Dense core secretory vesicles revealed as a dynamic Ca\(^{2+}\) store in neuroendocrine cells with a vesicle-associated membrane protein aequorin chimaera

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Introduction

In most mammalian cells, the ER (Streb et al., 1984; Rizzuto et al., 1993; Brini et al., 1993) and Golgi complex (Pinton et al., 1998) are believed to represent the major mobilizable intracellular Ca\(^{2+}\) stores (Rutter et al., 1998). Uptake of Ca\(^{2+}\) into these stores is mediated largely by sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs)* (Moller et al., 1996) and helps to maintain resting cytosolic Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_c\)) at levels (~10\(^{-7}\) M) some four orders of magnitude lower than in the extracellular space (~10\(^{-3}\) M). Release of Ca\(^{2+}\) from the ER and Golgi complex is provoked by hormones and other agonists which generate inositol 1,4,5 trisphosphate (IP\(_3\)) (Berridge, 1993) to open intracellular receptors (RyRs) activation with caffeine or 4-chloro-3-ethylphenol in intact cells, or cyclic ADP-ribose in permeabilized cells, causes a dramatic fall in [Ca\(^{2+}\)]\(_{sv}\). Thus, secretory vesicles represent a dynamic Ca\(^{2+}\) store in neuroendocrine cells, whose characteristics are in part distinct from the ER/Golgi apparatus. The presence of RyRs on secretory vesicles suggests that local Ca\(^{2+}\)-induced Ca\(^{2+}\) release from vesicles docked at the plasma membrane could participate in triggering exocytosis.

*Abbreviations used in this paper: cADPr, cyclic ADP ribose; CPA, cyclopiazonic acid; DHPG, dihydroxyphenylglycine; GFP, green fluorescent protein; HA, hemagglutinin; IB, intracellular buffer; IP\(_3\), inositol 1,4,5 trisphosphate; KRB, Krebs-Ringer bicarbonate buffer; RyR, ryanodine receptor; 4-CEP, 4-chloro-3-ethylphenol; SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; VAMP, vesicle-associated membrane protein.

Key words: calcium; secretory vesicle; insulin; ryanodine receptor; aequorin

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Bound and more rapidly exchanging pools of Ca\(^{2+}\); (c) inositol (1,4,5) trisphosphate has no impact on [Ca\(^{2+}\)]\(_{sv}\) in intact or permeabilized cells; and (d) ryanodine receptor (RyR) activation with caffeine or 4-chloro-3-ethylphenol in intact cells, or cyclic ADP-ribose in permeabilized cells, causes a dramatic fall in [Ca\(^{2+}\)]\(_{sv}\). Thus, secretory vesicles represent a dynamic Ca\(^{2+}\) store in neuroendocrine cells, whose characteristics are in part distinct from the ER/Golgi apparatus. The presence of RyRs on secretory vesicles suggests that local Ca\(^{2+}\)-induced Ca\(^{2+}\) release from vesicles docked at the plasma membrane could participate in triggering exocytosis.

The role of dense core secretory vesicles in the control of cytosolic-free Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{c}\)) in neuronal and neuroendocrine cells is enigmatic. By constructing a vesicle-associated membrane protein 2-synaptobrevin.aequorin chimera, we show that in clonal pancreatic islet \(\beta\)-cells: (a) increases in [Ca\(^{2+}\)]\(_c\) cause a prompt increase in intravesicular-free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{sv}\)), which is mediated by a P-type Ca\(^{2+}\)-ATPase distinct from the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase, but whose characteristics are in part distinct from the ER/Golgi apparatus. The presence of RyRs on secretory vesicles suggests that local Ca\(^{2+}\)-induced Ca\(^{2+}\) release from vesicles docked at the plasma membrane could participate in triggering exocytosis.

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41
showed the antibody used cross-reacted with insulin (Ravazzola et al., 1996). IP₃, as well as the receptor (RyR) agonist cyclic ADP ribose (cADPr) (Galiane, 1994) have been reported to release Ca²⁺ from individual acinar cell zymogen granules (Gerasimenko et al., 1996), although contamination of these preparations (e.g., with ER or Golgi apparatus-derived vesicles) is a potential problem (Yule et al., 1997). Finally, evidence for the direct participation of secretory granules in the control of cytoplasmic Ca²⁺ concentration was recently provided in intact goblet cells (Nguyen et al., 1998).

At present, the molecular mechanisms responsible for Ca²⁺ uptake into secretory vesicles are also a matter of controversy. At high Ca²⁺ concentrations (>50 μM), uptake into dense core vesicles in permeabilized hypophysial cells (Troade et al., 1998; Thirion et al., 1999) and into isolated chromaffin cell granules (Krieger-Brauer and Gratzl, 1982) can occur via Na⁺/Ca²⁺ exchange, while synaptic vesicles also accumulate Ca²⁺ via a Ca²⁺-H⁺ antiport system (Goncalves et al., 1998). However, the pump/exchange mechanisms active at physiological [Ca²⁺]c are uncertain.

To examine the pathways by which Ca²⁺ crosses the limiting membrane of the dense core secretory vesicle of living islet β-cells, we have developed a new approach to monitor the free Ca²⁺ concentration within the secretory vesicle matrix ([Ca²⁺]sv) using recombinant targeted aequorin (Rizzuto et al., 1995). Cloned from the jellyfish Aequorea victoria (Inouye et al., 1995), aequorin is a calcium-sensitive bioluminescent protein (Cobbold and Rink, 1987), previously used to measure free Ca²⁺ concentrations in a variety of subcellular organelles (Rutter et al., 1998). Importantly, aequorin activity is less severely inhibited at low pH values (<6.5; Blinks, 1989) than Ca²⁺ probes based on green fluorescent protein (GFP) (Miyawaki et al., 1997; Baird et al., 1999; Emmanouilidou et al., 1999). If appropriately targeted, this probe should allow Ca²⁺ concentrations to be measured in the acidic environment of the secretory granule interior (Orci et al., 1985).

Vesicle-associated membrane protein (VAMP)2/synaptobrevin (Sudhof et al., 1989) is a vesicle-specific SNARE with a single transmembrane-spanning region. Expression of chimeric cDNA encoding a fusion protein between VAMP2 and aequorin (VAMP.Aq) has therefore allowed the intravesicular Ca²⁺ concentration to be monitored dynamically in live MIN6 β-cells. With this approach, we show that Ca²⁺ is actively pumped into dense core vesicles when [Ca²⁺]c increases, and may be released via RyR, but not IP₃, receptors. This release may be important at sites of high intracellular Ca²⁺, including sites of exocytosis at the plasma membrane.

**Results**

**Subcellular targeting of recombinant VAMP.aequorin**

Chimeric cDNA encoding hemagglutinin (HA)1-tagged aequorin, fused to VAMP2 (Sudhof et al., 1989), was generated as shown in Fig. 1 A. Immunocytochemical analysis of MIN6 cells transfected with VAMP.Aq cDNA revealed close overlap with insulin staining (Fig. 1 B). Explored at a higher resolution by immunoelectron microscopy (Fig. 1 C), VAMP.Aq immunoreactivity was highly enriched in 61 of 148 (41.2%; n = 11 cells) vesicles colabeled for insulin (Fig. 1 C). Analyzed by single labeling for VAMP.Aq, staining of the ER, Golgi apparatus, and small synaptic-like microvesicles (Reetz et al., 1991) was very low, while reactivity was also present on the plasma membrane and in endosomes (see the legend to Fig. 1 and Discussion).

**Reconstitution and calibration of secretory vesicle and ER-targeted aequorins**

Given the high total Ca²⁺ content of secretory vesicles (Hutton et al., 1983), we used the approach adopted previously to measure Ca²⁺ in the ER lumen (Montero et al., 1995). Apoaequorin was reconstituted at a low free Ca²⁺ concentration (Montero et al., 1995), achieved by depleting cells of Ca²⁺ (Materials and methods). Depletion of vesicle Ca²⁺ had no marked effect on glucose or K⁺-stimulated insulin secretion, or on vesicle motility (Pouli et al., 1998b; Tsuboi et al., 2000; unpublished data).

To determine the response of the expressed aequorins to Ca²⁺ in situ, permeabilized cells were incubated at buffered...
Free \([\text{Ca}^{2+}]\) in insulin secretory vesicles

Mitchell et al.

Ca\(^{2+}\) concentrations in the presence of ionomycin and monensin (Fig. 2, C and E). The sensitivity to Ca\(^{2+}\) (at pH 7.0) of the VAMP.Aq chimera was similar to that reported previously for mutant (D\(^{119}\)A) aequorin (Montero et al., 1995). Intravesicular pH in intact cells was determined using a fusion construct between VAMP2 and a mutated, pH-sensitive GFP (pH.fluorin(e); Miesenbock et al., 1998), and gave a pH value of 6.3 ± 0.02 (n = 85 cells; Fig. 2 A). Confirming that this low intravesicular pH was unlikely to significantly affect VAMP.Aq, near identical calibration data were obtained at pH 5.7 (unpublished data). Therefore, we used the constants obtained in vitro (Montero et al., 1995) to calculate \([\text{Ca}^{2+}]\) from the fractional rate of aequorin consumption (F) according to: 

\[
[\text{Ca}^{2+}] = 1.44 \times 10^{(FG-3.4)}
\]

(Rutter et al., 1993).

Dynamic measurement of secretory vesicle and ER Ca\(^{2+}\) concentrations

To monitor uptake of Ca\(^{2+}\) into vesicles in living cells, we provoked rapid Ca\(^{2+}\) influx into cells through store-operated channels (Parekh and Penner, 1997). During reintroduction...
Table I. Kinetic parameters for Ca<sup>2+</sup> uptake into the ER versus secretory vesicles

|                | Steady state [Ca<sup>2+</sup>] ± SE | t<sub>1/2</sub> ± SE | K<sub>init</sub> n  |
|----------------|--------------------------------------|---------------------|-------------------|
| ER            | 249 ± 12.9 5.7 ± 0.2 29.8 ± 0.8 3  |
| + Thap        | 71 ± 12.3 6.1 ± 1.3 9.2 ± 2.7 3  |
| Vesicles      | 51 ± 7.5 4.2 ± 0.1 7.1 ± 1.1 5  |
| + Thap        | 40 ± 4.4 3.9 ± 0.6 7.4 ± 0.5 3  |

Kinetic values were calculated by fitting time course data (Fig. 3) to a simple first order exponential curve by nonlinear least squares regression (Microsoft Excel<sup>™</sup>).

Pathways of Ca<sup>2+</sup> uptake into dense core secretory vesicles

Both the rate and extent of the [Ca<sup>2+</sup>]<sub>SV</sub> increases in the secretory vesicles and the ER were strongly dependent on the presence of added ATP in permeabilized cells (Fig. 3, A and D). The increase in [Ca<sup>2+</sup>]<sub>SV</sub> upon reintroduction of Ca<sup>2+</sup> ions was completely inhibited by the P-type Ca<sup>2+</sup> pump inhibitor, orthovanadate, at >100 μM (Fig. 3 B), but was insensitive to 10 μM orthovanadate, a concentration which strongly inhibits the plasma membrane Ca<sup>2+</sup>-ATPase (Carafoli, 1991). Preincubation with the specific SERCA inhibitor, thapsigargin (Thastrup, 1990), and perfusion with cyclopiazonic acid (CPA) (Mason et al., 1991) had no effect on the changes in [Ca<sup>2+</sup>]<sub>SV</sub> (Fig. 3 C and Table I), but markedly (>85%) inhibited ER [Ca<sup>2+</sup>]<sub>ER</sub> increases (Fig. 3 E).

Arguing against vesicular Ca<sup>2+</sup> uptake via Ca<sup>2+</sup>/H<sup>+</sup> exchange, monensin, an Na<sup>+</sup>/H<sup>+</sup> exchanger, had no effect on [Ca<sup>2+</sup>]<sub>SV</sub> increases in permeabilized cells (Fig. 4 A). Similarly, ionomycin (a Ca<sup>2+</sup>/H<sup>+</sup> exchanger) decreased [Ca<sup>2+</sup>]<sub>SV</sub> marginally (~20%; Fig. 4 B), which is consistent with the inability of this ionophore to bind to and transport Ca<sup>2+</sup> against a prevailing H<sup>+</sup> gradient, but caused a rapid and complete decline in [Ca<sup>2+</sup>]<sub>SV</sub> in the additional presence of monensin (Fig. 4 B). Neither the vacular H<sup>+</sup>-ATPase inhibitor, bafilomycin (Fig. 4, C and D), nor the protonophore carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), had any effect on vesicular Ca<sup>2+</sup> uptake (Fig. 4 E).

[Ca<sup>2+</sup>]<sub>SV</sub> increases were also completely unaffected by raising [Na<sup>+</sup>] from 0, 2, or 140 mM (Fig. 4 F), and by simultaneous blockade of Na<sup>+</sup>/Ca<sup>2+</sup> exchange and Ca<sup>2+</sup>/H<sup>+</sup> exchange (zero Na<sup>+</sup>, 300 nM bafilomycin; unpublished data).

Effects of IP<sub>3</sub> on [Ca<sup>2+</sup>]<sub>SV</sub> and [Ca<sup>2+</sup>]<sub>ER</sub>

To achieve large changes in intracellular IP<sub>3</sub> levels, we coexpressed the metabotropic glutamate receptor, mGluR5, with VAMP.Aq or ER.Aq. Stimulation of mGluR5-transfected cells with the specific agonist, (S)-3,5-dihydroxyphenylglycine (DHPG) (Thomas et al., 2000) caused a significant de-

![Figure 4. Effects of collapse of H<sup>+</sup> and Na<sup>+</sup> gradients on vesicular Ca<sup>2+</sup> accumulation in permeabilized cells.](image-url)

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Vol. 155, No. 1, 2001
increase in \([\text{Ca}^{2+}]_{\text{ER}}\) (Fig. 5 E), but an increase in \([\text{Ca}^{2+}]_{\text{SV}}\) (Fig. 5 A). DHPG had no significant effect on the distribution of vesicles (imaged after expression of VAMP.GFP/pH-fluorin (r); Fig. 5 D), indicating that enhanced exocytosis and exposure of the VAMP.Aq to the extracellular medium was unlikely to contribute to the observed increases in \([\text{Ca}^{2+}]_{\text{SV}}\) (Fig. 5 A, and see Discussion).

Similarly, in permeabilized cells, IP\(_3\) had no effect on \([\text{Ca}^{2+}]_{\text{SV}}\) (Fig. 5, B and C), while causing a large decrease in \([\text{Ca}^{2+}]_{\text{ER}}\) (Fig. 5, F and G).

**RyR activation decreases \([\text{Ca}^{2+}]_{\text{SV}}\) and \([\text{Ca}^{2+}]_{\text{ER}}\)**

The RyR agonists caffeine or 4-chloro-3-ethylphenol (4-CEP) (Zorzato et al., 1993) provoked rapid decreases in \([\text{Ca}^{2+}]_{\text{SV}}\) and \([\text{Ca}^{2+}]_{\text{ER}}\) (Fig. 6, A and C) in intact cells. Similarly, cADP\(_r\) (Galiano, 1994) caused clear decreases in both parameters in permeabilized cells (Fig. 6, B and D), and the effects of cADP\(_r\) were strongly potentiated by palmitoyl CoA (Fig. 6, B and D), a coagonist of RyRs (Chini and Dousa, 1996).

**4-CEP mobilizes \(\text{Ca}^{2+}\) from an acidic, CPA-insensitive store in fura-2–loaded MIN6 cells**

The above results suggested that activation of RyR on vesicles should cause an increase in cytoplasmic \([\text{Ca}^{2+}]_c\), even after the depletion of the ER \([\text{Ca}^{2+}]_c\) pool. Added to fura-2–loaded MIN6 cells, 4-CEP caused a substantial increase in \([\text{Ca}^{2+}]_c\), in the absence of external \(\text{Ca}^{2+}\) (Fig. 7 A). This \([\text{Ca}^{2+}]_c\) increase was partly retained after depletion of SERCA-dependent stores, giving a peak \([\text{Ca}^{2+}]_c\), increase of 30–40% of that in control cells (Fig. 7 B versus A). The \([\text{Ca}^{2+}]_c\) increase elicited by 4-CEP was also partially retained after treatment of cells with ionomycin (Fig. 7 C), but completely abolished after treatment with ionomycin plus monensin, to deplete acidic \([\text{Ca}^{2+}]_c\) stores (Fig. 7 D). Demonstrating that the effects of 4-CEP on \([\text{Ca}^{2+}]_{\text{SV}}\) were likely mediated by RyR, immunoreactivity to these channels was revealed on vesicle membranes by direct immunoelectron microscopy (Fig. 7 E).

**Effects of nutrient secretagogues on \([\text{Ca}^{2+}]_{\text{ER}}\) and \([\text{Ca}^{2+}]_{\text{SV}}\)**

Exposure of islet \(\beta\)- or MIN6 cells to glucose or other nutrients causes an increase in intracellular-free [ATP] (Kennedy et al., 1999) closure of ATP-sensitive K\(_{\text{ATP}}\) (K\(_{\text{ATP}}\)) channels (Bryan and Aguilar-Bryan, 1997) and \(\text{Ca}^{2+}\) entry via voltage-sensitive (L-type) \(\text{Ca}^{2+}\) channels (Safayhi et al., 1997).

High (20 mM) glucose, or a combination of nutrient secretagogues (Ashcroft and Ashcroft, 1992), caused a large increase in steady state \([\text{Ca}^{2+}]_c\) both in the ER and in vesicles (Fig. 8, A and C), with no significant change in vesicular distribution (Fig. 8 B), which is consistent with the increases in \([\text{Ca}^{2+}]_c\), seen under these conditions (Grapengiesser et al., 1988).
Aequorin as a reporter of secretory vesicle–free Ca\(^{2+}\) concentration

We show here that aequorin can be targeted to the lumen of dense core secretory vesicles in living cells, with no incorporation into the ER, Golgi, or trans-Golgi network using the cell's own protein-sorting machinery (Fig. 1). However, a proportion of VAMP.Aq was also present on the plasma membrane and endosomes (Fig. 1 C). Since our recordings were made at either high (1.5 mM, intact cells) or low (400 nM, permeabilized cells, B and D), as indicated. Other additions were: caffeine, 10 mM; 4-CEP, 500 μM; cADPr, 5 μM; palmitoyl CoA, 50 μM.

Discussion

Aequorin as a reporter of secretory vesicle–free Ca\(^{2+}\) concentration

We show here that aequorin can be targeted to the lumen of dense core secretory vesicles in living cells, with no incorporation into the ER, Golgi, or trans-Golgi network using the cell's own protein-sorting machinery (Fig. 1). However, a proportion of VAMP.Aq was also present on the plasma membrane and endosomes (Fig. 1 C). Since our recordings were made at either high (1.5 mM, intact cells) or low (400 nM, permeabilized cells) external Ca\(^{2+}\) concentrations, the plasma membrane–located photoprotein should be either rapidly exhausted, or inactive, respectively. In any case, it would appear that plasma membrane–targeted aequorin is not reconstituted, since we would have expected to see a “spike” of luminescence upon readdition of high Ca\(^{2+}\) concentrations to intact cells (e.g., Fig. 3 C). None was seen, suggesting that the mistargeted protein may be inactivated by unknown mechanisms during recycling between the plasma membrane and endosomes.

Similarly, endosomal aequorin is likely to be inactive under our conditions, since (a) the total endosomal Ca\(^{2+}\) content is low in neuroendocrine cells (Pezzati et al., 1997) and (b) free [Ca\(^{2+}\)] is particularly low (~3.0 μM) in acidic endosomes (Gerasimenko et al., 1998); active VAMP.Aq was located carbachol, which caused a large increase in [Ca\(^{2+}\)], in untreated cells (unpublished data). In C, CPA and ionomycin (10 μM each) were added as indicated. Panel D was as C, but with the further addition of monensin (mon, 10 μM) to the perfusate. Data are the means (± SEM) on observations on a total of (A) 58, (B) 35, (C) 28, and (D) 40 single cells, imaged in 3–5 separate experiments.

(E) Immuno-electron microscopic localization of RyR (arrows) on dense core vesicles.
tions of nutrients shown for 2 min in complete KRB medium which pertains within the secretory vesicle, as well as the rest of the secretory pathway.

**Free \( \text{Ca}^{2+} \) concentration in the secretory vesicle lumen**

Despite possessing a larger total \( \text{Ca}^{2+} \) content (Andersson et al., 1982; Hutton et al., 1983; Nicaise et al., 1992), secretory vesicles displayed a significantly lower free \[ \text{Ca}^{2+} \] than the ER or Golgi apparatus. Our measured values for \[ [\text{Ca}^{2+}]_{SV} \] (\( \sim 50 \ \mu\text{M} \)) correspond fairly well to measurements using other techniques in isolated chromaffin granules (Krieger-Brauer and Gratzl, 1982; 24 \mu M; null point titration), respiratory tract goblet cells (24 \mu M; calcium orange 5 N fluorescence; Nguyen et al., 1998) and platelet \( \alpha \)-granules (12 \mu M; null point titration; Grinstein et al., 1983). Thus, free \( \text{Ca}^{2+} \) represents <0.05% of the total vesicular calcium content of \( \beta \)-cell secretory vesicles (assuming a total \( \text{Ca}^{2+} \) concentration of 50–100 mM; Hutton et al., 1983). Secretory vesicles appear to have the highest \( \text{Ca}^{2+} \)-buffering capacity of all subcellular organelles so far examined, with the percentage of free \( \text{Ca}^{2+} \) being much higher both in the cytosol (\( \sim 2\% \)) and in the ER (\( \sim 10\% \)); Pozzan et al., 1994). Importantly, resting \[ [\text{Ca}^{2+}]_{SV} \] was well below the \( K_M \) for \( \text{Ca}^{2+} \) of proinsulin-processing enzymes (Davidson et al., 1988).

The identity of the \( \text{Ca}^{2+} \) binding proteins (or other molecules) responsible for chelating free \( \text{Ca}^{2+} \) in these vesicles is unknown. Chromogranins (Yoo and Albanesi, 1990), or the mammalian homologue of *Tetrahymena thermophila* granule lattice protein 1 (Grlp1) (Chilcoat et al., 1996), are each strong possibilities. In addition, \( \text{Ca}^{2+} \) chelation by small molecules, such as ATP (Hutton et al., 1983), may also be involved. Finally, it is likely that in islet \( \beta \)-cells the insulin crystal itself also participates in chelating vesicular \( \text{Ca}^{2+} \) (Palmiéri et al., 1988; see below).

**Uptake of \( \text{Ca}^{2+} \) into secretory vesicles**

The dense core secretory vesicle pool of islet \( \beta \)-cells has previously been considered relatively inert (Howell et al., 1975; Prentki et al., 1984). We provide evidence here that net uptake of \( \text{Ca}^{2+} \) into the dense core secretory vesicle population occurs during activated \( \text{Ca}^{2+} \) influx (Figs. 2 and 3), and in response to a receptor agonist (Fig. 5) or to nutrients (Fig. 8). However, and as discussed below (see also Fig. 9), these measurements do not exclude the possibility that discrete vesicle pools may experience different \[ [\text{Ca}^{2+}]_{SV} \] changes.

We considered the possibility that the increases in \[ [\text{Ca}^{2+}]_{SV} \] observed upon challenge of cells with agonists (Fig. 5) or nutrients (Fig. 8) may be due in part to the activation of exocytosis, and thus the exposure of aequorin within the vesicle matrix to the extracellular \( \text{Ca}^{2+} \) concentrations (1.5 mM). However, this phenomenon is likely to contribute negligibly to the observed signals, since only a tiny fraction of the total vesicle population (the "primed" pool) in \( \beta \)-cells (5–20/13,000 per min; Rorsman, 1997) undergoes fusion.
The present data suggest that Ca²⁺ accumulation into vesicles is catalyzed at physiological [Ca²⁺], chiefly by a P-type Ca²⁺-ATPase. Previous studies have indicated that transport of Ca²⁺ into the Golgi apparatus is mediated partly by a SERCA pump, and partly by another, unidentified thapsigargin-insensitive system (Pinton et al., 1998). This second ATP-dependent Ca²⁺ uptake system may be closely related to ATP2C1 (Hu et al., 2000), the mammalian homologue of the yeast Golgi Ca²⁺ transport ATPase, PMR1 (Sorin et al., 1997). Supporting this view, mRNA-encoding ATP2C1 is abundant in MIN6 cells, and polyclonal antibodies raised to ATP2C1 reveal a punctate pattern of intracellular staining (unpublished data). Intriguingly, although patients defective in the ATP2C1 gene develop skin lesions (‘Hailey-Hailey’ disease; Hu et al., 2000), it is unclear whether these individuals also tend to suffer from neuroendocrine or other disorders (e.g., diabetes mellitus).

**Figure 9. Scheme: redistribution of organellar Ca²⁺ in secretory cells in response to G protein-coupled receptors (e.g., acetylcholine, AcCh) or glucose.** IP₃ generated in response to AcCh releases Ca²⁺ from the endoplasmic reticulum and Golgi apparatus, leading to an increase in cytosolic [Ca²⁺] and uptake of Ca²⁺ into dense core secretory vesicles distant from the plasma membrane (deep vesicles). Vesicular Ca²⁺ uptake is catalyzed by an undefined Ca²⁺-ATPase, with properties similar to PMR1/ATP2C1 (see Discussion). Increases in blood glucose lead to (1) the uptake of the sugar via glucose transporters, (2) enhanced ATP synthesis and closure of ATP-sensitive K⁺ channels, and (3) Ca²⁺ influx through L-type Ca²⁺ channels. The resultant increases in [Ca²⁺]c are likely to promote net Ca²⁺ uptake (reflecting the balance of uptake versus release) into vesicles distant from the cell surface (deep vesicles). For those vesicles (≤0.5% of total; Rorsman, 1997) located close to the plasma membrane and primed for exocytosis (primed vesicles), larger local [Ca²⁺]c increases (e.g., at the mouth of activated plasma membrane Ca²⁺ channels; Neher, 1998) may activate vesicular RyRs and provoke net Ca²⁺ release.

**Secretory vesicles are an IP₃-insensitive, but caffeine/cADPr-sensitive Ca²⁺ store**

The present results suggest that IP₃ is unlikely to stimulate the release of Ca²⁺ from dense core secretory vesicles directly (Fig. 5).

By contrast, we now show (Figs. 6 and 7) that functional RyR channels are present on the dense core vesicle membrane of MIN6 cells. Type II RyR mRNA is present in ob/ob mouse islets, mouse β-TC3 cells (Islam et al., 1998), and in rat islets (Holz et al., 1999). RyR II protein has been detected in INS1 β-cells (Gamberucci et al., 1999), and ryanodine binding sites revealed in human islets (Holz et al., 1999), mouse islets and MIN6 cells (Varadi and Rutter, 2001). Evidence for functional RyRs on the ER INS-1 β-cells (Maechler et al., 1999) has also recently been provided. The present data indicate that dense core vesicles may represent a large fraction (~30%) of the total RyR-accessible Ca²⁺ pool in MIN6 β-cells (Fig. 7 B). However, the proportion of the total vesicular Ca²⁺ pool which is mobilizable via RyR is small. Thus, the [Ca²⁺]c increases observed when the total acidic Ca²⁺ pool was emptied with CPA, ionomycin, and monensin (Fig. 7 D) were much larger than those apparent when cells were treated with 4-CEP after CPA and ionomycin (Fig. 7 C).

**Role of cADPr in β-cells**

In the present studies, cADPr caused an apparent release of Ca²⁺ from both the ER and from the secretory vesicles in permeabilized MIN6 β-cells. Moreover, we have recently observed (Varadi and Rutter, 2001) that photolysis of caged cADPr increases [Ca²⁺]c in MIN6 cells. These findings were surprising in light of earlier results using ob/ob mouse islets (Islam et al., 1993) or INS-1 β-cells (Rutter et al., 1994), where cADPr was ineffective in permeabilized cells. However, it should be noted that the present studies may provide greater sensitivity to small Ca²⁺ fluxes by measuring changes in [Ca²⁺]c inside the secretory vesicle or ER.
Could changes in intracellular cADPr concentration play a role in the regulation of insulin secretion as proposed by Takasawa et al. (1993)? Supporting this view, mice homozygous for inactivation of the CD38 (ADPribose cyclase) gene display impaired glucose tolerance (Kato et al., 1999). However, no change in the islet cADPr content was observed by others in response to acutely elevated glucose concentrations in vitro (Scruiel et al., 1998), suggesting further studies are necessary.

Conclusions
Dense core secretory vesicles rapidly take up Ca\(^{2+}\) ions via an ATP-dependent non-SERCA Ca\(^{2+}\) pump (see Scheme, Fig. 9). Ca\(^{2+}\) accumulated into vesicles may later be released via RyRs once these vesicles reach a high local Ca\(^{2+}\) concentration beneath the plasma membrane (Neher, 1998) or elsewhere in the cell. This release may further contribute to the high local Ca\(^{2+}\) in the vicinity of the submembrane vesicles (Emmanouilidou et al., 1999), and may be important to trigger exocytosis (Scheinen et al., 1998).

Materials and methods
Materials
Cell culture reagents were obtained from GIBCO BRL or Sigma-Aldrich and molecular biologicals from Roche Diagnostics. Fura-2 and cADPr were from Sigma-Aldrich and IP<sub>3</sub> was from Molecular Probes.

Construction of VAMP.Aq cDNA
VAMP2 cDNA was amplified by PCR using specific primers (forward: 5'-AAG CTG ACC ATG TCG ACC ACC ACC-3'; reverse: 5'-AAG CTT AGT GCC GAA GTA AAC CAG TTG-3'); HindIII sites are underlined). This fragment was then inserted upstream of the HindIII-EcoRI fragment encoding HA1-tagged D<sup>119-A</sup> aequorin (Kendall et al., 1992) in plasmid pBS<sup>/H11032</sup>. Chimeric VAMP.Aq cDNA was shuttled as a NotI-XhoI fragment into pcDNA3 (Invitrogen; Fig. 1 A).

Construction of adenoviral VAMP.Aq and ER.Aq
ER.Aq (Montero et al., 1995) and VAMP.Aq cDNAs were transferred into plasmid pShuttleCMV (He et al., 1998) as KpnI-XhoI and Xba1-HindIII fragments, respectively. The resultant constructs were recombined with vector pAdEasy-1, and viral particles were generated as described (Ainscow and Rutter, 2001).

Cell culture, transient transfection, and adenoviral infection
MIN6 β-cells (Miyazaki et al., 1990; passages #20 and #30) were cultured in DME, with additions as given previously (Ainscow and Rutter, 2001). Cells cultured on 13 mm poly-L-lysine-coated coverslips were transfected with VAMP.Aq or ER.Aq plasmid DNA (1 μg) using Lipofectamine (Promega) in Optimem I serum-free medium (GIBCO BRL), or infected with adenoviruses as described (Ainscow et al., 2000), using a Leica DM-IRBE inverted microscope (40× objective), and a Hamamatsu C4742-995 charge-coupled device camera driven by OpenLab (Improvision) software.

Imaging acridine orange fluorescence
Permeabilized cells were loaded with 3 μM acridine orange (Tsouli et al., 2000) before imaging (37°C) on a Leica DM-IRBE inverted microscope, at 493±10 excitation, 530 nm emission (filters were from Chroma Technology).

Immunocytochemistry
48 h after transfection or infection, cells were fixed in 3.7% (vol/vol) paraformaldehyde and probed with antibodies essentially as described (Pouli et al., 1998a).

Immunoelectron microscopy
Gelatin-embedded, aldehyde-fixed cells were frozen in liquid nitrogen and ultrathin cryosections were obtained with a Reichert-Jung Ultracut E. Immunogold localization was performed as described (Confalonieri et al., 2000). Sections were immunostained either with rabbit anti-HA antibody (Sigma-Aldrich), or with a sheep anti-RyR antibody (Molecular Probes) followed by 10-nm protein A-gold. For double labeling, anti-HA–labeled sections were incubated with 1% glutaraldehyde in 0.1 M Na<sub>2</sub>PO<sub>4</sub>, pH 7.0, followed by incubation with a guinea pig antisemrin antibody (Dako) and 15-nm protein A-gold. Sections were examined with a JEOL EM 912 electron microscope. No labeling was detected in control sections (unpublished data).

Confocal imaging
Cells were imaged at 37°C on a Leica SP2 confocal spectrophotometer, (488 nm excitation) using a 100× oil immersion objective.

Other methods and statistics
Free [Ca\(^{2+}\)] and [Mg\(^{2+}\)] were calculated using “METLIG” (Rutter and Denton, 1988). Data are presented as the mean ± SEM for the number of observations given, and statistical significance calculated using Student’s t test.

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