Biochem. J. (1990) 266, 189–194 (Printed in Great Britain)



# The size of inositol 1,4,5-trisphosphate-sensitive $Ca^{2+}$ stores depends on inositol 1,4,5-trisphosphate concentration

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An explanation of the complex effects of hormones on intracellular $Ca^{2+}$ requires that the intracellular actions of Ins(1,4,5)$P_3$ and the relationships between intracellular $Ca^{2+}$ stores are fully understood. We have examined the kinetics of $^{45}Ca^{2+}$ efflux from pre-loaded intracellular stores after stimulation with Ins(1,4,5)$P_3$ or the stable phosphorothioate analogue, Ins(1,4,5)$P_3$[S]$_3$, by simultaneous addition of one of them with glucose/hexokinase to rapidly deplete the medium of ATP. Under these conditions, a maximal concentration of either Ins(1,4,5)$P_3$ or Ins(1,4,5)$P_3$[S]$_3$ evoked rapid efflux of about half of the accumulated $^{45}Ca^{2+}$, and thereafter the efflux was the same as occurred under control conditions. Submaximal concentrations of Ins(1,4,5)$P_3$ or Ins(1,4,5)$P_3$[S]$_3$ caused a smaller rapid initial efflux of $^{45}Ca^{2+}$, after which the efflux was similar whatever the concentration of Ins(1,4,5)$P_3$ or Ins(1,4,5)$P_3$[S]$_3$ present. The failure of submaximal concentrations of Ins(1,4,5)$P_3$ and Ins(1,4,5)$P_3$[S]$_3$ to mobilize fully the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores despite prolonged incubation was not due either to inactivation of Ins(1,4,5)$P_3$ or to desensitization of the Ins(1,4,5)$P_3$ receptor. The results suggest that the size of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores depends upon the concentration of Ins(1,4,5)$P_3$.

## INTRODUCTION

The increase in cytoplasmic $Ca^{2+}$ concentration that follows activation of receptors that stimulate polyphosphoinositide hydrolysis is widely believed to be mediated by one of the products of that hydrolysis, namely Ins(1,4,5)$P_3$ (Berridge & Irvine, 1984, 1989). Specific high-affinity Ins(1,4,5)$P_3$ binding sites, now identified in many tissues (Spät *et al.*, 1986; Nahorski & Potter, 1989; Snyder & Supattapone, 1989; Nunn *et al.*, 1990), are thought to be the receptors that are activated by Ins(1,4,5)$P_3$ and then open $Ca^{2+}$ channels (Muallem *et al.*, 1985; Meyer *et al.*, 1988; Ehrlich & Watras, 1988) in the membranes of intracellular $Ca^{2+}$ stores, allowing $Ca^{2+}$ to leak into the cytosol. A purified cerebellar Ins(1,4,5)$P_3$ binding protein (Supattapone *et al.*, 1988) has recently been reconstituted into lipid vesicles that then release $Ca^{2+}$ in response to Ins(1,4,5)$P_3$ (Ross *et al.*, 1989; Ferris *et al.*, 1989), demonstrating that the protein, or a homo-oligomer of it, is both the functional Ins(1,4,5)$P_3$ receptor and the $Ca^{2+}$ channel. The relationship between this protein and the Ins(1,4,5)$P_3$ receptors of other tissues remains to be established.

From the first demonstration of Ins(1,4,5)$P_3$-induced $Ca^{2+}$ mobilization (Streb *et al.*, 1983) it was clear that $Ca^{2+}$ was released from non-mitochondrial stores. Although these stores have generally been assumed to be located within the endoplasmic reticulum (Streb *et al.*, 1984), more recent studies suggest that other organelles including the nuclear envelope, parts of the Golgi and perhaps a distinct organelle, the calciosome, may also be responsive to Ins(1,4,5)$P_3$ (Volpe *et al.*, 1988; Ross *et al.*, 1989). In intact cells, maximal hormonal stimulation releases only part of the intracellular $Ca^{2+}$ stores (Burgess *et al.*, 1984) and in permeabilized cells a similar fraction of the stores is released by Ins(1,4,5)$P_3$ (Taylor & Putney, 1985). These results provide further evidence in support of the view that a distinct $Ca^{2+}$-sequestering organelle is responsive to Ins(1,4,5)$P_3$.

The need to understand both receptor regulation of $Ca^{2+}$ entry at the plasma membrane and the complex oscillatory changes in cytoplasmic [$Ca^{2+}$] that can be elicited by hormonal stimulation (Woods *et al.*, 1986; Berridge & Galione, 1989; Jacob *et al.*, 1988) or by intracellular application of Ins(1,4,5)$P_3$ (Parker & Miledi, 1986; Taylor *et al.*, 1988; Wakui *et al.*, 1989) has prompted considerable interest in the relationships between different intracellular $Ca^{2+}$ stores. Two intracellular molecules, GTP and Ins(1,3,4,5)$P_4$, the product of phosphorylation of Ins(1,4,5)$P_3$ (Irvine *et al.*, 1986), have been proposed to regulate these relationships. GTP, first reported to enhance Ins(1,4,5)$P_3$-induced $Ca^{2+}$-mobilization (Dawson, 1985), is now believed to promote $Ca^{2+}$ transfer between intracellular stores that differ in their sensitivity to Ins(1,4,5)$P_3$ (Ghosh *et al.*, 1989). However, the mechanism underlying this effect and its role in cellular $Ca^{2+}$ homeostasis remain unclear. Ins(1,3,4,5)$P_4$ has been proposed to regulate $Ca^{2+}$ entry at the plasma membrane of some cells (Irvine & Moor, 1986; Morris *et al.*, 1987; Irvine, 1989), perhaps by allowing communication between Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores and the plasma membrane.

In the present study, the effects of Ins(1,4,5)$P_3$ and Ins(1,4,5)$P_3$ [S]$_3$ (DL-inositol 1,4,5-trisphosphorothioate) a stable analogue of Ins(1,4,5)$P_3$, on the kinetics of $Ca^{2+}$ efflux from intracellular stores were examined. Our results suggest that rather than altering the rate at which $Ca^{2+}$ leaks from a single Ins(1,4,5)$P_3$-sensitive store, different concentrations of Ins(1,4,5)$P_3$ rapidly release $Ca^{2+}$ stores of different sizes.

Abbreviation used: Ins(1,4,5)$P_3$[S]$_3$, DL-inositol 1,4,5-trisphosphorothioate.

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## MATERIALS AND METHODS

### $^{45}Ca^{2+}$ efflux from non-mitochondrial stores of permeabilized hepatocytes

Hepatocytes of male Wistar rats (180–250 g) were prepared and then stored on ice in $NaHCO_3$-buffered Eagle's medium containing 2% (w/v) bovine serum albumin (Taylor *et al.*, 1989; Nunn *et al.*, 1990). Cells were permeabilized by incubation with saponin (75 $\mu$g/ml) for 10 min at 37 °C in a $Ca^{2+}$-free cytosol-like medium (140 mM-KCl/20 mM-NaCl/2 mM-$MgCl_2$/1 mM-EGTA/20 mM-Pipes, pH 6.8). After permeabilization, the cells were washed and resuspended (0.1–0.4 mg of protein/ml) in the same medium with $CaCl_2$ added to give a final free [$Ca^{2+}$] of about 120 nM (Berridge *et al.*, 1984). Permeabilized cells were added to $^{45}CaCl_2$ (2 $\mu$Ci/ml), oligomycin (10 $\mu$M) and antimycin (10 $\mu$M), and after 1 min ATP (1.5 mM) and phosphocreatine (5 mM) were added to stimulate $^{45}Ca^{2+}$ accumulation into non-mitochondrial stores. Under these conditions, ATP-dependent $^{45}Ca^{2+}$ accumulation reached a steady state within 5 min. After 12 min, cells were added to glucose and hexokinase to give final concentrations of 10 mM and 25 units/ml respectively. Earlier experiments (Taylor & Putney, 1985) established that this depletes ATP sufficiently rapidly to allow $^{45}Ca^{2+}$ efflux from pre-loaded pools to be monitored free of residual ATP-dependent uptake. The effects of Ins(1,4,5)$P_3$, Ins(1,4,5)$P_3$[S]$_3$ or ionomycin on $^{45}Ca^{2+}$ efflux were examined by simultaneous addition of one of them with glucose/hexokinase.

Protein was measured by the method of Bradford (1976) with bovine serum albumin as standard. Ionomycin was from Calbiochem and hexokinase (type V from baker's yeast) was from Sigma. DL-Ins(1,4,5)$P_3$[S]$_3$ was synthesized according to Cooke *et al.* (1987). All other materials and methods were as described in earlier publications (Taylor & Putney, 1985; Taylor *et al.*, 1989; Nunn *et al.*, 1990).

### Analysis of results

The ATP-dependent $^{45}Ca^{2+}$ content of permeabilized hepatocytes was expressed as a percentage of the content at the time of addition of glucose/hexokinase; means and S.E.M.S were calculated and the exponential loss of $^{45}Ca^{2+}$ was analysed by non-linear least-squares curve fitting by the computer program KINETIC (MacPherson, 1985). In all experiments, $^{45}Ca^{2+}$ efflux after the first minute (see the Results section) was described by a single exponential, and attempts to fit the data to multiple exponentials gave no better fit. The results are plotted semi-logarithmically with the lines drawn from the equations describing the single exponential curve fit.

## RESULTS AND DISCUSSION

Permeabilized hepatocytes rapidly accumulated $^{45}Ca^{2+}$ when provided with ATP: their $^{45}Ca^{2+}$ content increased from 0.29 ± 0.02 nmol/mg of protein ($n$ = 18) to a steady state of 1.66 nmol/mg of protein. Addition of glucose/hexokinase to deplete the medium of ATP caused an initial rapid loss of 16 ± 5% of the ATP-dependent $^{45}Ca^{2+}$ pool and then a slower (half-time about 3 min) efflux of the remaining $^{45}Ca^{2+}$ that could be described by a single exponential function (Fig. 1, Table 1). These results confirm earlier findings (Taylor & Putney, 1985) and establish that with the protocol described the kinetics of $^{45}Ca^{2+}$ efflux from non-mitochondrial stores can be determined.

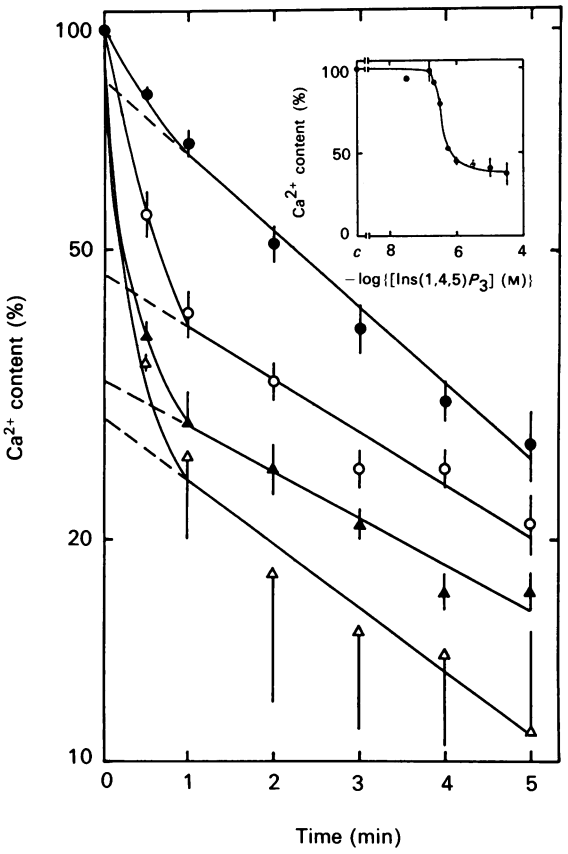


**Fig. 1. $^{45}Ca^{2+}$ efflux from permeabilized hepatocytes stimulated with Ins(1,4,5)$P_3$**

Permeabilized hepatocytes were loaded with $^{45}Ca^{2+}$ and after 12 min ($t$ = 0 in Figure), glucose (final concn. 10 mM) and hexokinase (final concn. 25 units/ml) were added together with various concentrations of Ins(1,4,5)$P_3$ (●, control; ○, 0.3 $\mu$M; ▲, 0.6 $\mu$M; △, 5 $\mu$M). The $^{45}Ca^{2+}$ contents of cells were determined and then expressed as a percentage of their contents at the time of addition of glucose/hexokinase/Ins(1,4,5)$P_3$. Results, the means ± S.E.M of duplicate determinations from three to six independent experiments, are plotted semilogarithmically. The lines are drawn from the equation derived from fitting the $^{45}Ca^{2+}$ contents for the period from 1 to 5 min to a single exponential (see the text for details). The inset shows the concentration–response relationship for Ins(1,4,5)$P_3$-induced $Ca^{2+}$ release 30 s after addition of Ins(1,4,5)$P_3$ measured under conditions identical with those used for $^{45}Ca^{2+}$ efflux studies (means ± S.E.M., $n$ = 4).

When glucose/hexokinase and a maximally effective concentration of Ins(1,4,5)$P_3$ (5 $\mu$M) were applied simultaneously, there was an initial rapid loss of accumulated $^{45}Ca^{2+}$ that was complete within 1 min; the kinetics of $^{45}Ca^{2+}$ efflux were then the same as occurred in the absence of Ins(1,4,5)$P_3$ (Fig. 1). In an earlier publication (Taylor & Putney, 1985), we concluded from very similar results that a maximal concentration of Ins(1,4,5)$P_3$ rapidly and totally emptied an Ins(1,4,5)$P_3$-sensitive fraction of the non-mitochondrial $Ca^{2+}$ stores, but had no effect on the remaining fraction of those stores. By

**Table 1. Effects of Ins(1,4,5)$P_3$, Ins(1,4,5)$P_3$[S]$_3$ and ionomycin on $^{45}Ca^{2+}$ efflux from permeabilized hepatocytes**

The Table summarizes the single exponential equations of the lines fitted to the $^{45}Ca^{2+}$ efflux results shown in Figs. 1–3. The rate of $^{45}Ca^{2+}$ efflux ($\lambda$) after the first 1 min is similar whatever the concentration of Ins(1,4,5)$P_3$ or Ins(1,4,5)$P_3$[S]$_3$, whereas the rate increases with increasing concentrations of ionomycin. By contrast, the intercept of the line at $t = 0$ ($c$) is unaffected by ionomycin concentration, implying that there is no additional effect beyond the sustained change in $^{45}Ca^{2+}$ efflux, whereas with Ins(1,4,5)$P_3$ and Ins(1,4,5)$P_3$[S]$_3$, the intercept decreases with increasing concentrations revealing the rapid concentration-dependent release of $^{45}Ca^{2+}$ from pools of different size. The final column lists $Ca^{2+}$ release calculated from the intercepts at $t = 0$ after correction for the initial rapid $^{45}Ca^{2+}$ release under control conditions. The Ca content (%) = $c$. $e^{-\lambda t}$.

| | $\lambda$ | $c$ (%) | $Ca^{2+}$ release (%) |
|---|---|---|---|
| Ins(1,4,5)$P_3$ | | | |
| 0 | 0.24±0.02 | 84±5 | – |
| 0.15 $\mu$M | 0.19±0.02 | 66±4 | 18 |
| 0.3 $\mu$M | 0.16±0.02 | 46±4 | 38 |
| 0.6 $\mu$M | 0.15±0.02 | 33±6 | 51 |
| 5 $\mu$M | 0.20±0.03 | 29±3 | 55 |
| Ins(1,4,5)$P_3$[S]$_3$ | | | |
| 0 | 0.21±0.02 | 84±5 | – |
| 1 $\mu$M | 0.19±0.02 | 68±4 | 16 |
| 2 $\mu$M | 0.17±0.02 | 55±3 | 29 |
| 10 $\mu$M | 0.19±0.01 | 41±1 | 43 |
| Ionomycin | | | |
| 0 | 0.18±0.02 | 89±6 | – |
| 0.3 nM | 0.24±0.02 | 82±4 | – |
| 1 nM | 0.47±0.01 | 83±2 | – |

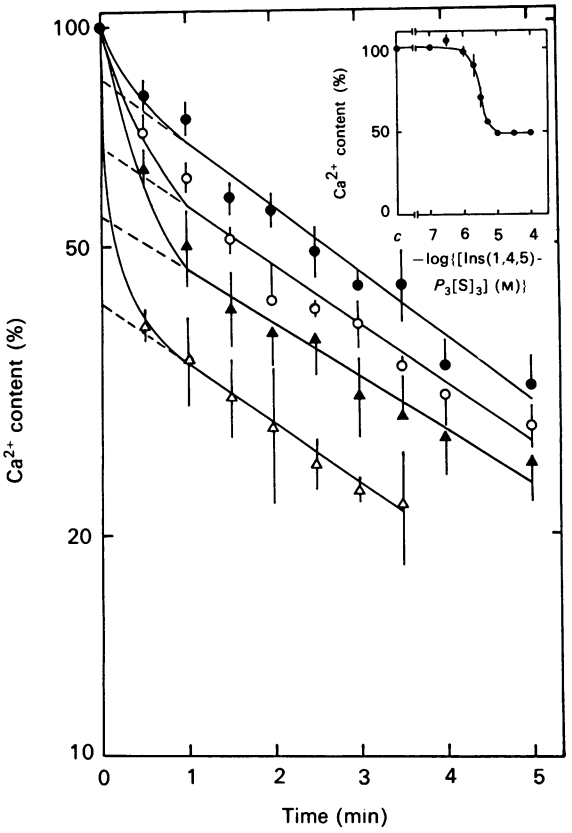


**Fig. 2. $^{45}Ca^{2+}$ efflux from permeabilized hepatocytes stimulated with Ins(1,4,5)$P_3$[S]$_3$**

Methods, analysis and presentation of the results are the same as for Fig. 1. Ins(1,4,5)$P_3$[S]$_3$ with glucose/hexokinase were added at $t = 0$ in Figure: ●, control; ○, 1 $\mu$M; ▲, 2 $\mu$M; △, 10 $\mu$M. Results are the means ± S.E.M. of duplicate determinations from three to six independent experiments. The inset shows the concentration–response relationship for Ins(1,4,5)$P_3$[S]$_3$-induced $Ca^{2+}$ release 30 s after its addition measured under conditions identical with those used for $^{45}Ca^{2+}$ efflux studies (means ± S.E.M., $n = 3$).

extrapolating the lines describing $^{45}Ca^{2+}$ efflux to the time of addition of glucose/hexokinase and Ins(1,4,5)$P_3$, we estimated that in that study of guinea pig hepatocytes about one third of the ATP-dependent non-mitochondrial $^{45}Ca^{2+}$ stores were sensitive to Ins(1,4,5)$P_3$. In the present study of rat hepatocytes, a similar analysis suggests that about 55% of the stores are Ins(1,4,5)$P_3$-sensitive (Fig. 1, Table 1).

We have now extended substantially the earlier experiments to examine the effects of submaximal concentrations of Ins(1,4,5)$P_3$ on $^{45}Ca^{2+}$ efflux (Fig. 1). Each concentration of Ins(1,4,5)$P_3$ caused a rapid concentration-dependent efflux of $^{45}Ca^{2+}$ that was complete within 1 min, but in each case the subsequent $^{45}Ca^{2+}$ efflux was the same irrespective of the Ins(1,4,5)$P_3$ concentration. The results therefore suggest that whatever the concentration of Ins(1,4,5)$P_3$, its effects on $^{45}Ca^{2+}$ efflux are complete within 1 min, and whereas a maximal concentration of Ins(1,4,5)$P_3$ completely empties the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool, submaximal concentrations empty only a fraction of it.

The failure of submaximal concentrations of Ins(1,4,5)$P_3$ to fully release all of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores could result from degradation of Ins(1,4,5)$P_3$ before the increased rate of efflux had fully emptied them. However, these experiments were performed at low cell density (less than 0.2 mg of protein/ml) to minimize Ins(1,4,5)$P_3$ metabolism, and in parallel incubations that included high specific radioactivity [$^3$H]Ins(1,4,5)$P_3$ (57 Ci/mmol; 20 nCi/ml) with the lowest concentration of Ins(1,4,5)$P_3$ examined (300 nM), 97±1% of the added label was recovered as Ins(1,4,5)$P_3$ after 1 min, 91±1% after 5 min, and 82±0.3% after 10 min (results not shown). Further evidence suggesting that Ins(1,4,5)$P_3$ metabolism cannot account for partial emptying of the $Ca^{2+}$ stores is provided by the results from similar experiments with Ins(1,4,5)$P_3$[S]$_3$. Our earlier studies have shown that this analogue of Ins(1,4,5)$P_3$ binds to the Ins(1,4,5)$P_3$ receptor and mobilizes intracellular $Ca^{2+}$ stores, but it is resistant to inactivation by the only enzymes known to metabolize Ins(1,4,5)$P_3$, namely Ins(1,4,5)$P_3$ 3-kinase and Ins(1,4,5)$P_3$ 5-phosphatase (Strupish *et al.*, 1988; Willcocks *et al.*, 1988; Taylor *et al.*, 1989; Nunn *et al.*, 1990). The metabolic stability of Ins(1,4,5)$P_3$[S]$_3$ allowed $^{45}Ca^{2+}$ efflux experiments to be performed at higher cell density (up to 0.4 mg of protein/ml), at which ATP-dependent $Ca^{2+}$ uptake per volume of incubation was increased, but at

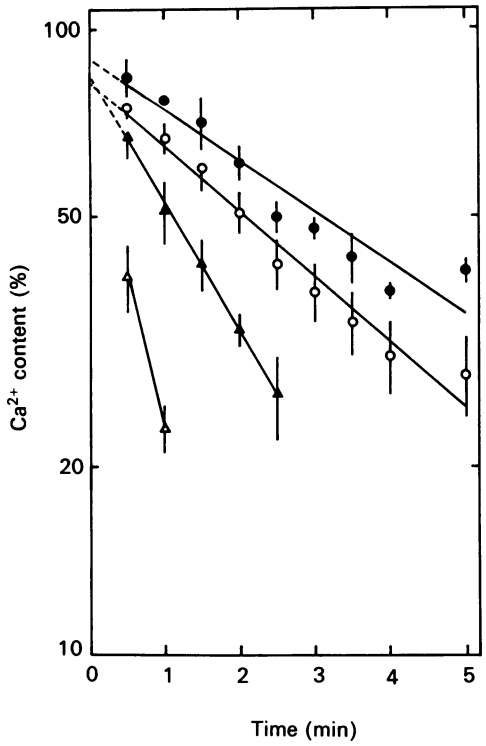


**Fig. 3. Effects of ionomycin on $^{45}Ca^{2+}$ efflux from permeabilized hepatocytes**

The experiments shown in Figs. 1 and 2 were repeated with various concentrations of ionomycin: ●, control [$(CH_3)_2SO$ vehicle]; ○, 0.3 nM; ▲, 1 nM; △, 10 nM. Results are shown as means ± S.E.M. of duplicate determinations from three or four independent experiments.

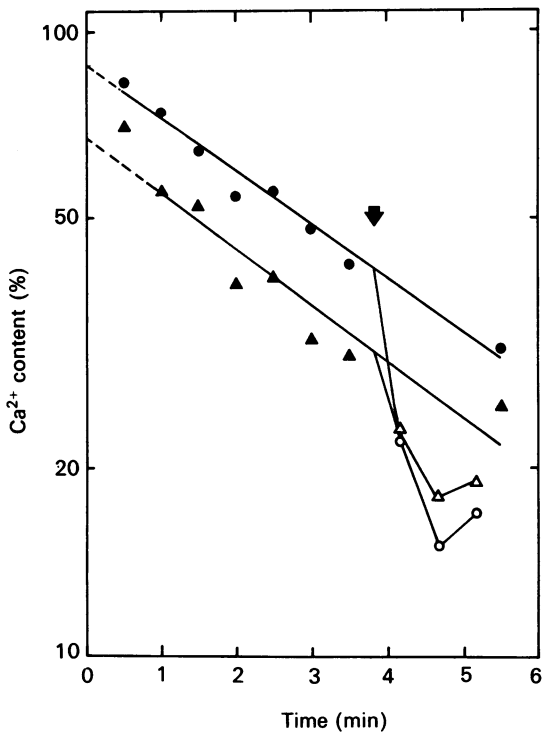


**Fig. 4. Effects of submaximal and then maximal concentrations of Ins(1,4,5)$P_3$[S]$_3$ on $^{45}Ca^{2+}$ efflux from permeabilized hepatocytes**

Permeabilized cells loaded with $^{45}Ca^{2+}$ were added to glucose/hexokinase either alone (●) or in combination with a submaximal concentration of Ins(1,4,5)$P_3$[S]$_3$ (2 μM, ▲). After 3 min 50 s (arrow) sufficient Ins(1,4,5)$P_3$[S]$_3$ was added to each incubation to bring its final concentration to 10 μM, and the effects of these additions are shown (○, △). The results show that although a submaximal concentration of Ins(1,4,5)$P_3$[S]$_3$ only partially depletes the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores, the cells retain full responsiveness to a maximal concentration of Ins(1,4,5)-$P_3$[S]$_3$. The results shown are the means of duplicate determinations from two independent experiments.

which degradation of Ins(1,4,5)$P_3$ would have posed problems. A maximal concentration of Ins(1,4,5)$P_3$[S]$_3$ (10 μM) rapidly stimulated complete emptying of about half of the ATP-dependent $Ca^{2+}$ stores and had no effect on the remaining half (Fig. 2), a very similar result to that obtained with a maximal concentration of Ins(1,4,5)$P_3$ (Fig. 1). Submaximal concentrations of Ins(1,4,5)$P_3$[S]$_3$ also stimulated rapid $^{45}Ca^{2+}$ efflux, but from a smaller fraction of the $Ca^{2+}$ pool and, as for Ins(1,4,5)$P_3$, the remaining fraction of the pool was unaffected by Ins(1,4,5)$P_3$[S]$_3$. Together, these results demonstrate that the failure of submaximal concentrations of Ins(1,4,5)$P_3$ to empty completely the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores is not due to inactivation of the added Ins(1,4,5)$P_3$.

Our results suggesting that the size of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ store is dependent on the concentration of Ins(1,4,5)$P_3$ were surprising; we therefore examined the effects of the $Ca^{2+}$ ionophore, ionomycin. The effects of various concentrations of ionomycin on $^{45}Ca^{2+}$ efflux were each described by a single exponential function where only the sustained rate of efflux and not the initial rapid emptying of the stores increased with increasing concentrations of ionophore (Fig. 3). These are the results expected if the entire ATP-dependent $Ca^{2+}$ store is sensitive to release by ionomycin and there is a concentration-dependent incorporation of the ionophore into the membranes of the stores that then causes an increased rate of $^{45}Ca^{2+}$ efflux throughout the time taken for the stores to empty. The results are, however, very different from those obtained with various concentrations of Ins(1,4,5)$P_3$ and Ins(1,4,5)$P_3$[S]$_3$ (Table 1).

A further concern was that partial emptying of the $Ca^{2+}$ stores could result from desensitization of the Ins(1,4,5)$P_3$ receptor. Such desensitization would perhaps not affect the rapid total emptying of the Ins(1,4,5)$P_3$-sensitive stores by a maximal concentration of Ins(1,4,5)$P_3$, but could limit the amount of $Ca^{2+}$-released by submaximal concentrations where the increased rate of efflux could be expected to be more prolonged. Earlier studies (Prentki *et al.*, 1985; Thomas, 1988; Taylor *et al.*, 1989) have suggested that prolonged incubation with Ins(1,4,5)$P_3$ does not cause desensitization of Ins(1,4,5)$P_3$-induced $Ca^{2+}$ mobilization. The results shown in Fig. 4 provide direct evidence that desensitization of the Ins(1,4,5)$P_3$ response is unlikely to explain partial emptying of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores. As with earlier experiments (Fig. 2), a submaximal concentration of Ins(1,4,5)$P_3$[S]$_3$ (2 μM, a half-maximally effective concentration) rapidly released about 20% of the ATP-dependent $Ca^{2+}$ stores, but thereafter the rate of $^{45}Ca^{2+}$

efflux was the same as in the control. Subsequent addition of sufficient Ins(1,4,5)$P_3[S]_3$ to increase its concentration to 10 $\mu$M, a maximally effective concentration, then stimulated rapid $^{45}Ca^{2+}$ efflux from both the control and the pre-stimulated cells, and the $^{45}Ca^{2+}$ content of each fell to the same level. Similar results were obtained when the experiments were repeated with submaximal (0.3 $\mu$M) and then maximal (5 $\mu$M) concentrations of Ins(1,4,5)$P_3$ (results not shown). The permeabilized hepatocytes that had been stimulated with submaximal concentrations of Ins(1,4,5)$P_3[S]_3$ or Ins(1,4,5)$P_3$ therefore retained Ins(1,4,5)$P_3$ receptors that were as responsive as those of cells that had not been stimulated previously.

Stimulation of pancreatic acini with submaximal concentrations of either carbachol or cholecystokinin appears to cause release of only a fraction of the intracellular $Ca^{2+}$ stores that are mobilized by maximal hormone concentrations (Muallem *et al.*, 1989). In these studies of cell populations, the different sensitivities of the intracellular $Ca^{2+}$ stores to hormonal stimulation could occur anywhere in the signalling pathway and could reflect heterogeneity within individual cells or merely differences in the sensitivity of different cells. The same study confirmed earlier results (e.g. Prentki *et al.*, 1985) by showing that, in the presence of ATP, the steady-state $Ca^{2+}$ content of permeabilized cells depends upon the concentration of Ins(1,4,5)$P_3$. Muallem *et al.* (1989) interpret their results as evidence in support of 'quantal release' of $Ca^{2+}$ by Ins(1,4,5)$P_3$, but a continuously graded response where submaximal concentrations of Ins(1,4,5)$P_3$ stimulate $Ca^{2+}$ efflux from the entire Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool that is counterbalanced by re-uptake into the stores could also explain the results. Indeed, the very rapid ($t_{\frac{1}{2}}$ about 30 s) loading of intracellular $Ca^{2+}$ stores in their experiments would be consistent with the latter explanation. However, they demonstrated that in the presence of vanadate to inhibit $Ca^{2+}$ re-uptake, the immediate Ins(1,4,5)$P_3$-induced $Ca^{2+}$ release was not enhanced, suggesting that re-uptake of $Ca^{2+}$ did not limit the immediate effects of Ins(1,4,5)$P_3$. Although this result argues against rapid resequestration of $Ca^{2+}$ and therefore in favour of their 'quantal release' model, that interpretation must be considered in the light of the considerable inhibition of Ins(1,4,5)$P_3$-induced $Ca^{2+}$ mobilization by vanadate [about 50% inhibition for 0.5 $\mu$M-Ins(1,4,5)$P_3$].

In our experiments with permeabilized hepatocytes, we have attempted to examine more directly the 'quantal release' phenomenon described by Muallem *et al.* (1989). Submaximal concentrations of Ins(1,4,5)$P_3$ or Ins(1,4,5)$P_3[S]_3$ evoke a rapid and concentration-dependent $Ca^{2+}$ efflux from permeabilized hepatocytes that is complete within 1 min, and thereafter efflux is the same as under control conditions. Our interpretation of these results is that whereas a maximal concentration of Ins(1,4,5)$P_3$ rapidly empties the entire Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool, submaximal concentrations rapidly empty only a fraction of it and have no effect on the remainder. Whether this response to Ins(1,4,5)$P_3$ results from heterogeneity between cells or within the stores of each cell does not lessen the problem of explaining how $Ca^{2+}$ stores that can be released immediately by a maximal concentration of Ins(1,4,5)$P_3$ are completely unaffected by prolonged incubation with a submaximal concentration.

We have established that desensitization of the Ins(1,4,5)$P_3$ receptor cannot account for partial emptying of the stores in response to submaximal stimulation, nor can formation of Ins(1,3,4,5)$P_4$, because under the conditions of our experiments there is no detectable formation of Ins(1,3,4,5)$P_4$ and similar results were obtained with Ins(1,4,5)$P_3$ and the analogue Ins(1,4,5)-$P_3[S]_3$ that cannot be phosphorylated. Neither a change in the medium free [$Ca^{2+}$] nor a change in the $Ca^{2+}$ content of the intracellular stores could explain the results, because the small amount of $Ca^{2+}$ released by a maximal concentration of Ins(1,4,5)$P_3$ (< 1% of medium $Ca^{2+}$ content) is buffered by a large excess of EGTA, and maximal concentrations of Ins(1,4,5)$P_3$ or Ins(1,4,5)$P_3$-$[S]_3$ stimulate release of a similar fraction of the stores before or after several minutes of passive $Ca^{2+}$ efflux.

We conclude that in permeabilized hepatocytes, the size of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores depends upon the concentration of Ins(1,4,5)$P_3$. The effect seems unlikely to be a consequence of the $Ca^{2+}$ release process and is more likely to be an inherent property of the intracellular $Ca^{2+}$ stores. In view of the co-operative effects of Ins(1,4,5)$P_3$ on the opening of $Ca^{2+}$ channels (Meyer *et al.*, 1988, 1989) and the importance attached to communication between discrete intracellular $Ca^{2+}$ stores in models proposed to account for $Ca^{2+}$ entry (Irvine, 1989), oscillatory changes in cytoplasm [$Ca^{2+}$] (Berridge & Galione, 1989), and the effects of GTP on intracellular $Ca^{2+}$ (Ghosh *et al.*, 1989), future studies must address the mechanisms whereby Ins(1,4,5)$P_3$ regulates the size of Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores.

This work was supported by grants from The Erna and Victor Hasselblad Foundation, The Nuffield Trust (C.W.T.) and the Science and Engineering Research Council (B.V.L.P.). C.W.T. was a Royal Society Locke Research Fellow and B.V.L.P. is a Lister Institute Fellow.

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Received 6 September 1989/13 October 1989; accepted 17 October 1989