The secretory compartment is characterized by low luminal pH and high Ca\textsuperscript{2+} content. Previous studies in several cell types have shown that the size of the acidic Ca\textsuperscript{2+} pool, of which secretory granules represent a major portion, could be estimated by applying first a Ca\textsuperscript{2+} ionophore followed by agents that collapse acidic pH gradients. In the present study we have employed this protocol in the insulin-secreting cell line Ins-1 to determine whether the Ca\textsuperscript{2+} trapped in the secretory granules plays a role in exocytosis. The results demonstrate that a high proportion of ionophore-mobilizable Ca\textsuperscript{2+} in Ins-1 cells resides in the acidic compartment. The latter pool, however, does not significantly contribute to the [Ca\textsuperscript{2+}], changes elicited by thapsigargin and the inositol trisphosphate-producing agonist carbachol. By monitoring membrane capacitance at the single cell level or by measuring insulin release in cell populations, we show that Ca\textsuperscript{2+} mobilization from nonacidic Ca\textsuperscript{2+} pools causes a profound and long lasting increase in depolarization-induced secretion, whereas breakdown of granule pH had no significant effect. In contrast, releasing Ca\textsuperscript{2+} from the acidic pool markedly reduces secretion. It is suggested that a high Ca\textsuperscript{2+} concentration in the secretory compartment is needed to sustain optimal exocytosis.

A rise in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) is necessary to induce regulated secretion in most cell types (1, 2). In neurons, [Ca\textsuperscript{2+}], increases up to several hundred \( \mu \)M are needed to trigger vesicle fusion, whereas in endocrine cells, granule exocytosis appears to require lower [Ca\textsuperscript{2+}], rises (3–7). The time course of exocytosis also appears different in the two cell types. Synaptic vesicle fusion is very fast (\( \mu \)s) and abrupt, whereas granule fusion is slower and more sustained (3, 8, 9).

Aside from these differences, important similarities exist between secretory vesicles and granules. Both secretory vesicles and granules contain large amounts of Ca\textsuperscript{2+} ions (10–13). The function traditionally attributed to the high Ca\textsuperscript{2+} content in the secretory compartment is the packaging and processing of intravesicular content (14, 15). More recently a granular localization of the type 3 InsP\textsubscript{3} receptor has been suggested, based on high resolution immunocytochemistry of pancreatic \( \beta \)-cells (16). In the excocrine pancreas, evidence has been provided suggesting that the intragranular Ca\textsuperscript{2+} content is released by opening of low affinity InsP\textsubscript{3} receptors (17). These conclusions, however, have recently been challenged (18, 19), and the role of granular Ca\textsuperscript{2+} remains elusive. Another line of evidence suggesting that intragranular Ca\textsuperscript{2+} is implicated in secretion comes from the recent identification of an acidic Ca\textsuperscript{2+}-binding protein, granule lattice Protein 1 (Gr11p), in dense core secretory granules of Tetrahymena thermophila that appears essential for regulated secretion (20).

Another common feature between secretory vesicles and granules is their low luminal pH. They share this characteristic with the lysosomal/endosomal compartment and the trans-Golgi network (21, 22). Indeed, the low pH of the lumen has proven a reliable means for determining the Ca\textsuperscript{2+} content of the so-called “acidic Ca\textsuperscript{2+} pool.” Since ionophores such as ionomycin or A23187 are largely ineffective in transporting Ca\textsuperscript{2+} from an acidic environment (28), the pH gradient between lumen and cytosol must be collapsed before they can effectively release the Ca\textsuperscript{2+} content of this pool into the cytoplasm (23–26).

The aim of the present study was to establish whether the Ca\textsuperscript{2+} stored within the acidic pool is important in the late steps of exocytosis. For this purpose we employed as a model system the \( \beta \)-cell line Ins-1, an insulin-secreting cell line established from a rat insulinoma that, among different lines, best retains the phenotype of \( \beta \)-cells (27–29). Among other properties, Ins-1 cells display temperature-dependent and glucose-responsive secretion and, as shown here, temperature-dependent increases in membrane capacitance upon depolarizing pulses. Therefore, they can be used as an alternative to \( \beta \)-cells for studying secretion at the single cell level. By using capacitance measurements in combination with agents that mobilize Ca\textsuperscript{2+} and/or collapse intracellular pH gradients, the role of different intracellular Ca\textsuperscript{2+} pools in secretion has been assessed. We here demonstrate that, although a low pH in the granules is not required for the last steps of secretion, the level of intragranular Ca\textsuperscript{2+} significantly affects the secretory profile.

**EXPERIMENTAL PROCEDURES**

Cells—Ins-1 cells were cultured as described previously (27, 29). Two days before the experiment, cells were trypanosized and plated on poly(t-Lysine)-coated coverslips (diameter 24 mm; 10\textsuperscript{5} cells/coverslip).

Ca\textsuperscript{2+} Measurements in Ins-1 Cells—Cells were loaded for 30 min at 37 °C with 2 \( \mu \)M fura-2/AM as described previously (26). Coverslips were then bathed in 1 ml of Ringer’s solution containing 140 mM NaCl, 3 mM KCl, 1.8 mM CaCl\textsubscript{2}, 1.2 mM MgCl\textsubscript{2}, 10 mM HEPES, 10 mM glucose, 20 mM tetraethylammonium chloride, 1 mM EGTA, and 4 mM 

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The abbreviations used are: InsP\textsubscript{3}, inositol trisphosphate; BCEO, 2,7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; Ca\textsuperscript{2+}, membrane capacitance; Gm, membrane conductance; Gs, series conductance; Tg, thapsigargin; CCh, carbachol; F, farads.
The Role of Secretory Granule Calcium in Secretion

2.8 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5.6 mM glucose, 10 mM HEPES, pH 7.4, at 33 °C. Cells were placed on the stage of an inverted microscope equipped with a 40× oil immersion objective (Zeiss, Germany) and connected to a digital video imaging system (Georgia Instruments, Roswell, GA). All experiments were performed at 31–33 °C. Excitation wavelengths were set at 340 and 380 nm. Fluorescence emission at 510 ± 15 nm was collected by a CCD camera, and 8 images were averaged/time point. Time series were acquired with a frame interval of 4 s, and images at both excitation wavelengths were stored on an optomagnetic disc recorder (Panasonic, Japan). All data were normalized to the first min base-line ratio.

The internal solution consisted of 145 mM glutamic acid, 2 mM MgCl₂, 0.1 mM fura-2 (or 0.1 mM BCECF), and 10 mM HEPES at pH 7.2.

Membrane Capacitance Measurements—Unless otherwise specified, during electrophysiological recordings, cells were perfused at 31–33 °C with an external solution containing 118 mM NaCl, 20 mM tetraethylammonium chloride, 5.6 mM CaCl₂, 1.2 mM MgCl₂, 2 mM CaCl₂, 5.7 mM glucose, 10 mM HEPES at pH 7.4. Electrophysiological recordings and fura-2 fluorescence were performed in the whole-cell configuration of the patch-clamp technique with a computer-based patch-clamp amplifier (EPC-9, HEKA, Lambrecht, Germany) controlled by the Pulse software (HEKA). The internal solution consisted of 145 mM glutamic acid, 155 mM CaOH, 1 mM MgCl₂, 8 mM NaCl, 2 mM MgATP, 0.1 mM cAMP, 0.1 mM fura-2 (or 0.1 mM BCFE), and 10 mM HEPES at pH 7.2. Membrane capacitance (Cm) was measured with the “sine+dc” mode of the “lock-in” extension of the Pulse software, based on the Lindau-Neher algorithm (30). An 800 Hz, 40 mV peak-to-peak sinusoid stimulus was applied to the DC holding potential of −80 mV. During a depolarizing pulse and 5 ms before and after the pulse, no sinusoid was applied. No leak subtraction was performed on the evoked currents in the calculations used. After the whole-cell configuration was established, Cm was recorded and canceled by the automatic capacitance compensation of the EPC-9. The procedure was repeated every 180 s to prevent a possible saturation of the lock-in signal (31).

For fast capacitance changes after depolarizing pulses, it has been reported that activation of a Na⁺ current can lead to transient increases in Cm not linked to exocytosis (ΔCt) (32). ΔCt contributed maximally 10% of the initial ΔCm and returned to zero within 100 ms after the depolarization. To reduce its contribution on ΔCm measurements, the first 100 ms of the trace was discarded. The capacitance values of the following 200 ms were averaged and compared with the capacitance values before the depolarization to obtain the ΔCm reported in Figs. 6 and 7.

The [Ca²⁺]i was monitored by a photometry equipment (T. I. L. Photonics, Germany) controlled by the fura-2 extension of the Pulse software (HEKA) as described previously (33). Calculation of [Ca²⁺]i was performed on the calibrated ratio values (360 nm/380 nm), where Rmin (0.86), Rmax (6.07), and K-factor (1.68 × 10⁻²) were obtained by an internal calibration procedure. The fura-2 fluorescence, the holding current, the lock-in, and other parameters were synchronously recorded and analyzed at low resolution (3 Hz) by the X-Chart extension of the Pulse software (HEKA). Time courses of Cm as shown in Figs. 3–5 were obtained from the Cm trace, recorded at low frequency by a point-by-point subtraction. Positive and negative values are indicative of, respectively, exocytotic and endocytotic events. The cytosolic pH was monitored by ratioing the BCECF fluorescence signal (510 nm), excited at 440 and 490 nm.

Ionomycin and monensin were prepared from stock solution in Me₃SO (or ethanol) (0.4% final concentration) in the Ca²⁺-free external solution containing 5 mM EGTA to prevent cells from loading with extracellular Ca²⁺ in the presence of ionomycin. All drugs were applied by local pressure from a wide-tipped micropipette (5–10 μm) positioned close to the cell.

Insulin Secretion Studies—Superfusion experiments on Ins-1 cell suspensions were performed as described previously (34). In short, cells were washed in 1 ml superfusion chambers filled with 106 cells/chamber at a density of 10⁶ cells/ml. The cells were superfused at a rate of 1 ml/min at 37 °C, and test substances were introduced with the buffer. One-min fractions were collected and subjected to an insulin radioimmunoassay. For presentation, the KCl-induced stimulation of the second pulse was integrated and normalized to that of the first pulse.

Materials—fura-2/AM, fura-2 free acid, BCFE free acid, and Pluronic F-127 were obtained from Molecular Probes (Eugene, OR, USA); culture media and sera were from Technogenetics (Milan, Italy); and other chemicals were from Sigma.

RESULTS

[Ca²⁺]i Dynamics in Ins-1 Cells—When [Ca²⁺]i was monitored by fura-2 in intact cells bathed in a medium containing CaCl₂ (2 mM) and glucose (5.6 mM), approximately 50% of the cells displayed synchronous oscillations of [Ca²⁺]i, whereas frequency and amplitude largely depended on the cell batch (29). In Fig. 1, the [Ca²⁺]i kinetics from several individual cells were averaged, leading to a partial masking of the initial oscillations. The addition of EGTA immediately abolished these oscillations and caused a decrease in the base-line ratio, indicating that they depend on Ca²⁺ influx. The presence of acidic
Ca²⁺ pools and their contribution to [Ca²⁺], rises were evaluated with the protocol previously employed in other cell lines (23–26). In Fig. 1A, after EGTA addition, the fast-exchangeable Ca²⁺ pool was released by the Ca²⁺ pump inhibitor thapsigargin (Tg) (1 μM). The subsequent addition of the Ca²⁺ ionophore ionomycin (1 μM) led to a small, further increase in [Ca²⁺], indicating that in these cells the large majority of the ionomycin-sensitive pool is represented by the Tg-sensitive one. Release of the acidic Ca²⁺ pool was then achieved by the addition of the Na⁺/H⁺ exchanger monensin (2 μM). Qualitatively similar data have been obtained by addition of the weak base chloroquine (40 μM), used in place of monensin to dissipate the intraluminal pH gradients. The increase in [Ca²⁺], after monensin (or chloroquine) application requires the pretreatment with ionomycin (26). In fact, addition of either drug alone was without appreciable effect on [Ca²⁺], (data not shown). Integrating peak areas showed that the amount of Ca²⁺ residing in acidic compartments was, on average, 51.3 ± 3.6% (n = 4) of total releasable Ca²⁺. The amount of Ca²⁺ released from the different Ca²⁺ pools was also assayed by atomic absorption spectrophotometry. In controls (unstimulated conditions), the total content of cellular Ca²⁺ was estimated to be 5.7 nmol of Ca²⁺/mg of protein (n = 3). Ionomycin alone or ionomycin and monensin together released 2.4 ± 1.1 and 4.5 ± 0.4 nmol of Ca²⁺/mg of protein, respectively (n = 3). Thus, of the total releasable Ca²⁺, about 54% was released by ionomycin alone; the remaining 46% was then attributed to the Ca²⁺ content selectively released from the acid pool.

We functionally tested for the existence of InsP₃ receptors on granules. Fig. 1B shows that InsP₃ production, induced by the muscarinic agonist carbachol (CCh, 0.5 mM), reduced the Tg-sensitive pool but had no effect on the size of the peak induced by monensin application. In fact, in the presence of CCh, the acidic pool represented 49 ± 1.7% (n = 7) of total mobilizable Ca²⁺. Finally, Fig. 1C shows that pretreatment with Tg abolished the peak in [Ca²⁺], induced by CCh, indicating that in this cell type, InsP₃- and Tg-sensitive pools fully overlap. Altogether the data demonstrate that acidic Ca²⁺ compartments in Ins-1 cells are depleted neither by InsP₃ produced by receptor stimulation nor by inhibition of Tg-sensitive pumps.

Capacitance Changes After Activation of Voltage Operated Ca²⁺ Channels—Fig. 2 shows fast changes in Cm, membrane conductance (Gm) as well as series conductance (Gs) before and after a 200-ms depolarizing pulse from −80 mV holding potential to 0 mV. With 200-ms pulses, individual cells displayed ΔCm ranging from 20 to 400 fF with an average of 49.2 ± 4.4 fF (mean ±S.E., n = 76). Increasing the pulse duration to 400 ms led to an increase in ΔCm of 60% when compared with a 200-ms depolarizing pulse in 4 of 9 cells (data not shown). However, these longer depolarizing pulses caused a rapid rundown of the evoked Ca²⁺ currents. We therefore decided to perform the experiments with 200-ms pulse duration.

By following Cm at a lower frequency (3 Hz) it can be seen that upon such a depolarization ΔCm remained constant for up to 4 min before a rundown in secretion was observed, as long as these pulses were at least 20 s apart (Fig. 3A). This result indicates that secretion in Ins-1 cells is not easily “exhaustible” by successive 200-ms depolarizing pulses. With this protocol, maximum secretion increased the initial membrane capacitance by about 10% and was equivalent to fusion of approximately 300 granules (n = 13) (assuming the mean diameter of the insulin-containing granules to be 250 nm and a membrane capacitance of 1 μF/cm²). However, since an interval of 20 s is too short to apply test substances between subsequent depolarizations, the interpulse duration was increased to 90 s. From the time course of ΔCm, we also noticed that, upon depolarization, the majority of the cells (65%) displayed only an increase in Cm; in the remaining 35%, slow endocytotic processes were observed after the first and, occasionally, the second pulse (see Fig. 3B), whereas large, abrupt endocytotic events were never observed under our experimental conditions.

In addition to Cm and Ca²⁺ current, the [Ca²⁺], was monitored as described under “Experimental Procedures.” As can be seen in Fig. 4A, the amplitude of the [Ca²⁺], peaks decreased upon subsequent depolarizations, whereas the integrated [Ca²⁺], peaks and Ca²⁺ currents (ΔIp) (Fig. 4C) as well as ΔCm (Fig. 4B) remained unchanged. The decrease in [Ca²⁺], peak amplitude is probably due to the relatively slow influx of furan-2 from the pipette, leading to changes in the Ca²⁺ buffering capacity of the intracellular medium during prolonged incubations (35).
Role of Granular Ca\textsuperscript{2+} Content in Secretion—We first tested if drug application by itself induced ΔCm. From the low frequency recording of Cm it can be seen that during application of ionomycin, monensin, or the combination of the two, an increase in Cm occurred (Fig. 5A–C, lower panels). Since similar increases were observed when the solvents ethanol or Me\textsubscript{2}SO were tested and when experiments were performed at room temperature to inhibit regulated secretion (36), we conclude that to a large extent the observed changes are due to the solvent. Chloroquine, on the other hand, being dissolved in Ringer's solution, had no effect. We also tested whether drug application changed the intracellular pH; therefore in some experiments BCECF was included in the intracellular solution instead of fura-2. These experiments showed that application of none of the drugs, applied alone or in combination, significantly altered the cytosolic pH when cells were kept in the whole-cell configuration (data not shown).

To determine the role of the granular Ca\textsuperscript{2+} content in granule fusion, the increases in Cm in response to 200-ms depolarizing pulses were thus monitored immediately before and after application of the different drugs. Changes in Cm at the second and subsequent depolarizing pulses were normalized to the change obtained in the first pulse. When ΔCm was monitored in untreated, control cells after this normalization protocol, it remained stable during the second depolarization (Fig. 6, n = 40). Figs. 5B and 6 show that, after a brief application of ionomycin, the subsequent depolarizing pulse caused a consistently larger increase in ΔCm (47 ± 12%, n = 10). The increase in ΔCm after the ionomycin pulse was relatively long lasting since it was maintained for at least 3 min and did not depend on larger Ca\textsuperscript{2+} currents during subsequent depolarizations (compare Figs. 7, A and B).

Application of chloroquine between the first and the second pulse had no effect (n = 6), whereas monensin led to a quite variable stimulation (Figs. 5A and 6). On average, however, the stimulation caused by monensin treatment between two successive depolarizing steps was not statistically significant (23 ± 21%, n = 9; see also Fig. 7).

A completely different pattern was observed when the Ca\textsuperscript{2+} ionophore and chloroquine (or monensin) were applied together in order to discharge the Ca\textsuperscript{2+} content of the acidic pools. The ΔCm increase following the depolarizing step, elicited after the discharge of the acidic Ca\textsuperscript{2+} pools (Figs. 5C and 6), was reduced not only with respect to the potentiation caused by ionomycin (46 ± 13%, n = 17, monensin/ionomycin, 54 ± 9%, chloroquine/ionomycin, n = 6) but was also reduced with respect to untreated control cells (21 ± 9%, monensin/ionomycin; 31 ± 2%, chloroquine/ionomycin).

The reduction in ΔCm following the depolarizing step was also observed with further test pulses, i.e. it was prolonged for up to 3 min (Fig. 7B). It is worth mentioning that there was no

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**Fig. 3.** Exocytosis in Ins-1 cells is not easily “exhaustible.” A, changes in Cm (ΔCm) after repetitive depolarizing pulses of 200-ms duration, delivered every 20 s. The time course of ΔCm was obtained by subtracting from each point of the Cm trace (recorded at low frequency (3 Hz)) the preceding value. B, enlargement of an exocytotic event that is followed by endocytosis. The shaded areas are an indication of the exocytosis (light gray) and endocytosis (dark gray).

**Fig. 4.** Measurements of [Ca\textsuperscript{2+}], ΔCm, and Ca\textsuperscript{2+} charge in control cells. A and B, [Ca\textsuperscript{2+}], and Cm changes were monitored as described under “Experimental Procedures.” ΔCm is plotted as described in Fig. 3. C, the integrated Ca\textsuperscript{2+} peak was obtained from the trace shown in panel A, whereas the Ca\textsuperscript{2+} charge (q) was obtained by integrating the Ca\textsuperscript{2+} current.
significant change in either Gm or Gs when the second depolarizing pulse was compared with the first pulse either in controls or treated cells (data not shown). Moreover, application of ionomycin, monensin, or the combination of ionomycin and monensin had no significant effect on changes in $\text{[Ca}^{2+}]_i$ ($\Delta\text{Ca}$) (Fig. 5) and $\Delta\text{Ip}$ of the subsequent depolarizing pulses (Fig. 7A).

The inhibition of secretion did not exceed more than 30% of the control values. A possible explanation for this incomplete inhibition is that a complete alkalinization by monensin or chloroquine (and therefore complete discharge of granule $\text{Ca}^{2+}$) takes longer than the application time of 20 s used in our electrophysiological experiments. To test this possibility, studies were performed where the H$^+$ exchanger was present for 5 min before the first depolarizing pulse was given, to ensure a complete breakdown of the pH gradient. After this prolonged incubation, secretion during the first depolarizing pulse was within the expected range of variability (43 ± 7.2 fF; $n = 7$).

**FIG. 5.** $[\text{Ca}^{2+}]_i$ and $\Delta\text{Cm}$ after application of test substances during depolarizing pulses. Depolarizing pulses of 200-ms duration were delivered every 90 s. During the first and the second pulses, test substances were puffed onto the cell for 20 s. $[\text{Ca}^{2+}]_i$ (upper panels) and Cm changes (lower panels) were measured as described in Fig. 4 in cells challenged, respectively, with 2 $\mu$M monensin/5 mM EGTA (A), 1 $\mu$M ionomycin/5 mM EGTA (B), 1 $\mu$M ionomycin/2 $\mu$M monensin/5 mM EGTA (C).

**FIG. 6.** Comparison of Cm changes in control and treated cells. Cells were repeatedly depolarized and challenged with test substances as described in Fig. 5. Changes in Cm immediately after the depolarizing pulses were monitored as described in Fig. 2. Values obtained in the second pulse were then normalized to the first pulse. *, $p < 0.05$; 2-tailed Student’s $t$ test. The effect of chloroquine (40 $\mu$M) and monensin (2 $\mu$M) alone was not statistically significant.

**FIG. 7.** Long lasting effect of the ionomycin-enhanced stimulation of regulated secretion. Cells were treated as described in Fig. 5. Between the first and the second depolarizing pulses, test substances were applied: $\blacksquare$, control; $\blacktriangle$, 1 $\mu$M ionomycin/5 mM EGTA; $\bullet$, 2 $\mu$M monensin/5 mM EGTA; $\times$, 1 $\mu$M ionomycin/2 $\mu$M monensin/5 mM EGTA. Changes in peak currents ($\Delta\text{Ip}$) (A) and $\Delta\text{Cm}$ (B), recorded as described in Fig. 2, were normalized to the changes obtained during the first depolarizing pulse. *, $p < 0.05$; 2-tailed Student’s $t$ test.
were depolarized with two pulses of 1 min duration of 30 m M KCl
sin) was employed, secretion during the second KCl pulse was
When a combination of ionomycin and chloroquine (or monen-
this compartment, i.e.
internal acidic pH gradients (monensin or chloroquine) after
acidic structures. In fact, these cells respond with a large
p
second KCl challenge was measured by radioimmunoassay as described
applied 5 min apart in Ringer’s solution. Insulin secretion during the
30 mM KCl of 1-min duration, 5 min apart. As summarized in
7; Fig. 6).
However, the prolonged treatment with monensin did not further
increase the level of inhibition obtained when ionomycin was
applied between the first and second pulses (24 ± 12%, n = 7; Fig. 6).
Insulin Secretion Studies—To determine whether the reduc-
tion in ΔCm caused by acidic Ca2+ pool depletion was attrib-
utable, at least in part, to fusion of insulin-containing granules, we
followed the release of insulin in populations of cells treated with
protocols that mimic those used in Fig. 5. Cell suspensions
obtained from monolayers were challenged with two pulses of 30 m M KCl of 1-min duration, 5 min apart. As summarized in
Fig. 8, secretion during the second depolarizing pulse was 25 ±
4% (n = 3) of that obtained during the first challenge. This
reduction in secretion probably reflects a reduction in readily
releasable insulin granules, although a rundown of the Ca2+
peak after depolarization may also contribute to this effect (34).
One-min stimulation with 1 μM ionomycin between the first and
second KCl pulses resulted in a less drastic reduction of
insulin secretion (62 ± 24% that of initially released, n = 3).
When a combination of ionomycin and chloroquine (or monen-
sin) was employed, secretion during the second KCl pulse was
25 ± 11 and 24 ± 4% (n = 3), respectively; i.e. the potentiating
effect of ionomycin was completely abolished.

**DISCUSSION**

In the β-cell line Ins-1, as in other secretory cells, a relatively
high proportion of intracellular Ca2+ appears to be stored in
acidic structures. In fact, these cells respond with a large
[Ca2+]i increase to the protocol previously employed to reveal
this compartment, i.e. the application of drugs that collapse
internal acidic pH gradients (monensin or chloroquine) after
addition of the Ca2+ ionophore ionomycin. The subcellular
localization of acidic Ca2+ pools has not been determined with
certainty, although it is likely that in Ins-1 cells, as in other cell
types, it is heterogeneous. A rough estimation of the contribu-
tion of insulin granules to the Ca2+ content of the acidic pool
can be obtained by considering the total releasable Ca2+ of
Ins-1 cells (4.5 nmol/mg of protein, this work), the cell volume
occupied by the granules (1.2%); and the releasable granule
Ca2+ (about 125 nmol/mg of granule protein, Ref. 10). By using
these parameters, intragranular Ca2+ mobilization could be as
high as 1.5 nmol/mg of protein. We have shown here that in
Ins-1 cells 46% (i.e. 2.4 nmol/mg of protein) of the total releas-
able Ca2+ is due to the acidic pool. Although based on a number
of assumptions, these values indicate that insulin granules
represent a major part (more than 60%) of the acidic compart-
ment in this cell type.

The main goal of this investigation was to establish the role
played by Ca2+ trapped in the secretory compartment in the
process of secretion. To address this question, we first investi-
gated whether or not the acidic compartment could contribute
to (i) the [Ca2+]i changes induced in Ins-1 cells by the musca-
rinic agonist CCh and (ii) the [Ca2+]i changes induced by de-
polarization. The finding that Ca2+ mobilization induced by
thapsigargin or InsP3 production through activation of musca-
rinic receptors does not affect the acidic pool is meaningful. In
fact, given that insulin granules represent a large proportion of
that pool, it confirms by a functional approach the conclusion of
Ravazzola et al. (18) that InsP3 receptors are not expressed on
the membrane of the secretory granules of β-cells. Similarly, a
role for the acidic pool (and thus for insulin granules) in Ca2+-
induced Ca2+ release is unlikely since the increase in [Ca2+]i
caused by depolarization was indistinguishable in controls and
cells whose acidic pool had been depleted (Fig. 5).
We next tested the possibility that intragranular Ca2+ plays
a role in the secretory process by monitoring membrane capac-
tance in single Ins-1 cells under different experimental condi-
tions. In untreated cells, the magnitude of ΔCm has a tendency
to decrease during a series of successive pulses; however up to
the fourth pulse (i.e. 300 s), ΔCm is fairly constant. On the
contrary, manipulation of intracellular Ca2+ in the time inter-
val between the first and the second depolarizing pulses sig-
nificantly changed the extent of secretion. In fact, depletion of
Ca2+ from nonacidic stores led to a prolonged stimulation of
secretion up to 50%. Such a priming action of ionomycin has
been described previously (4), but the fact that it can last for
several min at resting [Ca2+]i, is a novel observation. Releasing
Ca2+ from the ionomycin-sensitive compartments may favor
granule recruitment from a distant cytoskeletal-anchored pool
(38) or by promoting priming of granules at a late, post-docking
step (39). Such a priming has been previously described by
mechanisms that cause long lasting phases of moderately
elevated [Ca2+], (31).
In marked contrast with the potentiating effect of a brief
increase in [Ca2+], releasing Ca2+ from the acidic compart-
ments led to inhibition of secretion that reached 50% when
compared with cells treated only with ionomycin. Since break-
down of the intracellular pH gradients by itself was without
effect and the inhibition was observed with both monensin and
chloroquine (two agents that act on pH gradients by different
mechanisms), it can be concluded that the inhibitory effect is
due to the release of Ca2+ from the acidic organelles, including
insulin granules. Since our alkalinization protocol is by no
means specific for the granules, the question can be raised as to
whether the reduction in secretion is due to the decrease in
Ca2+ within the granules themselves or in other acidic com-
partments (trans-Golgi network or lysosomes). The observation
that inhibition is maximal within a few tens of seconds after

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2 This value has been estimated considering that Ins-1 cells contain
10% of the insulin content of β-cells (27) and that in the latter cell type,
the percentage volume occupied by granules is 11.5% based on morpho-
metric analysis (37).
acidic Ca\(^{2+}\) pool discharge argues for a distal site of action, i.e., the granules themselves.

It is probable that both insulin-containing granules and \(\delta\)-aminobutyric acid-containing vesicles contribute to the increases in \(\Delta m\) monitored by us and by other groups (7, 40, 41). However, the fact that Ca\(^{2+}\) depletion from nonacidic and acidic compartments affects both \(\Delta m\) increases and insulin secretion in radioimmunooassay suggests that at least part of the effects seen in our study reflects fusion of insulin-containing granules.

A high intragranular Ca\(^{2+}\) concentration may be important for docking or priming of the granules for the fusion process itself or for all of the steps. It is noteworthy that a protein called Grl1p, abundant in secretory granules of \(T.\ thermophila\) (20) is sensitive to both Ca\(^{2+}\) and pH.

In conclusion, our experiments show that in Ins-1 cells, a relatively large amount of the stored Ca\(^{2+}\) resides in acidic compartments. A high [Ca\(^{2+}\)] in this compartment but not a low pH is needed for optimal exocytosis.

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