Dynamics of Ca^{2+} and guanosine 5′-[γ-thio]triphosphate action on insulin secretion from α-toxin-permeabilized HIT-T15 cells

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The time course of Ca^{2+} and GTP-analogue effects on insulin secretion was investigated in HIT-T15 cells permeabilized with *Staphylococcus* α-toxin. These cells responded to Ca^{2+} in the range 0.1–10 μM and could be used in a dynamic perifusion system because of the minimal run-down of the secretory response. High Ca^{2+} (10 μM) elicited a monophasic ATP-dependent stimulation of insulin secretion that reached a peak within 5 min (~ 20-fold increase) and rapidly decreased during the subsequent 15 min to a plateau remaining above basal rates (0.1 μM Ca^{2+}). The decrease in Ca^{2+}-induced insulin secretion with time could not be attributed to decreased capacity to respond to Ca^{2+} after prolonged perfusion at low Ca^{2+} (run-down), nor to depletion of a particular secretory-granule pool. It was rather due to desensitization of the secretory machinery to Ca^{2+} that was not reversed by selective inhibition of the Ca^{2+}/calmodulin-dependent kinase II with KN-62. However, an intermediate Ca^{2+} concentration (2 μM) increased insulin secretion to stable level without causing any desensitization. Imposed oscillations of Ca^{2+} (0.1–10 μM) produced phasic oscillations of insulin secretion, but did not prevent desensitization to Ca^{2+}. Poorly hydrolysable GTP analogues increased insulin secretion at low Ca^{2+}, whereas they strongly inhibited Ca^{2+}-induced insulin secretion. By contrast, GTP did not affect basal secretion, and slightly increased Ca^{2+}-evoked secretion. These results indicate the following. (1) Oscillations of insulin secretion are tightly coupled to cytosolic Ca^{2+} oscillations. (2) Oscillations of Ca^{2+} do not prevent high-Ca^{2+}-induced desensitization to Ca^{2+}; this result does not support the idea of a greater efficiency of oscillations compared with sustained Ca^{2+} rises in triggering exocytosis. (3) Activation of G-proteins modulates exocytosis in a bimodal manner.

INTRODUCTION

Permeabilized-cell models have been widely used to study the molecular requirements of exocytosis in a variety of cell types (for reviews see Gomperts, 1990; Burgoyne and Morgan, 1993), including insulin-secreting cells (Jones et al., 1985; Vallar et al., 1987). Although ATP-dependency and Ca^{2+}-sensitivity are general properties of exocytosis in these models, the effects of guanine nucleotides greatly depend on the cell type, the size of pores generated by the permeabilization technique and the ionic composition of the medium (Knight et al., 1989; Ahnert-Hilger et al., 1992a,b; Burgoyne and Morgan, 1993).

In insulin-secreting cells (HIT-T15, RINm5F) permeabilized by high-voltage discharge or the pore-forming toxin streptolysin-O, exocytosis can be triggered either by Ca^{2+} or by poorly hydrolysable GTP analogues in a Ca^{2+}-independent manner (Vallar et al., 1987; Li et al., 1993a). Since these results were obtained in short-term (5–15 min) static incubations, little is known about the dynamics of Ca^{2+}- and guanine-nucleotide-induced insulin secretion in permeabilized cells. In particular, the interaction of these agents in a dynamic system could provide important information about the hierarchy of events controlling secretion.

In dynamic experiments with intact pancreatic islets, glucose triggers a biphasic increase in insulin secretion. Measurement of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) by microspectrofluorimetry has revealed a similar pattern of $[Ca^{2+}]_i$ rise in response to glucose (Santos et al., 1991; Gilon and Henquin, 1992). During the second phase, fast and/or slow $[Ca^{2+}]_i$ oscillations (frequency of ~ 2–3/min and 0.2–0.5/min respectively) have been reported in intact pancreatic islets (Valdeomillos et al., 1989; Longo et al., 1991; Gilon and Henquin, 1992) and in single pancreatic β-cells (Pralong et al., 1990; Herchuelz et al., 1991; Theler et al., 1992). Similarly, oscillations of insulin secretion were observed in single islets from *ob/ob* mice and rats (Opara et al., 1988; Longo et al., 1991; Bergsten and Hellman, 1993). However, the role of $[Ca^{2+}]_i$ oscillations in glucose-induced insulin secretion has not yet been clarified. For instance, it is still unclear whether $[Ca^{2+}]_i$ oscillations are more efficient at stimulating insulin secretion than is a sustained elevation of $[Ca^{2+}]_i$.

Thus the aim of this study was to characterize the dynamics of Ca^{2+}- and GTP-analogue-induced insulin secretion in permeabilized cells. We also evaluated whether oscillations of Ca^{2+} (i) produce phasic oscillations of insulin secretion; and/or (ii) would favour a sustained second phase of insulin secretion. We therefore used *Staphylococcus aureus* α-toxin to permeabilize HIT-T15 insulin-secreting cells.

The mechanism of pore formation by α-toxin has been well characterized (for review, see Bhakdi and Tranum-Jensen, 1991). Briefly, water-soluble α-toxin monomers (34 kDa) attach to the plasma membrane and, by hexamerization, form pores of 1–2 nm diameter. These pores allow the free passage of molecules ≤ 2–3 kDa, while preventing cytosolic protein leakage from cells. Similarly, α-toxin monomers do not enter cells, and leave intracellular membranes intact. This toxin, like other pore-forming toxins, has been successfully used to permeabilize a variety of cell types, including chromaffin, PC12 and RIN A2

Abbreviations used: GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; LDH, lactate dehydrogenase.

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cells (Ahnert-Hilger et al., 1985; Bader et al., 1986; Lind et al., 1987). Using α-toxin, we obtained permeabilized HIT cells that retain the ability to respond to Ca^{2+} for an extended period, allowing their use in a dynamic system.

EXPERIMENTAL

Materials

ATP, GTP, guanosine 5′-[γ-thio]triphosphate (GTP[S]) and guanosine 5′-[βγ-imido]triphosphate (p[NH]ppG) were purchased from Boehringer Mannheim, Mannheim, Germany. Stock solutions of guanine nucleotide analogues were prepared in 20 mM Hepes buffer, pH 7.0. KN-62 was supplied by Dr. H. Hidaka, Nagoya University School of Medicine, Nagoya, Japan.

α-Toxin

Staphylococcus aureus α-toxin was purified as described by Palmer et al. (1993) and reconstituted at a concentration of 3.6 mg/ml in 10 mM-phosphate-buffered saline (PBS), pH 7.0, containing 1 mg/ml BSA. The haemolytic activity of the toxin was determined in duplicate by using rabbit erythrocytes (Lind et al., 1987). Briefly, heparin-treated rabbit erythrocytes were washed twice and diluted 1:40 with ice-cold PBS. This 2.5% erythrocyte solution was then mixed with a serial dilution of α-toxin and incubated for 45 min at 37 °C. Total and non-specific haemolyses were determined in PBS alone or supplemented with 0.2% (w/v) SDS. After centrifugation for 2 min at 12000 *g*, 30 μl of supernatant was diluted in 1 ml of water, and released haemoglobin was measured spectrophotometrically at 412 nm. The dilution of α-toxin that produces 50% haemolysis of red cells (EC_{50}) was determined, and the reciprocal of this value was taken as the number of haemolytic units (H.U.) in 1 ml of the undiluted α-toxin solution. On average, the haemolytic activity of the toxin was 58000 ± 7800 H.U./mg ($n = 6$).

Cells

HIT-T15 cells (passages 71–79) were cultured in RPMI 1640 medium supplemented as described by Regazzi et al. (1990).

Permeabilization of attached cells and static insulin-release experiments

HIT-T15 cells were cultured for 2 days in microtitre plates at an initial density of 10^5 cells/well. Before use, the cells were washed twice with a Ca^{2+}-free modified Krebs–Ringer medium [buffer A, of composition (mM): Hepes 25, pH 7.4; NaCl 125; KCl 5; $MgSO_4$ 2; KH_2PO_4 1.2; EGTA 0.4; glucose 6; and 1 mg/ml BSA]. They were then permeabilized during 10 min at 37 °C with α-toxin (1000 H.U./ml) dissolved in buffer G [composition (mM): Hepes 20, pH 7.0; potassium glutamate 140; NaCl 5; $MgSO_4$ 7; Na_2ATP 5; EGTA 10.2] with sufficient $CaCl_2$ to give a free Ca^{2+} concentration of 0.1 μM. Cell permeabilization was assessed by eosin and Trypan Blue dye-uptake tests. Under these conditions, HIT cells did not detach from the well, and 95–100% of cells were permeable to eosin (624 kDa), whereas 15–20% of cells were coloured by Trypan Blue (960 kDa). A higher toxin concentration resulted in pronounced cell detachment from the well. Only a few (less than 5%) control cells incubated without α-toxin were stained with either dye.

The activity of lactate dehydrogenase (LDH; ~ 140 kDa), used as a marker for cytosolic proteins, was assessed in supernatants and cell lysates of α-toxin-permeabilized cells by a modification of the method described by Kornberg (1955). Briefly, the rate of consumption of NADH (20 μM) induced by a sample was measured in the presence of 2 mM pyruvate, 0.5 mg/ml BSA and 20 mM Hepes, pH 7.2, with a fluorescence spectrometer (excitation/emission wavelengths: 340/460 nm). The total LDH content was similar in control and treated cells [36 ± 1.3 and 35.7 ± 1.6 m-units/well ($n = 6$) respectively], and no LDH activity could be detected in supernatants (the limit of LDH detection using this assay was approx. 5% of cellular content).

After cell permeabilization, the medium was replaced for a 5 min incubation period by test medium (buffer G containing 0.1, 2 or 10 μM free Ca^{2+} and test substances). The amount of insulin secreted into the medium was assessed by radioimmunoassay, with human insulin as standard (Praz et al., 1983).

Permeabilization of cells in suspension and dynamic insulin-release experiments

On the day of the experiment, HIT-T15 cells were detached and transferred to spinner culture (Wollheim and Pozzan, 1984). After 3 h, the cells were washed twice in buffer A and resuspended at a concentration of 20×10^6 cells/ml in buffer E [buffer G, containing instead (mM): $MgSO_4$ 4, Na_2ATP 2 and EGTA 0.2; free Ca^{2+} approx. 0.1 μM]. This cell suspension was mixed 1:1 (v/v) with buffer E supplemented with α-toxin and incubated for 10 min at 37 °C at a final concentration of 3500 H.U./10^7 cells per ml. After centrifugation (5 min at 1000 *g*, 4 °C), the supernatant was discarded and cells were resuspended in buffer E (40×10^6 cells/ml). With this protocol, 97–100% of cells were permeable to Trypan Blue (control cells: 9.9 ± 1.2%, $n = 27$), and remained so for at least 2 h at 37 °C.

Permeabilized cells were transferred to perifusion chambers (100 μl, 4×10^6 cells; filter of 8 μm pore size) and were continuously perfused with buffer G (but containing 4 mM $MgSO_4$, 2 mM Na_2ATP) at a flow rate of ~ 600 μl/min, unless otherwise specified. After an initial 10 min washing period, effluent fractions were collected every 2 min, diluted with glycine buffer containing BSA (final concn. ~ 1 mg/ml) and kept at −20 °C until insulin assay. Before perifusion, a sample of permeabilized cell suspension was taken for measurement of insulin content.

Ca^{2+} concentration

The free Ca^{2+} concentrations in buffer G were measured with a Ca^{2+} electrode (Prentki et al., 1983) and corresponded to those calculated from the appropriate equilibrium constants (Sillén and Martell, 1964).

Statistical analysis

Results are expressed as means ± S.E.M. for the indicated number of experiments. The statistical significance of differences between groups was assessed by Student's *t* test.

RESULTS

Static insulin secretion

α-Toxin-permeabilized HIT-T15 cells incubated in the presence of 5 mM ATP at low free Ca^{2+} concentration (0.1 μM) secreted 2.67 ± 0.17% of their insulin content in 5 min ($n = 12$). This cell preparation was highly responsive to Ca^{2+}, since 2 μM and 10 μM free Ca^{2+} applied directly after cell permeabilization produced a 6- and 10-fold increase in insulin secretion respectively (5.98 ± 0.39 and 9.73 ± 0.47 versus 0.98 ± 0.05 ng/well per 5 min;

Table 1 Run-down of Ca^{2+}-stimulated insulin secretion from α-toxin-permeabilized HIT cells

Attached cells were permeabilized by incubation with α-toxin for 10 min. Cells were then incubated for 5 min in a medium containing 0.1 μM Ca^{2+} or 10 μM Ca^{2+}, either directly after cell permeabilization or after 10 or 20 min preincubation in the presence of 0.1 μM Ca^{2+}. Values are means ± S.E.M. for 12 observations from 3 independent experiments: * $P < 0.01$ compared with control group (without preincubation).

Preincubation period	Insulin secretion (ng/well)	
	0.1 μM Ca^{2+}	10 μM Ca^{2+}
–	1.56 ± 0.10	10.3 ± 0.72
10 min	1.65 ± 0.04	7.54 ± 0.65*
20 min	1.74 ± 0.06	6.47 ± 0.41*

$n = 28$). A 10 min preincubation of the permeabilized cells at 0.1 μM Ca^{2+} before stimulation significantly decreased Ca^{2+}-induced insulin secretion (7.54 ± 0.65 versus 10.3 ± 0.72 ng/well in the control), as shown in Table 1. No further decrease in Ca^{2+}-induced insulin secretion occurred when preincubation at low Ca^{2+} concentration was prolonged to 20 min. However, neither basal insulin secretion (Table 1) nor eosin-dye uptake was modified by the preincubation period.

We next evaluated the effects of GTP and its poorly hydrolysable analogues GTP[S] and p[NH]ppG on insulin secretion from α-toxin-permeabilized cells. As shown in Figure 1, 100 μM GTP[S] significantly increased insulin secretion in the presence of 0.1 μM Ca^{2+} and to a lesser extent in the presence of 2 μM Ca^{2+}, whereas it inhibited insulin secretion induced by 10 μM Ca^{2+} by 18.7 ± 3.3 %. Qualitatively similar effects were observed with 100 μM p[NH]ppG. By contrast, 100 μM GTP was completely ineffective on basal secretion, whereas it clearly potentiated Ca^{2+}-induced insulin secretion in the presence of both 2 and 10 μM Ca^{2+}.

The high responsiveness of α-toxin-permeabilized HIT cells to Ca^{2+} and the small rundown of Ca^{2+}-induced insulin secretion makes this permeabilized-cell preparation suitable for the study of insulin secretion in a dynamic perifusion system.

Dynamic insulin secretion

α-Toxin-permeabilized HIT cells perifused with a medium containing 2 mM ATP and a low free Ca^{2+} concentration (0.1 μM) secreted 0.022 ± 0.002 % of insulin content/min ($n = 12$). The dynamic secretory response to 10 μM Ca^{2+} is shown in Figure 2(a). Typically, a 30 min stimulation with Ca^{2+} induced a rapid monophasic increase in insulin secretion that reached a peak within 5 min (20-fold increase), then gradually decreased and tended to stabilize at a level ~ 3 times higher than the basal level. The total Ca^{2+}-induced insulin secretion (area under the curve) was 5.33 ± 1.56 % of insulin content/30 min, corresponding to an 8-fold increase in insulin secretion. Decreasing Ca^{2+} to 0.1 μM after 10 min stimulation was followed by the return of insulin secretion to basal level within 10 min (Figure 2a). In this case, secretion was lower than in the continuous presence of 10 μM Ca^{2+} (0.28 ± 0.06 and 0.69 ± 0.2 % of content from 40 to 50 min, in the absence and presence of high Ca^{2+} respectively; $P = 0.057$; $n = 3$). A second stimulation with Ca^{2+} still increased insulin release (Figure 2b). However, this Ca^{2+}-induced insulin secretion was 2.6 times lower than that occurring during the first stimulation (applied after 40 or 20 min perifusion with low Ca^{2+}). Thus the decrease in Ca^{2+}-induced insulin secretion in the continuous presence of Ca^{2+} or during a second stimulation was not due to the run-down of the response to Ca^{2+}, but rather to desensitization of the secretory machinery to Ca^{2+}.

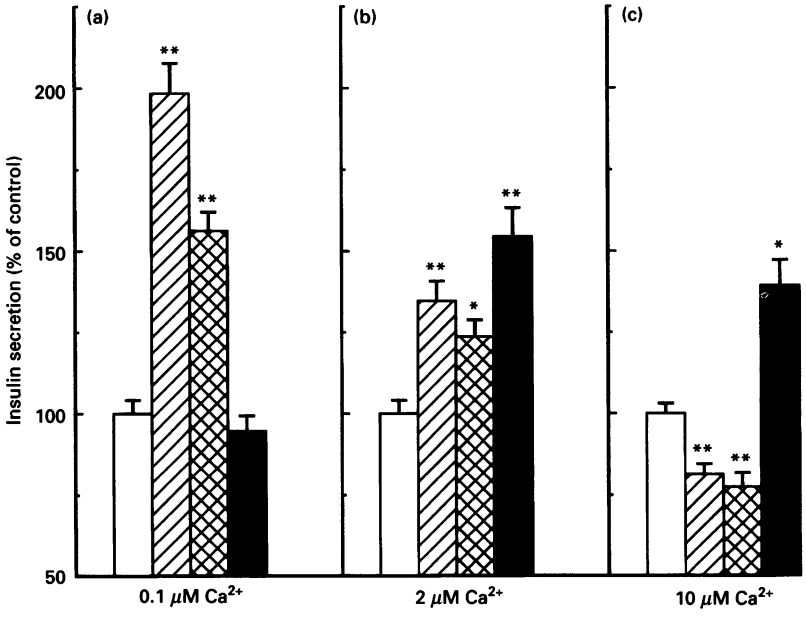

Figure 1 Effects of GTP analogues on insulin secretion from α-toxin-permeabilized HIT cells

After permeabilization, cells were incubated for 5 min in a medium containing (**a**) 0.1 μM Ca^{2+}, (**b**) 2 μM Ca^{2+} and (**c**) 10 μM Ca^{2+}, alone (□) or supplemented with 100 μM GTP[S] (▨), 100 μM p[NH]ppG (▩) or 100 μM GTP (■). Control secretion in the presence of 0.1 μM, 2 μM and 10 μM Ca^{2+} was 0.97 ± 0.05, 5.72 ± 0.52 and 9.57 ± 0.62 ng/well respectively. Values are means ± S.E.M. for 8–12 observations from 2–3 independent experiments: * $P < 0.005$ and ** $P < 0.001$ compared with control.

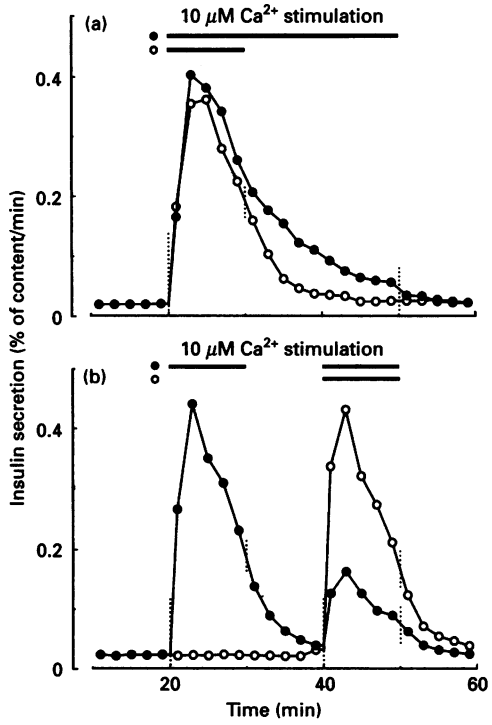

Figure 2 Dynamics of Ca^{2+}-induced insulin secretion in α-toxin-permeabilized HIT cells

Cells were permeabilized in suspension as described in the Experimental section and perifused during 20 min with basal medium (0.1 mM Ca^{2+}). (**a**) Exocytosis was triggered by 10 μM Ca^{2+} during 10 min (○) or 30 min (●), as indicated by the horizontal lines. (**b**) A 10 min stimulation with 10 μM Ca^{2+} was applied twice, from 20 to 30 and 40 to 50 min (●) or only once, from 40 to 50 min (○). Insulin secretion is expressed as a percentage of insulin content secreted per min, and results are corrected for dead volume of the perifusion system. Values are means for 3 experiments, and S.E.M. values are omitted for sake of clarity.

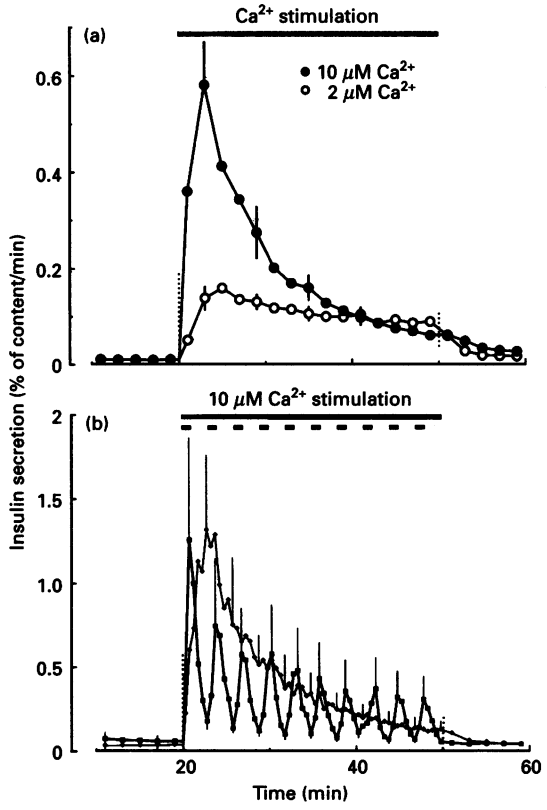

Figure 3 Effects of various patterns of Ca^{2+} addition on insulin secretion from α-toxin-permeabilized HIT cells

After permeabilization, cells were perifused during 20 min with basal medium (0.1 μM Ca^{2+}). (**a**) Exocytosis was then stimulated with 2 μM Ca^{2+} (○) or 10 μM Ca^{2+} (●). (**b**) A 10 μM Ca^{2+} stimulus was applied either continuously (◆, thin line) or in 1 min pulses every 3 min (■, thick line) during 30 min, as indicated by the horizontal lines. In (**a**) and (**b**), cells were perfused at a flow rate of ~ 1.2 ml/min, to accelerate the exchange of medium in the perfusion chambers, and effluent fractions were collected every 30 s, to increase the time resolution of the system. Insulin secretion is expressed as a percentage of insulin content secreted per min, and results are corrected for dead volume of the perifusion system. Results are means ± S.E.M. for 3–4 experiments.

We next studied the ATP-dependency of Ca^{2+}-induced insulin secretion. Cells were permeabilized in the absence of ATP, perifused with a medium containing 0 or 2 mM ATP and stimulated during 30 min with high Ca^{2+}. In the absence of ATP, basal secretion tended to be lower than in the presence of 2 mM ATP (0.12 ± 0.03 versus 0.24 ± 0.07 % of content/10 min; $n = 4$; $P = 0.06$ by paired t test). Under these conditions, Ca^{2+} stimulation failed to increase insulin secretion significantly above basal levels (0.17 ± 0.06 versus 0.12 ± 0.03 % of content/10 min; $n = 4$; $P = 0.16$ by paired t test).

We then evaluated the effect of an intermediate Ca^{2+} concentration ($EC_{50} \sim 2$ μM in static incubation) on insulin secretion. Compared with 10 μM, 2 μM Ca^{2+} caused a rapid and sustained increase in insulin secretion that was far less pronounced during the first 10 min (14- versus 36-fold increase), but was nearly identical at the end of stimulation (Figure 3a). The total insulin secretion was about 2 times lower with 2 μM than with 10 μM Ca^{2+} (3.19 ± 0.4 versus 6.24 ± 0.91 % of content/30 min; $n = 4$).

We also compared pulsatile and continuous application of 10 μM Ca^{2+}. Interestingly, oscillations of Ca^{2+} produced phasic oscillations of insulin secretion (Figure 3b). The first peak of insulin secretion was as high as that observed during sustained Ca^{2+} application. Nevertheless, there was a clear decrease in Ca^{2+}-induced insulin secretion (area under the curve) from pulse to pulse (30 and 24 % decrease from the first to the second and from the second to the third Ca^{2+} pulse respectively; $n = 3$). This was not due to changes in Ca^{2+} stimulus itself, since peak and nadir Ca^{2+} levels were identical throughout the experiment (7–10 μM and 0.1 μM Ca^{2+} respectively), as assessed by Ca^{2+} measurement in 15 s effluent collections from parallel experiments performed without cells. From the third Ca^{2+} pulse on, insulin secretion induced by each Ca^{2+} pulse tended to stabilize (at about 30 % of first pulse). Thus Ca^{2+} pulses were still capable of generating pulsatile insulin secretion. The peak insulin secretion in each transient during the first 15 min stimulation was roughly similar to the rate seen during continuous Ca^{2+} stimulation at corresponding time points. In contrast, during the last 15 min Ca^{2+} stimulation, the pulses produced higher rates of secretion (Figure 3b). However, during the last 10 min, the mean insulin secretion was similar during pulsatile, sustained 2 μM and 10 μM Ca^{2+} stimulation (0.91 ± 0.19, 1.01 ± 0.05 and 0.86 ± 0.10 % of content/10 min respectively; $n = 3$). Hence, oscillations of Ca^{2+} do not prevent desensitization of the secretory machinery to Ca^{2+}.

To evaluate the influence of low Ca^{2+}-interval duration between pulses, three pulses of Ca^{2+} (2 min) were applied at 14 min

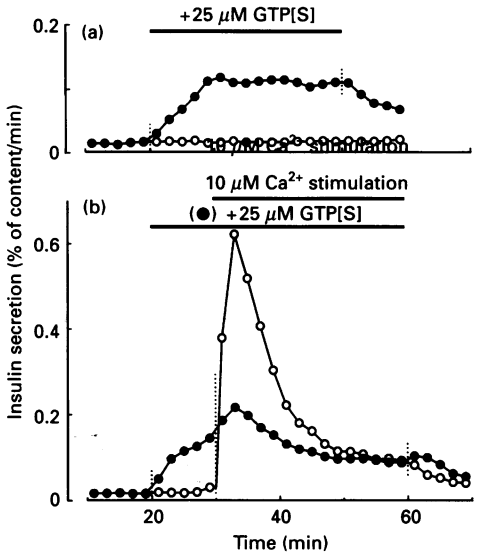

Figure 4 Dynamics of GTP[S] effects on basal and Ca^{2+}-stimulated insulin secretion from α-toxin-permeabilized HIT cells

(**a**) After permeabilization, cells were perifused with a medium containing 0.1 μM Ca^{2+} throughout the experiment (○) or stimulated with 25 μM GTP[S] as indicated by the horizontal line (●). (**b**) Cells were stimulated with 10 μM Ca^{2+} alone (○) or 10 μM Ca^{2+} and 25 μM GTP[S], the latter being added 10 min before the Ca^{2+} stimulus (●). Insulin secretion is expressed as a percentage of insulin content secreted per min, and results are corrected for dead volume of the perifusion system. Values are means for 3–6 experiments, and S.E.M. values are omitted for sake of clarity.

intervals. Under these conditions, Ca^{2+}-induced insulin secretion gradually decreased from the first to the third pulse (30 and 36% decreases from first to second and from second to third Ca^{2+} pulse respectively; $n = 2$). Thus, no recovery of Ca^{2+} sensitivity occurred as time increased between the Ca^{2+} pulses.

We also used KN-62, an inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) (Tokumitsu et al., 1990) to evaluate the role of the autophosphorylation of this enzyme in Ca^{2+}-induced desensitization to Ca^{2+}. KN-62 (5 μM), applied 10 min before and also during Ca^{2+} stimulation, did not modify the dynamics of basal and Ca^{2+} (10 μM)-evoked dynamic insulin secretion (8.73 ± 1.11% and 7.27 ± 1.13% of content/30 min in the presence and absence of KN-62 respectively; $n = 5$). This is consistent with the inability of KN-62 to affect Ca^{2+}-stimulated insulin secretion during static incubation of electropermeabilized and streptolysin-O-permeabilized HIT-T15 cells reported previously (Li et al., 1992). This result also reinforces the view that CaM kinase II may not be involved in the exocytosis elicited by Ca^{2+} in permeabilized insulin-secreting cells.

The effect of GTP[S] on basal and Ca^{2+}-induced insulin secretion was also tested in dynamic experiments. As shown in Figure 4(a), 25 μM GTP[S] clearly increased basal insulin secretion. Maximal effect (7-fold stimulation) was reached after 10 min and remained stable until GTP[S] was removed from the medium. Identical results (not shown) were obtained in the presence of a free Ca^{2+} concentration < 1 nM. Pre-exposure of cells to 25 μM GTP[S] for 10 min resulted in an inhibition of subsequent Ca^{2+}-induced insulin secretion by 87% (Figure 4b). The total increase in insulin secretion produced by Ca^{2+} (first 10 min) in the absence and presence of GTP[S] was 4.20 ± 0.65 and 0.55 ± 0.09% of content respectively ($n = 6$). By contrast, 20 μM GTP did not change basal insulin secretion and slightly increased Ca^{2+}-induced insulin secretion, in agreement with results from static incubations. The latter effect, however, was transient, as it was limited to the first 5 min of secretion (results not shown).

DISCUSSION

The present study indicates that α-toxin-permeabilized HIT-T15 cells are a good model to study the effect of low-molecular-mass substances on exocytosis in a dynamic perifusion system. The Ca^{2+}-sensitivity of insulin secretion was in the range 0.1–10 μM Ca^{2+} ($EC_{50} \sim 1$–2 μM), as described in other cell types (Knight et al., 1989; Holz et al., 1992) and in insulin-secreting cells permeabilized by other methods (Jones et al., 1985; Valler et al., 1987). The run-down of the response to Ca^{2+} was small and limited to the first 10–20 min after permeabilization, in contrast with the rapid run-down observed in streptolysin-O-permeabilized HIT cells (Li et al., 1993a). This difference is probably due to the leakage of different cytosolic components from cells, as expected from the different diameter of pores generated by the two toxins (1–2 nm for α-toxin and ⩾ 15 nm for streptolysin-O) (Bhakdi and Tranum-Jensen, 1987). Thus, PC12 and RIN A2 cells permeabilized with α-toxin allowed the free passage of nucleotides, but not of LDH (Lind et al., 1987; Ahnert-Hilger et al., 1989). Similar results were obtained with HIT cells in the present study, as indicated by dye uptake, comparison of cell LDH content before and after permeabilization and the strict ATP-dependency of Ca^{2+}-induced insulin secretion under dynamic conditions. This method of permeabilization, although yielding similar membrane pores, had some advantages over electroporation. First, we could easily permeabilize cells plated in 96 microwells, which is convenient for static incubations. Second, since cells did not reseal with time, we could perform lengthy dynamic experiments.

The typical time course of Ca^{2+}-induced insulin secretion reported herein is similar to that described for electropermeabilized islets (Jones et al., 1992), except for much higher amplitude in the HIT cells (see below). Although insulin secretion was already severely decreased after 20 min of Ca^{2+} stimulation, the ion still enhanced the rate of secretion even beyond this time point, as indicated by the following observations. First, perfusion of cells with low Ca^{2+} (0.1 μM) at the end of the experiment produced a rapid decrease in insulin secretion to the baseline. Second, when Ca^{2+} was decreased after 10 min of stimulation, insulin secretion was lower than in the continuous presence of high Ca^{2+}. In this case, a second stimulation with Ca^{2+} was followed by a second monophasic increase in insulin secretion, albeit of lower amplitude. Nevertheless, the decrease of insulin secretion in the presence of high Ca^{2+} or during a second stimulation could not be attributed to the run-down of the cell preparation, as insulin responses to early and late first Ca^{2+} stimulation were identical. Nor could it be explained by depletion of secretory granules, since insulin secretion during 30 min of high-Ca^{2+} stimulation did not exceed 10% of total insulin content. Rather, a desensitization of the secretory machinery to Ca^{2+} seems to occur. However, it should be emphasized that Ca^{2+} effects were never completely abolished within 30 min of perfusion, in contrast with results obtained with permeabilized islets (Jones et al., 1989, 1992). This difference could be due, at least in part, to the 10 times higher fold increase in insulin secretion elicited by Ca^{2+} in α-toxin-permeabilized HIT cells. A small residual increase in insulin secretion was therefore easily detectable. Alternatively, high-voltage discharge could have untoward effects on pancreatic islets.

It has been suggested that oscillations of Ca^{2+} could be more efficient in triggering insulin secretion than is a sustained elevation of Ca^{2+} (Hellman et al., 1992). Since α-toxin-permeabilized HIT cells are highly responsive to Ca^{2+}, we were able to test this hypothesis in our perfusion system. An important finding was that regular pulses of high Ca^{2+} produced phasic pulses of insulin secretion. This clearly demonstrates that oscillations of Ca^{2+} can trigger oscillations of insulin secretion, independent of oscillations in NAD(P)H, ATP or other metabolic coupling factors (Pralong et al., 1991; Longo et al., 1991). Nevertheless, this does not exclude an essential role for oscillations of these factors in the generation of pulsatile secretion from intact cells.

The oscillatory secretion persisted throughout the experiment, but the peak rates gradually decreased, as did the response triggered by the sustained elevation of Ca^{2+}. Considering that total insulin secretion was 2 times lower when Ca^{2+} was oscillating, these results reinforce the view of a true desensitization to Ca^{2+}, which is not due to the depletion of a particular pool of secretory granules. These observations also suggest that the so-called 'desensitization to Ca^{2+}' corresponds to a decrease in the maximal Ca^{2+}-induced insulin secretion, rather than to an impaired capacity of responding to Ca^{2+} changes. The decrease in peak insulin secretion from pulse to pulse was not modified by increasing the pulse interval, at least up to 14 min. These results indicate that the mechanism involved in the desensitization to Ca^{2+} is rapidly activated upon the rise in Ca^{2+} and is operative for a long time after Ca^{2+} has returned to the basal level. The CaM kinase II, recently identified also in pancreatic islets and RINm5F cells (Urquidi and Ashcroft, 1993), could be involved in the desensitization process. Indeed, upon binding of Ca^{2+}/calmodulin, CaM kinase II is autophosphorylated, leading to a Ca^{2+}-independent activated state of the kinase (Tokumitsu et al., 1990). Dephosphorylation by phosphatases is then required for kinase inactivation (for review, see Hanson and Schulman, 1992). However, inhibition of CaM kinase II by KN-62 before Ca^{2+} application did not prevent desensitization to Ca^{2+}. In agreement with these results, Jones et al. (1992) recently reported that desensitization to Ca^{2+} does not require the presence of ATP during the first Ca^{2+} pulse. Thus CaM kinase II autophosphorylation does not seem to be the cause of Ca^{2+}-induced desensitization to Ca^{2+}.

An intermediate Ca^{2+} concentration (2 μM) produced a stable increase in insulin secretion, the level of which was close to that observed 20 min after high-Ca^{2+} stimulation. Since Ca^{2+}-induced desensitization was observed at high, but not intermediate, Ca^{2+} concentration, this process could be due to the unphysiologically high Ca^{2+} concentration reached in the cytoplasm. However, there are at least two reasons to think that this process occurs under physiological conditions. First, Ämmälä et al. (1993) recently reported that desensitization to Ca^{2+} occurred within seconds during application of trains of voltage-clamp Ca^{2+} currents. These authors also provide evidence that the $[Ca^{2+}]_i$ beneath the plasma membrane reaches much higher levels than those reported by microfluorimetric techniques. Second, in mouse pancreatic islets depolarized with high K^+ in the absence of glucose, despite a stable elevation of $[Ca^{2+}]_i$, the time course of insulin secretion was very similar to that observed with high Ca^{2+} in permeabilized cells (Gembal et al., 1993). However, in the presence of even low concentrations of glucose, a sustained second phase of insulin secretion appeared. Therefore, glucose seems to alleviate the densensitization to Ca^{2+} occurring in glucose-deprived pancreatic islets.

The dynamic effects of GTP and its poorly hydrolysable analogues provide some more information about their mode of action. A Ca^{2+}-independent stimulatory effect of GTP[S] has previously been described in a variety of cell types (for review, see Burgoyne and Morgan, 1993). In insulin-secreting cells, this effect could clearly be dissociated from activation of phospholipase C, protein kinase C and protein kinase A (Vallar et al., 1987; Regazzi et al., 1989). In the present study, we show that this effect of GTP[S] was of slow onset and remained stable throughout the experiment. This argues for a progressive activation of GTP-binding protein(s) (G-protein) by slow exchange of endogenous guanine nucleotides with GTP[S] (Gilman, 1987). These findings also suggest that GTP[S] activates a stimulatory G-protein, the nature of which is unknown. It has been proposed that an as-yet unidentified heterotrimetric G-protein, termed G_E, could control a distal step of exocytosis (Gomperts, 1990; Ullrich and Wollheim, 1988). On the other hand, small GTP-binding proteins might also be involved in the control of exocytosis in insulin-secreting cells (Regazzi et al., 1992; Li et al., 1993a,b). However, GTP[S] seems to inhibit, rather than stimulate, processes controlled by small G-proteins, such as vesicular transport from the endoplasmic reticulum to the Golgi apparatus (Rothman and Orci, 1992). Therefore, GTP[S] was thought to interfere with the presumed cycling between membrane-bound and soluble form of these proteins. If this hypothesis is correct, the stimulatory effect of GTP[S] is best explained by activation of the putative trimeric G_E.

There is no doubt that GTP increased peak Ca^{2+}-induced insulin secretion in the present study. This may indicate that Ca^{2+} effects are accompanied by GTP hydrolysis and that GTP could be rate-limiting for exocytosis, at least in permeabilized cells. We also observed that 10 min perfusion with GTP[S] strongly inhibited the subsequent Ca^{2+}-induced insulin secretion, confirming the small inhibitory effect of GTP[S] and p[NH]ppG seen in static incubations. The difference in the degree of inhibition between static and dynamic experiments is most probably due to the slow onset of GTP[S] effects, rather than to a difference in permeabilization efficiency. Indeed, despite differences in Trypan Blue uptake between plated cells and cells in suspension, pores generated by α-toxin are of constant diameter because of their mode of formation (cf. the Introduction). Accordingly, simultaneous addition (as in static incubations) of GTP[S] and Ca^{2+} to the perfusion medium resulted in only slight inhibition of Ca^{2+}-induced insulin secretion (results not shown). In other cell types, GTP[S] either stimulates or inhibits Ca^{2+}-induced exocytosis (for review, see Holz et al., 1992; Burgoyne and Morgan, 1993). The inhibition of Ca^{2+}-induced insulin secretion by GTP[S] in α-toxin-permeabilized HIT cells is reminiscent of the ability of inhibitory neurohormones (acting via pertussis-toxin-sensitive G-proteins) to abolish Ca^{2+}-ionophore-induced insulin secretion from intact cells and to attenuate Ca^{2+}-induced insulin secretion from permeabilized cells (Jones et al., 1987; Ullrich and Wollheim, 1988; Ullrich et al., 1990). This effect of GTP[S] is thus probably mediated by inhibitory G-proteins (possibly of the G_i/G_o family). However, GTP[S] induced simulation rather than inhibition of Ca^{2+}-induced insulin secretion in electropermeabilized RINm5F cells (Vallar et al., 1987). These results are not necessarily contradictory, since GTP[S] is a non-selective activator of many G-proteins. Its effect will thus depend on the balance between stimulatory and inhibitory actions of G-proteins, whose level of expression varies between cell types (Birnbaumer, 1990).

In conclusion, in α-toxin-permeabilized HIT-T15 cells, oscillations of insulin secretion are tightly linked to oscillations of $[Ca^{2+}]_i$. Even a short rise in Ca^{2+} induces prolonged desensitization of the secretory machinery to Ca^{2+}, resulting in decreased amplitude of the protracted insulin secretion. Thus, pulsatile addition of Ca^{2+} does not avoid desensitization, sug-

gesting that, in addition to $[Ca^{2+}]_i$, other factors are required for optimal insulin secretion in intact cells. Activation of G-proteins exert a bimodal influence on insulin secretion: a Ca^{2+}-independent stimulation and an inhibition at a step distal to Ca^{2+} activation of the secretory process.

We thank Ms. A.-S. Annen and C. Bartley for technical assistance. We are also grateful to Dr. R. Regazzi and Dr. J. Lang for helpful discussions. This work was supported by Grant 32-32376.91 (to C.B.W.) from the Swiss National Science Foundation and by the Deutsche Forschungsgemeinschaft Grant SFB 311 (to M.P. and U.W.). J-C. J. is 'Aspirant' of the Fonds National de la Recherche Scientifique of Belgium.

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Received 22 November 1993/28 February 1994; accepted 7 March 1994

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