The EMBO Journal vol.11 no.13 pp.4795-4804, 1992

Multiple trimeric G-proteins on the trans-Golgi network exert stimulatory and inhibitory effects on secretory vesicle formation

Anja Leyte, Francis A.Barr, Ralph H.Kehlenbach and Wieland B.Huttner

Institute for Neurobiology, University of Heidelberg, Im Neuenheimer Feld 364, D-6900 Heidelberg, Germany

Communicated by W.B.Huttner

The role of heterotrimeric G-proteins on the formation of constitutive secretory vesicles (CSVs) and immature secretory granules (ISGs) from the trans-Golgi network (TGN) of PC12 cells was investigated. Using immunofluorescence and subcellular fractionation in conjunction with immunoblotting or ADP-ribosylation by either pertussis toxin or cholera toxin, TGN membranes were found to contain not only several αi/αo G-protein subunits including apparently αi3, but also αs. Pertussis toxin treatment of cells, which resulted in the stoichiometric ADP-ribosylation of αi/αo, a modification known to prevent their coupling to receptors, led to the stimulation of cell-free CSV and ISG formation, suggesting the presence of a guanine nucleotide exchange factor for αi/αo on the TGN. Mastoparan-7, a peptide known to mimic an activated receptor and to stimulate nucleotide exchange on αi/αo, inhibited cell-free vesicle formation, an effect abolished by pertussis toxin. In contrast, activation of αs by cholera toxin treatment of cells resulted in a stimulation of cell-free CSV and ISG formation. This stimulation could be reversed when the α subunits not activated by cholera toxin, i.e. αi/αo, were activated by GTPγS and $[AlF_4]^-$. Our results show that both inhibitory and stimulatory trimeric G-proteins on the TGN participate in the regulation of secretory vesicle formation.

Key words: cell-free system/cholera toxin/G-proteins/pertussis toxin/vesicular transport

Introduction

Substantial progress has been made over the past several years, using both biochemical and genetic approaches, in identifying molecules involved in vesicular transport (for reviews see Schekman, 1985; Rothman and Orci, 1992). This work has revealed that GTP binding proteins function at several steps of this process. Small GTP binding proteins of the rab family, typified by the yeast sec4 protein (Salminen and Novick, 1987), are found in association with specific vesicles and organelles and are thought to have a crucial role in the targeting of vesicles to, and their fusion with, the appropriate acceptor organelles (Chavrier *et al.*, 1990; Fischer von Mollard *et al.*, 1990; for review see Pfeffer, 1992). Another step in vesicular transport, the formation of vesicles from donor compartments, requires a different class of small GTP binding proteins, the arf/sar1 family (d'Enfert *et al.*, 1991; Taylor *et al.*, 1992), which also are localized to specific compartments (Stearns *et al.*, 1990).

However, the requirement of GTP hydrolysis in vesicle formation (Tooze *et al.*, 1990; Rexach and Schekman, 1991) does not only reflect the participation of arf and sar1 in this process. Recently, several lines of evidence obtained with mammalian systems have pointed to a role for heterotrimeric G-proteins in vesicle formation (Balch, 1992; Barr *et al.*, 1992; Burgoyne, 1992). The G-protein α subunit αi3 has been shown to be associated with the Golgi complex (Ercolani *et al.*, 1990; Stow *et al.*, 1991) and overexpression of this protein leads to an inhibition of constitutive secretion (Stow *et al.*, 1991). Using a cell-free system which reconstitutes the formation of constitutive secretory vesicles (CSVs) and immature secretory granules (ISGs) from the trans-Golgi network (TGN) (Tooze and Huttner, 1990), a trimeric G-protein has been shown to inhibit the secretory process at the level of vesicle formation (Barr *et al.*, 1991). Furthermore, a trimeric G-protein appears to regulate the binding to Golgi membranes of two components of the non-clathrin coat of Golgi-derived vesicles, β-COP and arf (Donaldson *et al.*, 1991; Ktistakis *et al.*, 1992), which are believed to be required for vesicle formation (Rothman and Orci, 1992). Together, these observations suggest that the molecular machinery mediating vesicle formation is regulated by trimeric G-proteins.

The receptor-mediated regulation of the classical effector system operating in signal transduction at the plasma membrane, adenylate cyclase, occurs via both stimulatory and inhibitory G-proteins (Gilman, 1987; Taylor, 1990). Given the existence of such a regulatory network of G-proteins, we explored the possibility that the same complexity may be true for the regulation of vesicle formation at the level of the TGN.

Results

Association of multiple pertussis toxin-sensitive G-protein α subunits and of β subunits with the TGN of PC12 cells

To operate in the regulation of vesicle formation, a trimeric G-protein must be associated, or interact, with the donor compartment. In the epithelial cell line LLC-PK1, the association of the G-protein subunit αi3 with the Golgi complex has previously been documented (Ercolani *et al.*, 1990; Stow *et al.*, 1991). We have recently reported that a pertussis toxin-sensitive α subunit cofractionates with the TGN of PC12 cells (Barr *et al.*, 1991). To investigate whether this α subunit was αi3 and to explore the possibility that, perhaps α subunits other than αi3 might also be associated with the TGN, a post-nuclear supernatant (PNS) of PC12 cells was fractionated by velocity sucrose gradient centrifugation (Tooze and Huttner, 1990) and the fractions were analysed by immunoblotting using various anti-G-

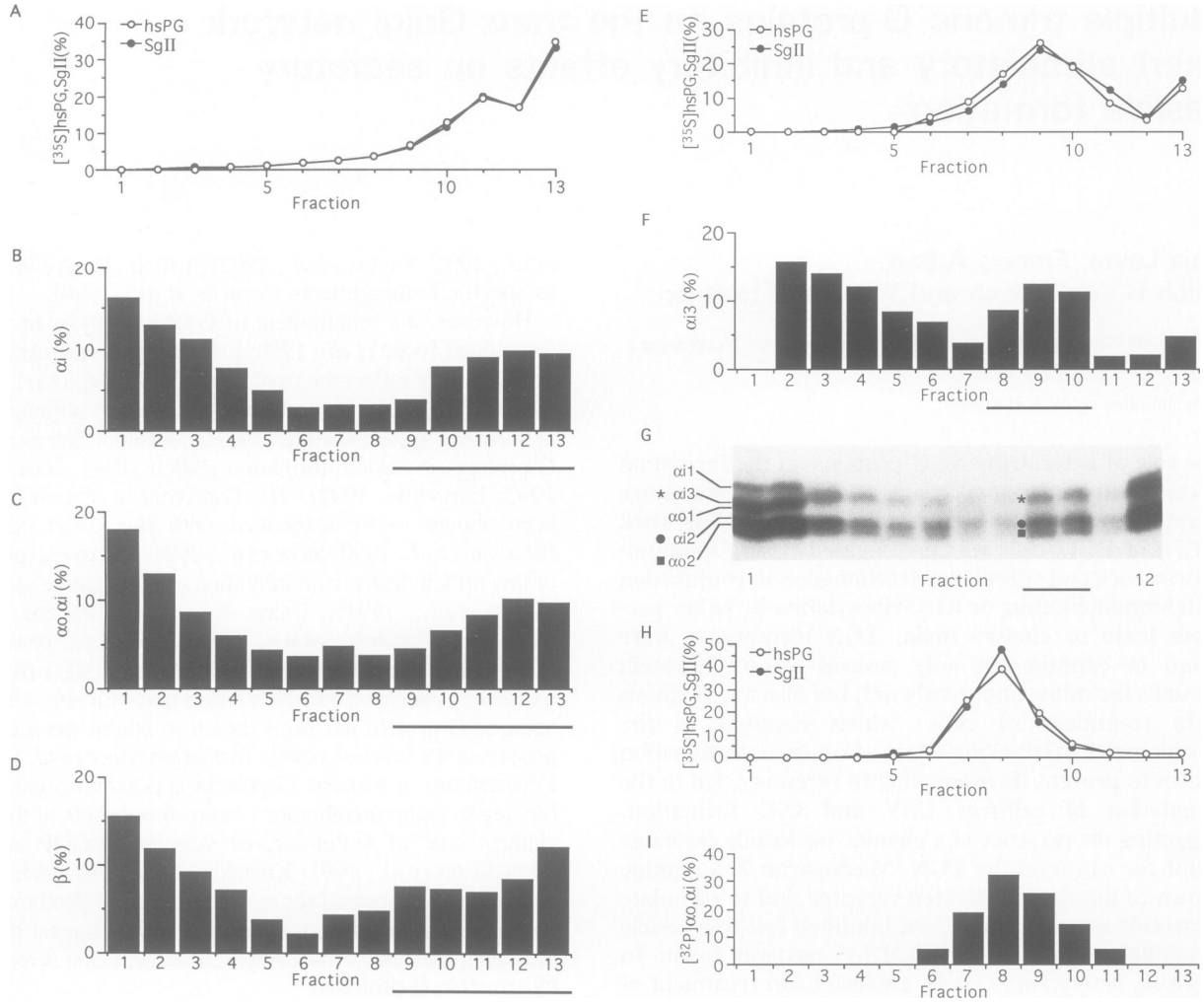

Fig. 1. Presence of multiple G-protein αi and αo subunits and of G-protein β subunits on TGN membranes of PC12 cells. PC12 cells were [^{35}S]sulfate-labelled for 5 min (A)−(F) or not labelled (G), and PNSs were prepared and incubated either at 4°C (A), (E) and (F) or at 37°C under cell-free vesicle formation conditions without (B)−(D) or with (G) pertussis toxin and [^{32}P]NAD, followed by velocity sucrose gradient centrifugation. Aliquots of the fractions (top of gradient = fraction 1) were analysed as follows. (A) and (E): SDS−PAGE followed by quantitation of [^{35}S]sulfate-labelled hsPG (open circles) and [^{35}S]sulfate-labelled SgII (filled circles). (B)−(D) and (F): SDS−PAGE using 10% minigels followed by immunoblotting with antiserum 136A recognizing αi1 and αi2 (B), antiserum GA/1 recognizing αi and αo (C), antiserum 1C recognizing αo and β; only the results for β are shown (D), and antiserum EC/2 recognizing αi3 (F). The data shown in (E) and (F) are from a different set of velocity sucrose gradients than those shown in (A)−(D). Note that in the experiment shown in (A)−(D), the TGN as identified by [^{35}S]sulfate-labelled hsPG and SgII is largely found in fractions 9−12 and in the pellet (fraction 13) [bars in (B)−(D)], whereas in the experiment shown in (E) and (F), the TGN peaks in fractions 8−10 (bar in F). (G): SDS−PAGE using urea gels followed by autoradiography. Only the 35−45 kDa region of the gel is shown. (H) and (I): fractions 9 and 10 of velocity gradients corresponding to (A) and (G), respectively, were pooled and subjected to equilibrium sucrose gradient centrifugation. Aliquots of the fractions of the two equilibrium gradients (top of gradient = fraction 1) were subjected to either SDS−PAGE followed by quantitation of [^{35}S]sulfate-labelled hsPG (panel H, open circles) and [^{35}S]sulfate-labelled SgII (panel H, filled circles), or SDS−PAGE using urea gels followed by quantitation of the sum of [^{32}P]ADP-ribosylated α subunits (I). (A)−(F), (H) and (I): data are expressed as percentage of total label or immunoreactivity recovered per gradient.

protein subunit antibodies (Figure 1). To identify fractions containing TGN, we pulse-labelled PC12 cells for 5 min with [^{35}S]sulfate without a subsequent chase, and then followed the distribution of two sulfated markers, hsPG and SgII, across the velocity gradient. [Under these conditions, the two markers are known to be localized in the TGN, whereas after chase the hsPG and SgII are found in CSVs and ISGs, respectively (Tooze and Huttner, 1990).] The comparison of the distribution of the [^{35}S]sulfate-labelled hsPG and SgII (Figure 1A) with that of the various G-protein α subunit immunoreactivities showed that αi, αo and β subunits were found not only in the top fractions of the gradient which are known to contain cytosol and organelles such as plasma membrane (Régnier-Vigouroux *et al.*, 1991), but also in the fractions enriched in TGN (Figure 1B−D, bars). Immunoblotting with an antibody against αi3 (for specificity, see Figure 3C below) suggested that one of the TGN-associated αi subunits was αi3 (Figure 1F). To characterize further the TGN-associated αi and αo subunits, we subjected a PC12 cell PNS to ADP-ribosylation with pertussis toxin in the presence of [^{32}P]NAD. The PNS was then fractionated by velocity sucrose gradient centrifugation, and the fractions were analysed by SDS−PAGE in the presence of 4 M urea, a condition which allows the separation of the various αi and αo subunits from one another (Gierschik *et al.*, 1989). The pattern of ADP-ribosylated α subunits obtained was compared with published data (Gierschik, 1992) and with immunoblots, using the above antibodies,

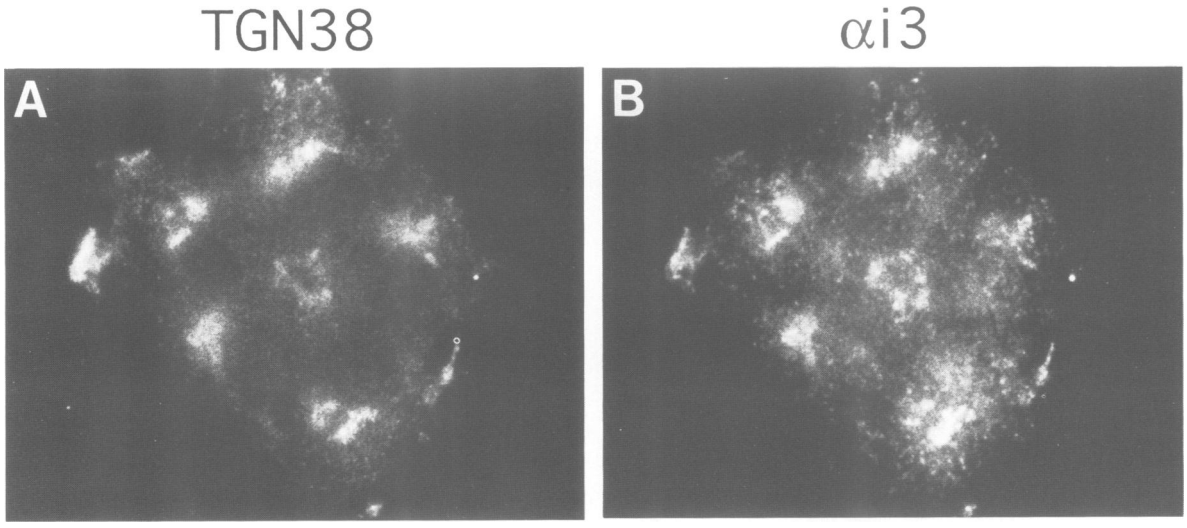

Fig. 2. Localization of αi3 to the TGN of PC12 cells by double immunofluorescence. PC12 cells were analysed using sheep anti-TGN38 antiserum (**A**) and rabbit antiserum EC/2 recognizing αi3 (**B**).

of α subunits separated by SDS−PAGE in the presence of 4 M urea (data not shown). This revealed that αi1, αi2, αi3, αo1 and αo2 were present in PC12 cells (see fraction 1, Figure 1G), of which at least αi2 and αo2 and apparently αi3 were associated with the TGN under the present conditions (Figure 1G, bar). The ADP-ribosylated αi/αo subunits found in the TGN-containing fractions of the velocity gradient comigrated with the TGN, again identified by [^{35}S]sulfate pulse-labelled hsPG and SgII (Figure 1H), upon equilibrium sucrose gradient centrifugation (Figure 1I).

The subcellular localization of the αi3 subunit in PC12 cells was also investigated by immunofluorescence using the anti-αi3 antiserum. To visualize the TGN, PC12 cells were stained with a sheep antibody against TGN38, an integral membrane protein found predominantly, though not exclusively, in the TGN (Luzio *et al.*, 1990). The immunoreactivity observed with this antibody in the perinuclear region of the cells (Figure 2A) showed the characteristic pattern of the TGN (Griffiths *et al.*, 1985) previously reported for PC12 cells (Rosa *et al.*, 1992). Comparison by double immunofluorescence of the TGN38 pattern (Figure 2A) with that observed with the anti-αi3 antibody (Figure 2B) revealed that a major portion of the αi3 immunoreactivity was clearly colocalized with the TGN structures stained with anti-TGN38. In addition to the staining of the TGN, the anti-αi3 antibody gave a punctate staining extending into the periphery of the cells (Figure 2B), consistent with the results shown in Figure 1F (presence of αi3 in fractions 2−6) and with observations (Birnbaumer *et al.*, 1990; Ausiello *et al.*, 1992) indicating that αi3 is not exclusively localized to the Golgi complex.

ADP-ribosylation of αi and αo subunits by pertussis toxin results in stimulation of cell-free formation of constitutive secretory vesicles and immature secretory granules

Pertussis toxin-catalysed ADP-ribosylation of αi and αo subunits blocks their ability to interact functionally with receptor proteins which, when activated, stimulate the exchange of GDP for GTP and thereby activate α subunits (Birnbaumer *et al.*, 1990; Gierschik and Jakobs, 1992). We used pertussis toxin as a tool to investigate a possible role of αi and αo subunits in the formation of secretory vesicles from the TGN. For this purpose, it was important to determine whether treatment of intact cells with pertussis toxin resulted in stoichiometric ADP-ribosylation of αi/αo subunits. This was investigated by 'back-ribosylation', i.e. the titration of unmodified αi/αo subunits by ADP-ribosylation with additional pertussis toxin and [^{32}P]NAD *in vitro*. In contrast to a PNS from untreated PC12 cells, which contained non-ADP-ribosylated αi/αo subunits as revealed by back-ribosylation (Figure 3A top, left lane), the PNS from pertussis toxin-treated cells contained no detectable unmodified αi/αo subunits (Figure 3A top, right lane). Immunoblotting showed that this was not due to the lack of αi/αo subunits in pertussis toxin-treated cells. In fact, pertussis toxin-treated cells contained higher levels of αi than untreated cells. Thus, pertussis toxin treatment of PC12 cells resulted in the stoichiometric ADP-ribosylation of αi/αo subunits.

Further support for this conclusion was obtained when the electrophoretic mobilities of metabolically labelled α subunits, immunoprecipitated from control and pertussis toxin-treated PC12 cells, were analysed (Figure 3C). Using SDS−polyacrylamide gels with half the normal concentration of bisacrylamide, which improves the separation of αi and αo subunits, it was observed that all of the αi3 from pertussis toxin-treated cells migrated more slowly than that from control cells, presumably because it had been modified stoichiometrically with ADP-ribose. In contrast, the migration of the β subunit, which does not become ADP-ribosylated by pertussis toxin (Gierschik and Jakobs, 1992), was unaffected. Consistent with the results of immunoblotting (Figure 3A, bottom), pertussis toxin-treated PC12 cells contained a higher level of metabolically labelled αi3 than control cells (Figure 3C).

ADP-ribosylation of αi and αo subunits by pertussis toxin requires that these α subunits are in the trimeric state (Gilman, 1987; Gierschik and Jakobs, 1992). Thus, GTPγS binding to α subunits, which results in their dissociation from the βγ subunits, prevents their ADP-ribosylation by pertussis toxin (Ribeiro-Neto *et al.*, 1987). Given our previous observation that GTPγS inhibits secretory vesicle formation from the TGN (Tooze *et al.*, 1990) and the data of Figure 1

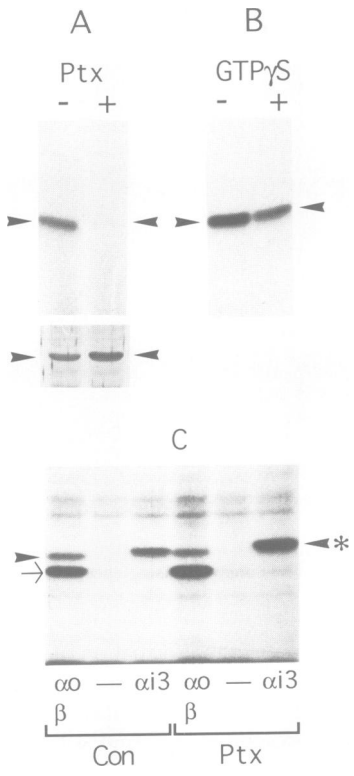

Fig. 3. Effects of pertussis toxin and of GTPγS on G-protein α subunits of PC12 cells. (**A**) PC12 cells were incubated without (−) or with (+) pertussis toxin (Ptx), and aliquots of the PNS prepared from the two sets of cells were subjected to either pertussis toxin-catalysed ADP-ribosylation with [^{32}P]NAD followed by SDS−PAGE and autoradiography (top), or SDS−PAGE followed by immunoblotting using antiserum 136A recognizing αi1 and αi2 and antiserum EC/2 recognizing αi3, both at 1:1000 dilution. Only the 40 kDa region of the immunoblot is shown (bottom). (**B**) A PC12 cell PNS was incubated in the absence (−) or presence (+) of 10 μM GTPγS, and a fraction enriched in TGN membranes was prepared and subjected to pertussis toxin-catalysed ADP-ribosylation with [^{32}P]NAD followed by SDS−PAGE and autoradiography. (**C**) PC12 cells were labelled for 16 h with [^{35}S]methionine in the absence (Con) or presence (Ptx) of pertussis toxin, and aliquots of the two cell lysates were subjected to immunoprecipitation using antiserum 1C recognizing αo and β subunits, normal rabbit serum (−), or antiserum EC/2 recognizing αi3, as indicated. Samples were analysed by SDS−PAGE using low bisacrylamide followed by fluorography. Note the slower electrophoretic mobility of αi3 upon pertussis toxin treatment (asterisk). Arrowheads indicate αo and/or αi subunits, the arrow indicates β subunits.

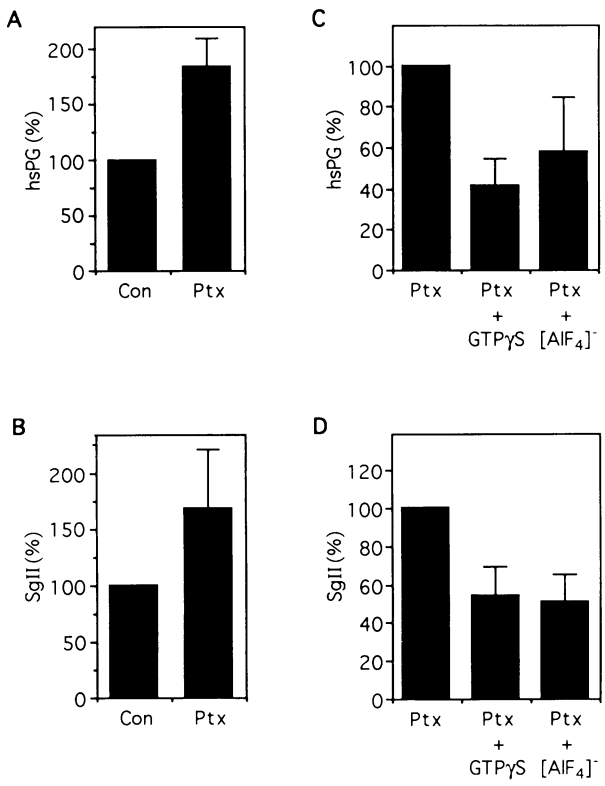

Fig. 4. Upon pertussis toxin treatment of intact PC12 cells, vesicle formation in the cell-free system is stimulated, and can still be inhibited by the trimeric G-protein activators GTPγS and $[AlF_4]^-$. PC12 cells were incubated without (Con) or with (Ptx) pertussis toxin, [^{35}S]sulfate-labelled for 5 min, and the PNS prepared from the two sets of cells was used for cell-free vesicle formation reactions in the absence or presence of 10 μM GTPγS or of 40 μM Al^{3+} plus 6 mM F^- ($[AlF_4]^-$), as indicated. The formation of hsPG-containing [(A) and (C)] and SgII-containing [(B) and (D)] post-TGN vesicles was quantitated. (A) and (B) vesicle formation upon pertussis toxin treatment is expressed as percentage of that observed in the absence of the toxin. The mean of five separate experiments is shown. Bars indicate the standard deviation. (C) and (D) vesicle formation in the presence of GTPγS or $[AlF_4]^-$ is expressed as percentage of that observed in the absence of these compounds; all reactions were carried out with a PNS from pertussis toxin-treated cells. The means of three separate experiments (GTPγS) or four separate reactions from two different experiments ($[AlF_4]^-$) are shown. Bars indicate the standard deviation.

that various αi and αo subunits are associated with the TGN of PC12 cells, it was of interest to determine whether addition of GTPγS actually leads to the activation of TGN-associated αi/αo under the conditions used for cell-free vesicle formation. As shown in Figure 3B, this appeared to be the case since GTPγS reduced by ~70% the amount of TGN-associated αi/αo subunits that were available for ADP-ribosylation by pertussis toxin, i.e. that were in the trimeric state.

The consequence of pertussis toxin-catalysed ADP-ribosylation of αi/αo subunits on the formation of secretory vesicles from the TGN was investigated using the previously established cell-free system (Tooze and Huttner, 1990, 1992). In this system, [^{35}S]sulfate-labelled hsPG and SgII are packaged into CSVs and ISGs, respectively, upon *in vitro* incubation of a PC12 cell PNS. As shown in Figure 4, pertussis toxin treatment of PC12 cells resulted in a stimulation of the cell-free formation of both hsPG-containing (panel A) and SgII-containing (panel B) post-TGN vesicles. This observation strongly suggested that in the control condition, at least some of the TGN-associated αi/αo subunits were in an active state, i.e. inhibitory for vesicle formation, as a consequence of stimulation of guanine nucleotide exchange by a component which acted similarly to a G-protein-coupled receptor.

Pertussis toxin-catalysed ADP-ribosylation, although blocking the interaction of αi/αo subunits with receptors, does not prevent the binding of GTPγS to these subunits (Gierschik and Jakobs, 1992). Since cell-free vesicle formation is performed at low magnesium concentration (Tooze and Huttner, 1992), there should be sufficient intrinsic exchange of GDP for GTPγS even when the αi/αo subunits are ADP-ribosylated by pertussis toxin. Receptor activation of α subunits is also not required in the case of $[AlF_4]^-$, which binds to α subunits in the GDP state (Chabre, 1990). One would therefore expect that both

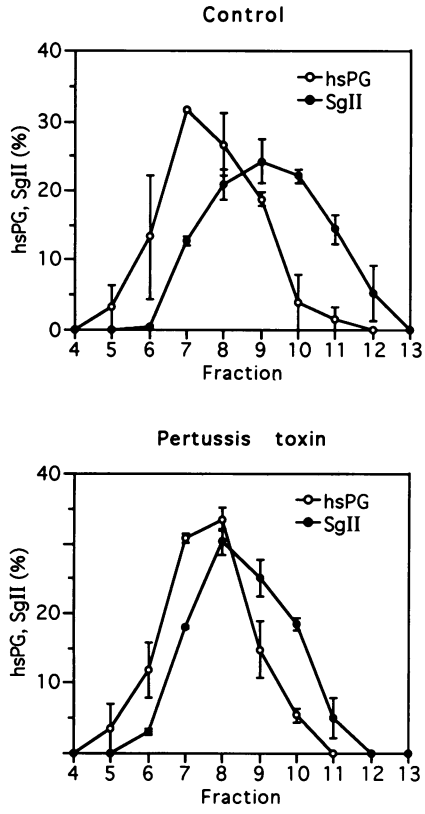

Fig. 5. Differential effect of pertussis toxin on the formation of CSVs and ISGs. PC12 cells were incubated without (Control) or with pertussis toxin, [^{35}S]sulfate-labelled for 5 min, and the PNS prepared from the two sets of cells was incubated to allow cell-free vesicle formation followed by velocity sucrose gradient centrifugation. Fractions 2–5, known to contain post-TGN vesicles (Tooze and Huttner, 1990), were pooled and subjected to a second, equilibrium sucrose gradient centrifugation. Aliquots of the fractions of the two equilibrium gradients (top of gradient = fraction 1) were subjected to SDS–PAGE and analysed for [^{35}S]sulfate-labelled hsPG (open circles) and [^{35}S]sulfate-labelled SgII (filled circles). Data are expressed as percentage of total labelled hsPG or SgII recovered per gradient. The mean of two independent experiments is shown; bars indicate the variation of the individual values from the mean.

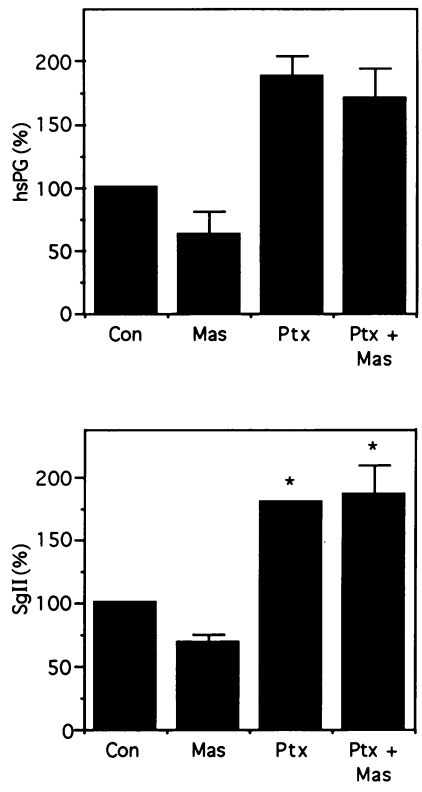

Fig. 6. Mastoparan inhibits cell-free vesicle formation in the PNS prepared from normal, but not pertussis toxin-treated, PC12 cells. PC12 cells were incubated without or with pertussis toxin (Ptx), [^{35}S]sulfate-labelled for 5 min, and the PNS prepared from the two sets of cells was used for cell-free vesicle formation reactions in the absence or presence of 1 μM mastoparan-7 (Mas). The formation of hsPG-containing (top panel) and SgII-containing (bottom panel) post-TGN vesicles was quantitated and is expressed as a percentage of that observed in the control condition (Con; no pertussis toxin, no mastoparan-7). The means of four (Con, Mas) or three (Ptx, Ptx+Mas) separate experiments are shown. Bars indicate the standard deviation. In the conditions indicated by the asterisks, the variation of the Ptx+Mas value from the Ptx value was calculated for each experiment and expressed as a percentage of the latter. The bar marked with the asterisk indicates the mean of this variation (12%) after multiplication by 1.8, i.e. the ratio of Ptx over Con.

GTPγS and $[AlF_4]^-$ should still inhibit the cell-free formation of post-TGN vesicles after pertussis toxin treatment. As shown in Figure 4, this was found to be the case for both CSVs (panel C) and ISGs (panel D).

Effect of pertussis toxin-catalysed ADP-ribosylation of αi and αo subunits on the biogenesis and/or properties of the immature secretory granules formed in the cell-free system

We previously reported that G-protein $\beta\gamma$ subunits, when added to the cell-free system, not only stimulate post-TGN vesicle formation, but in addition cause partial missorting of SgII into CSVs (Barr *et al.*, 1991). Both addition of $\beta\gamma$ subunits and pertussis toxin treatment, result in a reduction of the level of dissociated, active αi/αo subunits (Gilman, 1987; Birnbaumer *et al.*, 1990; Taylor, 1990). We therefore investigated whether pertussis toxin treatment, in addition to stimulating post-TGN vesicle formation, would also affect the sorting of SgII in the cell-free system. Confirming previous observations (Tooze and Huttner, 1990), the analysis of the post-TGN vesicles formed in the cell-free system by equilibrium sucrose gradient centrifugation showed that in the control condition, the hsPG and SgII had been packaged into two distinct populations of vesicles, the CSVs peaking in fraction 7 and the ISGs peaking in fraction 9, respectively (Figure 5, top). Upon treatment of PC12 cells with pertussis toxin, the distribution of SgII on the sucrose gradient changed significantly towards the position of the CSVs containing the hsPG, with the peak of SgII now being in fraction 8 (Figure 5, bottom). This effect of pertussis toxin treatment reflects either a partial missorting of SgII to CSVs or a reduction in the buyoant density of the ISGs formed in the cell-free system.

Activation of αi/αo via the receptor binding site inhibits secretory vesicle formation

The observation that pertussis toxin-catalysed ADP-ribosylation of αi/αo, which would prevent their functional interaction with receptors, stimulated the cell-free formation of secretory vesicles from the TGN (Figure 4, panels A and B) suggested that compounds which stimulate the guanine nucleotide exchange on αi/αo subunits might affect secretory vesicle formation in an opposite manner to pertussis toxin, i.e. inhibit this process. The peptide mastoparan, which mimics an activated, i.e. ligand-occupied, receptor

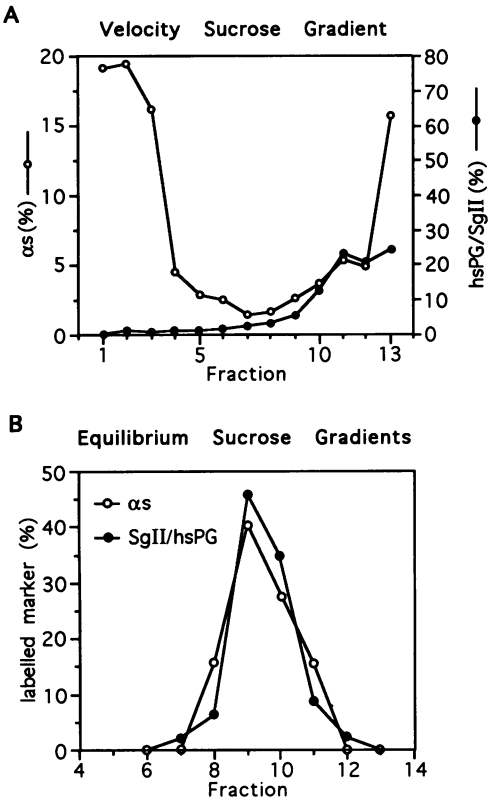

Fig. 7. Presence of a G-protein αs subunit on TGN membranes of PC12 cells. (**A**) PC12 cells were [^{35}S]sulfate-labelled for 5 min, and a PNS was prepared, incubated either at 4°C (filled circles) or at 37°C under cell-free vesicle formation conditions (open circles) and subjected to velocity sucrose gradient centrifugation. Aliquots of the fractions (top of gradient = fraction 1) were subjected to either SDS–PAGE followed by quantitation of [^{35}S]sulfate-labelled hsPG and [^{35}S]sulfate-labelled SgII, or SDS–PAGE using urea gels followed by immunoblotting using antiserum RM/1 recognizing αs subunits. Data are expressed as percentage of total labelled hsPG or SgII, or of total αs immunoreactivity (open circles) recovered per gradient. Since the hsPG and SgII curves were virtually identical, the mean of the two curves (filled circles) is shown for reasons of clarity. (**B**) PC12 cells were either [^{35}S]sulfate-labelled for 5 min, or not labelled, followed by the preparation of a PNS. The unlabelled PNS was incubated at 37°C under cell-free vesicle formation conditions with cholera toxin and [^{32}P]NAD, while the labelled PNS was kept at 4°C. Both PNSs were subjected to velocity sucrose gradient centrifugation, and fractions 9 and 10, known to contain TGN membranes (Tooze and Huttner, 1990), were pooled and subjected to a second, equilibrium sucrose gradient centrifugation. Aliquots of the fractions of the two equilibrium gradients (top of gradient = fraction 1) were subjected to SDS–PAGE and analysed for [^{35}S]sulfate-labelled hsPG, [^{35}S]sulfate-labelled SgII and [^{32}P]ADP-ribosylated αs subunits (open circles). Data are expressed as percentage of total label recovered per gradient. Since the hsPG and SgII curves were virtually identical, the mean of the two curves (filled circles) is shown for reasons of clarity.

(Higashijima *et al.*, 1990), is such a compound. We therefore investigated the effects of mastoparan on the formation of CSVs and ISGs in the cell-free system. Indeed, addition of 1 μM of mastoparan-7, a potent mastoparan analogue (Higashijima *et al.*, 1990), to the cell-free system inhibited the formation of both CSVs (Figure 6 top) and ISGs (Figure 6 bottom). A lesser inhibition of cell-free secretory vesicle formation was found with 0.1 μM mastoparan-7; results of an experiment using a higher concentration (10 μM) of mastoparan-7 could not be reliably interpreted because substantial amounts of SgII were found in fraction 1 of the velocity gradient, indicating partial lysis of membrane vesicles by the amphiphilic peptide (Higashijima *et al.*, 1990) (data not shown).

If the inhibitory effect of mastoparan-7 occurred via the receptor binding site of αi/αo, rather than being due to other effects of the peptide, one would expect that no inhibitory effect should be seen in a PNS prepared from pertussis toxin-treated cells because of the stoichiometric ADP-ribosylation, and hence structural alteration, of this specific site. As shown in Figure 6, this was found to be the case since the pertussis toxin-induced stimulation of cell-free vesicle formation was not reduced by the addition of mastoparan-7.

Association of a cholera toxin-sensitive αs subunit with the TGN of PC12 cells

The presence of multiple pertussis toxin-sensitive αi/αo subunits on the TGN of PC12 cells triggered the question whether another class of α subunit, the cholera toxin-sensitive αs subunit, might also be associated with TGN membranes. We investigated this question using the antibody RM/1 (Simonds *et al.*, 1989) which detected in PC12 cells, by both immunoblotting and immunoprecipitation, a single specific immunoreactive band corresponding to the heavy form of αs (data not shown). When a PNS of PC12 cells pulse-labelled with [^{35}S]sulfate was fractionated by velocity sucrose gradient centrifugation and the fractions analysed by immunoblotting with this antibody, the vast majority of immunoreactivity was found in the top fractions of the gradient (Figure 7A) which are known to contain the cytosol and organelles such as plasma membrane and early endosomes (Régnier-Vigouroux *et al.*, 1991). Interestingly, however, a small peak of αs immunoreactivity was detected in fractions 10–12 which in this particular velocity sucrose gradient contained the peak of [^{35}S]sulfate pulse-labelled hsPG and SgII indicative of the position of the TGN (Figure 7A). To obtain further evidence for the association of αs with the TGN, a PC12 cell PNS was subjected to cholera toxin-catalysed ADP-ribosylation with [^{32}P]NAD and subjected to velocity sucrose gradient centrifugation. Consistent with our previous report (Barr *et al.*, 1991), this type of analysis did not provide sufficient evidence for the TGN association of an ADP-ribosylated band corresponding to αs (data not shown). However, when velocity gradient fractions containing TGN were subjected to a second, equilibrium sucrose gradient centrifugation, the comparison of the distribution of ADP-ribosylated αs subunits across this second gradient with that of the TGN as identified by [^{35}S]sulfate pulse-labelled hsPG and SgII clearly showed comigration (Figure 7B). Thus, a small amount of the αs subunits of PC12 cells are associated with the TGN as indicated by subcellular fractionation.

The subcellular localization of the αs subunits in PC12 cells was also investigated by double immunofluorescence using the sheep anti-TGN38 antibody and the rabbit antibody RM/1 against αs. Consistent with the predominant localization of αs subunits in the cytosol as well as on the plasma membranes and early endosomes as observed by subcellular fractionation, the anti-αs antibody gave a diffuse, punctate staining of the cells (Figure 8, right). Interestingly, a small portion of the αs immunoreactivity was associated with structures which, by comparison with the pattern of immunoreactivity observed with anti-TGN38 (Figure 8, left), could be identified as TGN (arrowheads in Figure 8). Thus,

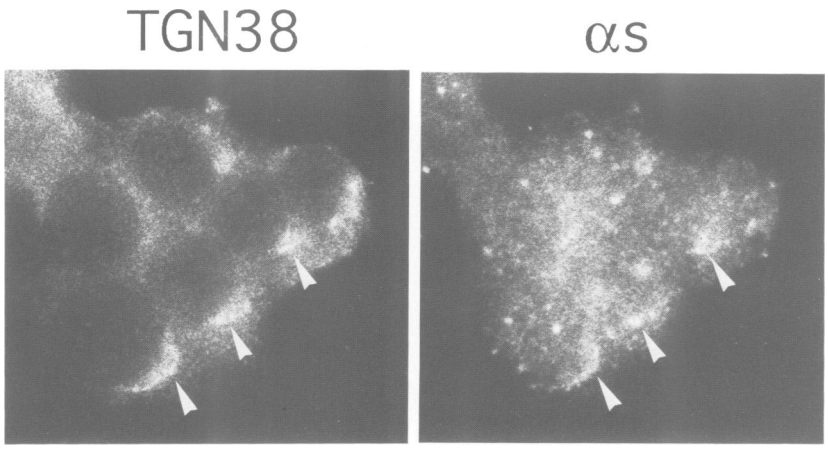

Fig. 8. Localization of αs to the TGN of PC12 cells by double immunofluorescence. PC12 cells were analysed using sheep anti-TGN38 antiserum (left) and rabbit antiserum RM/1 recognizing αs (right). Arrowheads indicate the TGN.

a small amount of the αs subunits of PC12 cells are associated with the TGN as indicated by double immunofluorescence.

Activation of αs subunits by cholera toxin stimulates cell-free formation of constitutive secretory vesicles and immature secretory granules

ADP-ribosylation of αs subunits by cholera toxin inhibits their intrinsic GTPase activity, thereby leading to their irreversible activation (Cassel and Pfeuffer, 1978; for review see Gilman, 1987). Upon treatment of PC12 cells with cholera toxin, we found that the level, in the PNS, of αs subunits that could serve as cholera toxin substrate in back-ribosylation experiments was <25% of that in a PNS from control cells, without a similar reduction in the αs level as determined by immunoblotting (data not shown). Given the association of αs subunits with the TGN of PC12 cells and the ability to activate them by cholera toxin-catalysed ADP-ribosylation, we investigated whether cholera toxin treatment of PC12 cells had an effect on secretory vesicle formation in the cell-free system. As shown in Figure 9, cholera toxin treatment resulted in the stimulation of the cell-free formation of CSVs (top panel) and, to a lesser extent, ISGs (bottom panel). In contrast to the effect of pertussis toxin (Figure 5), cholera toxin did not significantly change the distribution of SgII across the equilibrium gradient (data not shown).

The results described so far are consistent with the idea that the αs and αi/αo subunits associated with the TGN of PC12 cells, when activated, exert opposite regulatory effects on secretory vesicle formation, αs stimulating and αi/αo inhibiting this process. If this is the case, activation of αi/αo should, at least partially, antagonize the effect of cholera toxin on secretory vesicle formation. We therefore investigated the effect of GTPγS and $[AlF_4]^-$ on cell-free vesicle formation in a PNS prepared from cholera toxin-treated PC12 cells. Both GTPγS and $[AlF_4]^-$ reverted the stimulation by cholera toxin of the formation of CSVs (Figure 9 top) and ISGs (Figure 9 bottom). The relative degree of inhibition of cell-free vesicle formation by GTPγS and $[AlF_4]^-$ in this condition was similar to that seen in the PNS prepared from control cells (cf. Barr *et al.*, 1991), i.e. the absolute level of cell-free vesicle formation in the presence of GTPγS and $[AlF_4]^-$ was still higher in the PNS from cholera toxin-treated cells than that from control cells.

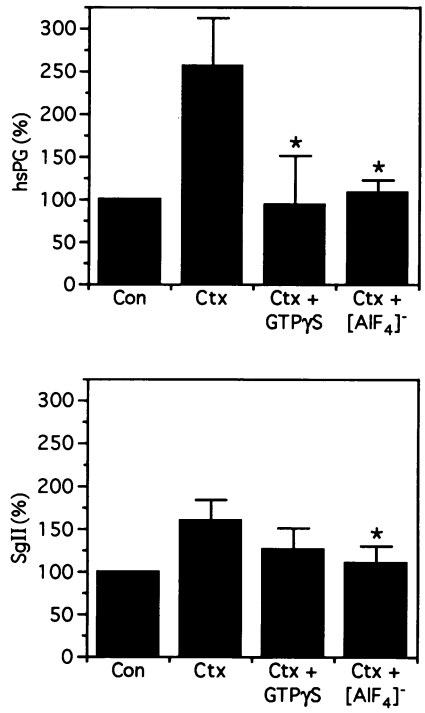

Fig. 9. Upon cholera toxin treatment of intact PC12 cells, vesicle formation in the cell-free system is stimulated, and can still be inhibited by the trimeric G-protein activators GTPγS and $[AlF_4]^-$. PC12 cells were incubated without (Con) or with (Ctx) cholera toxin, [^{35}S]sulfate-labelled for 5 min, and the PNS prepared from the two sets of cells was used for cell-free vesicle formation reactions in the absence or presence of 10 μM GTPγS or of 40 μM Al^{3+} plus 6 mM F^- ($[AlF_4]^-$), as indicated. The formation of hsPG-containing (top panel) and SgII-containing (bottom panel) post-TGN vesicles was quantitated and is expressed as percentage of that observed in the absence of the toxin (Con). The mean of three (no asterisks) or two (asterisks) separate experiments is shown; bars without asterisks indicate the standard error and bars with asterisks the variation of the individual values from the mean.

Discussion

Presence of αs and αi/αo on the TGN and their opposing effects on secretory vesicle formation

We have shown that both αs and αi/αo subunits regulate the formation of secretory vesicles from the TGN. The effects of these two classes of α subunit were opposite to

each other, αs stimulating and αi/αo inhibiting vesicle formation when activated. Thus, the picture emerging from this and previous studies (Barr *et al.*, 1991; Donaldson *et al.*, 1991; Stow *et al.*, 1991; Ktistakis *et al.*, 1992) is that secretory vesicle formation, which has been viewed as a constitutive event, is in fact a highly regulated process.

Since both αs and several αi/αo subunits were found to be associated with TGN membranes of PC12 cells, the simplest explanation for the effects of pertussis toxin and cholera toxin on vesicle formation is that they were the result of a direct action of these toxins on the TGN-associated α subunits. This conclusion is supported by several observations. First, as shown for pertussis toxin, virtually all αi/αo molecules present in the cells, and therefore also the TGN-associated αi/αo, were ADP-ribosylated upon toxin treatment of the cells. Second, after such treatment, the G-protein activator mastoparan-7 added to the cell-free system no longer inhibited vesicle formation. Since the action of mastoparan-7 on cell-free vesicle formation from the TGN requires that the αi/αo subunits on the TGN are in an activatable state, the loss of the mastoparan effect upon pertussis toxin treatment implies that the TGN-associated αi/αo subunits were the target of the toxin. Third, cyclic AMP, the second messenger generated upon cholera toxin treatment of cells (Gilman, 1987), and phosphodiesterase inhibitors had no effect on vesicle formation in the cell-free system (M.Ohashi and W.B.Huttner, unpublished observations).

The presence of multiple α subunits on the TGN extends previous work which had demonstrated the association of αi3 with the Golgi complex (Ercolani *et al.*, 1990; Stow *et al.*, 1991). It will be important to investigate the molecular basis for targetting the various α subunits to the TGN, and to determine whether the membrane association of these subunits is subject to regulation. It will also be interesting to study whether the αi/αo and the αs subunits associated with the TGN are TGN-resident proteins or associate with this compartment only transiently and leave it with the newly formed secretory vesicles. In this context, it should be mentioned that secretory granules have previously been shown to contain αi/αo and αs subunits (Toutant *et al.*, 1987; Rotrosen *et al.*, 1988; Watson *et al.*, 1992).

Given the opposing effects of activated αs and αi/αo subunits on cell-free vesicle formation, how can one explain that GTPγS and $[AlF_4]^-$, which activate all of these α subunits (Bourne *et al.*, 1991), inhibit vesicle formation? Our interpretation is that when both αs and αi/αo subunits are active, the αi/αo subunits are dominant with respect to vesicle formation, possibly because the levels of αi/αo subunits exceed those of αs (Gilman, 1987). This is supported by our observation that the stimulation of vesicle formation upon αs activation by cholera toxin was reverted by the addition of GTPγS or $[AlF_4]^-$, which activated the α subunits not activated by cholera toxin, i.e. αi/αo. The inhibition of vesicle formation by mastoparan-7, which presumably activated not only αi/αo but also αs, is also consistent with this interpretation.

Receptors and effectors

The trimeric G-protein activator $[AlF_4]^-$, while inhibiting vesicle formation from the TGN (Barr *et al.*, 1991), promotes the binding to Golgi membranes of β-COP and arf (Donaldson *et al.*, 1991) which are believed to be required for vesicle formation (Rothman and Orci, 1992). These observations are puzzling if one assumes that the basic machinery for vesicle formation is the same in the two systems in which these observations were made. The association of αs with TGN membranes may resolve this issue. Given the stimulatory effect of activated αs on vesicle formation, one possible explanation is that the increased β-COP and arf binding seen upon addition of $[AlF_4]^-$ may be related to the activation of αs rather than αi/αo. Although evidence exists that the binding of β-COP to Golgi membranes observed *in vitro* can be regulated by a pertussis toxin-sensitive trimeric G-protein (Ktistakis *et al.*, 1992), our interpretation would be consistent with data supporting a direct arf−Gs interaction (Kahn, 1990).

The regulation of secretory vesicle formation by multiple G-proteins poses two important questions. First, are there guanine nucleotide exchange factors which stimulate the TGN-associated α subunits, and, if so, what are they? Our observation that cell-free vesicle formation is increased upon pertussis toxin treatment implies that such guanine nucleotide exchange factors were acting on αi/αo in the cell-free system in the control condition. It will be interesting to investigate whether these factors are (i) seven transmembrane domain receptors (Taylor, 1990), (ii) single transmembrane domain receptors like the insulin-like growth factor II/mannose-6-phosphate receptor which couples to αi2 (Okamoto *et al.*, 1990), and/or (iii) proteins like GAP-43 which stimulates nucleotide exchange on αo (Strittmatter *et al.*, 1991).

Second, what are the effector systems mediating the stimulation and inhibition of vesicle formation by αs and αi/αo, respectively? One possibility is that there is one common effector system on which both αs and αi/αo act. Alternatively, αs and αi/αo may exert their effects on vesicle formation via distinct effector systems. This second possibility is consistent with the putative relationship between the binding of components of the non-clathrin coat to Golgi membranes and αs rather than αi/αo (see above), which might indicate that the latter α subunits exert their inhibitory effects on vesicle formation via an effector system other than coat proteins.

This possibility is also consistent with our observation that pertussis toxin treatment, besides stimulating cell-free vesicle formation, had a second effect, i.e. a decrease in the segregation of SgII from the hsPG as measured by equilibrium sucrose gradient centrifugation. The latter may indicate partial missorting of SgII to CSVs upon pertussis toxin treatment, as was previously observed upon addition of βγ subunits to the cell-free system (Barr *et al.*, 1991). On the other hand, since in contrast to these previous findings the distribution of SgII across the gradient still showed a single peak, it is also possible that pertussis toxin treatment resulted in a selective decrease in the buoyant density of the ISGs formed in the cell-free system. Whatever the explanation, the multiplicity of effects of trimeric G-proteins on secretory vesicle formation indicates that a regulatory network of unexpected complexity is involved in this process.

Materials and methods

Cell culture and metabolic labelling

PC12 cells were grown in DMEM supplemented with 10% horse serum and 5% fetal calf serum (growth medium) as previously described (Tooze and Huttner, 1990). Labelling of cells with carrier-free [^{35}S]sulfate (Amersham) and L-[^{35}S]methionine (Amersham) was performed as follows.

[^{35}S]*Sulfate pulse-labelling*. PC12 cells (15 cm dishes) were incubated for 30 min at 37°C in 10% CO_2 in 10 ml of sulfate-free DMEM (DMEM lacking sulfate with 1% of the normal methionine and cysteine concentration) supplemented with 1% dialysed horse serum and 0.5% dialysed fetal calf serum. Following medium change (new volume: 5 ml), the cells were placed on a rocker, received 4 mCi per dish of [^{35}S]sulfate and were incubated for another 5 min.

Long-term [^{35}S]methionine labelling. PC12 cells (15 cm dishes) were incubated for 30 min at 37°C in 10% CO_2 in 10 ml of methionine-free MEM supplemented with 2% horse serum and 0.1% fetal calf serum. Following medium change, the cells received 500 μCi per dish of [^{35}S]methionine and were incubated for 16 h.

Cell-free vesicle formation

A PNS was prepared from [^{35}S]sulfate pulse-labelled PC12 cells and incubated for 60 min at 37°C as described in detail previously (Tooze and Huttner, 1990, 1992), except that unlabelled PAPS was omitted. 10 μM GTPγS or 40 μM $AlCl_3$ plus 6 mM KF ($[AlF_4]^-$) were added as indicated in the figure legends. The mastoparan analogue mastoparan-7 (INLKALAALAKALL-NH_2) (Higashijima *et al.*, 1990) was added from a 1 mM stock in 10 mM HEPES–KOH (pH 7.2) to a final concentration of 1 μM.

Separation of CSVs and ISGs

CSVs and ISGs were separated from the TGN by velocity sucrose gradient centrifugation as described (Tooze and Huttner, 1990, 1992), and the gradient fractions were analysed by SDS–PAGE and fluorography. When indicated, fractions 2–5 of the velocity gradient, containing the bulk of post-TGN vesicles, or fractions 9 and 10, containing TGN, were subjected to equilibrium sucrose gradient centrifugation as described (Tooze and Huttner, 1990, 1992) using the modified sucrose gradient as described in Barr *et al.* (1991). The fractions from the equilibrium gradient were analysed by SDS–PAGE and fluorography.

Isolation of TGN membranes after GTPγS treatment of the PNS

A PNS prepared from [^{35}S]sulfate pulse-labelled PC12 cells as above was incubated for 20 min at 4°C with 100 μM ATP in the absence or presence of 10 μM GTPγS. TGN membranes were then isolated from the PNS as will be described in detail elsewhere (F.A.Barr and W.B.Huttner, manuscript in preparation). Briefly, the PNS was centrifuged for 30 min at 110 000 *g* on a 0.8/1.2 M sucrose step gradient, and the 0.8/1.2 M interphase was collected. After 16 h on ice, aliquots of the interphase were used for cell-free ADP-ribosylation by pertussis toxin as described below.

Treatment of PC12 cells with pertussis toxin or cholera toxin

PC12 cells (15 cm dishes) were incubated for 16 h at 37°C in 10% CO_2 in 40 ml growth medium, supplemented with 4 μg of either pertussis toxin (a gift from Dr M.Witvliet, Bilthoven, the Netherlands) or cholera toxin (Calbiochem). Pertussis toxin was added from a stock solution of 1.8 mg/ml in 4 M $MgCl_2$, 25 mM Tris–HCl, pH 7.8; cholera toxin was added from a stock that had been reconstituted to 1 mg/ml in 1 mM EDTA, 200 mM NaCl, 50 mM Tris–HCl, pH 7.5.

Cell-free ADP-ribosylation

For activation, the above stock solutions of pertussis toxin and cholera toxin were mixed 1:1 with 50 mM DTT and incubated for 30 min at 37°C. For some experiments, the above pertussis toxin stock was first diluted with 0.5 vol of water and then mixed with the DTT.

Back-ribosylations. Back-ribosylation reactions were performed using 10 μl aliquots of either PC12 cell PNS or TGN membranes in a final reaction volume of 100 μl. The final reaction mixture contained either: 9 μg/ml activated pertussis toxin, 0.25 mM DTT (from the activation reaction), 20 mM $MgCl_2$ (from the pertussis toxin stock), 20 mM EDTA, 1 mM ATP, 0.1 mM GTP, 20 μM NAD, 10 mM thymidine, 25 mM Tris–HCl, pH 7.5, 0.25 M sucrose and 100 μCi/ml [^{32}P]NAD (NEN, 800 Ci/mmol); or: 50 μg/ml activated cholera toxin, 2.5 mM DTT (from the activation reaction), 10 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, 0.1 mM GTP, 20 μM NAD, 10 mM thymidine, 25 mM Tris–HCl, pH 7.5, 100 mM potassium phosphate, pH 7.0, 0.25 M sucrose and 100 μCi/ml [^{32}P]NAD. Incubations were for 60 min at 37°C. Samples were analysed by SDS–PAGE and autoradiography.

Identification of toxin substrates by ADP-ribosylation. A PNS from unlabelled PC12 cells was subjected to cell-free vesicle formation as described (Tooze and Huttner, 1990, 1992), except that 10 mM thymidine was included in the homogenization buffer. In addition, for pertussis toxin reactions, magnesium acetate was omitted from the homogenization buffer and the EDTA concentration was raised to 2 mM. For cholera toxin reactions, sodium sulfate in the homogenization buffer was reduced from 1.6 to 0.8 mM and 0.8 mM sodium phosphate were added. In addition to the ingredients of the homogenization buffer, samples contained either: 1 μg/ml activated pertussis toxin, 42 μM DTT (from the activation reaction), 2.25 mM $MgCl_2$ (from the pertussis toxin stock; yielding a final free magnesium concentration in the reaction of ~0.25 mM), 10 μM NAD, and 20 μCi/ml [^{32}P]NAD; or: 4 μg/ml activated cholera toxin, 0.2 mM DTT (from the activation), 10 μM NAD and 20 μCi/ml [^{32}P]NAD. After incubation for 60 min at 37°C, samples were subjected to sequential velocity and equilibrium sucrose gradient centrifugation, and the distribution of [^{32}P]ADP-ribosylated G-protein α subunits in the gradient fractions was determined by SDS–PAGE and autoradiography.

Immunoprecipitation of G-protein subunits

Unless otherwise indicated, all steps were performed at room temperature. [^{35}S]methionine-labelled PC12 cells were washed at 4°C with PBS and lysed in lysis buffer (0.5% SDS, 50 mM sodium phosphate, pH 7.2, 1 mM DTT; 2 ml per 15 cm dish). Lysates were passed through a 20-gauge needle (10 times up and down), boiled for 2 min, and centrifuged for 5 min at 13 000 r.p.m. in an Eppendorf centrifuge in order to remove debris. Aliquots of 250 μl of the resulting supernatant (cleared lysate) were diluted with 1 ml of 1.25× detergent buffer, yielding (at 1× concentration) 150 mM NaCl, 10 mM sodium phosphate, pH 7.2, 1% sodium deoxycholate, 1% Nonidet P-40, 0.5% SDS and 1 mM DTT. For immunological preclearing, the following procedure was performed twice consecutively: the diluted cleared lysate received 2 μl of normal rabbit serum (giving an estimated final concentration of 15 μg/ml of total IgG) and was incubated for 30 min on a rotating wheel, followed by the addition of 10 μl of a 10% suspension of pansorbin cells (Calbiochem) in 1× detergent buffer, a further incubation for 60 min and centrifugation for 5 min at 13 000 r.p.m. in an Eppendorf centrifuge to remove the pansorbin cells. For specific precipitations, the resulting supernatant then received 2 μl of antiserum EC/2, antiserum 1C or normal rabbit serum and was incubated for 30 min on a rotating wheel, followed by the addition of 10 μl of a 10% suspension of pansorbin cells, a further incubation for 60 min and centrifugation for 5 min at 13 000 r.p.m. in an Eppendorf centrifuge. The pansorbin cell pellets were washed five times with 1× detergent buffer, and the immunoprecipitated material was eluted from the cells by boiling in Laemmli sample buffer and analysed by SDS–PAGE and fluorography.

SDS–PAGE and immunoblotting

SDS–PAGE was performed and gels were processed as described (Lee and Huttner, 1983), in some experiments with the following modifications. To achieve separation of G-protein αi and αo subunits, 10% polyacrylamide separating gels (but not stacking gels) were prepared using acrylamide stock solutions with half the normal bisacrylamide concentration (acrylamide:bisacrylamide, 30:0.4). To separate different G-protein αi and αo subunits from each other, long (32 cm separating gel) 4 M urea/8% polyacrylamide–SDS gels were used and run at 15 mA in the cold-room (Gierschik *et al.*, 1989). Immunoblotting was performed according to standard procedures, using 25 mM Tris/190 mM glycine containing 20% methanol as transfer buffer (minigels: 20 V, 1 h on ice, Genieblotter, Idea Scientific Company; long urea gels: 30 mA, 16 h in the cold-room). Blocking of the nitrocellulose sheets was performed either in PBS containing 10% low fat milk powder for 2 h at room temperature, or in PBS containing 5% BSA overnight at 4°C. The anti-G-protein antisera were used at 1:500 to 1:1000 dilutions. Immunoreactive G-protein α and β subunits were revealed either by incubation with [^{125}I]protein A (0.4 μCi/ml final; Amersham), or using the ECL system (Amersham) according to the manufacturer's instructions.

Antibodies

The following polyclonal rabbit antisera were used. From NEN (Simonds *et al.*, 1989): EC/2 [recognizing αi3; some cross-reactivity with αo has been reported but was not observed under our conditions of immunoprecipitation (see Figure 3C)], RM/1(recognizing αs) and GA/2 (recognizing αi1–3 and αo1 and 2). Antibody 136A (recognizing αi1 and αi2) was a gift from Dr P.Gierschik (Heidelberg, Germany), and antibody 1C (recognizing αo and β subunits) was a gift from Dr L.van der Voorn (Amsterdam, The Netherlands). Sheep antiserum against TGN38 was a gift of Dr G.Banting (Bristol, UK).

Quantitations

Quantitation of autoradiograms, fluorograms and immunoblots was performed by densitometric scanning using the Pharmacia–LKB ultroscan XL. Vesicle formation was determined using the hsPG as a marker for CSVs and SgII as a marker for ISGs and was calculated as described previously (Tooze and Huttner, 1990, 1992) with minor modifications as follows. For each marker, the values obtained in velocity gradient fractions were first expressed as percentage of total per gradient. The percentage values of fractions 1–5 of the 4°C sample were then subtracted from the percentage values of fractions 1–5 of the various 37°C incubations to give the budding efficiencies of CSVs and ISGs. The budding efficiencies were then expressed as percentages, with the budding efficiency of the control condition being set to 100 as indicated in the figure legends.

Immunofluorescence

Double immunofluorescence of paraformaldehyde-fixed PC12 cells was performed using standard procedures (see Rosa *et al.*, 1989). Sheep anti-TGN38 antiserum, rabbit antiserum EC/2 against αi3, and rabbit antiserum RM/1 against αs were used at 1:50 dilution, and the secondary antibodies were fluorescein-conjugated donkey anti-sheep and rhodamine-conjugated goat anti-rabbit (Dianova).

Acknowledgements

We are indebted to Dr Sharon Tooze for her generous support of the PhD thesis work of Francis A.Barr at the EMBL. We thank Drs G.Banting, P.Gierschik and L.van der Voorn for their kind gifts of antibodies, Dr M.Witvliet for a generous supply of pertussis toxin, and Drs L.Birnbaumer, J.Codina and P.Gierschik for their help in identifying G-protein α subunits, and Drs L.Birnbaumer, P.Gierschik, T.Pfeuffer, K.Spicher, S.Tooze and L.van der Voorn for advice and helpful discussion. We also wish to thank Ruth Jelinek for excellent technical assistance, Alan Summerfield for excellent photography and artwork, and Margret Shrady for secretarial help with the manuscript. A.L. was supported by an EMBO long-term fellowship, F.A.Barr is in the EMBL PhD programme, and W.B.Huttner was supported by the SFB 317 of the DFG.

References

Ausiello,D.A., Stow,J.L., Cantiello,H.F., de Almeida,J.B. and Benos,D.J. (1992) *J. Biol. Chem.*, **267**, 4759–4765.
Balch,W.E. (1992) *Current Biol.*, **2**, 157–160.
Barr,F.A., Leyte,A., Mollner,S., Pfeuffer,T., Tooze,S.A. and Huttner,W.B. (1991) *FEBS Lett.*, **294**, 239–243.
Barr,F.A., Leyte,A. and Huttner,W.B. (1992) *Trends Cell Biol.*, **2**, 91–93.
Birnbaumer,L., Abramowitz,J. and Brown,A.M. (1990) *Biochim. Biophys. Acta*, **1031**, 163–224.
Bourne,H.R., Sanders,D.A. and McCormick,F. (1991) *Nature*, **349**, 117–127.
Burgoyne,R.D. (1992) *Trends Biochem. Sci.*, **17**, 87–88.
Cassel,D. and Pfeuffer,T. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 2669–2673.
Chabre,M. (1990) *Trends Biochem. Sci.*, **15**, 6.
Chavrier,P., Parton,R.G., Hauri,H.-P., Simons,K. and Zerial,M. (1990) *Cell*, **62**, 317–329.
Donaldson,J.G., Kahn,R.A., Lippincott-Schwartz,J. and Klausner,R.D. (1991) *Science*, **254**, 1197–1199.
d'Enfert,C., Wuestehube,L.J., Lila,T. and Schekman,R. (1991) *J. Cell. Biol.*, **114**, 663–670.
Ercolani,L., Stow,J.L., Boyle,J.F., Holtzman,E.J., Lin,H., Grove,J.R. and Ausiello,D.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 4635–4639.
Fischer von Mollard,G., Mignery,G.A., Baumert,M., Perin,M.S., Hansen,T.J., Burger,P.M., Jahn,R. and Südhof,T.C. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1988–1992.
Gierschik,P. (1992) *Current Topics Microbiol. Immunol.*, **175**, 69–96.
Gierschik,P. and Jakobs,K.H. (1992) In Herken,H. and Hucho,F. (eds), *Handbook of Experimental Pharmacology*. Springer-Verlag, Berlin, Vol. 102, pp. 807–839.
Gierschik,P., Sidiropoulos,D. and Jakobs,K.H. (1989) *J. Biol. Chem.*, **264**, 21470–21473.
Gilman,A.G. (1987) *Annu. Rev. Biochem.*, **56**, 615–649.
Griffiths,G., Pfeiffer,S., Simons,K. and Matlin,K. (1985) *J. Cell Biol.*, **101**, 949–964.
Higashijima,T., Burnier,J. and Ross,E.M. (1990) *J. Biol. Chem.*, **265**, 14176–14186.
Kahn,R.A. (1990) In Iyengar,R. and Birnbaumer,L. (eds), *G Proteins*. Academic Press. San Diego, p. 201.
Ktistakis,N.T., Linder,M.E. and Roth,M.G. (1992) *Nature*, **356**, 344–346.
Lee,R.W.H. and Huttner,W.B. (1983) *J. Biol. Chem.*, **258**, 11326–11334.
Luzio,J.P., Brake,B., Banting,G., Howell,K.A., Braghetta,P. and Stanley,K.K. (1990) *Biochem. J.*, **270**, 97–102.
Okamoto,T., Katada,T., Murayama,Y., Ui,M., Ogata,E. and Nishimoto,I. (1990) *Cell*, **62**, 709–717.
Pfeffer,S.R. (1992) *Trends Cell Biol.*, **2**, 41–46.
Rexach,M. and Schekman,R. (1991) *J. Cell Biol.*, **114**, 219–229.
Régnier-Vigouroux,A., Tooze,S.A. and Huttner,W.B. (1991) *EMBO J.*, **10**, 3589–3601.
Ribeiro-Neto,F., Mattera,R., Grenet,D., Sekura,R.D., Birnbaumer,L. and Field,J.B. (1987) *Mol. Endocrinol.*, **87**, 472–481.
Rosa,P., Weiss,U., Pepperkok,R., Ansorge,W., Niehrs,C., Stelzer,E.H.K. and Huttner,W.B. (1989) *J. Cell Biol.*, **109**, 17–34.
Rosa,P., Mantovani,S., Rosboch,R. and Huttner,W.B. (1992) *J. Biol. Chem.*, **267**, 12227–12232.
Rothman,J.E. and Orci,L. (1992) *Nature*, **355**, 409–415.
Rotrosen,D., Gallin,J.I., Spiegel,A.M. and Malech,H.L. (1988) *J. Biol. Chem.*, **263**, 10958–10964.
Salminen,A. and Novick,P. (1987) *Cell*, **49**, 527–538.
Schekman,R. (1985) *Annu. Rev. Cell Biol.*, **1**, 115–144.
Simonds,W.F., Goldsmith,P.K., Codina,J., Unson,C.G. and Spiegel,A.M. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7809–7813.
Stearns,T., Willingham,M.C., Botstein,D. and Kahn,R.A. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 1238–1242.
Stow,J.L., de Almeida,J.B., Narula,N., Holtzman,E.J., Ercolani,L. and Ausiello,D.A. (1991) *J. Cell Biol.*, **114**, 1113–1124.
Strittmatter,S.M., Valenzuala,D., Sudo,Y., Linder,M.E. and Fishman,M.C. (1991) *J. Biol. Chem.*, **266**, 22465–22471.
Taylor,C.W. (1990) *Biochem. J.*, **272**, 1–13.
Taylor,T.C., Kahn,R.A. and Melançon,P. (1992) *Cell*, **70**, 69–80.
Tooze,S.A. and Huttner,W.B. (1990) *Cell*, **60**, 837–847.
Tooze,S.A. and Huttner,W.B. (1992) *Methods Enzymol.*, **219**, 81–93.
Tooze,S.A., Weiss,U. and Huttner,W.B. (1990) *Nature*, **347**, 207–208.
Toutant,M., Aunis,D., Bockaert,J., Homburger,V. and Rouot,B. (1987) *FEBS Lett.*, **215**, 339–344.
Watson,E.L., DiJulio,D., Kauffman,D., Iversen,J., Robinovitch,M.R. and Izutsu,K.T. (1992) *Biochem. J.*, **285**, 441–449.

Received on August 17, 1992; revised on September 9, 1992