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Luminal Ca^{2+} Increases the Sensitivity of Ca^{2+} Stores to Inositol 1,4,5-Trisphosphate

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SUMMARY

Ca^{2+} within intracellular stores has been proposed to act with cytosolic inositol 1,4,5-trisphosphate ($InsP_3$) to cause opening of the integral Ca^{2+} channel of the $InsP_3$ receptor, leading to mobilization of intracellular Ca^{2+} stores [*FEBS Lett.* **263**:5–9 (1990)]. We have tested that suggestion in saponin-permeabilized rat hepatocytes by manipulating the Ca^{2+} content of the stores and then determining their sensitivity to $InsP_3$, while keeping the cytosolic Ca^{2+} concentration constant. Stores depleted of Ca^{2+} by incubation with ionomycin were significantly less sensitive to $InsP_3$, an effect thought likely to result from the decrease in luminal free Ca^{2+} concentration rather than from direct effects of ionomycin on $InsP_3$ binding or Ca^{2+} permeability. The luminal free Ca^{2+} concentration of stores loaded in the presence of pyrophosphate appeared to be substantially reduced, and again there was a significant inverse correlation between the estimated free Ca^{2+} concentration of the stores and their sensitivity to $InsP_3$. By following the kinetics of $^{45}Ca^{2+}$ uptake into empty stores in the presence of inositol trisphosphorothioate, a stable $InsP_3$ analogue, we demonstrated that stores respond to inositol trisphosphorothioate only after their luminal free Ca^{2+} concentration exceeds a critical level. We conclude that $InsP_3$ and luminal Ca^{2+} together regulate Ca^{2+} mobilization from intracellular stores, and we discuss some of the implications of this interaction for the complex Ca^{2+} signals evoked by extracellular stimuli.

$InsP_3$ is a messenger responsible for mobilization of intracellular Ca^{2+} stores (1) and is probably thereby responsible for initiating the complex Ca^{2+} signals evoked by extracellular stimuli that cause activation of phosphoinositidase C (2). In a variety of cells, including hepatocytes, $InsP_3$ appears to cause essentially all-or-nothing emptying of Ca^{2+} stores that differ in their sensitivities to $InsP_3$ (3–7); Muallem *et al.* (3), in their initial report, described this as quantal Ca^{2+} mobilization. The mechanisms underlying such behavior are not yet understood. Irvine (8) proposed that the inability of low $InsP_3$ concentrations to cause complete emptying of the $InsP_3$-sensitive stores could result from an interaction between Ca^{2+} within the lumen of the stores and cytosolic $InsP_3$. He suggested that, if both $InsP_3$ and luminal Ca^{2+} were needed to allow opening of the integral Ca^{2+} channel of the $InsP_3$ receptor (9), then the effects of low $InsP_3$ concentrations could become limited as the luminal free Ca^{2+} concentration of the stores fell during Ca^{2+} mobilization. In other words, low concentrations of $InsP_3$ would cause partial emptying of the entire population of $InsP_3$-sensitive stores. Our recent results (7), and to some extent the earlier work of Muallem *et al.* (3), suggest that this is an unlikely explanation, because low $InsP_3$ concentrations appear to cause complete emptying of the most sensitive fraction of the $InsP_3$-sensitive stores without affecting the less sensitive stores. Nevertheless, the initial proposition that luminal Ca^{2+} and $InsP_3$ together regulate Ca^{2+} mobilization is attractive because the interaction could generate highly cooperative responses to $InsP_3$ and could contribute to the heterogeneous sensitivity of Ca^{2+} stores; both are essential requirements for quantal responses to $InsP_3$ (6, 10).

In the present study, we use a combination of techniques to manipulate the free Ca^{2+} concentration within the intracellular stores of permeabilized hepatocytes and then determine their sensitivity to $InsP_3$ in the presence of a constant cytosolic free Ca^{2+} concentration. Our results suggest that cytosolic $InsP_3$ and luminal Ca^{2+} together regulate Ca^{2+} mobilization.

Materials and Methods

$^{45}Ca^{2+}$ flux measurements. Rat hepatocytes were prepared from livers of male Wistar rats (175–200 g) by collagenase digestion (11). The cells were washed and resuspended in Ca^{2+}-free medium (140 mM KCl, 20 mM NaCl, 2 mM $MgCl_2$, 1 mM EGTA, 20 mM PIPES, pH 6.8), and their plasma membranes were permeabilized, as required, by incubation with saponin (25 μg/ml) for 10 min at 37°. After permeabilization, the cells were resuspended (1–2 × 10^6 cells/ml) in the same medium supplemented with Ca^{2+} (final free $[Ca^{2+}]$ = 120 nM), $^{45}CaCl_2$ (2 μCi/ml), and mitochondrial inhibitors (10 μM oligomycin and 10 μM

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ABBREVIATIONS: $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_3[S]_3$, DL-inositol 1,4,5-trisphosphorothioate; EC_{50}, concentration causing half-maximal response; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; PIPES, piperazine-*N,N′*-bis(2-ethanesulfonic acid).

antimycin). ATP (1.5 mM), creatine phosphate (5 mM), and creatine phosphokinase (5 units/ml) were added to stimulate Ca^{2+} uptake; after 5 min, the intracellular stores had loaded to steady state. In some experiments, Ca^{2+} uptake was enhanced by addition of tetrapotassium pyrophosphate (5 mM); to correct for the additional Mg^{2+} buffering (verified using chlortetracycline fluorescence), the total medium $MgCl_2$ concentration was increased to 4 mM; the free Ca^{2+} concentration (measured by using fluo 3) was unaffected. The $^{45}Ca^{2+}$ contents of the intracellular stores were determined by stopping incubations by addition of 5 ml of ice-cold 310 mM sucrose, 1 mM sodium citrate, and then rapidly filtering the samples through Whatman GF/C filters, using a Brandel receptor-binding harvester. With this rapid filtration method, we were able to obtain a temporal resolution of about 5 sec. Results are expressed after correction for ATP-independent $^{45}Ca^{2+}$ binding. In all experiments involving additions of $InsP_3$, its metabolism (assessed from parallel incubations with [^{3}H]$InsP_3$) (11) was negligible (<5%).

Data analysis. Concentration-response curves were fitted to a logistic equation using the nonlinear, least-squares, curve-fitting routine DRUG, in the EBDA program (12):

$$R = \frac{T - \mathrm{InS}}{1 + ([\mathrm{InsP_3}]/\mathrm{EC_{50}})^n}$$

where R is the size of the remaining $InsP_3$-sensitive Ca^{2+} stores, T is the size of the total Ca^{2+} stores, InS is the size of $InsP_3$-insensitive Ca^{2+} stores, [$InsP_3$] is the concentration of $InsP_3$, EC_{50} is the concentration of $InsP_3$ causing mobilization of half the $InsP_3$-sensitive stores, and n is an empirical value equivalent to the Hill coefficient.

The kinetics of $^{45}Ca^{2+}$ uptake were fitted to a monoexponential rate equation using the nonlinear, least-squares, curve-fitting program in GraphPAD InPlot (GraphPAD Software, San Diego, CA):

$$C = S(1 - e^{\lambda t})$$

where C is the $^{45}Ca^{2+}$ content of the cells at time t, S is their steady state $^{45}Ca^{2+}$ content, and λ is the efflux rate constant.

Materials. Ionomycin was from Calbiochem, $InsP_3$ and $InsP_3$[S]$_3$ were from RF Irvine (Babraham, UK) and BVL Potter (Bath, UK), respectively, fluo 3 was from Molecular Probes (OR), chlortetracycline was from Sigma, and other reagents were from suppliers listed earlier (13).

Results and Discussion

If luminal Ca^{2+} and $InsP_3$ together regulate Ca^{2+} mobilization from intracellular stores (8), we would expect stores depleted of Ca^{2+} to be less sensitive to $InsP_3$ than full stores. We tested this prediction by loading permeabilized cells to steady state with $^{45}Ca^{2+}$, in the presence of various concentrations of ionomycin. With this method, we were able to manipulate the Ca^{2+} content of the stores to between 10 and 100% of their control loading and so to examine the effects of varying luminal Ca^{2+} on $InsP_3$-stimulated Ca^{2+} mobilization. The results of many such experiments (Fig. 1) demonstrate a very significant inverse correlation ($p < 0.001$, Student's t test) between the initial $^{45}Ca^{2+}$ content of the stores and the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization; full stores are more sensitive to $InsP_3$. Ionomycin (≤1 μM) did not displace specific [^{3}H]$InsP_3$ binding (data not shown) from the sites in permeabilized hepatocytes that we have earlier shown to be the ligand-binding domains of the Ca^{2+}-mobilizing receptors (13).

When measuring the sensitivity of stores to $InsP_3$, we and others almost invariably express our results as the fraction of actively sequestered Ca^{2+} released by each $InsP_3$ concentration, because with the limited temporal resolution of most methods it is impossible to resolve the more useful initial rates of $InsP_3$-stimulated Ca^{2+} mobilization. With such a measure of $InsP_3$ sensitivity, it could be argued that the additional leak introduced into the stores by ionomycin would inevitably require the addition of more $InsP_3$ to cause the same fractional increase in leak as occurred before addition of ionomycin. Ionomycin would thereby decrease the sensitivity of the stores to $InsP_3$ irrespective of its effects on luminal free Ca^{2+} concentration. This would occur only if ionomycin introduced a leak comparable in magnitude to that caused by $InsP_3$. The fact that this is not the case in our experiments is demonstrated by comparing the leaks introduced by ionomycin or $InsP_3$ with the rate of ATP-dependent Ca^{2+} uptake. In stores that retain Ca^{2+} at steady state, as occurs during the preincubation with ionomycin, leak and pump rates must be equal. However, our earlier work, where we examined the effects of $InsP_3$ on unidirectional $^{45}Ca^{2+}$ efflux under conditions where the stores continued to pump $^{40}Ca^{2+}$, demonstrated all-or-nothing emptying of stores by $InsP_3$ (7); this implies that the leak caused by activation of $InsP_3$ receptors is massive relative to Ca^{2+} pumping. Therefore, within stores that respond to $InsP_3$ the leak created by activation of $InsP_3$ receptors is very much larger than the leak introduced in our experiments by the ionomycin used to manipulate their Ca^{2+} content.

The same argument, namely the relative magnitudes of Ca^{2+} pumping and $InsP_3$-stimulated leaks, suggests that any modest increase in Ca^{2+} pumping after loss of Ca^{2+} from the stores (14) is unlikely to appreciably counteract the effects of $InsP_3$ (6). We conclude that the effects of ionomycin on $InsP_3$ sensitivity are most likely to result from the change in luminal free Ca^{2+} concentration and are neither a direct consequence of the increased Ca^{2+} permeability of the stores nor a consequence of increased Ca^{2+} pumping.

In the presence of pyrophosphate (5 mM), cells continue to accumulate $^{45}Ca^{2+}$ at a near-linear rate for up to 60 min (data not shown); their $^{45}Ca^{2+}$ content is then up to 10-fold greater than that in control cells. However, during this loading period, the total amount of $^{45}Ca^{2+}$ released during a 2-min incubation with a maximal concentration of $InsP_3$ progressively declined from 3.2 nmol/10^6 cells after 5 min to 1.8 nmol/10^6 cells after 30 min. Under these conditions, it seems that the free Ca^{2+} concentration within the $InsP_3$-sensitive stores falls as Ca^{2+} precipitates within them, causing a decline in the amount of Ca^{2+} rapidly released after addition of $InsP_3$. This observation provides an additional means of examining the effects of luminal free Ca^{2+} concentration on $InsP_3$ sensitivity. In Fig. 2, we have used the amount of $^{45}Ca^{2+}$ released by a brief incubation with a maximal $InsP_3$ concentration as an index of the free Ca^{2+} concentration within the stores and compared that with the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization. Again, there is an inverse correlation ($r = -0.96$, $p < 0.05$) between the estimate of the free Ca^{2+} concentration of the $InsP_3$-sensitive stores and the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization (Fig. 2).

Ca^{2+} mobilization in response to a maximal concentration of $InsP_3$ is very rapid ($t_{0.5} < 200$ msec) (4, 15), relative to Ca^{2+} uptake ($t_{0.5}$ about 1 min) (Fig. 3). If $InsP_3$ could open the Ca^{2+} channel of the $InsP_3$ receptor in the absence of luminal Ca^{2+}, we would expect that as empty stores begin to load with Ca^{2+} the fraction that normally responds to $InsP_3$ (around 50%) would fail to accumulate any $^{45}Ca^{2+}$. In the presence of a maximal $InsP_3$ concentration, the rate of $^{45}Ca^{2+}$ uptake into empty stores would then be about half the rate seen for control

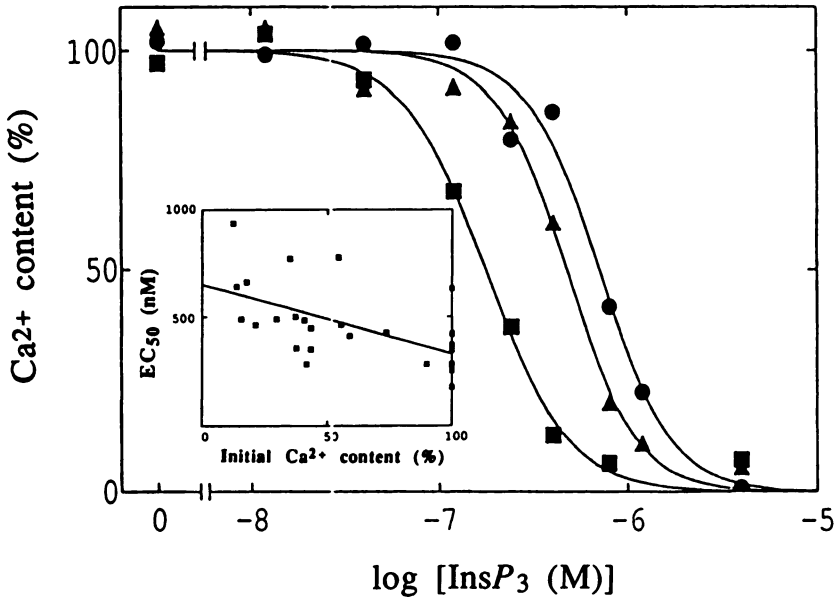

Fig. 1. Effects of ionomycin on the $InsP_3$ sensitivity of intracellular Ca^{2+} stores. The steady state $^{45}Ca^{2+}$ content of permeabilized hepatocytes was reduced by incubation with low concentrations of ionomycin (≤700 nM) before addition of $InsP_3$ and further incubation for 5 min. Concentration-response curves were fitted to a logistic equation from which the maximal Ca^{2+} release and EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization were determined. The main figure shows the effects of $InsP_3$ on $^{45}Ca^{2+}$ release (expressed as a fraction of that released by a maximal concentration of $InsP_3$) from the intracellular stores of cells in which the initial Ca^{2+} content was normal (100%) (■) or reduced by incubation with ionomycin to 73% (▲) or 54% (●) of the control content. Pooled results from similar experiments show a significant inverse correlation ($r = -0.61$, $p < 0.001$, 32 experiments) between the initial Ca^{2+} content of the stores and the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization (*inset*).

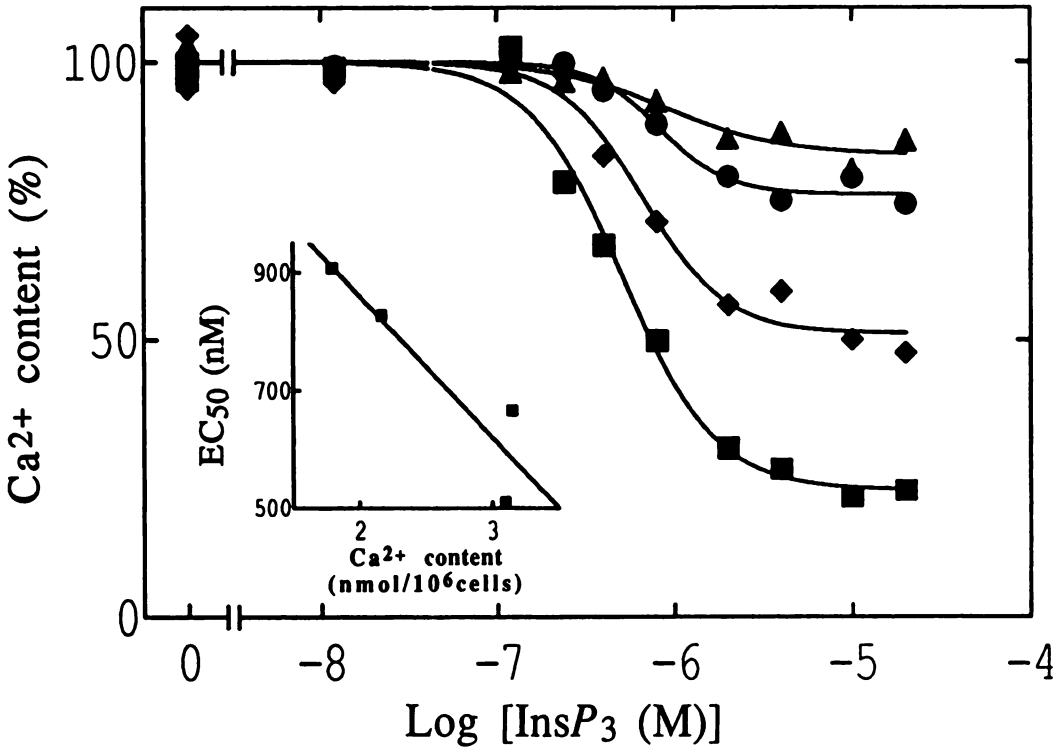

Fig. 2. Effects of loading stores with calcium pyrophosphate on $InsP_3$ sensitivity. Permeabilized cells were incubated with pyrophosphate (5 mM) in modified incubation medium (see Materials and Methods) to enhance $^{45}Ca^{2+}$ uptake. After loading for 5 min (■), 10 min (♦), 20 min (●), or 30 min (▲), cells were incubated for an additional 2 min with various concentrations of $InsP_3$. The amount of Ca^{2+} released by a maximal $InsP_3$ concentration (calculated after fitting curves to the logistic equation) was taken as an index of the free Ca^{2+} concentration of the $InsP_3$-sensitive stores (see Results and Discussion), and this was then plotted (*inset*) against the EC_{50} for $InsP_3$-stimulated Ca^{2+} mobilization. The results demonstrate a significant inverse correlation ($r = -0.96$; $p < 0.05$) between the estimate of luminal free Ca^{2+} concentration and EC_{50} for Ca^{2+} mobilization.

cells. Our results conflict with this suggestion. Fig. 3 demonstrates that, for about 30 sec after addition of ATP, the rate of $^{45}Ca^{2+}$ uptake into intracellular stores is not significantly different in the absence or presence of a maximal concentration of the stable $InsP_3$ analogue $InsP_3[S]_3$ (11). Only after a critical Ca^{2+} content is exceeded do the stores become sensitive to $InsP_3[S]_3$. They then accumulate Ca^{2+} more slowly and reach final steady state Ca^{2+} contents that decrease with increasing $InsP_3[S]_3$ concentrations (Fig. 3). These results suggest that empty Ca^{2+} stores cannot respond to $InsP_3$; only as their luminal free Ca^{2+} concentration exceeds a critical level can the $InsP_3$-regulated Ca^{2+} channel open.

Our results suggesting that luminal Ca^{2+} and $InsP_3$ together regulate Ca^{2+} mobilization prompts consideration of the nature of $InsP_3$-sensitive Ca^{2+} stores; are they anatomically discrete, as many authors suggest (16, 17), or are the $InsP_3$-insensitive Ca^{2+} pools merely the Ca^{2+} that remains within $InsP_3$-sensitive stores after their Ca^{2+} content falls to a level at which the channel closes? Our earlier work suggests that the latter is unlikely, because when a store responds to $InsP_3$ it releases its entire Ca^{2+} content (7). Additional evidence is provided by experiments where we examined the fraction of the stores releasable by $InsP_3$ after they had been depleted of Ca^{2+} to varying degrees. In these experiments, 34 ± 2% of Ca^{2+} could be released from control cells by $InsP_3$; we should, therefore, expect that, if luminal Ca^{2+} alone were preventing further Ca^{2+} mobilization, stores from which ionomycin had released 34% of their Ca^{2+} should also be insensitive to $InsP_3$. Our results

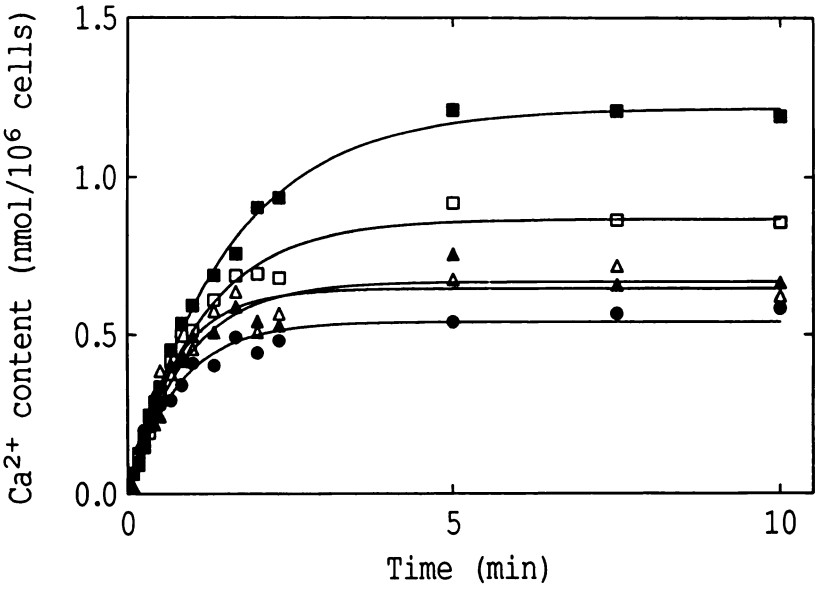

Fig. 3. $^{45}Ca^{2+}$ uptake into empty stores in the presence of $InsP_3[S]_3$. Permeabilized cells were incubated with ATP, to initiate $^{45}Ca^{2+}$ uptake, in the presence of various concentrations of $InsP_3[S]_3$. The *lines* were drawn after fitting data, corrected for ATP-independent $^{45}Ca^{2+}$ binding, to a monoexponential rate equation. $InsP_3[S]_3$ caused a concentration-dependent decrease in the steady state $^{45}Ca^{2+}$ content (by 47 ± 7%, three experiments, for maximal $InsP_3[S]_3$) by increasing the efflux rate constant without significantly affecting the initial rate of $^{45}Ca^{2+}$ uptake (0.71 ± 0.09 nmol/min/10^6 cells, three experiments, for control; 0.75 ± 0.13 nmol/min/10^6 cells, four experiments, with 10 μM $InsP_3[S]_3$). ■, 0; □, 4 μM; ▲, 6 μM; △, 8 μM; ●, 10 μM $InsP_3[S]_3$.

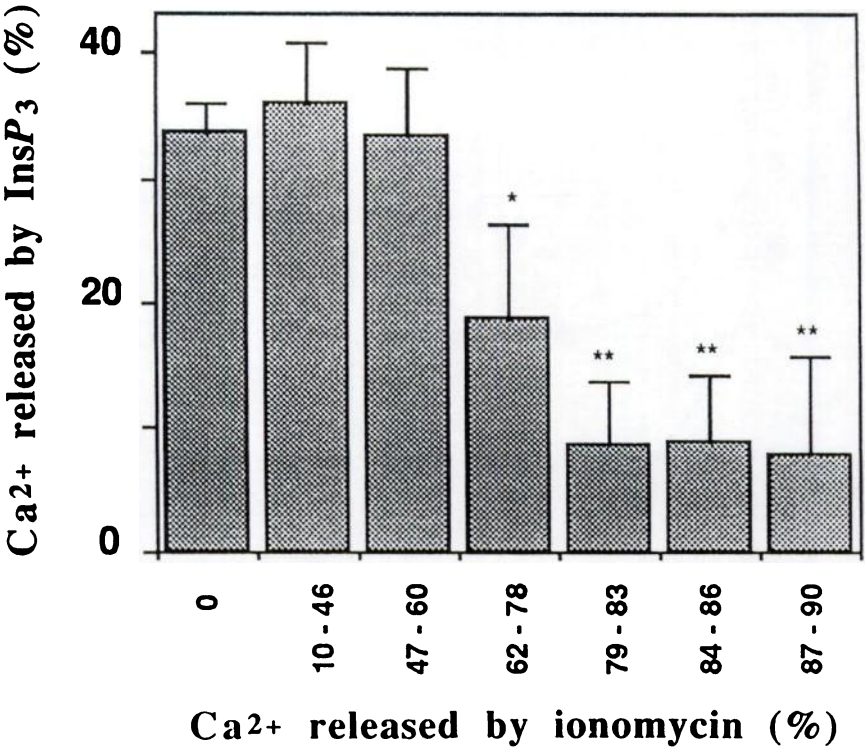

Fig. 4. Size of $InsP_3$-sensitive Ca^{2+} stores after reduction of their initial Ca^{2+} content. Various concentrations of ionomycin (≤700 nM) were used to reduce the steady state Ca^{2+} content of intracellular stores, and the fraction of the remaining stores released by addition of a maximal $InsP_3$ concentration was then determined, as described for Fig. 1. The fraction of the stores released by $InsP_3$ (percentage) is plotted against the reduction in Ca^{2+} content caused by prior incubation with ionomycin (percentage). About 35% of the Ca^{2+} stores were released by $InsP_3$ in control cells, and only when >60% of the stores had been released by ionomycin was the fraction released by $InsP_3$ diminished. In 12 of the 15 incubations in which ionomycin reduced the $^{45}Ca^{2+}$ content by >79%, $InsP_3$ had no effect. The failure of $InsP_3$ to mobilize Ca^{2+} does not result from the absence of a favorable Ca^{2+} gradient, because a maximal ionomycin concentration completely emptied the Ca^{2+} stores. Results are shown as mean ± standard error of five experiments, except for control (13 experiments). *, $p < 0.05$; **, $p < 0.001$, with respect to controls.

with ionomycin (Fig. 4) indicate that only after the initial Ca^{2+} content of the stores has been reduced by more than 60% is there a significant decrease in the fraction of the stores released by $InsP_3$. We conclude that it cannot be luminal Ca^{2+} alone that distinguishes $InsP_3$-sensitive from -insensitive Ca^{2+} stores. These results are consistent with subcellular fractionation (18) and $InsP_3$ receptor immunolocalization studies (19), suggesting that $InsP_3$-sensitive stores are anatomically discrete. However, stores capable of responding to $InsP_3$ may be functionally unresponsive if their luminal free Ca^{2+} is too low. Intracellular signals such as cyclic AMP, which appears to increase both the sensitivity of stores to $InsP_3$ and the amount of Ca^{2+} mobilized by a maximal $InsP_3$ concentration (20), may, therefore, do so by promoting Ca^{2+} uptake into stores or by increasing the sensitivity of the $InsP_3$ receptor to luminal Ca^{2+}.

The simplest interpretation of our data, namely that luminal Ca^{2+} and $InsP_3$ together regulate Ca^{2+} mobilization, suggests possible explanations for two additional aspects of the complex Ca^{2+} signals evoked by extracellular stimuli. The ability of luminal Ca^{2+} to sensitize stores to $InsP_3$ provides a mechanism whereby Ca^{2+} released from one store can, when sequestered by another, cause it to release Ca^{2+} in the presence of otherwise ineffective $InsP_3$ concentrations. Evidence supporting this mechanism, a modified form of the two-pool model for Ca^{2+} waves and Ca^{2+} spikes (2), has recently been presented (21). Substantial evidence (22, 23) suggests that the Ca^{2+} content of $InsP_3$-sensitive Ca^{2+} stores is a major control of Ca^{2+} entry

pathways, but the mechanism is unknown. A clear implication of our results is that the conformation of the $InsP_3$ receptor is modified by luminal Ca^{2+}; it is, therefore, possible, as Irvine (8) initially proposed, that the $InsP_3$ receptor senses the Ca^{2+} content of intracellular stores and, in communication with proteins in the plasma membrane, regulates Ca^{2+} entry.

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