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# Synthetic peptides of the effector-binding domain of rab enhance secretion from digitonin-permeabilized chromaffin cells

Jan Senyshyn[a], William E. Balch[b] and Ronald W. Holz[a]

[a]*Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109-0626, USA* and [b]*Department of Molecular Biology, Research Institute of Scripps Clinic, La Jolla, CA 92037, USA*

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There is evidence that the rab class of low molecular weight GTP-binding proteins is involved in vesicular transfer from endoplasmic reticulum to Golgi and between Golgi cisternae. To determine whether similar proteins play a role in regulated exocytosis, the effects of synthetic peptides derived from low molecular weight GTP-binding proteins on catecholamine secretion from digitonin-permeabilized chromaffin cells were investigated. The synthetic peptides represent the putative effector-binding domains of the rab, ras and ral classes of low molecular weight GTP-binding proteins and correspond to ras(33–48). Two rab peptides but neither a ras nor a ral peptide enhanced $Ca^{2+}$-dependent secretion by approximately 30%. Maximal secretion in response to $Ca^{2+}$ was increased. The enhancement was not blocked by the pseudosubstrate inhibitor of protein kinase C, PKC(19–31), thus indicating that activation of protein kinase C was not responsible for the enhancement of secretion. Similarly a rab peptide but neither a ras nor a ral peptide enhanced GppNHp-induced secretion 30–70%. The peptides had little or no effect in the absence of $Ca^{2+}$ or GppNHp. The data are consistent with a protein of the rab class playing a role in regulated exocytosis.

rab Peptide; Exocytosis; Chromaffin cell; Calcium; Guanine nucleotide

## 1. INTRODUCTION

The genetic analysis of protein secretion in yeast led to the identification and purification of SEC4p, which is necessary for fusion of post-Golgi vesicles with the plasma membrane [1,2], and YPT1p, a protein necessary for protein transfer from ER to Golgi [3]. Both SEC4p and YPT1p are 23-kDa, GTP-binding proteins homologous to the ras family of GTP-binding proteins. GTP-binding proteins are also involved in the transfer and fusion of a membrane-bound compartment formed from donor cisternae (ER or Golgi compartments) to subsequent cisternae in the mammalian biosynthetic pathway. A nonhydrolyzable analogue of GTP (GTPγS) prevents transfer of protein from ER to Golgi [4] and between Golgi compartments [5] and induces the accumulation of coated vesicles in the Golgi region [6] and the accumulation of tubular-vesicular membranes near the ER (see [7] for review). It has been postulated that GTP binding to vesicular protein and subsequent GTP hydrolysis are required for proper targeting and transfer of budding vesicles [8]. An antibody against yeast YPT1p cross-reacts with a protein in mammalian Golgi [3]. The effects of guanine nucleotides may be mediated by the rab class of low molecular weight GTP-binding proteins which are located on the ER and Golgi [6,8–12].

The compelling evidence for a role of guanine nucleotide binding proteins in the secretory biosynthetic pathway in yeast and mammalian cells has prompted investigation of the role of guanine nucleotides in final steps of regulated secretion in mammalian cells. Although $Ca^{2+}$ is the primary stimulus, there is also evidence for multiple roles for guanine nucleotides in regulated secretion in cells and neurons. Nonhydrolyzable guanine nucleotides can inhibit $Ca^{2+}$-dependent secretion [13–15], stimulate secretion in the absence of $Ca^{2+}$ [16–20], and enhance $Ca^{2+}$-dependent secretion [14]. These various effects are dependent upon cell type, species and protocol used during the secretion experiment.

In bovine adrenal chromaffin cells the entire spectrum of guanine nucleotide effects has been observed. The presence of guanine nucleotides in the medium is not necessary for $Ca^{2+}$-dependent secretion from electropermeabilized or digitonin-permeabilized bovine chromaffin cells [14,21,22]. Nonhydrolyzable guanine nucleotides stimulate a small amount of $Ca^{2+}$-independent secretion from digitonin-permeabilized bovine chromaffin cells [17,21]. GTPγS also inhibits $Ca^{2+}$-dependent secretion from electropermeabilized bovine chromaffin cells when added together with $Ca^{2+}$ [14] and from digitonin-permeabilized bovine chromaffin cells

*Abbreviations:* ER, endoplasmic reticulum; GppNHp, guanosine 5'-(β,γ-imido)triphosphate; KGEPM, potassium glutamate-, EGTA-, PIPES-, $MgCl_2$-, MgATP- and bovine serum, albumin-containing medium; PKC, protein kinase C; PSS, physiological salt solution.

*Correspondence address:* R.W. Holz, Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI 48109-0626, USA. Fax: (1)(313) 936 7787

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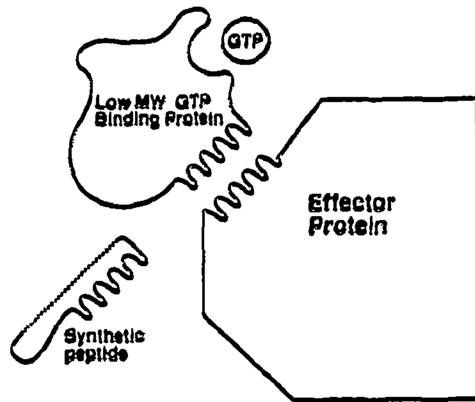


Fig. 1. Schematic of a low molecular weight GTP-binding protein showing the region which interacts with its effector. In the absence of bound GTP, the GTP-binding protein does not interact with its effector. The binding of GTP to the protein permits it to interact with an effector. The peptides used in the study correspond to the region of ras which likely interacts with the GTPase-activating protein (GAP).

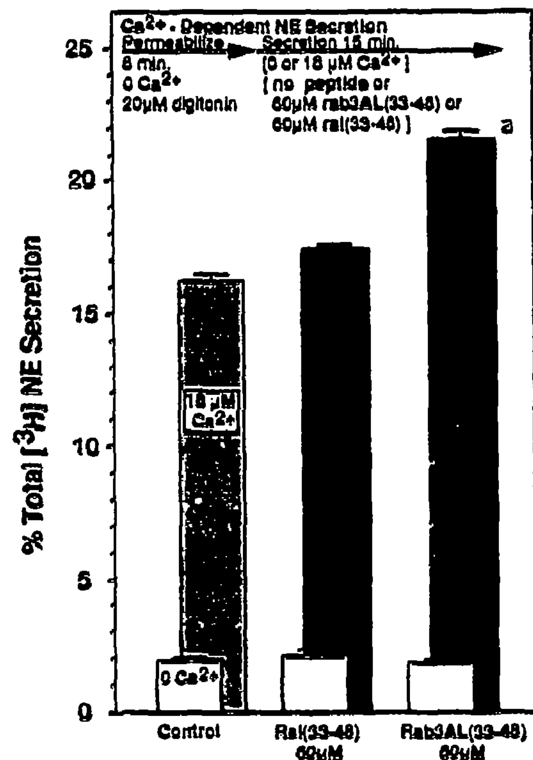


Fig. 2. Effects of rab3AL(33-48) on $Ca^{2+}$-dependent secretion. [$^3$H] Norepinephrine-labeled chromaffin cells were permeabilized in $Ca^{2+}$-free KGEPM with 2 mM MgATP and 2-μM digitonin with 0, 60 μM ral(33-48) or 60 μM rab3AL(33-48) for 8 min. Solutions were replaced with digitonin-free KGEPM (with 2 mM MgATP) in the continuing presence or absence of peptide with or without 18 μM $Ca^{2+}$. $Ca^{2+}$-dependent secretion was determined after 15 min. There were 3 wells/group. *$P<0.001$ vs. no peptide.

when a low concentration of GTPγS is incubated with permeabilized cells prior to stimulation with $Ca^{2+}$ [15].

Both chromaffin granules, the secretory vesicles in adrenal chromaffin cells [15,23–25], and bovine brain synaptic vesicles [15,26,27] have numerous membrane-bound GTP-binding proteins between 20 and 30 kDa. One of them is reported to be rab3A [25] although this is disputed [27]. It is possible that one or more of the 20–30 kDa GTP-binding proteins associated with chromaffin granules mediates one or more of the guanine nucleotide effects. Other pathways are also possible. Guanine nucleotides through trimeric GTP-binding proteins indirectly activate a host of enzymes (e.g. adenylate cyclase, phospholipase C, phospholipase $A_2$, protein kinase C) that could influence the secretory response. Indeed, nonhydrolyzable guanine nucleotides probably enhance $Ca^{2+}$-dependent secretion from *Staphylococcus aureus* α-toxin permeabilized chromaffin cells through a protein kinase C mediated mechanism activated by phospholipase C-induced formation of diacylglycerol [22].

Because of the myriad of possible interactions of guanine nucleotides, it has not been possible to determine the molecular basis of most the effects of guanine nucleotides on regulated secretion. To examine specifically the role of low molecular weight GTP-binding proteins on the final steps of regulated secretion, we have been guided by studies which demonstrated that synthetic peptides of a putative rab effector binding domain specifically inhibited ER to Golgi and intra-Golgi transfer of newly synthesized protein [28] (Fig. 1). ER to Golgi transport is inhibited after a $Ca^{2+}$-requiring step. We have explored the effects of one of these rab peptides that represents the putative effector region of rab3p and rab1p on catecholamine secretion from digitonin-permeabilized chromaffin cells. Synthetic peptides to a homologous region in ras inhibit ras-GTPase activating protein (GAP) interaction [29] but do not inhibit ER to Golgi or intra-Golgi transport [28]. In ras, this region (amino acids 32–40 [30]) probably interacts with GAP [31] as an exposed loop on the surface of the protein [32,33]. This region is highly conserved within each GTP-binding protein family but not between different families [30]. If rab peptide mimics the effector binding region in a low molecular weight GTP-binding protein involved in the final stages of exocytosis, the peptide could inhibit secretion if the endogenous GTP-binding protein is required for a cyclic process in which it must be turned on and off to have an effect[8]. Alternatively, if the endogenous GTP-binding protein need only to be turned on to stimulate secretion, the peptide may enhance secretion. We found that the peptide specifically enhances secretion.

## 2. MATERIALS AND METHODS

Primary dissociated cells from bovine adrenal medulla were prepared and maintained as monolayer cultures in Eagle's minimal essen-

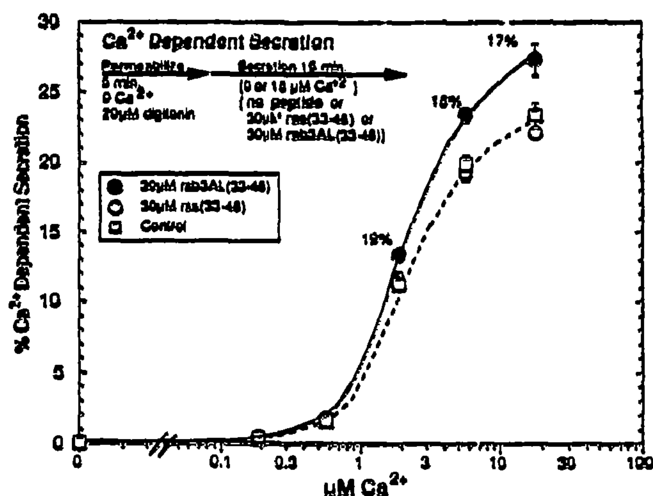


Fig. 3. Rab3AL(33–48) increases the maximal secretory response to $Ca^{2+}$. [$^3$H]Norepinephrine-labeled chromaffin cells were permeabilized in $Ca^{2+}$-free KGEPM containing 2 mM MgATP and 20 $\mu$M digitonin. After 5 min the solution was replaced with KGEPM with 2 mM MgATP containing various free $Ca^{2+}$ concentrations and either no addition (open squares), 30 $\mu$M ras(33–48) (open circles), or 30 $\mu$M rab3AL(33–48) (filled circles). Secretion was measured after 15 min. Percentages correspond to the enhancement of secretion compared to no peptide. $P<0.01$ at 1.9 $\mu$M $Ca^{2+}$ and $P<0.05$ at 5.5 and 18 $\mu$M $Ca^{2+}$ compared to no peptide. There were 3 wells/group.

tial medium (Gibco, Grand Island, NY) containing 10% heat-inactivated fetal calf serum as previously described [34,35]. Cells were cultured as monolayers in 6.4 mm diameter collagen-coated plastic culture wells (Costar, Cambridge, MA) at a density of 500,000 cells/cm$^2$. The release of [$^3$H]norepinephrine from cells prelabelled with [$^3$H]norepinephrine was measured. Secretion was expressed as the percentage of the total cellular [$^3$H]norepinephrine released into the medium [35]. Cells were permeabilized with 20 $\mu$M digitonin in potassium glutamate solution (KGEPM solution) containing 139 mM potassium glutamate, 20 mM PIPES (pH 6.6), 2 mM MgATP and 1 mM $MgCl_2$, 0.5% BSA, and 5 mM EGTA (without $Ca^{2+}$). The compositions of solutions with buffered free $Ca^{2+}$ concentrations of 0.6–18 $\mu$M were calculated [36] with constants from Martell and Smith [37]. Experiments were performed at 27°C.

Peptides were synthesized as previously described [28]. The sequences of rab3AL(33–48), rab3F(33–48), ras(33–48) and ral(33–48) are shown in Table I. Digitonin was purchased from Fluka Chemical Corp. (Hauppage, NY), l-[$^3$H]norepinephrine (21.4 Ci/mmol) from New England Nuclear (Boston, MA), and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Data are expressed as mean ± standard error of the mean unless otherwise indicated. Significance between groups was determined by Student's $t$-test unless otherwise indicated. Error bars smaller than symbols were omitted from figures.

## 3. RESULTS

### 3.1. *Rab3AL(33–48) and Rab3F(33–48) enhance $Ca^{2+}$-dependent secretion from permeabilized chromaffin cells*

The peptides used in the study are shown in Table I and are compared to homologous segments in various low molecular weight GTP-binding proteins. Rab3AL(33–48) is identical to a segment in rab3A except for alanine replacing threonine at position 35 and leucine replacing valine at position 36. The segment includes the putative effector binding region of rab3A based upon comparisons with the better understood ras proteins. The peptide inhibits vesicular transport between ER and Golgi and between Golgi cisternae [28]. Ral(33–48) and ras(33–48) are identical to respective regions in ral and Ki-ras. The segment in ras includes the effector binding region necessary for oncogenic effects. Both peptides are without effect on ER to Golgi and intra-Golgi transport.

Rab3AL(33–48) (60 $\mu$M) caused a 37% enhancement of secretion in response to 18 $\mu$M $Ca^{2+}$ (Fig. 2). Rab3F caused a similar enhancement (data not shown). The enhancement was specific since the peptide to the homologous region in ral, ral(33–48), had no effect. In other experiments, ras(33–48) (60 $\mu$M) also had little effect on $Ca^{2+}$-dependent secretion. Experiments from a large number of experiments are summarized in Table II. Although the effects of rab3AL(33–48) were small, they were reproducible and specific. Half-maximal responses were obtained at approximately 30 $\mu$M and maximal responses by 60 $\mu$M rab3AL(33–48) (data not shown). Rab3AL(33–48) (30 $\mu$M) caused similar relative enhancements of secretion over a range of 1.9–18 $\mu$M $Ca^{2+}$ (Fig. 3). Thus, the enhancement caused by rab3AL(33–48) represents an increase in the maximal response to $Ca^{2+}$ and not a change in $Ca^{2+}$ sensitivity. Ras(33–48) did not alter secretion at any of the $Ca^{2+}$ concentrations tested.

The effects of rab3A(33–48) were rapid and not readily reversed. The peptide enhanced secretion when it was introduced to the cells together with $Ca^{2+}$, indicating an effect as rapid as that of $Ca^{2+}$ (data not shown). The peptide also enhanced secretion when present only during permeabilization prior to the incubation with $Ca^{2+}$ (Fig. 4). The rapidity and irreversibility of effects of the peptide on the enhancement of exocytosis are similar to the characteristics of the peptide on inhibition of vesicular transport between ER and Golgi [28].

### 3.2. *The enhancement of $Ca^{2+}$-dependent secretion by rab3AL(33–48) is ATP dependent*

To determine whether the rab3AL(33–48) effect requires ATP, permeabilized cells were stimulated with 18 $\mu$m $Ca^{2+}$ in the presence or absence of ATP (2 mM). In 5 experiments, the peptide (60 $\mu$M) enhanced ATP-and $Ca^{2+}$-dependent secretion 29±4% ($P<0.05$ vs. no peptide). The peptide had no significant effect on $Ca^{2+}$-dependent secretion in the absence of ATP.

### 3.3. *Rab3AL(33–48) specifically enhances GppNHp-induced secretion*

Nonhydrolyzable guanine nucleotides cause a small amount of $Ca^{2+}$-independent secretion [17,21].

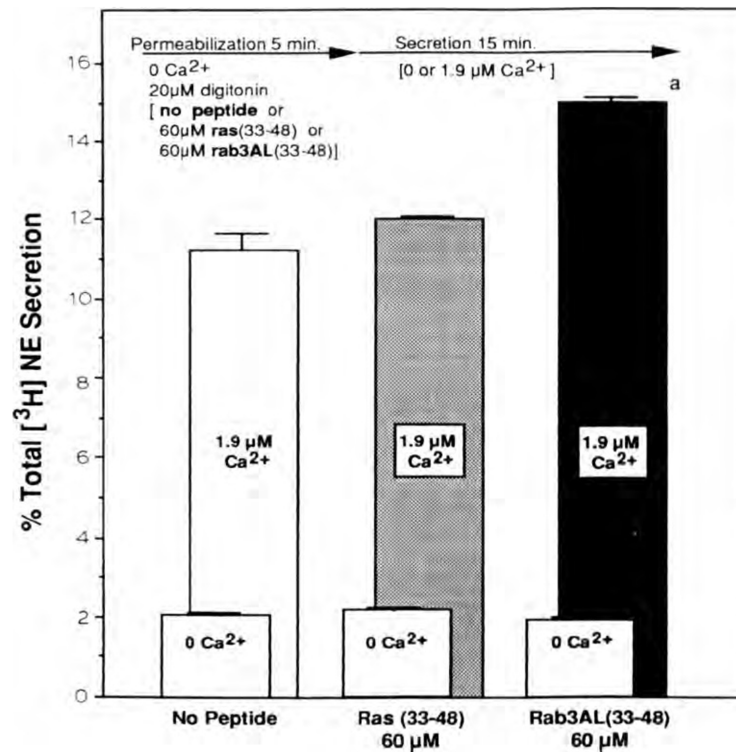


Fig. 4. Preincubation with rab3AL(33–48) enhances subsequent $Ca^{2+}$-dependent secretion. [$^{3}$H]Norepinephrine-labeled cells were incubated for 5 min in $Ca^{2+}$-free KGEPM with 2 mM MgATP and 20 μM digitonin and either no peptide or 60 μM rab3AL(33–48) or 60 μM ras(33–48). Cells were then incubated for 15 min without digitonin in the absence of peptide with 0 or 1.9 μM $Ca^{2+}$. Secretion after the 15 min incubation was determined. [$^{3}$H]Norepinephrine released during the first incubation was the same (4.0% of the total) for 0 peptide, rab3AL(33–48), and ras(33–48). There were 3 wells/group. $^{a}P<0.01$ vs. no peptide or ras(33–48).

Rab3AL(33–48), 60 μM, caused a 28% enhancement of GppNHp-dependent secretion (Fig. 5). Ral(33–48), 60 μM, had little effect. The effects of rab3AL(33–48) were reproducible in a large number of experiments (Table II). Ras(33–48), 60 μM, did not have significant effects on GppNHp-dependent secretion (Table II).

### 3.4. *Rab3AL(33–48) does not enhance $Ca^{2+}$-dependent secretion by activation of protein kinase C*

In *Staphylococcus aureus* α-toxin permeabilized chromaffin cells (which are not leaky to proteins) guanine nucleotides enhance $Ca^{2+}$-dependent secretion by activation of protein kinase C [38]. To determine whether rab3AL(33–48) enhances $Ca^{2+}$-dependent secretion through activation of protein kinase C, the ability of a pseudosubstrate inhibitor of protein kinase C to inhibit the effect of rab3AL(33–48) was examined. PKC(19–31) has no effect on $Ca^{2+}$-dependent secretion in digitonin-permeabilized cells in the absence of exogenous protein kinase C activators [39]. It specifically inhibits protein kinase C-mediated phosphorylation and enhancement of $Ca^{2+}$-dependent secretion induced by TPA in digitonin-permeabilized chromaffin cells [39]. PKC(19–31), 30 μM, did not alter the enhancement of secretion induced by 60 μM rab3AL(33–48) (data not shown). Thus, the enhancement of $Ca^{2+}$-dependent secretion by

Table I

Comparison of Amino Acid Sequences of Peptides and GTP-Binding Proteins

| | | |
|---|---|---|
| rab3AL(33-48) | V S A L G I D F K V K T I Y R N | |
| H-rab3A | V S T V G I D F K V K T I Y R N | (94%) |
| rab3F(33-48) | V S T F G I D F K V K T I Y R N | (88%) |
| H-rab3B | V S T V G I D F K V K T V Y R H | (88%) |
| SEC4 | I T T I G I D F K I K T V D I N | (81%) |
| H-rab1 | I S T I G V D F K I R T I E L D | (75%) |
| YPT1 | I S T I G V Q F K I K T V E L D | (69%) |
| H-rab2 | D L T I G V E F G A R M I T I D | (50%) |
| H-rab6 | Q A T I G I D F L S K T M Y L E | (50%) |
| H-rab4 | N H T I G V E F G S K I I N V G | (44%) |
| H-rab5 | E S T I G A A F L T Q T V C L D | (44%) |
| ral(33-48) | E P T K A D S Y R K K V V L D | (25%) |
| ras(33-48) | D P T I E D S Y R K Q V V I D | (19%) |

The amino acid residues include the ras effector binding domain (residues 32-40) that binds GAP [31] and is found on a loop of ras exposed on the surface of the molecule [32,33]. The ras sequence is for Ki-ras. The reference numbering corresponds to that of Ki-ras according to Zahraoui et al. [9]. Percentages in parenthese indicate relative amino acid homologies with rabAL(33-48) allowing for conservative replacements.

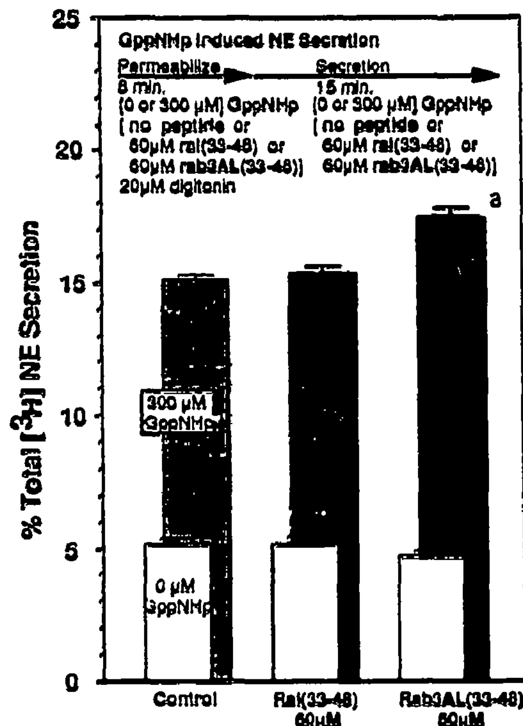


Fig. 5. Effects of rab3AL(33–48) GppNHp-induced secretion. [$^{3}$H]Norepinephrine-labeled chromaffin cells were permeabilized in $Ca^{2+}$-free KGEPM with 2 mM MgATP and 20 $\mu$M digitonin with 0, 60 $\mu$M ral(33–48) or 60 $\mu$M rab3AL(33–48) ± GppNHp (300 $\mu$M) for 8 min. Solutions were replaced with digitonin-free KGEPM (with 2 mM MgATP) in the continuing presence or absence of peptide and GppNHp and the incubation continued for 15 min. The sums of GppNHp-dependent secretion in the first and second incubations was determined and are plotted in the figure. There were 3 wells/group. $^{a}P<0.01$ vs. no peptide.

rab3AL(33–48) is not caused by activation of protein kinase C.

## 4. DISCUSSION

Rab3AL(33–48) and closely homologous peptides inhibit ER-Golgi transport at a step following $Ca^{2+}$, immediately before fusion of vesicles with *cis*-Golgi [28]. The blockade of vesicular transport has been postulated to be caused by the peptides binding to unknown effectors and preventing their cyclic interaction of the effector with a GTP-binding protein [8,28]. In the present experiments rab3AL(33–48) enhanced $Ca^{2+}$- and ATP-dependent secretion from permeabilized chromaffin cells. Rab3F(33–48) also enhanced $Ca^{2+}$-dependent secretion. The action of these peptides was specific since comparable effects were seen with neither ras(33–48) nor ral(33–48). These data suggest that a low molecular weight GTP-binding protein is involved in the regulation of $Ca^{2+}$-dependent secretion.

The stimulation of exocytosis by the rab peptides contrasts with the inhibition of transport in the Golgi. We suggest that because the assay for regulated secretion measures only one round of fusion and not a cyclic process only the stimulatory effects of activated effectors in the GTP-dependent pathway are observed.

Rab3AL(33–48) has considerable homology (80–90%) with comparable regions in H-rab3A, H-rab3B, H-rab1, and SEC4 (yeast) if conservative replacements of amino acids are considered (Table I). It also has substantial (approximately 50%) homology with regions in H-rab2, H-rab4 and H-rab6. Rab3AL(33–48) has much less homology (25 and 19%) to comparable regions in Ki-ras and ral which did not enhance secretion. Our data are consistent with a protein of the rab class playing a modulatory role in regulated exocytosis. Since several species of rab proteins have been identified, it is possible that rab3AL(33–48) and rab3F(33–48) only approximate the effector binding region of the putative rab protein active in the final steps of exocytosis. Another peptide more closely resembling the appropriate effector binding region may cause greater enhancements of secretion than observed in the present study.

Although rab3AL(33–48) alone did not cause significant amounts of secretion, it did enhance secretion induced by GppNHp. The result suggests that that more than one GTP-binding protein may be involved in the ability of guanine nucleotides to stimulate secretion. Rab3AL(33–48) may mimic the function of only one of these proteins.

Studies to evaluate the effects of rab peptides have recently been performed on SLO-permeabilized pancreatic acinar cells [40] and patch-clamped mast cells [41]. In pancreatic acinar cells both $Ca^{2+}$-dependent and guanine nucleotide-dependent amylase secretion are also

Table II

Comparison of Amino Acid Sequences of Peptides and GTP-Binding Proteins

| | Rab3AL(33-48) 60 $\mu$M | Ras(33-48) 60 $\mu$M | Ral(33-48) 60 $\mu$M |
|---|---|---|---|
| $Ca^{2+}$-dependent secretion | 1.31±0.02$^{a}$ (18) | 1.03±0.03$^{b}$ (16) | 1.05±0.02$^{b}$ (5) |
| GppNHp-induced secretion | 1.68±0.14$^{a}$ (10) | 1.19±0.08$^{b}$ (7) | 1.03±0.07 (1) |

Data are combined from a number of experiments (indicated in parentheses). In each experiment the ratio of secretionin the presence of peptide to secretion in the absence of peptide was determined. The data are summarized as the mean ± standard error of the mean of the ratios. The concentration of peptide was always 60 $\mu$K. Most of the $Ca^{2+}$-dependent secretion experiments were performed with 18 $\mu$M $Ca^{2+}$, although some were performed with 1.9 $\mu$M $Ca^{2+}$. GppNHp-induced secretion was determined with 30 or 300 $\mu$M GppNHp. Seven of the experiments with GppNHp were performed in solution in which 250 mM sucrose replaced 139 mM potassium glutamate. A paired t-test was performed on the differences between secretion in the presence and absence ofpeptide. $^{a}P<0.001$ vs. no peptide. $^{b}$not significant.

stimulated by rab3AL(33–48). Peptide alone causes significant amylase secretion. (Stimulation of secretion in chromaffin cells by peptide alone was only sporadically observed.) Most striking are the effects in mast cells where rab peptides triggered 100% secretion in the absence of other secretagogues. A comparison of the different secretory systems provides evidence that the rab peptides are, indeed, mimicking a pathway involving a guanine-nucleotide binding protein. Mast cells secrete vigorously to guanine nucleotides and respond strongly to rab peptides. SLO-permeabilized pancreatic acinar cells and digitonin-permeabilized chromaffin cells respond more temperately to guanine nucleotides and correspondingly less vigorously to the rab peptides.

The smaller effects on secretion of guanine nucleotides and rab peptides in chromaffin and pancreatic acinar cells than in mast cells could reflect a modulatory role of GTP-binding proteins in the secretory pathway in chromaffin and acinar cells and a major regulatory role in mast cells. However, it is also possible that both GTP-binding proteins and $Ca^{2+}$-dependent steps are necessary in all three systems. Chromaffin and pancreatic acinar cells may have an already high state of activation of GTP-dependent processes required for secretion which exogenous guanine nucleotides and rab peptides enhance only to a small degree. $Ca^{2+}$-dependent processes limit secretion. In mast cells, the activation of a rab-like protein limits secretion and elements of the $Ca^{2+}$-triggered pathway are already strongly activated.

In summary, a peptide resembling the putative effector region of the rab class of low molecular weight GTP-binding proteins enhances $Ca^{2+}$-and GppNHp-dependent secretion from permeabilized chromaffin cells. The data provide evidence for the involvement of a low molecular weight GTP-binding protein in the secretory pathway in adrenal chromaffin cells.

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