Use of Intracellular Ca^{2+} Stores from Rat Basophilic Leukemia Cells To Study the Molecular Mechanism Leading to Quantal Ca^{2+} Release by Inositol 1,4,5-Trisphosphate†

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Received September 18, 1992; Revised Manuscript Received November 19, 1992

ABSTRACT: Quantal Ca^{2+} release is a novel motif for the mediation of signal transduction in which the amplitude of a biological response following multiple stepwise increases in agonist concentration is retained. The release of Ca^{2+} from permeabilized cells in response to the second messenger inositol 1,4,5-trisphosphate ($InsP_3$) proceeds in this fashion. The mechanisms leading to quantal Ca^{2+} release are unknown. $InsP_3$ releases 50–90% of the Ca^{2+} sequestered within the intracellular stores of mammalian cells permeabilized with saponin. However, preparation of microsomes results in the loss of this sensitivity. In this report, functionally intact intracellular Ca^{2+} stores were isolated from rat basophilic leukemia (RBL) cells by osmotic lysis followed by differential and sucrose density gradient centrifugation. From this preparation, 64% of the stored Ca^{2+} is released by $InsP_3$. We demonstrate that quantal Ca^{2+} release is retained by isolated Ca^{2+} stores and is identical to that observed in permeabilized cells. Addition of a subsaturating (28 nM) concentration of $InsP_3$ to permeabilized cells at 37 °C results in the release of only a small fraction of the sequestered Ca^{2+}. When the cells are cooled to 11 °C, the remaining Ca^{2+} is rapidly released. Hence, the mechanism leading to the quantal nature of Ca^{2+} release is reversible and is thus not likely to be the result of a covalent modification of the channel protein or of the Ca^{2+} store. We also demonstrate that the nonphosphorylatable analog of $InsP_3$, 2,3,6-trideoxyinositol 1,4,5-trisphosphate, and the poorly hydrolyzed analogs of $InsP_3$, 2-deoxy-1,4,5-$InsP_3$ and 6-deoxy-1,4,5-$InsP_3$, also cause quantal Ca^{2+} release, indicating that metabolites of $InsP_3$ do not drive increment detection. We conclude that the molecular mechanisms leading to quantal Ca^{2+} release reside within or are tightly associated with the Ca^{2+} store. We propose a theoretical explanation for the mechanism of quantal Ca^{2+} release which fits the experimental data well. This model takes into account the highly cooperative nature of channel opening described for the RBL cell, and the molecular heterogeneity of the $InsP_3$-gated Ca^{2+} channel. Control at this fundamental level assures great flexibility and high fidelity in regulating the cellular response to activation of the phosphoinositide cascade by hormonal stimuli.

The actions of many hormones are mediated by the intracellular second messenger inositol 1,4,5-trisphosphate ($InsP_3$)[1] (Berridge, 1988). $InsP_3$ releases Ca^{2+} from intracellular stores by binding to a ligand-gated Ca^{2+} channel (Ferris et al., 1989; Bezprozvanny et al., 1991; Finch et al., 1991; Mayrleitner et al., 1991). In permeabilized rat basophilic leukemia (RBL) cells (Meyer & Stryer, 1990), and in pancreatic acinar cells (Muallem et al., 1989), as well as many peripheral cell types, Ca^{2+} release in response to subsaturating additions of $InsP_3$ does not occur as a first-order process. Under physiologic conditions, over a defined range of $InsP_3$ concentrations, addition of $InsP_3$ leads to the rapid, but partial, release of sequestered Ca^{2+}. The release terminates abruptly but can be reinitiated by an additional increment in $InsP_3$ concentration. *Increment detection*, or *quantal Ca^{2+} release*, is a unique signaling motif that allows the cell to retain complete responsiveness to *changes* in stimuli over a defined physiologic range. In contrast to either inactivation or adaptation, where the response to repeated stimuli is diminished, with quantal Ca^{2+} release repeated addition of the same amount of $InsP_3$ leads to the release of a similar burst of Ca^{2+}. Increment detection in RBL cells occurs at 37 and 23 °C but not at 11 °C (Meyer & Stryer, 1990), demonstrating the presence of a temperature-sensitive, perhaps catalytic, step in its genesis.

In this report, we demonstrate that functional Ca^{2+} stores can be isolated from RBL cells by osmotic lysis followed by differential and sucrose density gradient centrifugation. The stores retain the ability to release most of the sequestered Ca^{2+} upon addition of $InsP_3$. Quantal Ca^{2+} release is retained by Ca^{2+} stores in the absence of cytosolic factors and complex structure and is *identical* to that observed in permeabilized cells. Quantal Ca^{2+} release is rapidly reversed by cooling to 11 °C and is therefore not likely to be the result of a catalytic event alone. We investigate the effects of several poorly metabolized analogs of $InsP_3$ on Ca^{2+} release and observe quantal release by these compounds as well. We conclude that the molecular basis for quantal Ca^{2+} release resides within or is tightly associated with the Ca^{2+} store, and we have developed a mathematical model to describe its mechanism.

† L.A.K. was a recipient of a Merck Foundation–American College of Cardiology Fellowship and a Physician Scientist Award from the National Heart, Lung, and Blood Institute (1K11 HL02361). This work was supported in part by NIH Grants GM 24032 and MH 45324 to L. Stryer.

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[1] Abbreviations: $InsP_3$, inositol 1,4,5-trisphosphate; $InsP_4$, inositol 1,3,4,5-tetrakisphosphate; $InsP_2$, inositol 4,5-bisphosphate; K-HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid, potassium salt; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; RBL, rat basophilic leukemia cell.

0006-2960/93/0432-1270$04.00/0

MATERIALS AND METHODS

Reagents. $InsP_3$ and ATP were from Calbiochem, fluo-3 and A23187 were from Molecular Probes, Chelex 100 was from Bio-Rad, sucrose and HEPES were from BRL (ultrapure), ultrapure Tris was from BRL or from U.S. Biochemicals, and $MgCl_2$ and saponin were from Sigma. ^3H-$InsP_3$ was from Dupont-NEN. All other reagents were of the highest grade commercially available. The $InsP_3$ analogs 2,3,6-trideoxy-1,4,5-$InsP_3$, 6-deoxy-1,4,5-$InsP_3$, and 3-deoxy-1,4,5-$InsP_3$ were gifts of Dr. Mark Bednarski and Mr. J. Lyssikatos, Department of Chemistry, University of California, Berkeley.

Ca^{2+} Assays. Fluorometric determination of free ionized Ca^{2+} was made with the Ca^{2+} indicator fluo-3 as described previously (Meyer et al., 1990). All reagents used for fluorometric assays were depleted of Ca^{2+} by passage through the "Ca^{2+} sponge", a calcium-chelating column (Meyer et al., 1990). Briefly, 2.0 mL of Ca^{2+}-free buffer containing 20 mM K-HEPES, pH 7.40, 5 mM NaCl, 130 mM KCl, 2 mM $MgCl_2$, 0.4 mM Na^+ATP, and 1.3 μM fluo-3 was equilibrated to 37 °C in an SLM 8000C fluorometer. Excitation was at 488 nm, and emission of all light which passed through a combination of LL500 and FCG 083 (Corion) filters was monitored (effectively all light >515 nm). Experiments with intact cells also included 0.125 mg/mL saponin. Under the conditions of the assay, additions of less than 5 pmol of Ca^{2+} could be reliably and reproducibly detected. Following the addition of $InsP_3$, 10 μM Ca^{2+} ionophore A23187 was added to determine the quantity of the sequestered Ca^{2+} residing in the $InsP_3$-insensitive pool (2 μL of a 10 mM solution of A23187 dissolved in dimethyl sulfoxide). Under these conditions, contamination with Ca^{2+} and autofluorescence of the ionophore do not significantly contribute to the fluorescence signal.

Cell Culture and Isolation of Intracellular Ca^{2+} Stores. Rat basophilic leukemia cells were grown as described previously (Meyer et al., 1988), with minor modifications. The medium consists of minimal essential medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 0.01 mg of gentamycin (all from Gibco), and 1 vial of Mito+ serum extender (Collaborative Research) per liter. Cells were grown without supplemental CO_2 in sealed 75- or 175-cm^2 flasks until confluence. They were harvested in Dulbeccos's phosphate-buffered saline (Gibco) supplemented with 5 mM NaEDTA, pH 7.4. The cells were washed once with 20 mM K-HEPES, 5 mM NaCl, and 130 mM KCl, pH 7.40. This was followed by two washes in lysis buffer (5 mM Tris-HCl, 0.5 mM $MgCl_2$, and 5 mM sucrose, pH 7.40). The contents of each 75-cm^2 tissue culture flask ($\sim 2 \times 10^7$ cells) were resuspended in 1.0 mL of lysis buffer. This will be referred to as cell suspension 1. Cell suspension 1 was incubated at 20 °C for 40 min with gentle mixing every 10 min. The suspension was then made 6% in sucrose by the addition of 100 μL of Ca^{2+}-depleted 60% sucrose and 0.1 M K-HEPES, pH 7.40 (prepared as described below). This will be referred to as cell suspension 2. Cell suspension 2 was centrifuged at 1500*g* for 3 min at 4 °C. This removed >99% of all cellular forms from the supernatant, as assessed by light microscopy. Using a P1000 pipetman with the distal 3–4 mm of the tip cut off to minimize shear, the supernatant was then layered on top of a 10–60% sucrose step gradient. Three-milliliter steps in 33-mL acid-washed Beckman ultraclear centrifuge tubes or 1-mL steps in 13-mL acid-washed Beckman ultraclear tubes were used. Sucrose steps were prepared by the appropriate dilution of 60% Ca^{2+}-depleted sucrose in 0.1 M K-HEPES, pH 7.40 with "Ca^{2+}-sponged" 0.1 M K-HEPES, pH 7.40, to make 10, 20, 30, 40, 50, and 60% steps. The gradient was then centrifuged for 30 min at 10 °C at 7500*g* in SW28 or SW41 TI rotors (Beckman). Fractions were collected dropwise by puncturing the bottom of the tube with a 16-gauge needle. Typically, 6-drop fractions (~0.5–0.6 mL) were collected.

Calcium-depleted sucrose was prepared as follows. Sucrose (60% w/v) was made up in 0.1 M K-HEPES, pH 7.40. A 500-mL solution was then passed through a 100-mL bed volume column of Chelex 100 flowing at a rate of 1 mL/min. The final Ca^{2+} concentration of this solution was approximately 5 μM. The sucrose concentration in gradient fractions was determined by refractometry, using stock solutions of sucrose as standards. To prepare Ca^{2+} stores washed with EGTA, NaEGTA was added to make the supernatant 2 mM. This was layered on to a 10–60% sucrose gradient buffered with 0.1 M K-HEPES, pH 7.40, to which all steps had been supplemented with 2 mM NaEGTA. Following a 30-min centrifugation at 7500*g* at 10 °C, the 44% sucrose fraction, equivalent to fraction 10 of Figure 3, was identified by its typical opalescent appearance. It was diluted with 3 volumes of Ca^{2+}-depleted 0.1 M K-HEPES, pH 7.40, and layered onto a 20–60% sucrose step gradient without EGTA. Fractions were collected and assayed as described above.

Percoll was obtained from Pharmacia and dialyzed extensively against "Ca^{2+}-sponged" buffers or passed through a Chelex 100 column equilibrated with 0.1 M K-HEPES, pH 7.40. Five milliliters of Percoll appropriately diluted with Ca^{2+}-sponged 0.1 M K-HEPES, pH 7.40, was placed into a 13-mL tube, and 4 mL of supernatant was carefully layered on top, along with Percoll density standards (Pharmacia). The sample was centrifuged at 7500*g* for 30 min in an SW41 TI rotor (Beckman).

Electron microscopy was performed with a Phillips 201C electron microscope. The samples were applied to carbon-coated, glow-discharged grids and stained with 1% uranyl acetate.

Protein determinations were made by the method of Bradford (1976), using bovine serum albumin as a standard. DNA was determined using the fluorescent indicator Hoechst 33258 as described (Labarca & Pagan, 1980). Succinate–cyanide cytochrome *c* reductase (mitochondria) and rotenone-insensitive NADPH-cytochrome *c* reductase were determined using the method of Fleisher and Fleisher (1967). Lactate dehydrogenase (cytosol), and 5′ nucleotidase (plasma membrane) were determined using kits obtained from Sigma. $InsP_3$ binding was performed by the method of Worley et al. (1987).

Numerical Simulations. Mathematical modeling was performed using the QuickBasic programming language (Microsoft), version 4.5, with a Gateway 2000 486DX2/50 personal computer.

RESULTS

Osmotic Lysis of RBL Cells Yields Intact Ca^{2+} Stores. Cell lysis by sonication, nitrogen cavitation, or homogenization resulted in a drastic loss of functional Ca^{2+} stores. Much of the sequestered Ca^{2+} was released by these treatments, little Ca^{2+} reuptake occurred in the presence of ATP, and only a small fraction of the sequestered Ca^{2+} could be released by $InsP_3$ (not shown). In contrast, $InsP_3$-gated Ca^{2+} stores remained largely intact following osmotic lysis. Figure 1 demonstrates Ca^{2+} release by 2 μM $InsP_3$ during the lysis process. Following incubation of washed cells is osmotic lysis buffer (5 mM Tris-HCl, pH 7.40, 5 mM sucrose, and 0.5 mM $MgCl_2$), at 20 °C for 40 min, $InsP_3$-gated Ca^{2+} release in the absence of saponin (Figure 1b) is comparable to that of

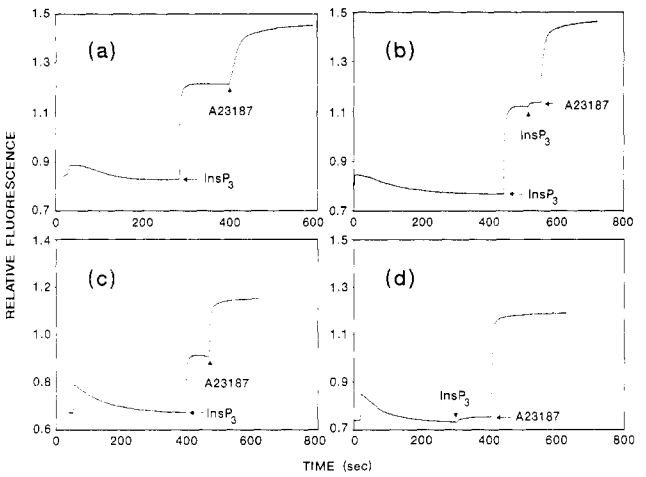

FIGURE 1: Osmotic lysis of RBL cells and susceptibility to shear. The four fluorometric tracings represent data from the same batch of cells. Assay procedures are described in the Materials and Methods section. In all panels, 2 μM $InsP_3$ and 10 μM A23187 are added at the indicated times. (a) An aliquot of 100 μL of freshly harvested cells washed into lysis buffer (cell suspension 1) is permeabilized by saponin and assayed for $InsP_3$- and A23187-releasable Ca^{2+} pools as described in the Materials and Methods section. (b) An aliquot was taken from cells incubated in lysis buffer for 40 min at 20 °C. Sucrose was added after the incubation to make the concentration 6% (cell suspension 2). Saponin is not added for this assay. Most of the stored Ca^{2+} remains in the $InsP_3$-sensitive pool. (c) An aliquot of the supernatant was taken following a 1-min 1500*g* centrifugation of osmotically lysed RBL cells treated as described in (b). The sequestered Ca^{2+} remains in the supernatant and is released upon the addition of $InsP_3$. (d) Following shearing with a Gilson P-200 pipetman using a Applied Scientific gel loading tip, active uptake of Ca^{2+} into stores still can be observed. However, only a small fraction of this Ca^{2+} can be released by $InsP_3$. The remaining stored Ca^{2+} is released by 10 μM A23187.

saponin-permeabilized cells (Figure 1a), indicating that the plasma membrane of most cells is disrupted. This was confirmed by the demonstration that >95% of cells take up trypan blue following incubation in the lysis buffer. Sixty-four percent of the $InsP_3$-sensitive Ca^{2+} store remains in the supernatant (Figure 1c) following centrifugation at 1500*g* for 1 min. By light microscopy, only about 5% of the initial number of cells remained in the supernatant. Figure 1d shows the effects of shearing of the supernatant by repeated pipetting. Only ~3% of the response to $InsP_3$ remains. While essentially all of the $InsP_3$ sensitivity is lost, the ability of the supernatant to store Ca^{2+} remains relatively intact. The homogenization steps utilized in the preparation of microsomes result in shear forces which typically are far in excess of those used to generate the result in Figure 1d (Finch et al., 1991; Brown et al., 1992). This observation suggests that the use of gentler isolation protocols might result in more intact Ca^{2+} stores from other tissues as well.

Sucrose Density Gradient Centrifugation Yields an Enriched Preparation of $InsP_3$-Sensitive Stores Which Retain Quantal Ca^{2+} Release. Sucrose density gradient centrifugation results in a significant enrichment expressed in terms of picomoles of Ca^{2+} stored per milligram of protein of the $InsP_3$-sensitive Ca^{2+} store (Table I). In a typical preparation, permeabilized cells store 1210 pmol of Ca^{2+}/mg of protein in an $InsP_3$-releasable form. The total ionophore-releasable pool is 1770 pmol/mg of protein. Isolated stores contain 10 000 pmol of $InsP_3$-releasable Ca^{2+}/mg of protein and a total of 15 900 pmol of ionophore-releasable Ca^{2+} (Table I). In this preparation, 63% of the Ca^{2+} stored in this preparation is releasable by 2 μM $InsP_3$. From separate experiments, the mean percentage ± SEM of Ca^{2+} released by $InsP_3$, relative to total ionophore-releasable Ca^{2+}, is 64% ± 1.7% (n = 10). This compares favorably to the 70–90% release typically observed from saponin-permeabilized RBL cells (Figure 1). The heaviest, functionally intact stores are found at a sucrose density of 44% (w/v) (Figure 2). Functional $InsP_3$-sensitive Ca^{2+} stores were found to have a density of ~1.013 g/mL by Percoll density gradient centrifugation.

Table I: Preparation of Cell-Free $InsP_3$-Releasable Ca^{2+} Stores[a]

	suspension 1	supernatant	stores
total $InsP_3$-relasable Ca^{2+} (pmol of $InsP_3$ released)	13 000	3700	2200
pmol (mg of protein)$^{-1}$	1210	650	10 000
recovery of $InsP_3$-releasable Ca^{2+} (%)	100	28	17
total Ca^{2+} stored ($InsP_3$ and A23187 released) [pmol (mg of protein)$^{-1}$]	1770	1340	15 900
total $InsP_3$ binding (cpm)	257 000	70 000	6800
cpm (mg of protein)$^{-1}$	23 500	12 400	77 000
total protein (mg)	10.9	5.7	0.22
total LDH (mmol min^{-1})	1.8×10^{-1}	8.8×10^{-2}	5.4×10^{-6}
mmol min^{-1} (mg of protein)$^{-1}$	1.6×10^{-2}	1.5×10^{-2}	2.5×10^{-5}
total DNA (mg)	3.9	0.8	0.02
mg of DNA (mg of protein)$^{-1}$	0.36	0.14	0.008
total 5′ nucleotidase (mmol min^{-1})	1.4×10^{-3}	6.0×10^{-4}	4.2×10^{-5}
mmol min^{-1} (mg of protein)$^{-1}$	1.2×10^{-4}	1.1×10^{-4}	1.9×10^{-4}
total rotenone-insensitive cytochrome *c* reductase (mmol min^{-1})	4.3×10^{-5}	3×10^{-5}	3.4×10^{-7}
mmol min^{-1} (mg of protein)$^{-1}$	3.9×10^{-6}	5.3×10^{-5}	1.5×10^{-5}
total succinate–cyanide cytochrome *c* reductase (mmol min^{-1})	1.0×10^{-3}	5.1×10^{-4}	1.2×10^{-4}
mmol min^{-1} (mg of protein)$^{-1}$	9.5×10^{-5}	9.0×10^{-5}	5.6×10^{-4}

[a] Cells (1.6×10^8) were prepared by osmotic lysis. The supernatant was layered onto a 10–60% sucrose step gradient composed of 3-mL steps as in Figure 2. Following centrifugation for 30 min at 7500*g* in an SW28 rotor, ~0.5–0.6-mL fractions were collected dropwise. The data presented are for the fraction containing the peak $InsP_3$-releasable Ca^{2+} (equivalent to fraction 10 of Figure 2).

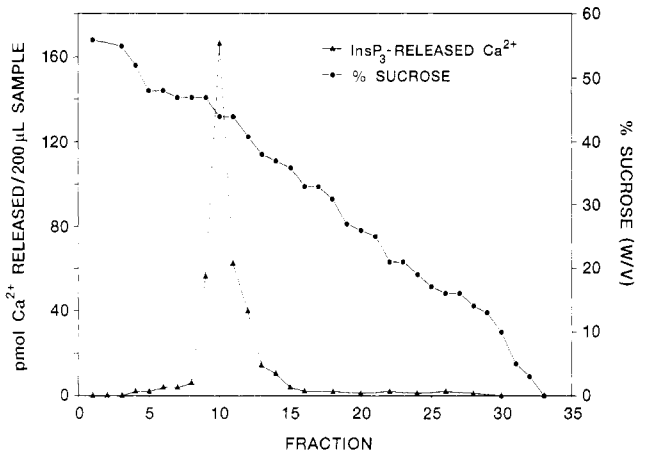

FIGURE 2: Sucrose density gradient profile of Ca^{2+} stores from RBL cells. The preparation of the sucrose density step gradient is described in the Materials and Methods section. The gradient was centrifuged for 30 min at 7500*g*. Similar results were obtained for runs of 60 min at 31000*g* and 90 min at 7500*g*, indicating a close approximation of equilibrium. The nominal free Ca^{2+} concentration at the start of each assay is approximately 0.2–0.4 μM. The peak of $InsP_3$- and A23187-released (2 and 10 μM, respectively) Ca^{2+} migrates to a density of 44% sucrose (w/v). It can be identified while still in the centrifuge tube by its opalescent appearance.

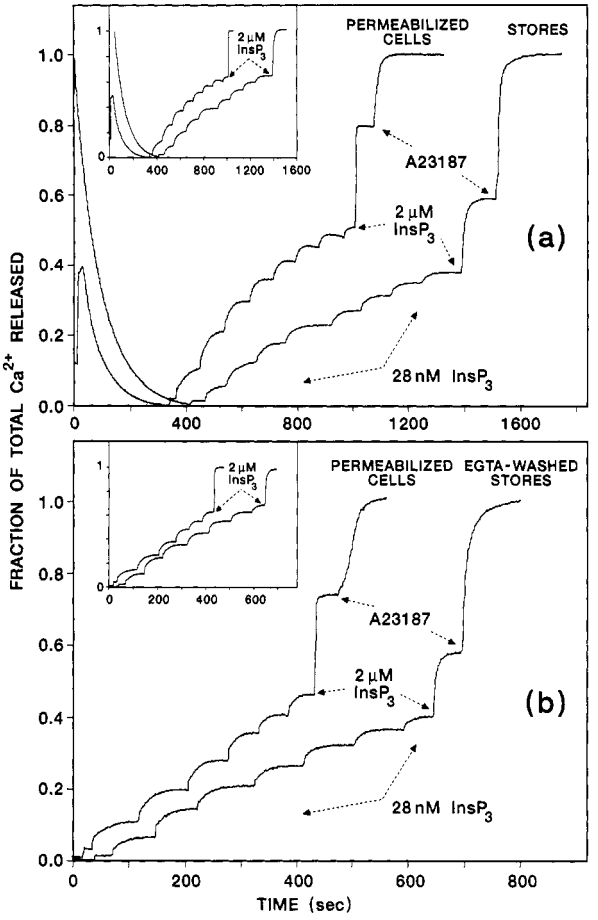

FIGURE 3: Quantal Ca^{2+} release by isolated Ca^{2+} stores. Ca^{2+} stores and EGTA-washed Ca^{2+} stores were isolated as described in the Materials and Methods section. Quantal Ca^{2+} release was assayed for by the repeated addition of 28 nM $InsP_3$. A last addition of 2 μM $InsP_3$ was made, followed by 10 μM of A23187. For comparison, in both panels, saponin-permeabilized cells, obtained along with the cells used to prepare the stores, were assayed for quantal Ca^{2+} release by an identical protocol. The inset in each panel is the same data redrawn and normalized to the fraction of stored Ca^{2+} released by $InsP_3$. (a) Calcium stores isolated by a sucrose gradient. (b) Ca^{2+} stores isolated from a sucrose gradient containing 2 mM EGTA, followed by a second sucrose gradient without EGTA.

Quantal Ca^{2+} release is retained by both sucrose- and Percoll-isolated material: sequential additions of 28 nM $InsP_3$ results in a quantized release of Ca^{2+} which is virtually identical to that of permeabilized cells (Figure 3). Quantal Ca^{2+} release is retained even after pretreatment with 2.0 mM EGTA, centrifugation through a 10–60% sucrose gradient supplemented with 2 mM EGTA, and recentrifugation through a 20–60% sucrose step gradient without EGTA (Figure 3). In 2 mM EGTA, the free Ca^{2+} associated with the stores is not detectable by the fluorescence methods employed here (with sensitivity in the nanomolar range). Despite being subjected to two density gradient centrifugations, the phenomenon of quantal Ca^{2+} release is still retained, indicating the mechanism regulating its occurrence must be tightly associated with the store.

Ca^{2+} Stores Do Not Retain High-Order Cellular Architecture. Table I characterizes a typical preparation by comparison of enzyme markers whose activities are associated with specific organelles. Density gradient centrifugation results in an essentially complete separation of Ca^{2+} stores from cytosol (measured by lactate dehydrogenase activity) as expected. The preparation differs from the "calciosome" preparation described by Volpe et al. (1988, 1991) in that the

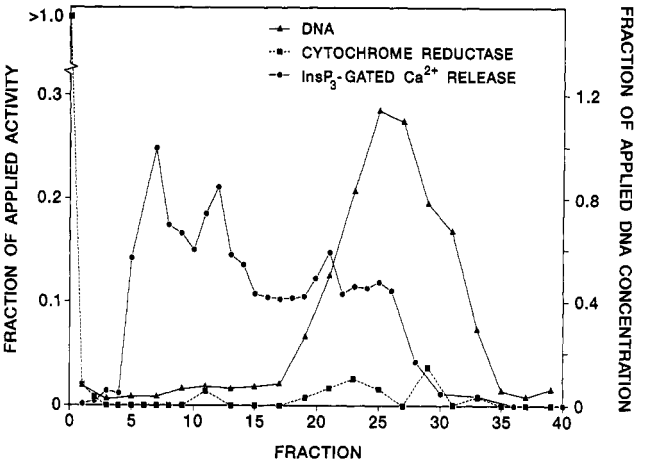

FIGURE 4: Ca^{2+} stores are not tightly associated with nuclei and mitochondria. Following osmotic lysis and low-speed centrifugation, supernatant is layered onto a 15-mL 35% sucrose cushion and centrifuged for 1 h at 31000*g* in an SW28 rotor. A thinly spread pellet at the bottom of the tube is observed. When scraped and resuspended, this pellet bears the succinate–cytochrome *c* reductase activity. Essentially no activity is found elsewhere in the profile. DNA remains near the top, indicating that intact nuclei are not present in this preparation. $InsP_3$-sensitive Ca^{2+} stores are found throughout the gradient, but essentially none reached the bottom in this run.

density of $InsP_3$-responsive RBL Ca^{2+} stores isolated by sucrose gradient is greater than that found in avian brain (Volpe et al., 1991). It is, however, closer to that observed by these authors for ryanodine-sensitive, $InsP_3$-insensitive Ca^{2+} stores. We have not observed functional evidence for ryanodine receptors in the RBL cell (unpublished observations) and hence have not sought their presence in these gradients. The differences between the calciosome preparation (Volpe et al., 1991) and the Ca^{2+} store preparation described here may be due to differences in methodology. Our work employs functional assays for $InsP_3$ sensitivity as well as the $InsP_3$ binding assays used by Volpe et al. to detect Ca^{2+} stores. However, the differences may be reflective of the functional differences between the tissue types investigated.

Osmotic lysis and density gradient centrifugation did not separate Ca^{2+} stores from succinate–cyanide cytochrome *c* reductase, a marker for mitochondria. DNA is also found in these fractions. This suggested that the calcium stores might be associated with mitochondria and nuclei and hence might still be part of a complex subcellular structure. Mitochondria and DNA were readily dissociated from the Ca^{2+} stores by means of velocity centrifugation (Figure 4). Supernatants of osmotically lysed cells were subjected to centrifugation onto a 35% sucrose cushion. Figure 4 demonstrates that the sedimentation profiles of $InsP_3$-gated Ca^{2+} stores, DNA, and succinate–cytochrome *c* reductase are distinct. Quantal Ca^{2+} release is observed from slow, intermediate, and rapidly sedimenting fractions. In separate experiments, electron microscopy of Ca^{2+} stores isolated by sucrose density gradient centrifugation were also performed. Preparations negatively stained with 1% uranyl acetate and studied by electron microscopy demonstrated individual vesicular structures with a heterogeneity in size ranging from <0.1 to ~0.5 μm (not shown). Hence, complex intracellular architecture does not contribute to the integrity of the Ca^{2+} stores and is not required for quantal Ca^{2+} release.

Effect of Rapid Cooling of Calcium Stores on $InsP_3$-Gated Ca^{2+} Release. At 11 °C, quantal Ca^{2+} release is not observed (Meyer & Stryer, 1990). At this temperature, kinetics of

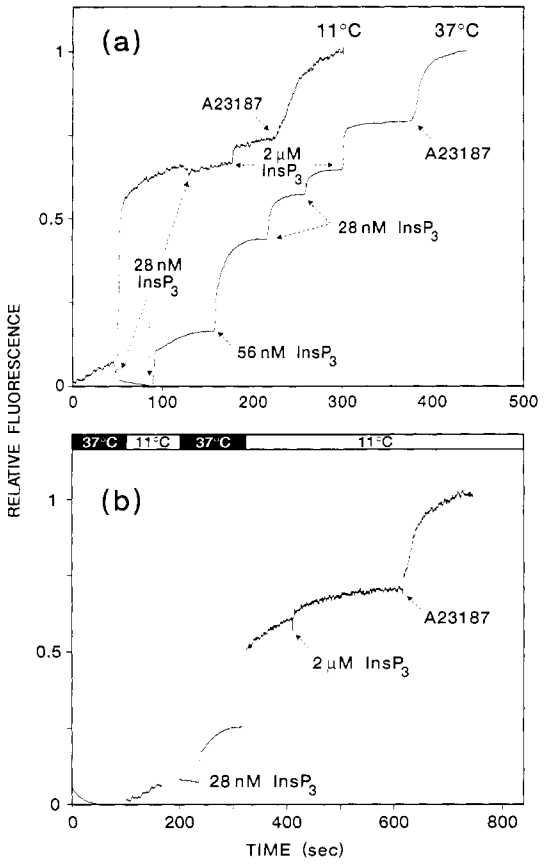

FIGURE 5: Increment detection is lost upon cooling to 11 °C. Permeabilized cells are incubated at 37 °C in the presence of 0.4 mM ATP and 2.0 mM Mg^{2+}. The cuvette was then rapidly cooled to 11 °C by dipping it into liquid N_2 or maintained at 37 °C as indicated. Studies at 11 °C were performed in a second, custom-built fluorometer. Data are normalized such that 0 is defined as the minimum emission, when Ca^{2+} stores are completely loaded, and 1 is defined as the maximum, following addition of 10 μM A23187. (a) Controls demonstrating Ca^{2+} release by 28 nM $InsP_3$ at 11 °C and increment detection at 37 °C by additions of 28 and 56 nM $InsP_3$, as indicated. (b) The bar across the top of the figure indicates the temperature of the sample at the time of the recording. The cuvette is cooled to 11 °C to establish a fluorescence emission baseline. It is then rewarmed to 37 °C, and 28 nM $InsP_3$ is added. The sample is again rapidly cooled to 11 °C and returned to the 11 °C equilibrated fluorometer. The $InsP_3$-sensitive Ca^{2+} store has been emptied within the 20 s required to cool the sample and transfer it to the second fluorometer, as confirmed by the subsequent addition of 2 μM $InsP_3$. The calcium ionophore A23187 is added at the end to demonstrate the full extent of the sequestered Ca^{2+}.

Ca^{2+} release is essentially a first-order process. Even the addition of 10 nM $InsP_3$ at 11 °C leads to the complete release of Ca^{2+} from Ca^{2+} stores (Meyer et al., 1990; Meyer & Stryer 1990). We sought to determine whether the mechanism leading to increment detection, when established at 37 °C, is preserved upon cooling to 11 °C. Figure 5a shows that addition of 28 nM $InsP_3$ leads to the rapid and almost complete release of calcium from $InsP_3$-sensitive pools at 11 °C. When permeabilized cells at 37 °C are treated with the same amount of $InsP_3$ (Figure 5b), only a small fraction of the stored Ca^{2+} is released. However, rapid cooling of these cells to 11 °C leads to the release of the remaining Ca^{2+} in the $InsP_3$-sensitive pool in less than 30 s (Figure 5b). In the absence of $InsP_3$, permeabilized cells may be cooled to 11 °C and rewarmed to 37 °C without loss of function (not shown). This result demonstrates that the quantal nature of Ca^{2+} release by $InsP_3$, established at 37 °C, can be reversed by cooling to 11 °C. In addition, it confirms the observation that significant hydrolysis of $InsP_3$ does not occur. Were this the case, further release of Ca^{2+} upon cooling would not be expected.

Metabolites of $InsP_3$ Are Not Involved in the Regulation of Increment Detection. Under the conditions of this assay, with RBL cells, it is known that $InsP_3$ is not extensively metabolized (Meyer & Stryer, 1990). However, the phosphorylation of a small amount of $InsP_3$ to inositol 1,3,4,5-tetrakisphosphate ($InsP_4$), or to another tetrakisphosphate derivative, could not be excluded. The $InsP_3$ analog 2,3,6-deoxy-1,4,5-$InsP_3$ lacks free hydroxyl groups and cannot be phosphorylated to $InsP_4$. This compound is a full agonist, with an EC_{50} of ~125 μM (Lyssikatos, Kindman, and Bednarski, in preparation). Twenty-five micromolar additions of 2,3,6-deoxy-1,4,5-$InsP_3$ also cause quantal Ca^{2+} release identical to that observed in Figure 3 (not shown).

The data presented in Figure 5, as well as our previous report (Meyer & Stryer, 1990), demonstrate that hydrolysis of $InsP_3$ to $InsP_2$ is not likely to be related to quantal Ca^{2+} release. To further examine this issue, we studied the effects of two putative inhibitors of the $InsP_3$ phosphatase, 6-deoxy-$InsP_3$ and 3-deoxy-$InsP_3$ (Safrany et al., 1991). Both of these compounds are also full agonists of Ca^{2+} release, with EC_{50} values of 68 and 8.5 μM, respectively (Lyssikatos, Kindman, and Bednarski, in preparation). By themselves, both compounds also lead to quantal Ca^{2+} release (not shown). This is further evidence against the hypothesis that the hydrolysis of $InsP_3$ to $InsP_2$ is the molecular mechanism which leads to quantal Ca^{2+} release.

A Quantitative Model for Quantal Ca^{2+} Release. Having established that quantal Ca^{2+} release results from an integral property of the Ca^{2+} store in the RBL cell, we sought to develop a theoretical explanation based on parameters which have been demonstrated to be physiologically relevant. It is known, for example, that $InsP_3$-gated channel opening in the RBL cell is highly cooperative, with a Hill coefficient of >3 at 11 °C and at higher temperatures (Meyer et al., 1988, 1990). The existence of multiple isoforms of the $InsP_3$-gated Ca^{2+} channel has also been established (Mignery et al., 1990; Südhof et al, 1991; Ross et al., 1992; Nakagawa et al., 1991). Because intracellular Ca^{2+} stores appear to be heterogeneous (Takei et al., 1992), functionally distinct pools of Ca^{2+}, each with its own receptor isoform, might exist. Finally, the EC_{50} for Ca^{2+} release by $InsP_3$ at physiologic temperature is on the order of 300 nM (Finch et al., 1991; Bezprozvanny et al., 1991, Meyer et al., 1990). Hence, the K_d under physiologic conditions (25–37 °C, 50–300 nM Ca^{2+}) may be much greater than K_d described at 4 °C in the absence of Ca^{2+} and Mg^{2+} (Worley et al., 1987; Volpe et al., 1990). The data presented in Figure 3, as well as those presented previously (Meyer & Stryer, 1990), can be approximated mathematically when these factors (multiple isoforms with distinct K_d, cooperative channel opening, and multiple pools each with a unique type of $InsP_3$-gated Ca^{2+} channel) are combined into one model. The description of the model starts with the defintion of the Ca^{2+} within each type of store. The absolute concentration of Ca^{2+} within the nth Ca^{2+} store can be defined at time t as a fraction of its maximal value. At time $t = 0$, then $X_n = 1$, and

$$-dX_n(t)/dt = X_n(t) + a_n[y^h/(y^h + K_n^h) + \text{leak}]X_n(t) - \text{pump}$$

where n is the number of distinct classes of channels, h is the Hill coefficient (4 for the RBL cell; Meyer et al., 1990), K_n is the dissociation constant for the nth species of $InsP_3$-gated channel, and y is the concentration of added $InsP_3$. The conductance of each nth type of channel is not known, nor is the number of nth channels. These two terms may be grouped

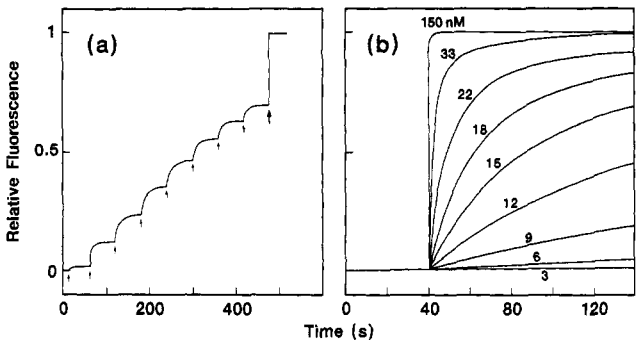

FIGURE 6: Numerical simulation of Ca^{2+} release. Computer modeling was carried out as described in the Results section. (a) Model of quantal Ca^{2+} release at 37 °C. The figure should be compared with the data presented in Figure 3 of this paper. Selected parameters are described in the text. The stores are fully loaded with Ca^{2+} at the start of the simulation. Additions of 25 nM $InsP_3$ are then simulated (small arrows). The final simulated addition is for 1 μM $InsP_3$ (bold arrow). (b) Model of Ca^{2+} release at 11 °C. This model fits data observed previously by our group [Figure 2 of Meyer et al. (1988)]. To model this experiment, the pump term was set to 0, because neither ATP nor Mg^{2+} had been included in the incubations. K_n is set to 75 nM. The family of curves represents 3, 6, 9, 12, 15, 22.5, 33, and 150 nM additions of $InsP_3$.

into the rate term a_n. The leak is the rate of Ca^{2+} release from the store in the absence of $InsP_3$. It has been shown to result largely from Ca^{2+} leakage through the $InsP_3$-gated Ca^{2+} channel (Meyer & Stryer, 1990). For the purposes of this model, Ca^{2+} leak is assumed to occur exclusively by this pathway. For simplicity, the leak is assumed to be proportional to the number of n channels. The expression "pump" represents the rate at which the Ca^{2+} ATPase pumps Ca^{2+} back to the store. Also for simplicity, we assume that the rate of the pump is constant:

$$\text{pump} = a_n(\text{leak})$$

At any time, the fraction of the system's total Ca^{2+} released into the medium is given by

$$D_n(1 - X_n)/(\sum_{1 \to n} D_n)$$

where D_n is the quantity of Ca^{2+} which is immediately accessible to the nth type of channel. When many variables are undefined, more than one solution may be identified which satisfies the equation. One possible solution is when $h = 4$, leak = 0.001 (Meyer et al., 1990) and all $X_n = 1$ (the store is filled); at $t = 0$ the following parameters give a good fit to the data of Figure 3: $n = 5$, $K_n = 240, 360, 480, 750$, and 1400 nM, respectively, and $a_n = 160, 80, 40, 40$, and 40 s^{-1}, respectively. The result of this simulation is given in Figure 6a. These observations suggest that quantal calcium release may be the result of an interaction of several defined biochemical variables.

As a control, we modeled conditions which would simulate the loss of quantal Ca^{2+} release at 11 °C. To varying extents, the factors most likely to be temperature dependent are K_n, the channel conductance (Champiel et al., 1989), the rate of the ATP-dependent pump (Meyer et al., 1988), and the leak. Cooperative channel opening is retained at 11 °C for the RBL cell (Meyer et al., 1988). To simulate Figure 2 of Meyer et al., (1988), we set the pump term equal to 0, because neither ATP nor Mg^{2+} was included in their incubations. We set a_n to be 1, 1, 5, 10, and 20. We then chose K_n to be 75 nM for all K_n. The result of this simulation is given in Figure 6b. With these modifications, the model very closely approximates the results presented by our earlier work. This suggests that the loss of quantal Ca^{2+} release at low temperature could be the result of a shift to a single higher affinity in the binding of $InsP_3$ to the $InsP_3$-gated Ca^{2+} channel, coupled with a diminished Ca^{2+} ATPase activity at this temperature. This also could explain why only single receptor populations are identified by careful binding studies (Supattapone et al., 1988). At 4 °C, the temperature under which the studies are performed, all species of receptors might have a similar affinity for $InsP_3$.

Thus, the mechanism of quantal Ca^{2+} release may be explained by a model which incorporates the cooperativity of channel opening, multiple stores, and molecular heterogeneity of the $InsP_3$-gated Ca^{2+} channel. In addition, consideration must be given to the action of the Ca^{2+} ATPase, which returns Ca^{2+} to the store even in the presence of $InsP_3$. This allows a new steady state to be reached following each small addition of $InsP_3$.

DISCUSSION

Quantal Ca^{2+} release is a novel model for intracellular signaling, distinct from adaptation and inactivation. Over a defined narrow range of $InsP_3$ concentrations, at physiologic temperature, termination of Ca^{2+} release from intracellular stores is accompanied by an offset in sensitivity to $InsP_3$, leaving the system responsive to the next rise in $InsP_3$ concentration. This allows the cell to detect *changes* in $InsP_3$ concentration. Biologically, this permits the titration of intracellular Ca^{2+} release following hormonal stimulation. Incremental detection of $InsP_3$ may also be important for the generation of Ca^{2+} waves and Ca^{2+} oscillations (Meyer, 1991). Ca^{2+} release from intracellular stores is tightly coupled to increases in $InsP_3$ concentration and rapidly terminates after each successive increase. The resulting local increase in free Ca^{2+} would be rapidly quenched by the Ca^{2+} ATPase.

We describe the isolation and characterization of functional intracellular Ca^{2+} stores. Osmotic lysis of RBL cells yields functionally intact Ca^{2+} stores with 64% of the stored Ca^{2+} releasable by $InsP_3$. This represents a significant improvement in $InsP_3$ responsiveness when compared with microsome preparations (cf. Finch et al., 1991; Burnett et al., 1990), which are prepared by homogenization of the source tissue. This improved responsiveness is likely to be the result of the gentler conditions employed during our preparation. We found that shear converts highly $InsP_3$-responsive stores to relatively unresponsive ones (Figure 1), with only 3% of the sequestered Ca^{2+} being released by $InsP_3$. Why should the $InsP_3$ responsiveness of Ca^{2+} stores be so sensitive to shear while the Ca^{2+} uptake and storage capacity is retained? One explanation which would account for these observations is a differential distribution of Ca^{2+} pumps and $InsP_3$-gated Ca^{2+} channels. Shearing has little effect on the store's capacity for Ca^{2+}. Shearing generates Ca^{2+} stores which are *functionally* devoid of $InsP_3$-gated Ca^{2+} channels. Relative to the ATPase, the $InsP_3$-gated Ca^{2+} channels are either sparsely or unevenly distributed (Satoh et al., 1990; Rossier et al., 1991; Takei et al., 1992). As a result, while all of the microsomes generated by the shearing process bear the Ca^{2+} ATPase, only a small fraction of the microsomes generated will bear $InsP_3$-gated Ca^{2+} channels.

This report demonstrates quantal Ca^{2+} release from isolated functionally intact Ca^{2+} stores to be *identical* to that observed in permeabilized cells. By differential centrifugation and by electron microscopy, we established that this preparation of Ca^{2+} stores is free of cytosol and of complex intracellular architecture. Isolated Ca^{2+} stores respond with quantal Ca^{2+}

release to incremental addition of $InsP_3$ in a fashion which is identical to that observed in permeabilized cells (Figure 3). Our findings indicate that the molecular components leading to incremental detection of $InsP_3$ reside within or are tightly associated with the Ca^{2+} store. In addition, we have found that Ca^{2+}- or temperature-dependent binding factors cannot be responsible for modulating Ca^{2+} release. Centrifugation of stores at 10 °C through 2 mM EGTA also had no effect on Ca^{2+} release properties. Events such as the autophosporylation of the protein (Ferris et al., 1992a) or the production of another second messenger occurring upon addition of $InsP_3$ are not likely, by themselves, to be the cause of quantal Ca^{2+} release. Incremental detection established with a small amount of $InsP_3$ at 37 °C is lost upon cooling to 11 °C, as illustrated by the complete and rapid efflux of Ca^{2+} from the $InsP_3$-sensitive pool (Figure 5). Finally, metabolism of a small amount of $InsP_3$ to $InsP_4$ cannot be implicated since the nonphosphorylatable analog 2,3,6-deoxy-1,4,5-$InsP_3$ also causes quantal Ca^{2+} release, as do analogs which are inhibitors of the $InsP_3$ phosphatase.

Recently, Swillens (1992) proposed a unique model which provides a good mathematical fit to our previously reported (Meyer & Stryer, 1990) Ca^{2+} release data. This model requires that a regulatory molecule rapidly scan a large number of channels and catalyze, in an energy-dependent process, the interconversion of open channels to closed ones. The number of active memory molecules must be tightly regulated by the fraction of channels that are open. Our finding of quantal Ca^{2+} release by isolated stores indicates that the memory molecule would have to be tightly associated with the stores.

Because our results demonstrate that the fundamental process driving quantal Ca^{2+} release must be an integral part of the Ca^{2+} store, we developed a model employing biochemical parameters which have already been established about the $InsP_3$-gated Ca^{2+} channel and the Ca^{2+} store. It is known that channel opening in the RBL cell is highly cooperative (Meyer et al., 1990). Molecular heterogeneity of the $InsP_3$-gated Ca^{2+} channel, resulting from alternative splicing of messenger RNA and from the expression of multiple genes encoding for distinct entities of the $InsP_3$-gated Ca^{2+} channel (Mignery et al., 1990; Südhof et al., 1991; Ross et al., 1992), has also been identified. Distinct isoforms are expressed in different quantities in different tissues (Südhof et al., 1991; Ross et al., 1992). The potential for functional diversity of the $InsP_3$-gated Ca^{2+} channel is increased by the observation that the protein may be phosphorylated at several sites, thereby modulating $InsP_3$ binding (Ferris et al., 1992a), and possibly Ca^{2+} gating. These observations by themselves could not account for quantal Ca^{2+} release. However, we have combined these factors into a model which fits the experimental data presented here and elsewhere by our group. Ferris et al. (1992b) have observed biphasic kinetics of Ca^{2+} flux into liposomes in which purified $InsP_3$-gated Ca^{2+} channels had been incorporated. They noted the highly cooperative nature of channel opening following small applications of $InsP_3$. The model proposed here provides results which are fundamentally consistent with their data. This indicates that quantal Ca^{2+} release indeed results from an intrinsic property of the channel. A high degree of cooperativity in channel opening at low $InsP_3$ concentration is essential for quantal Ca^{2+} release but is not sufficient to account for the observation in more intact systems. The Ca^{2+} ATPase, the Ca^{2+} leak, molecular and functional heterogeneity of the channel, and functional heterogeneity of the store are also required.

There may be several possible numerical solutions to the model presented. Proof of this model will require the precise determination of the numbers of unique functional $InsP_3$ receptor states (isoforms, phosphorylation products, etc.), their individual conductances and temperature dependence, the determination of their individual dissociation constants under *physiologic* conditions, and demonstration of their segregation to distinct Ca^{2+} stores. The most important assumptions of the model, the cooperativity (Meyer et al., 1988) and the multiple isoforms of $InsP_3$-gated Ca^{2+} channel, each with a unique copy number (Mignery et al., 1989; Südhof et al., 1991; Nakagawa et al., 1991; Ross et al., 1992), are well established. Because quantal Ca^{2+} release appears to proceed in an "all or none" fashion (Oldershaw et al., 1991), the hypothesis regarding segregation of stores into functionally distinct entities may also be correct. Our model provides a plausible hypothesis based on well-understood biochemical principles. Investigation of quantal Ca^{2+} release will provide a new perspective on cell signaling. The functionally intact Ca^{2+} stores described in this report will be a useful tool for the further molecular characterization of the mechanisms leading to quantal Ca^{2+} release and for the investigation of the biology of the Ca^{2+} stores.

ACKNOWLEDGMENT

The authors are most appreciative of the reagents provided by Dr. Mark Bednarski and Dr. Joe Lyssikatos of the Department of Chemistry, University of California at Berkeley. We also wish to thank L. Mercer and J. Wren for performing electron microscopy and N. Allbritton, D. Friel, P. Gardner, R. Hu, S. Ray, D. Stork, and L. Stryer for helpful conversations.

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