The EMBO Journal Vol.17 No.21 pp.6210–6218, 1998

Regulation of exocytosis from rat peritoneal mast cells by G protein βγ-subunits

Jef A.Pinxteren[1], Antony J.O'Sullivan[2], Peter E.R.Tatham and Bastien D.Gomperts

Secretory Mechanisms Group, Department of Physiology, University College London, University Street, London WC1E 6JJ, UK

[2]Present address: Department of Biological Sciences, University of Durham, South Road, Durham DH1 3LE, UK

[1]Corresponding author

We applied G protein-derived βγ-subunits to permeabilized mast cells to test their ability to regulate exocytotic secretion. Mast cells permeabilized with streptolysin-O leak soluble (cytosol) proteins over a period of 5 min and become refractory to stimulation by Ca^{2+} and GTPγS over ~20–30 min. βγ-Subunits applied to the permeabilized cells retard this loss of sensitivity to stimulation (run-down) and it can be inferred that they interact with the regulatory mechanism for secretion. While α-subunits are without effect, βγ-subunits at concentrations $>10^{-8}$ M enhance the secretion due to Ca^{2+} and GTPγS. Unlike the small GTPases Rac and Cdc42, βγ-subunits cannot induce secretion in the absence of an activating guanine nucleotide, and thus further GTP-binding proteins (likely to be Rho-related GTPases) must be involved. The enhancement due to βγ-subunits is mediated largely through interaction with pleckstrin homology (PH) domains. It remains manifest in the face of maximum activation by PMA and inhibition of PKC with the pseudosubstrate inhibitory peptide. Soluble peptides mimicking PH domains inhibit the secretion due to GTPγS and block the enhancement due to βγ-subunits. Our data suggest that βγ-subunits are components of the pathway of activation of secretion due to receptor-mimetic ligands such as mastoparan and compound 48/80.

Keywords: exocytosis/GTP-binding proteins/mast cells/PH domains/βγ-subunits

Introduction

Agents which stimulate the secretory reaction of mast cells fall into two main classes. Specific antigens (allergens) act indirectly to cross-link the cell surface receptors for IgE (Metzger, 1992), initiating a series of phosphorylations on tyrosine residues (Park *et al*., 1991). This leads to activation of phospholipase-Cγ (PLCγ) with consequent elevation of cytosol Ca^{2+} and activation of protein kinase C (PKC; Takai *et al*., 1979). The receptor-mimetic agents such as the wasp venom peptide mastoparan and compound 48/80, which are understood to bypass conventional receptors and activate GTP-binding proteins directly (Aridor *et al*., 1990; Higashijima *et al*., 1990), can induce exocytosis without activation of PLC and without elevation of cytosol Ca^{2+} (Aridor and Sagi-Eisenberg, 1991). Secretion from mast cells can also be induced by application of Ca^{2+}-carrying ionophores (Foreman *et al*., 1973; Bennett *et al*., 1980).

Regardless of the nature of the stimulating agent, exocytosis from these and from other cells of hematopoietic origin is understood to be mediated through the activation of GTP-binding proteins called G_E (reviewed in Gomperts, 1990). Depletion of guanine nucleotides by treatment of mast cells with inhibitors of IMP dehydrogenase (such as mycophenolic acid) suppresses the extent of secretion due to all forms of stimulation (Marquardt *et al*., 1987; Wilson *et al*., 1989). Conversely, exocytosis can be induced by the direct introduction of stable analogues of GTP (GTPγS) into the cytosol of permeabilized mast cells (Howell *et al*., 1987), by microinjection (Tatham and Gomperts, 1991) or in the whole cell patch–clamp configuration (Fernandez *et al*., 1984). Under some conditions, this can occur in the effective absence of Ca^{2+} (Lillie and Gomperts, 1992). However, only the stimulus due to the receptor-mimetic agents is suppressed in cells treated with pertussis toxin (Saito *et al*., 1987), and this indicates that a G_E protein active in this pathway is a substrate for ADP ribosylation.

The heterotrimeric G protein G_{i3} has been proposed as a regulator of exocytosis in the pathway initiated by the receptor-mimetic agents and is understood to operate at a site subsequent to the elevation of intracellular Ca^{2+} (Aridor and Sagi-Eisenberg, 1991; Aridor *et al*., 1993). Importantly, the identification of G_{i3} as the pertussis toxin-sensitive G_E transducing the signals from non-immunological receptor-mimetic agents was based on the finding of inhibition of secretion by reagents (peptides and antibodies) specific to the C-terminus of the α_{i3}-subunit applied to permeabilized cells (Aridor *et al*., 1993). Comparable reagents reactive at the C-terminus of G_{i2} were without effect. These procedures necessarily prevent coupling of the G protein to its receptor (Rasenick *et al*., 1994), and no information has so far been forthcoming regarding which of the subunits, α or βγ, convey the onward signals which induce exocytosis

Here we demonstrate that βγ-subunits introduced into permeabilized mast cells retard the loss of sensitivity to stimulation (run-down) which occurs during the period following permeabilization and amplify the extent of secretion induced by Ca^{2+} and GTPγS. In contrast, α_{i3}-subunits are without effect.

Results

At the outset of this work, we have had to deal with the problem that heterotrimeric GTP-binding proteins can only be released from membranes and then maintained in

Table I. Properties of some common detergents in the run-down assay

Detergent	c.m.c. (mM)	Concentration for 5% inhibition (mM)	Concentration for total inhibition (mM)	Concentration for total lysis (mM)
Cholate	9–15	0.01	0.1	10
Cholate (purified)[a]	9–15	0.03	0.5	>10
Lubrol-PX	0.1	0.01	0.1	0.3
Octyl-β-glucoside	20–25	0.06	10	>10
Triton X-100	0.2–0.9	0.002	0.1	0.3
Genapol C-100	–	0.002	0.03	0.3
CHAPS	6–10	0.3	3	>10

The c.m.c. values are as reported by the suppliers at 50 mM Na^+. The experimental concentrations referred to in the table are taken from dose–response curves of the respective detergent at 5 min run-down in the assay as described in the text.
[a]Sternweis and Pang (1990).

solution in the presence of detergents. The detergents used for solubilization, purification and storage of the heterotrimers and their α- and βγ-subunits (cholate and Lubrol) are not cell-friendly. They are incompatible with the maintenance of mast cells in secretion experiments. We have therefore surveyed a panel of detergents with the aim of discovering conditions under which we might be able to keep the subunits in solution while causing minimal disturbance to the secretory reaction. As shown in Table I, all the detergents tested caused total cell lysis when applied at high concentrations (apparent as 100% release of the contained hexosaminidase under non-stimulating conditions). We found that as the concentrations were reduced, the detergents all caused total inhibition of the response of permeabilized mast cells to GTPγS and Ca^{2+} (Table I). As pointed out by others (Chernomordik *et al*., 1993), such inhibition, which occurs below the critical micelle concentration (c.m.c.), is probably caused by the interference of the amphipathic molecules with the process of membrane fusion in exocytosis. For CHAPS, the concentration causing maximal inhibition (3 mM) approaches the c.m.c. (5 mM) most closely and, importantly, there is an extensive concentration range (up to 0.1 mM; see Figure 1) within which the effect on the secretory response is minimal, but which is sufficient to maintain G protein-derived subunits in solution (Logothetis *et al*., 1988).

In all subsequent work, we have isolated G proteins in solutions containing cholate (1%) and then performed detergent exchange using an anion exchange column (MonoQ). The proteins were eluted in CHAPS 0.3% (4.9 mM) just prior to applying them to the permeabilized mast cells. The elution profile of βγ-subunits is illustrated in Figure 2. After allowing unbound material to wash through, βγ-subunits were eluted with a salt gradient (0–1 M NaCl made up in 0.3% CHAPS, pH 8). The high resolution system also causes some separation of different βγ-subtypes so that protein eluting after the main peak appears to be enriched with β_2 and protein eluting earlier is enriched with β_1. When tested on partially run-down cells, all fractions containing βγ-subunits were found to enhance the level of secretion stimulated by Ca^{2+} and GTPγS (see Figure 2) but it appears that the specific activity of those fractions enriched in β_2 may be more active in the support of secretion than those enriched in β_1. This variation in specific activity could arise from the separation of modified or denatured proteins, or co-purification of minor peptides which are invisible by our detection methods. Alternatively, it could represent a real difference in the activity of different βγ-subunits. However, as reported in Table II, while the response to the recombinant proteins $\beta_1\gamma_2$ and $\beta_2\gamma_3$ was of a magnitude similar to that obtained with βγ-subunits purified from brain, we were unable to distinguish any significant differences between these two individual classes. In all further run-down experiments involving proteins purified from bovine brain, the fractions containing βγ-subunits were combined and then diluted (to give a final concentration of 50 μM CHAPS) into suspensions of mast cells just before permeabilization.

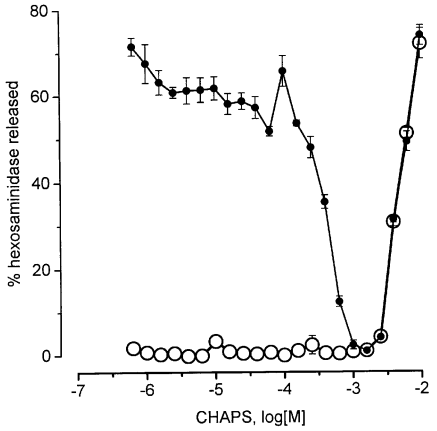


Fig. 1. Effect of the detergent CHAPS on secretion from permeabilized mast cells.The cells, pre-treated with SL-O at 0°C, were transferred into a buffered salts solution, pH 6.8, containing Mg·ATP (100 μM), Ca·EGTA (3 mM, formulated to set pCa5) and GTPγS (100 μM). CHAPS was provided at the concentrations indicated. After incubation at 30°C for 10 min, the systems were quenched by addition of ice-cold buffered salts solution containing EGTA (5 mM) and the cells were sedimented by centrifugation. Samples of the supernatants were removed for measurement of secreted hexosaminidase. ○, unstimulated cells; ●, cells stimulated with Ca^{2+} and GTPγS. Data points are means ± SEM (n = 4 determinations of secretion).

Enhancement of secretion from permeabilized mast cells by purified βγ-subunits

Mast cells stimulated by application of Ca^{2+} and GTPγS at the time of permeabilization secrete approaching 100% of their contained hexosaminidase and histamine. However, as shown in Figure 3, when the stimulus is provided to the permeabilized cells after a delay of some minutes, the extent of secretion is reduced and then declines progressively to negligible levels over a period of ~30 min.

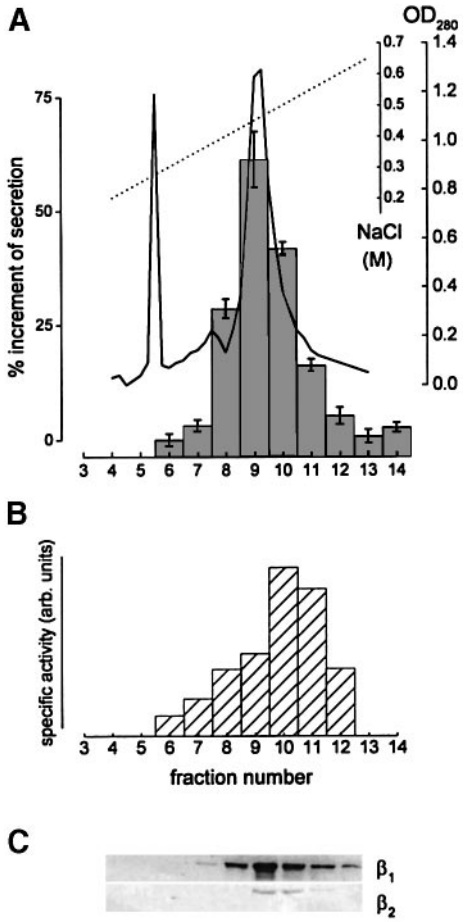


Fig. 2. Chromatography of βγ-subunits on MonoQ Sepharose: exchange of cholate for CHAPS. (**A**) Elution profile (OD_{280}) and run-down assays of the fractions. βγ-Subunits, generated by activation of brain G proteins (mainly a mixture of G_i and G_o) and solubilized in cholate, were separated by hydrophobic interaction chromatography on phenyl-Sepharose. They were then loaded onto a MonoQ column which was eluted with a gradient of NaCl (0–1 M) in buffer containing 0.3% CHAPS, and 0.5 ml fractions were collected. (**B**) The specific activity (% increment) of stimulated secretion divided by the protein concentration. Note that the peak specific activity of the fractions is to the right of the main protein peak. (**C**) Western blotting analysis of the fractions using specific antibodies to β_1- (Ab 4-49; 1:1000) and β_2-subunits (Ab B2N1; 1:1000). This indicates that the high resolution MonoQ column is causing some separation of different βγ-subtypes, with enrichment of β_1 over β_2 in the fractions leading up to the peak and enrichment of β_2 relative to β_1 in the tail fractions.

The experiment illustrated in Figure 3 indicates that mixed βγ-subunits (10^{-7} M, isolated from bovine brain) provided at the time of permeabilization can retard run-down. This had the result that cells stimulated with Ca^{2+} and GTPγS after a delay of 10 min were capable of releasing twice the amount of hexosaminidase (44% secretion) as the control cells and were still capable of 15% secretion when stimulated after a delay of 30 min. The effect of the βγ-subunits upon cell responsiveness always took a few minutes to become apparent (see Figure 3). This appears to be a general characteristic of all proteins modulating the run-down process, both negatively and positively (O'Sullivan *et al.*, 1996; Brown *et al.*, 1998), and most probably registers the time taken for diffusion into the cells through the permeability lesions and then for interaction with intracellular targets.

The concentration–effect relationship for mixed brain

Table II. Fractional enhancement of secretion by recombinant βγ-subunits

Subtype	3×10^{-8} M	10^{-7} M
$\beta_1\gamma_2$	18.0 ± 2.1%	45.4 ± 5.3%
$\beta_2\gamma_3$	18.8 ± 3.3%	46.3 ± 4.7%

The values express the fractional enhancements of secretion (± SEM, $n = 4$ separate determinations) due to the presence of the βγ-subtype at the indicated concentrations and under the same conditions as in the experiment illustrated in Figure 4.

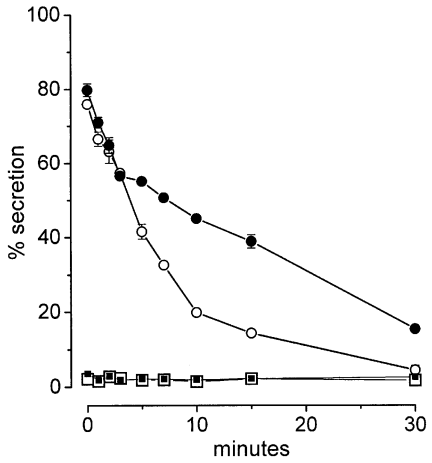


Fig. 3. Time course of run-down of permeabilized mast cells in the presence of native or boiled βγ-subunits. Mast cells, pre-treated with SL-O at 0°C, were permeabilized by transfer to a buffer (pH 6.8) containing Mg·ATP (100 μM) with sufficient EGTA (0.1 mM) to suppress Ca^{2+} below 10^{-8} M, and in the presence of βγ-subunits (10^{-7} M). The concentration of CHAPS was 50 μM. At the indicated times, the cells were stimulated to secrete by addition of a solution containing GTPγS (100 μM) and Ca·EGTA (3 mM) formulated to regulate pCa5. After a further 10 min incubation, the systems were quenched by addition of ice-cold salts buffer containing EGTA (5 mM). The cells were sedimented and the supernatants were sampled and assayed for secreted hexosaminidase. ○ and ●, cells stimulated with GTPγS and Ca^{2+}, □ and ■, unstimulated cells; filled symbols indicate the presence of native protein. Data points are means ± SEM ($n = 4$ determinations).

βγ-subunits in the run-down experiment is illustrated in Figure 4. In this, and all other experiments of this form, we tested the effect of the exogenous proteins on the secretory response induced by Ca^{2+} and GTPγS provided 10 min after permeabilization. As in this experiment, at the optimal concentration, the additional extent of secretion due to the provision of βγ-subunits is typically ~30%. The reduced extent of enhancement which occurs at still higher concentrations of βγ-subunits (see Figure 4) is unlikely to be due to the presence of excess detergent since this was always adjusted to a constant concentration (50 μM) and all conditions were referred to controls containing boiled protein and detergent. In contrast, α-subunits, mainly G_o and G_i derived from brain, are without effect. Furthermore, recombinant *N*-myristoylated α_{i3}-subunits (pre-activated with $[AlF_4]^-$) presented in the range 10^{-8}–10^{-5} M were also without effect, neither as a stimulus nor on the extent of secretion induced by GTPγS (see inset, Figure 4).

Although βγ-subunits behave in many systems as an activated component of G proteins (Neer and Clapham, 1988; Clapham and Neer, 1993), their effect on secretion from mast cells depends absolutely on the presence of an

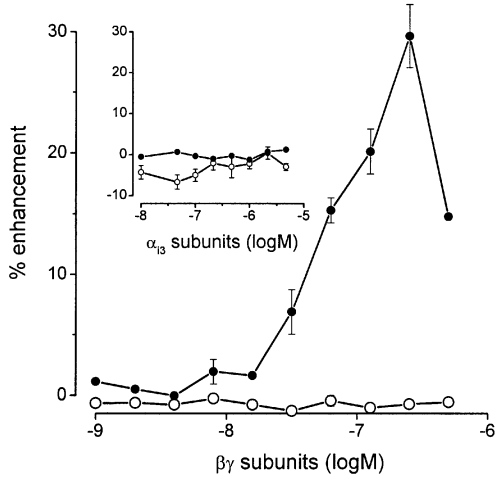


Fig. 4. Concentration–effect relationship for the enhancement of secretion by βγ-subunits. In this experiment, the cells were allowed to run down in the presence of βγ-subunits for 10 min before the stimulus was applied. The final concentration of CHAPS was 50 μM throughout. For other details, see the legend to Figure 3. Results are expressed as mean enhancements of secretion ± SEM (n = 4 determinations) (i.e. the difference due to native minus boiled protein). Similar results were obtained on three separate occasions. The data presented in the inset illustrates the result of a similar experiment in which permeabilized mast cells were allowed to run down in the presence of $[AlF_4]^-$-pre-activated α_{i3}-subunits. Open symbols represent release from unstimulated cells.

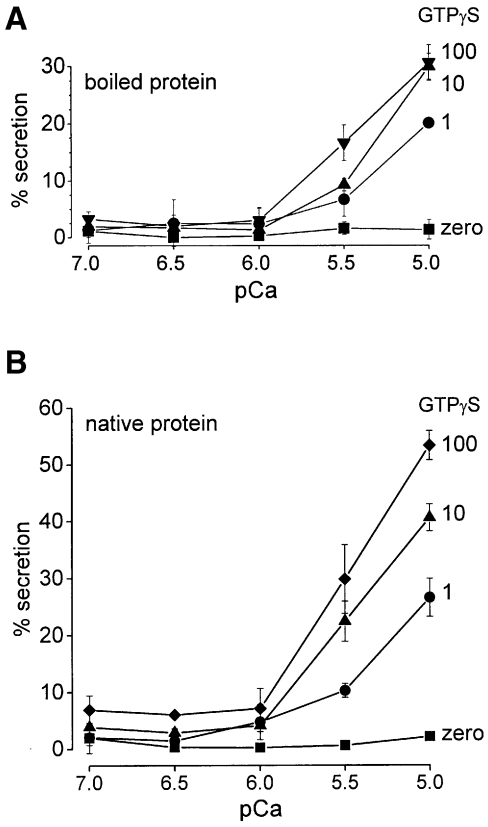
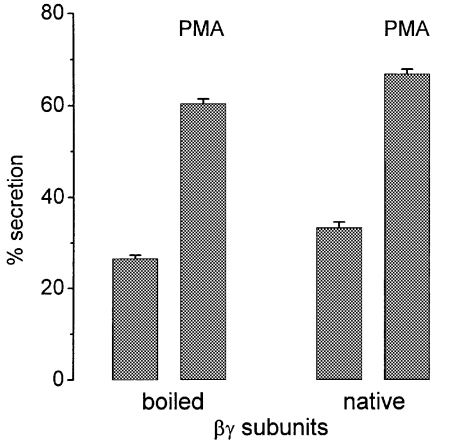


Fig. 5. Dependence of secretion on Ca^{2+} and GTPγS: enhancement by βγ-subunits. Permeabilized mast cells were allowed to run down for 10 min in the presence of βγ-subunits (2×10^{-7} M) as outlined in the legend to Figure 4. They were then transferred to solutions containing Ca^{2+} and GTPγS as indicated, and incubated for a further 10 min. After centrifugation, samples of supernatant were withdrawn for measurement of secreted hexosaminidase. GTPγS (μM): zero, ■; 1, ●; 10, ▲; 100, ◆.

activating guanine nucleotide. We measured the enhancement by βγ-subunits of the secretion induced by a wide range of concentrations of Ca^{2+} and GTPγS. The results of the experiment illustrated in Figure 5 indicate that βγ-subunits cause an elevation in the extent of secretion induced by all activating concentrations of GTPγS. Even in the presence of substimulatory concentrations of Ca^{2+}, the low levels of GTPγS-induced secretion generally are doubled (although this effect is not significant below pCa6) by the presence of βγ-subunits. It follows from this that the enhancement of secretion due to βγ-subunits must be mediated through the action of other GTP-binding proteins**.**

Although there is no requirement for ATP when mast cells are stimulated to secrete by application of Ca^{2+} and GTPγS at the time of permeabilization, the rate of run-down is far more rapid in its absence. The system becomes fully dependent on the presence of ATP after ~5 min (Churcher *et al*., 1990). The effect of ATP in maintaining the responsiveness of permeabilized cells is understood to be mediated by phosphorylations catalyzed by PKC (Howell *et al*., 1989). We have considered the possibility that the effect of βγ-subunits could be mediated by a phosphorylating enzyme, in particular PKC, due to the generation of diglyceride as a consequence of the activation of phospholipase C. We therefore investigated the effect of reagents known to cause stimulation or inhibition of PKC in the presence of either native or heat-inactivated βγ-subunits. The experiment illustrated in Figure 6 ('boiled') confirms that treatment of run-down cells with phorbol myristate acetate (PMA) enhances the extent of secretion. The amount of extra release due to PMA (100 nM), ~30% in the experiment shown, is comparable with that induced by an optimal concentration of βγ-subunits (see Figure 4). In this experiment, the βγ-subunits were applied at a suboptimal concentration (5×10^{-8} M) which enhanced the extent of secretion by 6.9% (P = 0.00019, one-way ANOVA). When the two reagents were applied together, their combined effect was greater (6.4%, P = 0.0002) than that of PMA alone, and thus the enhancements were approximately additive (see Figure 6, 'native'). We conclude that although the enhancement of secretion by βγ-subunits may be due, in part, to activation of the PLC–PKC cascade, a significant component of the effect must have another origin. This conclusion is supported by our finding that the pseudosubstrate inhibitor peptide ($PKC_{19–36}$, K_i = 15 μM) has no effect on the extent of secretion induced by Ca^{2+} and GTPγS after 10 min run-down when tested at concentrations up to 100 μM (not shown). These experiments suggest that PKC itself plays only a minor role in the regulation of secretion in these cells.

We have also investigated inhibitors of phosphatidylinositol 3-kinase (PI-3 kinase) but this had no noticeable effect. Compound LY294002 [reported IC_{50} = 1.4 μM (Vlahos *et al*., 1994)] applied at 10 μM did not significantly alter the extent of secretion induced by Ca^{2+} and GTPγS applied 10 min after permeabilization (55.4 ± 3.6% compared with control 61.7 ± 6.9%). Similarly, there was no effect on the secretion from cells stimulated at the time of permeabilization. Likewise, wortmannin was without effect.



				PMA		
βγ	mean	variance	n	mean	variance	n
boiled	26.5	4.4	8	60.5	13.1	12
native	33.4	10.5	8	66.9	12.0	12
p = 0.00019				p = 0.0002		

Fig. 6. Enhancement of secretion by PMA and βγ-subunits. A suboptimal concentration (5×10^{-8} M) of βγ-subunits was applied together with PMA (100 nM) and the cells were allowed to run down for 10 min before stimulation. Boiled protein was used as control. The results of an analysis of variance (ANOVA), presented in the box, indicate that additional stimulation of secretion by βγ-subunits was highly significant both in the absence (6.9% secretion) and presence of PMA (6.4% secretion). Similar results were obtained on five separate occasions.

Effect of the pleckstrin homology domain (PH)-containing β-adrenergic receptor kinase 1 C-terminal peptide (βARK$_1$-Cp)

We investigated the effect of the soluble PH domain protein βARK$_1$ C-terminal peptide on the enhancement of secretion by βγ-subunits. In a typical experiment, we compared the secretion from cells that had run down for 10 min in the presence of 5×10^{-7} M βγ-subunits, with that from cells additionally supplemented with an equimolar concentration of βARK$_1$-Cp. The βγ-subunit-treated cells secreted 45.0 ± 2.7% of their total hexosaminidase (compared with 38.5 ± 2.3% for control cells), whereas when βARK$_1$-Cp was additionally present, the level of secretion was only 38.9 ± 2.2%.

At higher concentrations, βARK$_1$-Cp also inhibits secretion due to Ca^{2+} and GTPγS. The experiment illustrated in Figure 7 shows that, in the absence of βγ-subunits, βARK$_1$-Cp inhibits the secretion due to Ca^{2+} and GTPγS when presented at concentrations in the range 10^{-7}–10^{-5} M. This suggests that an endogenous PH domain protein is an integral component of the normal regulatory pathway. In the knowledge that the PH domain of βARK$_1$ binds not only to βγ-subunits but also to polyphosphoinositides (Harlan *et al*., 1994), we have tested mutants which display selectivity in their interactions with βγ-subunits and phosphatidylinositol-4,5-bisphosphate (PIP_2) (Touhara *et al*., 1995). The mutants L647G and WAA (two Ala residues inserted after Trp643) which have a reduced (20-fold) affinity for βγ-subunits, and K645E, which binds βγ-subunits with an intermediate affinity, all suppressed the secretion induced by Ca^{2+} and GTPγS (Figure 8) to the same extent as the wild-type βARK$_1$-Cp (see Figure 7). Since all these mutants have comparable binding affinities for PIP_2, we conclude that besides βγ-subunits, the presence of PIP_2 is also necessary for secretion. Phosducin, a βγ-binding protein with negligible PIP_2-binding activity (Xu *et al*., 1995) was also found to cause inhibition of secretion to an extent similar to the βARK$_1$ peptides (Figure 8).

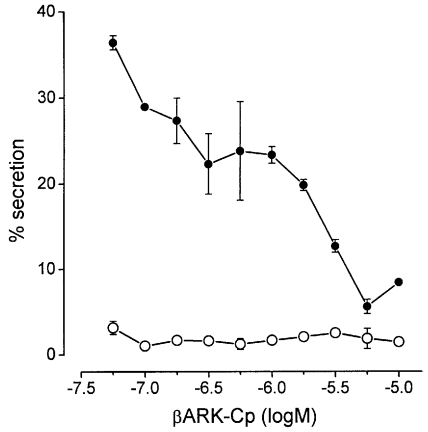


Fig. 7. Concentration–effect relationship for the inhibition of secretion by βARK$_1$-Cp. In this experiment, the cells were run down for 10 min in the presence of the peptide (as a GST fusion protein) before the stimulus was applied. Results are expressed as means ± SEM (n = 4 determinations). Similar results were obtained on three separate occasions. ●, cells stimulated with Ca^{2+} (pCa5) and GTPγS; ○, unstimulated cells.

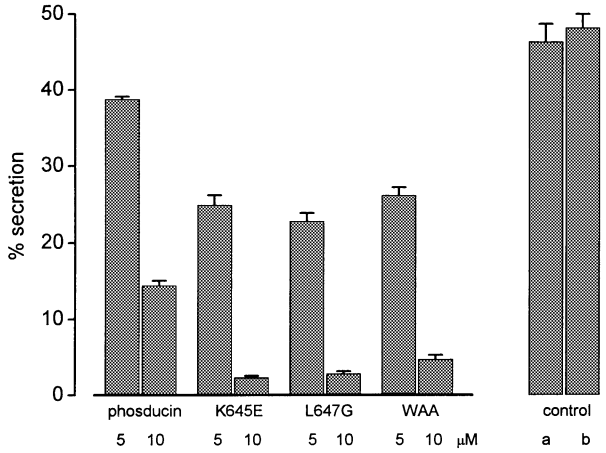


Fig. 8. Inhibition of secretion by phosducin and by mutants of the βARK$_1$-Cp. Cells were run down for 10 min in the presence of 5 or 10 μM phosducin or one of the βARK$_1$-Cp mutants L647G, K645E and WAA (as GST fusion proteins) before the stimulus (as in Figure 7) was applied. Results are expressed as means ± SEM (n = 4 determinations). No protein (a) or 10^{-5} M GST (b) were used as controls.

Discussion

Secretory cells, permeabilized by reagents such as streptolysin O (SL-O) or digitonin, leak soluble proteins (monitored as lactate dehydrogenase) within minutes but their propensity to respond to stimulation generally declines over a much longer time period. This so-called 'run-down' has been ascribed to the loss of tethered proteins which may act as essential regulators or even as components of

the fusion mechanism leading to exocytosis (Howell and Gomperts, 1987; Ali and Burgoyne, 1990; Nishizaki *et al*., 1992; Matsuda *et al*., 1994; O'Sullivan *et al*., 1996). Such preparations provide the opportunity to investigate the effects of exogenous proteins, which might replace soluble proteins lost by leakage, or others which modulate the activity of endogenous regulators retained in the permeabilized cells.

We recently have reported that the Rho-related GTPases Rac and Cdc42 can retard the rate of run-down of permeabilized mast cells (Brown *et al*., 1998). When pre-activated by binding GTPγS, they are both capable of inducing substantial secretion in the absence of free guanine nucleotide, and this indicates that they can assume the role of the endogenous GTPases which normally mediate the activating signals for exocytosis. Confidence in this conclusion is found in the inhibition of exocytosis by RhoGDI (Mariot *et al*., 1996; O'Sullivan *et al*., 1996) which binds to all of the Rho-related GTPases including Rac and Cdc42 (Tanaka *et al*., 1995). When permeabilized mast cells are treated with pre-activated Rac or Cdc42 together with free GTPγS, a further increment in secretion can be induced (Brown *et al*., 1998). Depending on which Rho-related GTPase is applied as the stimulus, the effect of free GTPγS either amplifies the response, as with Rac, or is interactive, sensitizing the system so that secretion occurs at a reduced concentration of the applied GTPase, as with Cdc42. Either way, the extra response to GTPγS indicates that there are additional GTP-binding proteins which are retained in the permeabilized cells and which are operative in the regulatory pathway for secretion.

The reaction pathway initiated by the receptor-mimetic agents mastoparan and compound 48/80 can be inhibited by pertussis toxin (Saito *et al*., 1987) and by reagents which interact or interfere with the function of the C-terminal domain of the α-subunits of the heterotrimeric G protein G_{i3} (Aridor *et al*., 1993). Necessarily, because of the indirect nature of these approaches, it has not been possible to distinguish which subunit, the α or the βγ, is instrumental in transmitting the signal for secretion. Furthermore, because of the need to provide an activating guanine nucleotide, it has not been possible to rule out the possibility that further GTPases might be involved in the control pathway for secretion.

Our work reported here shows that exogenous βγ-subunits are capable of enhancing the extent of secretion in partially run-down cells but, unlike the monomeric GTPases Rac and Cdc42, they are unable to induce secretion in the absence of an activating guanine nucleotide. In contrast to Rac and Cdc42, βγ-subunits cannot induce secretion in their own right. Because of the nature of their attachment to the plasma membrane, it is improbable that the exogenous βγ-subunits are replacing any that have been lost from the cell. What our experiments show is that they act to enhance the secretion induced by other GTPases. In view of the essential roles of the Rho-related GTPases, the βγ-subunits are therefore likely to function as upstream activators or modulators of Rac and/or Cdc42.

The binding and activation of effectors by βγ-subunits appear to be linked in many situations to the presence of PH domains (the interaction with α-subunits is an obvious exception). These are found on several key enzymes in cell signalling, including PLC, Cdc24, βARK_1, phosducin and PKCμ (Saraste and Hyvonen, 1995). βARK_1-Cp, which is in effect a soluble PH domain, binds βγ-subunits with high affinity (Touhara *et al*., 1994) and we find that it abolishes the enhancement of secretion induced by βγ-subunits. More importantly, βARK_1-Cp inhibits the secretion induced by GTPγS, as does phosducin, another βγ-binding peptide (Xu *et al*., 1995). This raises the possibility of an interaction involving a PH domain in the normal pathways regulating exocytosis. In view of the finding that mutants of the βARK_1-Cp having reduced affinities for βγ-subunits still cause inhibition of GTPγS-induced secretion, it is likely that other interactions of PH domains, notably with polyphosphoinositides, are of importance. Previous work has demonstrated that maintenance of polyphosphoinositides is an essential requirement for exocytosis from neuroendocrine cells (Eberhard *et al*., 1990; Hay *et al*., 1995).

Our work extends the understanding of mechanisms by which GTP-binding proteins act to regulate secretory processes. G protein-mediated exocytosis is by no means limited to mast cells, nor even to the cells of hematopoietic origin (Gomperts, 1990; Lindau and Gomperts, 1991). A good case in point is the pancreatic β-cell and its associated cell lines (Lang *et al*., 1995). Here too, secretion can be induced by receptor-mimetic ligands such as mastoparan and, following permeabilization (Jones *et al*., 1993; Komatsu *et al*., 1993), by application of GTPγS (Vallar *et al*., 1987). Insulin-secreting tumour cells overexpressing βγ-subunit-binding proteins have a reduced secretory response to bombesin, and an activating role for βγ-subunits has been inferred (Zhang *et al*., 1998). In the light of the finding that free GTPγS enhances the affinity of pre-activated Cdc42, but not Rac, in the secretory reaction of mast cells (Brown *et al*., 1998), it will be worth considering if the signal emanating from the receptor-mimetic ligands, is channelled through a pathway involving interaction of βγ-subunits with Cdc42. A plausible point of communication could involve the products of the Lfc and Dbs proto-oncogenes (Whitehead *et al*., 1995a,b), homologues of Cdc24, both of which express PH domains in tandem with putative guanine nucleotide exchange factor domains. A similar pathway linking G proteins and Cdc42 and/or Rac has been implicated in the activation of Jun kinase in NIH 3T3 cells (Coso *et al*., 1996).

Materials and methods

Materials

Deep-frozen bovine brains were obtained from First Link UK Ltd (Brierley Hill, West Midlands, UK). SL-O was from Murex Diagnostics (Dartford, Kent, UK). CHAPS, Genapol C-100, LY294002, wortmannin, $PKC_{19–36}$ and all commercial primary antibodies were from Calbiochem (Nottingham, UK). Lubrol PX was from ICN, octyl-β-glucoside and dithiothreitol (DTT) were from Alexis (Läufelfingen, Switzerland). Secondary antibodies were from Bio-Rad. Triton X-100 and cholate were from Sigma. Protein concentrations were determined using BCA assay kits from Pierce (Chester, UK). Insect cell culture media, fetal bovine serum, Pluronic F68 and all other supplements were from Gibco-BRL (Life-Technologies; Paisley, UK). [^{35}S]GTPγS (462.5 MBq/ml) was from Du Pont-NEN (Stevenage, UK). All other chemicals were of the highest quality from standard commercial sources.

All chromatographic columns and equipment were obtained from Pharmacia (Uppsala, Sweden). Membrane-supported microtitre test plates

(Event detect) and filtration unit (Event 4160) were from Eppendorf (Merck Ltd, Poole, UK). Phosphorescence imaging plates (Fuji), phosphorescence imaging apparatus (FujiBAS1000) and software (Tina) were from Raytek (Sheffield, UK).

Escherichia coli strains containing plasmids for α_{i3} and NMT were provided by Jeffrey Gordon (St Louis, MO). Baculoviruses encoding the β_1, β_2, γ_2 and γ_3 heterotrimeric G protein subunits were provided by James Garrison (Charlottesville, VA). Plasmids encoding βARK_1-Cp, mutants thereof, and phosducin were provided by Robert Lefkowitz (Durham, NC). Antibodies against β_1-subunits (4–49) and β_x-subunits (B-600) were provided by Susanne Mumby (Dallas, TX), and antibodies against β_1- (BN1), β_2- (B2N1), γ_2- (BG) and γ_3-subunits (CG) were provided by Narasimhan Gautam (St Louis, MO).

Mast cells

Cells were isolated by peritoneal lavage of male Sprague–Dawley rats (>300 g) and mast cells were purified (>98%) by centrifugation through a Percoll cushion as previously described (Gomperts and Tatham, 1992).

Calcium buffers

Calcium/EGTA buffers were prepared by mixing solutions, made up at identical concentrations adjusted to pH 6.8, of EGTA and end-point titrated Ca·EGTA, according to a computer program as previously described (Gomperts and Tatham, 1992).

Secretion measurements

Cells, suspended in an iso-osmotic salts buffer solution (137 mM NaCl, 2.47 mM KCl, 1 mM $MgCl_2$, 20 mM PIPES, pH 6.8) supplemented with bovine serum albumin (BSA; 1 mg/ml), were incubated with metabolic inhibitors [2-deoxyglucose (0.6 mM) and antimycin A (10 μM)] for 5 min at 30°C, then cooled on ice and added to SL-O (final concentration 1.6 IU/ml) in the same buffer. After 5 min, unbound SL-O was washed away by dilution and centrifugation (Larbi and Gomperts, 1996). Run-down was initiated by transferring the cells to pre-warmed (30°C to cause permeabilization) iso-osmotic buffer supplemented with Ca·EGTA (0.3 mM to regulate pCa 8) and MgATP (100 μM) together with proteins under test contained in the wells of 96-well microtitre plates. Cell concentration was ~3×10^4 per well. After allowing predetermined times for run-down, the cells were stimulated to secrete by adding pre-warmed solutions containing Ca·EGTA buffer (3 mM final) formulated to regulate pCa5 (or pCa7 as control) and GTPγS to a final concentration of 100 μM. After 10 min to allow secretion to proceed to completion, the incubations were quenched by addition of an equal volume of ice-cold iso-osmotic buffer supplemented with 5 mM EGTA. The cells were sedimented by centrifugation and the supernatants sampled for measurement of secreted hexosaminidase as previously described (Larbi and Gomperts, 1996). In all experiments with G protein-derived subunits, samples of heat-inactivated (>95°C, 10 min) protein containing detergent were used as control.

Secretion is expressed as the percentage of total cellular hexosaminidase released, calibrated by reference to appropriate reagent blanks and the total cell content released by 0.2% Triton X-100. All determinations were in quadruplicate unless otherwise stated, and all experiments have been repeated at least three times.

Brain βγ-subunits

Heterotrimeric GTP-binding proteins and their α- and βγ-subunits were prepared by an adaptation of published methods (Sternweis and Pang, 1990). All procedures (apart from activation) were carried out at 0°C. Normally one brain was homogenized in 1.5 l of sucrose buffer [0.3 M sucrose, 1 mM EDTA, 10 mM Tris, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.5] and a membrane fraction was prepared as described. The membranes were subjected to a mild salt extraction (133 mM NaCl made up in TEDP which comprised 20 mM Tris, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, pH 8.0) and then sedimented before extraction with cholate (1%), purified and made up in TEDP. After centrifugation (142 000 *g* for 1 h), the supernatant was loaded immediately onto a 400 ml DEAE–Sephacel (anion exchange) column, equilibrated with cholate (1%) made up in TEDP. The unbound material was removed by washing with the same buffer, and then a salt gradient (0–250 mM NaCl) was applied to elute bound proteins and the fractions (12 ml) were assayed for the presence of GTPγS-binding activity (see below). The active material was pooled, concentrated on a YM30 (Amicon) membrane and loaded onto a Sephacryl S-200 HR (gel filtration) column equilibrated with TEDP containing 1% cholate and 100 mM NaCl. The fractions containing GTPγS-binding activity were combined, diluted in 3 vol. of TEDP buffer containing 100 mM NaCl, to reduce the concentration of cholate to 0.25%, and, after addition of GDP (10 μM), loaded onto a phenyl-Sepharose HP (hydrophobic interaction) column, equilibrated with the same buffer made up with GDP (10 μM). The column was eluted using a double gradient system in TEDP buffer, commencing with 300 mM NaCl and 0.25% cholate, finishing with 50 mM NaCl and 1% cholate. Fractions were assayed and combined as before. The active material was again diluted to reduce the cholate concentration and the heterotrimeric G proteins were activated by addition of NaF (10 mM), $MgCl_2$ (10 mM) and $AlCl_3$ (50 μM) and incubation for 30 min at 30°C. The dissociated α- and βγ-subunits were separated by chromatography on the phenyl-Sepharose HP column, equilibrated with the same buffer and eluted with a double gradient, starting this time with 200 mM NaCl and 0.2% cholate, finishing with 50 mM NaCl and 0.9% cholate. The fractions were sampled and assayed for GTPγS-binding activity and were analysed by SDS–PAGE, electrophoretically transferred to nitrocellulose and probed with antibodies to α-, β- and γ-subunits. Fractions containing the βγ-subunits (free of α-subunits) were pooled, diluted 5-fold in TEDP and concentrated to 5 ml on a YM10 membrane. This material was stored at 4°C and always used within 3 days of preparation.

Before application to the cells, the βγ-subunits (made up with cholate) were loaded onto a 1 ml MonoQ anion exchange column and eluted by application of a salt gradient (0–1 M NaCl in TEDP, 0.3% CHAPS, pH 8). This served to exchange detergents and also to remove remaining impurities. Fractions containing protein were combined and the protein concentration was determined using the BCA protein assay procedure (Pierce, Chester, UK). The protein was diluted with TEDP buffer containing 0.3% CHAPS to ensure that the final concentration of the detergent presented to the cells was always 50 μM (0.003%). α-Subunits were exchanged into CHAPS by the same procedure and similarly diluted before use.

Recombinant proteins

Recombinant βγ-subunits. Recombinant βγ-subunits were expressed using the baculovirus–insect cell (Sf9) system (Graber *et al*., 1992). Generally, 5×10^8 Sf9 cells were resuspended in 50 ml of fresh medium and incubated for 1 h with 2 p.f.u./cell of baculoviruses containing the appropriate β- and γ-subunit. The cells were then diluted with fresh medium containing Pluronic F68 (final concentration 0.1%), and transferred to a 500 ml spinner flask. Cells were maintained at 27°C and stirred at a rate of 90 r.p.m. under air. Infected cells were harvested after 48 h by centrifugation at 800 *g* for 5 min at 4°C. They were washed three times with ice-cold phosphate-buffered saline (PBS), resuspended in 1 ml of homogenization buffer (10 mM Tris, 25 mM NaCl, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 0.1 mM benzamidine, pH 8.0) and then snap-frozen in liquid nitrogen.

Frozen, harvested cells (typically 2–5 g wet weight) were thawed in 25 ml of ice-cold homogenization buffer and lysed by means of sonication, and the proteins were then purified according to established methods (Muller *et al*., 1993; Graber *et al*., 1996). The detergent Genapol C-100 (10% stock solution) was added to a final concentration of 0.2% and the mixture was stirred gently for 1 h at 4°C. The extracts were then centrifuged at 100 000 *g* for 1 h. The supernatant was diluted 10-fold in TEDP/AlF_4^- buffer (20 mM Tris, 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 5 mM $MgCl_2$, 10 mM NaF, 50 μM $AlCl_3$, pH 8.0) containing 0.15% cholate, and applied to a phenyl–Sepharose column, equilibrated in the same buffer. The proteins were eluted from the column with a double gradient system in TEDP/AlF_4^- buffer, starting with 250 mM NaCl and 0.15% cholate, finishing with 25 mM NaCl and 2% cholate. The fractions containing βγ-subunits were pooled and diluted 10-fold in TEDP buffer and then loaded onto a DEAE–Sephacel column equilibrated in TEDP plus 0.15% cholate. The protein was eluted with a salt gradient (0–500 mM NaCl) made up in the same buffer, and the fractions containing βγ-subunits were pooled, diluted 5-fold in TEDP and concentrated to 5 ml on a YM10 membrane. This material was then applied to a MonoQ column to exchange cholate for CHAPS as described above for the brain βγ-subunits.

PH domain-containing peptides. βARK_1-Cp, mutants of this peptide (WAA, L647G, K645E) and phosducin were expressed as glutathione *S*-transferase (GST) fusion proteins in *E.coli*. The proteins were purified using glutathione–Sepharose 4B beads according to standard protocols. All proteins were subjected to gel filtration on a Superdex G-75 column to remove remaining impurities and high molecular weight aggregates.

Recombinant α_{i3}. Recombinant, myristoylated α_{i3} was expressed and purified as described (Mumby and Linder, 1994). The cells were harvested 8 h after induction, sedimented, flash-frozen in liquid nitrogen

and stored at –80°C. The frozen cell paste was thawed in TEDP and the cells were lysed using lysozyme combined with $MgSO_4$ and DNase I. The proteins were purified by chromatography on DEAE–Sephacel, phenyl-Sepharose and MonoQ columns, respectively. Remaining impurities were removed by gel filtration on Superdex G75.

Electrophoresis and immunoblotting

SDS–PAGE (Bio-Rad) was performed according to established methods (Laemmli, 1970; Schägger and von Jagow, 1987). Proteins were transferred to nitrocellulose membranes (Bio-Rad) using the Multiphor II Novablot system (LKB-Pharmacia) and further treated according to established methods (Towbin *et al*., 1979). Detection of primary antibody binding was performed with peroxidase- or alkaline phosphatase-conjugated secondary antibodies and appropriate detection techiques (Bio-Rad).

GTPγS binding assay

In order to screen large numbers of column fractions for the presence of GTP-binding proteins, we adapted and modified an established method (Northup *et al*., 1982). Samples were diluted 50-fold with 'dilution buffer' (20 mM HEPES, 1 mM EDTA, 1 mM DTT, 0.1% Lubrol-PX, pH 8.0) and 30 µl were then transferred to 96-well plates. An equal volume of 'binding mix' (50 mM HEPES, 2 mM $MgCl_2$, 2 mM EDTA, 200 mM NaCl, 2 mM DTT, 2 MBq/ml [^{35}S]GTPγS, 1 µM GTPγS, pH 8.0) was added and the plates were incubated for 1 h at 30°C. The reaction was stopped by adding 100 µl of ice-cold 'stop solution' (20 mM Tris, 25 mM $MgCl_2$, 100 mM NaCl, pH 8.0) to each well. The mixtures were then transferred to 96-well filtration plates (Event plates, Eppendorf), equilibrated with 'stop solution'. Suction was applied by the use of an automatic vacuum system (Event 4160, Eppendorf) and the wells were washed through five times with 200 µl of 'stop solution'. The membranes were allowed to dry in the air for 5 min and then exposed to a Fuji phosphorescence imaging plate (Raytek, Sheffield, UK) for 30 min. The plate was read using a FujiBAS1000 phosphorescence imaging apparatus and the data were processed using the software package TINA (Raytek). This method allowed us to identify fractions of interest binding GTPγS within 2 h.

Acknowledgements

We want to thank James Garrison, Robert Lefkowitz, Susanne Mumby and Narasimhan Gautam for provision of essential reagents and much invaluable advice. We would also like to thank Carl Stone, Bob Stoffel, Julie Pitcher, Martin Lohse and our colleagues Steve Moss and David Jones for advice and assistance. J.A.P. held a long-term EMBO fellowship (ALTF 1994). This work has been supported further by the Wellcome Trust (051189/Z/97/ZMP/RD/JF), the Medical Research Council (G9619112), the Vandervell Foundation and the Gower Street Secretory Mechanisms Group.

References

Ali,S.M. and Burgoyne,R.D. (1990) The stimulatory effect of calpactin (annexin II) on calcium-dependent exocytosis in chromaffin cells: requirement for both the N-terminal and core domains of p36 and ATP. *Cell Signalling*, **2**, 265–276.

Aridor,M. and Sagi-Eisenberg,R. (1991) Neomycin is a potent secretagogue of mast cells that directly activates a GTP-binding protein involved in exocytosis. *J. Cell Biol.*, **111**, 2885–2891.

Aridor,M., Traub,L.M. and Sagi-Eisenberg,R. (1990) Exocytosis in mast cells by basic secretagogues: evidence for direct activation of GTP-binding proteins. *J. Cell Biol.*, **111**, 909–917.

Aridor,M., Rajmilevich,G., Beaven,M.A. and Sagi-Eisenberg,R. (1993) Activation of exocytosis by the heterotrimeric G protein G_{i3}. *Science*, **262**, 1569–1572.

Bennett,J.P., Cockcroft,S. and Gomperts,B.D. (1980) Ionomycin stimulates mast cell histamine secretion by forming a lipid soluble calcium complex. *Nature*, **282**, 851–853.

Brown,A.M., O'Sullivan,A.J. and Gomperts,B.D. (1998) Induction of exocytosis from permeabilized mast cells by the guanosine triphosphatases Rac and Cdc42. *Mol. Biol. Cell*, **9**, 1053–1063.

Chernomordik,L.V., Vogel,S.S., Sokoloff,A., Onaran,H.O., Leikina,E.A. and Zimmerberg,J. (1993) Lysolipids reversibly inhibit Ca^{2+}-, GTP- and pH-dependent fusion of biological membranes. *FEBS Lett.*, **318**, 71–76.

Churcher,Y., Kramer,I.M. and Gomperts,B.D. (1990) Evidence for protein dephosphorylation as a permissive step in GTP-gamma-S induced exocytosis from permeabilised mast cells. *Cell Regul.*, **1**, 523–530.

Clapham,D.E. and Neer,E.J. (1993) New roles for G-protein βγ-dimers in transmembrane signalling. *Nature*, **365**, 403–406.

Coso,O.A., Teramoto,H., Simonds,W.F. and Gutkind,J.S. (1996) Signaling from G protein-coupled receptors to c-Jun kinase involves βγ subunits of heterotrimeric G proteins acting on a Ras and Rac1-dependent pathway. *J. Biol. Chem.*, **271**, 3963–3966.

Eberhard,D.A., Cooper,C.L., Low,M.G. and Holz,R.W. (1990) Evidence that the inositol phospholipids are necessary for exocytosis. Loss of inositol phospholipids and inhibition of secretion in permeabilized cells caused by a bacterial phospholipase C and removal of ATP. *Biochem. J.*, **268**, 15–25.

Fernandez,J.M., Neher,E. and Gomperts,B.D. (1984) Capacitance measurements reveal stepwise fusion events in degranulating mast cells. *Nature*, **312**, 453–455.

Foreman,J.C., Mongar,J.L. and Gomperts,B.D. (1973) Calcium ionophores and movement of calcium ions following the physiological stimulus to a secretory process. *Nature*, **245**, 249–251.

Gomperts,B.D. (1990) G_E: a GTP-binding protein mediating exocytosis. *Annu. Rev. Physiol.*, **52**, 591–606.

Gomperts,B.D. and Tatham,P.E.R. (1992) Regulated exocytotic secretion from permeabilized cells. *Methods Enzymol.*, **219**, 178–189.

Graber,S.G., Figler,R.A. and Garrison,J.C. (1992) Expression and purification of functional G protein α-subunits using a baculovirus expression system. *J. Biol. Chem.*, **267**, 1271–1278.

Graber,S.G., Lindorfer,M.A. and Garrison,J.C. (1996) Expression and purification of G protein βγ subunits using baculovirus expression system. *Methods Neurosci.*, **29**, 207–226.

Harlan,J.E., Hajduk,P.J., Yoon,H.S. and Fesik,S.W. (1994) Pleckstrin homology domains bind to phosphatidylinositol-4,5-biphosphate. *Nature*, **371**, 168–170.

Hay,J.C., Fisette,P.L., Jenkins,G.H., Fukami,K., Takenawa,T., Anderson, R.A. and Martin,T.F.J. (1995) ATP-dependent inositide phosphorylation required for Ca^{2+}-activated secretion. *Nature*, **374**, 173–177.

Higashijima,T., Burnier,J. and Ross,E.M. (1990) Regulation of G_i and G_o by mastoparan, related amphiphilic peptides and hydrophobic amines. *J. Biol. Chem.*, **265**, 14176–14186.

Howell,T.W. and Gomperts,B.D. (1987) Rat mast cells permeabilised with streptolysin-O secrete histamine in response to Ca^{2+} at concentrations buffered in the micromolar range. *Biochim. Biophys. Acta*, **927**, 177–183.

Howell,T.W., Cockcroft,S. and Gomperts,B.D. (1987) Essential synergy between Ca^{2+} and guanine nucleotides in exocytotic secretion from permeabilised mast cells. *J. Cell Biol.*, **105**, 191–197.

Howell,T.W., Kramer,I.M. and Gomperts,B.D. (1989) Protein phosphorylation and the dependence on Ca^{2+} for GTP-γ-S stimulated exocytosis from permeabilised mast cells. *Cell Signalling*, **1**, 157–163.

Jones,P.M., Mann,F.M., Persaud,S.J. and Wheeler,J.C. (1993) Mastoparan stimulates insulin secretion from pancreatic β-cells by effects at a late stage in the secretory pathway. *Mol. Cell. Endocrinol.*, **94**, 97–103.

Komatsu,M., McDermott,A.M., Gillison,S.L. and Sharp,G.W. (1993) Mastoparan stimulates exocytosis at a Ca^{2+}-independent late site in stimulus–secretion coupling. Studies with the RINm5F β-cell line. *J. Biol. Chem.*, **268**, 23297–23306.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**, 680–685.

Lang,J., Nishimoto,I., Okamoto,T., Regazzi,R., Kiraly,C., Weller,U. and Wollheim,C.B. (1995) Direct control of exocytosis by receptor-mediated activation of the heterotrimeric GTPases G_i and G_o or by the expression of their active G α-subunits. *EMBO J.*, **14**, 3635–3644.

Larbi,K.Y. and Gomperts,B.D. (1996) Practical considerations regarding the use of streptolysin-O as a permeabilising agent for cells in the investigation of exocytosis. *Biosci. Rep.*, **16**, 11–21.

Lillie,T.H.W. and Gomperts,B.D. (1992) Guanine nucleotide is essential, Ca^{2+} is a modulator, in the exocytotic reaction of permeabilised rat mast cells. *Biochem. J.*, **288**, 181–187.

Lindau,M. and Gomperts,B.D. (1991) Techniques and concepts in exocytosis: focus on mast cells. *Biochim. Biophys. Acta*, **1071**, 429–471.

Logothetis,D.E., Kim,D.H., Northup,J.K., Neer,E.J. and Clapham,D.E. (1988) Specificity of action of guanine nucleotide-binding regulatory protein subunits on the cardiac muscarinic K^+ channel. *Proc. Natl Acad. Sci. USA*, **85**, 5814–5818.

Mariot,P., O'Sullivan,A.J., Brown,A.M. and Tatham,P.E.R. (1996) Rho-guanine nucleotide dissociation inhibitor protein (Rho-GDI) inhibits exocytosis in mast cells. *EMBO J.*, **15**, 6476–6482.

Marquardt,D.L., Gruber,H.E. and Walker,L.L. (1987) Ribavirin inhibits mast cell mediator release. *J. Pharmacol. Exp. Ther.*, **240**, 145–149.
Matsuda,M., Okabe,T., Sugimoto,N., Senda,T. and Fujita,H. (1994) Tetanus toxin and *Clostridium perfringens* enterotoxin as tools for the study of exocytosis. *Ann. NY Acad. Sci.*, **710**, 94–106.
Metzger,H. (1992) The receptor with high affinity for IgE. *Immunol. Rev.*, **125**, 37–47.
Muller,S., Hekman,M. and Lohse,M.J. (1993) Specific enhancement of β-adrenergic receptor kinase activity by defined G-protein β and γ subunits. *Proc. Natl Acad. Sci. USA*, **90**, 10439–10443.
Mumby,S.M. and Linder,M.E. (1994) Myristoylation of G-protein α subunits. In Iyengar,R. (ed.), *Heterotrimeric G proteins*. Academic Press, NY, pp. 254–268.
Neer,E.J. and Clapham,D.E. (1988) Roles of G protein subunits in transmembrane signalling. *Nature*, **333**, 129–134.
Nishizaki,T., Walent,J.H., Kowalchyk,J.A. and Martin,T.F.J. (1992) A key role for a 145-kDa cytosolic protein in the stimulation of Ca^{2+}-dependent secretion by protein kinase C. *J. Biol. Chem.*, **267**, 23972–23981.
Northup,J.K., Smigel,M.D. and Gilman,A.G. (1982) The guanine nucleotide activating site of the regulatory component of adenylate cyclase: identification by ligand binding. *J. Biol. Chem.*, **257**, 11416–11423.
O'Sullivan,A.J., Brown,A.M., Freeman,H.N.M. and Gomperts,B.D. (1996) Purification and identification of FOAD-II, a cytosolic protein that regulates secretion in streptolysin-O permeabilised mast cells, as a rac/rhoGDI complex. *Mol. Biol. Cell*, **7**, 397–408.
Park,D.J., Min,H.K. and Rhee,S.G. (1991) IgE-induced tyrosine phosphorylation of phospholipase C-gamma1 in rat basophilic leukemia cells. *J. Biol. Chem.*, **266**, 24237–24240.
Rasenick,M.M., Watanabe,M., Lazarevic,M.B., Hatta,S. and Hamm,H.E. (1994) Synthetic peptides as probes for G protein function. Carboxyl-terminal $G\alpha_s$ peptides mimic G_s and evoke high affinity agonist binding to β-adrenergic receptors. *J. Biol. Chem.*, **269**, 21519–21525.
Saito,H., Okajima,F., Molski,T.F.P., Sha'afi,R.I., Ui,M. and Ishizaka,T. (1987) Effects of ADP-ribosylation of GTP-binding protein by pertussis toxin on immunoglobulin E-dependent and -independent histamine release from mast cells and basophils. *J. Immunol.*, **138**, 3927–3934.
Saraste,M. and Hyvonen,M. (1995) Pleckstrin homology domains: a fact file. *Curr. Biol.*, **5**, 403–408.
Schägger,H. and von Jagow,G. (1987) Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.*, **166**, 368–379.
Sternweis,P.C. and Pang,I.-H. (1990) Preparation of G-proteins and their subunits. In Hulme,E.C. (ed.), *Receptor–Effector Coupling: A Practical Approach*. IRL Press, Oxford, UK, pp. 1–30.
Takai,Y., Kishimoto,A., Iwasa,Y., Kawahara,Y., Mori,T. and Nishizuka,Y. (1979) Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. *J. Biol. Chem.*, **254**, 3692–3695.
Tanaka,K., Sasaki,T. and Takai,Y. (1995) Purification and properties of recombinant Rho-GDP dissociation inhibitor. *Methods Enzymol.*, **256**, 41–49.
Tatham,P.E.R. and Gomperts,B.D. (1991) Rat mast cells degranulate in response to microinjection of guanine nucleotide. *J. Cell Sci.*, **98**, 217–224.
Touhara,K., Inglese,J., Pitcher,J.A., Shaw,G. and Lefkowitz,R.J. (1994) Binding of G protein βγ-subunits to pleckstrin homology domains. *J. Biol. Chem.*, **269**, 10217–10220.
Touhara,K., Koch,W.J., Hawes,B.E. and Lefkowitz,R.J. (1995) Mutational analysis of the pleckstrin homology domain of the β-adrenergic receptor kinase. *J. Biol. Chem.*, **270**, 17000–17005.
Towbin,H., Staehelin,T. and Gordon,J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl Acad. Sci. USA*, **76**, 4350–4354.
Vallar,L., Biden,T.J. and Wollheim,C.B. (1987) Guanine nucleotides induce Ca^{2+} independent secretion from permeabilized RINm5F cells. *J. Biol. Chem.*, **262**, 5049–5056.
Vlahos,C.J., Matter,W.F., Hui,K.Y. and Brown,R.F. (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J. Biol. Chem.*, **269**, 5241–5248.
Whitehead,I., Kirk,H. and Kay,R. (1995a) Retroviral transduction and oncogenic selection of a cDNA encoding Dbs, a homolog of the Dbl guanine nucleotide exchange factor. *Oncogene*, **10**, 713–721.
Whitehead,I., Kirk,H., Tognon,C., Trigo,G.G. and Kay,R. (1995b) Expression cloning of *lfc*, a novel oncogene with structural similarities to guanine nucleotide exchange factors and to the regulatory region of protein kinase C. *J. Biol. Chem.*, **270**, 18388–18395.
Wilson,B.S., Deanin,G.G., Standefer,J.C., Vanderjagt,D. and Oliver,J.M. (1989) Depletion of guanine nucleotides with mycophenolic acid suppresses IgE receptor-mediated degranulation in rat basophilic leukemia cells. *J. Immunol.*, **143**, 259–265.
Xu,J., Wu,D., Slepak,V.Z. and Simon,M.I. (1995) The N terminus of phosducin is involved in binding of βγ subunits of G protein. *Proc. Natl Acad. Sci. USA*, **92**, 2086–2090.
Zhang,H., Yasrebi-Nejad,H. and Lang,J. (1998) G-protein βγ-binding domains regulate insulin exocytosis in clonal pancreatic β-cells. *FEBS Lett.*, **424**, 202–206.

Received July 29, 1998; revised September 8, 1998; accepted September 9, 1998