Dynamic Properties of an Inositol 1,4,5-Trisphosphate– and Thapsigargin-insensitive Calcium Pool in Mammalian Cell Lines

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Abstract. The functional characteristics of a nonacidic, inositol 1,4,5-trisphosphate– and thapsigargin-insensitive Ca^{2+} pool have been characterized in mammalian cells derived from the rat pituitary gland (GH3, GC, and GH3B6), the adrenal tissue (PC12), and mast cells (RBL-1). This Ca^{2+} pool is released into the cytoplasm by the Ca^{2+} ionophores ionomycin or A23187 after the discharge of the inositol 1,4,5-trisphosphate–sensitive store with an agonist coupled to phospholipase C activation and/or thapsigargin. The amount of Ca^{2+} trapped within this pool increased significantly after a prolonged elevation of intracellular Ca^{2+} concentration elicited by activation of Ca^{2+} influx. This pool was affected neither by caffeine-ryanodine nor by mitochondrial uncouplers. Probing mitochondrial Ca^{2+} with recombinant aequorin confirmed that this pool did not coincide with mitochondria, whereas its homogeneous distribution across the cytosol, as revealed by confocal microscopy, and its insensitivity to brefeldin A make localization within the Golgi complex unlikely. A proton gradient as the driving mechanism for Ca^{2+} uptake was excluded since ionomycin is inefficient in releasing Ca^{2+} from acidic pools and Ca^{2+} accumulation/release in/from this store was unaffected by monensin or NH_4Cl, drugs known to collapse organelle acidic pH gradients. Ca^{2+} sequestration inside this pool, thus, may occur through a low-affinity, high-capacity Ca^{2+}–ATPase system, which is, however, distinct from classical endo-sarcoplasmic reticulum Ca^{2+}–ATPases. The cytological nature and functional role of this Ca^{2+} storage compartment are discussed.

T HE cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$)[1] of eukaryotic cells rests in the range of 50–200 nM, i.e., at a very low level, if compared to the Ca^{2+} concentration of physiological media (2 mM). However, the total cellular Ca^{2+} content is closer to this latter value (1–3 mmol/l of cell water). In other words, eukaryotic cells sequester large amounts of Ca^{2+} mainly by uptake inside intracellular Ca^{2+} stores (~90%) (for reviews see Pozzan et al., 1994; Clapham, 1995).

The complexity of intracellular Ca^{2+} stores has been intensively investigated in recent years (for reviews see Meldolesi et al., 1990; Pozzan et al., 1994; Simpson et al., 1995). Attention has been focused mainly on Ca^{2+} stores that are highly dynamic because of their ability to rapidly take up and release Ca^{2+}. Ca^{2+} sequestration into these pools depends on Ca^{2+}–ATPases, known as sarco/endoplasmic reticulum Ca^{2+}–ATPases (SERCAs) (Burk et al., 1989; Bobe et al., 1994; Wuytack et al., 1994). All the SERCA isoforms share the property of being selectively inhibited by thapsigargin (Tg), a tumor-promoting sesquiterpene lactone (Lytton et al., 1991). Tg acts with both high affinity, at nanomolar concentrations, and high specificity, with virtually no effect on the Ca^{2+}– or Na^+/K^+–ATPase of the plasmalemma.

Other drugs, such as 2,5-di(tert-butyl)-1,4-benzohydroquinone (tBHQ) and cyclopiazonic acid (CA), also block SERCAs, albeit with a significantly lower affinity (Mason et al., 1991). Ca^{2+} release, on the other hand, depends mainly on two types of Ca^{2+} release channels named inositol 1,4,5-trisphosphate ($InsP_3$) and ryanodine receptors (for reviews see Mikoshiba, 1993; Sorrentino and Volpe, 1993; Ehrlich, 1995). These channels are expressed in variable proportions in different cell types and couple extracellular stimuli to the release of Ca^{2+}, with possible ensuing generation of Ca^{2+} waves and spikes (for reviews see Amundson and Clapham, 1993; Taylor, 1994; Bootman

1. *Abbreviations used in this paper*: BFA, brefeldin A; CA, cyclopiazonic acid; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; $[Ca^{2+}]_m$, intramitochondrial free Ca^{2+} concentration; cytAEQ, cytosolic aequorin; FCCP, *p*-(trifluoro-methoxy) phenylhydrazone; $InsP_3$, inositol 1,4,5-trisphosphate; mKRB, modified Krebs Ringer Buffer; mtAEQ, mitochondrial aequorin; SERCAs, sarco/endoplasmic reticulum Ca^{2+}–ATPases; SIC, stimulus-induced-calcium; tBHQ, 2,5-di(tert-butyl)-1,4-benzohydroquinone; Tg, thapsigargin; TRH, thyrotropin-releasing hormone; VOCCs, voltage-operated Ca^{2+} channels.

and Berridge, 1995). The relationship between these types of Ca^{2+}-release channels is still largely debated. The ryanodine-sensitive channel is also activated by caffeine, and ryanodine- and caffeine-sensitive stores are generally regarded to comprise the same pool (Zacchetti et al., 1991; Barry and Cheek, 1994; but also see Giannini et al., 1992; McNulty and Taylor, 1993).

In the vast majority of cell types so far investigated, the $InsP_3$- (and/or the ryanodine-) sensitive stores almost completely overlap with those sensitive to Tg (Zacchetti et al., 1991; Gamberucci et al., 1995) and are thus referred to also as Tg-sensitive Ca^{2+} pools. From the cytological point of view, the $InsP_3$-/Tg-sensitive Ca^{2+} pool is identified with the ER or with a subfraction of it (Hashimoto et al., 1988).

The complexity of the relationships between the $InsP_3$- and ryanodine/caffeine-sensitive stores does not cover the entire issue of intracellular Ca^{2+} pool heterogeneity. Other types of Ca^{2+} pools are known to exist, the size of which varies considerably among different cell types. These latter Ca^{2+} stores account for roughly half of all sequestered Ca^{2+} (Chandra et al., 1991; Fasolato et al., 1991; Shorte et al., 1991; Bastianutto et al., 1995; Mery et al., 1996). They have been identified through the increase in $[Ca^{2+}]_i$ upon application of Ca^{2+} ionophores, after depletion of the Tg-sensitive pool with a combination, or a sequence, of $InsP_3$-generating agonists, Tg, and caffeine. These residual Tg-insensitive pools appear rather heterogeneous in terms of cytological identity and pharmacological sensitivity. Part of these pools shows an acidic lumenal pH and is discharged only by a combination of a Ca^{2+} ionophore and of agents that collapse internal acidic pH gradients (such as monensin and NH_4Cl). $^{45}Ca^{2+}$ labeling of Tg-insensitive pools is slower than that of the Tg-sensitive store, and, for this reason, they have been generally indicated as slowly exchanging Ca^{2+} pools (Fasolato et al., 1991). As far as their identification is concerned, the acidic pool seems largely identifiable with secretory compartments and lysosomes, while very little is known yet about the rest of the Tg-insensitive store.

Here we demonstrate that a nonacidic, $InsP_3$- and Tg-insensitive Ca^{2+} pool rapidly accumulates large amounts of Ca^{2+} when high and sustained increases of $[Ca^{2+}]_i$ are induced by opening of voltage- or store-operated Ca^{2+} channels. This Ca^{2+} storage compartment is insensitive to mitochondrial uncouplers and appears diffusely distributed in the cell cytosol. The possibility is discussed that this low-affinity, high-capacity Ca^{2+} pool represents a previously unidentified subcompartment of the ER expressing a Tg-insensitive Ca^{2+}–ATPase.

Materials and Methods

Materials

Tissue culture medium and complements were purchased from Technogenetics (Milan, Italy); indo-1 and indo-1/AM were from Molecular Probes (Eugene, OR); S202791 was a gift of Dr. D. Pietrobon (University of Padova); and all other reagents were from Sigma Chemical Co. (St. Louis, MO).

Cell Culture and Transfection

Rat pituitary GH3 cells (from Dr. Hescheler, University of Berlin, Germany) were grown in Ham's F-10 medium supplemented with 15% horse serum, 2.5% FCS, nonessential amino acids, and penicillin/streptomycin. The clonal cell lines GC and GH3B6 (from Dr. Argenton, University of Padova), derived from GH3 cells, were maintained in the same conditions (with the addition of 10^{-7} M estradiol for the latter cell type). The pheochromocytoma cell line PC12 was grown in RPMI supplemented with 12.5% horse serum, 2.5% FCS, and gentamycin. The rat basophilic leukemia cell line RBL-1 (from Dr. Penner, Max-Planck-Institut, Göttingen, Germany) was cultured in DME supplemented with 10% FCS and penicillin/streptomycin. For Ca^{2+} measurements, all cells were allowed to attach to poly-L-lysine–coated glass coverslips and grow for 1 d.

Transient transfection with recombinant cytosolic or mitochondrial aequorin (cytAEQ or mtAEQ) (Rizzuto et al., 1992) was made by electroporation (Kodak, IBI, Rochester, NY). Cells were harvested and resuspended in fresh medium in 4-mm cuvettes in the presence of 10 μg of cytAEQ/VR1012 or mtAEQ/VR1012 plasmid/10^6 cells. Cells were subjected to a single pulse characterized by an electric field of 300 V, 1,500 μF. Transfected cells were transferred to 13-mm-diam poly-L-lysine–coated glass coverslips (10^6 cells/coverslip), and after an overnight incubation, the medium was changed and the incubation continued under the same conditions. After 2 d, the cells were used for Ca^{2+} measurements, according to the procedure described in detail elsewhere (Rizzuto et al., 1994).

Ca^{2+} Measurements

Cells, grown on coverslips (24-mm-diam), were loaded with indo-1 by incubation with 5 μM indo-1/AM at 37°C for ~30 min in Ham's F-10 medium containing 3% FCS and 0.04% pluronic acid. After washing the cells with a modified Krebs-Ringer medium (mKRB: in mM, 125 NaCl, 5 KCl, 1 $MgSO_4$, 1 Na_3PO_4, 1 $CaCl_2$, 20 Hepes, 5.5 glucose, pH 7.4), the coverslips were mounted in a chamber and placed on the stage of an inverted microscope (model Diaphot 300; Nikon Europe B.V., Badhoevedorp, The Netherlands), equipped with a 40× water immersion objective (NA=1.1; Nikon) and connected with a real-time UV confocal system (model RCM-8000; Nikon). The 351-nm band of the argon ion laser was used for excitation and the emitted light, separated into its two components (405 and 485 nm) by a dichroic mirror, was collected by two photomultipliers. The ratio of the intensity of the light emitted at the two wavelengths (F405/F485), a function of $[Ca^{2+}]_i$, was displayed as a pseudocolor scale. Unless otherwise indicated, all experiments were performed at room temperature and time series were acquired with a frame interval of 2 s, averaging 16 images for each frame. Online analysis of the ratio was obtained from the signals of individual cells. For presentation, ratios were off-line averaged and normalized to the average value obtained in Ca^{2+}-free medium before ionomycin addition.

For measurements of cytosolic or mitochondrial free Ca^{2+} concentration ($[Ca^{2+}]_m$) with recombinant aequorins, cells were transiently transfected with cytAEQ or mtAEQ and seeded onto 13-mm-diam poly-L-lysine–coated glass coverslips (10^6 cells/coverslip) 2 d before the experiment. Aequorin was reconstituted by adding 5 μM coelenterazine to the culture medium 1–2 h before the experiment. During the experiment, cells were continuously perfused with mKRB containing different stimuli. At the end of each experiment, cells were lysed by perfusion with an ipoosmotic solution containing only digitonin (100 μM) and 10 mM $CaCl_2$ to expose all the cellular aequorin to a high $[Ca^{2+}]$. Light emission was measured by a purpose-built luminometer and calibrated in terms of $[Ca^{2+}]_i$ or $[Ca^{2+}]_m$ as described (Rizzuto et al., 1994; Brini et al., 1995).

The total content of cellular Ca^{2+} was assayed by atomic absorption spectrophotometry. Cells were suspended in mKRB (10^7 cells/ml) and challenged with 30 mM KCl (GH3 cells) or 1 μM Tg (RBL-1 cells) for 3 min before addition of 4 mM EGTA. Control experiments were carried out under the same conditions in Ca^{2+}-free mKRB containing 1 mM EGTA. After centrifugation, cells were resuspended in Ca^{2+}-free mKRH containing 1 mM EGTA and centrifuged for 1 min at 14,000 rpm in Eppendorf tubes containing 100 μl sucrose 12.5% and 400 μl silicon oil. The pellet was resuspended in 0.05% Triton plus 0.2 N NaOH before measurement.

Immunolocalization of TGN38

GH3 cells were fixed and immunostained as described previously (Brini et al., 1995). The antibody, used at 1:200 dilution (kind gift of Dr. O. Rossetto, University of Padova), was against the type I membrane protein TGN38, a marker of the *trans*-Golgi network (Reaves et al., 1996). Binding of the antibody was revealed with an FITC-labeled anti–rabbit IgG antibody. Fluorescence was analyzed with a microscope (model RCM 8000; Nikon)

and photographed (Technical Pan film; Kodak) after digital subtraction of the background.

Results

The Ionomycin-sensitive Ca^{2+} Pool in GH3 Cells Increases after Depolarization, and Its Size in the Cell Population Is Heterogeneous

The existence of different Ca^{2+} storage compartments whose content can be discharged in the cytoplasm and/or in the extracellular medium by treatment with a variety of agents appears to be a widespread characteristic of eukaryotic cells. Initially in the neuroendocrine cell line PC12 (Fasolato et al., 1991; Zacchetti et al., 1991), and later in several other cell types, we have functionally distinguished three types of intracellular Ca^{2+} pools: (*a*) an InsP3-sensitive pool, largely identified with the ER and endowed with a Tg-sensitive Ca^{2+}–ATPase; (*b*) an acidic pool, whose Ca^{2+} content could be released into the cytoplasm by Ca^{2+} ionophores (A23187 or ionomycin), but only after neutralization of the luminal acidic pH with monensin or NH_4Cl; and (*c*) a Ca^{2+} pool that could be discharged by treatment only with Ca^{2+} ionophores. This last Ca^{2+} store, presumably heterogeneous, is the most mysterious in terms of cytological nature, mechanism of loading, and physiological role. The experiments presented below were aimed at the characterization of this elusive Ca^{2+} compartment, using as a model system primarily the rat pituitary cell line GH3.

Fig. 1, *a* and *b*, shows the typical protocol used to ascertain the existence of the different Ca^{2+} pools in GH3 cells. Cells, loaded with the fluorescent Ca^{2+} indicator indo-1, were incubated in mKRB medium and analyzed by confocal microscopy. The cells were treated in sequence with EGTA, to chelate extracellular Ca^{2+}; thyrotropin-releasing hormone (TRH), an agonist coupled to InsP3 generation; a SERCA inhibitor, such as Tg; ionomycin, a Ca^{2+} ionophore; and monensin, a Na^+-K^+/H^+ ionophore. Addition of TRH induced a rapid and transient increase in $[Ca^{2+}]_i$, while Tg, after TRH, caused a barely detectable rise. The subsequent challenge with ionomycin resulted in a further small increase of $[Ca^{2+}]_i$, and finally monensin discharged the acidic pool.

The kinetics of the $[Ca^{2+}]_i$ peaks reflect the balance between Ca^{2+} efflux from the stores, pumping out of the cell and reuptake into the stores themselves. However, the integral of the curve underlying the $[Ca^{2+}]_i$ rise, induced by each stimulus, mirrors, to a reasonable level of approximation, the Ca^{2+} content of the different stores as estimated by $^{45}Ca^{2+}$ measurements (Fasolato et al., 1991; Bastianutto et al., 1995; Mery et al., 1996) and can be used to calculate the size of the different pools. Accordingly (see Table I), the TRH- and Tg-releasable pools represent altogether 38%, the ionomycin-pool 17%, and the monensin-pool 45% of total mobilizable Ca^{2+}. In Fig. 1 *a*, the trace is the mean of 30 cells, while in *b* the kinetic changes of $[Ca^{2+}]_i$ in four typical cells are presented. As previously reported, in Ca^{2+}-containing medium, GH3 cells underwent spontaneous oscillations that ceased immediately upon addition of EGTA (Schlegel et al., 1987). Noteworthily, the size of the various Ca^{2+} pools was quite homogeneous in the cell population.

No $[Ca^{2+}]_i$ increase was detected upon application of 10 mM caffeine (not shown), although in another pituitary cell line (GH4C1), a caffeine-sensitive store has been identified (Law et al., 1990; Tanaka and Tashjian, 1993).

Fig. 1, *c* and *d*, shows that 1-min depolarization with 30 mM KCl in Ca^{2+}-containing medium affected the size of the different pools. In particular, *c*, mean of 30 cells, shows that addition of KCl induced a sharp increase of $[Ca^{2+}]_i$
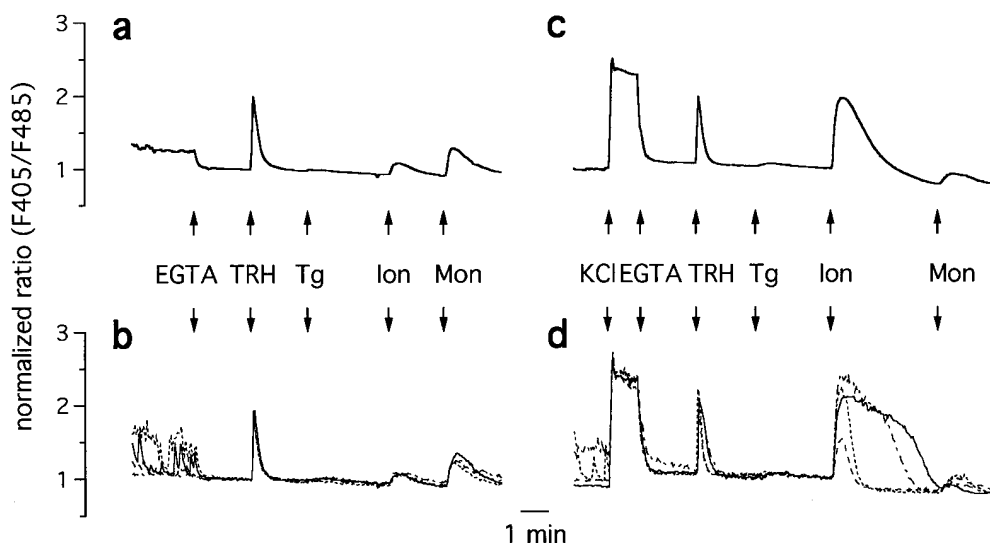

Figure 1. Dynamic properties of Ca^{2+} pools in GH3 cells. Experiments were carried out on monolayers of GH3 cells, loaded with indo-1, as described in Materials and Methods. The traces in *a* and *c* are an average of 30 cells, while in *b* and *d*, four typical single cell traces of the same experiment are presented. In this and the following figures, representative of at least three experiments carried out in different cell batches, the normalized ratio of the intensity of the light emitted at the two wavelengths (F405/F485), a function of $[Ca^{2+}]_i$, is displayed on the left-hand side. Where indicated, KCl (30 mM), EGTA (4 mM), TRH (1 μM), Tg, (1 μM), ionomycin (*Ion*; 1 μM) and monensin (*Mon*; 1 μM) were added.

Table I. Analysis of Releasable Ca^{2+} in GH3 Cells

	Δ peak*		Integral of the peak*	
	Control	KC1 treated	Control	KC1 treated
TRH	1.15 ± 0.3	1.03 ± 0.2	18.42 ± 5.7	12.92 ± 4.1
Tg	ND	ND	1.89 ± 1.2	3.17 ± 2.2
Ion	0.18 ± 0.6	0.98 ± 0.3	9.12 ± 2.1	88.42 ± 46.1
Mon	0.42 ± 0.1	0.13 ± 0.1	24.10 ± 5.8	10.24 ± 2.6

*Values, expressed as arbitrary ratio units (mean ± SD, $n = 30$), were measured from the single cell traces of the experiment shown in Fig. 1. Data analysis and integral of the peaks, after baseline subtraction, have been obtained with Igor Pro 2.0 (Wavemetrics, Lake Oswego, Oregon).

that rapidly subsided upon chelation of extracellular Ca^{2+} with EGTA (or addition of an L channel blocker such as nifedipine; not shown). The kinetics and amplitude of TRH- and Tg-induced [Ca^{2+}]$_i$ rise were hardly different from those of control cells (Fig. 1 *a* and Table I), while a massive increase occurred in the ionomycin-releasable pool. When the other Ca^{2+} ionophore A23187 was used instead of ionomycin, the results were indistinguishable (see below). This Ca^{2+} ionophore-sensitive pool induced by KCl stimulation will be referred to, for simplicity, as stimulus-induced-calcium (SIC) pool. Finally, the monensin pool appeared decreased by KCl-induced depolarization, as expected if it is accounted for, at least in part, by Ca^{2+} sequestered within secretory compartments. Similar results for both controls and treated cells have been obtained when the experiments were carried out at 37°C (not shown).

Fig. 1 *d* shows the behavior of four typical cells. While all the examined cells reacted to KCl stimulation with high and sustained [Ca^{2+}]$_i$ rises, the increase of the SIC pool was consistently observed in >50% of the cells. In particular, out of the 30 cells analyzed in this experiment, in a fraction of them (10%) the ionomycin peak and the integrated area were indistinguishable from those of controls, while in ∼30% the increase in the integrated area was two to sixfold higher than in controls, and the increase in the remaining 60% was between 6- and 15-fold. On average, in this experiment, there was a 10-fold increase in the area of the SIC pool (Fig. 1, *a* and *c*). In a series of similar experiments ($n = 33$), in 16 trials the averaged area of this pool was between 6–10-fold the control value, in 15 trials the area was three to fivefold, and the area hardly increased in the remaining two trials.

The possibility was considered that the increase of the SIC pool was only apparent and due to an impairment of the Ca^{2+} extrusion mechanisms. Two types of evidence argue against this possibility: (*a*) The peaks and kinetics of decay of the TRH-induced [Ca^{2+}]$_i$ increase were similar in cells that have been treated with KCl when compared to controls (1.90 ± 0.17 and 1.88 ± 0.04 normalized ratio U for the peak, and 14.9 ± 0.9 and 9.8 ± 0.3 s for the half time of decay in control and KCl-treated cells, respectively [mean ± SD]) and (*b*) the substitution of Na$^+$ with *N*-methyl-glucamine$^+$ in the medium (to inhibit any contribution of the Na$^+$/Ca^{2+} exchanger) resulted in no alteration of the kinetics and amplitude of the [Ca^{2+}]$_i$ transients caused by the different stimuli (not shown). These results suggest that, overall, extrusion of Ca^{2+} into the medium or reaccumulation into the stores is not grossly affected by depolar-

ization. In addition, these experiments indicate that, in GH3 cells, the Na$^+$/Ca^{2+} exchanger has minor relevance in Ca^{2+} extrusion, at least under our experimental conditions.

Ca^{2+} indicators, such as indo-1, when loaded as acetoxy-methyl-esters, are not only found in the cytosol but also trapped within intracellular organelles. In addition, given that their final cytosolic concentration is in the order of 20–100 μM, they substantially increase the cytosolic Ca^{2+} buffering capacity. To determine whether the appearance of the SIC pool was at least in part an artifact of the methodology used, the [Ca^{2+}] in the cytosol was measured with recombinant aequorin. In this latter case, the increase in Ca^{2+} buffering capacity is negligible and the transfected
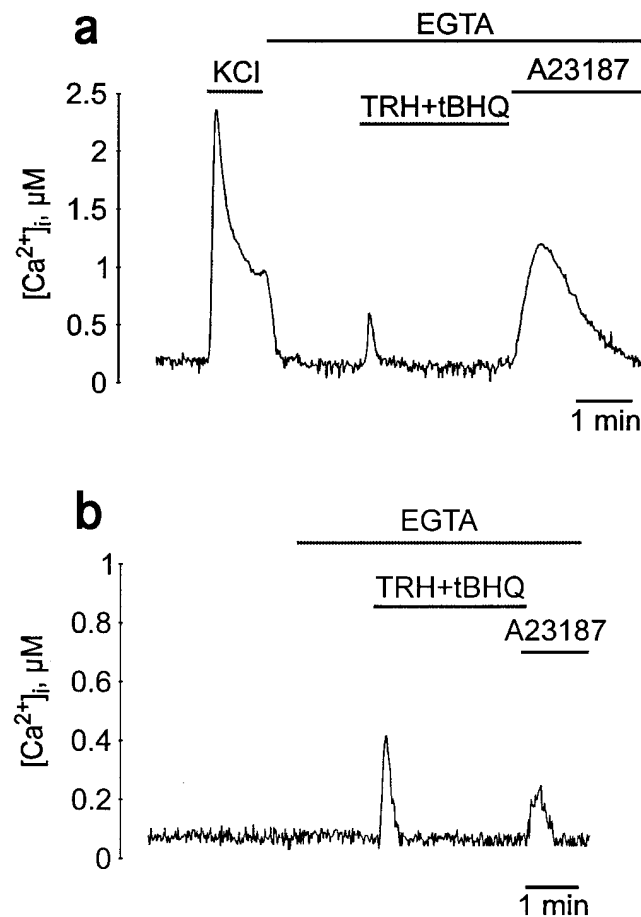

Figure 2. Ca^{2+} pools in GH3 cells revealed by cytAEQ transfection. Reconstitution with coelenterazine, as well as detection and calibration of the luminescence signal into [Ca^{2+}]$_i$ values, were performed as described in Materials and Methods. These experiments were carried out in mKRB at 37°C, under perfusion; similar results were obtained at room temperature. tBHQ and A23187 were preferred to Tg as a SERCA blocker and to ionomycin as a Ca^{2+} ionophore because the latter compounds tend to adhere to the plastic tubes of the perfusion system. For the same reason, TRH was used at 10 times higher concentration compared to confocal experiments. Indeed, in confocal experiments, tBHQ, A23187, and TRH at high doses induced [Ca^{2+}]$_i$ rises comparable to those measured using Tg (1 μM), ionomycin (1 μM), or TRH (1 μM). Where indicated, the cells were stimulated with KCl (30 mM), EGTA (4 mM), TRH (10 μM), tBHQ, (50 μM), and A23187 (20 μM).

protein is excluded from organelles (Brini et al., 1995). Fig. 2 shows that, while in control cells, A23187 added after TRH and tBHQ caused a marginal increase in $[Ca^{2+}]_i$ (Fig. 2 b), a very large rise was caused by the ionophore in cells pretreated with KCl (Fig. 2 a). In the experiments with recombinant aequorin (see also below), tBHQ and A23187 were used instead of Tg and ionomycin because the latter compounds tend to adhere strongly to the tubes of the perfusion system (Rizzuto et al., 1994).

To further confirm that the increase in the ionomycin response was indeed due to Ca^{2+} accumulation and not to impairment of the extrusion mechanisms, the total cellular Ca^{2+} content was measured by atomic absorption (see Materials and Methods). After 3 min of exposure to 30 mM KCl in mKRB medium, the total Ca^{2+} content increased by 43% compared to controls (5.65 ± 0.74 and 3.94 ± 0.4 nmol/mg protein, respectively; $n = 5$ for each condition, $P < 0.002$, Student's t test).

The SIC Pool Is Not Identifiable with Known Ca^{2+} Stores

One of the key features of the SIC pool, besides its InsP$_3$ insensitivity, is its resistance to unloading by classical SERCA inhibitors. Fig. 3 a demonstrates that Tg, when added before depolarization, even at doses as high as 10 μM, while inhibiting the TRH-induced $[Ca^{2+}]_i$ changes, did not significantly decrease the amplitude and kinetics of the
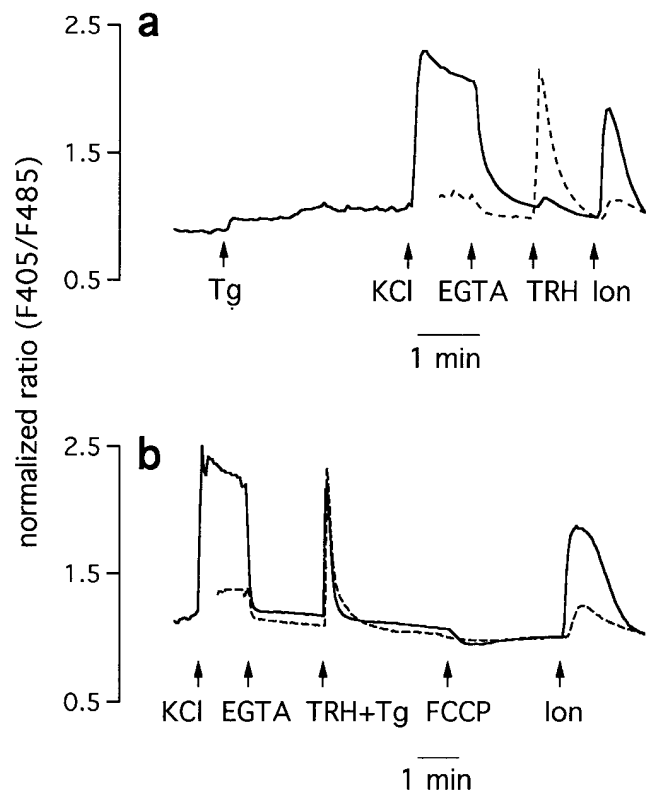

Figure 3. Tg and FCCP insensitivity of the SIC pool. Effect of Tg (*a*) and FCCP (*b*). Conditions as in Fig. 1; in both panels, the dashed trace represents control cells. Where indicated, KCl (30 mM), EGTA (4 mM), TRH (1 μM), Tg (10 μM in *a*, 1 μM in *b*), ionomycin (*Ion*; 1 μM), and FCCP (10 μM) were added.

Ca^{2+} rise induced by ionomycin. Other SERCA inhibitors such as tBHQ (30 μM) and CA (10 μM) were similarly ineffective (not shown). Thus, not only are SERCA inhibitors unable to deplete this pool, but they are also ineffective in preventing its loading.

The SIC pool was also unaffected by caffeine (10 mM) or ryanodine (10 μM), whether added before or after KCl depolarization (not shown). Last but not least, preincubation with monensin resulted in an increase in the ionomycin-dependent $[Ca^{2+}]_i$ rise of both controls and KCl-treated cells. The integrals of the curves, however, were equivalent to the sum of the individual ionomycin- and monensin-induced $[Ca^{2+}]_i$ increases. Thus, alkalinization of luminal pH does not prevent loading of the SIC pool.

The insensitivity to SERCA inhibitors and caffeine/ryanodine, as well as the ability to load efficiently only as a consequence of large $[Ca^{2+}]_i$ increases, appear compatible with the identification of the SIC pool with mitochondria (for reviews see Pietrobon et al., 1990; Gunter et al., 1994; Pozzan et al., 1994). Two lines of evidence, however, argue against this hypothesis. Fig. 3 b shows that in cells subjected to the same protocol of Fig. 1 c (i.e., KCl for 1 min followed by EGTA, TRH, and Tg), the addition of the mitochondrial uncoupler *p*-(trifluoro-methoxy)phenylhydrazone (FCCP, 10 μM) caused no increase of $[Ca^{2+}]_i$ per se and, more importantly, hardly affected the amplitude and kinetics of the ionomycin-induced $[Ca^{2+}]_i$ rise. The area of the SIC pool was 57.1 ± 12.3 and 60.5 ± 24.8 normalized ratio U (mean $\pm$ SD, $n = 3$) in the presence and absence of FCCP, respectively. At these concentrations, FCCP completely collapses the mitochondrial membrane potential and, in isolated organelles, causes rapid release of accumulated Ca^{2+}. Accordingly, the inefficacy of the uncoupler in reducing the size of the SIC pool strongly argues against the possibility that mitochondria represent a significant part of this store. Similar results were obtained when the mitochondrial membrane potential was collapsed by a combination of the ATP-synthase inhibitor oligomycin (2 μM) and the mitochondrial complex–I inhibitor rotenone (4 μM) (not shown).

The role of mitochondria was directly tested in the experiment presented in Fig. 4. GH3 cells were transiently transfected with the cDNA coding for recombinant mtAEQ (Rizzuto et al., 1992). Fig. 4 shows that the mean resting $[Ca^{2+}]_m$ of GH3 cells was somewhat higher than that reported previously in other cell types, such as HeLa or endothelial cells ($\sim$500 vs. $\sim$200 nM) (Rizzuto et al., 1994; Lawrie et al., 1996). This, however, was not unexpected since, as shown in Fig. 1 b, GH3 cells undergo spontaneous $[Ca^{2+}]_i$ oscillations. Addition of KCl resulted in a very sharp and large increase of $[Ca^{2+}]_m$, with a peak around or above 10 μM. After the peak, $[Ca^{2+}]_m$ rapidly returned toward basal and addition of EGTA caused a further decrease to, and below, resting level. While perfused with Ca^{2+}-free, EGTA-containing medium, addition of TRH + tBHQ resulted in an increase of $[Ca^{2+}]_m$ to $\sim$3 μM, and finally the Ca^{2+} ionophore A23187 caused a further slow and reproducible increase. Thus, the ionophore caused an increase in $[Ca^{2+}]_m$ (presumably by equilibrating the Ca^{2+} concentration of the cytosol with that of the mitochondrial matrix) and not a decrease, as expected if mitochondria represented a significant part of the SIC pool.
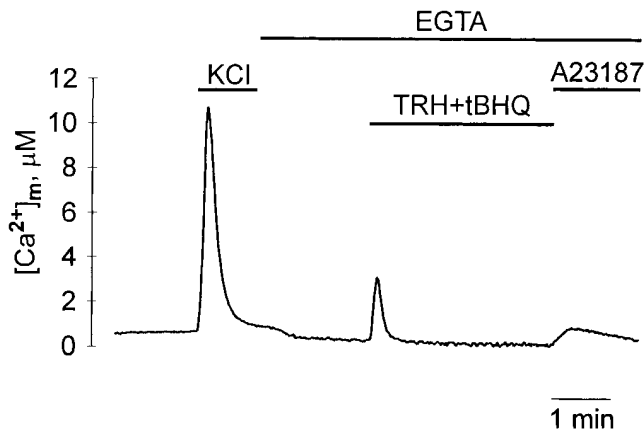
EGTA

KCl

A23187

TRH+tBHQ

12
10
8
6
4
2
0

$[Ca^{2+}]_m$, μM

1 min

Figure 4. $[Ca^{2+}]_m$ values in GH3 cells after mtAEQ transfection. GH3 cells were transfected with mtAEQ (Materials and Methods). After reconstitution with coelenterazine, the experiments were carried out as described in Fig. 2. Where indicated, the cells were stimulated with KCl (30 mM), EGTA (4 mM), TRH (10 μM), tBHQ (50 μM), and A23187 (20 μM).

Loading and Unloading Properties of the SIC Store

The experiments shown in Fig. 5 were undertaken to define the kinetics of loading and unloading of the SIC pool. A 10-s exposure to 30 mM KCl (Fig. 5 *a, continuous trace*) failed to induce a reproducible increase, compared to controls, of the ionomycin-induced rise of $[Ca^{2+}]_i$, while a 20-s depolarization caused, in 50% of the trials ($n = 13$), a significant increase in the size of this store (Fig. 5 *a, dotted trace*). After 60 s of depolarization, the increase of $[Ca^{2+}]_i$ caused by the Ca^{2+} ionophore was similar to that observed after 5 min of KCl exposure (Fig. 5 *a, long- and short-dashed traces*, respectively). In addition, the SIC pool loaded faster and was larger if the depolarization was carried out in high external $[Ca^{2+}]$ (5 mM) (compare Fig. 5 *b*

vs. *a*). In nondepolarizing conditions, the size of the pool was not appreciably affected by the increase in $[Ca^{2+}]_{out}$ (not shown).

In the experiments presented in Figs. 1–4, loading of the SIC pool was caused by a prolonged depolarization with KCl. The question then arises as to whether other stimuli causing Ca^{2+} influx can similarly induce an increase in the size of this Ca^{2+} store. The amplitude and kinetics of the ionomycin-sensitive pool were thus investigated in cells pretreated for 3 min with TRH in Ca^{2+}-containing medium, a condition that discharges the $InsP_3$-sensitive store and activates voltage-operated Ca^{2+} channels (VOCCs) (Gollasch et al., 1991; Mantegazza et al., 1995) and, presumably, store-dependent Ca^{2+} influx. Fig. 5 *c* shows that TRH addition in Ca^{2+}-containing medium resulted in a biphasic Ca^{2+} transient, a sharp rise followed by a sustained plateau that is much smaller, however, than that induced by KCl. Upon chelation of extracellular Ca^{2+} with EGTA, the addition of ionomycin, after Tg, resulted in a transient $[Ca^{2+}]_i$ increase, which was, however, indistinguishable from that of cells treated with TRH and Tg in Ca^{2+}-free, EGTA-containing medium (Fig. 5 *c, continuous and dashed traces*, respectively). Thus, the influx of Ca^{2+} induced by TRH is insufficient to induce a significant increase in the size of the SIC pool.

The kinetics of the unloading of the SIC pool were next investigated. The protocol was the following: the pool was first loaded with Ca^{2+} through a 1-min exposure to KCl depolarization, followed by EGTA addition, to block Ca^{2+} influx through VOCCs. The cells were then washed and kept, for different times, in Ca^{2+}-containing medium. Finally, after readdition of EGTA, the size of the pools was tested, as before, by addition of TRH + Tg followed by ionomycin. Fig. 5 *d* shows that complete unloading of the SIC pool required a total of 20–30 min between the end of KCl to the ionomycin treatment.

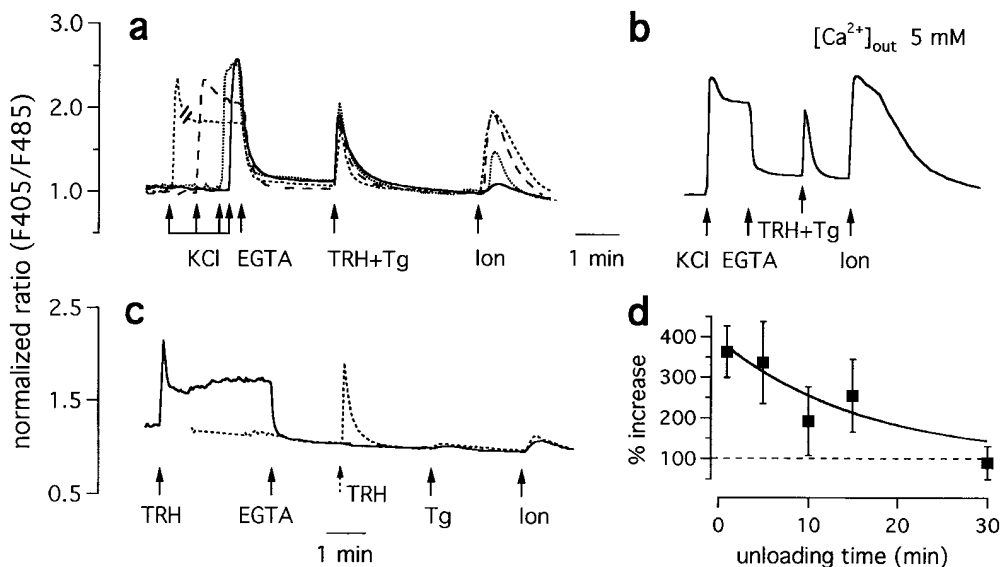The insensitivity of the SIC pool to Tg and monensin in-

Figure 5. Kinetic properties of the SIC pool. (*a*) Time course of Ca^{2+} uptake into the SIC pool (*continuous trace*, 10 s; *dotted trace*, 20 s; *long-dashed trace*, 60 s; *short-dashed trace*, 5 min). (*b*) Dependence of the SIC pool on external Ca^{2+} concentration. Conditions as in Fig. 1, but with 5 mM external Ca^{2+}. (*c*) A prolonged $[Ca^{2+}]_i$ rise was achieved by stimulating cells with TRH (1 μM) in mKRB (*continuous trace*). Control cells were stimulated with TRH in EGTA-containing mKRB (*dashed trace*). (*d*) Unloading kinetics of the Ca^{2+} pool released by ionomycin. Cells were depolarized with 30 mM KCl for 60 s, washed with fresh medium, and incubated for the indicated time in mKRB medium. Data are presented as percentage increase in the area of the SIC pool with respect to the value measured in control unstimulated cells (100%) (mean ± SEM of three experiments). In *a–c*, where indicated, KCl (30 mM), TRH (1 μM), EGTA (4 mM), Tg (1 μM), and ionomycin (*Ion*; 1 μM) were added.

dicates that Ca^{2+} uptake in this pool is not driven by H^+ or Na^+ gradients and does not use classical SERCAs. We considered the possibility that Ca^{2+} accumulation in the SIC pool occurred by a mechanism of vesicle endocytosis after KCl-induced secretion. However, the lack of a significant uptake of Lucifer yellow, a marker of fluid phase endocytosis, in KCl-treated cells (not shown) makes this possibility unlikely. If it occurred through endocytosis, the amount of Ca^{2+} increase ($\cong 0.4$ mmol/l of cell water) caused by depolarization would have, in fact, implied an accumulation of extracellular medium that was not only rapid but also exceedingly vast, i.e., corresponding to about 1/4 of the cell volume.

Characteristics of the SIC Pool in Other Cell Types

A Ca^{2+} pool with characteristics similar to those identified in GH3 cells was found also in other neuroendocrine cell lines, i.e., two pituitary cell lines, GC and GH3B6 (not shown), and in PC12 cells, derived from a rat pheochromocytoma. In this latter cell line, however, the SIC pool was detectable only when depolarization with KCl was carried out in the presence of an agonist of L type VOCCs, S202791 (1 μM) (Fig. 6, *a* and *b*). In the absence of this drug, in fact, KCl-induced depolarization resulted in a plateau level of $[Ca^{2+}]_i$ consistently lower than in GH3 cells. Thus, considerable Ca^{2+} accumulation in the SIC pool requires a $[Ca^{2+}]_i$ rise of large amplitude and long duration.

A low-affinity, high-capacity SIC pool was also found in nonexcitable cells, such as the rat basophilic leukemia cell line, RBL-1. In these cells, large loading of this pool could not be achieved by membrane potential depolarization, since they do not possess VOCCs, but rather through activation of another Ca^{2+}-selective current, named I_{CRAC}, for Ca^{2+} release-activated Ca^{2+} current (Hoth and Penner, 1992; Fasolato et al., 1993), maximally activated by Tg (for review see Fasolato et al., 1994). Fig. 6 *d* shows that Tg induced a long-lasting elevation of $[Ca^{2+}]_i$ that rapidly returned to basal level upon EGTA addition. Under these conditions, ionomycin induced a large, transient increase in $[Ca^{2+}]_i$, severalfold higher and more prolonged than that caused by the ionophore in cells treated with Tg in EGTA-containing medium (*c*). Similarly to GH3 cells, the increase in total cellular Ca^{2+} content, as estimated by atomic absorption, accounted for ~33% ($n = 3$). Moreover, in these cells addition of monensin (1 μM) after ionomycin never elicited a $[Ca^{2+}]_i$ rise, either in control or stimulated cells (not shown). Finally, also in the RBL-1 cells the SIC pool was insensitive to caffeine (10 mM) and ryanodine (10 μM), added either before or after Tg stimulation, and to the mitochondrial uncoupler FCCP (10 μM) (not shown).

Intracellular Localization

A final property of the SIC pool here investigated is its intracellular distribution. In both GH3 and RBL-1 cells, high-resolution (67 ms/ratio frame) analysis by confocal microscopy failed to reveal a localized origin of the ionomycin-induced response. In fact, Fig. 7 shows that, upon ionomycin addition in both GH3 (*a*) and RBL-1 cells (*b*), Ca^{2+} increased rather homogeneously in the cytosol and quickly diffused to the central nuclear region, with no evidence for a localized origin of the $[Ca^{2+}]_i$ rise. These observations would tend to exclude the Golgi complex, which has been previously suggested to accumulate Ca^{2+} (Virk et al., 1985; Chandra et al., 1991; Wahl et al., 1992) as the major source of the ionomycin-releasable Ca^{2+}. As confirmed by immunofluorescence, the Golgi network is, in fact, specifically localized to a distinct perinuclear region (Fig. 8 *c*). It may be argued, however, that the increase in $[Ca^{2+}]_i$ caused by the ionophore is relatively slow, i.e., 8.1 ± 2.9 s to the peak (mean ± SD, in 12 similar experiments with GH3 cells), and thus the localization of the increase may be masked by diffusion of Ca^{2+} and/or of the indicator.

To obtain further insights into the possible involvement of the Golgi complex, induction of the SIC pool was monitored in the continuous presence of brefeldin A (BFA) (10 μg/ml, after a pretreatment of 15 min at 37°C). BFA is known to disassemble the Golgi network (Lippincott-Schwartz et al., 1989) and to cause a major intermixing of membrane and lumenal Golgi components with the ER. Thus, the prediction will be that if the SIC pool was largely identifiable with the Golgi, BFA treatment should make it largely sensitive to Tg. Fig. 8 shows that in both RBL-1 (*a*) and GH3 cells (*b*), BFA did not significantly affect the size of the SIC pool, although it caused the known fragmentation of the Golgi complex (*c*).
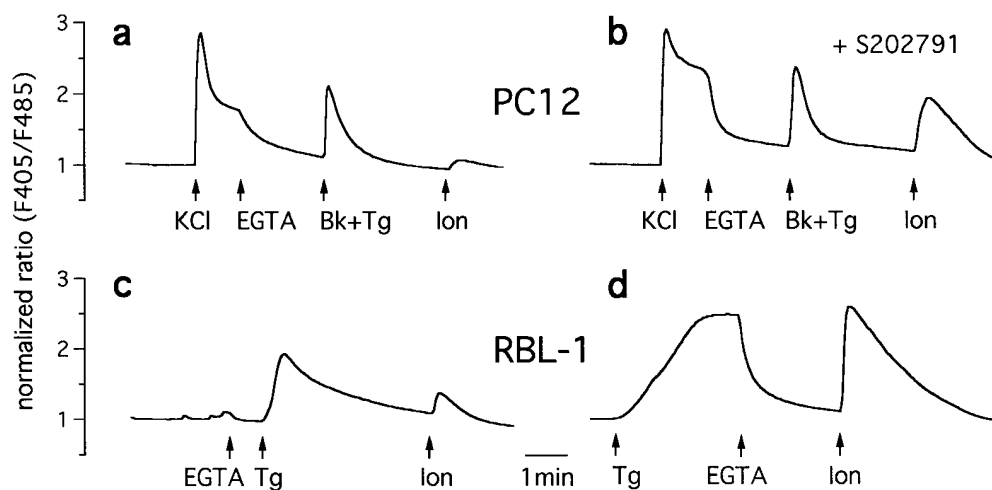

Figure 6. Detection of the SIC pool in other cell lines. Cells from the neuroendocrine cell line PC12 were stimulated with a protocol similar to that described in Fig. 1 *c*, in the absence (*a*) or in the presence (*b*) of the agonist of L-VOCCs, S202791. *c* and *d* show the behavior of the rat basophilic leukemia cell line RBL-1. Where indicated, KCl (60 mM), EGTA (4 mM), bradykinin (*Bk*; 1 μM), Tg (1 μM), ionomycin (*Ion*; 1 μM), and the L-VOCCs agonist S202791 (1 μM) were added.
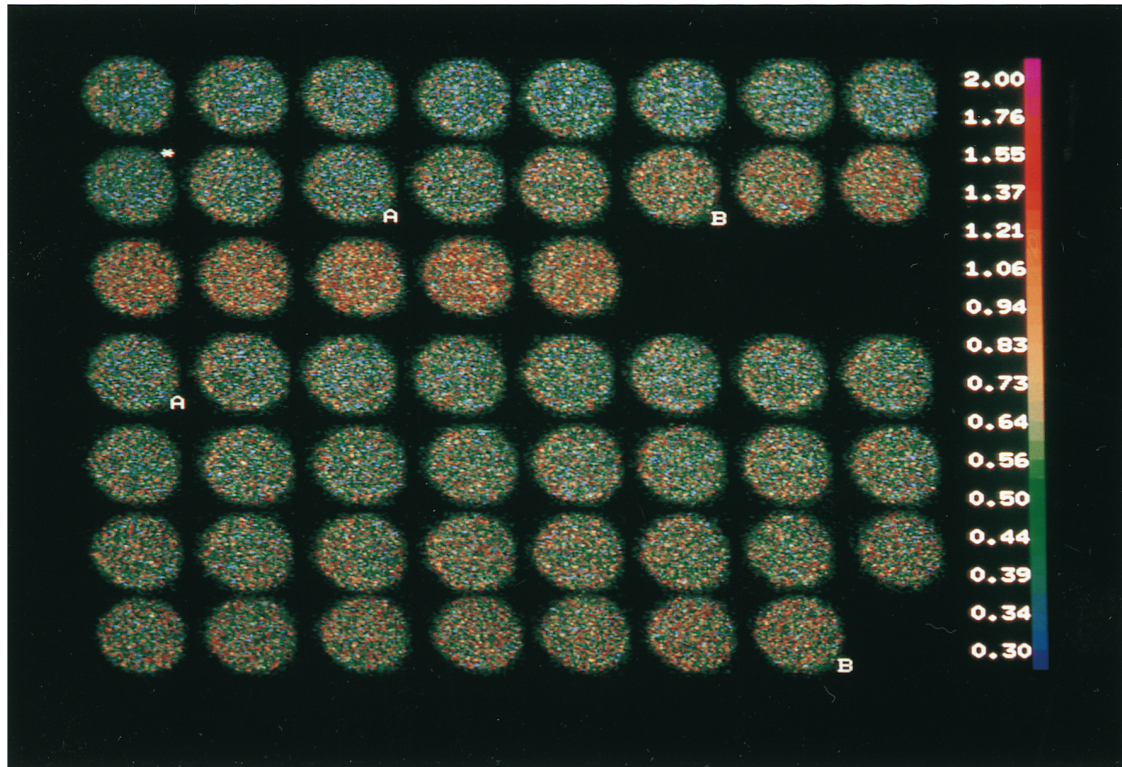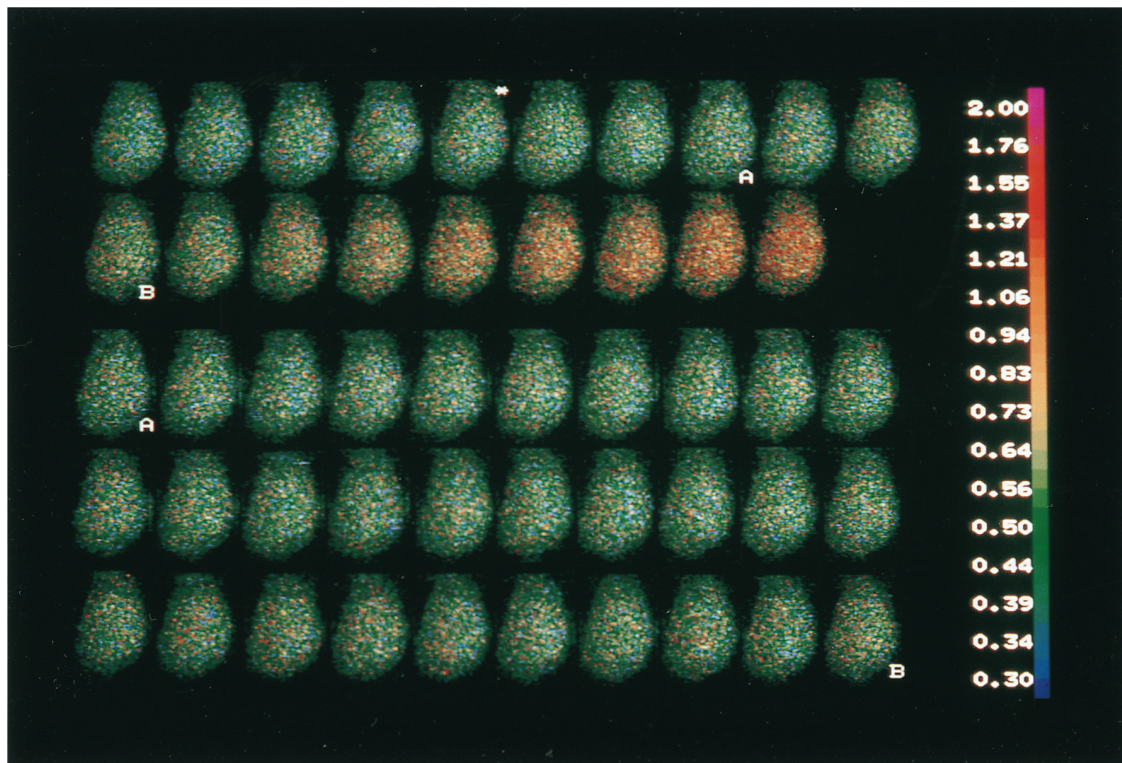
Figure 7. High-resolution confocal imaging of $[Ca^{2+}]_i$ rises induced by ionomycin. GH3 (*a*) and RBL-1 cells (*b*) were treated as described in Figs. 1 *c* and 6 *c*, respectively, to induce the SIC pool. Before ionomycin addition (*), the acquisition was changed from 2 s/ratio frame (16 images/frame) to 67 ms/ratio frame (1 image/frame). The first group of pictures (on the top of each panel) presents pseudocolor ratio images with a frame interval of 670 ms. The second group of pictures (on the bottom of each panel) represents images of the same cell (from *A* to *B*) with a frame interval of 67 ms. The ratio of the intensity of the light emitted at the two wavelengths (F405/F485), a function of $[Ca^{2+}]_i$, is displayed as a pseudocolor scale on the right side of each panel.
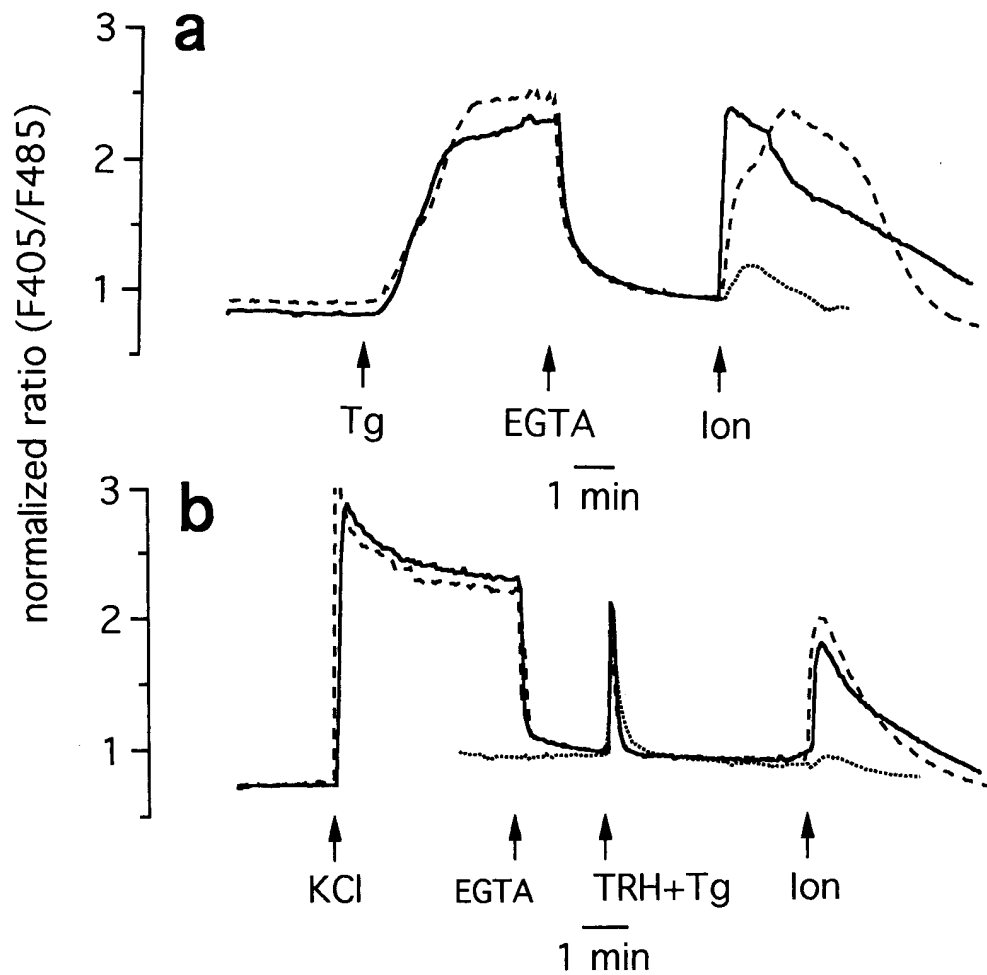
a — normalized ratio (F405/F485) axis (values 1, 2, 3)
Arrows: Tg, EGTA, Ion
1 min

b — normalized ratio axis (values 1, 2, 3)
Arrows: KCl, EGTA, TRH+Tg, Ion
1 min

c

control   + BFA

Figure 8. Effect of BFA on the SIC pool. RBL-1 (*a*) and GH3 cells (*b*) were pretreated with BFA (10 μg/ml) at 37°C during the last 15 min of the loading period with indo-1/AM. Experiments were carried out as described in Figs. 1 *c* and 6 *c* for GH3 and RBL-1 cells, respectively, in the absence (*dashed traces*) or in the continuous presence of BFA (10 μg/ml; *continuous traces*). The dotted traces represent unstimulated control cells not treated with BFA. For clarity, in *a*, only the ionomycin addition to control cells (*dotted trace*) is shown. Where indicated, KCl (30 mM), TRH (1 μM), EGTA (4 mM), Tg (1 μM), and ionomycin (*Ion*; 1 μM) were added. Immunolocalization of the *trans*-Golgi network protein TGN38 (*c*; see Materials and Methods for details) in GH3 cells, with or without BFA. Bar, 4.5 μm.
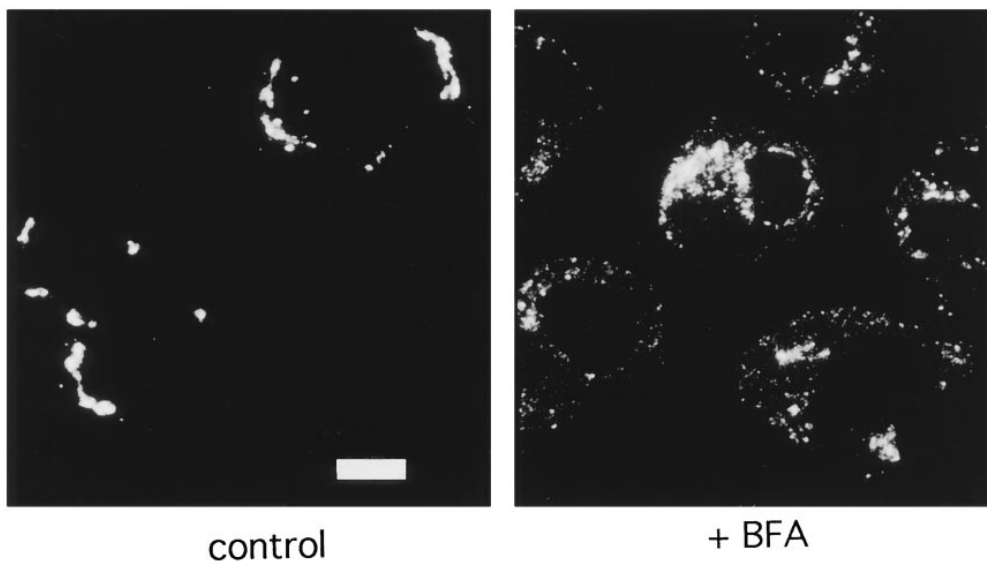
Discussion

The Ca^{2+} concentration in the cytoplasm controls a variety of cellular functions and is in dynamic equilibrium with that of intracellular organelles. Within the lumen of differ-

ent compartments, Ca^{2+} plays different roles, such as that of a reservoir to be mobilized upon cell activation (e.g., in the ER and its subcompartments), of a modulator of enzymatic reactions (e.g., in the mitochondria), and of a cofac-

tor in the packaging of secretory products (e.g., in the Golgi complex and secretory vesicles). With the exception of the ER and mitochondria, however, the mechanism of uptake into and release from other organelles is still largely obscure. In the cell lines PC12, HeLa, mouse fibroblasts, and human lymphocytes, three major pools (or groups of pools) have been functionally characterized on the basis of their loading and release properties: the first released by $InsP_3$ and Tg, the second insensitive to the above agents and released by Ca^{2+} ionophores, and the third released by the combination of the latter compounds with a drug that dissipates H^+ gradients, such as monensin and NH_4Cl (Fasolato et al., 1991; Clementi et al., 1994; Bastianutto et al., 1995; Mery et al., 1996).

The Ca^{2+} pool released by ionomycin in the presence of monensin was undetectable in RBL-1 cells, while it was consistently observed in the neurosecretory cell types. In GH3 cells and, as previously observed, in PC12 cells (Fasolato et al., 1991), the size of the acidic compartment is reduced after stimulation of secretion and may suggest its identification, at least in part, with secretory compartments.

The key observation of this contribution is that only a fraction of the nonacidic, $InsP_3$- and Tg-insensitive pool, which in unstimulated cells is rather heterogeneous (including mitochondria, Golgi, etc.), is highly dynamic and dramatically increases its Ca^{2+} content upon prolonged rises of $[Ca^{2+}]_i$.

The two major questions generated by our data concern, on the one hand, the cytological nature of the SIC pool, and on the other hand, the physiological role of this compartment. As to the first question, our approach has been that of comparing the pharmacological sensitivity and subcellular distribution of the Ca^{2+} uptake and release into and from this pool with that of known organelles. The first compartment that we considered as a candidate was the mitochondrion, since some of the characteristics of the SIC pool, i.e., low affinity and high capacity, resemble those traditionally attributed to this organelle. In particular, several authors have suggested that mitochondria play a key role in buffering large and prolonged increases in $[Ca^{2+}]_i$ (Carafoli and Crompton, 1976; Bygrave, 1978; Brinley et al., 1979; Friel and Tsien, 1994; Werth and Thayer, 1994; Budd and Nicholls, 1996; Herrington et al., 1996;). However: (*a*) The Ca^{2+} accumulated in the SIC pool cannot be released into the cytosol by mitochondrial uncouplers; and (*b*) direct monitoring of $[Ca^{2+}]_m$ with targeted recombinant aequorin demonstrates that Ca^{2+} ionophores cause an increase and not a decrease in $[Ca^{2+}]_m$. Thus, though mitochondria may well contribute to the ionomycin-sensitive pool of resting cells, they do not represent a major part of the pool that accumulates Ca^{2+} after prolonged $[Ca^{2+}]_i$ increases. Rather than Ca^{2+} sinks, mitochondria appear therefore to be in dynamic equilibrium with the changes in $[Ca^{2+}]_i$, with dramatic transient increases of their matrix $[Ca^{2+}]$ during cell activation.

Similarly, identification of the SIC pool with acidic compartments of the cells, i.e., secretory vesicles, granules, lysosomes, endosomes, etc., appears unlikely since the kinetics, amplitude, and duration of the $[Ca^{2+}]_i$ increases induced by ionophores are unaffected by agents such as monensin or NH_4Cl, which collapse internal pH gradients.

Moreover, Ca^{2+} ionophores, such as A23187 and even more ionomycin, are highly inefficient at releasing Ca^{2+} from acidic compartments (Liu and Hermann, 1978; Fasolato and Pozzan, 1989).

Another possible candidate is the Golgi complex, given that several lines of evidence, though mainly indirect, suggest that this compartment may contain large amounts of Ca^{2+} (Chandra et al., 1991; Cui et al., 1995). Since neither the mechanism of Ca^{2+} uptake nor that of release from the Golgi complex are known to date, we have tested this idea by taking advantage of the high spatial and temporal resolution of the confocal microscope. In fact, if the Ca^{2+} released by ionomycin was largely coming from the Golgi, the response was expected to get started in a region of high Ca^{2+} close to the nucleus, where the Golgi complex is known to be localized. On the contrary, the $[Ca^{2+}]_i$ increase caused by the ionophore appeared from the very beginning to distribute in the whole cytosol. In addition, the increase in $[Ca^{2+}]_i$ caused by ionomycin was unaffected by BFA pretreatment. This drug is known to cause dissolution of the Golgi complex and intermixing of its membrane and content with the ER (Lippincott-Schwartz et al., 1989).

Among the other known organelles, peroxisomes were considered unlikely candidates since they represent at the most 1% of the cell volume (Lazarow, 1989), while the increase in total Ca^{2+} in GH3 cells treated with KCl is about 0.4 mmol/l of cell water. Thus, if peroxisomes accounted for the majority of the SIC pool, their Ca^{2+} content should exceed 40 mM, a value in the order of that estimated for the cell organelle with the highest Ca^{2+} content reported so far: the terminal cysternae of sarcoplasmic reticulum (Volpe and Simon, 1991; Grohovaz et al., 1996).

As far as the ER is concerned, the insensitivity to SERCA inhibitors, $InsP_3$, caffeine, and ryanodine would tend to exclude also this compartment as the candidate organelle. SERCAs are in fact believed to be widespread in this endomembrane system. However, several lines of evidence indicate not only that the ER itself may be heterogeneous (for review see Sitia and Meldolesi, 1992), but also that Ca^{2+} pumps other than those sensitive to the classical inhibitors (Tg, CA, and tBHQ) may be present in this organelle (Bian et al., 1991, Chen et al., 1993). Ca^{2+}–ATPases with molecular weight different from those of classical SERCAs have been described by Burgoyne et al. (1989) in adrenal chromaffin cells (but see also Poulsen et al., 1995) and by Rooney and Meldolesi (1996) in PC12 cells. Finally, in subcellular fractionation experiments, the existence of a microsomal fraction whose Ca^{2+} content is insensitive to $InsP_3$, caffeine-ryanodine, and also to SERCA inhibitors has been repetitively reported (Nori et al., 1993, 1996; Hussain et al., 1995). If indeed the SIC pool represents a subcompartment of the ER, it must be concluded, however, that this part of the organelle equilibrates very slowly, or not at all, with the rest of the membrane network. Until now, its existence might have gone unnoticed given that under resting conditions the Ca^{2+} content of this subcompartment is relatively small and cannot be released into the cytosol by known physiological stimuli. It should be mentioned that a low-affinity Ca^{2+} storage compartment, which loaded only upon large and prolonged increases in $[Ca^{2+}]_i$ caused by KCl depolarization, had been

previously described in a clone of PC12 cells by Reuter's group. Unlike the experiments reported here, however, this compartment was sensitive to caffeine, while its Tg sensitivity was not tested (Reber and Reuter, 1991; Reber et al., 1993).

As to the physiological role of the SIC pool, the interest depends not only on its large capacity (it can account, on average, for >40% of total cell Ca^{2+}) but also on the fact that it is inducible (Clementi et al., 1994; Hussain et al., 1995), and its expression and loading properties appear widespread, if not ubiquitous, as documented by its presence in numerous cell types of different origin (GH3, GC, GH3B6, PC12, RBL-1, and lymphocytes; Clementi et al., 1994; Pizzo, P., C. Fasolato, and T. Pozzan, unpublished).

Admittedly, the conditions needed for loading of this Ca^{2+} pool may appear extreme. However, long-lasting increases in $[Ca^{2+}]_i$ can be elicited by high agonist doses or under pathological conditions (Choi, 1989). Furthermore, the observation that loading and unloading of this Ca^{2+} pool can be relatively fast suggests the possibility that this store plays a role also under physiological conditions, but its high-capacity is unraveled by large and long-lasting $[Ca^{2+}]_i$ increases, when its size overwhelms that of the other Ca^{2+} storage compartments. In other words, it can be suggested that a Ca^{2+} pool, endowed with a Ca^{2+} accumulation mechanism distinct from that of mitochondria and acidic compartments and independent from classical SERCAs, is a normal component of the Ca^{2+} homeostatic machinery of the cell. The role of this previously unrecognized compartment could be twofold: on the one hand, by accumulating part of the Ca^{2+} coming from the extracellular medium or released from other stores, it becomes the most relevant Ca^{2+} sink under conditions of stress, a function traditionally attributed to mitochondria; on the other hand, by slowly releasing its Ca^{2+} content into the cytosol after a prolonged stimulation, it may also participate in signaling integration.

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