Ca^{2+}- and GTP-dependent exocytosis in mouse pancreatic β-cells involves both common and distinct steps

Peter Proks*, Lena Eliasson†, Carina Ämmälä†, Patrik Rorsman† and Frances M. Ashcroft*

*University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK and †Department of Physiology and Pharmacology, Division of Medical Biophysics, Medicinaregatan 11, S-413 90 Göteborg, Sweden

1. The effects of GTP and Ca^{2+} on secretion from single pancreatic β-cells were studied using capacitance measurements as an indicator of exocytosis.

2. GTP or GTPγS produced a concentration-dependent increase in cell capacitance in the absence of intracellular calcium. There was no effect of cyclic AMP or BAPTA on GTP-induced secretion.

3. In the absence of GTP, the relationship between intracellular calcium concentration and the maximum rate of secretion was fitted by the Hill equation with a slope factor of 2·5 and half-maximal activation at 1·6 μM intracellular Ca^{2+}. Similar values were obtained in the presence of GTPγS, suggesting GTP does not alter the sensitivity of the secretory machinery to Ca^{2+}.

4. GDPβS alone had no effect on cell capacitance but caused a dose-dependent inhibition of exocytosis induced by infusion of either GTPγS or Ca^{2+}, suggesting both stimuli involve G-protein activation. GDPβS was without effect on exocytosis evoked by depolarization-mediated Ca^{2+} entry.

5. The time course of exocytosis following rapid elevation of GTPγS by photolysis of a caged precursor was dependent on the intracellular Ca^{2+} and cyclic AMP concentrations.

6. Our results are interpreted in terms of a model in which the secretory pathways stimulated by Ca^{2+} and GTP contain both common and separate parts.

In a variety of cell types, secretion can be elicited both by a rise in intracellular Ca^{2+} and by Ca^{2+}-independent mechanisms. It is well documented that elevation of the intracellular calcium concentration ($[Ca^{2+}]_i$) stimulates exocytosis (Burgoyne & Morgan, 1993; Neher & Zucker, 1993). However, in many cell types, secretion can be evoked in the complete absence of Ca^{2+} by GTP or its non-hydrolysable analogue GTPγS (Gomperts, 1990; Lindau & Gomperts, 1991). The pancreatic β-cell constitutes an example of a cell in which both Ca^{2+}-dependent and GTP-dependent secretion occur (see review by Ashcroft & Rorsman, 1995). Under physiological conditions, insulin secretion is initiated by an increase in $[Ca^{2+}]_i$ resulting from voltage-gated Ca^{2+} influx (Gillis & Misler, 1992; Ämmälä, Eliasson, Bokvist, Larsson, Ashcroft & Rorsman, 1993*b*). Studies on permeabilized insulinoma cells have also revealed that GTP is able to support secretion in the absence of intracellular calcium (Wollheim, Ullrich, Meda & Vallar, 1987; Vallar, Biden & Wollheim, 1987; Regazzi, Li, Deshusses & Wollheim, 1989). Although the mechanism by which GTP triggers exocytosis is not yet fully understood, there is evidence that it does not involve activation of protein kinases or liberation of Ca^{2+} from intracellular stores: instead GTP appears to act at a late stage in the secretory process, probably by directly interacting with the secretory machinery. The interactions between the GTP-dependent and Ca^{2+}-dependent secretory pathways remain unclear. The capacitance method of measuring exocytosis both enables the study of secretion at the single-cell level and allows greater temporal resolution than biochemical methods (Neher & Marty 1982; Ämmälä *et al.* 1993*b*). We have utilized this method to examine the kinetics of GTP-stimulated secretion and to compare it with that of Ca^{2+}-dependent release in single isolated pancreatic β-cells.

METHODS

Cell preparation

NMRI mice were killed by cervical dislocation, the pancreas was removed and pancreatic islets isolated by collagenase digestion. Islets were then dispersed into single cells by low-Ca^{2+} treatment (Ämmälä *et al.* 1993*b*). Cells were plated onto plastic Petri dishes and maintained for 1–3 days in RPMI 1640 tissue culture medium supplemented with 10% fetal calf serum, 10 U ml^{-1} penicillin and 10 μg ml^{-1} streptomycin at 37 °C in a humidified atmosphere. All tissue culture media were obtained from Life Technologies (Paisley, UK) or Northumbria Biologicals (UK).

Recording methods

Patch pipettes were pulled from borosilicate glass capillaries, coated with Sylgard at their tips and fire polished immediately before use. They had resistances of 2–6 MΩ when filled with pipette solution.

Whole-cell currents and changes in cell capacitance and conductance were recorded using an EPC-7 patch-clamp amplifier (List Electronic). All experiments were carried out using the standard whole-cell configuration and a holding potential of −70 mV. The zero current potential of the pipette was adjusted before establishment of the seal, with the pipette in the bath. In the series of experiments described here the cells had an initial capacitance of around 4 pF. Only cells which had an initial series resistance of < 10 MΩ were accepted and no series resistance compensation was used. Changes in cell capacitance and conductance were measured as previously described (Ämmälä *et al.* 1993*b*). Briefly, a 20 mV r.m.s. 800 Hz sine wave was added to the holding potential (−70 mV) and ten cycles averaged for each data point. The resulting current was analysed at two orthogonal phase angles with a resolution of 100 ms per point. The phase angle was determined empirically for each experiment by varying the G_{series} and C_{slow} knobs on the amplifier. The phase angle was recalibrated several times throughout the course of the experiment; in most figures the capacitance changes associated with this recalibration have been removed from the records for clarity. Capacitance changes were evoked by infusion of Ca^{2+}- and/or GTP-containing solution, by voltage-clamp depolarization, or by photoliberation of caged compounds. Measurements were carried out using a Digidata or Labmaster A/D converter (Axon Instruments) with a 486 PC computer and in-house software written in Axobasic (Axon Instruments).

Solutions

The standard extracellular (bath) solution contained (mM): 140 NaCl, 5·6 KCl, 1·2 $MgCl_2$ and 5–10 Hepes (pH 7·4 with NaOH). The extracellular Ca^{2+} concentration was 2·6 mM for the perforated patch experiments, but external Ca^{2+} was usually absent for experiments in which GTP was infused. The pipette was filled with (mM): 125 potassium glutamate, 1 $MgCl_2$, 10 EGTA, 0–10 $CaCl_2$ (to produce free Ca^{2+} concentrations of between < 1 nM and 30 μM) and 5 Hepes (pH 7·15 with KOH, adjusted after addition of all salts). Unless otherwise indicated, 3 MgATP was added to this solution fresh each day and the pH readjusted as required. Where indicated, 0·1 mM cyclic AMP (cAMP) was also included in the intracellular solution. The free Ca^{2+} concentration of the pipette solutions was calculated using the program PERTEM (written by Peter Griffiths, University Laboratory of Physiology, Oxford, UK) and the binding constants given by Martell & Smith (1971), as described by Perrin & Sayce (1967): all ligands and cations were included in these calculations. For calculated free Ca^{2+} concentrations >10 μM, the actual concentration was measured using a Ca^{2+} electrode (Corning 746041) and the measured value (rather than theoretical) used in calculations. The bath was continuously perfused and all experiments were carried out at 30–34 °C.

Rapid elevation of the cytoplasmic GTPγS concentration was produced by photoliberation of caged GTPγS (K^+ salt; Molecular Probes), which was included in the pipette solution at a concentration of 50 μM. Photolysis was effected by a flash of UV light as previously described (Ämmälä *et al.* 1991).

GTPγS and 5′-adenylimidodiphosphate (AMP-PNP) were obtained as tetralithium salts from Sigma. In some experiments BAPTA (20 mM; Molecular Probes) was added to the < 1 nM Ca^{2+} solution in place of EGTA.

Data analysis

In most infusion experiments, the rate of cell capacitance change (dC/dt) was measured during the linear phase of the capacitance increase, over a period of 10–50 s during which the conductance was stable. The rate of change of cell capacitance was measured as the change in cell capacitance divided by the time interval (usually >60 s). In some experiments where the capacitance increase was sigmoidal rather than linear, a polynomial was fitted to the data and the polynomial then differentiated to obtain the maximum rate of capacitance increase. In AMP-PNP solution the rate of capacitance increase was measured 2 min after establishment of the whole-cell configuration, which is sufficient for endogenous ATP to wash out of the cell. The delay times for secretion elicited by photorelease of caged GTPγS were measured with respect to the start of the flash.

Data are expressed as mean values ± S.E.M. of the indicated number of cells and statistical significance evaluated using Student's *t* test.

RESULTS

Effects of GTP

Figure 1 shows that in the absence of intracellular Ca^{2+} (< 10 nM) the infusion of GTP (1 mM) produces an increase in cell capacitance, indicative of exocytosis, within ~1 min of obtaining the whole-cell configuration. Similar effects were observed with the poorly hydrolysable analogue of GTP, GTPγS, but at a much lower concentration (40 μM), presumably because GTP is rapidly hydrolysed within the cell. The fact that GTPγS induces secretion suggests that hydrolysis of GTP is not required for its stimulatory action. In the absence of guanine nucleotides, the cell capacitance remained constant with time (mean rate, 0·08 ± 0·01 fF s^{-1}, $n = 13$).

In most cells dialysed with GTP or its analogues, the cell capacitance did not reach a steady-state level but continued to increase throughout the recording period. To enable quantitative comparison between β-cells we have therefore measured the maximum rate of capacitance change (dC/dt). As shown in Fig. 2, GTPγS was more effective than GTP and induced a faster increase in the rate of exocytosis. With 40 μM GTPγS the average rate of secretion was 10·8 ± 1·0 fF s^{-1} ($n = 23$), corresponding to the release of around 5 granules s^{-1}. This rate of exocytosis is similar to that observed in melanotrophs (6 fF s^{-1} in the presence of 0·1 mM GTPγS; Okano, Monck & Fernandez, 1993). The mean cell capacitance increased about 1·5-fold from a mean value of 4·2 ± 0·9 pF to 6·3 ± 1·4 pF ($n = 17$) after 10 min, indicating that about 1000 secretory granules were released during this period.

The relationship between the mean maximum rate of change in cell capacitance and GTPγS concentration is shown in Fig. 2*B*. The relationship is dome shaped, with a maximal response between 40 and 200 μM GTPγS, and exocytosis was actually less when GTPγS was elevated further to 2 mM. One explanation for this finding is that GTPγS has both stimulatory and inhibitory actions on

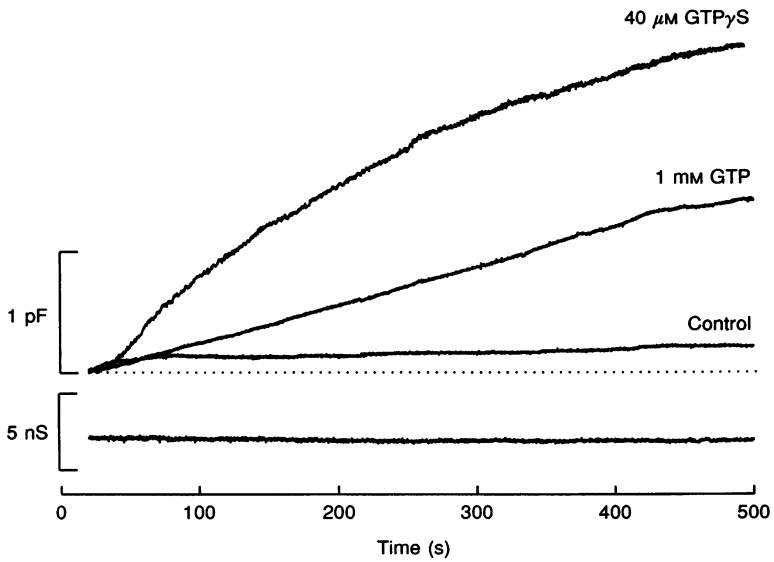

Figure 1. GTP stimulates exocytosis in the absence of Ca^{2+}

A, changes in capacitance (above) and conductance (below) produced by intracellular dialysis of three different β-cells with a control solution containing $< 0{\cdot}3$ nM Ca^{2+} and lacking guanine nucleotides, or with the same solution plus either 1 mM GTP or 40 μM GTPγS. The whole-cell configuration was established at time zero. The dotted line indicates the zero capacitance level. Only one conductance trace, corresponding to the capacitance trace recorded with 1 mM GTP is shown, for clarity.

exocytosis, and that the former is preferentially activated by lower GTPγS concentrations. An alternative possibility is that Li^+ may inhibit exocytosis, since GTPγS is added as the tetralithium salt.

The physiological concentration of GTP in islets is believed to be about 1·3 mM (Meglasson, Nelson, Nelson & Ericinska, 1989), a concentration which would be expected to stimulate secretion (Fig. 2*A*). In the presence of a physiological GDP concentration (0·6 mM), however, the change in cell capacitance was not significantly different from that observed in control cells ($0{\cdot}26 \pm 0{\cdot}18$ fF s^{-1}, $n = 11$, as compared with $0{\cdot}08 \pm 0{\cdot}01$ fF s^{-1}, $n = 13$). The ability of GDP to inhibit exocytosis stimulated by GTP argues that a

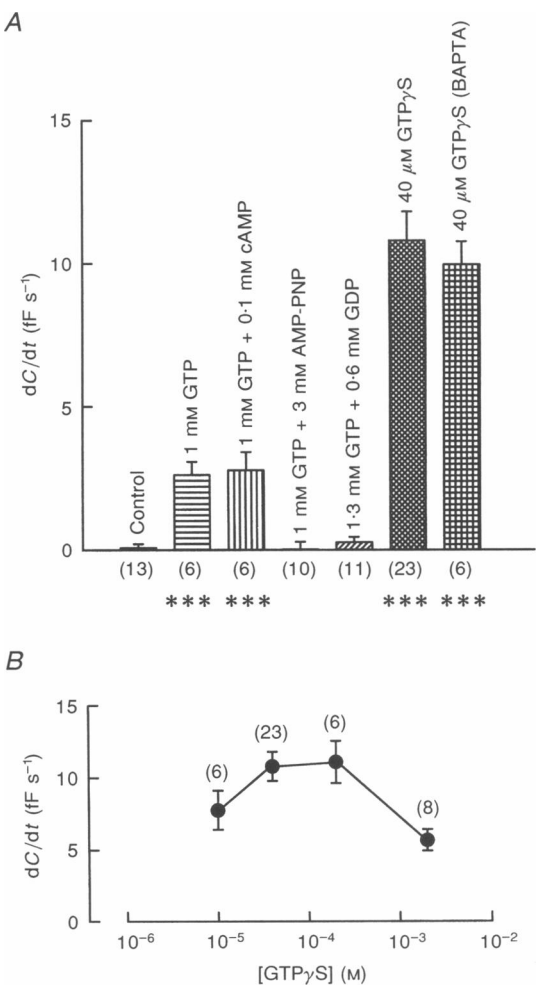

Figure 2. Properties of GTP-mediated capacitance changes

A, mean rate of capacitance change measured in β-cells dialysed with intracellular solutions containing the additions indicated. ATP was omitted from the solution containing 3 mM AMP-PNP. Values of n given in parentheses; *** $P < 0{\cdot}001$ against control. *B*, relationship between the maximum rate of exocytosis and intracellular GTPγS concentration.

GTP-binding protein is involved in mediating the effect of GTP, and that secretion will not be activated at physiological GTP/GDP concentrations.

We next examined the mechanism by which GTP induces exocytosis. Figure 2 shows that there was no effect of 0·1 mM cAMP on secretion induced by GTP, in contrast to the potentiatory effect of this nucleotide on Ca^{2+}-dependent secretion (Ämmälä, Ashcroft & Rorsman, 1993*a*). This is in agreement with earlier work on permeabilized RINmF cells (Wollheim *et al.* 1987) and confirms that the effect of GTP on exocytosis is not mediated by activation of adenylate cyclase. In platelets, GTPγS activates both exocytosis and phospholipase D (PLD) and it has therefore been proposed that PLD may mediate the stimulatory action of GTP on secretion (Coorssen & Haslam, 1993). The Ca^{2+} chelator BAPTA has also been shown to block PLD activity and to inhibit GTPγS-elicited secretion, via a mechanism independent of its ability to buffer Ca^{2+} (Coorssen & Haslam, 1993). Although PLD activity is present in β-cells and can stimulate insulin release (Dunlop & Metz, 1989; Metz & Dunlop, 1990), GTPγS-induced secretion from β-cells was not significantly affected when 20 mM BAPTA was used as the Ca^{2+} chelator rather than EGTA (Fig. 2). If GTPγS blocks PLD in β-cells, as it does in platelets (Coorssen & Haslam, 1993), this finding would suggest that PLD may not be involved in mediating the stimulatory effect of GTP on secretion in β-cells.

Finally, Fig. 2 shows that replacement of ATP with the non-hydrolysable analogue AMP-PNP abolished GTP-stimulated exocytosis (mean rate of capacitance change: $0·01 \pm 0·26$ fF s^{-1}, $n = 10$). This indicates that ATP hydrolysis is required for secretion.

We next explored the relationship between Ca^{2+}-stimulated and GTP-stimulated exocytosis. Figure 3 shows that infusion of the β-cell with an intracellular solution containing 2 μM Ca^{2+} causes a much larger and more rapid increase in cell capacitance than 40 μM GTPγS. In addition, secretion elicited by Ca^{2+} usually followed a sigmoidal time course, whereas the cell capacitance generally increased linearly for >5 min when GTP was infused. The maximum rate of the Ca^{2+}-induced capacitance increase was usually attained within 1–2 min of obtaining the whole-cell configuration. The mean maximal rate of exocytosis elicited by 2 μM Ca^{2+} was 39 ± 5 fF s^{-1} ($n = 10$) and ranged between 20 and 120 fF s^{-1}. These values compare with a maximum rate of around 40 fF s^{-1} with 10 μM Ca^{2+} in chromaffin cells (Burgoyne & Handel, 1994) and 15 fF s^{-1} with 2 μM Ca^{2+} in melanotrophs (Okano *et al.* 1993). On average, the β-cell capacitance increased about 3-fold in response to 2 μM Ca^{2+}, corresponding to the release of around 4000 secretory granules.

In the simultaneous presence of 40 μM GTPγS and 2 μM Ca^{2+}, the rate of exocytosis was slightly greater than that observed in the presence of either secretagogue alone (Fig. 3). Figure 4 plots the relationship between $[Ca^{2+}]_i$ and the cell capacitance change in the absence and presence of 40 μM GTPγS. In each case, the relationship was fitted to a modified form of the Hill equation which takes into account the fact that exocytosis is not zero at the lowest $[Ca^{2+}]_i$:

$$V = V_{back} + V([Ca^{2+}]_i), \quad (1)$$

where

$$V([Ca^{2+}]_i) = \frac{V_{sat}}{1 + (K_d/[Ca^{2+}]_i)^n}, \quad (2)$$

and V is the peak rate of secretion at the test Ca^{2+} concentration ($[Ca^{2+}]_i$), V_{back} is the peak rate of secretion at 10^{-7} M intracellular Ca^{2+} (background secretion), V_{sat} is the maximum rate of secretion (i.e. the saturating rate of secretion, as at 10^{-4} M intracellular Ca^{2+}, minus the background secretion), K_d is the $[Ca^{2+}]_i$ concentration at which secretion is half-maximal and n is the Hill coefficient (slope factor). In the absence of GTPγS, $K_d = 1·6$ μM,

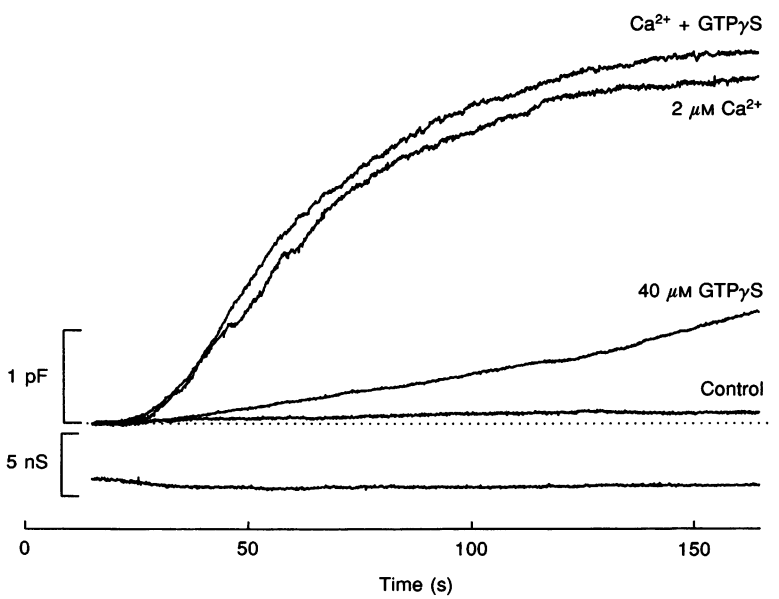

Figure 3. Interactions between cytoplasmic Ca^{2+} and GTPγS

Capacitance change produced by intracellular dialysis with a control solution lacking both Ca^{2+} and guanine nucleotides, and with solutions containing 40 μM GTPγS, or 2 μM Ca^{2+}, or 40 μM GTPγS + 2 μM Ca^{2+}. The whole-cell configuration was established at time zero. The dotted line indicates the zero capacitance level. Only one conductance trace, corresponding to the capacitance trace recorded with 2 μM Ca^{2+}, is shown for clarity.

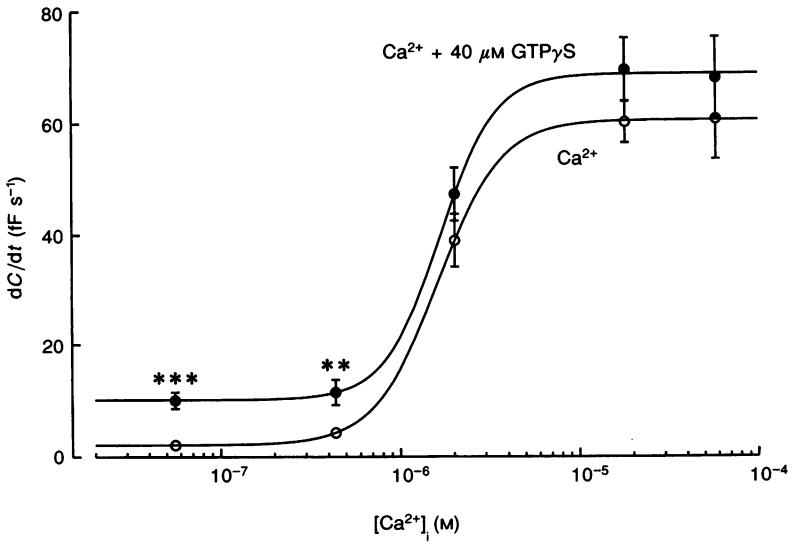

Figure 4. Ca^{2+} dependence of exocytosis in the presence and absence of GTPγS
Relationship between the maximum rate of exocytosis and $[Ca^{2+}]_i$ measured in the absence (○) or presence (●) of 40 μM GTPγS ($n = 6$–10). At the two lowest intracellular Ca^{2+} concentrations in the absence of GTPγS (○), the error bars are smaller than the data points ($n = 6$ in both cases). *** $P < 0.001$, ** $P < 0.02$ between the data points at the same $[Ca^{2+}]_i$ in the presence and absence of GTPγS.

$n = 2.5$, $V_{sat} = 59$ fF s^{-1} and the background secretion was 2 fF s^{-1}, whereas in the presence of 40 μM GTPγS, $K_d = 1.7$ μM, $n = 2.8$, $V_{sat} = 59$ fF s^{-1} and the background secretion was 10 fF s^{-1}. The Hill coefficient of >2 indicates that the co-operative action of more than one Ca^{2+} ion is required to initiate exocytosis. The similarity in both the K_d and the Hill coefficient in the absence and presence of GTPγS, indicates that GTP does not alter the sensitivity of the secretory machinery to Ca^{2+}. This finding, together with the fact that the maximal rate of Ca^{2+}-dependent secretion, V_{sat}, was unaltered by GTPγS whereas the background secretion was increased, suggests that GTPγS and Ca^{2+} may act via separate secretory pathways. In this case, one would expect that the effects of Ca^{2+} and GTPγS on the rate of secretion should be additive at all Ca^{2+}concentrations. However, although GTPγS caused secretion in the absence of Ca^{2+} and significantly potentiated the rate of Ca^{2+}-dependent secretion at low Ca^{2+}concentrations, the effects of GTPγS on Ca^{2+}-dependent secretion at higher Ca^{2+} concentrations did not reach significance.

We next tested the effect of the GTP analogue GDPβS, which is expected to lock GTP-binding proteins in an inactive state. GDPβS had no effect on the cell capacitance when infused alone (Fig. 5). However, it caused a dose-dependent inhibition of the exocytosis induced by GTPγS, with 1 mM GDPβS producing an 86% block and 40 μM GDPβS reducing secretion by 16%. Secretion induced by infusion of 2 μM intracellular Ca^{2+} was also inhibited 85% by 1 mM GDPβS. These results indicate that the effect of GTPγS is mediated by activation of a GTP-binding protein,

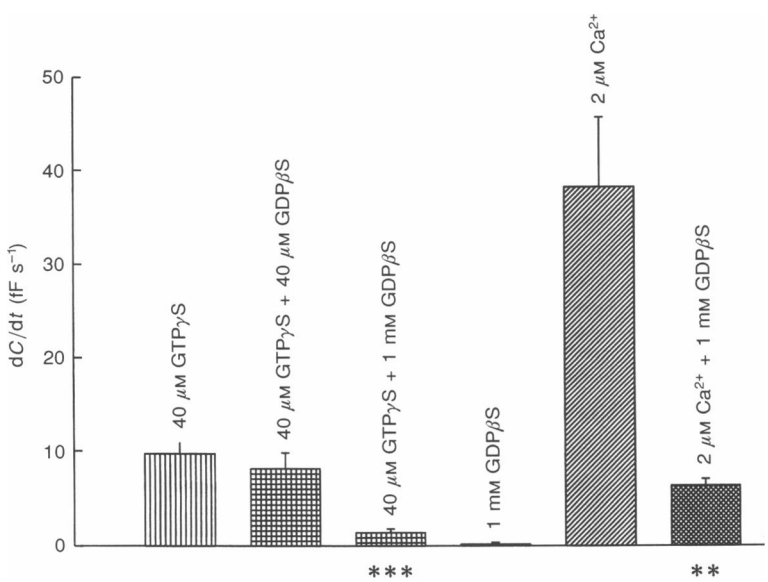

Figure 5. GDPβS inhibits exocytosis induced by infusion with either GTP or calcium
Mean rate of capacitance change measured in β-cells dialysed with intracellular solutions containing the additions indicated ($n = 6$–8). *** $P < 0.001$ for 40 μM GTPγS alone, ** $P < 0.002$ for 2 μM Ca^{2+} alone.

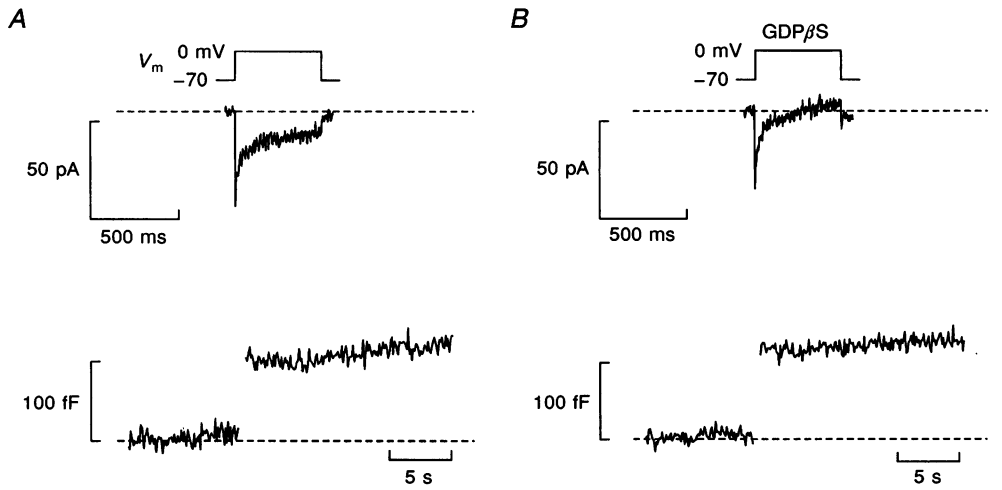

Figure 6. GDPβS does not inhibit exocytosis induced by depolarization-mediated Ca^{2+} entry
Ca^{2+} currents (above) and capacitance changes (below) recorded in response to a depolarization from −70 to 0 mV in a control cell (*A*) and in a different cell dialysed with 0·5 mM GDPβS (*B*).

and further, that at least part of the the secretory pathway initiated by Ca^{2+}also involves G-protein activation. It also implies that sufficient endogenous GTP must be present to support exocytosis in β-cells dialysed with Ca^{2+} in the absence of GTP: it is possible that transphosphorylation from exogenous ATP may contribute to the endogenous GTP levels.

Although GDPβS inhibited the cell capacitance change induced by infusion with Ca^{2+}, it did not affect exocytosis mediated by voltage-gated Ca^{2+} influx. Figure 6 shows Ca^{2+} currents and associated changes in cell capacitance evoked by depolarization from −70 to 0 mV, in a control cell and a cell dialysed with 0·5 mM GDPβS. Clearly, a brief voltage-clamp depolarization is capable of eliciting exocytosis even

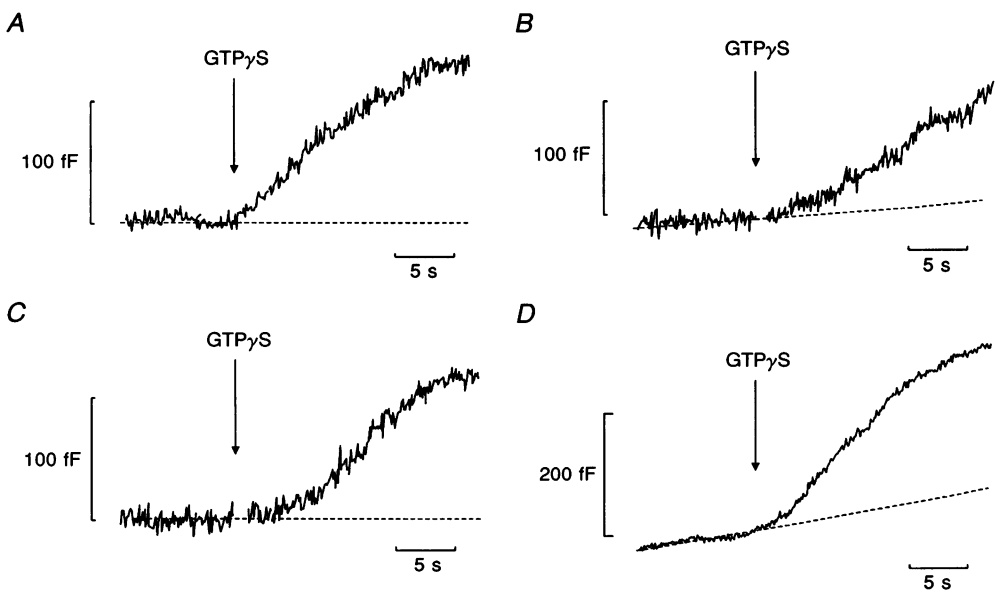

Figure 7. Kinetics of GTP-mediated exocytosis
Capacitance changes elicited by photorelease (arrow) of approximately 40 μM GTPγS from a caged precursor. *A*, the intracellular solution contained < 1 nM Ca^{2+}. The rate of exocytosis increased from 0·26 to 8·43 fF s^{-1} after the flash. The latency was < 0·1 s. *B*, the intracellular solution contained 170 nM Ca^{2+}. The rate of exocytosis increased from 1·04 to 6·79 fF s^{-1} after the flash. The latency was 3 s. *C*, the intracellular solution contained < 1 nM $Ca^{2+}$and 0·1 mM cAMP. The rate of exocytosis increased from 0·16 to 10·69 fF s^{-1} after the flash. The latency was 4 s. *D*, the intracellular solution contained 170 nM Ca^{2+} and 0·1 mM cAMP. The rate of exocytosis increased from 3·2 to 18·4 fF s^{-1} after the flash. The latency was 1·3 s.

Table 1. Flash release of GTPγS

Solution	0 GTPγS	40 μM GTPγS	Latency (s)	n
0 cAMP, < 1 nM Ca^{2+}	1·4 ± 0·7	7·3 ± 0·8**	<0·1	4
0 cAMP, 170 nM Ca^{2+}	1·6 ± 0·7	7·4 ± 1·6**	3·0 ± 1·0	8
0·1 mM cAMP, < 1 nM Ca^{2+}	1·3 ± 0·4	7·5 ± 1·7**	5·4 ± 0·9	11
0·1 mM cAMP, 170 nM Ca^{2+}	2·9 ± 0·6	20·1 ± 3·2**	1·4 ± 0·3	7

All solutions contained 3 mM ATP. ** $P < 0·01$.

in the presence of GDPβS. As discussed below this argues that there is a subset of granules which are released by Ca^{2+} in a GTP-independent fashion.

To determine the latency between application of GTPγS and the onset of secretion we rapidly elevated the GTPγS concentration by photorelease from a caged, biologically inert precursor included in the intracellular solution. Figure 7*A* shows that in the absence of Ca^{2+}, GTPγS initiated exocytosis with a delay of < 100 ms (the sample interval). The mean rate of exocytosis following elevation of GTPγS was 7·3 ± 0·8 fF s^{-1} ($n = 4$; Table 1), similar to that found in infusion experiments (10·8 ± 1·0 fF s^{-1}, $n = 23$). When Ca^{2+} was increased to 170 nM, there was no change in the rate of exocytosis elicited by GTPγS but the latency was increased to 3 s (Fig. 7*B*; Table 1). A longer delay between elevation of GTPγS and the onset of exocytosis was also observed at < 1 nM Ca^{2+} when 0·1 mM cAMP was included in the pipette solution (Fig. 7*C*; Table 1). As discussed below, we attribute these differences to the extent of filling of a readily releasable pool of granules.

The high rate of exocytosis found in the presence of both Ca^{2+} and cAMP is transient and subsequently declines to a rate similar to that observed prior to liberation of caged GTPγS. This decline is likely to result from depletion of GTPγS as another, similar, burst of exocytosis can be elicted by a subsequent flash which releases more GTPγS (Fig. 8).

DISCUSSION

Our results demonstrate that Ca^{2+} and GTPγS are both independently capable of eliciting exocytosis from single pancreatic β-cells, and confirm and extend earlier studies of permeabilized β-cell populations (Wollheim *et al.* 1987; Vallar *et al.* 1987; Regazzi *et al.* 1989). We can discount the possibility that G-protein activation stimulates secretion simply by mobilizing Ca^{2+} from intracellular stores, for example by activation of the phospholipase C or protein kinase C pathway(s) (Ämmälä *et al.* 1991), because GTP is able to elicit release in the presence of 20 mM intracellular BAPTA, a rapid and effective Ca^{2+} chelator. In addition, extracellular Ca^{2+} was absent in most of the experiments in which secretion was elicited by GTP infusion and intracellular Ca^{2+} stores should therefore have been depleted. It has previously been shown for permeabilized insulinoma cells that GTPγS-evoked secretion does not involve activation of protein kinases since it is unaffected by inhibitors of such kinases and no concomitant changes in phosphoprotein pattern are observed (Vallar *et al.* 1987; Regazzi *et al.* 1989). Furthermore it takes place in the complete absence of Ca^{2+} and is unaffected by protein kinase A activation. Our results are in agreement with these studies. Like Ca^{2+}-dependent secretion (Vallar *et al.* 1987), ATP hydrolysis is needed for GTP-dependent exocytosis in β-cells, but the precise role of ATP remains unclear.

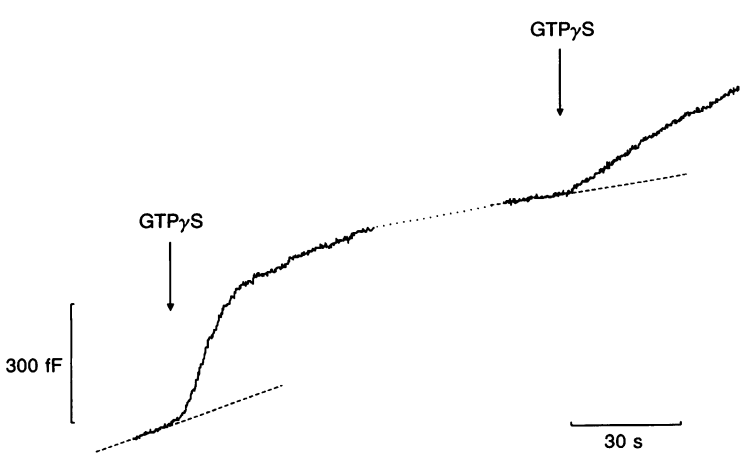

Figure 8. Effects of two successive liberations of caged GTPγS

Capacitance changes elicited by photorelease (arrows) of approximately 40 μM GTPγS from a caged precursor. The intracellular solution contained 170 nM Ca^{2+} and 0·1 mM cAMP.

The simplest explanation for the ability of GDPβS to inhibit secretion induced by infusion but not that induced by voltage-gated Ca^{2+} influx is that Ca^{2+} acts at (at least) two steps in the secretory pathway. We postulate Ca^{2+} influx rapidly elevates the submembrane Ca^{2+} concentration close to the release sites and releases vesicles from the readily releasable pool by a mechanism that does not require GTP. The decline in secretion observed during repetitive stimulation in standard whole-cell recordings (Ämmälä *et al.* 1993*b*) may result from the failure of this pool to be replenished. We hypothesize that, by contrast, Ca^{2+} infusion may mobilize stored granules in a GTP-dependent fashion. GTP-binding proteins are known to be involved in vesicle trafficking and docking in many secretory systems (Fischer von Mullard, Stahl, Li, Südhof & Jahn, 1994). The fact that Ca^{2+}-dependent exocytosis proceeds in the absence of GTP in the pipette solution, yet is inhibited by GDPβS, suggests that endogenous GTP must be present; it is possible that GTP levels are maintained in the dialysed cell by transphosphorylation from ATP.

In order to explain the ability of GDPβS to inhibit exocytosis produced by infusion of either Ca^{2+} or GTP, both secretory pathways must involve GTP-dependent processes. As discussed above, this is most easily explained by a common GTP-dependent pathway involved in granule mobilization. However, there is also evidence in favour of the idea that the more distal steps in the secretory process are different for Ca^{2+}-dependent or GTP-dependent release. Firstly, it is unlikely that GTP activates the Ca^{2+}-dependent pathway since GTP is active even when Ca^{2+} is buffered to vanishingly low levels using high concentrations of BAPTA or EGTA. Secondly, GDPβS does not inhibit exocytosis elicited by voltage-gated Ca^{2+} entry. Thirdly, the *maximum* rate of exocytosis elicited by GTP is < 10 fF s^{-1} or around 5 granules s^{-1}, whereas that elicited by Ca^{2+} (i.e. in response to a voltage-clamp depolarization, as in Fig. 6, or photo-release of caged Ca^{2+}) is 1–2 pF s^{-1} corresponding to about 500–1000 granules s^{-1} (Ämmälä *et al.* 1993*b*; Bokvist, Eliasson, Ämmälä, Renström & Rorsman, 1995). Fourthly, cAMP is able to stimulate Ca^{2+}-dependent, but not GTP-dependent, exocytosis. Furthermore, a separate secretory step is also suggested by the fact that tetanus toxin can inhibit GTP-dependent but not calcium-dependent release (Regazzi *et al.* 1995). Taken together, these data therefore suggest that the final steps in Ca^{2+}-dependent and GTP-dependent exocytosis may involve two separate pathways.

The time course of exocytosis following release of caged GTPγS was influenced by the intracellular Ca^{2+} and cAMP concentrations (Table 1). We suggest this reflects the extent to which a readily releasable pool of granules is filled prior to the application of GTPγS. Thus, in the absence of cAMP and Ca^{2+} the rate of exocytosis is maximal immediately following GTPγS liberation, because the readily releasable pool has not been depleted and secretory granules are available for immediate exocytosis. By contrast, when the internal solution contained either 0·1 mM cAMP or 170 nM Ca^{2+}, or both, the rate of exocytosis did not reach a maximum until ~6–8 s after stimulation. This suggests that no granules are available for immediate release and that the delay represents the time taken for granule mobilization. We speculate that this depletion of the readily releasable pool results from exocytosis without granule mobilization and pool refilling. For example, the submembrane Ca^{2+} concentration immediately after establishment of the whole-cell configuration may be high enough for dialysed cAMP to potentiate exocytosis initially, but subsequent dialysis with

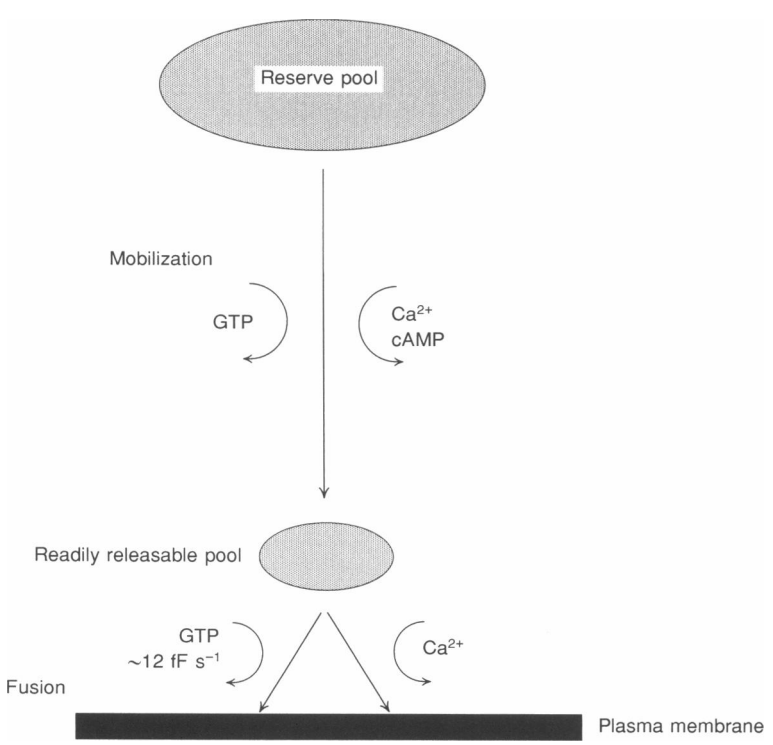

Figure 9. Model for Ca^{2+} and GTP-dependent exocytosis in the pancreatic β-cell

The sites of action of GTP, cAMP and Ca^{2+}, and the rates of transitions between pools, are indicated.

EGTA buffers Ca^{2+} to < 1 nM, and thereby prevents both exocytosis and replenishment of the readily releasable pool. Likewise, the time delay between the application of GTPγS and the maximum rate of exocytosis observed in the absence of cAMP but presence of Ca^{2+} suggests that 170 nM Ca^{2+} causes the depletion of the readily releasable pool but is insufficient by itself to promote its refilling. This is consistent with other data suggesting that cAMP is needed for granule mobilization in pancreatic β-cells (Gillis & Misler, 1993; Smith, Duchen & Ashcroft, 1995; Renström, Eliasson, Bokvist & Rorsman, 1996). Finally, when both Ca^{2+} and cAMP are present, continuous exocytosis prior to the release of GTPγS means that the readily releasable pool is never completely filled. However, mobilization is greatly accelerated by the simultaneous presence of Ca^{2+} and cAMP accounting for the fact that the highest rate of exocytosis is observed under these conditions.

The steady-state rate of exocytosis elicited by GTPγS in the absence of both Ca^{2+} and cAMP was ~7 fF s^{-1}, similar to that observed in the infusion experiments, which suggests that this may represent the rate of refilling of the readily releasable pool of granules. The initial rate, however, was faster (~12 fF s^{-1}) indicating that the rate-limiting step in GTP-dependent release is the rate at which granules are mobilized. The rate of granule mobilization in the presence of GTPγS was not substantially altered by the presence of either 0·1 mM cAMP or 170 nM Ca^{2+}. This suggests that cAMP alone, or 170 nM Ca^{2+} alone, does not enhance granule mobilization.

The maximum capacitance increase we observe in response to Ca^{2+} influx evoked by a train of depolarizations before depression occurs is 1–2 pF (Ämmälä *et al.* 1993*b*). This corresponds to a readily releasable pool of 500–1000 secretory granules with a unit amplitude of 2 fF. By contrast, the maximum number of granules released by Ca^{2+} infusion was larger, being around 3000 granules or 25% of the total granule population (Dean, 1973). This suggests that Ca^{2+} acts at two sites to elicit secretion: it causes the rapid exocytosis of the readily releasable pool and the recruitment of further granules from the reserve pool. The magnitude of the change in cell capacitance elicited by GTPγS was ~2 pF after a 10 min infusion. This is equivalent to the fusion of 1000 secretory granules. The different magnitudes of the capacitance increases elicited by GTPγS or Ca^{2+} infusion may simply reflect the slower rate of GTPγS-induced secretion combined with the washout of essential cytosolic constituents.

Figure 9 represents an attempt to incorporate all our observations into a simple model of secretion. By analogy with what has been suggested for other neuroendocrine cells (Neher & Zucker, 1993), we propose that the secretory granules may exist in two main pools: a reserve pool which is not immediately available for release and a readily releasable pool of mobilized granules. Both these pools participate in both Ca^{2+}- and GTP-dependent secretion. However, the final steps in which the readily releasable granules undergo exocytosis are different, being regulated in the one case by Ca^{2+} and in the other case by GTP (see above for arguments in support of this idea). We hypothesize that GTP is required for the transition of the granules from the reserve pool to the readily releasable pool, which accounts for the ability of GDPβS to block both Ca^{2+}-dependent and GTP-dependent secretion. In the case of GTPγS-induced secretion, the rate of mobilization from the reserve pool is 7 fF s^{-1} and that of exocytosis of the readily releasable pool of granules is 12 fF s^{-1}. This is suggested by the infusion experiments and the time course of exocytosis to rapid elevation of GTPγS. The higher rates of secretion observed in response to Ca^{2+} infusion suggest that Ca^{2+} is also able to mobilize granules from the reserve pool. There is evidence that this process is enhanced by cAMP in the presence of Ca^{2+}, but our data suggest that this is not the case in the absence of Ca^{2+}.

Ämmälä, C., Ashcroft, F. M. & Rorsman, P. (1993*a*). Ca^{2+}-independent potentiation of insulin release by cyclic AMP in single β-cells. *Nature* **363**, 356–358.

Ämmälä, C., Eliasson, L., Bokvist, K., Larsson, O., Ashcroft, F. M. & Rorsman, P. (1993*b*). Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. *Journal of Physiology* **472**, 665–689.

Ämmälä, C., Larsson, O., Berggren, P. O., Bokvist, K., Juntti-Berggren, L., Kindmark, H. & Rorsman, P. (1991). Inositol trisphosphate dependent periodic activation of a Ca^{2+}-activated K^+-conductance in glucose-stimulated pancreatic β-cells. *Nature* **353**, 849–852.

Ashcroft, F. M. & Rorsman, P. (1995). Electrophysiology of pancreatic islet cells. In *The Electrophysiology of Neuroendocrine Cells*, ed. Heschler, J. & Scherubl, H., pp. 207–245. CRC Press, Boca Raton, FL, USA.

Bokvist, K., Eliasson, L., Ämmälä, C., Renström, E. & Rorsman, P. (1995). Co-localisation of L-type Ca^{2+} channels and insulin-containing secretory granules and its significance for the initiation of exocytosis in mouse pancreatic β-cells. *EMBO Journal* **14**, 50–57.

Burgoyne, R. D. & Handel, S. (1994). Activation of exocytosis by GTP analogues in adrenal chromaffin cells revealed by patch-clamp measurements. *FEBS Letters* **344**, 139–142.

Burgoyne, R. D. & Morgan, A. (1993). Regulated exocytosis. *Biochemical Journal* **293**, 305–316.

Coorssen, J. R. & Haslam, R. J. (1993). GTPγS and phorbol ester act synergistically to stimulate both Ca^{2+}-independent secretion and phospholipase D activity in permeabilised human platelets. *FEBS Letters* **316**, 170–174.

Dean, P. M. (1973). Ultrastructural morphometry of the pancreatic beta-cell. *Diabetologia* **9**, 115–119.

Dunlop, M. & Metz, S. A. (1989). A phospholipase D-like mechanism in pancreatic islet cells: stimulation by calcium ionophore, phorbol ester and sodium fluoride. *Biochemical and Biophysical Research Communications* **163**, 922–928.

Fischer von Mullard, G., Stahl, B., Li, C., Südhof, T. C. & Jahn, R. (1994). Rab proteins in regulated exocytosis. *Trends in Biochemical Sciences* **19**, 164–168.

Gillis, K. D. & Misler, S. (1992). Single cell assay of exocytosis from pancreatic islet B-cells. *Pflügers Archiv* **420**, 121–123.

GILLIS, K. D. & MISLER, S. (1993). Enhancers of cytosolic cAMP augment depolarisation-induced exocytosis from pancreatic β-cells: evidence for events distal to Ca^{2+} entry. *Pflügers Archiv* **424**, 195–197.

GOMPERTS, B. D. (1990). G_E: a GTP-binding protein mediating exocytosis. *Annual Review of Physiology* **52**, 591–606.

LINDAU, M. & GOMPERTS, B. D. (1991). Techniques and concepts in exocytosis: focus on mast cells. *Biochimica et Biophysica Acta* **1071**, 429–471.

MARTELL, A. E. & SMITH, R. M. (1971). *Critical Stability Constants*, vol. 1, *Amino Acids*, and vol. 2, *Amines*. Plenum Press, New York.

MEGLASSON, M. D., NELSON, J., NELSON, D. & ERECINSKA, M. (1989). Bioenergetic response of pancreatic islets to stimulation by fuel molecules. *Metabolism* **38**, 1188–1195.

METZ, S. A. & DUNLOP, M. (1990). Stimulation of insulin release by phospholipase D. *Biochemical Journal* **270**, 427–435.

NEHER, E. & MARTY, A. (1982). Discrete changes of cell capacitance observed under conditions of enhanced secretion in bovine adrenal chromaffin cells. *Proceedings of the National Academy of Sciences of the USA* **79**, 6712–6716.

NEHER, E. & ZUCKER, R. S. (1993). Multiple calcium dependent processes related to secretion in bovine chromaffin cells. *Neuron* **10**, 21–30.

OKANO, K., MONCK, J. R. & FERNANDEZ, J. M. (1993). GTPγS stimulates exocytosis in patch-clamped rat melanotrophs. *Neurone* **11**, 165–172.

PERRIN, D. D. & SAYCE, I. G. (1967). Computer calculation of equilibrium concentrations in mixtures of metal and complexing species. *Talanta* **14**, 833–842.

REGAZZI, R., LI, G. D., DESHUSSES, J. & WOLLHEIM, C. B. (1989). Different requirements for protein kinase C activation and Ca^{2+}-independent insulin secretion in response to guanine nucleotides. *Journal of Biological Chemistry* **265**, 639–644.

REGAZZI, R., WOLLHEIM, C. B., LANG, J., THELER, J. M., ROSSETTO, O., MONTECUCCO, C., SADOUL, K., WELLER, U., PALMER, M. & THORENS, B. (1995). VAMP-2 and cellubrevin are expressed in pancreatic β-cells and are essential for Ca^{2+} but not for GTPγS induced insulin secretion. *EMBO Journal* **14**, 2723–2730.

RENSTÖM, E., ELIASSON, L., BOKVIST, K. & RORSMAN, P. (1996). Cooling inhibits exocytosis in single mouse pancreatic B-cells by supression of granule mobilization. *Journal of Physiology* **494**, 41–52.

SMITH, P. A., DUCHEN, M. R. & ASHCROFT, F. M. (1995). A fluorimetric and amperometric study of secretion in isolated mouse pancreatic β-cells. *Pflügers Archiv* **430**, 808–818.

VALLAR, L., BIDEN, T. J. & WOLLHEIM, C. B. (1987). Guanine nucleotides induce Ca^{2+}-dependent insulin secretion from permeabilised RINm5F cells. *Journal of Biological Chemistry* **262**, 5049–5056.

WOLLHEIM, C. B., ULLRICH, S., MEDA, P. & VALLAR, L. (1987). Regulation of exocytosis in electrically permeabilised insulin-secreting cells – evidence for Ca^{2+} dependent and independent secretion. *Bioscience Reports* **7**, 443–454.

Acknowledgements

We thank Dr B. Gomperts for his critical advice and insightful comments on the manuscript. Studies in the Oxford group were supported by grants from The Wellcome Trust, the British Medical Research Council and the British Diabetic Association, whom we thank. The Swedish group were supported by the Juvenile Diabetes Foundation International, the Swedish Medical Research Council, the Swedish Diabetes Association, the Nordic Insulin Foundation Committee, the Åke Wibergs Stiftelse and Novo-Nordisk A/S, Bagsvaerd, Denmark. C. Ä. was supported by a Swedish Medical Research Council Fellowship.

Authors' present addresses

C. Ämmälä: University Laboratory of Physiology, Parks Road, Oxford OX1 3PT, UK.

P. Rorsman: Department of Islet Cell Physiology, Novo Nordisk A/S, Fruebjergvej 3, DK-2100 Copenhagen, Denmark.

Author's permanent address

P. Proks: Institute of Molecular Physiology and Genetics, Vlarska 5, 83334 Bratislava, Slovakia.

Received 16 April 1996; accepted 10 June 1996.