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The heterotrimeric Gi_3 protein acts in slow but not in fast exocytosis of rat melanotrophs

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SUMMARY

Besides having a role in signal transduction some trimeric G-proteins may be involved in a late stage of exocytosis. Using immunocytochemistry and confocal microscopy we found that Gi_3-protein resides mainly in the plasma membrane, whereas $Gi_{1/2}$-protein is preferentially associated with secretory granules. To study the function of trimeric Gi_3- and $Gi_{1/2}$-proteins, secretory responses in single rat melanotrophs were monitored by patch-clamp membrane capacitance measurements. We report here that mastoparan, an activator of trimeric G-proteins, enhances calcium-induced secretory activity in rat melanotrophs. The introduction of synthetic peptides corresponding to the C-terminal domain of the α-subunit of Gi_3- and $Gi_{1/2}$-proteins indicated that Gi_3 peptide specifically blocked the mastoparan-stimulated secretory activity, which indicates an involvement of a trimeric Gi_3-protein in mastoparan-stimulated secretory activity. Flash photolysis of caged Ca^{2+}-elicited biphasic capacitance increases consisting of a fast and a slower component. Injection of anti-Gi_3 antibodies selectively inhibited the slow but not the fast component of secretory activity in rat melanotrophs. We propose that the plasma membrane-bound Gi_3-protein may be involved in regulated secretion by specifically controlling the slower kinetic component of exocytosis.

Key words: Kinetic component, Membrane capacitance, Regulated exocytosis, Trimeric G-protein

INTRODUCTION

Studies on diverse secretory cell types have highlighted the importance of GTP-binding proteins in calcium-regulated exocytic fusion of vesicles or granules with the plasma membrane (see reviews, Südhof, 1995; Calakos and Scheller, 1996; Martin, 1997; Burgoyne and Morgan, 1998). Proteins able to bind and hydrolyze GTP can be subdivided into different families including trimeric G proteins and *ras*-related low molecular mass G proteins. Trimeric G proteins have been found associated with the membrane of secretory granules in various secretory cells (Ahnert-Hilger et al., 1994; Konrad et al., 1995; Vitale et al., 1996). Thus, besides playing a role in the signal transduction cascade operating at the plasma membrane, trimeric G-proteins may also be involved in the regulation of calcium-evoked exocytosis. Indeed, the participation of Gi and Go proteins in the late stages of exocytosis has been demonstrated in mast cells (Aridor et al., 1993), insulin-secreting cells (Lang et al., 1995) and chromaffin cells (Vitale et al., 1993, 1994a; Gasman et al., 1997). Taken together these observations suggest that regulated exocytosis may represent a possible effector pathway for trimeric G proteins although the precise role of this class of G proteins at specific stages of the exocytotic machinery remains to be determined.

Subunits of trimeric G-proteins have been previously described on the plasma membrane and on the membrane of several intracellular compartments involved in the secretory pathway in rat pituitary (Muller et al., 1994). By immunocytochemistry, we provide here evidence that in rat melanotrophs Gi_3 resides mainly in the plasma membrane whereas $Gi_{1/2}$ is preferentially associated with the membrane of secretory granules. Melanotrophs secrete α-melanocyte stimulating hormone (α-MSH) and β-endorphin (Mains and Eipper, 1979). To study the function of Gi_3 and $Gi_{1/2}$ in α-MSH and β-endorphin regulated secretion, we used patch-clamp membrane capacitance measurements to monitor exocytotic activity in single rat melanotrophs (Neher and Marty, 1982). Mastoparan, a specific activator of trimeric G-proteins was found to enhance calcium-dependent secretory activity in rat melanotrophs, in agreement with our previous observations (Rupnik et al., 1997). Peptides, corresponding to the C-

terminal domain of the α-subunit of Gi and Go have been shown to prevent the stimulation of G-proteins by their respective receptors or by mastoparan (Hamm et al., 1988; Palm et al., 1990). Therefore, the introduction of synthetic peptides corresponding to the C-terminal domain of the α-subunit of Gi_3 and $Gi_{1/2}$ should inhibit mastoparan-enhanced changes in membrane capacitance elicited by a rise in intracellular [Ca^{2+}], if α-subunits of these Gi proteins are involved in the regulation of secretory activity of melanotrophs. We show that Gi_3 but not $Gi_{1/2}$ C-terminal peptides selectively inhibit mastoparan-enhanced changes in membrane capacitance.

In a series of experiments, cells were stimulated by increasing cytosolic calcium quickly and uniformly using flash photolysis of caged Ca^{2+} (Neher and Zucker, 1993). Photolysis of caged Ca^{2+} activated a biphasic membrane capacitance response, revealing an early and a slower phase of exocytosis, as reported previously (Neher and Zucker, 1993; Thomas et al., 1993a,b; Heinemann et al., 1994; Kasai et al., 1996; Takahashi et al., 1997; Ninomiya et al., 1997; Xu et al., 1998). Injection of specific antibodies against the α-subunits of Gi_3-protein selectively affected the slow but not the fast component of the membrane capacitance response. We propose that the plasma membrane-bound Gi_3-protein may be involved in regulated secretion by specifically controlling the slow kinetic component of exocytosis.

MATERIALS AND METHODS

Cell cultures

Rat pituitary melanotrophs were isolated and dispersed mechanically and enzymatically according to methods described previously (Rupnik and Zorec, 1992), seeded on poly-L-lysine covered glass coverslips, and cultured in an incubator at 36°C, 95% humidity and 5% CO_2 for up to a week before being used in the experiments.

Immunofluorescence microscopy

Melanotrophs were washed with PBS (phosphate-buffered saline) and then fixed for 15 minutes in 4% paraformaldehyde in PBS. The following 10 minutes cells were kept in fixative containing 0.1% of Triton X-100 and then four times washed with PBS. Non-specific staining was reduced by incubating cells in 3% BSA (bovine serum albumin) and 10% normal goat serum in PBS. Cells were then incubated with primary antibodies for 2 hours at 37°C. We used 50 μg/ml of rabbit affinity-purified anti-$G\alpha i_{1/2}$ and anti-$G\alpha i_3$ antibodies, prepared against a synthetic peptide corresponding to the C-terminal domain of Gi_3 and $Gi_{1/2}$ respectively as described (Vitale et al., 1996). We used mouse anti-Golgi antibody (Jasmin et al., 1989) undiluted, mouse anti-syntaxin I (Leveque et al., 1994; El Far et al., 1995) and anti-synaptotagmin I (Takahashi et al., 1991; Leveque et al., 1992) diluted 1:2000 in PBS containing 3% BSA. Cells were then washed and incubated in PBS containing Cy3-labelled anti-rabbit and FITC labelled anti-mouse secondary antibodies (1:2000) and 3% BSA for 45 minutes. Cells were washed and mounted in Light Antifade Kit (Molecular Probes, Netherlands). Stained cells were monitored using the Zeiss laser scanning microscope (LSM 410 invert) with a planapo oil immersion lens (×63, NA=1.4). FITC was excited using the argon laser (488 nm) and Cy3 using the He/Ne laser (543 nm). The FITC emission signal was filtered with a Zeiss 515-565 nm filter, and Cy3 emission signal was filtered using 595 nm long pass filter. Colocalization of the emission signal was evaluated as described (Chasserot-Golaz et al., 1996).

Membrane capacitance measurements

Compensated and uncompensated membrane capacitance measurements were used (Neher and Marty, 1982; Lindau and Neher, 1988; Zorec et al., 1991) employing a SWAM CELL or SWAM IIB patch clamp/lock-in amplifier (Henigman, Piran, Slovenia), operating at 1.6 kHz lock-in frequency.

Compensated C_m measurements were used to monitor fast changes in membrane capacitance. Upon establishment of the whole-cell configuration C_m and G_a (access conductance) were compensated by C_{slow} and G_a compensation controls. Sine voltage of 11 to 111 mV_{rms} was applied. The phase angle setting was determined by a 100 fF or 1 pF pulse and monitoring the projection of the pulse from the C (signal proportional to C_m) to G output of the lock-in amplifier. These two signals were stored unfiltered (C-DAT4 recorder, Cygnus, USA) for off-line analysis. Simultaneously we recorded filtered (300 Hz, 4 pole Bessel) C and G signals, the fluorescence intensity from a C660 photon counter (Thorn EMI UK) and membrane current (0-10 Hz, low pass). PhoCal program (LSR, UK) was used to acquire signals every 5 milliseconds. For high temporal resolution measurements of C_m, the records on DAT were played back and a 10 second epoch of the signal enveloping each flash was digitized at 50 kHz using a CDR program (J. Dempster). Signals were digitally filtered at 1 kHz (2-way 150^{th} order FIR filter, Math Works MATLAB) and resampled at 10 kHz. The pipette solution contained: 110 mM KCl, 10 mM TEACl, 40 mM KOH/Hepes (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid), 2 mM Na_2ATP, 2 mM $MgCl_2$, 4 mM K_4-NP-EGTA [*o*-nitrophenyl ethyleneglycol-bis-(b-aminoethyl-ether)-*N*,*N*,*N*′,*N*′-tetrapotassium salt], 3.6 mM $CaCl_2$, 0.5 mM furaptra, pH 7.2. The bath contained: 131.8 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 10 mM Na Hepes, 10 mM D-glucose, 1.8 mM $CaCl_2$, pH 7.2.

Uncompensated C_m measurements were used to monitor slow changes in C_m as described (Rupnik and Zorec, 1992). A sine wave voltage (1 mV rms, 1600 Hz) was applied to the holding potential. Output signals: DC current, holding potential, real and imaginary admittance (Bessel, low pass 10 Hz, −3 dB, 6-pole) were digitized by a CED 1401 A/D converted (Cambridge, UK) and analysed by the computer software (CAP3, J. Dempster, University of Strathclyde, UK). Software computed passive cell parameters: access conductance (G_a), membrane conductance (G_m) and membrane capacitance (C_m). The recording bath solution consisted of: 131.8 mM NaCl, 1.8 mM $CaCl_2$, 5 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes/NaOH, 10 mM D-glucose, 0.5 mM $NaH_2PO_4.2H_2O$, 5 mM $NaHCO_3$ (pH 7.2). The pipette solution consisted of: 150 mM KCl, 2 mM $MgCl_2$, 10 mM Hepes/KOH, 0.65 mM EGTA, 4.35 mM Ca^{2+}-saturated EGTA ($[Ca^{2+}]_i$ = 1 μM), 2 mM Na_2ATP, pH 7.2). Intracellular [Ca^{2+}] was calculated assuming an apparent dissociation constant (K_d) for the Ca-EGTA complex of 150 nM (Grynkiewicz et al., 1985), and assuming that the cytosol equilibrates with the pipette solution upon the establishment of the whole-cell recording. Total EGTA concentration was 4 mM, which exceeds the buffering capacity of melanotrophs (Thomas et al., 1990). All recordings were made at room temperature. All salts were obtained from Sigma. Synthetic C-terminal peptides $G\alpha i_{1/2}$ (KNNLKDCGLF) and $G\alpha i_3$ (KNNLKECGLY) were obtained from Neosystem (Strasbourg, France), mastoparan was obtained from Sigma. Peptides were added to the pipette solution short before the experiment. Final concentration of $G\alpha_i$ peptides and mastoparan was 100 μM and 1 μM, respectively. Cells were voltage clamped at a holding potential of –70 mV. The reversal potential of the whole-cell current was –50 mV and did not change during recordings. Recordings were made at room temperature with pipette resistance between 1 to 4 MΩ (measured in KCl-rich solution), giving access conductance of more than 80 nS. The pipette and bath solutions were of similar osmolarity (within 5%) measured by freezing point depression (Camlab, Cambridge, UK).

Flash photolysis and $[Ca^{2+}]_i$ measurements

We used NP-EGTA (*o*-nitrophenyl ethyleneglycol-bis-(b-aminoethyl-ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid, Molecular Probes, Oregon, USA or Calbiochem, Switzerland) to manipulate $[Ca^{2+}]_i$ (Ellis-Davies and Kaplan, 1994). A UV flash from a Xe arc flash lamp (Rapp and Güth, 1988) was delivered to cells through a ×40 fluor oil immersion objective of a Nikon Diaphot microscope. The same optical pathway as in flash photolysis was used to illuminate the fluorescent $[Ca^{2+}]$ indicator furaptra (Molecular Probes, Oregon, USA). A combination of two dichroic mirrors was used. The first one was a 390 nm dichroic positioned at 45° which passed through the 420 nm light for furaptra excitation from a Xe arc lamp and reflected the light below 390 nm for NP-EGTA flash photolysis from a Xe arc flash lamp. The second 430 nm dichroic reflected both lights through the objective to the cell under experiment and allowed only furaptra fluorescent light to pass back to the photomultiplier through a 510 nm barrier filter. $[Ca^{2+}]_i$ measurements were performed as described by Carter and Ogden (1994). The equation used in calculation is:

$$[Ca^{2+}]_i = K_d \frac{F - F_{min}}{F_{max} - F}, \quad (1)$$

where F_{max} is the autofluorescence in the cell-attached configuration, F_{min} is fluorescence in a resting whole-cell recording and F is fluorescence during the flash.

Microinjection of antibodies

Transjector 4657 with a micromanipulator (Eppendorf, Germany) was used. Pulses (8-12 hPa, 0.3 to 1 second) were applied with a compensation pressure set to 1 hPa. Microinjection solution consisted of: 150 mM K-gluconate, 2 mM $MgCl_2$, 10 mM Hepes. Pipettes were prepared with a horizontal puller (P-87, Sutter Instruments, CA, USA). Microinjected cells were identified with co-injection of rhodamine-labeled dextran (5 mg/ml, Sigma Chemical Co., USA, 25 kDa). Injection of dextran marker alone did not affect secretory responses per se. The same affinity-purified anti-Gαi_3 antibody was microinjected into cells as in immunofluorescent studies. The control antibodies (preimmune antibody) were purified using Protein A-Sepharose to prepare immunoglobulin fractions from sera of nonimmune.

Antibodies were injected into cells (50 μg/ml) together with the dextran marker, then cells were placed in an incubator for 1 to 2 hours at 37°C before experiments were performed.

RESULTS

Subcellular localisation of Gαi_3 and G$\alpha i_{1/2}$ in rat melanotrophs

The subcellular distribution Go and Gi proteins in cultured rat melanotrophs was investigated by immunofluorescence and confocal microscopy using affinity-purified anti-Gαo, anti-Gαi_3 and anti-G$\alpha i_{1/2}$ antibodies (Vitale et al., 1996). As illustrated in Fig. 1, the anti-Gαi_3 immunoreactivity was restricted to the subsplasmalemmal region, suggesting that the majority of Gi_3 protein resides probably in the plasma membrane. A comparable distribution for Gi_3 was previously reported in chromaffin cells (Vitale et al., 1996) and mast cells (Aridor et al., 1993). In contrast, the anti-G$\alpha i_{1/2}$ antibody decorated some intracellular compartments distributed over the whole cell body excluding the nucleus (Fig. 2). We could not detect any specific immunostaining with the anti-Gαo antibodies (data not shown).

To verify the association of Gαi_3 with the plasma membrane, double immunofluorescent experiments were performed with antibodies against syntaxin I, a marker for plasma membranes and against synaptotagmin I, a secretory granule marker (Söllner and Rothman, 1994). As shown in Fig. 1A,B, the pattern of anti-Gαi_3 immunoreactivity was identical to that obtained with anti-syntaxin I but differed from the anti-synaptotagmin I labelling (Fig. 1D,E). The cytofluorogram representing the pixels measured in the Gαi_3 and syntaxin images and the mask constructed from the colocalized pixels in the cytofluorogram (Fig. 1C) confirmed that Gαi_3 is entirely present on the plasma membrane in rat melanotrophs. Note the partial colocalization of synaptotagmin I with Gαi_3 at the cell periphery (Fig. 1F), most likely representing the subpopulation of secretory granules docked at the plasma membrane.

Double labelling experiments performed with the anti-

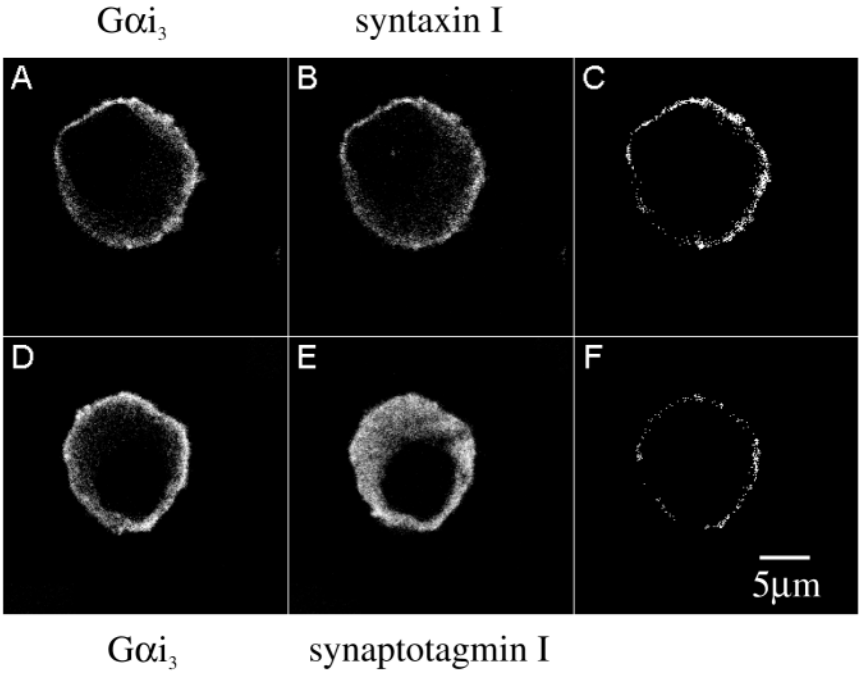

Fig. 1. Subcellular distribution of α subunit of heterotrimeric Gi_3-protein in rat melanotrophs**.** Double immunofluorescence confocal micrographs obtained with rabbit anti-Gαi_3 antibodies (A) and mouse anti-syntaxin I antibodies (B) or rabbit anti-Gαi_3 antibodies (D) and mouse anti-synaptotagmin I antibodies (E). Immunoreactivity was detected with Cy3-conjugated anti-rabbit and fluorescein-conjugated anti-mouse antibodies. Optical sections were taken trough the center of the nucleus. Cell diameter is approximately 12 μm. Two-dimensional scatter histograms of grey values were obtained from Gαi_3-syntaxin or Gαi_3-synaptotagmin labellings recorded simultaneously in the same section by a double exposure procedure. (C,F) illustrate the masks obtained after selection of the pixels double-labelled with Cy3 and fluorescein from the two-dimensional scatter histograms of grey values. Note the colocalization of Gαi_3 with syntaxin I in the cell periphery, suggesting the preferential association of Gi_3 with the plasma membrane.

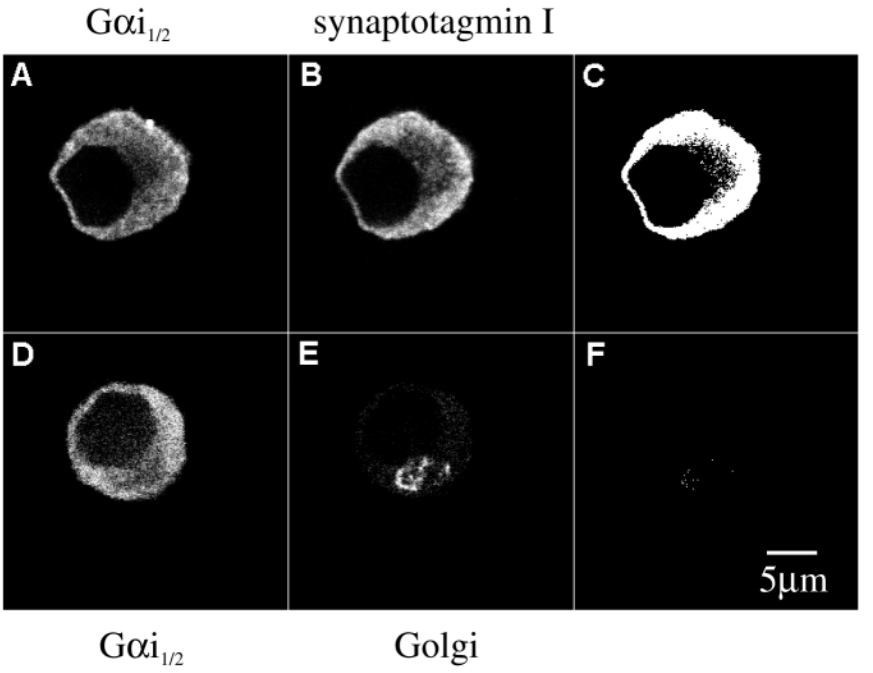

Fig. 2. Subcellular distribution of α subunits of heterotrimeric $Gi_{1/2}$-protein, secretory granules and Golgi apparatus in rat melanotrophs. Cells were double-labelled with rabbit anti-$G\alpha i_{1/2}$ antibodies (A,D) and either mouse anti-synaptotagmin I (B) or mouse anti-Golgi antibodies (E). Immunoreactivity was detected with Cy3-conjugated anti-rabbit antibodies and fluorescein-conjugated anti-mouse antibodies. Optical sections were made trough the center of the cell. Cell diameter is approximately 12 μm. Horizontal pairs of images represent the same cell. The dots corresponding to the co-localized pixels were used to construct the masks shown in C and F. $G\alpha i_{1/2}$ colocalizes with the secretory granule marker synaptotagmin I in cultured rat melanotrophs.

$G\alpha i_{1/2}$ and anti-synaptotagmin I antibodies or anti-$G\alpha i_{1/2}$ and anti-Golgi antibodies revealed the co-localization of $Gi_{1/2}$ with intracellular organelle(s) bearing synaptotagmin I (Fig. 2A-C). No apparent $Gi_{1/2}$ labelling could be detected on the Golgi apparatus (Fig. 2D,E), an observation confirmed by the absence of colocalized pixels in the mask illustrated in Fig. 2F. Thus, $G\alpha i_{1/2}$ appears to be preferentially associated with the membrane of secretory granules in melanotrophs.

Effect of mastoparan and synthetic C-terminal peptides corresponding to $G\alpha i_3$ and $G\alpha i_{1/2}$ on secretory activity of rat melanotrophs

Previous studies suggest a role of pertussis toxin-sensitive trimeric G proteins in regulated secretory activity of rat melanotrophs (Rupnik and Zorec, 1995). To further precise the function of the pertussis toxin-sensitive Gi_3- and $Gi_{1/2}$-proteins in the exocytotic pathway, we measured changes in membrane capacitance in rat melanotrophs by the whole-cell patch-clamp technique. Peptides corresponding to the C-terminal sequence of $G\alpha i_3$ and $G\alpha i_{1/2}$ were introduced into the cytosol with the patch-pipette. In cells dialysed with Ca^{2+}, membrane capacitance (C_m) increased by 16±3% after 5 minutes of recording (Fig. 3). In the presence of the $G\alpha i_3$ peptide, responses were not significantly different from controls (Fig. 4). In contrast, the inclusion of the synthetic $G\alpha i_{1/2}$ peptide resulted in a slight but significant increase of the extent of capacitance increase (Fig. 4). We then studied whether the Gα peptides may interfere with the effect of mastoparan on melanotroph secretory activity. Mastoparan, an amphiphilic tetradecapeptide from wasp venom, is a potent guanine nucleotide exchange promoter (Higashijima et al., 1990) thought to activate trimeric G proteins by interacting with the C-terminal domain of the α-subunit (Weingarten et al., 1990). Addition of 1 μM mastoparan increased C_m by 62±5%, which is significantly higher in comparison to controls (Figs 3 and 4). Addition of the synthetic $G\alpha i_3$ peptide completely neutralised this stimulatory effect of mastoparan peptide whereas the $G\alpha i_{1/2}$ peptide had no effect (Figs 3 and 4). Control experiments performed with a $G\alpha i_3$ peptide with a scrambled sequence confirmed that the $G\alpha i_3$ peptide specifically reversed the mastoparan-stimulated changes in membrane capacitance (Fig. 4). We also preinjected anti-$G\alpha i_3$-antibody to test its effect on mastoparan-induced increase of C_m. Anti-$G\alpha i_3$-antibody completely neutralized the effect ofmastoparan (Fig. 4). These results suggest that Gi_3 mediates the stimulatory effect of mastoparan on exocytosis in rat melanotrophs.

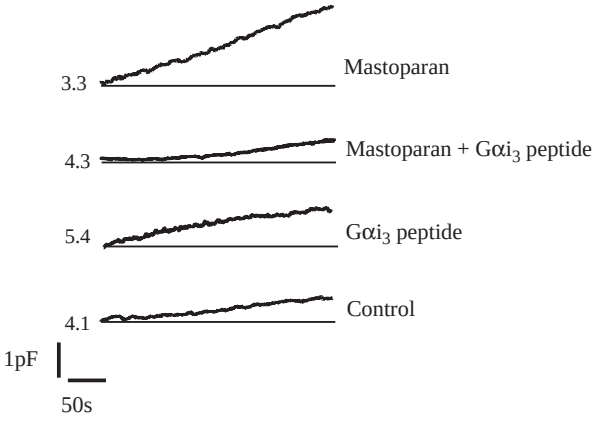

Fig. 3. Representative time-dependent changes in membrane capacitance in cells dialysed with a patch-pipette solution containing either 1 μM [Ca^{2+}] (Control), 1 μM [Ca^{2+}] with 1 μM mastoparan (Mastoparan), 1 μM [Ca^{2+}] with 1 μM mastoparan and 100 μM $G\alpha i_3$ synthetic peptide (Mastoparan + $G\alpha i_3$ peptide) or 1 μM [Ca^{2+}] with 100 μM $G\alpha i_3$ synthetic peptide ($G\alpha i_3$ peptide). Numbers adjacent to traces indicate resting membrane capacitance in pF. Traces were filtered at 10 Hz (4 pole Bessel, –3dB).

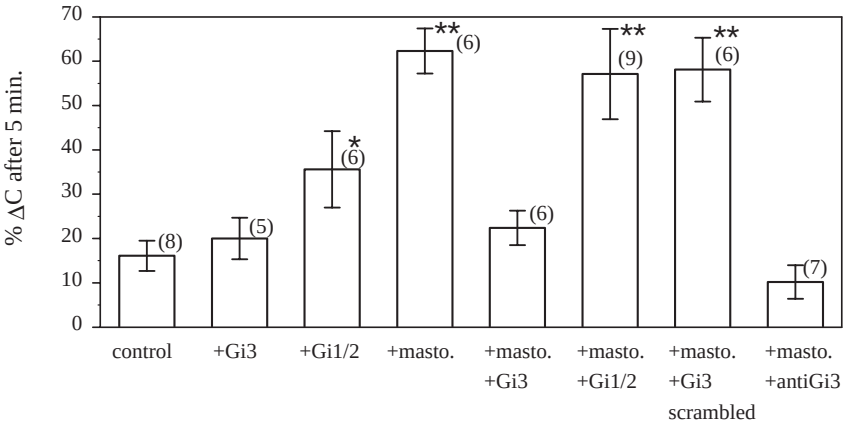

Fig. 4. Mean secretory responses in single melanotrophs measured as a relative change in membrane capacitance 300 seconds after the start of whole-cell recording relative to the resting membrane capacitance (% ΔC). Cells were dialysed with a patch-pipette solution containing 1 μM [Ca^{2+}] in the absence (control) or presence of either 100 μM Gαi_3 synthetic peptide (+Gi3), 100 μM G$\alpha i_{1/2}$ synthetic peptide (+Gi1/2), 1 μM mastoparan (+masto.), 1 μM mastoparan and 100 μM Gαi_3 synthetic peptide (+masto.+Gi_3), 1 μM mastoparan and 100 μM G$\alpha i_{1/2}$ synthetic peptide (+masto.+$Gi_{1/2}$), 1 μM mastoparan and 100 μM Gαi_3 synthetic peptide with a scrambled sequence (+masto +Gi3 scrambled), or 1 μM mastoparan in a cell preinjected with the anti-Gαi_3-antibody (+masto +antiGi3). Error bars represent standard error of the mean. Number of cells recorded is indicated in brackets. Asterisks indicate significant differences compared to the control (*P<0.05, **P<0.01, Student's t-test).

Anti-Gαi_3 antibodies affect the slow but not the fast kinetic component of exocytosis in rat melanotrophs

Whole cell dialysis with 1 μM Ca^{2+} elicits a slow gradual rise in C_m indicative of a net increase in exocytosis over endocytosis (Fig. 3, see Zupančič et al., 1994). In contrast, a rapid multiphasic exocytotic response is triggered by increasing cytosolic [Ca^{2+}] quickly and uniformly using flash photolysis of caged Ca^{2+} (Neher and Zucker, 1993). A UV light flash that photolyzed~20% of the loaded Ca^{2+}-bound NP-EGTA activated a biphasic membrane capacitance response (Fig. 5A,B) similar to that reported previously (Neher and Zucker, 1993; Thomas et al., 1993a,b; Heinemann et al., 1994; Kasai et al., 1996; Takahashi et al., 1997; Ninomiya et al., 1997; Xu et al., 1998) The flash discharge (arrow, Fig. 5A) transiently increased [Ca^{2+}] to 24 μM, which returned to baseline exponentially with a time constant of around 5 seconds. Membrane C_m rapidly increased by 805 fF and subsequently decreased due to slow endocytosis. The prominent C_m increase (termed slow exocytosis, labelled B in Fig. 5A, lower trace) was preceded by a smaller (148 fF) transient rise in C_m (termed fast exocytosis, labelled A, see inset in Fig. 5A, lower trace), which was clearly resolved at higher time resolution (Fig. 5A, inset). When NP-EGTA loaded into the cell lacked Ca^{2+}, a UV flash changed

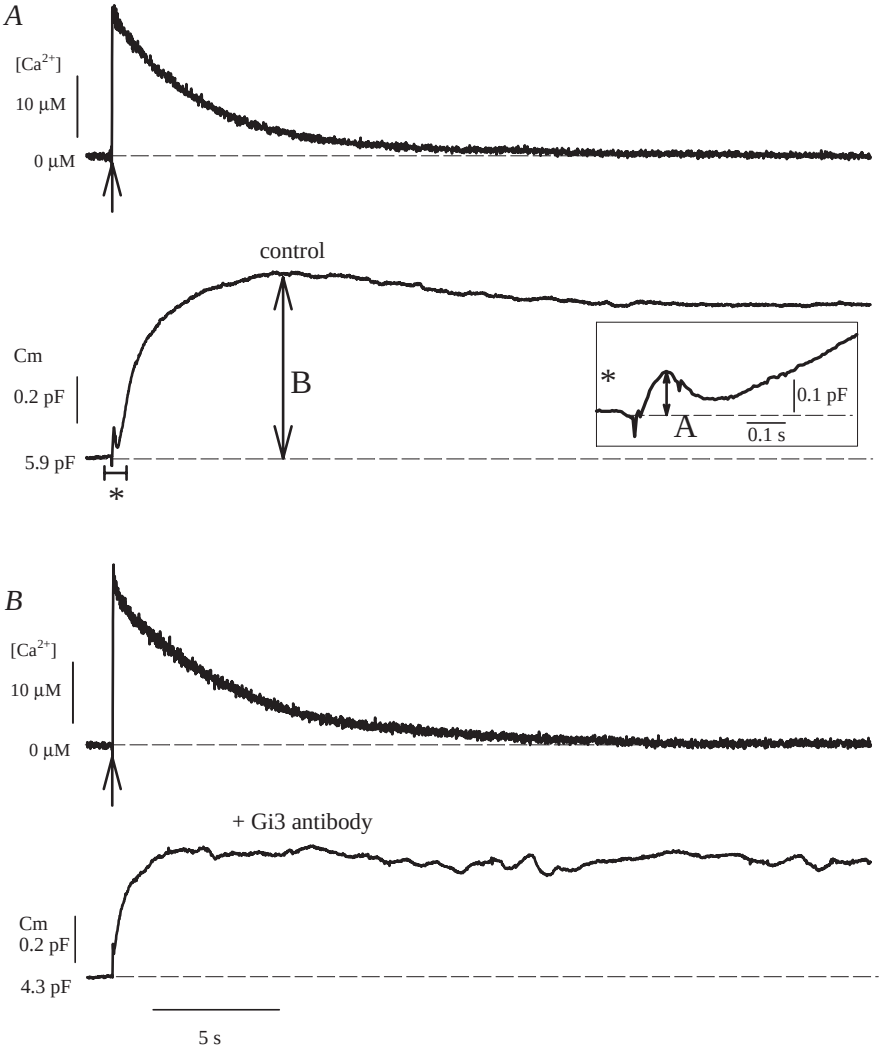

Fig. 5. (A) Representative time-dependent changes in $[Ca^{2+}]_i$ (top trace) and membrane capacitance (C_m, lower trace) after UV flash photolysis of Ca^{2+}-loaded NP-EGTA (arrow). Insert in the right part of the lower trace illustrates a high-time resolution of the C_m trace indicated by the asterisk. The amplitude of the fast response in C_m is labelled as A and the amplitude of the slow response is labelled as B. (B) A representative trace of $[Ca^{2+}]_i$ (upper trace) and membrane capacitance (lower trace) elicited by a transient increase in $[Ca^{2+}]_i$ in a cell preinjected with the anti-Gαi_3 antibody. Numbers adjacent to records in C_m indicate the resting membrane capacitance.

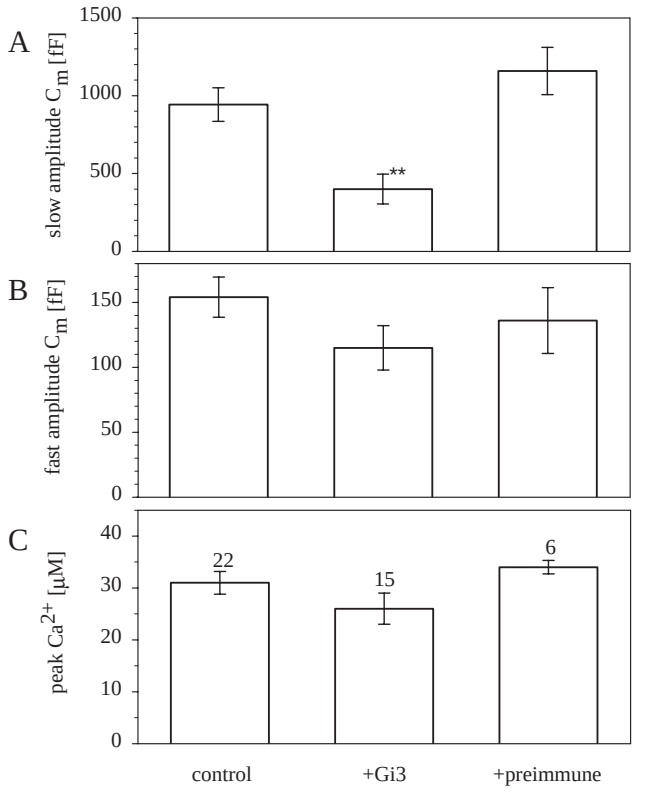

Fig. 6. Mean amplitude of the slow kinetic component (A) and mean amplitude of the fast kinetic component (B) of the biphasic secretory response elicited by a transient increase in $[Ca^{2+}]_i$ by UV flash photolysis. (C) Average peak response in $[Ca^{2+}]_i$ elicited by UV flash photolysis of Ca^{2+}-loaded NP-EGTA. Numbers adjacent to columns indicate the numbers of cells tested. Error bars indicate standard error of the mean and asterisks indicate significant differences compared to the control (**P<0.01, Student's t-test).

neither $[Ca^{2+}]$ nor C_m (not shown). The amplitude and rates of both the fast and slow components of the C_m increase were dependent upon the $[Ca^{2+}]$ levels achieved by uncaging of Ca^{2+}-NP-EGTA (M. Rupnik et al., unpublished).

Cells injected with anti-Gαi_3 antibodies exhibited C_m responses that were different to control cells or to cells injected with a preimmune serum (Fig. 6). The amplitude of the slow component of the UV flash-triggered capacitance response was significantly attenuated (Figs 5B, 6A). This decrease was not due to a reduction in the UV flash elicited cytosolic calcium as illustrated in Fig. 6C. Interestingly, the amplitude of the fast exocytosis was not affected by the anti-Gi_3 antibody pretreatment. Taken together, these results suggest that Gi_3-protein acts in the slow but not in the fast exocytosis of rat melanotrophs.

DISCUSSION

The aim of the present work was to study the role of trimeric G-proteins in regulated secretion of rat pituitary *pars intermedia* cells. Previous work suggested that subunits of various trimeric G-proteins reside on both intracellular and plasma membranes in pituitary cells (Muller et al., 1994) and that a pertussis toxin-sensitive G protein may play a role in regulated secretion from rat *pars intermedia* cells (Rupnik and Zorec, 1995). Using confocal immunocytochemistry to study the subcellular localization of the pertussis toxin-sensitive Gi_3 and $Gi_{1/2}$ proteins, we show here that Gαi_3 appears to be exclusively present in the plasma membrane (Fig. 1), an observation which correlates well with the distribution previously observed in secretory chromaffin cells (Vitale et al., 1996) and mast cells (Aridor et al., 1993). In contrast, we found that G$\alpha i_{1/2}$ is mainly associated with some intracellular compartment (Fig. 2), most likely the membrane of secretory granules.

To elucidate the function of Gi_3 and $Gi_{1/2}$ in regulated secretion of rat melanotrophs, we used the patch-clamp technique to monitor changes in membrane capacitance, a parameter linearly related to changes in membrane surface area. A net increase in surface area over the period of minutes is indicative of a net increase in exocytosis (Neher and Marty, 1982; Zupančič et al., 1994). Mastoparan, an activator of Gi proteins, enhanced the extent of capacitance increase by a factor of 4 to 5 (Figs 3 and 4). If the increase in membrane capacitance is due to inhibited endocytosis, then mastoparan should produce a similar effect at virtually free cytosolic Ca^{2+}. Dialysis of Ca^{2+}-free pipette solution resulted in an increase in membrane capacitance of a few % (Rupnik et al., 1997). Therefore, the mastoparan-enhanced increase in membrane capacitance at high cytosolic Ca^{2+} is very likely due to stimulated exocytosis. Inclusion of a synthetic peptide corresponding to the C terminus of Gαi_3 neither affected the time course nor the extent of membrane capacitance increase in rat melanotrophs. However, mastoparan-enhanced secretory responses were significantly attenuated in the presence of the Gαi_3 peptide. This observation cannot be attributed to some nonspecific effect of the Gαi_3 peptide since a scrambled version of the same peptide did not affect mastoparan-enhanced secretory responses (Fig. 4). Furthermore, the C terminus peptide corresponding to G$\alpha i_{1/2}$, which has a high sequence similarity with the Gαi_3 synthetic peptide, did not modify the mastoparan induced-secretory responses. Moreover, preinjection of anti-Gαi_3-antibodies abolished mastoparan-induced secretory responses (Fig. 4).These findings strongly suggest that mastoparan stimulates secretory activity in rat melanotrophs through the activation of the plasma membrane-associated Gi_3 protein. Interestingly, the participation of a plasma membrane-bound Gi_3 in the late stages of exocytosis has been previously reported in mast cells (Aridor et al., 1993) and in chromaffin cells (Vitale et al., 1996). In agreement with the present results, mastoparan triggers exocytosis in mast and chromaffin cells and the introduction of anti-Gi_3 antibodies or a synthetic peptide corresponding to the C-terminal sequence of Gi_3 into these cells specifically blocks mastoparan-evoked secretion (Aridor et al., 1993; Vitale et al., 1996). Thus, regulated exocytosis of large dense core vesicles in endocrine and neuroendocrine cells appears to involve a common molecular switch i.e. the activation of a trimeric Gi_3 protein located at the plasma membrane. Considering that mastoparan is mimicking membrane G protein-coupled receptors (Weingarten et al., 1990), it remains to be determined whether the Gi_3-mediated regulation of exocytosis is coupled to a plasma membrane-associated receptor or to some unknown endogenous

pseudoreceptor controlling the activation/inactivation cycle of Gi_3 (Vitale et al., 1994b).

Studies in diverse secretory systems indicate the ATP is required to prime the exocytotic apparatus whereas Ca^{2+} triggers the final ATP-independent fusion event (Bittner and Holz, 1992; Hay and Martin, 1992). In permeabilized chromaffin and mast cells, the mastoparan-activated Gi_3 stimulates the ATP-independent component of secretion (Vitale et al., 1996; Aridor et al., 1993), suggesting that Gi_3 is involved in the late fusion step of exocytosis. On the other hand, we show here that anti-Gαi_3 antibodies attenuate the slow but not the fast component of membrane capacitance biphasic responses triggered by a quick and spatially homogeneous rise in $[Ca^{2+}]_i$. Since slow kinetic component has been attributed to vesicles that need to advance to a state from which they can rapidly undergo exocytosis (Henkel and Almers, 1996), our results would suggest that Gi_3 may control early stages of the exocytotic pathway in melanotrophs. Alternatively, it has also been suggested that these multiple kinetic components of calcium-induced capacitance changes may represent distinct pathways of regulated exocytosis (Kasai et al., 1996; Ninomiya et al., 1997; Takahashi et al., 1997; Xu et al., 1998). Thus, the plasma membrane-associated Gi_3 may as well play a crucial role in distinct pathways of regulated exocytosis, which warrants further analysis to be carried out.

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