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Stimulation of insulin release from permeabilized HIT-T15 cells by a synthetic peptide corresponding to the effector domain of the small GTP-binding protein rab3

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A synthetic peptide (rab3AL) corresponding to the effector domain of rab3, a small GTP-binding protein, stimulated basal and potentiated Ca^{2+}-as well as GTPγS-evoked insulin secretion about 2-fold from streptolysin-O permeabilized HIT cells. This effect was specific, since the analogous peptides of ras or rab1 did not affect the exocytotic event. The more than additive effect of rab3AL on Ca^{2+} or GTPγS stimulation indicates a distinct mode of action of the peptide. The partial loss of cytosolic proteins from permeabilized cells was accompanied by a faster run-down of the secretory response to Ca^{2+} than the one to GTPγS. The persistent effect of rab3AL under these conditions points to a membrane localization of its target. These results suggest that rab3 and its effector are involved in the regulation of insulin secretion.

GTP-binding protein; Insulin secretion; Calcium; Rab protein; Exocytosis

1. INTRODUCTION

The ras and ras-related proteins belong to a large family of small molecular weight GTP-binding proteins (SMG), which regulate many functions such as cell growth and intracellular vesicle transport [1–5]. The activity of these proteins depends on whether they bind GDP or GTP. These SMGs have an intrinsic, albeit weak, GTPase activity [1–5].

The interconversion of the inactive, GDP-bound form and the active form of the SMGs is regulated by several proteins [1–4]. One of these, GDP-dissociation inhibitor (GDI), is only present in the cytosol and combines with the GDP-bound form of the SMG, thereby inhibiting its activation. Another regulatory protein, GDP-dissociation stimulator (GDS), activates SMGs by accelerating the release of GDP and the binding of GTP. This will result in a conformational change in the SMG and cause the interaction, via the effector domain, with its target. The GTP-bound form of SMGs is inactivated by GTPase-activating protein (GAP), promoting the hydrolysis of GTP to GDP. The GDI removes the GDP form of the SMG from membranes.

All ras-related SMGs contain five conserved regions which are important for the binding of guanine nucleotides and the hydrolysis of GTP, as well as for the interaction with GDS and GAP [1–3]. Most SMGs are post-translationally modified by isoprenylation at the C-terminus [6]. This lipidation is essential for their function, since it ascertains their correct targeting to membranes as well as the interaction with GDI [3,6]. The GAP-binding part is also denoted effector domain, since mutations in this region result in loss of function [7]. This region is conserved within a given subfamily but diverges substantially between different SMG subfamilies [1–3]. Therefore, one of the strategies for examining SMG function is the use of their effector domain peptides to explore their potential effects.

The rab proteins are a subfamily of SMGs, containing at least 15 members [4,5,8], all of which have been implicated in one or more of the vesicular transport steps from endoplasmic reticulum to Golgi complex, between Golgi cisternae, and in endocytosis and exocytosis [4,5]. One of them, rab3A, is particularly interesting since this protein is present only in brain and secretory cells [3–5], including insulin-secreting cells [9]. Several regulatory proteins for the rab3 subfamily have been discovered, including GDI [10], GAP [11,12] and GDS [12]. The potential function of rab3A is suggested by its predominant location on the membrane of secretory granules and by its similarity with the sec4 protein

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Abbreviations. G-protein, GTP-binding protein; SMGs, small molecular weight GTP-binding proteins; GAP, GTPase-activating protein; GDI, GDP-dissociation inhibitor; GDS, GDP-dissociation stimulator; GDPβS, guanosine 5′-*O*-(2-thiodiphosphate); GTPγS, guanosine-5′-*O*-(3-thiotrisphosphate); SL-O, streptolysin-O.

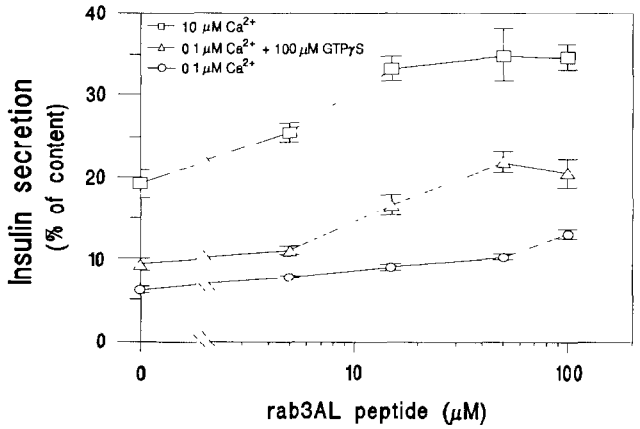

Fig. 1. Dose–response of rab3AL peptide on insulin secretion from SL-O permeabilized HIT cells. Cells were permeabilized by incubation with 1.5 U/ml SL-O for 5 min. Thereafter the medium was changed to one containing different concentrations of rab3AL peptide. After 5 min, the stimulating solution was added and the cells were incubated for another 5 min. Insulin in supernatants and attached cells was measured for the assessment of secretion and cellular content, respectively. Values are from one representative experiment in quadruplicate. Similar results were obtained in two other independent experiments.

which controls secretion in yeast [13]. It has thus been reported that the rab3A protein may participate in neurotransmitter release [14,15]. A synthetic peptide corresponding to the putative effector domain of this SMG, except for the exchange of two amino acids (denoted rab3AL), was shown to affect intracellular vesicle transport [16].

Previous work has demonstrated that poorly hydrolizable GTP analogs can trigger exocytosis via a pathway independent of phospholipase C and protein kinase C in insulin-secreting cells [17]. The underlying mechanism is still poorly understood. The involvement of a G-protein directly controlling exocytosis and named G_E has been postulated [18]. The identity of this protein remains, however, elusive. In the present study, the possible role of the small GTP-binding protein rab3 in the regulation of insulin secretion is examined. For this purpose we used the synthetic peptide rab3AL to investigate its action on exocytosis in permeabilized HIT-T15 cells.

2. MATERIALS AND METHODS

Peptides corresponding to the effector domain of rab3, ras and rab1 were synthesized as previously described [16]. GTPγS and GDPβS were purchased from Boehringer Mannheim, Mannheim, Germany and streptolysin-O from Wellcome Diagnostics, Dartford, UK.

The insulin-secreting cell line, HIT-T15, was originally provided by Dr. A.E. Boyd III (Tufts University, Boston, MA, USA). The cells (passages 70-79) were cultured in RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% fetal calf serum. Cells were seeded on microtiter (0.28 $cm^2 \times 96$ wells) or multiwell (1.77 $cm^2 \times 24$ wells) plates at a density of 3×10^5 cells per cm^2 and cultured for two days.

For the measurement of insulin secretion from permeabilized cells [19,20], the cells seeded in culture plates were washed twice with Ca^{2+}-free Krebs–Ringer bicarbonate-HEPES buffer and once with potassium glutamate buffer [21] at room temperature. Thereafter the cells were rendered permeable in the potassium glutamate buffer containing 1–1.5 U/ml of streptolysin-O (SL-O) for 5–8 min at 37°C, after which the permeabilizing solution was removed. Cells were then preincubated in the potassium glutamate buffer with different concentrations of the synthetic peptides for 5–8 min and incubated for another 5–8 min with different concentrations of Ca^{2+} and GTPγS in the continuous presence or absence of the peptides. The supernatants were removed, centrifuged at 4°C and kept at −20°C until insulin assay. The attached cells were extracted with acid/ethanol for insulin content determination [20]. Insulin was measured by radioimmunoassay using rat insulin as standard. Cell permeability was examined by the trypan blue exclusion test. After SL-O treatment, more than 95% of the cells were permeable to the dye.

For detection of SMGs, the cells were homogenized and the SMGs were resolved by PAGE and blotted on nitrocellulose membranes, and revealed by the [^{32}P]GTP-overlay technique exactly as previously described in detail [9,22]. Protein was determined with the method of Bradford using bovine serum albumin as standard [23].

The results are expressed as mean ± S.E.M. and analysed by two-tail unpaired Student's t-test.

3. RESULTS

The dose–response effect of rab3AL on insulin secretion from SL-O permeabilized HIT cells is shown in Fig. 1. This peptide stimulated insulin secretion at basal (0.1 μM) Ca^{2+} and clearly potentiated the secretory responses both to 100 μM GTPγS, a poorly hydrolizable

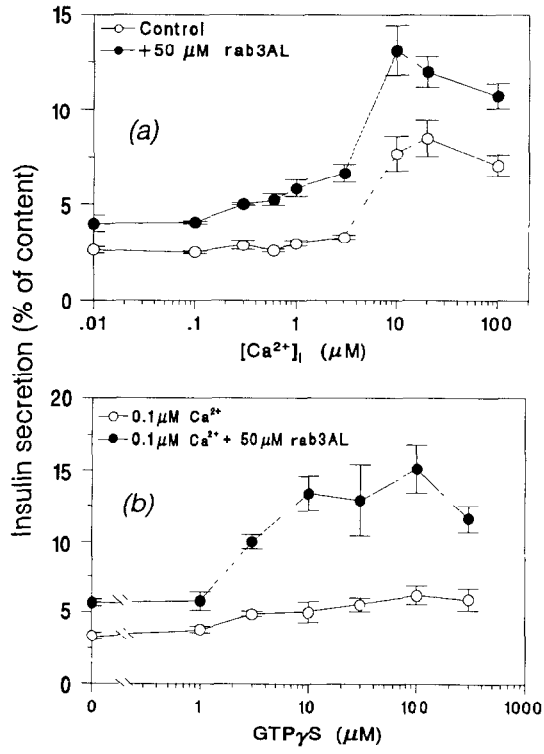

Fig. 2. Effect of rab3AL peptide on Ca^{2+}- and GTPγS-induced insulin secretion from SL-O permeabilized HIT cells. Values are mean ± S.E.M. of four experiments. For experimental detail, see legend to Fig. 1.

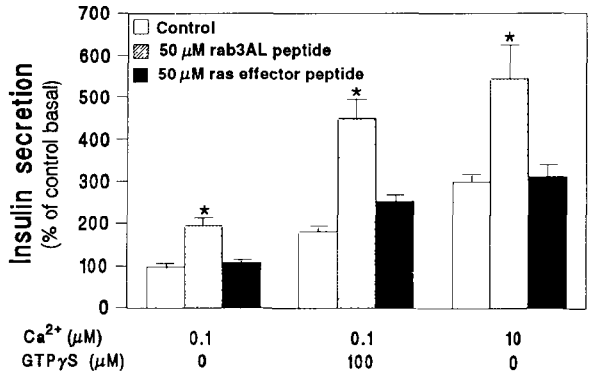

Fig 3. Comparison of rab3AL and ras effector-domain peptides on insulin secretion from SL-O permeabilized HIT cells. Values are mean ± S.E M of three replicates. For experimental detail, see legend to Fig. 1. *$P < 0.01$ compared to control.

GTP analog, and to 10 μM Ca^{2+} in a dose-dependent manner. The maximal effect, more than 2-fold, was seen at 50 μM. The EC_{50} of the peptide on the secretion induced by GTPγS and 10 μM Ca^{2+} was between 10 and 15 μM. The effects of rab3AL on different concentrations of Ca^{2+} and GTPγS-stimulated insulin secretion was further examined. Ca^{2+} dose-dependently stimulated insulin release from permeabilized HIT cells with an EC_{50} of about 4 μM. The peptide rab3AL (50 μM) potentiated the Ca^{2+} effect 2-fold at all Ca^{2+} concentrations from 10 nM to 100 μM (Fig. 2a). 50 μM rab3AL also potentiated the action of different concentrations of GTPγS on insulin release (Fig. 2b). GTPγS itself elicited secretion of the hormone in a dose-dependent manner with an EC_{50} of about 10 μM. At the higher GTPγS concentrations, the secretion was increased more than 2-fold by the peptide.

To test for the specificity of rab3AL, we also examined two other synthetic peptides corresponding respectively to the effector domain of ras and rab1 (for details including amino acid sequences, see [16]). As shown in Fig. 3, the same concentration (50 μM) of ras effector-domain peptide did not significantly affect the insulin release either at basal or under the stimulation of GTPγS and high Ca^{2+}. Similar negative results were obtained with 50 μM rab1 effector-domain peptide (not shown). Furthermore, rab3AL had no effect on insulin release from intact cells incubated in Krebs–Ringer bicarbonate-HEPES buffer (not shown).

The site of action of rab3AL was investigated by stimulating insulin secretion at various times after the permeabilization of the cells. The holes in the plasma membrane in SL-O permeabilized cells are large enough to allow cytosolic proteins to leak out [19]. The relative importance of cytosolic and membrane-associated proteins in the regulation of secretion could thus be evaluated.

The secretory response to Ca^{2+} stimulation displayed a quick run-down after SL-O permeabilization, while the run-down rate under either resting or GTPγS stimulation conditions was much slower (Fig. 4A). At 8 and 16 min after permeabilization, the Ca^{2+} (10 μM) effect was decreased respectively by 45% and 80% but the GTPγS action was only slightly affected, indicating that Ca^{2+} action is more dependent on cytosolic factors. The peptide rab3AL (50 μM) did not cause any substantial insulin release during the first 8 min (Fig. 4B, compared to the control secretory rate in Fig. 4A), possibly due to the incomplete penetration of the peptide and the retained endogenous rab3 protein during this simultaneous permeabilization and stimulation period. However, GTPγS and Ca^{2+} induced insulin secretion was still potentiated to the same extent, 2-fold, by this peptide under the run-down conditions at 8 min and 16 min after permeabilization, although the maximal response to Ca^{2+} stimulation was not restored (Fig. 4B). These results suggest that the target of rab3AL is associated with the membranes and its mode of action is mainly one of potentiation.

The profiles of protein and SMG distribution between cytosol and membranes after SL-O permeabilization were also examined. It was found that about 30% of the cytosolic proteins had leaked out while there was no change in the protein content in the membranes

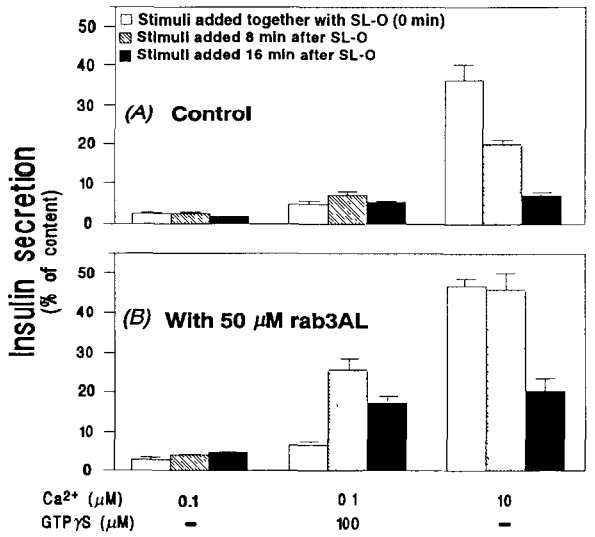

Fig. 4. Effect of rab3AL peptide on insulin secretion at various times after permeabilization of HIT cells. Cells were rendered permeable with 1 U/ml SL-O for 8 min. Open bars: stimuli were added together with SL-O and supernatants were removed for insulin measurement after 8 min. Hatched bars: after 8 min of SL-O permeabilization, the medium was exchanged for one containing stimuli and the cells were incubated for another 8 min. Closed bars: after 8 min of SL-O permeabilization, the cells were incubated with fresh potassium glutamate buffer for 8 min. Thereafter, the medium was replaced by stimulating buffer and cells were incubated for another 8 min. (A) Control incubations in the absence of rab3AL peptide. (B) 50 μM rab3AL was included during the 8 min stimulation period. Values are mean ± S.E.M. of four observations of one representative experiment. Similar results were obtained in two other independent experiments.

measured after 24 min permeabilization. These values were (mean ± S.E.M. μg/well; $n = 3$): the cytosol protein contents were 704 ± 18 and 483 ± 28 in intact and 24 min permeabilized cells while the protein contents in the membrane fraction were 400 ± 4 and 397 ± 18 in the intact and permeabilized cells, respectively. Using the GTP-overlay technique, the SMGs migrating in the range of 21–27 kDa could be detected (Fig. 5), confirming our previous work [9,22]. These SMGs were differently distributed between cytosol and membranes. 24 min after SL-O permeabilization, there was also about a 30% loss of these SMGs in the cytosol but no decrease was detected in the pool associated with the membranes. The inclusion of GTPγS (100 μM) during the last 8 min incubation period did not alter the distribution profiles of either total protein or SMGs. There was no detectable preferential reduction of a specified SMG, or group of SMGs, either in the cytosol or membranes.

4. DISCUSSION

Here we report that a synthetic peptide corresponding to the putative effector domain of rab3 affects the exocytosis of insulin-containing granules from a permeabilized β-cell line. The peptide has a weak stimulating effect on insulin release at nonstimulatory concentrations of Ca^{2+} (10–100 nM). The main action observed is, however, the potentiation of Ca^{2+}- and GTPγS-evoked insulin secretion. Although the net amount of increased insulin release by rab3AL is different under the various conditions, the maximal augmentation was always a doubling of the secretion obtained in the absence of the peptide. This effect is also more than additive, taking the sum of the peptide plus Ca^{2+} or GTPγS, indicating a distinct mode of action of the rab3AL peptide. It has been reported that the same peptide stimulates exocytosis of secretory granules in permeabilized pancreatic acinar cells and chromaffin cells as well as in patch-clamped mast cells [24–26]. In those studies, rab3AL only enhanced the secretion stimulated with Ca^{2+} and GTPγS in chromaffin cells, while in acinar cells the peptide increased the release under non- or substimulatory conditions. The native effector domain peptide of rab3 also affected exocytosis albeit with less efficacy [24,26]. The action of rab3AL on insulin release is specific, since two other peptides corresponding to the effector domain of ras and rab1 did not stimulate the secretion and the peptide had no effect on intact cells, confirming the observations on the specificity of rab3AL in other studies [24–26].

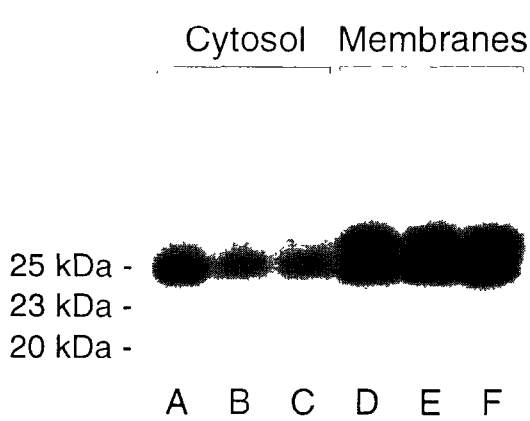

Fig. 5. Subcellular distribution of small G-proteins at various times after permeabilization of HIT cells. The cells were washed twice and either directly homogenized (A,D) or treated with 1 U/ml SL-O for 8 min (B,C,E,F) Before homogenization, the permeabilized cells were incubated for 24 min at 100 nM free Ca^{2+}, with (C,F) or without (B,E) 100 μM GTPγS included during the last 8 min incubation. The cytosolic and crude membrane fractions were then prepared as previously described [9,22]. The SMGs present in the two fractions were revealed using the GTP-overlay technique [9,22]. Two bands detected on the films were quantified by densitometric scanning. The values obtained for the 23 kDa and 25 kDa bands were respectively (arbitrary units, mean of two determinations): lane A, 1,680 and 4,829; lane B, 1,125 and 3,154; lane C, 1,261 and 3,277, lane D, 2,713 and 4,964, lane E, 2,300 and 4,981; and lane F, 2,522 and 5,284

The ras protein exerts its function through the interaction between its effector domain and its targets. The candidates for this role include GAP and other proteins [2,3]. It is postulated, assuming the same mechanism of action as for the ras protein, that the effector domain peptide rab3AL mimics native rab3 proteins and interacts with GAP or other target proteins. rab3A is mainly present in brain synaptic vesicles and neurosecretory cells [4,5]. Stimulation of neurotransmitter release was found to be accompanied by the dissociation of rab3A from the synaptic vesicles [14,15]. In HIT-T15 cells, rab3A is not detectable and only rab3B and rab3C are present ([27], and R. Regazzi, unpublished observations). However, since the four subtypes (A, B, C and D) of rab3 proteins share an identical sequence in the effector domain [8,28,29], rab3AL could mimic functions exerted by all these SMGs.

Under normal conditions, most of the native rab3 may be present in a GDP-bound form due to the activity of GAP. When rab3AL is added, it would bind to the rab3-specific GAP and the negative influence of the GAP on rab3 would be relieved. The endogenous rab3 could now be activated by GTP for a longer time period and exert a stronger effect on exocytosis. In contrast, GTPγS (poorly hydrolizable) alone only caused a small increase in secretion and did not enhance the Ca^{2+}-induced maximal insulin secretion ([21], and unpublished observations). In view of the potentiation of this maximal insulin secretion by rab3AL, it appears that the G-protein mediating the action of GTPγS is not the endogenous rab3 protein. Thus, GAP may neither represent a rate-limiting step nor be the main target of rab3AL in this case. It is more likely that the peptide acts on another rab3 target which regulates exocytosis. The fusion between isolated granules and plasma membrane from pancreatic acinar cells has also been reported to be promoted differently by GTPγS and rab3AL [30]. Whether rab3AL enhances secretion by

increasing the recruitment (targeting) of secretory granules to the plasma membrane or by facilitating the interaction between the two membranes to allow Ca^{2+} and GTPγS to trigger their fusion, remains to be clarified.

A putative target protein for rab3A has been identified in membranes from rat brain [31] and rab3AL is able to induce directly the fusion between the granules and the plasma membrane isolated from pancreatic acinar cells [30]. Therefore the target of rab3AL seems to be located on membranes. Accordingly, the action of rab3AL was persistent under conditions in which the secretory responses to Ca^{2+} were diminished, probably due to the partial loss of cytosolic proteins from the permeabilized cells. The extent of cytosolic protein leakage might be underestimated in our determinations, since the extraction procedure could have detached some loosely membrane-bound proteins into the cytosolic fractions.

The fast run-down of the secretory response to Ca^{2+} stimulation could be due to the loss of crucial cytosolic factor(s) or to modifications of the secretory machinery (e.g. phosphorylation/dephosphorylation) after permeabilization. The persistence of the GTPγS effect suggests that the former may be the case. It has been reported that several proteins are able to restore the secretory response in reconstitution experiments in other cells [32,33]. Such experiments have not yet been performed in insulin-secreting cells. Although the rab3AL was less potent during the initial period of permeabilization in the present study, possibly due to an inefficient penetration of the peptide into the cells, the rab3AL was not capable of restoring the secretion to the maximal levels after run-down. Since most SMGs were still present in the cells, it is unlikely that a rab3-like protein is the lost factor causing the run-down of the secretory response.

In conclusion, a synthetic peptide corresponding to the rab3 effector domain enhances insulin secretion from permeabilized HIT cells. The peptide acts mainly by potentiating GTPγS- and Ca^{2+}-elicited release. These results suggest that a rab3-like protein and its effector may be involved in the regulation of insulin secretion and that one of the rab3 proteins participates in the control of exocytosis.

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