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Direct control of exocytosis by receptor-mediated activation of the heterotrimeric GTPases G_i and G_o or by the expression of their active Gα subunits

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The exocytotic release of potent hormones is a tightly controlled process. Its direct regulation without the involvement of second messengers would ensure rapid signal processing. In streptolysin O-permeabilized insulin-secreting cells, a preparation allowing dialysis of cytosolic macromolecules, activation of α_2-adrenergic receptors caused pertussis toxin-sensitive inhibition of calcium-induced exocytosis. This inhibition was mimicked very efficiently by the use of specific receptor-mimetic peptides, indicating the involvement of G_i and, to a lesser extent, of G_o. The regulation was exerted beyond the ATP-dependent step of exocytosis. In addition, low nanomolar amounts of pre-activated G_i/G_o directly inhibited exocytosis. As transient overexpression of constitutively active mutants of $G\alpha_i1$, $G\alpha_i2$, $G\alpha_i3$ and $G\alpha_o2$ but not of $G\alpha_o1$ reproduced this regulation, the Gα subunit alone is sufficient to induce inhibition. These results define exocytosis as an effector for heterotrimeric G-proteins and delineate the properties of the transduction pathway.

Keywords: calcium/epinephrine/exocytosis/G-proteins/insulin

Introduction

The fusion of secretory vesicles with the plasma membrane and the release of their contents into the extracellular space constitutes exocytosis, the final step in the vesicular transport of hormones. This process requires ATP (Holz *et al.*, 1989; Heinemann *et al.*, 1993) and, in the case of regulated secretion, exocytosis is triggered by a rise in intracellular calcium (Burgess and Kelly, 1987). Although rapid progress has been made in the elucidation of the protein components of the fusion machinery (Rothman, 1994), its regulation is still a matter of debate (O'Connor *et al.*, 1994). The concentration of potent hormones in the extracellular space needs to be finely tuned, which necessitates tight regulation of their release. In the case of insulin secretion, such tight control is a prime requirement both for glucose homeostasis and stress adaptation mediated by neurohormones such as epinephrine (Havel and Taborsky, 1989).

Vesicular transport is regulated by GTPases in a wide variety of cells and unicellular organisms (Gomperts, 1990; Ferronovick and Novick, 1993). Most of the GTPases identified belong to the rab or ARF family of monomeric small molecular weight G-proteins (Serafini *et al.*, 1991; Fischer von Mollard *et al.*, 1994). Recent evidence points to a regulatory role also for the heterotrimeric GTPases (Bomsel and Mostov, 1992). These proteins consist of α, β and γ subunits and are known to be involved in the signal transmission from cell surface receptors to second messenger systems (Kaziro *et al.*, 1991). They have previously been functionally classified according to their sensitivity to cholera toxin (G_s), pertussis toxin (G_i/G_o) or toxin resistance (G_q). The discovery of a number of isoforms has added further complexity to the system. In addition, these GTPases dissociate into activated α subunits and βγ dimers upon stimulation, each capable of regulating several effectors such as adenylyl cyclases, phospholipases and ion channels (Birnbaumer, 1992).

The demonstration that distinct heterotrimeric G-protein subunits are involved in intracellular transport has increased the pleiotropy of these molecular switches. Thus $G\alpha_i3$ retards the constitutive transport through the Golgi in LLC-PK1 renal cells (Stow *et al.*, 1991) whereas G_i3 stimulates histamine release from mast cells (Aridor *et al.*, 1993). Moreover, $G\alpha_s$ and βγ subunits stimulate transcytosis in renal MDCK cells (Bomsel and Mostov, 1993) while inhibiting endosome fusion in macrophages (Colombo *et al.*, 1992, 1994). In each case, the putative receptor or regulatory event and the precise transport step controlled remain unknown. In exocytosis, a direct receptor-mediated control could favourably combine the features of transmembrane receptors and heterotrimeric G-proteins in terms of rapid on/off switching and signal amplification. Indeed, previous studies in electropermeabilized cells indicated such an inhibitory control exerted downstream from the calcium signal and from the action of the protein kinases PKA and PKC (Ullrich and Wollheim, 1988; Lang, *et al.*, 1993). However, hormone secretion was inhibited only to a limited extent and the molecular nature of the process remains ill defined.

The present study was undertaken to establish this new putative signal transduction pathway. Streptolysin O (SLO)-permeabilized cells offer a convenient model for the study of exocytosis. This system permits the dialysis of cytosolic macromolecules and retains the physiological stimulation of hormone release in response to imposed calcium concentrations normally occurring in intact cells (Ahnert Hilger *et al.*, 1989; Li *et al.*, 1993). We demonstrate here that activation of the heterotrimeric G-proteins G_i and G_o by either a functionally important receptor or by receptor-mimetic peptides leads to direct inhibition of exocytosis without transduction by diffusable second messengers. The inhibition takes place after vesicle priming at the calcium-dependent fusion step and is highly

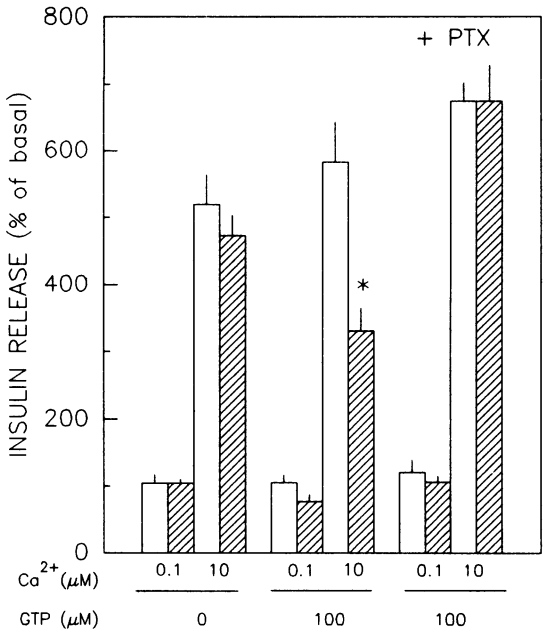

Fig. 1. Epinephrine inhibits insulin release from SLO-permeabilized HIT-T15 cells in a pertussis toxin (PTX)-sensitive fashion. HIT-T15 cells were permeabilized with SLO, pre-incubated at 0.1 μM calcium in the absence (open bars) or presence (hatched bars) of epinephrine (10 μM) and subsequently exposed to 0.1 μM or 10 μM Ca^{2+} as indicated, in the absence or presence of epinephrine (10 μM, open and hatched bars, respectively). GTP 100 μM was added as indicated and 0.3 mM free magnesium was present in all conditions. PTX pre-treatment was conducted at 0.1 μg/ml for 24 h. Basal insulin release at 0.1 μM Ca^{2+} amounted to 3.92 ± 0.3 ng/well (3.7 ± 0.28% of content). n = 6–18; *, $2P$ <0.05 versus the absence of epinephrine.

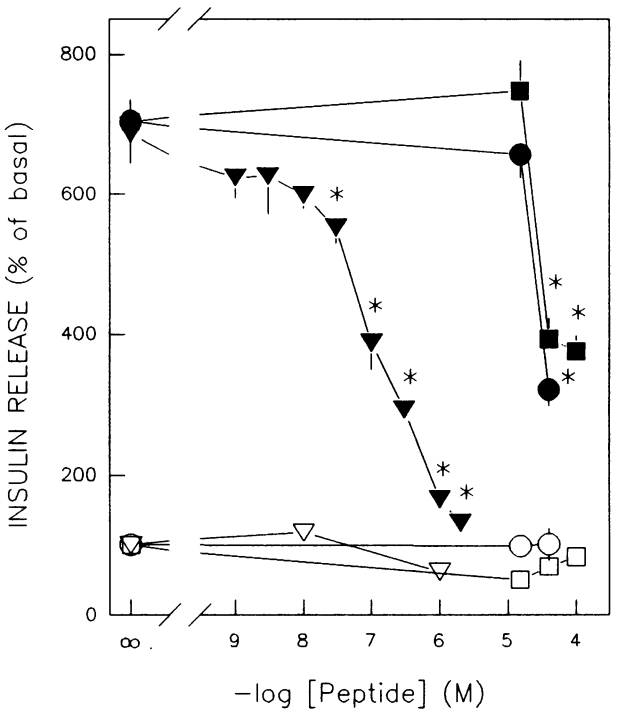

Fig. 2. Receptor-mimetic peptides inhibit insulin release in SLO-permeabilized HIT-T15 cells. SLO-permeabilized cells were pre-incubated with the indicated concentrations of synthetic G_i-stimulating peptides P14 (circles), TMP14 (triangles) or the G_o-stimulating peptide TMP20 (squares) at 0.1 μM Ca^{2+} and subsequently exposed to 0.1 (open symbols) or 10 μM Ca^{2+} (closed symbols) in the presence of the peptides. n = 7–22; *, $2P$ <0.05 versus the absence of peptides.

sensitive to pre-activated G-proteins. The G-protein α subunits alone, including all currently known isotypes of $G\alpha_i$ as well as $G\alpha_o2$, are sufficient to cause inhibition.

Results

Receptor-mediated inhibition of exocytosis

Activation of α_2-adrenergic receptors by epinephrine in insulin-secreting cells, including HIT-T15 cells, strongly inhibits insulin secretion from intact cells induced by a rise in cytosolic calcium (Wollheim and Sharp, 1981; Hsu *et al.*, 1991; Gilon *et al.*, 1993). We first investigated if this receptor effect could be reproduced in SLO-permeabilized cells, a preparation permitting the effective dialysis of cytosol including macromolecules such as lactate dehydrogenase (Ahnert Hilger *et al.*, 1989). A rise in the concentration of free calcium from 0.1 to 10 μM induced a 4- to 8-fold increase in the exocytosis of insulin from these permeabilized cells (Figure 1, see also Figure 2). In the presence of epinephrine (10 μM), exocytosis was inhibited by 40%, but only if GTP (100 μM) was added (Figure 1). In addition, this effect requires a free Mg^{2+} concentration between 0.01 and 1 mM (data not shown). As can be seen in Figure 1, pre-treatment of intact cells with pertussis toxin (PTX), which abolishes the interaction between the receptor and the G-proteins G_i or G_o, abrogated the receptor-mediated effect. Therefore, this process involves a G_i/G_o-like heterotrimeric GTPase. Furthermore, as cytosolic molecules are dialysed against a defined buffer, this signal transduction does not require diffusable second messengers or cytosolic macromolecules. Indeed, in this cell preparation, the addition of second messengers like cAMP in the presence of 3-isobutyl-1-methylxanthine (IBMX) or like inositol 1,4,5-triphosphate (IP_3), as well as addition of the protein kinase C-activator phorbol myristate acetate (PMA), does not alter exocytosis of insulin (Table I).

Table I. cAMP/IBMX, IP_3 or PMA do not alter insulin release from SLO-permeabilized HIT-T15 cells

Pre-incubation	Insulin release (% of content)	
	0.1 μM Ca^{2+} (mean ± SE)	10 μM Ca^{2+} (mean ± SE)
None	4.5 ± 0.3	21.0 ± 0.9
cAMP 100 μM + IBMX 100 μM	4.2 ± 0.4	19.0 ± 1.1
PMA 100 nM	4.6 ± 0.4	21.0 ± 1.4
IP_3 10 μM	4.8 ± 0.2	19.9 ± 1.7

Cells were permeabilized with SLO, pre-incubated for 7 min with the indicated agents and subsequently shifted to 0.1 or 10 μM Ca^{2+} in the presence of the various agents. Insulin release is indicated as percent of cellular content. n = 8 from two separate experiments.

Receptor-mimetic peptides activating G_i or G_o inhibit exocytosis

It is known that the coupling of plasma membrane receptors to heterotrimeric G-proteins is impaired by high potassium concentrations as used here to mimic the intracellular ion composition (Costa *et al.*, 1990). Since, in addition, receptors will couple to a number of different G-proteins

Table II. Pre-treatment with pertussis toxin attenuates the effect of receptor-mimetic peptides

PTX	Peptides	0.1 μM Ca^{2+}			10 μM Ca^{2+}		
		Mean ± SE	*n*	% inhibition	Mean ± SE	*n*	% inhibition
–	None	100.0 ± 3.6	6	0	744.6 ± 70.2	7	0
–	TMP14 (1 μM)	73.0 ± 7.9	5	28	127.4 ± 15.9	4	92[a,b]
–	TMP20 (100 μM)	87.5 ± 9.4	4	14	350.2 ± 33.4	4	59[a,b]
+	None	112.3 ± 9.1	6	0	649.5 ± 24.1	8	0
+	TMP14 (1 μM)	102.1 ± 6.2	8	9	574.8 ± 60.3	4	12
+	TMP20 (100 μM)	83.9 ± 12.2	4	25[a]	469.9 ± 43.7	4	30[a]

HIT-T15 cells were pre-incubated with PTX (200 ng/ml, 6 h), permeabilized with SLO, pre-incubated in the absence or presence of the indicated peptides and subsequently exposed to 0.1 or 10 μM Ca^{2+}. Values are expressed as percent of basal release in untreated cells and were obtained from two independent experiments.
[a]$2P < 0.05$ in comparison with the absence of peptides.
[b]$2P < 0.05$ in comparison with cells treated with pertussis toxin.

(Schmidt *et al.*, 1991), we used more specific tools to delineate which GTPases are involved in direct inhibition of exocytosis in a receptor-dependent manner. Defined cytoplasmic domains of the IGF-II/mannose-6-phosphate receptor (IGF-II/M6PR) or of the amyloid precursor polypeptide (APP) interact mainly with G_i or exclusively with G_o, respectively; this effect is retained by micromolar concentrations of receptor-mimetic peptides corresponding to these regions (Okamoto *et al.*, 1990; Nishimoto *et al.*, 1993). Indeed, the peptide P14, a receptor-mimetic peptide corresponding to the G-protein-activating domain (amino acids 2410–2423) of the IGF-II/M6PR, caused 50% inhibition of calcium-stimulated exocytosis in SLO-permeabilized cells when used at 50 μM (Figure 2). The efficacy was greatly enhanced by the coupling of the corresponding transmembrane domain from the IGF-II/M6PR to the receptor-mimetic peptide P14, yielding the peptide TMP14 (Figure 2). These transmembrane domains probably favour interactions with membrane lipids and allow peptide localization in the proximity of the membrane-bound GTPases G_i/G_o (Okamoto *et al.*, 1990). In contrast, TMP20, a peptide corresponding to the G_o-activating domain of APP and a part of its transmembrane-spanning domain (amino acids 639–648, 657–676), inhibited exocytosis only at a concentration similar to P14 (Figure 2). The action of the receptor-mimetic peptides TMP14 and TMP20 was not limited to the cell line under study, as they also induced a marked inhibition in SLO-permeabilized primary pancreatic β-cells (Table II). To test whether receptor-mediated activation of G_i/G_o is indeed mimicked by the peptides, cells were exposed to PTX before permeabilization with SLO, to interrupt the interaction between receptors and G_i/G_o. Indeed, the inhibition of calcium-stimulated exocytosis by the peptides TMP14 and TMP20 was almost completely abolished by PTX (Table II). Similar results were observed in HIT-T15 cells (Table III) where the action of TMP14 was almost completely abolished and that of TMP20 largely attenuated. A short pre-incubation (7 min) of the permeabilized cells with the peptides was necessary for full expression of their effects. When present only during the pre-incubation phase, TMP20 was inactive and the efficacy of 1 μM TMP14 was reduced from 98.2 ± 3.5% ($n = 3$) inhibition of Ca^{2+}-stimulated release to 23.3 ± 13.1% ($n = 3$). The peptides did not alter insulin release significantly during the pre-incubation period and were inactive on intact HIT-T15 cells (data not shown). These results indicate that the peptides must enter the cells in order to induce a reversible effect and that they do not induce granule lysis.

Table III. Effect of receptor-mimetic peptides on insulin release from SLO-permeabilized primary rat β-cells

PTX	Peptides	0.1 μM Ca^{2+}		10 μM Ca^{2+}	
		Mean ± SE	*n*	Mean ± SE	*n*
–	None	100.0 ± 8.4	12	485.4 ± 46.6	12
–	TMP14 (1 μM)	104.9 ± 15.6	8	177.3 ± 37.0	6[a]
–	TMP20 (100 μM)	76.4 ± 10.1	6	271.4 ± 81.9	6[a]
+	None	101.4 ± 6.0	6	363.8 ± 15.6	8
+	TMP14 (1 μM)	100.1 ± 11.9	4	318.3 ± 25.9	4
+	TMP20 (100 μM)	107.0 ± 7.5	4	406.5 ± 60.4	4

Primary cells were permeabilized with SLO, pre-incubated with peptides and subsequently shifted to 0.1 or 10 μM calcium. PTX pre-treatment was conducted at 300 ng/ml for 6 h.Values were obtained from at least two independent experiments and are given as percent of basal release in the absence of peptides and PTX.
[a]$2P < 0.05$ versus control.

As had been described for neuroendocrine cells (Holz *et al.*, 1989; Hay and Martin, 1992), exocytosis from SLO-permeabilized insulin-secreting cells requires ATP to express calcium-stimulated secretion fully (see Table IV). Since ATP was added together with the peptides, the experiments presented above (Figure 2) do not allow the distinction between an action of the receptor-mimetic peptides on the effect of ATP and an action on the effect of calcium. We therefore tested the peptides after pre-incubation of permeabilized cells with hexokinase and glucose to hydrolyse endogenous ATP without addition of any exogenous ATP. Under these conditions (Table IV), a 1.8-fold increase in exocytosis due to a rise in calcium can still be observed. Again, TMP14 and TMP20 inhibited exocytosis to a similar extent to when ATP was present only before (during SLO permeabilization) or after the pre-incubation with the peptides (during stimulation). Hence the Ca^{2+}-sensitive step, rather than the ATP-dependent step, constitutes the target for G-proteins activated by receptor-mimetic peptides.

Effect of pre-activated G_i/G_o on exocytosis

Activation of plasma membrane receptors or the use of receptor-mimetic peptides mirror the affinity of agonists or peptides but cannot indicate the actual sensitivity of

Table IV. Receptor-mimetic peptides inhibit insulin release from SLO-permeabilized HIT-T15 cells independent from the action of ATP

		MgATP (5 mM)					
		None		During SLO permeabilization		During stimulation	
		Mean ± SE	*n*	Mean ± SE	*n*	Mean ± SE	*n*
0.1 μM Ca^{2+}	Control	100.0 ± 6.7	6	100.0 ± 8.1	6	100.0 ± 14.2	6
	TMP14 (1 μM)	109.1 ± 5.3	6	59.8 ± 23.1	6	85.4 ± 3.9	6
	TMP20 (100 μM)	125.8 ± 16.1	6	91.8 ± 5.2	6	99.0 ± 4.3	6
10 μM Ca^{2+}	Control	178.5 ± 8.8	7	316.1 ± 42.5	5	361.6 ± 20.1	6
	TMP14 (1 μM)	105.3 ± 8.7	5[a]	84.5 ± 29.9	6[a]	73.8 ± 6.1	6[a]
	TMP20 (100 μM)	117.5 ± 4.6	6[a]	186.3 ± 19.5	6[a]	174.4 ± 31.3	6[a]

HIT-T15 cells were permeabilized with SLO, pre-incubated in the absence or presence of the indicated peptides and subsequently exposed to 0.1 or 10 μM Ca^{2+}. MgATP (5 mM) was either absent, or added only during cell permeabilization with SLO (prior to peptides), or added only during the final incubation phase (after peptides). When ATP was omitted or added only during the final stimulation phase, hexokinase (20 U/well) with glucose (10 mM) was added during the permeabilization with SLO to hydrolyse endogeneous ATP. Values are expressed as percent of basal insulin release which was not significantly altered for the different conditions and were obtained from two independent experiments.
[a]$2P < 0.05$ as compared with the control.

exocytosis to activated G-proteins. Yet, for this signal transduction pathway to be significant, G-proteins should be effective at least in the low micromolar range (Taussig *et al.*, 1993). Since the SLO-permeabilized cells allow the exchange of macromolecules, G-proteins purified from bovine brain were added directly to the system (Figure 3). Whereas non-activated G_i/G_o did not alter insulin secretion, G_i/G_o pre-activated with the slowly hydrolysable GTP analogue GTPγS reduced calcium-stimulated exocytosis by >50% with an IC_{50} at 10 nM. In the absence of βγ subunits, $G\alpha_o$ pre-activated with GTPγS also inhibited calcium-stimulated exocytosis. However, a higher concentration of this protein was required to attain a comparable effect (Figure 3).

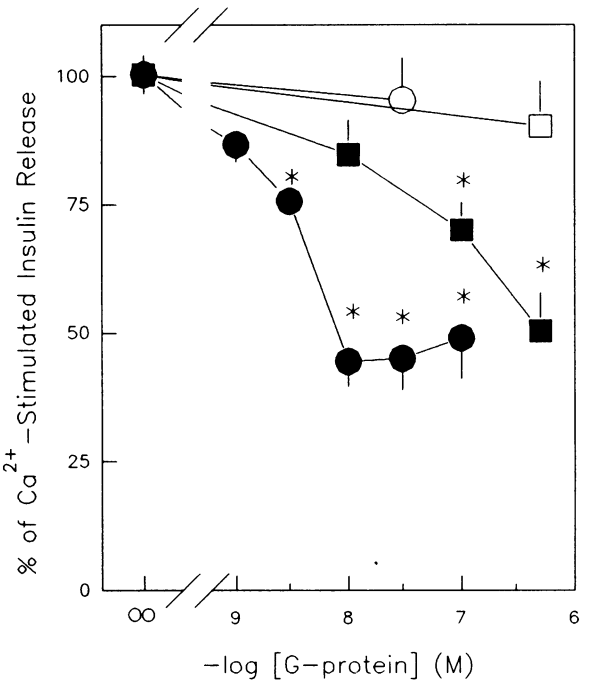

Fig. 3. Exogenous pre-activated G_i/G_o inhibits insulin release in SLO-permeabilized HIT-T15 cells. Cells were permeabilized with SLO, exposed to G-proteins purified from bovine brain (G_i/G_o: circles; $G\alpha_o$: squares) either added as such (open symbols) or pre-activated with GTPγS (closed symbols). Values are expressed as percent of calcium-induced release in comparison with the control. For experimental details see Figure 1. n = 4–19; *, $2P < 0.05$ versus absence of exogenous G-proteins or the presence of native G-proteins.

Human insulin C-peptide as reporter signal for transient transfection

The pronounced sensitivity of exocytosis to the presence of activated PTX-sensitive G-proteins prompted us to express $G\alpha_i$ and $G\alpha_o$ transiently and to investigate their effect on exocytosis in the hamster β-cell line HIT-T15. In view of the low efficacy of transient transfection, we co-expressed a reporter signal for exocytosis in the form of human insulin. The human C-peptide connecting the insulin A and B chains displays only 51% homology to the hamster sequence, allowing its selective measurement by radioimmunoassay (RIA; see Materials and methods).

Firstly, the subcellular location of transiently expressed human insulin C-peptide was determined in cells co-transfected with pBRhINS encoding human insulin and pCDNAI, the vector to be used for co-transfection with constitutively active mutants of Gα. The subcellular distribution of total (human and hamster) insulin was followed by an RIA recognizing insulin and, to a lesser extent, proinsulin, but none of the other hormone precursors or conversion intermediates found throughout the secretory pathway. As can be seen in Figure 4 (lower panel), the distribution of total insulin peaked at a density of ~1.3 M sucrose, indicating the location of secretory granules in agreement with previous findings (Reetz *et al.*, 1991). Approximately 70% of the total C-peptide immunoreactivity co-localizes to these fractions. As, in contrast to the insulin RIA, the C-peptide RIA also measures the insulin conversion intermediates, a minor part of the immunoreactivity was found throughout the gradient. Comparison with marker proteins for several cellular organelles (Figure 4, upper panels) demonstrates, in addition, that the distribution of endogenous insulin and transiently expressed human C-peptide did not coincide with the distribution of Na^+/K^+-ATPase, α-mannosidase, the heat shock protein BiP or arylsulphatase as indicators of plasma membranes, Golgi, endoplasmic reticulum or lysosomes, respectively. A comparable distribution for insulin and human C-peptide was also found when cells were co-transfected with pBRhINS and pCDNAI-α_i1Q204L instead of control plasmid pCDNAI (data not shown).

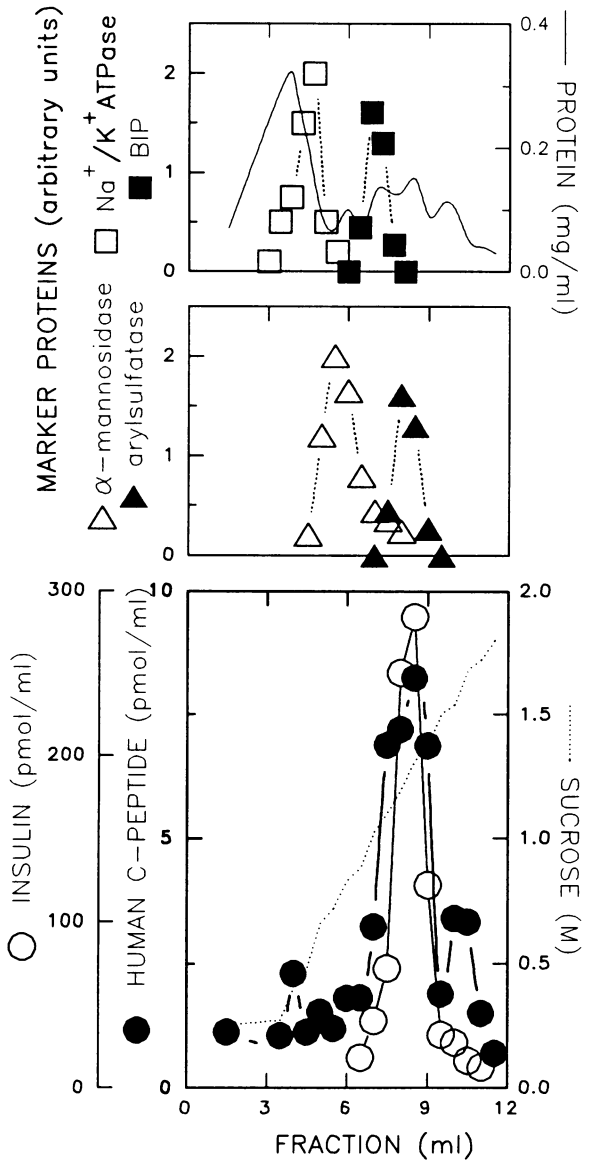

Fig. 4. Subcellular localization of endogenous hamster insulin and transiently expressed human insulin in HIT-T15 cells. Cells (10^7) were harvested 3 days after co-transfection with pBRhINS and pCDNAI, homogenized by nitrogen cavitation and centrifuged on a 0.25–2 M sucrose gradient at 40 000 r.p.m. for 19 h. Aliquots were taken from the top and analysed. Upper panel: protein (continuous line) Na^+/K^+-ATPase (plasma membranes), arylsulphatase (lysosomes); Middle panel: α-mannosidase (Golgi), BiP (endoplasmic reticulum). Lower panel: sucrose content (dotted line), total insulin (open circles) or human C-peptide (closed circles).

Effect of transiently expressed constitutively active mutants of $G\alpha_i$ or $G\alpha_o$ isoforms

A single amino acid exchange in the Gα-protein induces a GTPase-deficient state and renders the protein constitutively active (Hermouet *et al.*, 1991). We employed these mutants of $G\alpha_i1$–3 and $G\alpha_o1$–2 to determine the effect of activated Gα on exocytosis from SLO-permeabilized HIT-T15 cells. The overexpression of the different mutant proteins upon transfection with pBRhINS and pCDNAI carrying the respective inserts is documented in Figure 5. The levels of overexpression after 48 h ($G\alpha_i$ 1–3) or 80 h ($G\alpha_o$ 1–2) range from 2- to 6-fold of immunoreactivity

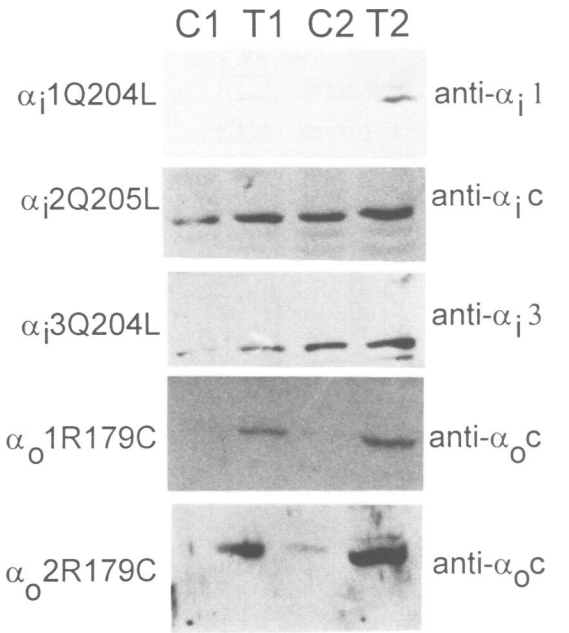

Fig. 5. Transient overexpression of constitutively active mutants of $G\alpha_i/G\alpha_o$ isoforms in HIT-T15 cells. The insulin-secreting hamster cells HIT-T15 were co-transfected with pBRhINS encoding human insulin and with either control vector pCDNAI (C1 and C2) or pCDNAI encoding the constitutively active mutants of $G\alpha_i/G\alpha_o$ isoforms (T1 and T2) as indicated to the left. 48 h ($G\alpha_i1$–3) or 80 h ($G\alpha_o1$–2) after transfection, cells were detached and subjected to SDS–PAGE/immunoblot using the antisera indicated to the right. In the case of co-transfection with plasmids encoding mutant α_i, 0.5×10^6 cells (C1, T1) or 1.5×10^6 cells (C2,T2) were loaded on the gel. In the case of co-transfection with mutants of α_o, 10^4 (C1, T1) or 3×10^4 cells (C2, T2) were applied. Densitometric evaluation of immunoreactive bands demonstrated a 3.1 ± 0.7 (α_i1Q204L), 2.1 ± 0.4 (α_i2Q205L), 2.9 ± 0.6 (α_i3Q204L), 3.9 ± 1.2 (α_o1R179C) and 6.1 ± 2.5 (α_o2R179C) fold overexpression in this and other blots as compared with controls (n = 3–5).

observed in control cells (transfected with pBRhINS and pCDNAI only).

Basal secretion of both total insulin and human C-peptide from intact cells was similar upon co-transfection with pBRhINS and vector pCDNAI when expressed as a percentage of cellular content (38.3 ± 4.5 versus 33.1 ± 1.2% of content after 48 h, n = 6–8). This fractional release did not change significantly when vector pCDNAI was replaced by constructs encoding the constitutively active mutants of $G\alpha_i$ or $G\alpha_o$ isoforms (data not shown). In addition, a comparable release of total insulin and of human C-peptide (expressed as percentage of content) was observed at 0.1 and 10 μM calcium from SLO-permeabilized cells co-transfected with the control vector only (Figures 6 and 7). In contradistinction, transient expression of constitutively active mutants of all three $G\alpha_i$ isoforms caused a marked inhibition of exocytosis of human C-peptide in calcium-stimulated SLO-permeabilized cells (Figure 6, cross-hatched versus full bars). Under the same conditions there was, as expected, no significant alteration detectable for total insulin, due to the limited number of cells expressing the exogenous genes after transient transfections (Figure 6, open versus hatched bars). Thus, expression of all three active isoforms of $G\alpha_i$ can inhibit exocytosis. Co-transfection with constitutively active mutants of $G\alpha_o1$ or $G\alpha_o2$ and pBRhINS did not

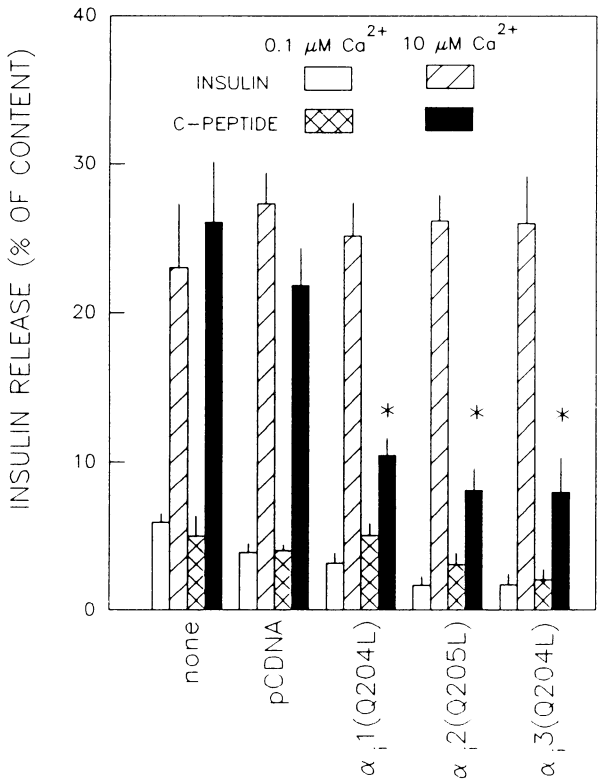

Fig. 6. Transient expression of constitutively active mutants of $G\alpha_i$ isoforms inhibits insulin release from SLO-permeabilized HIT-T15 cells. The insulin-secreting hamster cells HIT-T15 were transfected with pBRhINS encoding human insulin alone (none) or co-transfected with control vector pCDNAI or pCDNAI encoding the indicated constitutively active mutants of $G\alpha_i$ isoforms. Forty eight hours after transfection, cells were permeabilized with SLO and subsequently exposed to either 0.1 μM Ca^{2+} (open and cross-hatched bars) or 10 μM Ca^{2+} (diagonal-hatched and filled bars). Total insulin (open and diagonal hatched bars) and human C-peptide (cross-hatched and filled bars) were measured by radioimmunoassay; n = 6–14; *, $2P < 0.05$ versus co-transfection with control plasmid pCDNAI. The mean content of human C-peptide and total insulin in co-transfected cells (pBRhINS and vector) was 1.288 ± 0.210 C-peptide and 102 ± 25 pmol of total insulin per 10^6 cells, respectively, after 48 h.

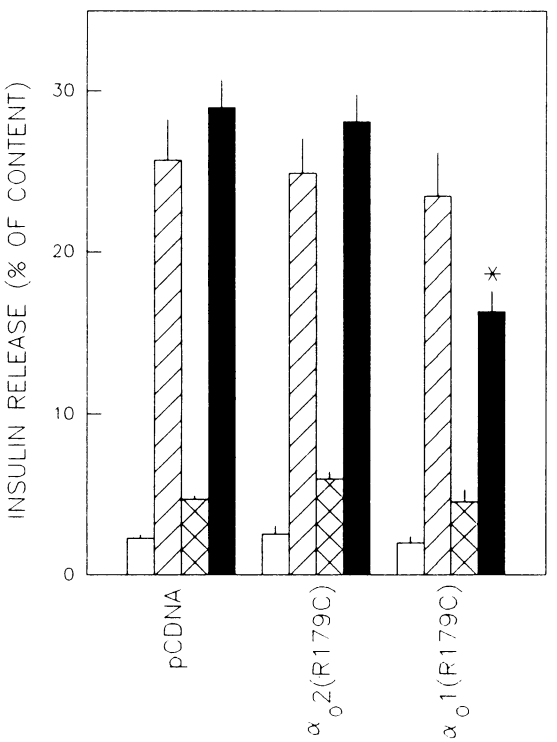

Fig. 7. Effect of transient expression of constitutively active mutants of $G\alpha_o$ isoforms on insulin release from SLO-permeabilized HIT-T15 cells. The insulin-secreting hamster cells HIT-T15 were co-transfected with pBRhINS encoding human insulin and control vector pCDNAI or pCDNAI encoding the indicated constitutively active mutants of $G\alpha_o$ isoforms. Exocytosis was measured in cells permeabilized with SLO and subsequently exposed to either 0.1 μM Ca^{2+} (open and cross-hatched bars) or 10 μM Ca^{2+} (diagonal-hatched and filled bars). Total insulin (open and diagonal-hatched bars) and human C-peptide (cross-hatched and filled bars) were measured by radioimmunoassay; n = 6–14; *, $2P < 0.05$ versus co-transfection with control plasmid pCDNAI. The mean content of human C-peptide and total insulin in co-transfected cells (pBRhINS and vector) was 3.09 ± 0.31 and 151 ± 42 pmol/10^6 cells respectively after 80 h.

alter exocytosis of human C-peptide when assessed after 48 h. The prolongation of the post-transfection time to 80 h induced ~50% inhibition of the calcium-stimulated exocytosis of human C-peptide, albeit only for $G\alpha_o2$ (Figure 7, full bars). As comparable levels of both mutants were expressed (see Figure 5), the functional difference cannot be explained by different amounts of mutant proteins. In addition, further prolonging the post-transfection time up to 6 days did not reveal any effect of $G\alpha_o1$ expression on calcium-stimulated exocytosis (data not shown).

Discussion

Release of hormones and neurotransmitters is induced by a rise in cytosolic calcium and potentiated by cAMP (Wollheim and Sharp, 1981; Burgess and Kelly, 1987). Plasma membrane receptor-mediated inhibition of secretion may be exerted at diverse steps regulating these well studied second messengers. However, previous work has suggested that inhibition of insulin secretion by α_2-adrenergic or other neurohormone receptors cannot be explained solely by altered generation of these second messengers (Ullrich and Wollheim, 1988; Ullrich *et al.*, 1990; Gilon *et al.*,1993). We now provide firm evidence for a direct and efficient receptor-activated regulation of exocytosis by PTX-sensitive GTPases.

First, in SLO-permeabilized cells exocytosis is diminished by the α-adrenergic receptor agonist epinephrine. The reduced efficacy of this agonist compared with its activity in intact cells is probably due to impaired receptor–ligand binding by high potassium concentrations (Costa *et al.*, 1990), as used here to approximate the intracellular ion composition. Second, receptor-mimetic peptides were used to circumvent this impairment. Indeed, the peptide TMP14 reproduces the complete inhibition of secretion observed in intact cells with epinephrine and other receptor agonists (Ullrich *et al.*, 1990; Wollheim and Sharp, 1981). The peptides TMP14 and TMP20, activating G_i or G_o respectively, mimic receptor-mediated activation, as their effect is attenuated by PTX. In addition, the apparent efficacy observed here for P14, TMP14 and TMP20 correlates well with those reported for their activation of purified G-proteins (Okamoto *et al.*, 1990; Nishimoto *et al.*, 1993). The specificity of the peptides is underlined

further by the reported failure of TMP20 to interfere with another fusion event of biological membranes, the G_s-regulated endosome fusion (Colombo *et al.*, 1994). Our results obtained with these peptides clearly demonstrate that efficient inhibitory regulation of exocytosis can take place without any regulation of protein kinase A, protein kinase C or transmembrane ion fluxes. In fact, these ion fluxes are only transiently affected in intact cells despite prolonged inhibition of secretion (Nilsson *et al.*, 1989; Gilon *et al.*, 1993). Third, the effects not only of the receptor-mimetic peptides but also of the exogenously added G-proteins indicate an inhibitory role in exocytosis for G_i and G_o and also suggest Gα as the potential mediator. Fourth, the isoform and subunit selectivity was characterized by transiently expressing GTPase-deficient, constitutively active mutants of Gα. To monitor the consequences of G-protein expression on exocytosis, we took advantage of the distinct immunoreactivity between endogenous hamster insulin and human insulin C-peptide to use the latter as a physiological reporter signal. The endogenous and the transiently expressed secretory product exhibited similar subcellular localization and release patterns. The versatility of this approach is underscored by the recent description of a similar method, using human growth hormone, to study exocytosis from chromaffin cells (Holz *et al.*, 1994). The transiently expressed constitutively active mutants of all $G\alpha_i$ isoforms and, to a lesser extent, of $G\alpha_o2$, blocked exocytosis of insulin from the SLO-permeabilized cells. It should be noted that transient expression of constitutively active Gα *per se* does not perturb exocytosis, as cells expressing the mutant of $G\alpha_o1$ behaved as control cells. The wild-type forms of the mutant G-protein α subunits investigated here are normally expressed in HIT-T15 cells (Seaquist *et al.*, 1992). Their activation by receptor-mimetic peptides resulted in inhibition of calcium-stimulated exocytosis comparable with that seen after transient transfection. Thus, direct regulation of exocytosis by $G\alpha_i$ and $G\alpha_o$ most certainly constitutes a physiological mechanism.

It cannot be excluded that the similar potency of all three mutant isoforms of $G\alpha_i$ results from their overexpression. However, this apparent promiscuity of the isoforms is not surprising as classical effector systems such as adenylyl cyclases are also regulated by all three currently known isoforms of $G\alpha_i$ and by $G\alpha_o$ (Taussig *et al.*, 1994). Furthermore, the effects observed during transient expression may take place at steps prior to exocytosis. Indeed several transport steps in the biosynthetic pathway are known to be regulated by trimeric GTPases (Donaldson *et al.*, 1991; Stow *et al.*, 1991; Leyte *et al.*, 1992), yet the unaltered basal levels of exocytosis argue against a reduced delivery of secretory granules. In addition, the effects of epinephrine as well as of the peptides were observed on a short time scale, clearly demonstrating a role for G_i/G_o in exocytosis itself and excluding an interference with earlier steps in the biosynthetic pathway. As distinct receptors activate different isoforms of G_i/G_o, the involvement of different isoforms of Gα may ensure selective signal transduction. It should also be pointed out that this system is apparently highly cell specific. As we previously demonstrated, G_i may be linked to inhibition or stimulation of secretion, depending on the cell type under study (Lang *et al.*, 1993). It is noteworthy that a stimulatory role in exocytosis has been reported for G_i3 in mast cells (Aridor *et al.*, 1993) although the subunit(s) involved have not been characterized.

A role for heterotrimeric G-proteins has been demonstrated in a number of intracellular transport phenomena; however, the signal activating these G-proteins remains to be identified. As our results with receptor-mimetic peptides and receptor activation demonstrate, this signal can be conveyed not only by seven transmembrane-spanning receptors but also by single membrane-spanning proteins, as IGF-II/M6PR is capable of conferring the regulation. Similarly, the precise transport step controlled, and consequently the localization of the functionally important heterotrimeric GTPases, are ill defined. Heterotrimeric G-proteins co-localize with secretory vesicles upon subcellular fractionation and confocal microscopy in neuronal and neuroendocrine cells (Vitale *et al.*, 1993; Ahnert-Hilger *et al.*, 1994). These vesicle-associated GTPases have been implemented in the regulation of exocytosis. As we show now in insulin-secreting cells, direct activation of a plasma membrane receptor and subsequent coupling to a PTX-sensitive GTPase is sufficient to inhibit exocytosis directly. In the permeabilized cell system this represents a *bona fide* plasma membrane delimited event. Although this does not exclude a role in exocytosis itself for G-proteins on hormone-containing vesicles, they may perhaps be involved in other transport steps such as vesicle budding or coat formation (Donaldson *et al.*, 1991; Leyte *et al.*, 1992) and regulation of vesicle 'priming' by ATP as demonstrated in chromaffin cells (Vitale *et al.*, 1994).

The persistence of inhibition of exocytosis after dialysis of large cytosolic macromolecules in the SLO-permeabilized cells indicates a tight coupling between G-proteins and the effector(s), which may underlie the only partial response to exogenously added G-proteins. Although subnanomolar concentrations of G-proteins achieve regulation of effectors such as Ca^{2+} or K^+ channels (Yatani *et al.*, 1987; Toselli *et al.*, 1989), the inhibition of exocytosis required amounts of G-proteins still below those necessary to observe an effect on the classical effector adenylyl cyclase (Taussig *et al.*, 1993). Even in reconstitution experiments of the latter enzyme, micromolar concentrations of G-proteins were necessary for maximal inhibition which, as in the case of exocytosis, is at ~50%. In addition, the exogenous G-proteins used in our study may not contain the most suitable isoforms in sufficient amounts. A sensitivity comparable with that observed here was found for another transport event, the transcytosis in MDCK cells, which is activated by nanomolar amounts of $G\alpha_s$ or Gβγ (Bomsel and Mostov, 1993). Hence, according to current knowledge, the putative effector(s) of heterotrimeric GTPases involved in membrane transport exhibits an intermediate affinity for the signal transducer.

We have not yet identified the molecular nature of the putative effector(s), nonetheless some conclusions can be drawn. Clearly the β-cell is endowed with at least some of the components of the neuronal exocytotic machinery (Jacobsson *et al.*, 1994; Sadoul *et al.*, 1995; Regazzi *et al.*, 1995). As in neuronal and neuroendocrine cells, ATP is required to achieve effective exocytosis in β-cells. In the former this event has been termed 'priming' of the vesicles and has been shown to take place before the effect

of calcium (Holz *et al.*, 1989; Hay and Martin, 1992; Heinemann *et al.*, 1993). The capacity of receptor-mimetic peptides to inhibit exocytosis without addition of ATP or after the exposure of cells to the nucleotide allows G_i/G_o to be located functionally to the final, calcium-dependent step of exocytosis. G_i/G_o could interfere with components of the fusion machinery itself or with non-dialysable factors mediating the calcium sensitivity of regulated exocytosis. The first hypothesis is supported by the various reports on heterotrimeric G-proteins regulating constitutive intracellular transport (Bomsel and Mostov, 1992), which is not subject to regulation by calcium (Burgess and Kelly, 1987). The molecular characterization of the effector of heterotrimeric GTPases in exocytosis should help to distinguish between the two postulates.

Materials and methods

Materials

The following receptor-mimetic peptides were synthesized by the solid-phase method and purified by HPLC to >95% purity (transmembrane regions are underlined): the 30mer TMP20 TVIVITLVMLHHGVVE-VDAAVTPEERHLSK; the 25mer TMP14 LTACLLTLLLYRVGLVR-GEKARKGK and P14 (TMP14 lacking the transmembrane domain) RVGLVRGEKARKGK. Heterotrimeric G-proteins were purified from bovine brain as described (Lang, 1989). Plasmid pBRhINS, derived from pBR322 and containing the human preproinsulin gene under the control of the cytomegalovirus promoter was kindly provided by Dr Jean-Claude Irminger (Geneva). Sources of pCDNAI vectors encoding the constitutively active mutants of PTX-sensitive Gα subunits α_i1-Q204L, α_i2-Q205L, α_i3-Q204L and α_o1R179C were as described (Ikezu *et al.*, 1994). To obtain pCDNAI- α_o2 R179C, an *Sph*I–*Sal*I fragment of the $G\alpha_o$2 gene (Tsukamoto *et al.*, 1991) was first subcloned in pUC19, which generated an *Xba*I site at the 3′ end of this gene. Subsequently, the *Sph*I–*Xba*I fragment of the $G\alpha_o$2 gene was prepared and ligated with a 4.5 kb fragment of α_o1R179C cDNA which was gel-purified after digestion with those enzymes. Recombinant SLO was produced in *Escherichia coli* (Weller and Bhakdi, unpublished). All nucleotides used were obtained from Boehringer (Rotkreuz, Switzerland).

Cell culture and transfection

HIT-T15 cells were cultured as described (Wollheim *et al.*, 1990a). Primary β-cells obtained by collagenase digestion of pancreatic islets (Wollheim *et al.*, 1990b) were cultured for 3 days in monolayers on microwells coated with fibroblast matrix (ELDAN-Tech, Jerusalem, Israel). For transient transfections, HIT-T15 cells were plated at 10^6 cells/well in a 24-well plate and were washed twice with magnesium-free phosphate-buffered saline (PBS) the following day. The lipopolyamine TRANSFECTAM (Promega) was diluted at 3 μl/well in double concentrated Dulbecco's modified Eagle's medium by vigorous vortexing. Ten minutes later this solution was compacted for 10 min with plasmids (2.5 μg of plasmid pBRhINS and 12.5 μg pCDNAI/well) before adding the same volume of sterile double-distilled water. After 8–16 h, transfecting solution was replaced by normal medium. This approach proved to be superior to electrotransfection or the use of DEAE–dextran and resulted in 5–10% of transfected cells as judged by immunofluorescence of human C-peptide or by galactosidase activity when pBRhINS was replaced by pCMV-βGAL. Culture was continued in normal medium for 48–80 h after transfection, the cells were permeabilized with SLO and secretion assayed as described below. For SDS–PAGE, permeabilized cells were detached with PBS–EDTA (10 mM) at 37°C, centrifuged and lysed in sample buffer. Gels were blotted on PVDF membranes and incubated overnight with anti-peptide antiserum specific for $G\alpha_o$1 and $G\alpha_o$2 (anti-α_oc, diluted at 1:5000) (Lang, 1989), for α_i1 or α_i3 (anti-α_i1 and anti-α_i3, both at 1:500; Calbiochem, Luzern, Switzerland), or anti-peptide antiserum recognizing α_i1,2,3 (anti-α_ic, 1:3000; Santa Cruz, Basel, Switzerland). Immunostaining was revealed using an ECL system (Amersham) and quantified by the use of a densitometer.

Cell permeabilization and insulin secretion assays

HIT-T15 cells grown for 2–3 days in microwells (0.2×10^6 cells/well) were washed with Krebs–Ringer buffer (136 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_5, 5 mM $NaHCO_3$ and 25 mM HEPES, pH 7.4) and KG (140 mM potassium glutamate, 5 mM NaCl, 7 mM $MgSO_4$, 5 mM Na_2ATP, 0.4 mM EGTA, 20 mM HEPES, pH 7.0). These and all subsequent steps were performed at 37°C. For experiments using epinephrine, $MgSO_4$ in KG was replaced by $MgCl_2$ and the free Mg^{2+} level kept at 100 μM as calculated by a computer program (METLIGAND). GTP (100 μM) was always present and alone did not alter insulin release. Permeabilization was performed with recombinant SLO in KG for 7 min, resulting in at least 95% of cells being permeable to trypan blue. Unless stated otherwise, cells were then pre-incubated for 7 min at 0.1 μM free calcium with KG/EGTA/$CaCl_2$ buffer containing 5 mM ATP and the agents to be tested. Subsequently, cells were transferred to 0.1 or 10 μM free calcium in KG/EGTA/$CaCl_2$ buffer for 7 min in the presence of agents to be tested and 5 mM ATP. Levels of free Ca^{2+} were determined by a microelectrode (Prentki *et al.*, 1984). For the determination of insulin, supernatants were removed, centrifuged at 4°C and kept at –20°C until RIA. Levels of human C-peptide were determined by the use of a commercial kit (Novo-Nordisk, Küssnacht, Switzerland), which does not cross-react with endogenous hamster insulin under the conditions used. Indeed no measurable signal could be observed for total insulin up to 10 pmol/ml, which represents double the amount of total insulin present in the transfected cells at the lowest dilution step used.

Pre-activation of G-proteins was achieved by incubation of 100 μM heterotrimer or α subunit at 30°C for 20 min in activation buffer (10 mM Tris–HCl, pH 8,10 mM $MgCl_2$, 20 mM NaCl, 0.5 mM DTT, 200 μM GTPγS, 10 μg/ml aprotinin and 10 μg/ml of leupeptin). Subsequently, proteins were diluted at least 200-fold. The incubation buffer (with GTPγS) did not alter secretion, as was the case for GTPγS (up to 5 μM) in KG/EGTA/$CaCl_2$ buffer, when present during pre-incubation and incubation. In addition, G-proteins incubated in incubation buffer without GTPγS did not alter insulin release (see Results).

The receptor-mimetic peptides used were suspended at 20 mM in DMSO. The final concentrations used did not change the pH or the concentration of free calcium present in the buffers, nor did the resulting DMSO concentration influence basal or calcium-stimulated release.

Subcellular fractionation

Cells were washed with KRB, resuspended in 250 mM sucrose, 10 mM HEPES–KOH pH 7.0, 0.5 mM EGTA, 0.3% PMSF, 40 μg/ml leupeptin and 10 μg/ml aprotinin and homogenized by nitrogen cavitation (15 bar, 20 min). The homogenate was loaded on a 0.4–2 M sucrose gradient, centrifuged in an SW-41 rotor for 20 h at 40 000 r.p.m. and fractions taken from the top of the tube. The sucrose concentration of the fractions was determined by diffraction, protein was measured by Coomassie Blue binding, total insulin and C-peptide were determined by RIA. The marker proteins α-mannosidase and arylsulphatase were determined enzymatically (Storrie and Madden, 1990); the distribution of Na^+/K^+-ATPase and BIP was detected by immunoblot.

Statistical analysis

Results are presented as mean ± SE from experiments performed on at least two (primary cell) or three independent cell preparations (HIT-T15 cells) unless stated otherwise. Statistical analysis was performed by the Student's two-tailed *t*-test for unpaired data (2*P*).

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