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# Quantal $Ca^{2+}$ mobilization stimulated by inositol 1,4,5-trisphosphate in permeabilized hepatocytes

Katherine A. OLDERSHAW, David L. NUNN and Colin W. TAYLOR*
Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1QJ, U.K.

In several cell types, including hepatocytes, submaximal concentrations of Ins(1,4,5)$P_3$ stimulate an initial rapid mobilization of intracellular $Ca^{2+}$ stores that is followed by either no further $Ca^{2+}$ release or very much slower release. Further additions of Ins(1,4,5)$P_3$ then evoke further $Ca^{2+}$ mobilization. Such 'incremental' responses [Meyer & Stryer (1990) Proc. Natl. Acad. Sci. U.S.A. **87**, 3841–3845] could result from all-or-nothing emptying of stores that differ in their sensitivities to Ins(1,4,5)$P_3$ or from partial emptying of stores that are more uniformly sensitive, but unable to release all of their $Ca^{2+}$ because the response to Ins(1,4,5)$P_3$ rapidly attenuates. By measuring unidirectional $^{45}Ca^{2+}$ efflux from intracellular stores stimulated with Ins(1,4,5)$P_3$ under conditions where they continue to sequester $^{40}Ca^{2+}$, we provide evidence suggesting that Ins(1,4,5)$P_3$ stimulates all-or-nothing emptying of stores that differ in their sensitivities to Ins(1,4,5)$P_3$, a quantal response pattern.

## INTRODUCTION

The effects of receptors that stimulate polyphosphoinositide hydrolysis on intracellular $Ca^{2+}$ mobilization are mediated by Ins(1,4,5)$P_3$, which interacts with a specific receptor in the membranes of intracellular $Ca^{2+}$ stores (Spät *et al.*, 1986; Berridge & Irvine, 1989). Two important aspects of the $Ca^{2+}$ signal evoked by hormonal stimulation of intact cells, stimulated $Ca^{2+}$ entry and $Ca^{2+}$ spiking, are not readily explained by known properties of the isolated Ins(1,4,5)$P_3$ receptor, although Ins(1,4,5)$P_3$ appears to be involved in both responses (Berridge & Irvine, 1989). Recent studies of pancreatic acinar cells (Muallem *et al.*, 1989), hepatocytes (Taylor & Potter, 1990) and basophilic leukaemia cells (Meyer & Stryer, 1990) suggest an additional complexity, because low concentrations of Ins(1,4,5)$P_3$ rapidly release only a fraction of the Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores. Muallem *et al.* (1989) in their initial report suggested that this reflected heterogeneity between individual Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores, with low concentrations of Ins(1,4,5)$P_3$ completely emptying the most sensitive stores and higher concentrations emptying the less sensitive ones (Fig. 1*a*). They therefore concluded that Ins(1,4,5)$P_3$- stimulated $Ca^{2+}$ mobilization was a quantal process. Another possibility is that stores are more uniformly sensitive to Ins(1,4,5)$P_3$, but the response rapidly attenuates and prevents submaximal concentrations of Ins(1,4,5)$P_3$ from releasing the entire $Ca^{2+}$ content of the store (Champeil *et al.*, 1989; Irvine, 1990) (Fig. 1*b*). Here we describe experiments that more fully characterize the effects of submaximal concentrations of Ins(1,4,5)$P_3$ and provide evidence that stores differing in their sensitivities to Ins(1,4,5)$P_3$ discharge $Ca^{2+}$ in an all-or-nothing fashion.

## MATERIALS AND METHODS

Isolated hepatocytes were prepared by collagenase digestion of the livers of male Wistar rats as previously described (Nunn & Taylor, 1990), and were stored on ice in $HCO_3^-$-buffered Eagle's medium for up to 24 h. The cells were permeabilized in a $Ca^{2+}$-free cytosol-like medium by incubation with saponin (25 $\mu$g/ml) at 37 °C for 10 min. After permeabilization the cells were resuspended (0.85 or $8.5 \times 10^6$ cells/ml) in cytosol-like medium {140 mM-KCl/20 mM-NaCl/2 mM-$MgCl_2$/1 mM-EGTA/0.2 mM $CaCl_2$ (free $[Ca^{2+}]$ about 120 nM)/20 mM-Pipes, pH 6.8}.

Permeabilized cells were loaded to steady state by incubation at 37 °C with $^{45}CaCl_2$ (2 $\mu$Ci/ml) in the presence of mitochondrial inhibitors (oligomycin, 10 $\mu$M; antimycin, 10 $\mu$M), ATP (1.5 mM), phosphocreatine (5 mM) and creatine kinase (5 units/ml). The latter was omitted from experiments involving hexokinase. After appropriate additions, cells were separated from the incubation medium by rapid filtration through Whatman GF/C filters. Active accumulation of $^{45}Ca^{2+}$ by the permeabilized cells was calculated by subtracting the $^{45}Ca^{2+}$ bound in the absence of ATP

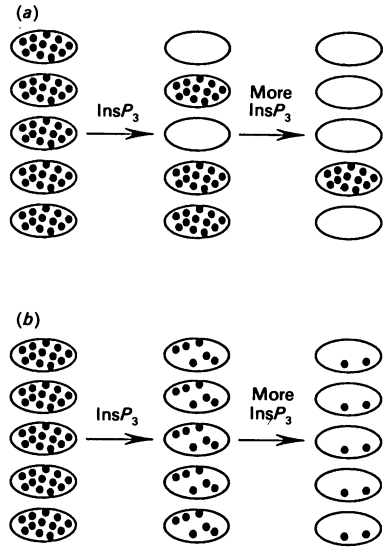


**Fig. 1. Partial emptying of intracellular $Ca^{2+}$ stores by low Ins$P_3$ concentrations**

Partial emptying of Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores by low concentrations of Ins(1,4,5)$P_3$ may result from (*a*) store heterogeneity, with low concentrations of Ins(1,4,5)$P_3$ completely emptying the most sensitive stores, a 'quantal response', or (*b*) modulation of the response such that more uniformly sensitive stores are only partially emptied by low concentrations of Ins(1,4,5)$P_3$.

* To whom correspondence should be addressed.

or after addition of a maximal concentration of ionomycin (10 $\mu$M); both methods gave similar results. In experiments requiring addition of Ins(1,4,5)$P_3$, its degradation, monitored by inclusion of trace amounts of [$^3$H]Ins(1,4,5)$P_3$, never exceeded 10% of that added.

Unidirectional $^{45}Ca^{2+}$ efflux from pre-loaded stores was measured by either adding glucose (10 mM) and hexokinase (25 units/ml) (Taylor & Potter, 1990) or by rapid 10-fold dilution of cells at high density ($8.5 \times 10^6$ cells/ml) into an identical medium at 2 °C (Nunn & Taylor, 1990). $^{40}Ca^{2+}/^{45}Ca^{2+}$ exchange was measured by preloading cells ($8.5 \times 10^6$ cells/ml) to steady state with $^{45}Ca^{2+}$ in cytosol-like medium and then diluting them 8-fold into similar medium at 37 °C with the same free [$Ca^{2+}$] but substantially more total $Ca^{2+}$ (2 mM) and EGTA (10 mM). The method allowed an immediate 71-fold decrease in the specific radioactivity of the $^{45}Ca^{2+}$ of the incubation medium with no detectable change (measured by fura-2 fluorescence) in its free [$Ca^{2+}$]. Reagents were from the suppliers listed in earlier publications (Nunn & Taylor, 1990; Taylor & Potter, 1990).

### Analysis of results

The ATP-dependent $^{45}Ca^{2+}$ content of permeabilized cells was expressed as a fraction of the content immediately before chilling, the decrease in the $^{45}Ca^{2+}$ specific radioactivity or addition of glucose/hexokinase. The $^{45}Ca^{2+}$ contents were then fitted to a single exponential equation using least-squares non-linear regression in the program GraphPAD InPlot (GraphPAD Software, SanDiego, CA, U.S.A.):

$$C_t = C_0 \cdot e^{-\lambda t} + C_b$$

where $C_t$ = $^{45}Ca^{2+}$ content at time $t$, $C_0$ = $^{45}Ca^{2+}$ content at $t_0$ or, for cells in which efflux was stimulated, the intercept of the fitted line at $t_0$, and $C_b$ is the small amount of $^{45}Ca^{2+}$ accumulated after addition of ATP, but not then readily released after its removal; it presumably reflects $^{45}Ca^{2+}$ tightly bound to the inside of the stores. $C_b$ has been subtracted from all data.

## RESULTS AND DISCUSSION

Our earlier experiments (Taylor & Potter, 1990) demonstrated that when $Ca^{2+}$ re-accumulation is prevented, submaximal concentrations of either Ins(1,4,5)$P_3$ or its stable analogue, inositol 1,4,5-trisphosphorothioate, rapidly mobilize a fraction of the intracellular $Ca^{2+}$ stores, but then fail to affect $Ca^{2+}$ efflux from the remaining stores, even though the stores remain sensitive to further additions of Ins(1,4,5)$P_3$. The results shown in Fig. 2 confirm that result, and the more detailed time course demonstrates that the effects of each concentration of Ins(1,4,5)$P_3$ on $^{45}Ca^{2+}$ efflux are complete within 10 s. Similar responses, termed 'incremental responses' by Meyer & Stryer (1990), have been observed in other cells (Muallem *et al.*, 1989; Meyer & Stryer, 1990), but in basophilic leukaemia cells the response is temperature-sensitive. At 11 °C low concentrations of Ins(1,4,5)$P_3$ fully empty the Ins(1,4,5)$P_3$-sensitive stores, albeit more slowly than a maximal concentration (Meyer *et al.*, 1990), whereas at 37 °C they cause only partial emptying (Meyer & Stryer, 1990). Our results with hepatocytes (Fig. 2; Taylor & Potter, 1990) indicate that at 2 °C or 37 °C the response to low concentrations of Ins(1,4,5)$P_3$ is similar, i.e. a rapid partial emptying of Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores, although the stores are about 20 times more sensitive to Ins(1,4,5)$P_3$ at 2 °C than at 37 °C (Nunn & Taylor, 1990).

One possible explanation for these results is that Ins(1,4,5)$P_3$-induced $Ca^{2+}$ mobilization occurs only in the short time taken for the cells either to cool after dilution into cold medium or to lose their ATP after addition of glucose and hexokinase (Smith *et al.*, 1985). Incomplete emptying of the stores by low concentrations of Ins(1,4,5)$P_3$ could then result if complete chilling or removal of ATP blocked the effects of low concentrations of Ins(1,4,5)$P_3$ before they had been fully exerted. However, the same pattern of response was observed when Ins(1,4,5)$P_3$ was added at the same time as $^{45}Ca^{2+}$ uptake was inhibited, or when glucose and hexokinase were added 1 min before Ins(1,4,5)$P_3$ (results not shown), or when the cells were chilled 4 min before addition of Ins(1,4,5)$P_3$ (Fig. 3). In each case, the effects of submaximal concentrations of Ins(1,4,5)$P_3$, i.e. partial emptying of the Ins(1,4,5)$P_3$-sensitive stores, were complete within 5 s. These results show that incomplete emptying of the stores by low concentrations of Ins(1,4,5)$P_3$ is not an artefact of the procedure used to block $Ca^{2+}$ uptake. Earlier results have established that it is not a result of an increase in free [$Ca^{2+}$] of the medium, nor does it result from depletion of Ins(1,4,5)$P_3$ as it binds to its receptor (Nunn & Taylor, 1990) or from Ins(1,4,5)$P_3$ metabolism (Taylor & Potter, 1990). We conclude, therefore, that low

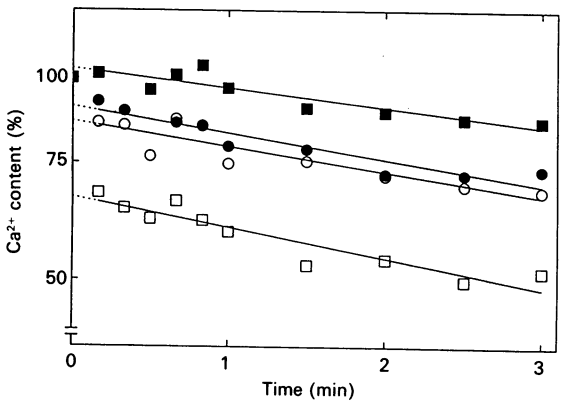


**Fig. 2. Unidirectional $^{45}Ca^{2+}$ efflux from intracellular stores stimulated with Ins(1,4,5)$P_3$**

Unidirectional $^{45}Ca^{2+}$ efflux from permeabilized cells is shown (mean of three experiments) after chilling ($t$ = 0) and simultaneous addition of 300 nM- (□), 30 nM- (○), 10 nM- (●) or no Ins(1,4,5)$P_3$ (■). The lines are fitted as described in the text.

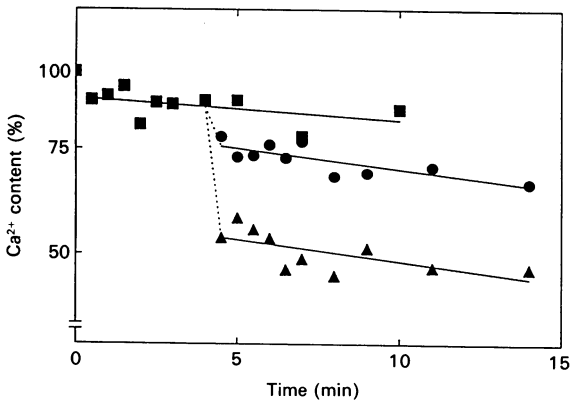


**Fig. 3. Partial emptying of Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores after pre-chilling**

Partial emptying of Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ stores by low concentrations of Ins(1,4,5)$P_3$ is not an artefact of progressive inhibition of the release process as the cells cool. Similar results were obtained when the cells were chilled at the same time as addition of Ins(1,4,5)$P_3$ (Fig. 2) or when they were pre-cooled for 4 min before addition of Ins(1,4,5)$P_3$ (■ control; ● 10 nM; ▲ 1 $\mu$M). Shown are typical results from experiments repeated at least three times.

**Table 1. Kinetics of $^{45}Ca^{2+}$ efflux after rapid dilution of $^{45}Ca^{2+}$ specific radioactivity**

Results from experiments similar to those shown in Fig. 4 and again performed at 37 °C are summarized. $^{45}Ca^{2+}$ contents of cells after dilution of the medium $^{45}Ca^{2+}$ specific radioactivity (at $t = 0$) were fitted to a single exponential equation as described in the text. The intercepts (at $t = 0$) and rate constant ($\lambda$) from 5–18 independent experiments were averaged and are shown as means ± S.E.M. The intercept provides a measure of the $Ca^{2+}$ content of the stores immediately after addition of Ins(1,4,5)$P_3$, and $\lambda$ is a measure of the sustained rate of $^{45}Ca^{2+}$ efflux from 10 s to 5 min after Ins(1,4,5)$P_3$ addition. The results show that even when the stores continue to sequester $Ca^{2+}$, the effects of Ins(1,4,5)$P_3$ on $^{45}Ca^{2+}$ exchange are complete within a few seconds, and thereafter Ins(1,4,5)$P_3$ has no further effect on the rate at which $^{45}Ca^{2+}$ leaks from the stores.

| [Ins(1,4,5)$P_3$] ($\mu$M) | Intercept (%) | $\lambda$ ($min^{-1}$) |
|---|---|---|
| 0 | 101 ± 3 | 0.990 ± 0.096 |
| 0.05 | 90 ± 6 | 0.948 ± 0.078 |
| 0.1 | 77 ± 4 | 1.014 ± 0.090 |
| 0.4 | 75 ± 6 | 0.978 ± 0.138 |
| 0.5 | 69 ± 4 | 0.948 ± 0.096 |
| 5 | 56 ± 2 | 0.852 ± 0.096 |

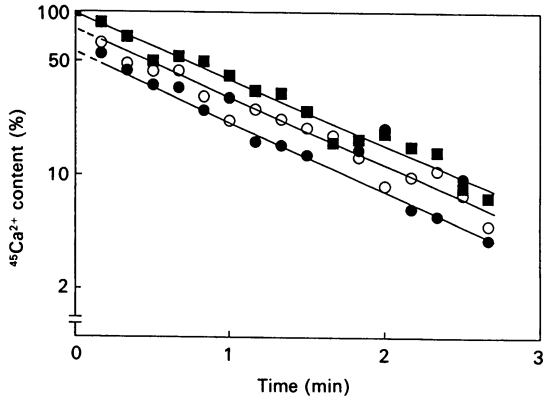


**Fig. 4. Ins(1,4,5)$P_3$-stimulated $^{45}Ca^{2+}$ efflux after a decrease in $^{45}Ca^{2+}$ specific radioactivity**

Permeabilized cells were loaded to steady state with $^{45}Ca^{2+}$ before (at $t = 0$ in the Figure) simultaneous 71-fold dilution of the $^{45}Ca^{2+}$ specific activity and addition of Ins(1,4,5)$P_3$ [● 5 $\mu$M; ○ 400 nM; ■ no added Ins(1,4,5)$P_3$]. The temperature throughout was 37 °C. The $^{45}Ca^{2+}$ contents of the cells are plotted semi-logarithmically as a fraction of their contents at $t = 0$. Lines are fitted to a single exponential equation as described in the text. Results from a typical experiment are shown. Table 1 summarizes data from many similar experiments.

concentrations of Ins(1,4,5)$P_3$ are unable to mobilize the entire Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ pool.

Muallem *et al.* (1989) proposed that individual $Ca^{2+}$ stores differed in their sensitivity to Ins(1,4,5)$P_3$, with low concentrations completely emptying the most sensitive stores of $Ca^{2+}$ and higher concentrations emptying the less sensitive stores as well, a quantal response (Fig. 1). The heterogeneity between stores could result from receptors with different affinities for Ins(1,4,5)$P_3$ or from different numbers of receptors. In either case, Ins(1,4,5)$P_3$-stimulated $Ca^{2+}$ mobilization from individual stores would have to be steeply co-operative to allow each to empty in an all-or-nothing manner. Earlier studies do not directly address this aspect, because Ins(1,4,5)$P_3$ binding measured at equilibrium may not represent binding to the active conformation of the receptor (Pietri *et al.*, 1990), and steeply co-operative responses of individual stores may not be detected in studies of cell populations (Meyer *et al.*, 1990).

Incremental responses could also arise from stores that are more uniformly sensitive to Ins(1,4,5)$P_3$ if the response rapidly attenuates. Low concentrations of Ins(1,4,5)$P_3$ would then partially empty each Ins(1,4,5)$P_3$-sensitive $Ca^{2+}$ store and higher concentrations would more completely empty them (Fig. 1*b*). A rapid switch to a conformation in which the receptor has higher affinity for Ins(1,4,5)$P_3$ but a much lowered permeability to $Ca^{2+}$ could explain such a response pattern (Champeil *et al.*, 1989). This attractive analogy with the nicotinic acetylcholine receptor is, however, difficult to reconcile with the observation that pre-incubation with Ins(1,4,5)$P_3$ does not affect the response to a subsequent Ins(1,4,5)$P_3$ addition (Meyer & Stryer, 1990). Nor can it readily explain the different effects of Ins(1,4,5)$P_3$ under conditions when $Ca^{2+}$ re-uptake is prevented (Fig. 2) and under steady-state conditions where $Ca^{+}$ can be re-sequestered. When intracellular stores are allowed to re-sequester $Ca^{2+}$, the Ins(1,4,5)$P_3$ receptor cannot be completely desensitized, because the stores remain depleted for as long as the receptor is occupied, but then rapidly refill when the receptor is blocked by an antagonist (Cullen *et al.*, 1988) or after removal or degradation of Ins(1,4,5)$P_3$ (Taylor *et al.*, 1989). However, when $Ca^{2+}$ re-uptake is prevented, the response to each concentration of Ins(1,4,5)$P_3$ is complete within the 5–10 s temporal resolution of our experiments (Fig. 2). Irvine (1990) has suggested another model in which luminal $Ca^{2+}$ and cytosolic Ins(1,4,5)$P_3$ together regulate opening of the $Ca^{2+}$ channel. The effects of submaximal concentrations of Ins(1,4,5)$P_3$ might then become limited by the luminal free [$Ca^{2+}$] as it falls after addition of Ins(1,4,5)$P_3$.

We have attempted to discriminate between the two models: all-or-nothing emptying of stores differing in their sensitivities to Ins(1,4,5)$P_3$, or incomplete emptying that becomes limited by the luminal $Ca^{2+}$ concentration. Stores were loaded to steady state with $^{45}Ca^{2+}$, and unidirectional $^{45}Ca^{2+}$ efflux was then monitored while the stores continued to pump $Ca^{2+}$ by rapidly diluting the $^{45}Ca^{2+}$ specific activity at the same time as addition of various concentrations of Ins(1,4,5)$P_3$. As expected, loss of $^{45}Ca^{2+}$ from control cells is described by a mono-exponential equation (Fig. 4). Computer modelling (Tregear *et al.*, 1991) cannot yet quantitatively predict the differences in $^{45}Ca^{2+}$ efflux rates expected in the two models, because the parameters needed to test the models have not yet been accurately measured. However, under our conditions, where the stores continue to pump $Ca^{2+}$, if the fall in luminal $Ca^{2+}$ were limiting the action of a low concentration of Ins(1,4,5)$P_3$, the $Ca^{2+}$ pump would begin to replenish the $Ca^{2+}$ stores and at least partially restore the sensitivity of the Ins(1,4,5)$P_3$ receptor. We would therefore expect the initial rapid efflux of $^{45}Ca^{2+}$ from the stores, reflecting a decrease in their total $Ca^{2+}$ content, to be followed by an enhanced rate of efflux as $^{45}Ca^{2+}$ continues to leak through the Ins(1,4,5)$P_3$-gated channel, because its opening would no longer be completely prevented by the fall in luminal free [$Ca^{2+}$]. By contrast, if low concentrations of Ins(1,4,5)$P_3$ completely empty the most sensitive $Ca^{2+}$ stores and do not affect less sensitive stores, the initial $^{45}Ca^{2+}$ efflux will be followed by a rate of $^{45}Ca^{2+}$ efflux that is insensitive to the presence of Ins(1,4,5)$P_3$.

Under steady-state conditions in the presence of Ins(1,4,5)$P_3$, $Ca^{2+}$ must be rapidly cycled through the depleted $Ca^{2+}$ stores, because the $Ca^{2+}$ released by Ins(1,4,5)$P_3$ is re-sequestered within seconds of addition of the Ins(1,4,5)$P_3$ receptor antagonist heparin (Cullen *et al.*, 1988). In our experiments Ins(1,4,5)$P_3$ was added at the same time as the change in $^{45}Ca^{2+}$ specific radioactivity that allowed $^{45}Ca^{2+}$ efflux to be measured, and our

recordings of $^{45}Ca^{2+}$ efflux were rapid relative to the rates of passive $^{45}Ca^{2+}$ efflux (Fig. 4). We should therefore be able to detect, within the relevant time scale, any change in the rate of $^{45}Ca^{2+}$ efflux in the interval following the net $Ca^{2+}$ release triggered by submaximal Ins(1,4,5)$P_3$ concentrations. Our results (Fig. 4, Table 1) show that, after the initial $Ca^{2+}$ mobilization, $^{45}Ca^{2+}$ efflux occurs at the same rate whatever the concentration of Ins(1,4,5)$P_3$. These results therefore suggest that submaximal concentrations of Ins(1,4,5)$P_3$ stimulate all-or-nothing emptying of intracellular $Ca^{2+}$ stores that differ in their sensitivity to Ins(1,4,5)$P_3$ (Fig. 1*a*).

In pancreatic acinar cell populations, submaximal concentrations of $Ca^{2+}$-mobilizing hormones stimulate release of only a fraction of the $Ca^{2+}$ stores that can be mobilized by higher hormone concentrations (Muallem *et al.*, 1989). This response could be a consequence of individual cells differing in their abilities to produce Ins(1,4,5)$P_3$, or their internal $Ca^{2+}$ stores may differ in their sensitivities to Ins(1,4,5)$P_3$. Evidence that the latter is at least part of the explanation is provided by studies of single cells. Flash photolysis of caged Ins(1,4,5)$P_3$ in *Xenopus* oocytes suggests that stores differing in their sensitivities to Ins(1,4,5)$P_3$ release $Ca^{2+}$ in an all-or-nothing manner (Parker & Ivorra, 1990). Similarly, single cell video imaging of human endothelial cells (Jacob *et al.*, 1988) or HeLa cells (M. D. Bootman, M. J. Berridge & C. W. Taylor, unpublished work) has shown that low histamine concentrations mobilize only a fraction of the internal $Ca^{2+}$ stores that are released after addition of a maximal concentration of histamine.

Our results and other studies of single cells suggest that quantal mobilization of $Ca^{2+}$ stores by Ins(1,4,5)$P_3$ may be a widespread phenomenon that is likely to play an important role in governing the spatial organization of $Ca^{2+}$ signals evoked by extracellular stimuli (Rooney *et al.*, 1990). However, the interactions between Ins(1,4,5)$P_3$ and its receptor that lead to responses so steeply dependent on Ins(1,4,5)$P_3$ concentration are not yet understood.

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