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Mastoparan and Rab3AL peptide potentiation of calcium-independent secretory activity in rat melanotrophs is inhibited by GDPβS

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Abstract The whole-cell patch-clamp membrane capacitance measurement was used to monitor secretory activity in rat melanotrophs, while rab3AL, putative effector domain peptides of Rab3 small GTPases (20–30 kDa), were introduced into cytosol. In melanotrophs dialyzed with calcium free solutions membrane capacitance tends to decrease slightly. This decrease is further potentiated with GDPβS (500 μM). We found that rab3AL (100 μM) stimulated secretory activity in the absence of calcium. The rab3AL response was qualitatively comparable to the response to mastoparan (1 μM), an activator of certain heterotrimeric GTP-binding proteins. Interestingly, inclusion of GDPβS (500 μM) resulted in a blockade of both rab3AL and mastoparan induced responses. We conclude that rab3AL and mastoparan induce calcium-independent stimulation of secretory activity in rat melanotrophs by activation of a downstream heterotrimeric GTP-binding protein.

Key words: Rat melanotroph; Membrane capacitance; GTP-binding protein; Exocytosis; Rab3 protein; Mastoparan

1. Introduction

Small (20–30 kDa) GTP-binding proteins, Rab3, are believed to be involved in regulated exocytosis [1,2]. These proteins were found on membranes of synaptic vesicles and secretory granules [3,4], and appear to dissociate from membranes upon exocytosis [3]. An isoform of Rab3, Rab3B, was shown to play a role in calcium-dependent exocytosis of anterior pituitary cells [5], whereas an inhibitory role for Rab3A isoform was described in chromaffin cells [6,7]. Additional evidence to support a role for Rab3 proteins in exocytosis comes from experiments where peptides of the Rab3 putative effector domain stimulated secretory activity in pancreatic acinar [8], chromaffin [9], insulin-secreting cells [10] and in mast cells [11]. These experiments indicate that G_E, the elusive GTP-binding protein believed to be involved in the control of exocytosis [12], may be a Rab3 protein. Edwardson et al. [13] have used an in vitro assay to monitor fusion of pancreatic zymogen granules with plasma membrane in the presence of Rab3 effector domain peptides. They suggested that the fusion may be under the control of more than one type of GTP-binding proteins. Aridor et al. [14] have shown that exocytosis in mast cells is activated by the heterotrimeric G_{i3} protein. Interestingly, the pertussis toxin sensitive GTP-binding protein was suggested to be a target for the Rab3 effector domain peptide in stimulating exocytosis [15].

We have studied the effects of Rab3 peptides on the secretory activity in patch-clamped rat melanotrophs. The results show that rab3AL potentiates secretory activity independently of calcium ions and that this effect is inhibited by GDPβS. Mastoparan, a potent guanine nucleotide exchange promoter [16], potentiates the secretory activity in a similar fashion to rab3AL. These findings are consistent with the model where downstream of regulation of secretion by Ca^{2+} there is a requirement for some activity of an unknown GTP binding protein whose functional identity is sensitive to mastoparan and rab3AL, and can be blocked by GDPβS.

2. Materials and methods

Peptide synthesis was performed on an SMPS Multiple Peptide Synthesis machine (Zinsser Analytic, Maidenhead, UK) using Fmoc chemistry. Purity was demonstrated to be 95% by analytical HPLC (Waters, Milford, MA, USA). Sequence identity was verified by Edman degradation on an automated gas-phase protein sequencer (Applied Biosystems, Warrington, UK).

Cell cultures were prepared by standard methods of enzymatic and mechanical dispersion as modified by Rupnik and Zorec [17], harvested on poly-L-lysine covered glass coverslips, and kept in an incubator at 36°C, 95% humidity and 5% CO_2 for 1–14 days before experimentation. Cell-covered coverslips were transferred into the recording chamber on the inverted microscope (Opton IM 35, Oberkochen, Germany) in the recording medium consisting of (in mM): NaCl, 131.8; KCl, 5; $MgCl_2$, 2; NaH_2PO_4, 0.5; $NaHCO_3$, 5; HEPES, 10; D-glucose, 10; $CaCl_2$, 1.8; pH = 7.2/NaOH).

The cells were voltage-clamped [18] at a holding potential of −70 mV. Membrane capacitance (C_m) was recorded using a two-phase lock-in amplifier (1600 Hz, 1 mV peak to peak) incorporated into a patch-clamp amplifier (SWAM IIA, Ljubljana, Slovenia) [19]. The DC current (low pass, 1–10 Hz, −3 dB), holding potential and the real and imaginary admittance signals (low pass, 1 Hz, −3 dB) were used in calculations. The plots of the passive cell parameters, access conductance (G_a), parallel combination of leak and membrane conductance (G_m) and membrane capacitance (C_m) were derived by a computer-aided reconstruction using an analog-to-digital converter (CED 1401, Cambridge, UK) to record the signals in digital format on the computer hard disk (IBM compatible). The software was written by Dr. J. Dempster (University of Strathclyde, Glasgow, UK).

Recordings were made at room temperature with pipette resistances between 2 and 4 MΩ (measured in KCl-rich solution), giving access conductances of more than 150 nS. Solution in the pipette contained (in mM); $MgCl_2$, 2; HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid], 10; EGTA [ethylenglycol-bis-(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 4; KCl, 150; Na_2ATP, 2; pH 7.2 (KOH)). EGTA concentration of 4 mM exceeds the buffering capacity of melanotrophs [20]. Pipette-filling solutions were of similar osmolarity to bathing solution (within 5%) measured by freezing point depression (Camlab, Cambridge, UK). Rab3 peptides were prepared as stock solutions in distilled water (1 to 10 mM) and diluted to the final concentration before experimentation. Statistics are in the format mean ± standard error of the mean.

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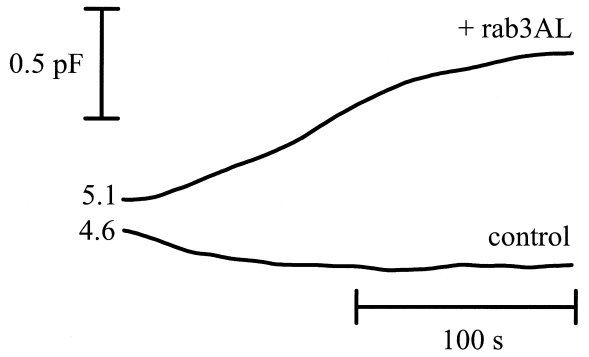

Fig. 1. Representative time-dependent changes in membrane capacitance in a control cell (bottom) and a cell dialyzed with a pipette-filling solution containing 100 μM rab3AL (top trace). Numbers adjacent to traces indicate resting membrane capacitance in pF. Traces were filtered at 0.1 Hz in this and all other figures.

3. Results

Rat melanotrophs were dialyzed with a pipette-filling solution containing virtually no free calcium (4 mM EGTA). As observed previously [17], the secretory responses were characterized by a small steady decline in C_m (Fig. 1, Table 1). The inclusion of rab3AL (100 μM) in the pipette solution resulted in a potentiation of secretory responses. Also, the C_m measured 200 s after the start of dialysis, expressed as a percentage of resting C_m, was increased (Table 1). These results show that rab3AL peptides potentiate secretory activity in the absence of cytosolic calcium. This potentiation may be explained by the competition between the rab3AL and the native Rab3 proteins with a common target(s). To demonstrate that this effect is specific, we studied whether peptides with a random sequence affected the secretory activity of melanotrophs as well.

A peptide with the same amino acids as the rab3AL was synthesized, in which the amino acids were oriented randomly (Table 1). The responses recorded with this peptide were not significantly different from controls (Fig. 2, Table 1). Moreover, with a truncated peptide (5-mer), corresponding to the N-terminus region of the rab3AL, responses in C_m were also not significantly different from controls (Fig. 2, Table 1). These results suggest that the rab3AL mediated potentiation of secretory activity is specific.

Interestingly, in the presence of mastoparan (1 μM) in the cytosol, we observed a similar potentiation of secretory responses as in the presence of 100 μM rab3AL (Fig. 2, Table 1). These results indicate that the mechanism of potentiation

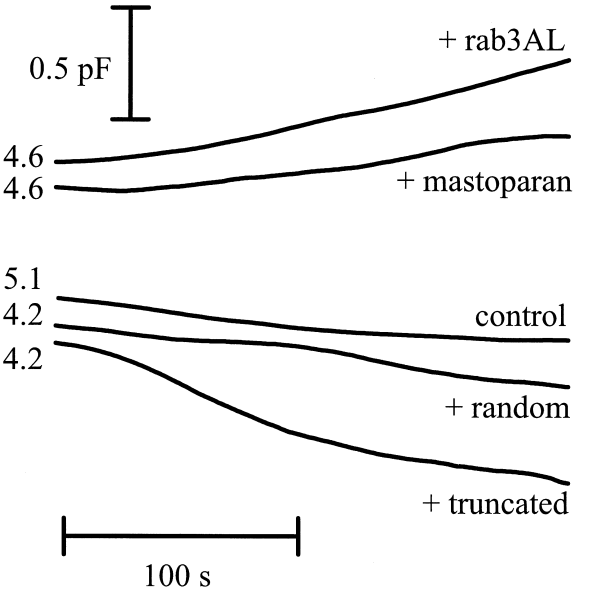

Fig. 2. Time-dependent changes in membrane capacitance recorded in a control cell and in cells dialyzed with: 100 μM rab3AL, 1 μM mastoparan, 100 μM random and truncated Rab3 peptide. See peptide structure in the footnote to Table 1. Numbers adjacent to traces indicate resting capacitance in pF.

by the two agents may be alike, as previously suggested by Law et al. [15]. It may involve an activation of a downstream GTP-binding protein, since mastoparan activates certain heterotrimeric GTP-binding proteins [16,21]. If this was true it should be possible to inhibit rab3AL- and mastoparan-induced responses by the addition of GDPβS. This presumption was tested and the results are presented in Fig. 3 and Table 1. In the presence of GDPβS (500 μM), the secretory responses were characterized by a net decrease in C_m. The rate of decline changed from around −2 fF/s (controls) to around −8 fF/s and was so significantly faster then controls. This is likely due to a complete inhibition of exocytosis [22]. In the presence of mastoparan or rab3AL and GDPβS, the secretory responses were not significantly different from those recorded in the presence of only GDPβS (Table 1).

Our results support the hypothesis that the potentiation of secretory responses by rab3AL and mastoparan involves a GTP-binding proteins. Furthermore, since mastoparan activates certain heterotrimeric GTP-binding proteins, our results also suggest that rab3AL may activate a downstream heterotrimeric GTP-binding protein [15].

Table 1
Secretory responses in single melanotrophs measured as the maximal rate (dC_m/dt) in membrane capacitance and as a relative change in membrane capacitance 200 s after the start of whole-cell recording relative to the resting membrane capacitance (% C_m)

	dC_m/dt [fF/s]	% C_m after 200 s	Number of cells
Control	−2 ± 0	−4 ± 1	27
+rab3AL	4 ± 0**	3 ± 1**	10
+mastoparan	4 ± 1**	2 ± 0**	5
+random	−4 ± 1	−2 ± 1	5
+truncated	−4 ± 2	−5 ± 2	6
+GDPβS	−8 ± 1$^{\Delta\Delta}$	−7 ± 3$^{\Delta}$	7
+GDPβS+rab3AL	−7 ± 1$^{\Delta\Delta}$	−6 ± 2$^{\Delta\Delta}$	6
+GDPβS+mastoparan	−7 ± 1$^{\Delta\Delta}$	−6 ± 2$^{\Delta\Delta}$	5

Control responses and responses in the presence of 100 μM rab3AL (VSALGIDFKVKTIYRN), 1 μM mastoparan, 100 μM truncated (VSALG) and random (YNVLSADFKVGKIRIT) rab3 peptides in the cytosol were recorded. Also responses in the presence of GDPβS (500 μM) were measured. Statistics are in the format mean ± standard error of the mean. Asterisks indicate significant differences compared to the control ($^{*}P < 0.01$, $^{**}P < 0.001$, Student's t-test). The statistical differences between GDPβS-treated and respective control cells are indicated with triangles ($^{\Delta}P < 0.01$, $^{\Delta\Delta}P < 0.001$, Student's t-test).

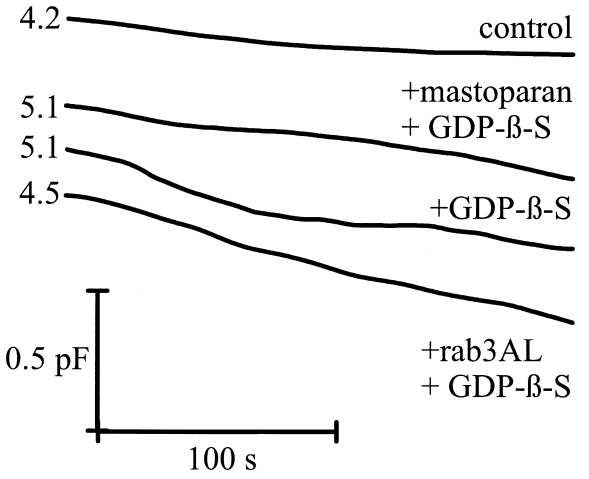

Fig. 3. Inhibition of melanotroph secretory activity by GDPβS. Time-dependent changes in membrane capacitance in a control cell and in the presence of GDPβS (500 μM), GDPβS (500 μM)+ rab3AL (100 μM) and GDPβS (500 μM)+mastoparan (1 μM). Numbers adjacent to traces indicate resting membrane capacitance in pF.

4. Discussion

The aim of this paper was to study the effects of Rab3-like peptides on secretory activity of rat melanotrophs. We found that rab3AL potentiated the secretory responses recorded as changes in membrane capacitance. The potentiation of secretory activity was calcium independent (Fig. 1, Table 1), which is in agreement with previous reports [8,11]. The activity of rab3AL appeared to be remarkably specific as it was absent in a random and truncated form of this peptide (Fig. 2, Table 1). It was thus likely that the specific effects of rab3AL peptides reported here were related to the activity of Rab3 proteins in cells. However, this interpretation has been questioned by an in vitro study, where interactions of recombinant Rab3A proteins with GAP or p85 proteins were studied in the presence of rab3AL [23]. It may be that the in vitro assay is not comparable to the experiments where cells are used. It is also possible that rab3AL and Rab3 interact with additional targets in cells.

It was shown that GTPγS stimulates secretory activity in patch-clamped rat melanotrophs [24,25] and that this effect was independent of calcium ions [26]. This suggests that GTP-binding proteins may mediate release in the absence of calcium ions. Potentiation of secretory activity was demonstrated with a well known activator of G proteins mastoparan [16]. This stimulation of release was absent in the presence of GDPβS. These data provide further evidence for a requirement of a GTP-binding protein in the secretory process. We found that the secretory response of patch-clamped melanotrophs to rab3AL was remarkably similar to that observed with mastoparan and that regulation of a G-protein is a possible mechanism of action for these two polycationic peptides [27].

In order to explain the stimulation of secretory activity by rab3AL in rat melanotrophs previous work has been assumed [8–11,13] that there may be an interaction between this surrogate effector domain of native Rab3 proteins and a target molecule on the plasma membrane [10,13]. One possible target molecule might be a heterotrimeric GTP-binding protein because the effects of rab3AL are sensitive to guanine nucleotides (Fig. 3) [11], pertussis toxin [15] and also because rab3AL was found to stimulate IP_3 production in cells [28]. A role for heterotrimeric GTP-binding proteins was already suggested to modulate secretory activity in melanotrophs [24–26] and chromaffin cells [29]. This paper provides new data to support a role of GTP-binding proteins in the final control of secretory activity in rat melanotrophs.

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