biochem. J.* **210**, 405–410
- Brattin, W. J. and Waller, R. L. (1983) *J. Biol. Chem.* **258**, 6725–6729
- Ferris, C. D., Haganir, R. L. and Snyder, S. H. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2147–2151
- Bezprozvanny, L., Watras, J. & Ehrlich, B. E. (1991) *Nature* (London) **351**, 751–754
- Finch, E. A., Turner, T. J. & Goldin, S. M. (1991) *Science* **252**, 443–446