THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 266, No. 30, Issue of October 25, pp. 20159-20167, 1991
Printed in U.S.A.

Intracellular Ca^{2+} Pools in PC12 Cells

THREE INTRACELLULAR POOLS ARE DISTINGUISHED BY THEIR TURNOVER AND MECHANISMS OF Ca^{2+} ACCUMULATION, STORAGE, AND RELEASE*

(Received for publication, February 21, 1991)

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Three, non-cytosolic Ca^{2+} pools were characterized in intact PC12 cells. The first pool, sensitive to both inositol 1,4,5-trisphosphate and caffeine (Zacchetti, D., Clementi, E., Fasolato, C., Zottini, M., Grohovaz, F., Fumagalli, G., Pozzan, T., and Meldolesi, J. (1991) *J. Biol. Chem.* 266, 20152–20158) accounts for ≅200 μM of Ca^{2+}/liter of cell water (< 30% of total exchangeable Ca^{2+}) and takes up Ca^{2+} from the cytosol via a Ca^{2+}-ATPase, blocked by thapsigargin. A second pool, ≅400 μM/liter, is insensitive to both inositol 1,4,5-trisphosphate, caffeine, and thapsigargin and is released by the Ca^{2+} ionophore ionomycin. This pool is probably heterogeneous and its intracellular localization and physiological roles remain undefined. The third pool, ≅170 μmoles of Ca^{2+}/liter, was discharged by the combination of ionomycin together with a substance that collapsed intracellular pH gradients, such as monensin or NH_4Cl. This indicates that the pool is acidic, at variance with the first two. When exocytosis was stimulated, the size of this pool declined, indicating its primary residence within secretory granules. In the conditions of our experiments no major transfer of Ca^{2+} among the pools seemed to occur. This is the first comprehensive description of non-cytosolic Ca^{2+} pools investigated in intact neurosecretory cells by non-invasive procedures.

PC12 is a cell line originally developed from a rat pheochromocytoma (and therefore similar to chromaffin cells of that species) which is widely used, especially for studies of neurosecretion and nerve cells differentiation (2). In the preceding article (1) we have demonstrated that in these cells a single rapidly exchanging intracellular Ca^{2+} pool accounts for the $[Ca^{2+}]_i$[1] responses triggered on the one hand by the generation of inositol 1,4,5-trisphosphate, Ins-P_3, at the receptor level, and on the other hand by caffeine, a drug believed to act by decreasing the threshold of the process of Ca^{2+}-induced Ca^{2+} release. Based on these results we have concluded that this rapidly exchanging Ca^{2+} pool (from here on indicated as Ins-P_3-sensitive) resides in an intracellular structure which expresses in its limiting membrane both Ins-P_3 receptors and receptors sensitive to caffeine and ryanodine. This result is at variance with those obtained in neurons and in several different secretory cell types, including rat chromaffin cells, where the two receptors are believed to be located in separate Ca^{2+} stores (3–7). General Ca^{2+} homeostasis appears therefore simplified in PC12 cells, thus making easier the interpretation of studies on additional Ca^{2+} pools, insensitive to both Ins-P_3 and caffeine. These pools are apparently ubiquitous but have so far been little characterized in both their cytological and physiological properties. In addition to the Ins-P_3-sensitive pool, the present results identify in PC12 cells at least two other Ca^{2+} pools. The first, probably heterogeneous, has an intralumenal neutral (or alkaline) pH, is insensitive to the Ca^{2+}-ATPase inhibitor thapsigargin, and resides in structures that remain to be identified. The second, of very slow turnover, has an acidic content and is shown to correspond primarily to secretory granules. Under the conditions of our experiments, neither of these two pools was found to interact with the Ins-P_3-sensitive pool to an appreciable extent. This suggests that the function of the Ins-P_3-insensitive pools has little to do with the rapid regulation of $[Ca^{2+}]_i$ which occurs under the control of surface channels and of the Ins-P_3-sensitive pool. Data concerning the mechanism of Ca^{2+} uptake in the Ins-P_3-sensitive and -insensitive pools are also discussed.

EXPERIMENTAL PROCEDURES

Materials—The experiments presented here were carried out on the parent PC12 line, subcultured in our laboratory as described (8). In addition, most of the experiments were duplicated using the 16A clone described by Zacchetti *et al.* (1). Preliminary experiments were carried out also at the single cell level (4, 9). In both cases the results were qualitatively very similar to those obtained with suspensions of the parent cell line. Fura-2 and quin2 (esters and free acids) were obtained from Boehringer (Mannheim, Federal Republic of Germany), $^{45}Ca^{2+}$ from Amersham Int. (United Kingdom), ionomycin from Calbiochem (San Diego, CA), thapsigargin from LC Services Corp. (Wouburn, MA), carbonylcyanide *p*-(trifluoromethoxy) phenylidrazone (FCCP), bradykinin, and monensin from Sigma. All other materials were analytical or of the highest available grade.

Ca^{2+} Measurements—Loading with fura-2 (in some experiments with quin2) and measurement of $[Ca^{2+}]_i$ are described in detail in Refs. 8–10. The incubation medium (Krebs-Ringer-Hepes, KRH) contained (mmol/liter): NaCl, 125; KCl, 5; KH_2PO_4, 1.2; $MgSO_4$, 1;

* This work was supported in part by the Italian Ministery of University and Scientific Research, from the Consiglio Nazionale delle Ricerche (Target Projects, Biotechnology and Bioinstrumentation, and Biotechnology-Molecular Biology Committee Project on the Biology and Pathology of Ca^{2+}, and from the Italian Association for Cancer Research (AIRC) (to T. P. and J. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; Ins-P_3, inositol 1,4,5-trisphosphate; EGTA, [ethylenebis(oxyethylenenitrilo)]tetracetic acid; FCCP, carbonylcyanide *p*-(trifluoromethoxy)phenylidrazone; Bk, bradykinin; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KRH, Krebs-Ringer-Hepes buffer.

glucose, 6; Hepes-NaOH buffer, pH 7.4 (at 37 °C), 20. In most experiments the medium contained no added $CaCl_2$ and was supplemented with 0.2 mM EGTA, KRH-EGTA. In fura-2 experiments 250 μM sulphynpirazone was also added to prevent dye leakage (10, 11). The cellular fura-2 (quin2) content was determined by comparing the total cellular fluorescence, after subtraction of cell autofluorescence, with that of a fresh sample of fura-2 (quin2) free acid. Autofluorescence was <5% and about 25% of total fluorescent signal with fura-2 and quin2, respectively. PC12 volume was calculated to be 500 μm^3/cell (12), *i.e* = 0.4 μl of water/10^6 cells.

Since the present investigation was mainly concerned with the amount of Ca^{2+} released upon various treatments, it was more appropiate to express the data as percent of the total, Ca^{2+}-sensitive, intracellular fura-2 signal (13), rather than as $[Ca^{2+}]_i$. Only in some experiments, *i.e* in cells loaded with fura-2 in EGTA-containing medium, the values of $[Ca^{2+}]_i$ were also included. In the quin2 experiments, the amount of quin2-Ca^{2+} complex formed (at the peak) was directly recalculated in terms of μmol of Ca^{2+}/liter of cell water on the basis of $[quin2]_i$. Although not entirely accurate, because it ignores the contribution of endogenous buffers, this procedure can be employed in quin2-loaded cells since the dye represents the major Ca^{2+} buffer of the cytoplasm (14).

Measurements of $^{45}Ca^{2+}$ release were performed as follows, PC12 cells monolayers were incubated for different times (18–190 h) with 4 μCi/ml $^{45}Ca^{2+}$ under standard culture conditions. After this period, the cells were detached and washed with non-radioactive, KRH-EGTA (maintained at 4 °C). An aliquot of the cell suspension was immediately centrifuged and the ensuing pellet used for the measurement of total $^{45}Ca^{2+}$ content. The rest of the cells were incubated at 37 °C in KRH-EGTA. At different times, aliquots of the cells (10^6 cells/point) were centrifuged and the $^{45}Ca^{2+}$ released into the medium measured.

All experiments were carried out at 37 °C and the results in the figures, as well as those mentioned in the text as "not shown," are representative of data obtained in at least three separate experiments.

Other Assays—ATP was measured by the luciferase assay and the protein by the Lowry procedure, as previously described (15). Other experimental conditions are described in detail in the accompanying paper (1).

RESULTS

Ins-P_3-sensitive and -insensitive Ca^{2+} pools

Unless otherwise specified, the experiments described below were carried out in the Ca^{2+}-free KRH medium supplemented with 0.2 mM EGTA (KRH-EGTA). Fig. 1 shows the effect on $[Ca^{2+}]_i$ of sequential or simultaneous addition of the nonapeptide bradykinin, Bk, and of the Ca^{2+} ionophore, ionomycin. Even in cells treated with a supramaximal concentration of Bk (2 μM), addition of ionomycin still induced a large increase in $[Ca^{2+}]_i$ (Fig. 1*a*), though smaller than in untreated cells (Fig. 1*b*). In contrast, pretreatment with the ionophore completely abolished the response to the peptide (Fig. 1*b*). Simultaneous addition of ionomycin and Bk caused a rise in $[Ca^{2+}]_i$ indistinguishable from that of the ionophore alone (Fig. 1*c*). With either type of protocol, no significant change was observed if the second stimulus (ionomycin in *a* and Bk in *b*) was added 2 or 10 min after the first agonist.

The experiments of Fig. 2 were carried out to investigate the nature of the $[Ca^{2+}]_i$ response caused by ionomycin after Bk treatment. Fig. 2*a* demostrates that, under the experimental conditions of Fig. 1*a*, only a minor proportion of the Ca^{2+} released by ionomycin from Bk-pretreated cells originated from refilled Ins-P_3-sensitive stores. Indeed, cells maintained in KRH-EGTA during the treatment with Bk, washed and resuspended in the same medium, responded with a very small increase in $[Ca^{2+}]_i$ to restimulation with the peptide, while a large increase was still caused by the ionophore. This latter result was not due to desensitization of Bk receptors because a brief pulse of extracellular Ca^{2+} resulted in a rapid recovery of the Bk response (see below). Additional results were obtained by combining Bk and ionomycin with thapsigargin, a

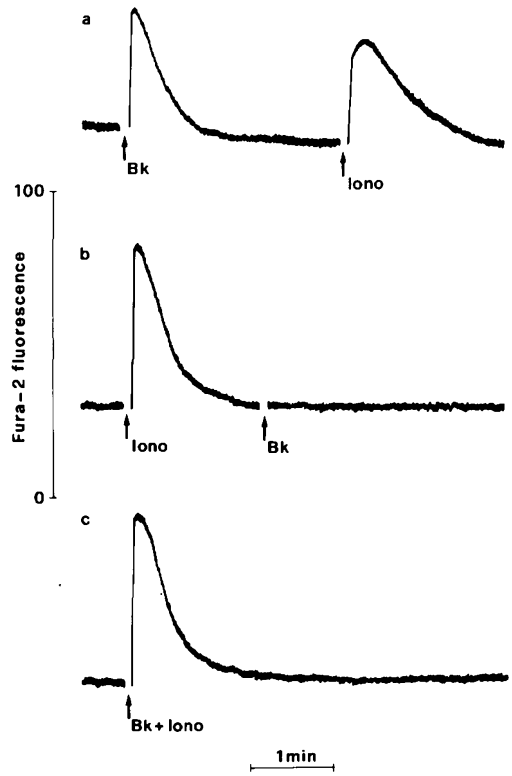

FIG. 1. **Effects of bradykinin (*Bk*) and ionomycin (*iono*) on Ca^{2+} release from intracellular stores.** PC12 cells were loaded with fura-2 as described under "Experimental Procedures." Immediately before use, aliquots (1–2 × 10^6 cells) were centrifuged (3 min, 1,000 × *g*) and resuspended in 2 ml of Ca^{2+}-free KRH medium containing 0.2 mM EGTA (KRH-EGTA). Where indicated, bradykinin (2 μM) and ionomycin (1 μM) were added. In this and the following experiments the total, Ca^{2+}-sensitive, fura-2 (quin2) signal (which refers to all traces in the figure) is shown on the *left- hand side* and presented as a linear scale from 0 to 100%. The resting $[Ca^{2+}]_i$ under the normal loading conditions described above was 90 ± 5 nM (n = 60) and corresponds to 30% of total, Ca^{2+}-sensitive, fura-2 fluorescence.

blocker of the Ca^{2+}-ATPase in the Ins-P_3-sensitive store (1, 16). Thapsigargin, when added after Bk treatment at concentrations causing complete discharge of the Ins-P_3-sensitive store (1), caused an increase in $[Ca^{2+}]_i$ somewhat larger than that observed upon restimulation with Bk (compare Fig. 2*a* with *b*), but a large release was still observed after application of ionomycin (Fig. 2*b*). Finally, addition of Bk and thapsigargin together caused a rise in $[Ca^{2+}]_i$ only marginally larger than that caused by Bk alone (compare Fig. 2*c* with *a* and *b*).

The experiments presented in Fig. 3 were carried out to investigate the role of intravesicular pH in the mechanism of Ca^{2+} release from intracellular pools. Fig. 3*a* shows that addition of monensin, which induces an electroneutral $H^+/Na^+(K^+)$ exchange across membranes, caused a negligible increase in $[Ca^{2+}]_i$ and did not affect the increase induced by a subsequent administration of Bk. In contrast, the response to ionomycin was increased appreciably by monensin pretreatment (compare *continuous* (monensin) with *dashed* (control) *trace* in Fig. 3*a*). Addition of monensin after Bk (Fig. 3*b*) again had no appreciable effect on $[Ca^{2+}]_i$, whereas after ionomycin it caused a large increase in $[Ca^{2+}]_i$ (Fig. 3*c*. Taken together these results indicate the existence in PC12 cells of at least two ionomycin-sensitive Ca^{2+} pools, one dischargeable by the Ca^{2+} ionophore alone, the other (of acidic content) which needs pH gradient neutralization in order for ionomycin-induced Ca^{2+} transport to be effective. Results similar to

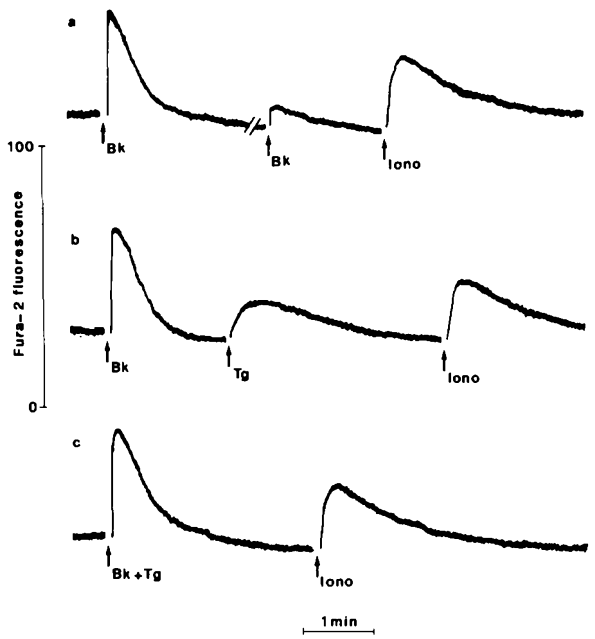

FIG. 2. **Effects of thapsigargin (*Tg*) on bradykinin (*Bk*)- and ionomycin (*iono*)-induced Ca^{2+} release.** Conditions as in Fig. 1, *a–c*, KRH-EGTA medium. Where indicated Bk (2 μM), ionomycin (1 μM), and thapsigargin (0.1 μM) were added. In *panel a* // indicates a 3-min interruption of the trace necessary to wash the cells with KRH-EGTA medium supplemented with 5% bovine serum albumin.

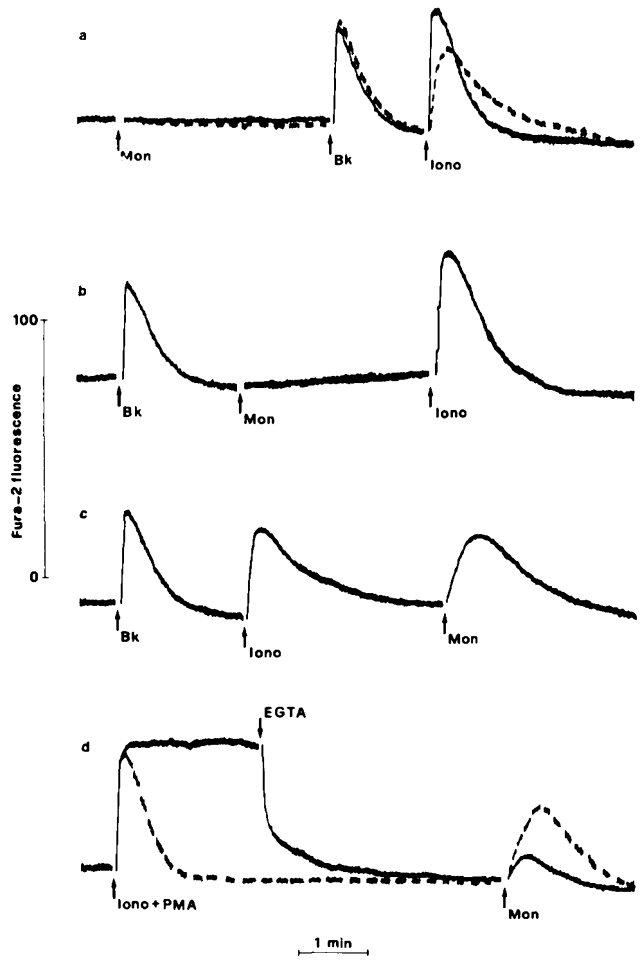

FIG. 3. **Effects of intralumenal pH and of stimulated secretion on Ca^{2+} release from internal pools.** *Panels a–c*, effects of monensin (*Mon*). Conditions as in Fig. 1. The *dashed trace* in *panel a* represents control cells, *i.e.* not treated with monensin. *Panel d*, effect of stimulated secretion. *Continuous trace*, EGTA was initially omitted from the KRH medium, which was instead supplemented with 1 mM $CaCl_2$. Under these condition secretion of [^{3}H]dopamine was 70% of total content (8). *Dashed trace*, the cells were incubated from the beginning in KRH-EGTA. Secretion of [^{3}H]dopamine under these conditions was ≅10% of total content (8). Where indicated monensin (1 μM), Bk (2 μM), ionomycin (1 μM), phorbol myristate acetate (*PMA*) (0.1 μM), and EGTA (4 mM) were added.

those obtained with monensin were observed with NH_4Cl (data not shown).

To investigate the possibility that the acidic pool is localized within secretory granules, the cells were exposed to pretreatments known to markedly stimulate exocytotic secretion, the black spider venom α-latrotoxin (17) or a combination of phorbol myristate acetate (PMA) and ionomycin (8). Fig. 3*d* shows the results obtained using this latter protocol. Cells were incubated in KRH medium containing 1 mM $CaCl_2$ (*continuous trace*). Addition of ionomycin (+ *PMA*) induced a rapid increase in $[Ca^{2+}]_i$ which remained elevated until excess EGTA was added. Chelation of extracellular Ca^{2+} allowed $[Ca^{2+}]_i$ to return to basal level. Compared to cells receiving the same treatment, but in the continuous presence of EGTA (*dashed trace*), *i.e.* conditions that cause little or no secretion (8, 17), the cells with stimulated exocytosis (*continuous trace*) exhibited a drastic reduction of the $[Ca^{2+}]_i$ effect of monensin, as expected for a mostly granular localization of the acidic Ca^{2+} pool. Treatment with α-latrotoxin in Ca^{2+}-containing KRH medium similarly caused a 70% inhibition of the Ca^{2+} rise due to monesin.

Role of Mitochondria and Cellular ATP Levels

The role of mitochondria was investigated by the use of two drugs, rotenone (an inhibitor of the respiratory chain) and FCCP (an uncoupler of oxidative phosphorylation) administered in a medium with or without glucose. With glucose, the two drugs reduced cellular ATP only by 20–30%. Without glucose present, both drugs could reduce ATP to less than 20% of controls within 5 min. Since the effects of rotenone and FCCP on $[Ca^{2+}]_i$ were practically identical, despite their different targets in mitochondria, only the results obtained with rotenone are shown in figures. Fig. 4 demonstrates that rotenone, added in glucose-containing medium, hardly affected resting $[Ca^{2+}]_i$ and $[Ca^{2+}]_i$ increases caused by Bk (compare *panel a* (control) with *b* (rotenone)). In contrast, without glucose in the medium (*panel c*) rotenone caused a considerable, though relatively slow, increase in $[Ca^{2+}]_i$ and a clear, progressively increasing reduction of the response to Bk. The latter was completely abolished after 10 min of rotenone treatment. Last, addition of rotenone in glucose-free medium, after Bk treatment, hardly affected $[Ca^{2+}]_i$ (*panel d*).

Ca^{2+} Uptake into the Various Pools

The Ca^{2+} content of the Ins-P_3-sensitive and -insensitive pools is likely the result of an equilibrium between continuously ongoing processes of uptake and release. To investigate the different mechanisms of Ca^{2+} uptake, in the experiments described below the Ca^{2+} pools were first depleted and then reuptake was allowed to take place in Ca^{2+} medium for different periods of time under carefully controlled experimental conditions. The rates and extents of reuptake were estimated by comparing the release of Ca^{2+} caused by the different stimuli before and after the depletion-refilling protocols.

Ins-P_3-sensitive Store—As already shown in Fig. 2 and in

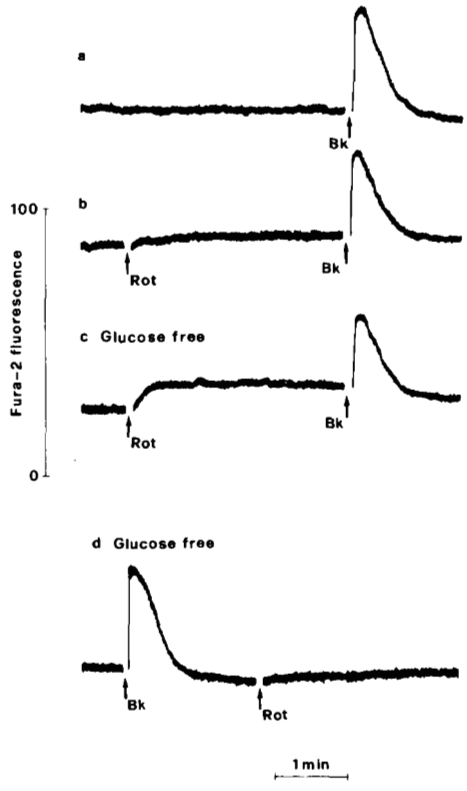

FIG. 4. **Effects of rotenone (*Rot*) on bradykinin (*Bk*)-induced Ca^{2+} release.** Conditions as in Fig. 1. *Panels a* and *b*, KRH-EGTA; *panels c* and *d*, as *panel a* and *b*, but without glucose in the medium. Rotenone (4 μM) and Bk (2 μM) were added where indicated.

the preceding article (1), this store can be largely depleted by the simple exposure to a maximal concentration of Bk administered in KRH-EGTA medium. In predepleted cells, reintroduction of Ca^{2+} into the medium resulted in the rapid refilling of the pool, as revealed by the effect of Bk and/or thapsigargin administered at various time intervals after Ca^{2+} readdition. Refilling was time dependent and faster the higher the extracellular Ca^{2+} concentration (see below). Various treatments were applied prior and/or during Ca^{2+} reintroduction to characterize the reuptake process.

Comparison of the experiments shown in Fig. 5 (*panels a* and *b*, *continuous traces*) demonstrates that the presence of monensin during the refilling process did not alter the extent of refilling of the Bk dischargeable Ca^{2+} pool. The *dashed trace* in Fig. 5, *panel a*, represents the rise in $[Ca^{2+}]_i$ caused by Bk in control, non-depleted cells. In four similar experiments the extent and rate of Ca^{2+} refilling was indistinguishable in cells treated with monensin compared to controls. Furthermore, the mitochondrial inhibitors, rotenone and FCCP, when added in glucose-containing medium, caused no appreciable reduction in the refilling of the Ins-P_3-sensitive Ca^{2+} pool (not shown). Thus, intralumenal acidity and mitochondria appear not to be directly involved with Ca^{2+} uptake in this pool.

In contrast, refilling of the Ins-P_3-sensitive store was found to be markedly reduced by a variety of treatments, in particular (*a*) low $[Ca^{2+}]_i$, (*b*) rotenone or FCCP in the absence of glucose, *i.e.* low intracellular ATP concentration, (*c*) high cytosolic Ca^{2+} buffering, and (*d*) low (depolarized) plasma membrane potential.

(*a*) In order to decrease the $[Ca^{2+}]_i$, fura-2 loading of the cells was carried out in Ca^{2+}-free, EGTA-containing medium. Under these conditions the cells are unable to compensate for

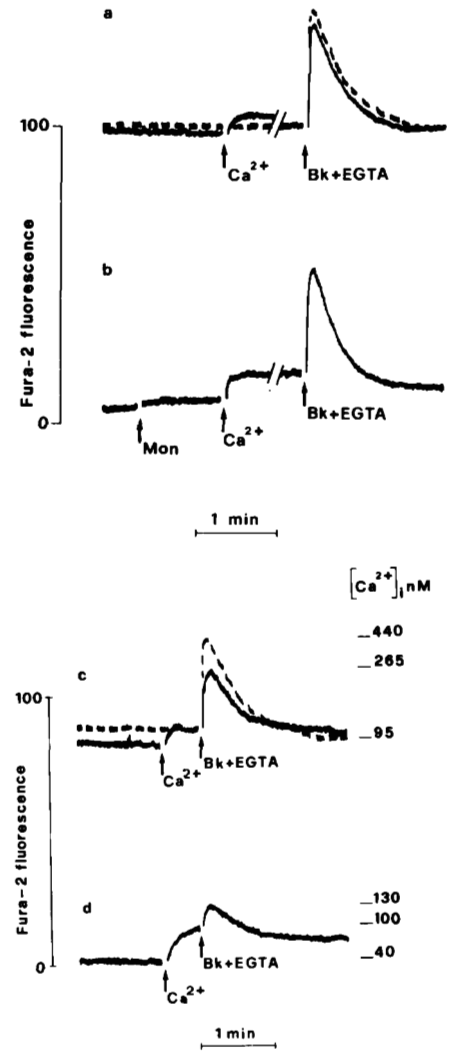

FIG. 5. **Effects of intralumenal pH and $[Ca^{2+}]_i$ on the refilling of the Ins-P_3-sensitive store.** *Panels a* and *b*, effects of monensin (*Mon*) on refilling. The InsP-$_3$-sensitive pool was depleted by exposure of the cells to 2 μM Bk, administered in KRH-EGTA. After 2 min at 37 °C under these conditions, the cell suspension was diluted 5-fold with KRH-EGTA supplemented with 5% bovine serum albumin and centrifuged for 3 min at 1000 × *g*. The pellet was finally resuspended in KRH-EGTA, prewarmed at 37 °C. Refilling was initiated by the addition of 0.3 mM $CaCl_2$ and blocked by an excess EGTA (4 mM). The extent of refilling of the store was calculated by measuring the $[Ca^{2+}]_i$ response elicited by Bk (2 μM) administered together with the excess EGTA. The $[Ca^{2+}]_i$ rise caused by 2 μM Bk in untreated cells, incubated for 3 min in KRH-EGTA, was taken as a measure of 100% refilling (*dashed trace* in *a*). The incubation in KRH-EGTA for up to 5 min did not modify the response to Bk compared to controls incubated in KRH medium containing 1 mM $CaCl_2$ and treated with EGTA just before addition of Bk. Other conditions as in Fig. 1. *Panels c* and *d*, effect of $[Ca^{2+}]_i$ on refilling. Cells were loaded with fura-2 in EGTA (1 mM)-containing RPMI medium, supplemented with 3% fetal calf serum. Where indicated monesin (1 μM), $CaCl_2$ (0.3 mM), and Bk (2 μM) plus EGTA (4 mM) were added. // indicates 4 min interruption of the trace. *Panel c*, *continuous trace*: after loading in EGTA medium, the cells were allowed to recover normal $[Ca^{2+}]_i$ by 10 min incubation in KRH medium containing 1 mM $CaCl_2$. Depletion of the Ins-P_3-sensitive pool was then performed as described in the legend of *a* and *b*. Note that the resting $[Ca^{2+}]_i$ of these cells is 95 nM, *i.e.* a value indistinguishable from that of PC12 cells loaded with fura-2 under normal conditions (1). *Panel c*, *dashed trace*: as above, but without the depletion protocol. *Panel d*, after loading in EGTA, the cells were maintained and depleted in KRH-EGTA. Note that in these cells $[Ca^{2+}]_i$ was reduced to 40 nM, *i.e.* well below normal resting values. The values of $[Ca^{2+}]_i$ are shown on the *right-hand side*.

the formation in the cytoplasm of the Ca^{2+} chelator (fura-2) and therefore $[Ca^{2+}]_i$ declines below normal resting values (in our conditions from ≅ 90 to 40 nM) (for a detailed discussion see Refs. 8, 13, 18–20). This treatment is not toxic in as much as cells transferred to the complete, Ca^{2+}-containing KRH medium regain their normal $[Ca^{2+}]_i$ and respond normally to stimulation (8, 20). Fig. 5 (*panels c and d*) shows the results of experiments performed with cells loaded with fura-2 in Ca^{2+}-free, EGTA-containing medium. After loading in these latter conditions, an aliquot of the cells was allowed to recover normal resting $[Ca^{2+}]_i$ by incubation for 10 min in KRH medium containing 1 mM $CaCl_2$. The rest of the cells were maintained in KRH-EGTA. Depletion of the Ins-P_3-sensitlve Ca^{2+} pool was then obtained, as described in the legend of Fig. 5, *a* and *b*, by treatment with Bk in KRH-EGTA. In depleted cells, but with a normal resting $[Ca^{2+}]_i$, exposure for 30 s to Ca^{2+}-containing medium (*panel c, continuous trace*) was sufficient to allow the recovery of about 60% of the control response to Bk (*panel c, dashed trace*). On the contrary, when this same protocol was applied to cells maintained at low $[Ca^{2+}]_i$ (*panel d*), the refilling obtained during the 30 s of exposure to Ca^{2+}-containing medium was largely reduced. Complete refilling was eventually obtained (after 5 min) even in cells with low $[Ca^{2+}]_i$, indicating that the treatment was not in itself toxic. The experiment clearly demonstrates that the level of $[Ca^{2+}]_i$ during the refilling process regulates the rate of Ca^{2+} uptake into the Ins-P_3-sensitive pool.

(*b*) Refilling was largely inhibited also by rotenone, when administered in glucose free medium, *i.e.* under conditions causing a dramatic reduction of cellular ATP levels. Fig. 6*a* shows that in glucose-free medium refilling of the InsP_3-sensitive store occurs normally (compare Fig. 6*a* with Fig. 5*a*). On the contrary, treatment of the cells in glucose-free medium with rotenone drastically reduced the response to subsequently administered Bk (Fig. 6*b*). This experiment demonstrates that a high intracellular ATP level is required to allow refilling of the Ins-P_3-sensitive Ca^{2+} pool.

(*c*) Likewise, refilling of the InsP_3-sensitive store was inhibited when the cytosolic Ca^{2+} buffering was increased. In fact, in cells loaded with fura-2 (intracellular dye concentration 80 μM) the refilling was about three times faster than in cells loaded with quin2 (intracellular dye concentration 600 μM) (Fig. 6*c*, compare *filled* with *empty circles*).

(*d*) A drastic reduction in the rate of refilling was also observed when the electrochemical gradient of Ca^{2+} across the plasma membrane was reduced. In fact (Fig. 6*c*, *triangles*) in cells loaded with fura-2 and resuspended in a medium where Na^+ was isoosmotically substituted with K^+ the rate of refilling was much slower than in controls. In this latter experiment, to avoid contribution by voltage-activated Ca^{2+} channels, the medium was supplemented with verapamil, a potent blocker of those channels.

Ins-P_3-insensitive Stores—In order to characterize unambiguously these stores, it was critical to avoid interference from the InsP_3-sensitive pool, due to either incomplete depletion or partial refilling. For these reasons from here on Bk was always applied in conjunction with thapsigargin. Fig. 7 demonstrates that the response to Bk and thapsigargin was completely abolished even without prior administration of the peptide, if the cells were loaded with fura-2 in EGTA-KRH and maintained in the same medium for 4 h (compare control, *panel a*, with cells loaded in EGTA, *panel b*). Note that under these latter conditions $[Ca^{2+}]_i$ was reduced to about 30 nM, *i.e.* 3-fold lower than in normal resting conditions. In cells loaded with fura-2 in EGTA and kept in EGTA medium, on the other hand, the $[Ca^{2+}]_i$ increase induced by ionomycin

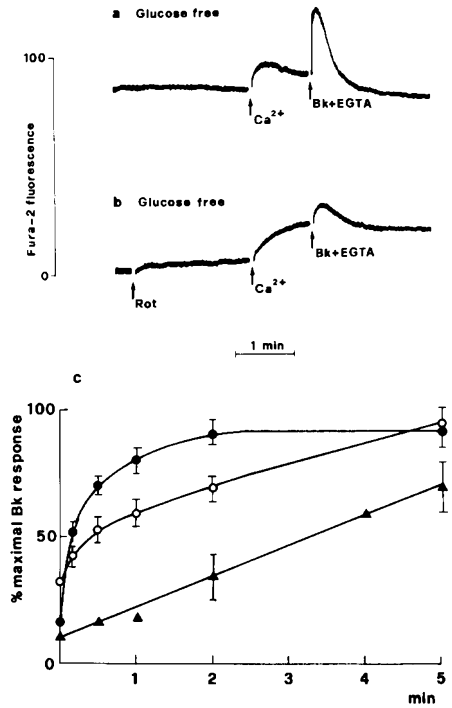

FIG. 6. **Effects of rotenone, internal Ca^{2+} buffering, and membrane potential on the refilling of the Ins-P_3-sensitive store.** *Panels a* and *b*, effect of rotenone (*Rot*), glucose-free, KRH-EGTA. The depletion protocol is described in detail in the legend of Fig. 5, *a* and *b*. Where indicated rotenone (4 μM), $CaCl_2$ (0.3 mM), and Bk (2 μM) plus EGTA (4 mM) were added. *Panel c*, effect of internal Ca^{2+} buffering and of membrane potential. Cells were loaded with fura-2 (●) or quin2 (○) under standard conditions. The Ins-P_3-sensitive store was depleted as described in the legend of Fig. 5, *a* and *b*. ▲, cells loaded with fura-2 and depleted as above were finally resuspended in medium where NaCl was isoosmotically substituted with KCl and supplemented with 20 μM verapamil (to inhibit voltage-dependent Ca^{2+} channels). In parallel batches of cells, incubated in NaCl medium, it was demonstrated that verapamil had no effect on the rate of refilling. Refilling was initiated by addition of 0.3 mM $CaCl_2$ and blocked at different times by addition of EGTA (4 mM) + Bk (2 μM), to determine the extent of refilling. The data are expressed as percent of the maximal Bk response, as obtained in non-depleted cells. The values at time 0 represent the response to restimulation with Bk without refilling in Ca^{2+}-containing medium. Mean ± S.E. of three independent experiments.

was only 50% reduced and that due to monensin indistinguishable from that of controls (compare *panel a* with *b*).

In the experiments described in Fig. 7*c*, depletion of ionomycin-sensitive pools was obtained by treating the cells in KRH-EGTA with 500 nM ionomycin. After washing away the ionophore, the Bk + thapsigargin response was completely abolished and that induced by ionomycin reduced to less than 20% of that in control cells (compare *dashed trace* in Fig. 7*c* with *continuous trace* in Fig. 7*a*). Addition of 1 mM Ca^{2+} for 5 min (Fig. 7*c*, *continuous trace*) allowed complete recovery of the response to Bk + thapsigargin; in contrast, that due to ionomycin was still distinctly reduced (about 50%), although the complete recovery of the Bk response indicates that ionomycin has been completely removed. The reduction of the ionomycin response was still distinctly visible even after 15 min of refilling in Ca^{2+}-containing medium.

A similar depletion protocol (pretreatment with ionomycin and monensin followed by washing and resuspension in KRH-EGTA) was employed to deplete also the acidic pool. Some refilling of this Ca^{2+} store was obtained by exposure of the cells to the Ca^{2+}-containing medium, but only at extremely slow rates. A first appreciable response to monensin, admin-

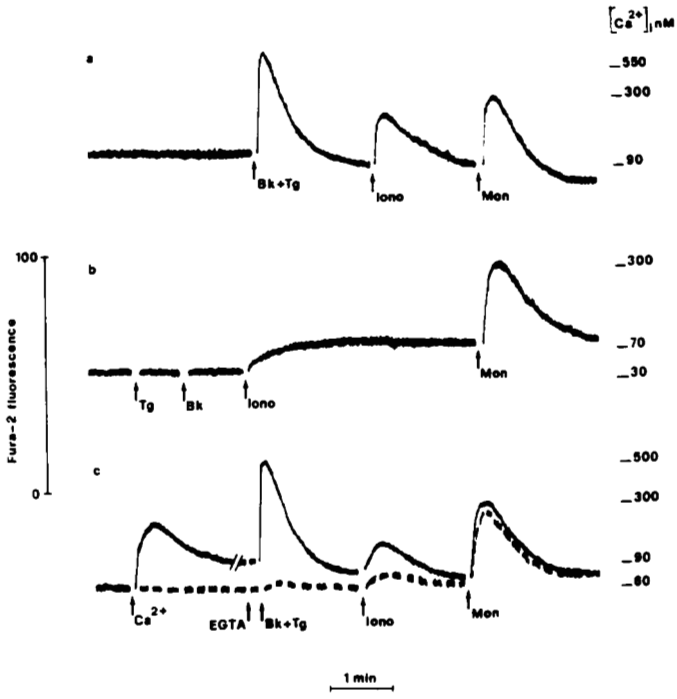

FIG. 7. **Refilling of the Ins-P_3-sensitive and -insensitive Ca^{2+} stores.** *Panel a*, control, non-depleted cells; conditions as in Fig. 1. The incubation in KRH-EGTA for 3 min did not affect the rise in $[Ca^{2+}]_i$ elicited by the different stimuli compared to controls incubated in KRH medium containing 1 mM $CaCl_2$ and treated with 2 mM EGTA just before addition of Bk + thapsigargin (*Tg*). *Panel b*, effect of low $[Ca^{2+}]_i$. Cells were loaded with fura-2 in EGTA containing medium, as described in Fig. 5, *c* and *d*, and left for 4 h in the same medium at room temperature. *Panel c*, refilling of Ins-P_3-insensitive stores. Ins-P_3-and ionomycin-sensitive pools were depleted by using a protocol identical to that described in Fig. 5, *a* and *b*, but with 0.5 μM ionomycin instead of Bk. *Continuous trace* where indicated $CaCl_2$ (1 mM) was added; *dashed trace*, as above but $CaCl_2$ was omitted. Where indicated Bk (2 μM), thapsigargin (0.1 μM), ionomycin (1 μM), monensin (1 μM) and EGTA (4 mM) were added. On the *right- hand side* $[Ca^{2+}]_i$ is presented. Note that in cells loaded and maintained in EGTA-containing medium (*panel b*), the $[Ca^{2+}]_i$ before any stimulation is about 30 nM, *i.e.* 2–3-fold lower than in cells loaded under normal conditions (*panel a* and *c*). // indicates a 3-min interruption of the trace.

istered after ionomycin, was detected only after 5 min in a medium containing 1 mM $CaCl_2$. After 15 min in 1 mM $CaCl_2$, the recovery of monensin response did not exceed 35% of control levels.

Size and Interactions of the Various Ca^{2+} Pools

The two last problems addressed concerned the size, *i.e.* the actual Ca^{2+} content of the various pools we have identified, and their mutual interactions, in particular the possibility that at least part of the Ca^{2+} released from the Ins-P_3-sensitive store was accumulated by the Ins-P_3-insensitive stores. For the size assessment of the various pools, two techniques were employed. First, $[Ca^{2+}]_i$ measurements were made in cells loaded with high concentrations of quin2. Second, the cells were labeled near isotopic equilibrium with $^{45}Ca^{2+}$, and the counts released into the medium upon various treatments were measured. Quin2 was used because it can be loaded within the cytoplasm at concentrations higher than those obtainable with fura-2 (13, 18). High $[quin2]_i$ were advantageous in our experiments for at least two reasons. (*a*) Rises in $[Ca^{2+}]_i$ were blunted, so that the speed of Ca^{2+} extrusion-sequestration was reduced. (*b*) In the loaded cells, the bulk of the cytosolic Ca^{2+} buffering was accounted for by the dye. The contribution of the endogenous Ca^{2+} buffers could thus be overlooked, at least as a first approximation. Given that, in absolute terms, $[Ca^{2+}]_i$ is negligible compared to $[quin2]_i$, the changes in $[quin2\text{-}Ca^{2+}]_i$ complex upon various treatments could be directly expressed in moles of Ca^{2+} released into the cytoplasm (14).

Fig. 8 shows the kinetics of the $[Ca^{2+}]_i$ increases induced in parallel batches of quin2-loaded cells by the sequential addition of Bk (+ thapsigargin), ionomycin, and monensin (*trace a*) or ionomycin and monensin only (*trace b*). The relative size of each pool, calculated as described under "Experimental Procedures," is shown by the histograms in Fig. 8*c*. The size of the total ionomycin-releasable Ca^{2+} pool was found to correspond to the sum of that released separately by Bk (+ thapsigargin) and ionomycin added in sequence (compare *filled* with *empty bars*). The kinetics of the $[Ca^{2+}]_i$ transients in quin2-loaded cells were, however, clearly different from those of the experiments with fura-2 (compare the trace of Fig. 8*a* with that of Fig. 7*a*). In particular, in quin2-loaded cells the rapid $[Ca^{2+}]_i$ rise caused by ionomycin (after Bk) was followed by a steady state level of about 1–2 min and, finally, by a slow decline toward the resting level. The existence of a

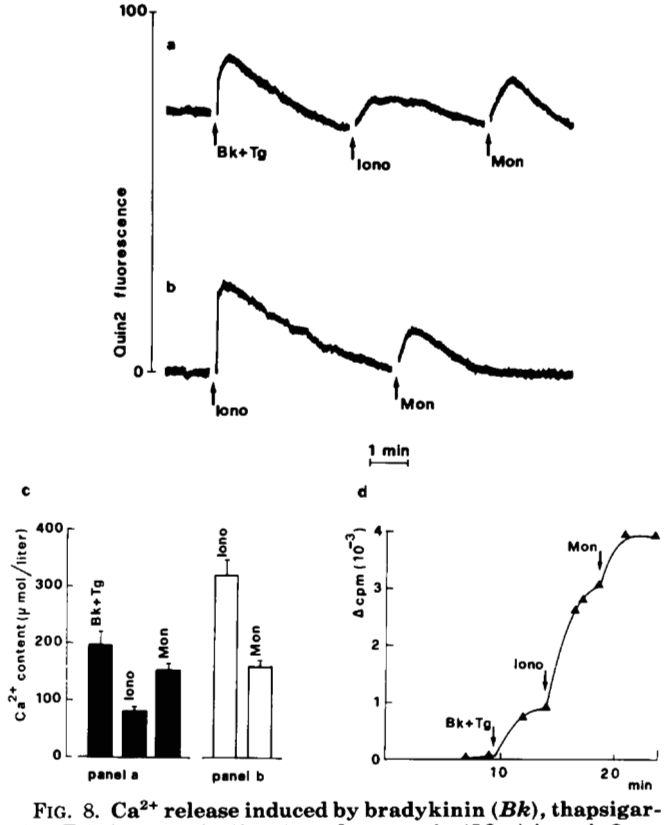

FIG. 8. **Ca^{2+} release induced by bradykinin (*Bk*), thapsigargin (*Tg*), ionomycin (*iono*), and monensin (*Mon*) in quin2- or $^{45}Ca^{2+}$-loaded cells.** Cells were loaded with quin2 as described under "Experimental Procedures." *Panels a* and *b*, KRH-EGTA medium. Where indicated Bk (2 μM) plus thapsigargin (0.1 μM), ionomycin (1 μM), and monensin (1 μM) were added. *Panel c*, the Ca^{2+} content of the Ins-P_3-sensitive and -insensitive pools was calculated from the quin2-Ca^{2+} complex formed (at the peak) according to the protocols shown in *panel a* (*filled bars*) or *panel b* (*empty bars*). Data are expressed in μmol/liter of cell water. Mean ± S.E. of three independent experiments. *Panel d*, $^{45}Ca^{2+}$ release from the three pools. Cells were loaded for 52 h with $^{45}Ca^{2+}$ (= 10^4 dpm/nmol) as described under "Experimental Procedures." Data were subtracted of the counts/minute present in the medium after 5 min incubation in KRH-EGTA. Unstimulated $^{45}Ca^{2+}$ release was negligible in the period investigated (10–30 min). Where indicated Bk (2 μM) plus thapsigargin (0.2 μM), ionomycin (5 μM), and monensin (5 μM) were added.

prolonged plateau phase suggests that during this period Ca^{2+} was slowly and continuously released from intracellular store(s) by the ionophore, but no net increase in $[Ca^{2+}]_i$ occurred because the continuous release of the cation was compensated by its extrusion into the medium at the same rate. Were this the case, one would expect the total ionomycin releasable pool to be higher than that calculated with the Ca^{2+} indicator.

In order to obtain independent evidence on the size of the various pools, we carried out experiments with $^{45}Ca^{2+}$. Fig. 8*d* shows the results obtained with cells loaded for 52 h with $^{45}Ca^{2+}$. The amount of $^{45}Ca^{2+}$ released into the medium by Bk + thapsigargin was about a half of that released by ionomycin added in sequence. The amount released by monensin, added after the other two stimuli, was similar to that released by the first treatment. Assuming that each Ca^{2+} pool was labeled to isotopic equilibrium (see below), these values correspond to 206 ± 27, 388 ± 35, and 174 ± 15 (mean of four experiments, S.E.) μmol/liter of cell water for the Ins-P_3-, ionomycin-, and monensin-sensitive pools, respectively. Further information (not shown in figures) was obtained by additional experiments with $^{45}Ca^{2+}$. (*a*) FCCP, added after Bk and thapsigargin, released 8% of total cell $^{45}Ca^{2+}$ into the medium. (*b*) The three pools were loaded with $^{45}Ca^{2+}$ at different rates. In fact the Ins-P_3-sensitive pool was labeled at equilibrium at the earliest time point investigated, 18 h, while the ionomycin-sensitive pool was ≅50% and the monensin-sensitive pool ≅10% labeled. Maximum labeling of these last two pools was observed after 48 h of incubation. Prolonging the incubation with $^{45}Ca^{2+}$ from 48 to 190 h did not increase the labeling of the three pools any further.

The results reported so far have been obtained by exposing the cells in sequence to the three drugs. A basic limitation of this approach is that these treatments induce an increase in $[Ca^{2+}]_i$. This rise can in turn stimulate accumulation of the cation into the subsequently investigated pools and thus lead to their overestimation. In order to establish whether the values obtained from the experiments described in Fig. 8 reflect the situation in resting cells, we had to exclude major transfer of Ca^{2+} among the pools, particularly from the first to the others. Evidence against this possibility already emerged from previous results (see Fig. 1). Additional evidence was obtained from the experiments described in Fig. 9, *a* and *b*. The rationale of these experiments was the following: if a sizable transfer of Ca^{2+} from the Ins-P_3-sensitive to the other pools occurred during Bk stimulation, it is expected that the $[Ca^{2+}]_i$ rise caused by ionomycin and/or monensin (or the amount of $^{45}Ca^{2+}$ released) should increase after a depletion-refilling protocol. The experiments shown in Fig. 9, *a* and *b*, demonstrate that this was not the case. In the experiment of Fig. 9*a*, parallel batches of fura-2-loaded cells were first incubated in KRH-EGTA, with or without Bk, washed, and resuspended in Ca^{2+} medium to allow refilling of the Ins-P_3-sensitive store. Finally, the cells were exposed (after chelation of extracellular Ca^{2+}) to ionomycin (to reveal the Ca^{2+} accumulated in the Ins-P_3-sensitive and in the ionomycin-sensitive stores) and to monensin (to reveal the Ca^{2+} accumulated in the acidic pool). An increase of the response induced by the latter two drugs in the Bk-pretreated cells would demonstrate a transfer of Ca^{2+} from the Ins-P_3-sensitive to the other pools. As can be seen no effect of the Bk pretreatment on the amplitude of the $[Ca^{2+}]_i$ rise induced by ionomycin and monensin was observed, indicating that transfer, if it occurred, was of minor extent.

A similar approach was utilized in the experiment described in Fig. 9*b*. In cells loaded with $^{45}Ca^{2+}$ to isotopic equilibrium,

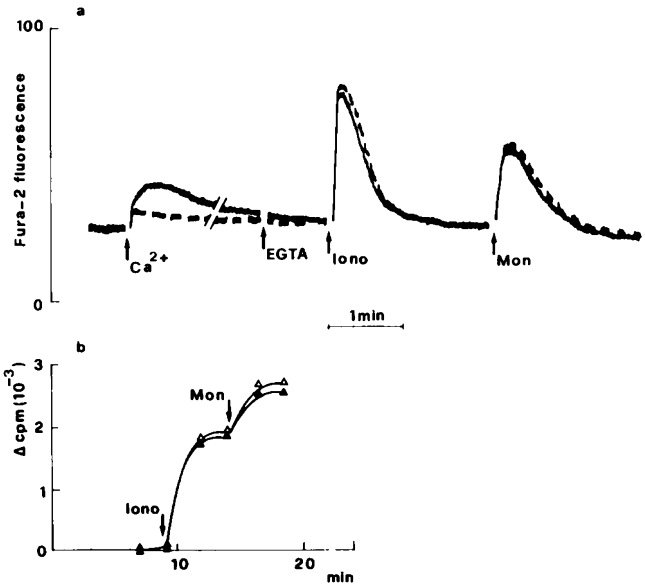

FIG. 9. **Effect of the depletion-refilling protocol on the size of the Ins-P_3-insensitive pools.** *Panel a*, $[Ca^{2+}]_i$ increases in depleted-refilled cells. The Ins-P_3-sensitive pool was depleted by Bk pretreatment, as described in the legend of Fig. 5, *a* and *b*. *Continuous trace*, predepleted cells; *dashed trace*, control non-depleted cells. Where indicated $CaCl_2$ (1 mM) EGTA (4 mM), ionomycin (1 μM) and monensin (1 μM) were added. // indicates a 3-min interruption of the trace. *Panel b*, $^{45}Ca^{2+}$ release in depleted-refilled cells. Conditions as in *panel a*. ▲, the Ins-P_3-sensitive pool was emptied by Bk pretreatment, as described in the legend of Fig. 5, *a* and *b*. Refilling of the Ins-P_3-sensitive pool was carried out as shown in *panel a*, but in the presence of $^{45}Ca^{2+}$ (10^4 dpm/nmol). △, control cells, *i.e.* without Bk pretreatment.

the Ins-P_3-sensitive pool was first depleted by applying Bk in KRH-EGTA. Refilling was then performed, as described for the fura-2 experiments, but in medium containing $^{45}Ca^{2+}$. Again, if a significant transfer of $^{45}Ca^{2+}$ had occurred during the Bk-induced $[Ca^{2+}]_i$ rise, cells refilled in the presence of $^{45}Ca^{2+}$ were predicted to contain more ionomycin and monensin-releasable counts than untreated controls. In contrast Fig. 9*b* shows that the amount of $^{45}Ca^{2+}$ released by the two drugs from cells with and without Bk pretreatment was indistinguishable.

DISCUSSION

In the present study we have provided evidence for the existence in PC12 cells of at least three separate, membrane-bound Ca^{2+} pools, distinguishable for their different rates of turnover and their mechanisms of Ca^{2+} accumulation, storage, and release. As shown in the preceding article (1), the cells contain a single rapidly exchanging Ca^{2+} pool, sensitive to both Ins-P_3 and caffeine-ryanodine. Of the two other pools identified, one (Ins-P_3-insensitive and ionomycin-sensitive) is probably heterogeneous. This pool is characterized by intermediate turnover and remains largely mysterious in its subcellular localization and function. The other, the acidic pool, resides primarily within secretory granules, is loaded very slowly and, once established, seems to have minimal leakage to the cytosol.

The evidence we have obtained indicates that within the time resolution of our experiments the various membrane-bound Ca^{2+} pools of PC12 cells operate independently from each other. In particular, no appreciable accumulation of Ca^{2+} was detected in the Ins-P_3-insensitive pools when the Ins-P_3-sensitive pool was rapidly and persistently discharged into the cytosol by the combination of Bk + thapsigargin. In the rest

of this discussion the pools will therefore be independently considered.

The Ins-P_3-sensitive Pool—As discussed extensively elsewhere (5, 22), we believe this pool to be located not in the entire endoplasmic reticulum but in specialized regions (possibly the calciosomes) equipped for the rapid uptake, storage, and regulated release of Ca^{2+}. For the study of this pool, and particulary for its quantitative assessment, we found essential the combination of the stimulatory receptor agonist Bk, with the blocker of the specific ATPase thapsigargin, administered in the KRH-EGTA medium. In fact the blocker, on the one hand, prevented the (moderate) reuptake of Ca^{2+} into the pool which follows Ins-P_3 metabolism. On the other hand, thapsigargin compensated for the fact that not all but only 80–90% of the cells express Bk receptors (of the B_2 subtype) and are thus stimulated by the peptide (22).

By the two procedures we have employed for size assessment, the measurement of quin2-Ca^{2+} complex formed and $^{45}Ca^{2+}$ released after stimulation, the Ins-P_3-sensitive pool was estimated to account for ≅200 μmol/liter of cell water. This represents ≅25% of the total cell exchangeable Ca^{2+}. This pool is in continuous, rapid exchange with the cytoplasm, as indicated by its rapid discharge into the cytosol upon both reduction of ATP levels and/or blockade of the Ca^{2+}-ATPase with thapsigargin. From the initial rates of $[Ca^{2+}]_i$ rises triggered by thapsigargin, one can get a minimum estimate of the steady state turnover across the membrane of this pool, *i.e.* about 150–200 μmol/liter/min. Such a rapid turnover of the pool is not typical of PC12 cells only since comparable values can be calculated for other cell types as well (Ref. 16).[2]

The results on Ca^{2+} refilling into depleted cells, coupled with the effects of thapsigargin and ATP depletion, lead us to the conclusion that this pool is loaded by a Ca^{2+}-ATPase working with the Ca^{2+} of the cytosolic pool. Such a conclusion is strengthened by the observation that the rate of Ca^{2+} refilling is slowed down by (*a*) increasing the cytosolic Ca^{2+} buffering capacity, (*b*) low $[Ca^{2+}]_i$, (*c*) depolarization with high K^+, which decreases the driving force for Ca^{2+} flux across the plasma membrane. Together with additional, consistent evidence recently reported by others (23), these results concur in ruling out the possibility of a "private" direct pathway from the extracellular space to the Ins-P_3-sensitive store, as proposed by a popular model (24, 25).

The lack of effect of both monensin and NH_4Cl on $[Ca^{2+}]_i$ and on the refilling of the Ins-P_3-sensitive store indicates that in PC12 cells cytoplasmic alkalinization does not severely affect Ca^{2+} handling (see, however, Ref. 26 about endothelial cells) and argues against the Ca^{2+}/H^+ exchange mechanism of refilling recently suggested by Thevenod *et al.* (27) for the Ins-P_3-sensitive pool in pancreatic acinar cells.

The Ins-P_3-insensitive, Ionomycin-sensitive Pool—Ca^{2+} released by ionomycin from cells whose Ins-P_3-sensitive pool had already been depleted by treatment with Bk + thapsigargin remains the least precisely defined. Doubts as to whether this pool preexisted receptor stimulation or simply was the result of Ca^{2+} transfer from the Ins-P_3-sensitive pool can be given unambiguous response. In fact (*a*) in control cells the responses to ionomycin were always greater than those to Bk and thapsigargin, alone or in combination, and (*b*) a ionomycin response was obtained in cells depleted of the Ins-P_3-sensitive pool not only by the above mentioned treatments, but also by prolonged incubations in media containing excess EGTA.

Unlike the Ins-P_3-sensitive pool, a major discrepancy is observed in the quantitation of this pool with the two procedures employed, *i.e.* Ca^{2+} indicators and $^{45}Ca^{2+}$. In fact the former method underestimated the size of the Ins-P_3-insensitive pool probably because discharge by ionomycin is slow and occurs concomitantly with the stimulated efflux through the plasma membrane. Two independent lines of evidence indicate that no major transfer of Ca^{2+} occurs from the Ins-P_3-sensitive to the Ins-P_3-insensitive pool. In fact (*a*) neither the peak rise in $[Ca^{2+}]_i$ nor (*b*) the $^{45}Ca^{2+}$ release induced by ionomycin were appreciably changed in cells depleted by Bk treatment in EGTA and then refilled in Ca^{2+}-containing medium, with respect to untreated controls.

In quantitative terms, the Ins-P_3-insensitive, ionomycin-sensitive pool is the largest in PC12 cells, about 400 μmol/liter, but its subcellular localization is still undefined. Among the multiple intracellular structures presumably belonging to this pool there are obviously the mitochondria. The results obtained with the two mitochondrial poisons, rotenone and FCCP, however, suggest that in intact PC12 cells mitochondria account for only a small fraction of this pool. In fact (*a*) rotenone and FCCP had practically the same effect on $[Ca^{2+}]_i$, while their effect on mitochondrial Ca^{2+} handling is expected to be very different (28). (*b*) Both drugs caused $[Ca^{2+}]_i$ increases only in glucose-free medium, while, at least in the case of FCCP, the release of mitochondrial Ca^{2+} should be unaffected by the glycolitic production of ATP. (*c*) Most important, neither rotenone nor FCCP appreciably increased $[Ca^{2+}]_i$ when added after Bk, *i.e.* after depletion of the Ins-P_3-sensitive pool. Taken together, these results suggest that the main effect of the two mitochondrial inhibitors on $[Ca^{2+}]_i$ is due to their capacity of reducing cellular ATP levels, thus causing the release of Ca^{2+} from ATP-dependent pools (primarily the Ins-P_3-sensitive one). The small release of $^{45}Ca^{2+}$ induced by FCCP in glucose-containing medium puts an upper limit to the amount of mitochondrial mobilizable Ca^{2+}, *i.e.* 11% of the ionomycin-sensitive pool. If one considers that in PC12 cells mitochondria account for 7.7% of the volume,[3] their content of exchangeable Ca^{2+} is estimated to be, at the most, 0.57 mmol/liter, a value close to those measured by electron probe x-ray microanalysis in mitochondria of freeze-dried cryosections from various tissues (see, *inter alia*, Refs. 29 and 30).

About the remaining part(s) of the ionomycin-sensitive pool we only know that uptake is acid-independent and thapsigargin-insensitive. The existence of organelles (Golgi complex, part of the endoplasmic reticulum, etc.) endowed with thapsigargin-insensitive Ca^{2+}-ATPases appears a plausible hypothesis, given that various isoforms of this latter enzyme are known to be expressed in non-muscle cells (see Ref. 21 for a review).

The Acidic Pool—The acidity of this pool (and, conversely, the lack of acidity of the others) is clearly demonstrated by the fact that ionomycin was unable to discharge it unless the pH gradient across its limiting membrane was dissipated (by monensin or NH_4Cl). The inability of ionomycin to bind Ca^{2+} in an acidic enviroment is in fact very well known (31).

The possibility that acidity within the pool was established by H^+/Ca^{2+} exchange catalyzed by the ionophore (*i.e.* that the acidic pool constitutes the remaining part of the ionomycin-sensitive pool, undischarged, however, because of the progressive accumulation of H^+) appears unlikely. This is because monensin (or NH_4Cl) was needed to discharge this pool, even when depletion of the other stores was obtained by the combination of fura-2 loading and prolonged incubation of the cells in EGTA-containing medium.

Various intracellular organelles are known to enclose an

[2] C. Fasolato, unpublished observations.

[3] J. Meldolesi, unpublished observations.

acid content: lysosomes, endosomes, the trans Golgi network and, in PC12 and other secretory cells, also secretory granules. The large decrease in the size of the acidic pool in cells where robust exocytosis was stimulated demonstrates that this pool is mainly represented by secretory granules. The existence of a high Ca^{2+} content in chromaffin granules of adrenal gland cells, the normal counterparts of tumoral PC12 cells, is well known (32, 33). So far, however, studies were carried out with subcellular fractions, an experimental approach which cannot yield direct physiological information because it precludes the study of organelles in their natural enviroment, the cytosol. To our knowledge, this is the first time that Ca^{2+} of secretory granules (of any type) is identified and studied in intact, living cells.

Two properties we have characterized for the granule-located acidic Ca^{2+} pool of PC12 cells should be emphasized. First, the granule pool accounts for about 170 μmol/liter of cell water, *i.e.* it is as large as the the Ins-P_3-sensitive pool. Since in PC12 cells secretory granules account for 0.56% of the cell volume (9), this corresponds to a Ca^{2+} granule content of $\cong$30 mmol/liter, right in the range of values measured in isolated chromaffin granules (20–40 mmol/liter) (32, 33). Second, the pool is extraordinarily stable: no appreciable reduction of its size was in fact observed after several hours of incubation of the cells in EGTA-containing medium. Furthermore, labeling of this pool with $^{45}Ca^{2+}$ required incubation with the isotope much longer than the other pools. We conclude therefore that the acidic Ca^{2+} pool represents a dead end for cellular Ca^{2+}, destined not to participate in cytosolic regulation, but (presumably) to keep granule content components together and be ultimately discharged to the extracellular space by exocytosis.

Acknowledgments—We are indebted to G. Ronconi and L. Di Giorgio for excellent technical and secretarial assistances, respectively, to Dr. D. Milani for performing ATP measurements, and to Drs. F. Di Virgilio, P. Pizzo, and A. Simpson for stimulating discussions.

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