Calcium Release in HSY Cells Conforms to a Steady-state Mechanism Involving Regulation of the Inositol 1,4,5-trisphosphate Receptor Ca^{2+} Channel by Luminal $[Ca^{2+}]$

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Abstract. In many cell types, low concentrations of inositol 1,4,5-trisphosphate (IP_3) release only a portion of the intracellular IP_3-sensitive Ca^{2+} store, a phenomenon known as "quantal" Ca^{2+} release. It has been suggested that this effect is a result of reduced activity of the IP_3-dependent Ca^{2+} channel with decreasing calcium concentration within the IP_3-sensitive store ($[Ca^{2+}]_S$). To test this hypothesis, the properties of IP_3-dependent Ca^{2+} release in single saponin-permeabilized HSY cells were studied by monitoring $[Ca^{2+}]_S$ using the Ca^{2+}-sensitive fluorescent dye mag-fura-2. In permeabilized cells, blockade of the sarco/ER Ca^{2+}-ATPase pump in stores partially depleted by IP_3 induced further Ca^{2+} release via an IP_3-dependent route, indicating that Ca^{2+} entry via the sarco/ER Ca^{2+}-ATPase pump had been balanced by Ca^{2+} loss via the IP_3-sensitive channel before pump inhibition. IP_3-dependent Mn^{2+} entry, monitored via quenching of luminal mag-fura-2 fluorescence, was readily apparent in filled stores but undetectable in Ca^{2+}-depleted stores, indicating markedly reduced IP_3-sensitive channel activity in the latter. Also consistent with reduced responsiveness of Ca^{2+}-depleted stores to IP_3, the initial rate of refilling of these stores was unaffected by the presence of 0.3 μM IP_3, a concentration that was clearly effective in eliciting Ca^{2+} release from filled stores. Analysis of the rate of Ca^{2+} release at various IP_3 concentrations indicated a significant shift of the IP_3 dose response toward higher $[IP_3]$ with decreasing $[Ca^{2+}]_S$. We conclude that IP_3-dependent Ca^{2+} release in HSY cells is a steady-state process wherein Ca^{2+} efflux via the IP_3 receptor Ca^{2+} channel is regulated by $[Ca^{2+}]_S$, apparently via changes in the sensitivity of the channel to IP_3.

THE physiologic effects of numerous hormones, growth factors, and neurotransmitters are mediated by a rise in intracellular (cytosolic) calcium concentration (Berridge, 1993; Pozzan et al., 1994; Clapham, 1995). Many of these agents act by binding to G protein–coupled or tyrosine kinase–linked receptors on the cell surface, resulting in the receptor-dependent activation of phospholipase C. Phospholipase C in turn hydrolyzes the lipid precursor phosphatidylinositol 4,5-bisphosphate to produce the intracellular messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP_3).[1] IP_3 then releases Ca^{2+} from intracellular stores by binding to its receptor which is a Ca^{2+} release channel (Marshall and Taylor, 1993; Ferris and Snyder, 1992; Mikoshiba, 1993). This Ca^{2+} release is usually followed by a sustained elevation of intracellular (cytosolic) calcium concentration due to increased Ca^{2+} entry from the extracellular solution. At present little is known about this Ca^{2+} entry pathway. However, there is good evidence from a variety of cell types that the Ca^{2+} permeability of the plasma membrane is regulated by the degree of depletion of the IP_3-sensitive intracellular Ca^{2+} store (Clapham, 1995; Putney and Bird, 1993), and thus indirectly by intracellular levels of IP_3 and the activity of the IP_3 receptor.

Owing to their central roles in intracellular signaling, the Ca^{2+} uptake and release pathways of the IP_3-sensitive Ca^{2+} store have received considerable experimental attention. Ca^{2+} uptake is mediated by sarco/ER Ca^{2+}-ATPase (SERCA) pumps of molecular weight ~110,000 (Pozzan et al., 1994). These transporters are structurally distinct from plasma membrane Ca^{2+}-ATPases and are specifically and irreversibly inhibited by the tumor-promoting sesquiterpene lactone, thapsigargin (Thastrup et al., 1990). The IP_3 receptor is a homotetramer of ~310 kD subunits. Based on molecular cloning and other analyses, each sub-

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1. *Abbreviations used in this paper*: $[Ca^{2+}]_S$, calcium concentration within the IP_3-sensitive store; CaS, calcium sponge-treated medium; ICM, intracellular-like medium; IP_3, inositol 1,4,5-trisphosphate; PSS, physiological salt solution; SERCA, sarco/ ER Ca^{2+}-ATPase.

unit is thought to consist of a transmembrane domain localized at the COOH terminus of the molecule and a long NH_2-terminal cytosolic region consisting of the IP_3 binding site and a regulatory domain. This regulatory domain contains the sites of action of a number of agents that modulate Ca^{2+} transport activity, e.g., protein kinases, adenine nucleotides, and intracellular (cytosolic) calcium concentration itself (Marshall and Taylor, 1993; Ferris and Snyder, 1992; Mikoshiba, 1993).

In 1989, Muallem et al. made an intriguing discovery concerning the nature of intracellular Ca^{2+} release. Using pancreatic acini whose intracellular Ca^{2+} stores had been loaded with $^{45}Ca^{2+}$, they showed that low concentrations of Ca^{2+}-mobilizing hormone applied to intact acini, or low concentrations of IP_3 applied to permeabilized acini, apparently resulted in only a partial release of the available Ca^{2+} store. The remaining $^{45}Ca^{2+}$ could, however, be released by higher levels of hormone or IP_3, respectively. These observations were surprising in that they are not consistent with the hypothesis that the Ca^{2+} channel activity of the IP_3 receptor simply increases monotonically with increasing IP_3 concentration. If this were the case, both high and low doses of IP_3 would result in the complete emptying of the IP_3-sensitive store of $^{45}Ca^{2+}$, the former simply at a faster rate than the latter. Similar or related observations to those of Muallem et al. (1989) have now been made in a variety of intact and permeabilized cells (for review see Missiaen et al., 1994; Bootman, 1994). Hence, this phenomenon, commonly referred to as "quantal" or "incremental" Ca^{2+} release, appears to be a common feature of the IP_3-sensitive Ca^{2+} stores of many, and possibly all, cell types.

The functional significance of this characteristic of intracellular Ca^{2+} release is still uncertain. Its effect is to allow cells to respond to different levels of stimulation in a graded fashion, and it has been suggested that this may be preferable to producing a maximal response at differing rates (Irvine, 1990). It has also been argued that this phenomenon provides a mechanism for "increment detection"; i.e., it allows cells to respond rapidly to increases in agonist concentration that follow a previous lower level of stimulation (Meyer and Stryer, 1990). It is nevertheless clear that this behavior is a direct manifestation of the complexity of Ca^{2+} handling by intracellular stores and that its explanation will greatly enhance our understanding of this process.

In the present paper, we study the properties of Ca^{2+} release in single saponin-permeabilized HSY cells, a continuous salivary ductal cell line from human parotid (Yanagawa et al., 1986). In our experiments, we have taken advantage of the fact that under appropriate loading conditions, certain Ca^{2+}-sensitive fluorescent dyes can accumulate in intracellular organelles and be used to monitor their Ca^{2+} content (Hofer and Machen, 1993; Short et al., 1993; Renard-Rooney et al., 1993; Glennon et al., 1992; Hajnoczky and Thomas, 1994). In this case, we have used mag-fura-2, which, as we demonstrate, accumulates in the IP_3-sensitive Ca^{2+} stores of HSY cells. A particular advantage of our experimental system is that these IP_3-sensitive stores can be repeatedly loaded and discharged in an essentially reproducible fashion, thus allowing comparisons of various experimental maneuvers in the same cell. Briefly stated, our results indicate that IP_3-dependent intracellular Ca^{2+} release by HSY cells conforms well to a steady-state process wherein Ca^{2+} efflux via the IP_3 receptor Ca^{2+} channel is regulated by the calcium concentration within the store ($[Ca^{2+}]_S$). In particular, we provide direct and convincing evidence that the permeability of the IP_3-sensitive Ca^{2+} channel decreases as $[Ca^{2+}]_S$ decreases.

Materials and Methods

Media

The physiological salt solution (PSS) contained 135 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, 0.73 mM NaH_2PO_4, 11 mM glucose, 2 mM glutamine, 20 mM Hepes, pH 7.4 with NaOH, and 1% BSA (Sigma Chemical Co., St. Louis, MO). Intracellular-like medium (ICM) contained 125 mM KCl, 19 mM NaCl, 10 mM Hepes, pH 7.3 with KOH, 3 mM ATP, 1.4 mM $MgCl_2$, 330 μM $CaCl_2$, and 1 mM EGTA (free Ca^{2+} and Mg^{2+} concentrations, 50 nM and 0.1 mM, respectively). Calcium sponge-treated medium (CaS) was made from a solution containing 125 mM KCl, 19 mM NaCl, 10 mM Hepes, pH 7.3 with KOH, and 1 mM ATP. To remove Ca^{2+}, ~60 ml of this solution was passed twice over a column containing 0.3 g Calcium Sponge S (Molecular Probes, Eugene, OR), regenerating the column after each passage by washing with the same solution (without ATP) adjusted to pH 4.0. The final Ca^{2+} concentration of CaS was ~50 nM (determined fluorescently using fura-2).

Cell Culture

The HSY cells (Yanagawa et al., 1986), a generous gift from Dr. Mitsunobu Sato (Tokushima University, Japan), were cultured as described previously (Moran and Turner, 1993) in a 50:50 mixture of DME and Ham's F12 medium supplemented with 10% newborn calf serum, 2 mM glutamine, and 100 μg/ml each of penicillin and streptomycin (all from Biofluids, Inc., Rockville, MD).

Fluorescence experiments were carried out on HSY cells growing in glass sample chambers consisting of 10 × 10 mm cloning cylinders (Bellco Glass, Inc., Vineland, NJ) glued to round glass coverslips (25-mm diameter) with RTV silicone rubber adhesive (General Electric Co., Waterford, NY). Sample chambers were precoated with fibronectin by incubation for 1 h with 100 μl of Ca^{2+}/Mg^{2+}-free PBS (Biofluids, Inc.) containing 1 mM EGTA and 20 μg/ml human fibronectin (Collaborative Research, Inc., Bedford, MA). Fibronectin was stored above liquid nitrogen in aliquots as a 50× stock solution in 100 mM 3-(cyclohexylamino)-1-propanesulfonic acid, 150 mM NaCl, and 1.0 mM $CaCl_2$, pH 11.5.

Cells were cultured for experiments as follows. A single cell suspension was made by incubating confluent monolayers of HSY cells with Ca^{2+}/Mg^{2+}-free PBS containing 1 mM EGTA for 15–20 min at 37°C and then vigorously pipetting. This suspension was diluted to 50,000 cells/ml in culture medium, and 200-μl aliquots were plated into fibronectin-coated sample chambers. The cells in these chambers were then returned to the tissue culture incubator for at least 14 h before use.

Mag-Fura-2 Loading

Cells in glass sample chambers were washed with PSS and then incubated with 6 μM mag-fura-2/AM (Molecular Probes) in the same medium for 30 min at 37°C in the dark. After washing with PSS, these mag-fura-2–loaded cells were kept at room temperature (~25°C) until use (10–60 min).

Permeabilization

Sample chambers were washed with BSA-free PSS and then with Mg^{2+}/ATP-free ICM. Cells were permeabilized by incubation with Mg^{2+}/ATP-free ICM containing 50 μg/ml (wt/vol) saponin (Calbiochem-Novabiochem Corp., San Diego, CA) for 2.5–3 min at room temperature. Permeabilization was monitored by looking for the release of (fluorescent) cytosolic mag-fura-2 (see *Results*) using the same microscope employed for microfluorimetic measurements (see below). After permeabilization cells were washed with Mg^{2+}/ATP-free ICM, and then switched to complete ICM and left for at least 5 min to allow for complete filling of their intracellular stores.

Mag-Fura-2 Fluorescence Measurements

Mag-fura-2 fluorescence from saponin-permeabilized HSY cells was monitored using a microfluorometer (ARCM-MIC; Spex Industries, Inc., Edison, NJ) coupled to a microscope (Diaphot; Nikon Inc., Garden City, NY) equipped with a 40× objective (CF Fluor; Nikon Inc.). Fluorescence emission at 510 nm was measured with a photomultiplier tube attached to the side port of the microscope. Unless otherwise stated, a pinhole aperture placed in front of the photomultiplier tube restricted its view to a field 50 μm in diameter containing a single permeabilized HSY cell that was alternately excited with light at 340 nm and 360 nm at 1-s intervals. In some experiments, 344 nm excitation (the isosbestic wavelength of mag-fura-2) was used instead of 340 nm.

A vacuum line was placed in the sample chamber and adjusted so that the volume was maintained at ~50 μl. Solution changes were accomplished by the addition of 600–1,200 μl of the new solution to the sample chamber. All experiments were carried out at room temperature.

The ratio of mag-fura-2 fluorescence at 340 (or 344) nm excitation divided by that at 360 nm is a measure of free $[Ca^{2+}]$ (or $[Mg^{2+}]$; see text and Raju et al., 1989) and is illustrated in most experiments. In some experiments, free calcium concentrations were calculated from mag-fura-2 fluorescence ratios according to the procedure of Grynkiewicz et al. (1985) (Raju et al., 1989). The fluorescence ratios at saturating and zero free $[Ca^{2+}]$ (R_{max} and R_{min}, respectively) required for these calculations were determined from 5-μM mag-fura-2 solutions in Mg^{2+}/ATP-free ICM containing no added $CaCl_2$ or 9 mM $CaCl_2$, respectively. In our experimental system, using excitation wavelengths of 344 and 360 nm, $R_{max} = 10.0 \pm 0.6$ and $R_{min} = 0.733 \pm 0.003$ ($n = 3$) for mag-fura-2.

Data Presentation

Unless otherwise stated, results of single experiments from single HSY cells are illustrated. In each case, the result shown is representative of three or more experiments carried out under the same experimental conditions and yielding the same result. Quantitative results are given as means ± SEM.

Results and Discussion

Predictions of Proposed Mechanisms for Quantal Ca^{2+} Release

Initially two explanations were suggested to account for quantal Ca^{2+} release (Muallem et al., 1989; Irvine, 1990; Missiaen et al., 1994; Bootman, 1994). The first proposes that there are multiple intracellular Ca^{2+} stores with differing sensitivities to IP_3 that release their Ca^{2+} in an "all-or-none" fashion (Muallem et al., 1989). Thus, at a given submaximal IP_3 level, only a fraction of the stores, those most sensitive to IP_3, discharge their Ca^{2+} content. This model requires both the existence of IP_3 receptor isoforms with differing IP_3 sensitivities and their sequestration into distinct intracellular compartments. The second mechanism proposes that all intracellular Ca^{2+} stores are equally sensitive to IP_3, but that their sensitivity is regulated by their Ca^{2+} content. This regulation may be the result of an effect of the $[Ca^{2+}]_S$ on the IP_3 receptor itself or some less direct effect. One possibility is the existence of a Ca^{2+} binding site on the luminal side of the receptor (Irvine, 1990). Thus, as $[Ca^{2+}]_S$ falls after IP_3-induced release, this (putative) site would act as a sensor closing the channel until a new steady state is reached between Ca^{2+} entry and Ca^{2+} loss. Both of these hypotheses have received considerable attention in the literature (Missiaen et al., 1994; Bootman, 1994), and more recently several additional explanations have been proposed (see below). At present, however, there is no consensus concerning the mechanism of this phenomenon.

These two possible mechanisms for quantal Ca^{2+} release make quite different predictions for the behavior of the IP_3-sensitive Ca^{2+} channels after IP_3-induced Ca^{2+} release. In the case of multiple stores that respond to IP_3 in an all-or-none fashion, those channels that open in response to a given dose of IP_3 remain open as long as IP_3 is present (if they were to close, the stores would refill, and this is not observed). On the other hand, in the case of stores whose sensitivity to IP_3 is regulated by $[Ca^{2+}]_S$, the channels initially open in response to IP_3 but then close with decreasing $[Ca^{2+}]_S$ until IP_3-dependent Ca^{2+} loss is balanced by Ca^{2+} entry on the SERCA pump. The differences between the predictions of these two models would be further exaggerated with blockade of the SERCA pump. No effect on the status of the Ca^{2+} channels would be expected in the former all-or-none model. However, the resulting fall in $[Ca^{2+}]_S$ expected in the latter steady-state model would presumably lead to a further closing of Ca^{2+} channels.

More recently, two additional mechanisms have been proposed to account for quantal Ca^{2+} release. Hajnoczky and Thomas (1994) have presented evidence that IP_3-dependent Ca^{2+} channels are inactivated by IP_3 in a time-dependent manner in permeabilized hepatocytes ($t_{1/2}$ ~15 s). Thus, they suggest that Ca^{2+} release by submaximal doses of IP_3 terminates before completely emptying intracellular stores, giving rise to quantal release. This model is also necessarily of the steady-state type since complete closure of the channels would result in refilling of the stores. In this regard, the inactivation documented by Hajnoczky and Thomas is, in fact, incomplete and reversible ($t_{1/2}$ ~30 s). Hirose and Iino (1994), on the other hand, have argued that quantal release in permeabilized vascular smooth muscle cells is due to heterogeneity of intracellular Ca^{2+} stores, but that this heterogeneity is due to differing densities of equally IP_3-sensitive Ca^{2+} channels in different stores. Thus, the extent of IP_3-induced Ca^{2+} release varies from store to store owing to their differing pump/leak ratios, giving rise to an apparent heterogeneity in IP_3 sensitivity. This model is therefore also of the steady-state type but, like the all-or-none model discussed above, predicts that the IP_3-sensitive Ca^{2+} channels remain open throughout the response to IP_3.

In what follows, we characterize IP_3-dependent Ca^{2+} release from the intracellular stores of permeabilized HSY cells. In our experiments, we place particular emphasis on the open or closed status of the IP_3-sensitive Ca^{2+} channels since this characteristic figures heavily in the distinctions among the above models.

Ca^{2+} Release in Permeabilized HSY Cells

When intact HSY cells loaded with mag-fura-2 as described in *Materials and Methods* were examined visually under the fluorescence microscope, the dye appeared to be uniformly distributed throughout the cytoplasm. After several minutes of exposure to 50 μg/ml saponin (see *Materials and Methods*), ~75% of the intracellular fluorescence was lost, presumably as a result of permeabilization of the plasma membrane and release of cytosolic dye (~50% of the cells were permeabilized within 2.5–3-min exposure to saponin). The remaining fluorescence appeared as a diffuse ring encircling the (darker) cell nucleus

(not shown). Subsequent washing had little effect on the remaining dye, consistent with the hypothesis that it was trapped in a membrane-bound compartment. In addition, in the absence of stimuli, this fluorescent signal changed little with time (see examples below), indicating that the compartment(s) being monitored were stable and that there was negligible dye leakage or bleaching under our experimental conditions. Since mag-fura-2 is sensitive to both $[Ca^{2+}]$ and $[Mg^{2+}]$ ($K_d \approx 53$ μM and 1.5 mM, respectively) (Raju et al., 1989), the mag-fura-2 fluorescent ratio could reflect the concentrations of either or both of these ions in the compartment(s) it monitors.

Fig. 1 *A* shows the effects of IP_3 and heparin on the mag-fura-2 fluorescence ratio recorded from a saponin-permeabilized HSY cell bathed in ICM (see *Materials and Methods*). The application of 0.3 μM IP_3 causes a rapid and marked decrease in the ratio that is largely reversed by heparin (100 μg/ml). As already mentioned, it is well documented (Hofer and Machen, 1993; Short et al., 1993; Renard-Rooney et al., 1993; Glennon et al., 1992; Hajnoczky and Thomas, 1994) that under appropriate conditions, Ca^{2+}-sensitive dyes such as mag-fura-2 can accumulate in intracellular organelles and be used to monitor their Ca^{2+} content. These observations, together with the well-known behavior of heparin as a competitive inhibitor of the IP_3 receptor, strongly suggest that the effects on the mag-fura-2 signal seen in Fig. 1 *A* are due to changes in the calcium concentration in the IP_3-sensitive store. More specifically, this experiment is consistent with the release of Ca^{2+} from this store due to the IP_3-induced opening of the Ca^{2+} channel, followed by its reuptake via the SERCA pump after the closing of the channel in response to heparin. Further evidence for the Ca^{2+} dependence of the IP_3-sensitive component of the mag-fura-2 signal is shown in Fig. 1 *B*. Here we monitor the mag-fura-2 ratio in a permeabilized HSY cell after treatment with a near-maximal concentration of IP_3 (10 μM; see below), followed by the removal of IP_3, Mg^{2+}, and Ca^{2+} from ICM. The readdition of Mg^{2+} to ICM (free $[Mg^{2+}] \approx 0.1$ mM) has no effect on the mag-fura-2 ratio (solutions with free $[Mg^{2+}]$ up to 1.4 mM were tested with no effect; not shown), but readdition of Ca^{2+} causes a complete recovery to the level seen before the application of IP_3, again consistent with the refilling of the IP_3-sensitive store via the SERCA pump. Taken together, these results indicate that the IP_3-sensitive changes in the mag-fura-2 signal recorded from these cells is a direct measure of changes in $[Ca^{2+}]_S$.

In Fig. 1 *C* we demonstrate that permeabilized HSY cells exhibit the now familiar pattern associated with quantal Ca^{2+} release. Here, a series of increasingly larger concentrations of IP_3 were applied to a single permeabilized mag-fura-2–loaded HSY cell. After each application, the mag-fura-2 ratio ($[Ca^{2+}]_S$) rapidly falls to a new lower value where it remains until the next increase in $[IP_3]$. Upon removal of IP_3, the ratio quickly recovers to its prestimulus value.

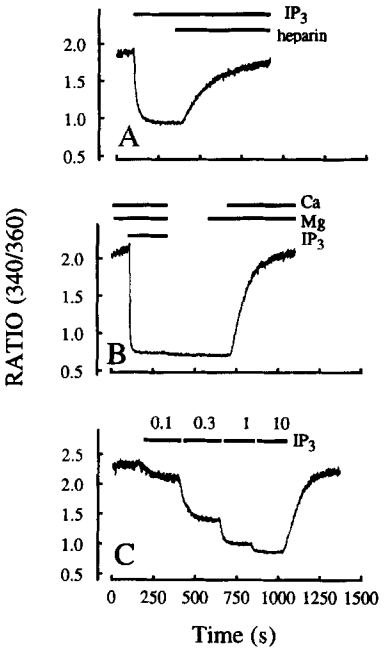

Figure 1. Responses of single, permeabilized HSY cells to IP_3 and other agents. Mag-fura-2 fluorescence, displayed in ratio mode, was monitored in permeabilized HSY cells as described in *Materials and Methods*. The presence of various agents in the medium bathing the cells is indicated by the horizontal bars at the top of each panel. (*A*) A single cell bathed in ICM was exposed to 0.3 μM IP_3 and then to to 0.3 μM IP_3 plus 100 μg/ml heparin as indicated. (*B*) A single cell initially bathed in ICM was exposed to 10 μM IP_3 and then successively to Ca^{2+}/Mg^{2+}-free ICM, Ca^{2+}-free ICM, and complete ICM as indicated (the free Ca^{2+} and Mg^{2+} concentrations in complete ICM are 50 nM and 0.1 mM, respectively). (*C*) A single cell in ICM was exposed to successively larger concentrations of IP_3 as indicated.

Evidence that Ca^{2+} Release in HSY Cells Is a Steady-state Process

When the SERCA pump inhibitor thapsigargin was applied to permeabilized HSY cells in complete ICM, little change in the mag-fura-2 ratio was observed (Fig. 2 *A*), indicating that the passive Ca^{2+} permeability of the IP_3-sensitive store is relatively low in this system. However, when thapsigargin was applied after a submaximal concentration of IP_3 (0.3 μM; see also Fig. 1 *C* and below), a decrease in the mag-fura-2 ratio toward a new lower value was seen (Fig. 2 *B*). As already discussed, this behavior is consistent with a steady state model for intracellular Ca^{2+} release. In this same experiment, the addition of 10 μM IP_3 induced further Ca^{2+} release, and no recovery was observed after IP_3 removal consistent with a complete (irreversible) inhibition of the SERCA pump by thapsigargin. The experiments shown in Fig. 2, *C* and *D*, demonstrate that the thapsigargin-induced decrease in the mag-fura-2 ratio seen in Fig. 2 *B* is IP_3-dependent. In these experiments, the SERCA pump was inhibited by Mg^{2+} removal. A similar result to that seen with thapsigargin was observed when Mg^{2+} was removed in the presence of IP_3 (Fig. 2 *C*), but in contrast, when both IP_3 and Mg^{2+} were removed from ICM, the mag-fura-2 ratio was unaffected (Fig. 2 *D*). In this latter experiment, however, the ratio does fall as before when IP_3 is readded, consistent with the hypothesis that this Ca^{2+} loss does indeed occur via the IP_3-sensitive Ca^{2+} channel.

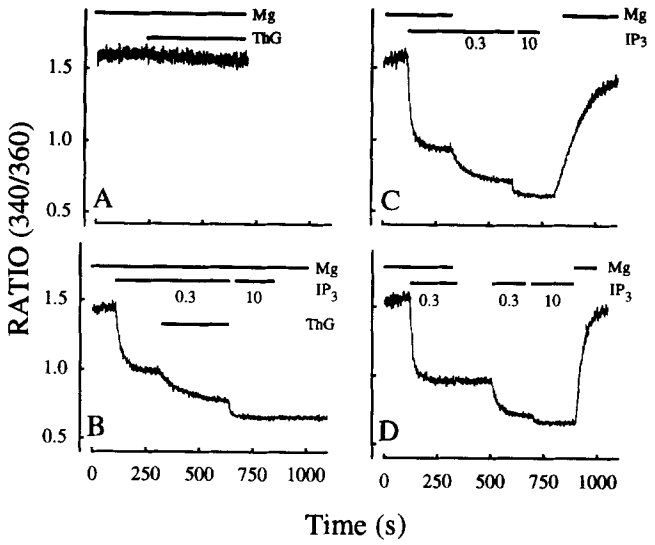

Figure 2. Responses of permeabilized HSY cells to SERCA pump inhibition. Mag-fura-2 fluorescence was monitored as in Fig. 1. Single cells in ICM were exposed to thapsigargin (*ThG*; 1 μM), Mg^{2+}-free ICM, and/or various concentrations of IP_3 (0.3 μM or 10 μM) as indicated. The presence of agents in the medium is indicated by the horizontal bars at the top of each panel.

Evidence that IP_3 Channels Close as $[Ca^{2+}]_S$ Decreases

The left panel of Fig. 3 shows the results of an experiment that was designed to study the effect of the presence of IP_3 on the rate of refilling of depleted IP_3-sensitive stores. Here, a series of maneuvers were carried out on the same permeabilized HSY cell. First (trace *a*, which has been superimposed on trace *b*), IP_3-sensitive Ca^{2+} stores were depleted with a transient exposure to 10 μM IP_3 in Mg^{2+}-free ICM, and then 90 s later Mg^{2+} was added and the cell was allowed to recover to its original $[Ca^{2+}]_S$. Next (trace *b*), stores were again emptied with 10 μM IP_3, but this time Mg^{2+} was added in the presence of 0.3 μM IP_3. Several of the models discussed above propose that Ca^{2+} channels remain open in the presence of IP_3 when IP_3-sensitive stores are depleted. If this is the case, one would expect a slower initial rate of filling in the presence of 0.3 μM IP_3 than in its absence, since the size of the fillable Ca^{2+} pool would be decreased by those stores whose Ca^{2+} channels are open. But it is clear from comparison of traces *a* and *b* that the initial rates of increase of the mag-fura-2 ratio seen after Mg^{2+} addition in the presence and absence of 0.3 μM IP_3 are indistinguishable (the relevant time period is shown in expanded format in the right-hand panel of Fig. 3; in five experiments of this type, no detectable difference in initial recovery rates in the presence and absence of IP_3 was observed). As the stores fill, however, the mag-fura-2 ratio monitored in the presence of 0.3 μM IP_3 does begin to deviate from that measured in its absence, consistent with a gradual opening of Ca^{2+} channels. Finally, as expected, in the presence of 0.3 μM IP_3 the mag-fura-2 ratio stabilizes at a value consistent with a partial reloading of IP_3-sensitive stores. Similar results have been previously obtained with $^{45}Ca^{2+}$ fluxes into liver microsomes (Tregear et al., 1991) and permeabilized rat hepatocytes (Nunn and Taylor, 1992).

The above observations are consistent with the hypothesis that the Ca^{2+} channels activated by 0.3 μM IP_3 are closed in Ca^{2+} stores depleted by 10 μM IP_3 and only open after the stores have partially refilled. In the experiment shown in Fig. 4, we test directly whether Ca^{2+} channels are opened by IP_3 in Ca^{2+}-depleted stores. In this experiment, we take advantage of earlier demonstrations that Mn^{2+} is also a substrate for the IP_3-sensitive Ca^{2+} channel (Renard-Rooney et al., 1993; Short et al., 1993; Hajnoczky and Thomas, 1994), and of the fact that Mn^{2+} entry into a mag-fura-2–loaded compartment can be monitored by its quenching of mag-fura-2 fluorescence. These measurements were carried out in an EGTA-free medium (CaS) (see *Materials and Methods*) to avoid the changes in $[Ca^{2+}]$ induced by displacement of Ca^{2+} from EGTA by Mn^{2+}. In the cell studied in Fig. 4, we first partially depleted intracellular Ca^{2+} stores by adding 0.3 μM IP_3 to CaS. This addition dramatically increases mag-fura-2 fluorescence at 360 nm

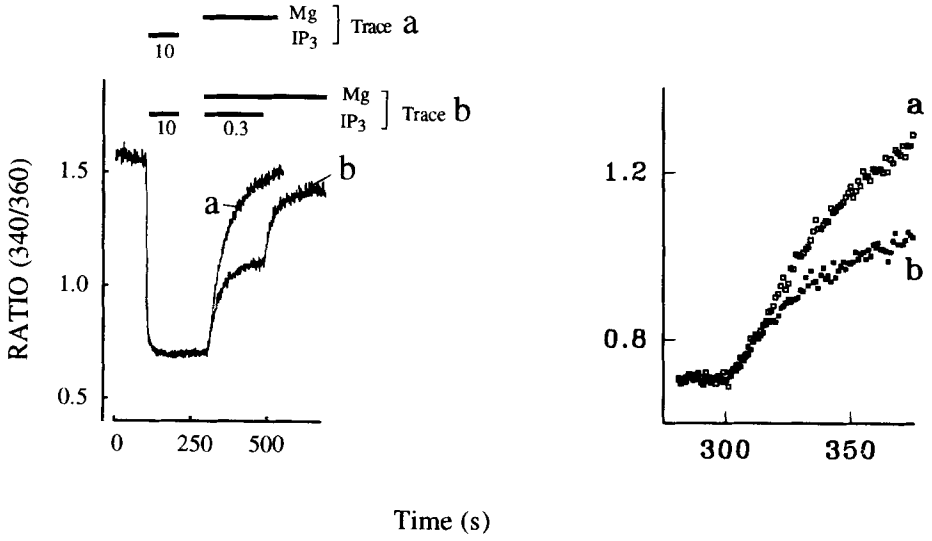

Figure 3. Effect of IP_3 on Ca^{2+} uptake into IP_3-sensitive stores in permeabilized HSY cells. (*Left*) Mag-fura-2 fluorescence from a single permeabilized HSY cell was monitored as in Fig. 1. (Trace *a*) The cell was initially equilibrated with Ca^{2+} in ICM and then successively switched to Mg^{2+}-free ICM (beginning of trace), Mg^{2+}-free ICM plus 10 μM IP_3, and then complete ICM, as indicated by the horizontal bars above the figure. Trace *a* has been superimposed on trace *b*. (Trace *b*) The same cell used to obtain trace *a* was exposed to Mg^{2+}-free ICM or complete ICM in the presence or absence of the concentrations of IP_3 indicated. (*Right*) An expanded view of traces *a* and *b* over the time interval indicated. All experimental points are shown (1-s intervals).

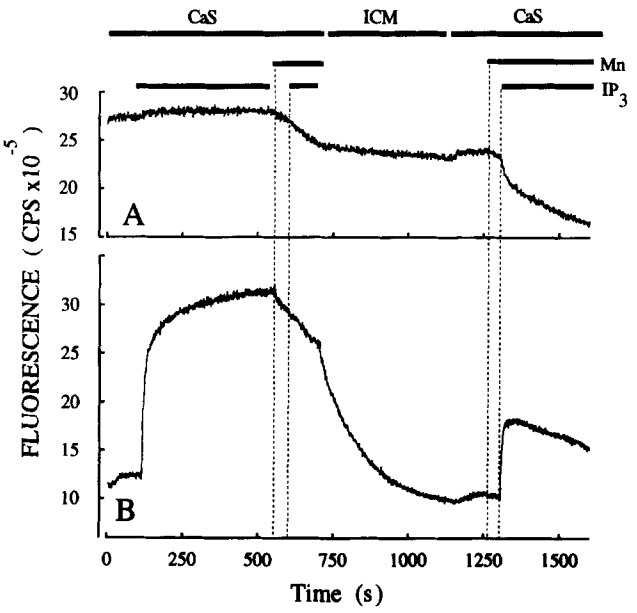

Figure 4. IP_3-dependent Mn^{2+} entry into filled and partially depleted IP_3-sensitive Ca^{2+} stores in permeabilized HSY cells. Mag-fura-2 fluorescence was monitored in a single permeabilized HSY cell as described in *Materials and Methods.* The signals recorded with excitation wavelengths of 344 nm (isosbestic wavelength) and 360 nm are shown in *A* and *B*, respectively. The cell was initially equilibrated with Ca^{2+} in ICM and then exposed to CaS, CaS plus 0.3 μM IP_3, CaS plus 0.15 mM $MnCl_2$, CaS plus both IP_3 and $MnCl_2$, or to ICM as indicated by the horizontal bars above *A*.

excitation (Fig. 4 *B*) but has little effect on the signal at 344 nm (Fig. 4 *A*). When IP_3 is removed from CaS and replaced by Mn^{2+}, a slow decrease in the mag-fura-2 signals at both 344 and 360 nm is seen (Fig. 4) with no change in their ratio (not shown). This result is consistent with the slow permeation of Mn^{2+} into mag-fura-2–loaded compartment(s) and the concomitant quenching of mag-fura-2 fluorescence. Under these Ca^{2+}-depleted conditions, the later readdition of IP_3 in the presence of Mn^{2+} produces no change in the rate of fluorescence quenching, indicating that IP_3 induces no detectable component of Mn^{2+} entry. However, in the remainder of the experiment, we demonstrate that when IP_3-sensitive stores are reloaded with Ca^{2+} and then reexposed to 0.3 μM IP_3 in the presence of Mn^{2+}, a dramatic quenching of mag-fura-2 fluorescence at 344 nm excitation is observed (Fig. 4 *A*). This latter result indicates that this concentration of IP_3 is indeed capable of inducing Mn^{2+} entry into mag-fura-2–loaded stores when these stores are filled with Ca^{2+}.

It is also worth emphasizing that mag-fura-2 fluorescence quenching is, in fact, expected to be more sensitive to Mn^{2+} entry in Ca^{2+}-depleted stores than in Ca^{2+}-filled stores since in the former case, there is less Ca^{2+} available to compete with Mn^{2+} for the dye. Thus, the experiment shown in Fig. 4 provides strong evidence that IP_3-sensitive Ca^{2+} channels in permeabilized HSY cells close in response to depletion of internal Ca^{2+} stores by IP_3.

Estimate of $[Ca^{2+}]_S$ in Permeabilized HSY Cells

Determination of $[Ca^{2+}]_S$ in permeabilized HSY cells is complicated by the possibility that some mag-fura-2 could be sequestered into non–IP_3-sensitive compartments, and by the fact that this dye is sensitive to both $[Ca^{2+}]$ ($K_d \approx 53$ μM) and $[Mg^{2+}]$ ($K_d \approx 1.5$ mM). The experimental protocol illustrated in Fig. 5 was devised in an attempt to examine these problems. The details of the experimental procedure are described in the figure legend, but the essence of the experiment is as follows. The internal Ca^{2+} stores of a single permeabilized HSY cell were first loaded with Ca^{2+} in ICM, and then the cell was exposed to 10 μM IP_3 in the absence of ATP and Mg^{2+}. Points *a* and *b* indicated in Fig. 5 *B* thus correspond to filled and depleted IP_3-sensitive stores, respectively. The cell was then switched back to complete ICM to refill and then briefly exposed to Mn^{2+} in the presence of IP_3 to allow Mn^{2+} entry into IP_3-sensitive stores and a partial quenching of the mag-fura-2 therein. The cell was next switched back to ICM to reload and then reexposed to 10 μM IP_3. Thus, the points labeled *c* and *d* in Fig. 5 *B* again correspond to filled and depleted stores, respectively, this time with some quenching of mag-fura-2 by Mn^{2+}.

The magnitudes of the IP_3-sensitive and IP_3-insensitive mag-fura-2 signals at points *a*, *b*, *c*, and *d* in the above experiment can now be calculated by making the following assumptions: (*i*) the Ca^{2+} and Mg^{2+} concentrations in the IP_3-sensitive store at points *a* and *c* are the same, (*ii*) the Ca^{2+} and Mg^{2+} concentrations in the IP_3-sensitive store at points *b* and *d* are the same, and (*iii*) the magnitude of the IP_3-insensitive signal is constant over the course of the experiment. In particular, we assume that Mn^{2+} quenching of the IP_3-insensitive signal is small. The results shown in Fig. 5 *A* confirm that there is relatively little fluorescence quenching by Mn^{2+} in the absence of IP_3. The effect of relaxing assumption (*iii*) are discussed below.

Analysis[2] of three experiments of the type shown in Fig. 5 yields average mag-fura-2 fluorescence ratios of 5.52 ± 0.17 and 0.80 ± 0.04 for filled and depleted IP_3-sensitive stores, respectively. This latter value is very close to R_{min} for mag-fura-2 in our experimental system ($R_{min} \approx 0.73$; see *Materials and Methods*) indicating that the $[Mg^{2+}]$ in the IP_3-sensitive stores of these cells is well below the K_d of mag-fura-2 for Mg^{2+}. These calculations also indicate that the IP_3-sensitive store contains 56 ± 6% of the sequestered mag-fura-2, and that the mag-fura-2 ratio for the IP_3-insensitive store is 1.11 ± 0.05 (the magnitudes of the calculated mag-fura-2 signals from IP_3-insensitive

2. Expressing the problem as an algebraic one: at each point *a*, *b*, *c*, and *d*, there are four unknown quantities (the fluorescent signals from the IP_3-sensitive and IP_3-insensitive compartments at 344 and 360 nm, respectively), making 16 unknowns in all. Using *s* and *i* to denote the IP_3-sensitive and IP_3-insensitive compartments, respectively, we denote these four signals at point *a* as F(*a*,344,*s*), F(*a*,344,*i*), F(*a*,360,*s*), and F(*a*,360,*i*). Thus, the total fluorescent signal at point *a* with 344 nm excitation is simply F(*a*,344,*s*) + F(*a*,344,*i*). From assumption (*iii*) we have that the IP_3-insensitive signals are constant, i.e., F(*a*,344,*i*) = F(*b*,344,*i*) = F(*c*,344,*i*) = F(*d*,344,*i*) and F(*a*,360,*i*) = F(*b*,360,*i*) = F(*c*,360,*i*) = F(*d*,360,*i*) reducing the problem to 10 unknowns. From assumptions (*i*) and (*ii*) we have F(*a*,344,*s*)/F(*a*,360,*s*) = F(*c*,344,*s*)/F(*c*,360,*s*) and F(*b*,344,*s*)/F(*b*,360,*s*) = F(*d*,344,*s*)/F(*d*,360,*s*), respectively. The above two equations, together with the eight values of the total 344- and 360-nm fluorescent signals at each point, results in a system of 10 equations in 10 unknowns that is easily solved by algebraic methods. Note that it is not necessary to assume that the Mn^{2+} content of the IP_3-sensitive store is the same at points *c* and *d*.

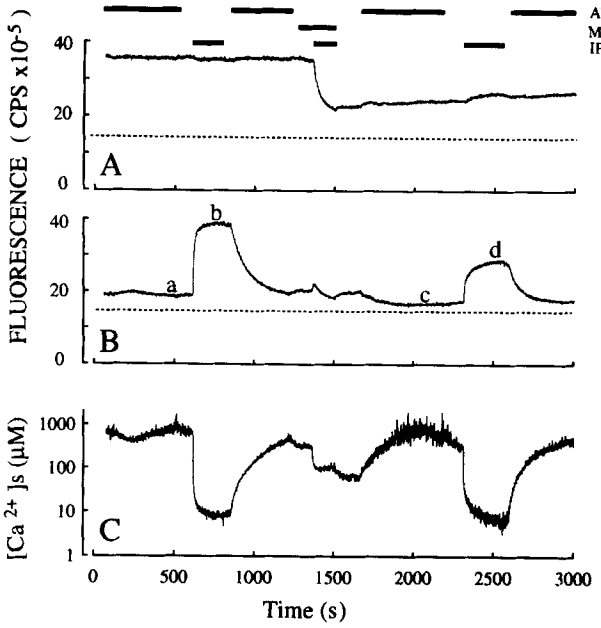

Figure 5. Estimation of $[Ca^{2+}]_S$. Mag-fura-2 fluorescence was monitored in a single permeabilized HSY cell as described in *Materials and Methods.* The signals recorded with excitation wavelengths of 344 nm and 360 nm are shown in *A* and *B*, respectively. The cell was initially equilibrated with Ca^{2+} in complete ICM and then exposed to Mg^{2+}/ATP-free ICM, Mg^{2+}/ATP-free ICM plus 10 μM IP_3, Mg^{2+}/ATP-free ICM plus 1.05 mM $MnCl_2$, or Mg^{2+}/ATP-free ICM plus both IP_3 and $MnCl_2$, as indicated by the horizontal bars above *A* ($CaCl_2$ was omitted from Mn^{2+}-containing media). The significance of the dashed lines and points labeled *a*, *b*, *c*, and *d* in *A* and *B* are explained in the text. *C* shows $[Ca^{2+}]_S$ calculated as described in the text and in *Materials and Methods.*

stores in the experiment illustrated in Fig. 5 are indicated as dashed lines in *A* and *B*).

Assuming, as indicated above, that most of the mag-fura-2 signal from IP_3-sensitive stores is due to Ca^{2+}, we illustrate in Fig. 5 *C* the calculated $[Ca^{2+}]_S$ corresponding to the traces shown in Fig. 5, *A* and *B*. By this method the average value of $[Ca^{2+}]_S$ in fully loaded IP_3-sensitive stores of permeabilized HSY cells was found to be 694 ± 52 μM (n = 3). The effect of possible quenching of the IP_3-insensitive signal by Mn^{2+} (relaxation of assumption [*iii*]) on this calculation can be estimated as follows. Consider first the mag-fura-2 fluorescent signal at 360 nm excitation shown in Fig. 5 *B*. Since $[Ca^{2+}]_S$ is the same at points *a* and *c* (assumption [*i*]), the decrease in this signal between these two points (11 ± 3%; n = 3) must be solely due to the effects of Mn^{2+} quenching. It is clear from Fig. 5 *A* that there is considerable quenching of the IP_3-sensitive signal, so it seems reasonable to assume that this quenching accounts for a large part of this decrease. If we assume, for example, that two-thirds of the decrease in the 360-nm signal between points *a* and *c* is due to quenching in the IP_3-sensitive compartment, while one-third is due to quenching in the IP_3-insensitive compartment, we find that the average mag-fura-2 fluorescence ratios for filled and depleted IP_3-sensitive stores are now 7.26 ± 0.17 and 0.79 ± 0.05, respectively. The latter value is not significantly changed from the case where assumption (*iii*) holds (see above), while the former corresponds to a $[Ca^{2+}]_S$ of 1545 ± 130 μM (n = 3). Thus, any breakdown in assumption (*iii*) will lead to higher estimates of $[Ca^{2+}]_S$ in filled IP_3-sensitive stores.

The IP_3 Dose Response

Taken together, the results shown in Figs. 2, 3, and 4 are consistent with a steady-state model of IP_3-induced Ca^{2+} release in HSY cells in which Ca^{2+} efflux via the IP_3 receptor Ca^{2+} channel is regulated by $[Ca^{2+}]_S$. In his original paper proposing this mechanism to explain quantal Ca^{2+} release, Irvine (1990) suggested that the effect of reduced $[Ca^{2+}]_S$ might be manifested as reduced sensitivity of the IP_3-sensitive Ca^{2+} channel to IP_3. A test of this hypothesis in HSY cells is given in the experiment illustrated in Fig. 6. In Fig. 6 *A* we show a typical dose–response pattern to IP_3 in a small cluster of permeabilized cells (see figure legend for details). This experiment was carried out in the absence of Mg^{2+} and thus is indicative of IP_3-dependent Ca^{2+} efflux in the absence of the SERCA pump. The data are illustrated as the mag-fura-2 ratio vs. time; however, numerical calculations (not shown) demonstrate that this ratio is approximately a linear function of log($[Ca^{2+}]$) over the range 0.1–10 times the K_d of mag-fura-2 for Ca^{2+} (53 μM), which is the expected range of $[Ca^{2+}]_S$ in this type of experiment (see Fig. 5 *C*). The IP_3 dose response was quantitated by determining the rate of change of the mag-fura-2 ratio with time (which is proportional to the rate of change of $[Ca^{2+}]_S$) at four levels of $[Ca^{2+}]_S$ labeled *10%*, *20%*, *40%*, and *60%* in Fig. 5 *A*. These represent 10, 20, 40, and 60% decreases, respectively, in the IP_3-sensitive component of the mag-fura-2 ratio (the IP_3-sensitive component was taken to be the decrease in the ratio induced by >50-s incubation with 10 μM IP_3). From the estimation of $[Ca^{2+}]_S$ in Fig. 5, it can be shown that these levels correspond to ~40, 60, 80, and 90% decreases, respectively, in $[Ca^{2+}]_S$ (our numerical calculations indicate that these estimates of percent Ca^{2+} loss are relatively independent of the size of the IP_3-insensitive store).

The rates determined for the various $[IP_3]$ (traces *a*–*e*) at each level of $[Ca^{2+}]_S$ were normalized to the rates observed at 10 μM IP_3 at that level (trace *e*). If the dose response for Ca^{2+} release were the same for each level of $[Ca^{2+}]_S$, one would expect these normalized dose responses to be identical. However, analysis of the averaged data from six experiments of this type (Fig. 5 *B*) using a paired t test indicates that the IP_3 dose response shifts significantly toward higher values of $[IP_3]$ at each decreased level of $[Ca^{2+}]_S$. More specifically, all data points labeled *d* in Fig. 5 *B* are significantly different from one another ($P < 0.015$) decreasing from right to left, as are all points labeled *c* ($P < 0.03$), *b* ($P < 0.05$), and *a* ($P < 0.05$) except for the two points marked with asterisks for which P = 0.08 (marginally significantly different).

Applicability of Proposed Mechanisms for Quantal Ca^{2+} Release to IP_3-dependent Ca^{2+} Release from HSY Cells

As already stated, our results provide strong evidence in favor of the steady-state model for intracellular Ca^{2+} release originally proposed by Irvine (1990). In contradiction to the model suggesting that intracellular Ca^{2+} stores

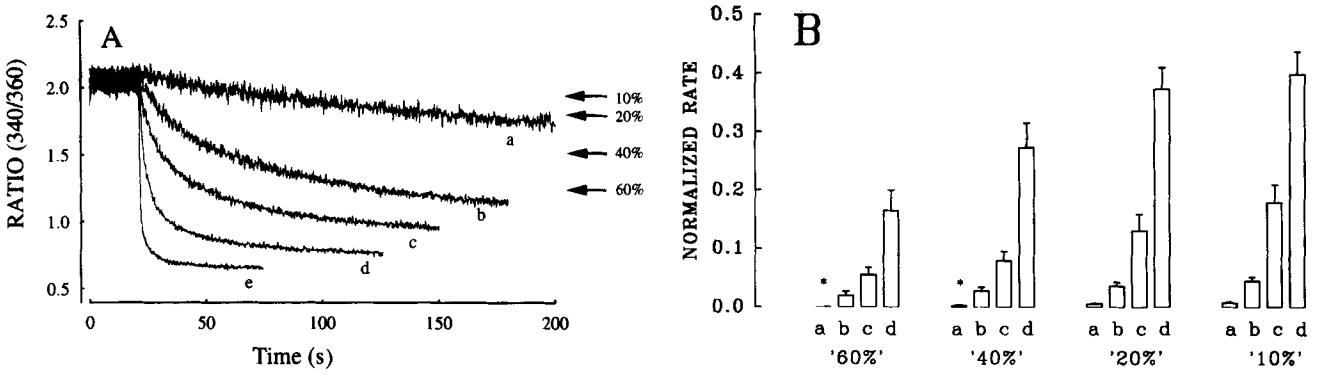

Figure 6. [IP_3] dependence of Ca^{2+} loss from permeabilized HSY cells. (*A*) Mag-fura-2 fluorescence was monitored at 0.2-s intervals from a cluster of four permeabilized HSY cells using a pinhole aperture 125 μm in diameter. This time resolution was necessary in order to resolve the initial rates of mag-fura-2 fluorescence change at higher concentrations of IP_3, and several cells were needed in order to have a sufficient signal. The cells were repeatedly equilibrated with Ca^{2+} in ICM and then exposed to various concentrations of IP_3 in Mg^{2+}-free ICM (*a*, 0.1 μM; *b*, 0.3 μM; *c*, 0.5 μM; *d*, 1.0 μM; *e*, 10 μM). In experiments of this type, a second exposure to the same concentration of IP_3 yielded an almost identical response to the first (not shown). The experiment illustrated is typical of six experiments carried out on clusters of three to five permeabilized HSY cells. (*B*) The derivatives of the curves illustrated in *A* were determined using the smoothing procedure of Savitzky and Golay (see below). The derivatives of traces *a*, *b*, *c*, and *d* at each of the values of the mag-fura-2 ratio labeled *10%*, *20%*, *40%*, and *60%* in *A* were normalized to the values observed for trace *e* (see text). The results shown are the averages of six experiments analyzed and normalized in this way. Thus, each group of bars corresponds to a normalized IP_3 dose response (*a*, 0.1 μM; *b*, 0.3 μM; *c*, 0.5 μM; *d*, 1.0 μM) at the mag-fura-2 ratio value indicated. The significance of the asterisks is given in the text.

Briefly stated, the method of Savitsky and Golay (Press et al., 1992) involves fitting a window of data points to a polynomial to determine the smoothed value of the slope at the central point of the window. In our calculations, we used a second degree polynomial and a window whose size varied from 5 points at early times after the application of IP_3 (when the response to IP_3 was rapid) to 25 points at longer times.

respond to IP_3 in an all-or-none fashion, and to the more recent model of Hirose and Iino (1994), our data indicate that IP_3-sensitive Ca^{2+} channels close as intracellular stores are depleted (Figs. 3 and 4). The model of Hirose and Iino also predicts that the IP_3 dose response should be independent of $[Ca^{2+}]_S$ in the absence of the SERCA pump (Hirose and Iino, 1994), which is likewise not the case in our experimental system (Fig. 6).

Hajnoczky and Thomas (1994) propose partial closure of IP_3-sensitive Ca^{2+} channels to explain quantal Ca^{2+} release, but they argue that this is due to the time-dependent inactivation of these channels by IP_3 itself. However, several observations indicate that this explanation is not applicable to our results. First, the inactivation documented by Hajnoczky and Thomas was reversible upon IP_3 removal. But in our experiments we have seen no evidence of this phenomenon. For example, in experiments like that shown in Fig. 5, when IP_3 is removed after partial depletion of intracellular stores and then later readded, we see no evidence of the enhanced IP_3-dependent Ca^{2+} loss one would expect if the receptor were resensitized in the absence of IP_3 (not shown). Very similar observations have recently been reported by Parys et al. (1995) who monitored IP_3-dependent $^{45}Ca^{2+}$ release from permeabilized A7r5 cells. A number of other groups have also failed to detect desensitization of the IP_3 receptor by IP_3 (Meyer and Stryer, 1990; Ferris et al., 1992; Finch et al., 1991; Bootman et al., 1994; Oldershaw et al., 1992; Bezprozvanny and Ehrlich, 1994). Secondly, as already emphasized in the discussion of Fig. 3, our results indicate that the Ca^{2+} channels activated by 0.3 μM IP_3 are closed in previously depleted Ca^{2+} stores and slowly open with time as $[Ca^{2+}]_S$ increases. This sequence of events is exactly the opposite of what would be expected if IP_3 acts to inactivate the channel with time. Finally, as demonstrated directly in the experiment illustrated in Fig. 4, the differences in channel permeability we observe at high and low $[Ca^{2+}]_S$ are quite dramatic; IP_3-dependent Mn^{2+} entry was obvious and substantial in filled stores but undetectable in depleted stores. In contrast, the inactivation observed by Hajnoczky and Thomas (1994) was rather small (~10%) at the free calcium concentration of our ICM (50 nM; see *Materials and Methods*). Even at 300 nM Ca^{2+}, where most of their measurements were carried out, these authors found that IP_3-sensitive channels retained ~40% of their activity after prolonged incubation with IP_3 (Hajnoczky and Thomas, 1994). These levels of residual channel activity in depleted stores should have been easily detectable in Fig. 4 of the present study. Thus, aside from the other points raised above, we conclude that it is extremely unlikely that the effect observed by Hajnoczky and Thomas (1994) can account for our results.

Concluding Remarks

The quantal or incremental release of Ca^{2+} from intracellular stores appears to be a rather general phenomenon and thus to reflect an intrinsic characteristic or characteristics of the IP_3-sensitive compartment. The two models originally proposed to account for this effect—the steady-state model of Irvine and the "all-or-none" model—have dominated most of the experimental work and discussion (Missiaen et al., 1994; Bootman, 1994). Several authors have presented evidence that IP_3-induced Ca^{2+} release is a steady-state process and that the sensitivity of IP_3-sensitive Ca^{2+} stores is modulated by $[Ca^{2+}]_S$ as required by the

model of Irvine (Nunn and Taylor, 1992; Loomis-Husselbee and Dawson, 1993; Tregear et al., 1991; Missiaen et al., 1992; Parys et al., 1993). Others, however, have failed to observe an effect of luminal Ca^{2+} loading on IP_3-dependent release (Shuttleworth, 1992; Combettes et al., 1993; Combettes et al., 1992; Sayers et al., 1993; Hirose and Iino, 1994). Because of these latter observations, as well as for other reasons, a number of investigators have proposed all-or-none Ca^{2+} release as an explanation for their data (Taylor and Potter, 1990; Oldershaw et al., 1991; Bootman et al., 1992; Muallem et al., 1989; Shuttleworth, 1992; Combettes et al., 1993; Combettes et al., 1992; Sayers et al., 1993). A discussion of the differences between these results obtained using a wide variety of cell types and experimental methodologies is beyond the scope of the present article. However, one possible significant difference between many of these earlier reports and our own is that the signal we have monitored is a direct reflection of $[Ca^{2+}]_S$, the central parameter in the model of Irvine. Measurements of Ca^{2+} uptake or release reported in some previous studies may not provide an accurate reflection of changes in $[Ca^{2+}]_S$ owing to the (as yet uncharacterized) Ca^{2+} buffering capacity of the IP_3-sensitive store.

It should also be pointed out that direct experimental evidence for intracellular Ca^{2+} stores with differing sensitivities to IP_3 has been found (Parker and Ivorra, 1990; Kasai et al., 1993; Thorn et al., 1993; Tortorici et al., 1994). However, there is at present no evidence that these stores release Ca^{2+} in an all-or-none manner. Indeed, the existence of these stores is quite consistent with the steady-state model of Irvine. In this model, stores with differing $[Ca^{2+}]_S$, due, for example, to varying levels of SERCA pumping capacity or passive Ca^{2+} leak, would be expected to have different sensitivities to IP_3.

In conclusion, our data provide strong evidence that IP_3-sensitive Ca^{2+} release in HSY cells conforms to the steady-state model of Irvine. Our results demonstrate that Ca^{2+} release in these cells is a steady-state phenomenon (Fig. 2) in which the permeability of the IP_3 channels markedly decreases with decreasing $[Ca^{2+}]_S$ (Figs. 3 and 4). Consistent with this observation a pattern resembling quantal release is seen in permeabilized HSY cells even in the absence of SERCA pump activity (Fig. 6 *A*). Also, as suggested by Irvine, we find that the sensitivity of IP_3-induced Ca^{2+} release decreases with decreasing $[Ca^{2+}]_S$ (Fig. 6). Finally, our data indicate that $[Ca^{2+}]_S$ in fully loaded stores approaches extracellular levels (Fig. 5), consistent with similar pumping capacities for the SERCA pump and the plasma membrane Ca^{2+}-ATPase.

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