Biochem. J. (1992) 284, 321–326 (Printed in Great Britain)

Exocytosis from permeabilized bovine adrenal chromaffin cells is differently modulated by guanosine 5′-[γ-thio]triphosphate and guanosine 5′-[βγ-imido]triphosphate

Evidence for the involvement of various guanine nucleotide-binding proteins

Gudrun AHNERT-HILGER,*§ Ulrike WEGENHORST,† Brigitte STECHER,† Karsten SPICHER,‡ Walter ROSENTHAL‡ and Manfred GRATZ†

* Institut für Neuropsychopharmakologie der Freien Universität Berlin, Ulmenallee 30, D-1000 Berlin 19,
† Abteilung Anatomie und Zellbiologie der Universität Ulm, D-7900 Ulm,
and ‡ Institut für Pharmakologie der Freien Universität Berlin, D-1000 Berlin 33, Germany

1. In bovine adrenal chromaffin cells made permeable either to molecules $\leqslant$ 3 kDa with alphatoxin or to proteins $\leqslant$ 150 kDa with streptolysin O, the GTP analogues guanosine 5′[βγ-imido]triphosphate (p[NH]ppG) and guanosine 5′-[γ-thio]triphosphate (GTP[S]) differently modulated Ca^{2+}-stimulated exocytosis. 2. In alphatoxin-permeabilized cells, p[NH]ppG up to 20 μM activated Ca^{2+}-stimulated exocytosis. Higher concentrations had little or no effect. At a free Ca^{2+} concentration of 5 μM, 7 μM-p[NH]ppG stimulated exocytosis 6-fold. Increasing the free Ca^{2+} concentration reduced the effect of p[NH]ppG. Pretreatment of the cells with pertussis toxin prevented the activation of the Ca^{2+}-stimulated exocytosis by p[NH]ppG. 3. In streptolysin O-permeabilized cells, p[NH]ppG did not activate, but rather inhibited Ca^{2+}-dependent catecholamine release under all conditions studied. In the soluble cytoplasmic material that escaped during permeabilization with streptolysin O, different G-protein α-subunits were detected using an appropriate antibody. Around 15% of the cellular α-subunits were detected in the supernatant of permeabilized control cells. p[NH]ppG or GTP[S] stimulated the release of α-subunits 2-fold, causing a loss of about 30% of the cellular G-protein α-subunits under these conditions. Two of the α-subunits in the supernatant belonged to the G_o type, as revealed by an antibody specific for $G_o\alpha$. 4. GTP[S], when present alone during stimulation with Ca^{2+}, activated exocytosis in a similar manner to p[NH]ppG. Upon prolonged incubation, GTP[S], in contrast to p[NH]ppG, inhibited Ca^{2+}-induced exocytosis from cells permeabilized by either of the pore-forming toxins. This effect was resistant to pertussin toxin. 5. The p[NH]ppG-induced activation of Ca^{2+}-stimulated release from alphatoxin-permeabilized chromaffin cells may be attributed to one of the heterotrimeric G-proteins lost during permeabilization with streptolysin O. The inhibitory effect of GTP[S] on exocytosis is apparently not mediated by G-protein α-subunits, but by another GTP-dependent process still occurring after permeabilization with streptolysin O.

INTRODUCTION

Permeabilized bovine adrenal chromaffin cells kept in short-term culture are an excellent model with which to analyse the molecular requirements for exocytosis and its modulation. In order to gain control over the cytosolic composition we use the well-defined pores generated by bacterial toxins: alphatoxin from *Staphylococcus aureus* yields small pores which allow the passage of molecules of $\leqslant$ 3 kDa, whereas streptolysin O (SLO) permeabilizes cells to proteins of $\leqslant$ 150 kDa [1,2].

Poorly hydrolysable analogues of GTP, guanosine 5′-[βγ-imido]triphosphate (p[NH]ppG) and guanosine 5′-[γ-thio]triphosphate (GTP[S]), are able to permanently stimulate GTP-binding proteins. Effects of GTP[S] and p[NH]ppG may therefore be taken as evidence for an involvement of a GTP-binding protein. So far two classes of GTP-binding proteins have been distinguished: the plasma membrane-associated heterotrimeric G-proteins responsible for various signal transduction pathways [3], and a growing family of low-molecular-mass GTP-binding proteins [4] thought to be involved in intracellular membrane sorting and fusion processes [5,6], as well as in axonal transport [7].

Secretion by exocytosis which involves vesicle transport and fusion of two membranes may also be controlled or modulated by the various GTP-binding proteins. Histamine release from mast cells [8–11] and exocytosis from neutrophils [12,13] is drastically enhanced by guanine nucleotides. Likewise, exocytosis from permeabilized cytotoxic T-lymphocytes [14,15] or insulin release from rat insulinoma cells [16] can be fully activated in the presence of GTP[S] or p[NH]ppG. On the other hand, catecholamine release from rat pheochromocytoma (PC 12) cells is inhibited by GTP[S], probably due to the activation of a pertussis toxin-sensitive G-protein [17]. Furthermore, a receptor-coupled pertussis toxin-sensitive G-protein has been found to modulate exocytosis downstream from the increase in Ca^{2+} in the insulin-secreting cell line RINmF5 [18,19].

These contrasting results may not only reflect differences in the secretory mechanism of the cell types investigated, since even the data obtained with bovine adrenal chromaffin cells are contradictory. Both stimulation [20–24] and inhibition [20] of exocytosis by guanine nucleotides has been described. So far, it is unclear whether these differing results are due to different kinds of cell preparations (freshly isolated cells versus cells in short-term culture), to the permeabilization technique used

Abbreviations used: KG buffer, potassium glutamate buffer; NTA, nitrilotriacetic acid; SLO, streptolysin O; GTP[S], guanosine 5′-[γ-thio]triphosphate; p[NH]ppG, guanosine 5′-[βγ-imido]triphosphate.

§ To whom correspondence should be sent, at present address: Medizinische Klinik und Poliklinic, Abt. Gastroenterologie, Klinikum Steglitz FU Berlin, Hindenburgdamm 30, D-1000 Berlin 45, Germany.

(electrical, detergent or alphatoxin), or to the various protocols used to stimulate exocytosis. The different data obtained for bovine adrenal chromaffin cells may also be explained by the involvement of different GTP-binding proteins which either activate or inhibit exocytosis. Both heterotrimeric [25] as well as low-molecular-mass [26,27] GTP-binding proteins have been found in cultured bovine adrenal chromaffin cells.

We compared the intracellular actions of GTP[S] and p[NH]ppG on exocytosis in adrenal chromaffin cells permeabilized either with alphatoxin or with SLO. The effects of both guanine nucleotides markedly depended on the permeabilization procedure and on the Ca^{2+} concentration used to stimulate exocytosis. Evidence is provided that heterotrimeric G-proteins which are lost by the cells after permeabilization with SLO might be involved in the modulation of exocytosis.

MATERIALS AND METHODS

Materials

GTP[S] and p[NH]ppG were purchased from Sigma, Deisenhofen, Germany. Alphatoxin was purified from the culture medium of *Staphylococcus aureus* [28]. SLO was purified [29] and kindly provided by S. Bhakdi (Institut für Medizinische Mikrobiologie, Mainz, Germany). Pertussis and cholera toxins were from List Laboratories, Campbell, CA, U.S.A. G_i and G_o were purified from porcine brain [30] and used as standards in SDS/PAGE and immunoblotting. Antibodies against the purified subunits of the G-proteins were characterized previously [31,32]. All other materials were from commercial sources.

Methods

Chromaffin cells from bovine adrenal medulla were prepared and cultivated for up to 7 days as described [33]. They were preloaded with [^{3}H]noradrenaline and washed as described previously [34]. Cells were permeabilized either for 30 min at 36 °C with alphatoxin, or for 2 min at 36 °C with SLO [34] in KG buffer [potassium glutamate 150 mM, Pipes 10 mM, EGTA 1 mM, nitrilotriacetic acid (NTA) 5 mM, Mg^{2+}/ATP 2 mM, free Mg^{2+} 1 mM, pH 7, supplemented with 0.1% BSA]. A total of 300–500 haemolytic units (h.u.) of either pore-forming toxin [35] was applied to 10^7 cells. The permeabilization buffer was exchanged for fresh buffer containing various amounts of free Ca^{2+}. If not stated otherwise, guanine nucleotides were present 20–30 min before and during the 10 min stimulation with Ca^{2+}. The presence of guanine nucleotides up to 200 μM did not change the free Ca^{2+} concentration under these conditions, as determined by the use of a Ca^{2+}-sensitive electrode (see below). [^{3}H]Noradrenaline release was determined in the supernatant and in the cells after lysis with 0.2% SDS [2,36].

Free Ca^{2+} concentrations were calculated using the stability constants given in [37] and controlled with a Ca^{2+}-sensitive electrode [38,39]. The neutral carrier was kindly provided by W. Simon, ETH Zürich, Switzerland.

SDS/PAGE of the various cell fractions was performed using 9% polyacrylamide gels supplemented with 6 M-urea [40]. Immunoblotting followed a protocol given earlier [32], using the ECL detection system (Amersham). For quantification of the G-protein α-subunits, blots were analysed with a laser densitometer (2202 Ultroscan; LKB, Rockeville, MD, U.S.A.).

RESULTS

p[NH]ppG enhances Ca^{2+}-dependent exocytosis from alphatoxin-permeabilized adrenal chromaffin cells

p[NH]ppG activated Ca^{2+}-stimulated exocytosis in alphatoxin-permeabilized adrenal chromaffin cells. The nucleotide was usually present 20–30 min before and during the 10 min Ca^{2+} stimulation (Figs. 1*a* and 1*b*; see also Tables 3 and 4). If p[NH]ppG was only present during the 10 min Ca^{2+} stimulation, similar results were obtained (Table 1). Under the latter conditions, GTP[S] also enhanced Ca^{2+}-induced exocytosis from alphatoxin-permeabilized adrenal chromaffin cells (Table 1). However, when present 20–30 min before as well as during the 10 min Ca^{2+} stimulation, GTP[S], in contrast to p[NH]ppG, inhibited exocytosis (Tables 2 and 3).

The stimulatory effect on Ca^{2+}-dependent exocytosis was maximal at p[NH]ppG concentrations of 10–20 μM. At higher concentrations the effect was less pronounced or did not occur (Figs. 1*a* and 1*b*). The free Ca^{2+} concentration used to stimulate exocytosis was also crucial for the modulation of this process by p[NH]ppG: an effect was only observed when free Ca^{2+} concentrations below 50 μM were applied. Activation of exocytosis by p[NH]ppG was most prominent with free Ca^{2+} concentrations

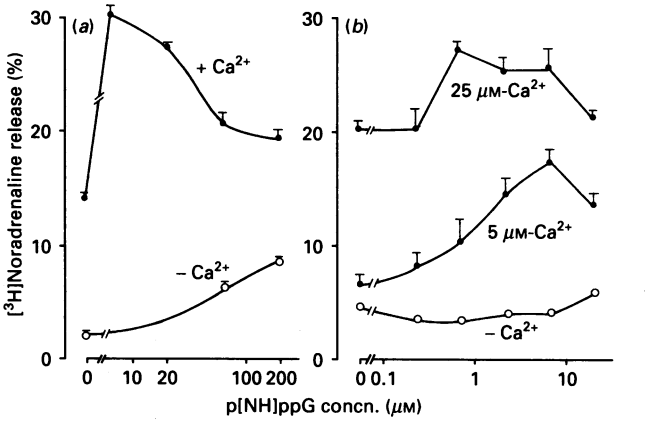

Fig. 1. Dose–response curve for effects of p[NH]ppG on Ca^{2+}-stimulated exocytosis from alphatoxin-permeabilized bovine adrenal chromaffin cells

(*a*) [^{3}H]Noradrenaline-preloaded bovine adrenal chromaffin cells were permeabilized with alphatoxin for 30 min with the given concentrations of p[NH]ppG. Cells were stimulated for 10 min with fresh buffer containing no (○) or 15 μM (●) free Ca^{2+} and p[NH]ppG as indicated. (*b*) The experimental procedure followed the protocol given in (*a*), with p[NH]ppG concentrations between 0.4 and 20 μM. Cells were stimulated with 5 or 25 μM free Ca^{2+} and the indicated amount of p[NH]ppG. Values are means of either two (controls without Ca^{2+} in *b*) or three samples ±S.D.

Table 1. Short-term effects of GTP[S] and p[NH]ppG on Ca^{2+}-stimulated noradrenaline release from alphatoxin-permeabilized bovine adrenal chromaffin cells

Bovine adrenal chromaffin cells were permeabilized for 30 min with alphatoxin. The buffer was replaced by fresh buffer containing the given free Ca^{2+} concentration and the nucleotide indicated. Release in the absence of Ca^{2+} does not vary under these conditions. Values are the means ± S.D. of three determinations. Note that only at the lower Ca^{2+} concentration was a significant activation observed by either nucleotide.

Free Ca^{2+} concentration (μM)	[^{3}H]Noradrenaline release (%)		
	Control	p[NH]ppG (10 μM)	GTP[S] (10 μM)
0	4.9±0.6	4.3±0.8	4.6±0.2
4.5	10.1±1.0	18.2±0.7	14.1±1.6
15	23.5±2.2	25.9±0.8	22.0±1.4

Table 2. Long-term effects of GTP[S] on basal and Ca^{2+}-stimulated exocytosis from alphatoxin-permeabilized adrenal chromaffin cells

Preloaded bovine adrenal chromaffin cells were permeabilized with alphatoxin for 30 min at 30 °C with or without the given amount of GTP[S]. The buffer was exchanged for fresh buffer containing no or 24 μM free Ca^{2+} and GTP[S] as indicated. Values represent the means ± S.D. of three samples. Note that GTP[S] only slightly affected the basal release, whereas it drastically reduced the Ca^{2+}-stimulated one.

GTP[S] concn. (μM)	[^{3}H]Noradrenaline release (%)	
	No Ca^{2+}	Ca^{2+} (24 μM)
0	5.4±1.0	44.2±2.2
50	8.9±0.6	23.8±0.8
10		28.4±1.8
2		37.3±3.9
0.4		43.3±1.2

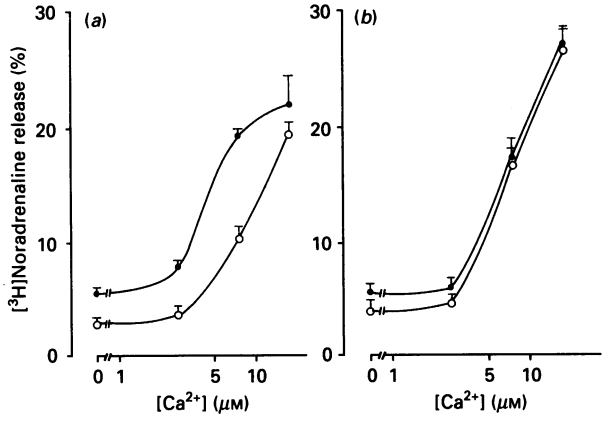

Fig. 2. Effect of pertussis toxin on the p[NH]ppG-induced stimulation of Ca^{2+}-dependent exocytosis from alphatoxin-permeabilized adrenal chromaffin cells

Adrenal chromaffin cells were loaded for 3 h with [^{3}H]noradrenaline in either the absence (*a*) or the presence (*b*) of 5 μg of pertussis toxin/ml. The subsequent washing and permeabilization with alphatoxin in either the presence or the absence of 20 μM-p[NH]ppG followed the protocol given in Fig. 1. The cells were then stimulated with the free Ca^{2+} concentrations indicated, supplemented (●) or not (○) with 20 μM-p[NH]ppG. Values are means ± S.D. of three determinations.

around 5 μM. Effects started at 0.5 μM- and were maximal (6-fold stimulation of Ca^{2+}-dependent exocytosis) at 7 μM-p[NH]ppG. On using a higher Ca^{2+} concentration (25 μM), the stimulatory effect was less pronounced (Fig. 1*b*).

In order to find out whether a heterotrimeric G-protein was involved, adrenal chromaffin cells were pretreated with pertussis toxin for 3 h. After this treatment, p[NH]ppG failed to enhance Ca^{2+}-stimulated exocytosis (Figs. 2*a* and 2*b*). The pretreatment with pertussis toxin, however, slightly increased basal and Ca^{2+}-stimulated release (see also [41]). A similar observation was made with alphatoxin-permeabilized PC 12 cells [17].

G-protein α-subunits escape from SLO-permeabilized adrenal chromaffin cells

In contrast to the situation seen in alphatoxin-permeabilized adrenal chromaffin cells, p[NH]ppG had either no effect on or inhibited Ca^{2+}-stimulated exocytosis in cells permeabilized by SLO (Tables 3 and 4). One possible explanation for the failure of

Table 3. Direct comparison of the effects of guanine nucleotides on Ca^{2+}-stimulated exocytosis from chromaffin cells permeabilized with either alphatoxin or SLO

Preloaded bovine adrenal chromaffin cells were permeabilized with alphatoxin in the presence of 20 μM-p[NH]ppG or 20 μM-GTP[S] for 30 min. The buffer was exchanged for fresh buffer containing 15 μM free Ca^{2+} and the indicated guanine nucleotide. Alternatively, SLO-treated (2 min) cells were incubated for 25 min in the absence or the presence of either nucleotide. The buffer was exchanged for fresh buffer containing 15 μM free Ca^{2+} and the indicated guanine nucleotide. Values are the means ± S.D. of three samples.

	[^{3}H]Noradrenaline release (%)	
	Alphatoxin	SLO
Buffer	2.4±0.3	2.4±0.7
Ca^{2+}	21.6±1.0	17.1±0.7
p[NH]ppG (20 μM) plus Ca^{2+}	31.3±1.7	13.7±1.3
GTP[S] (20 μM) plus Ca^{2+}	17.8±0.5	8.3±0.3

Table 4. Effects of p[NH]ppG on Ca^{2+}-stimulated exocytosis from SLO-permeabilized adrenal chromaffin cells

Preloaded bovine adrenal chromaffin cells were permeabilized for 2 min with SLO and then incubated for 25 min with or without the indicated concentration of p[NH]ppG. The buffer was exchanged for fresh buffer containing no or 15 μM free Ca^{2+} and the guanine nucleotide. Release (means ± S.D. of three samples) was measured after 10 min.

	[^{3}H]Noradrenaline release (%)
Buffer	3.9±1.1
Ca^{2+}	15.3±1.3
p[NH]ppG (200 μM) plus Ca^{2+}	12.9±1.0
p[NH]ppG (20 μM) plus Ca^{2+}	12.8±1.0
p[NH]ppG (2 μM) plus Ca^{2+}	15.1±0.8

p[NH]ppG to enhance exocytosis under these conditions is the loss of an intracellular compound through the large pores generated by SLO. We therefore investigated whether G-protein α-subunits could be detected in the incubation medium of SLO-permeabilized cells. Using an antibody raised against a confined region common to various G-protein α-subunits (α_{common} antibody), different α-subunits were found in the supernatant of the cells after SLO treatment (Fig. 3*a*). By contrast, in alphatoxin-permeabilized cells, a release of G-protein α-subunits was not observed (results not shown). As a consequence, p[NH]ppG could stimulate Ca^{2+}-induced exocytosis in these cells (see Figs 1 and 2, and Tables 1 and 3).

The three bands between 43 and 36 kDa in the supernatant (Fig. 3*a*, lanes 1–3) and in the cell lysate (Fig. 3*a*, lanes 4–6) may represent α-subunits of the G_o or G_i subtypes; the middle one could in fact be a mixture of the α-subunits of G_{o1} and G_{i2}, which often co-migrate [42]. Two of the proteins were also recognized by an antibody against a confined region of G_o α-subunits ($\alpha_{o,common}$ antibody) (Fig. 3*a*). The protein band in the 50 kDa region recognized by the α_{common} antibody (Fig. 3*a*) may be attributed to an α-subunit of G_s, but its identity has to be further clarified. The other proteins in the 40 kDa region could be α-subunits of G_i, but also need further clarification, since an antibody against a confined region of G_i α-subunits ($\alpha_{i,common}$ antibody) failed to stain them. However, membranes of chromaffin cells contain at least two α-subunits of G_i besides two G_o α-

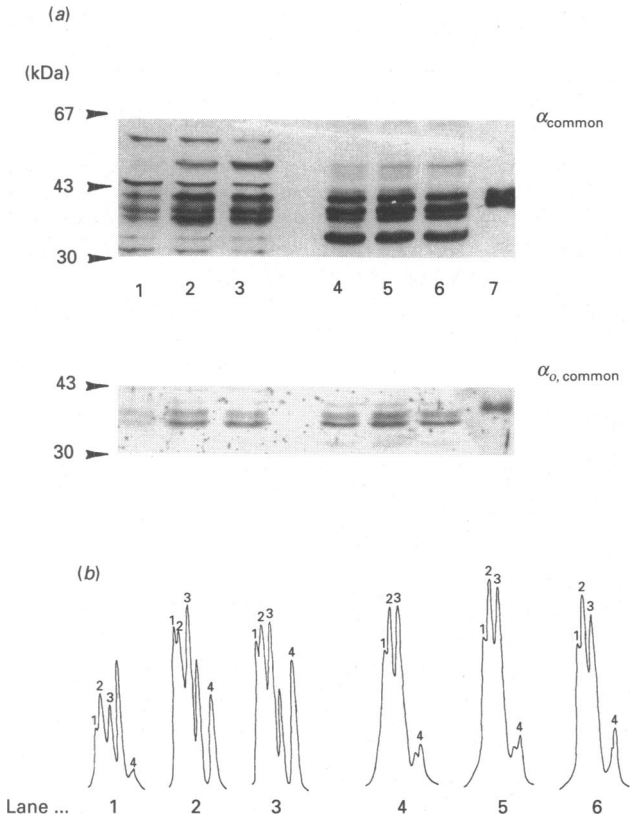

Fig. 3. p[NH]ppG and GTP[S] provoke the release of G-protein α-subunits from SLO-permeabilized cells

(*a*) Cells were permeabilized with SLO for 2 min, and then treated with Ca^{2+}-free KG buffer (lanes 1 and 4), 100 μM-p[NH]ppG (lanes 2 and 5) or 100 μM-GTP[S] (lanes 3 and 6) in Ca^{2+}-free KG buffer. The supernatants (lanes 1–3) as well as the Lubrol extracts (lanes 4–6) from about 3×10^6 cells were each precipitated with acetone. The precipitated material was dissolved in electrophoresis buffer and subjected to SDS/PAGE in the presence of 6 M-urea and immunoblotting, using an affinity-purified α_{common} antibody. From the cell extract only half of the material was loaded on the gel. The α_{common} antibody recognized G-protein α-subunits in the 40 kDa region which co-migrate with a G_i/G_o standard preparation from porcine brain (lane 7). A protein in the 50 kDa region may represent a G_s α-subunit. In addition, the α_{common} antibody reacted with proteins of unknown identity at 48 kDa and in the 34 kDa region. The α_{common} antibody was removed by incubating the nitrocellulose filter in a solution containing 2-mercaptoethanol (100 mM), SDS (2%), Tris/HCl (62.5 mM), pH 6.7, for 30 min at 50 °C. The stripped filter was re-probed by an $\alpha_{o,common}$ antiserum. Two faster-migrating proteins in the 39/40 kDa region were stained. The upper band of the G_i/G_o standard had also disappeared. However, the porcine brain G_o α-subunit migrated a bit slower than the respective bovine adrenal chromaffin $G_{\alpha o}$ subunits, probably due to species differences in the post-translational modification. The release of both G_o α-subunits was stimulated by guanine nucleotides. (*b*) Scan of the α_{common} blot from (*a*). Peaks 1–3 correspond to the three bands in the 40 kDa region, and peak 4 to the protein at 50 kDa. The release of these α-subunits was stimulated by guanine nucleotides.

subunits [42]. The α_{common} antibody also reacted with a protein at 48 kDa of so far unknown identity, which was also found in RINmF5 cells (W. Rosenthal, unpublished work). Besides the G-protein α-subunits, two proteins of unknown identity, migrating in the 34 kDa region, were recognized by the α_{common} antibody.

p[NH]ppG and GTP[S] increased the release of G-protein α-subunits in the 39/40 kDa region, as well as the release of a 50 kDa protein, probably $G_s\alpha$ (Fig. 3*a*). For quantification, blots from the supernatant and the cell lysate stained with the α_{common} antibody were scanned (Fig. 3*b*). From these scans the percentage release of G-protein α-subunits in the 40 kDa region was calculated. In control cells, 13.8±4.1% (mean±S.D., $n = 5$) of the G-protein α-subunits were detected in the supernatant. Addition of 100 μM guanine nucleotide stimulated the release of α-subunits in the 40 kDa region 2-fold, to give 27.4±4.4% release ($n = 5$) for p[NH]ppG- and 24.3% release ($n = 2$) for GTP[S]-treated cells. Release of G-protein α-subunits depended on the applied guanine nucleotide concentrations between 8 and 200 μM (results not shown). The proteins migrating at 48 kDa and 34 kDa were also detected in the supernatant of the cells, but their release was unaffected by the addition of guanine nucleotides (Fig. 3*a*).

GTP[S] inhibits Ca^{2+}-induced exocytosis from adrenal chromaffin cells permeabilized by alphatoxin or SLO

GTP[S] dose-dependently inhibited Ca^{2+}-induced exocytosis when the nucleotide was present prior to the stimulation with Ca^{2+}. Cells permeabilized by either pore-forming toxin gave similar results (Tables 2 and 3). Half-maximal inhibition occurred at 10 μM-GTP[S]. The inhibitory action of GTP[S] increased with the free Ca^{2+} concentration (results not shown). Pretreatment of the cells with either pertussis or cholera toxin or the direct application of their activated forms to the permeabilized cells did not alter GTP[S]-induced inhibition of exocytosis (results not shown).

DISCUSSION

The present study demonstrates that GTP[S] and p[NH]ppG differently affect exocytosis in permeabilized bovine adrenal chromaffin cells in primary culture. The pore size, the time of incubation with the respective guanine nucleotide, its concentration and the free Ca^{2+} concentration used for stimulation are crucial for the observed effects. At least three steps in the exocytotic process are modulated by the GTP analogues: (i) stimulation of exocytosis in the absence of Ca^{2+}, (ii) enhancement of Ca^{2+}-dependent exocytosis, and (iii) inhibition of Ca^{2+}-dependent exocytosis.

p[NH]ppG elevates basal, Ca^{2+}-independent exocytosis in adrenal chromaffin [21,24] and PC 12 [36,43] cells. The guanine nucleotide-regulated component is not lost after permeabilization with SLO [36] or digitonin [21,43]. The effects of GTP[S] on basal catecholamine release from both types of chromaffin cells are less pronounced [17,21,36]. It remains to be determined whether a heterotrimeric G-protein or one of the low-molecular-mass GTP-binding proteins is involved in this process.

In alphatoxin-permeabilized adrenal chromaffin cells, p[NH]-ppG increased Ca^{2+}-induced catecholamine release. Low concentrations, which do not change basal release, were more effective than high concentrations. The p[NH]ppG-induced activation was also more pronounced when low concentrations of free Ca^{2+} were used to stimulate exocytosis, whereas at higher concentrations of Ca^{2+}, a further activation did not occur. An increased free Ca^{2+} concentration may therefore act as a negative feed-back control to the p[NH]ppG-induced activation of exocytosis.

The activating effect of p[NH]ppG on Ca^{2+}-stimulated exocytosis may be attributed to a heterotrimeric G-protein for two reasons. First, after pretreatment of the cells with pertussis toxin, p[NH]ppG no longer stimulates Ca^{2+}-dependent exocytosis in alphatoxin-permeabilized cells. Secondly, in SLO-permeabilized cells p[NH]ppG fails to stimulate, but rather inhibits, Ca^{2+}-dependent exocytosis. The permeabilization with SLO leads to the loss of various α-subunits, tentatively identified as $G_s\alpha$

(50 kDa region), $G_i\alpha$, and two $G_o\alpha$-subunits (40 kDa region). In membranes of adrenal chromaffin cells two α-subunits of G_o and two of G_i have been identified, amongst them G_{o1}, G_{i1} and G_{i2} [42]. The pattern of G-protein α-subunits released into the medium after permeabilization and extraction of the cells resembles that detected in the membranes. The applied guanine nucleotides do not discriminate between the α-subunits of the various G-proteins. Further studies are necessary to clarify the individual roles of the different G-proteins during secretion by exocytosis.

It is tentative to speculate that the release of the pertussis toxin-sensitive G-protein α-subunits from SLO-permeabilized cells may be responsible for the failure of p[NH]ppG to enhance Ca^{2+}-induced exocytosis. The activated α-subunits, now extremely diluted in the extracellular medium, might not find their target within the cell. Incubation with GTP analogues before the stimulation of exocytosis by Ca^{2+} should intensify the loss of α-subunits. Under the latter conditions up to 30% of the cellular α-subunits of the G_i or G_o type could be detected in the supernatant. The loss of the 50 kDa protein, which might represent an α-subunit of G_s, was more pronounced than the loss of the α-subunits in the 40 kDa region. Probably, at least in bovine adrenal chromaffin cells, this protein is found more in the cytoplasm than attached to membranes.

GTP analogues may also stabilize the solubilized G-protein α-subunits against proteolytic attack, as shown for the α-subunits of G_o and G_i from bovine brain [44]. In this case GTP analogues would increase the detectable amounts of α-subunits in the supernatant by protecting them from degradation. This could mimic an increased release. However, the experimental conditions under which we observed a p[NH]ppG- or GTP[S]-induced stimulation of the release of G-protein α-subunits from permeabilized cells resembled the conditions in intact cells (except for the dilution of the cytoplasmic proteins) and may not favour degradation by endogenous enzymes (e.g. proteases). Permeabilization by SLO is restricted to the plasma membrane, leaving other intracellular membranes unaffected [2]. The G-protein α-subunits were collected from the supernatant, in which the free Ca^{2+} concentration was buffered below 100 nM, and before the cells were stimulated with Ca^{2+}.

Our results are in line with the detection of pertussis toxin-sensitive G-proteins in the supernatant of bovine adrenal chromaffin cells after treatment with digitonin [41]. However, the detergent digitonin may induce dissociation of G-protein α-subunits from the membrane, and the loss of pertussis toxin-sensitive substrates was not directly correlated with secretion data.

One possibility is that the G-protein involved does not directly stimulate exocytosis, but modulates it by activating the phospholipase/protein kinase C pathways, as reported for mast cells [12]. Protein kinase C has been shown to increase the Ca^{2+}-sensitivity of exocytotic membrane fusion in various secretory cells [16,17,23,45,46]. Alternatively, a G-protein not directly coupled to a plasma membrane receptor may be activated by GTP and Ca^{2+} during exocytosis [18,19,47].

Short incubations with GTP[S] mimic the p[NH]ppG-induced activation of Ca^{2+}-stimulated catecholamine release from alphatoxin-permeabilized adrenal chromaffin cells ([22]; the present paper). However, the short-term effects of GTP[S] and p[NH]ppG cover only one aspect of the rather complex pattern of effects that guanine nucleotides exert during exocytosis.

The GTP[S]-induced inhibition of exocytosis in adrenal chromaffin cells occurs irrespective of the permeabilizing toxin applied. The incubation time is the crucial parameter. Inhibition occurs only when GTP[S] is present before the cells are challenged with Ca^{2+} ([20]; the present paper). The GTP[S]-induced inhibition is unaffected by cholera toxin or pertussis toxin. This contrasts with the pertussis toxin-sensitive inhibition of exocytosis from alphatoxin-permeabilized PC 12 cells [17]. Probably different steps are affected in PC 12 and adrenal chromaffin cells which result in the same cellular response, i.e. the inhibition of exocytosis.

Like p[NH]ppG, GTP[S] stimulates the release of a G-protein α-subunit from SLO-permeabilized cells. However, this does not affect the inhibition of exocytosis observed under conditions whereby guanine nucleotides are expected to stimulate Ca^{2+}-dependent exocytosis (permeabilization with alphatoxin). Since there is no obvious connection with the heterotrimeric G-proteins, GTP[S] might affect one of the low-molecular-mass GTP-binding proteins involved in transport and membrane traffic, which apparently exist in membrane-bound as well as soluble forms [5,6]. Examples are the secp4 and ypt1 proteins from yeast which are involved in either the fusion of secretory vesicles with the plasma membrane or vesicular transport through the Golgi complex [48]. The proteins may be subjected to a constant cycle of activation (by GTP) and inactivation [6,47]. Binding of a poorly hydrolysable GTP analogue might interrupt the cycle. As a consequence, vesicle transport or membrane fusion may be inhibited [48,49].

Low-molecular-mass GTP-binding proteins have been found in the vesicular and plasma membranes of chromaffin cells [26]. p[NH]ppG and GTP[S] probably activate these GTP-binding proteins with different sensitivities. Interestingly, a GTP-binding protein involved in vesicular transport through the Golgi complex has a 100-fold greater sensitivity to GTP[S] than to p[NH]ppG [48].

We thank M. Rudolf (Ulm), M. Bigalke, and I. Reinsch (Berlin) for expert technical assistance. Work was supported by the Deutsche Forschungsgemeinschaft and the state of Baden-Württemberg.

REFERENCES

1. Bhakdi, S. & Tranum-Jensen, J. (1987) Rev. Physiol. Biochem. Pharmacol. **107**, 147–223
2. Ahnert-Hilger, G., Mach, W., Föhr, K. J. & Gratzl, M. (1989) Methods Cell Biol. **31**, 63–90
3. Birnbaumer, L., Yatani, A., Codina, J., VanDongen, R., Graf, R., Mattera, R., Sanford, J. & Brown, A. M. (1989) in Molecular Mechanisms of Hormone Action (Gehring, U., Helmreich, E. & Schultz, G., eds.), pp. 146–176, Springer Verlag, Berlin, Heidelberg
4. Matsui, Y., Kikuchi, A., Kondo, J., Hishida, T., Teraniski, Y. & Takai, Y. (1988) J. Biol. Chem. **263**, 11071–11074
5. Goud, B., Salminen, A., Walworth, N. C. & Novick, P. J. (1988) Cell **53**, 753–768
6. Bourne, H. R. (1988) Cell **53**, 669–671
7. Bielinski, D. F., Morin, P., Dickey, B. & Fine, R. (1989) J. Biol. Chem. **264**, 18363–18367
8. Fernandez, J. M., Neher, E. & Gomperts, B. D. (1984) Nature (London) **312**, 453–455
9. Howell, T. W., Cockcroft, S. & Gomperts, B. (1987) J. Cell Biol. **105**, 191–197
10. Gomperts, B. D., Cockcroft, S., Howell, T. W., Nüsse, O. & Tatham, P. (1987) Biosci. Rep. **7**, 369–381
11. Koopmann, W. R. & Jackson, R. C. (1900) Biochem. J. **225**, 365–373
12. Barrowman, M. M., Cockcroft, S. & Gomperts, B. (1986) Nature (London) **319**, 504–507
13. Nüße, O. & Lindau, M. (1988) J. Cell Biol. **107**, 2117–2123
14. Schrezenmeier, H., Ahnert-Hilger, G. & Fleischer, B. (1988) J. Exp. Med. **168**, 817–822
15. Schrezenmeier, H., Ahnert-Hilger, G. & Fleischer, B. (1988) J. Immunol. **141**, 3785–3790
16. Vallar, L., Biden, T. J. & Wollheim, C. B. (1987) J. Biol. Chem. **262**, 5049–5056
17. Ahnert-Hilger, G., Bräutigam, M. & Gratzl, M. (1987) Biochemistry **26**, 7842–7848
18. Ullrich, S. & Wollheim, C. (1988) J. Biol. Chem. **263**, 8615–8620
19. Ullrich, S. & Wollheim, C. (1989) FEBS Lett. **247**, 401–404

20. Knight, D. E. & Baker, P. F. (1985) FEBS Lett. **189**, 345–349
21. Bittner, M. A., Holz, R. W. & Neubig, R. R. (1986) J. Biol. Chem. **261**, 10182–10188
22. Bader, M.-F., Sontag, J.-M., Thierse, D. & Aunis, D. (1989) J. Biol. Chem. **264**, 16426–16434
23. Burgoyne, R., Morgan, A. & O'Sullivan, A. (1988) FEBS Lett. **238**, 151–155
24. Morgan, A. & Burgoyne, R. (1990) Biochem. J. **269**, 521–526
25. Toutant, M., Aunis, D., Bockaert, J., Homburger, V. & Rouot, B. (1987) FEBS Lett. **215**, 339–344
26. Doucet, J.-P., Fournier, S., Parulekar, M. & Trifaro, J.-M. (1989) FEBS Lett. **247**, 127–131
27. Burgoyne, R. & Morgan, A. (1989) FEBS Lett. **245**, 122–126
28. Lind, I., Ahnert-Hilger, G., Fuchs, G. & Gratzl, M. (1987) Anal. Biochem. **164**, 84–89
29. Bhakdi, S., Roth, M., Sziegoleit, A. & Tranum-Jensen, J. (1984) Infect. Immun. **46**, 394–400
30. Rosenthal, W., Koesling, D., Rudolph, U., Kleuss, C., Pallast, M., Yajima, M. & Schultz, G. (1986) Eur. J. Biochem. **158**, 255–263
31. Hinsch, K. D., Rosenthal, W., Spicher, K., Binder, T., Gansepohl, H., Frank, R., Schultz, G. & Joost, H. G. (1988) FEBS Lett. **238**, 191–196
32. Offermanns, S., Schäfer, R., Hoffmann, B., Bombien, E., Spicher, K., Hinsch, K.-D., Schultz, G. & Rosenthal, W. (1990) FEBS Lett. **260**, 14–18
33. Livett, B. (1984) Physiol. Rev. **64**, 1103–1161
34. Stecher, B., Gratzl, M. & Ahnert-Hilger, G. (1989) FEBS Lett. **248**, 23–27
35. Ahnert-Hilger, G., Bader, M. F., Bhakdi, S. & Gratzl, M. (1989) J. Neurochem. **52**, 1751–1758
36. Ahnert-Hilger, G., Stecher, B., Beyer, C. & Gratzl, M. (1992) Methods Enzymol., in the press
37. Martell, A. E. & Smith, R. M. (1974) Critical Stability Constants. Vol. 1, Plenum Press, New York
38. Ammann, D., Bührer, T., Schäfer, U., Müller, M. & Simon, W. (1987) Pflügers Arch. **409**, 223–228
39. Föhr, K.-J., Warchol, W. & Gratzl, M. (1992) Methods Enzymol., in the press
40. Ribeiro-Neto, F. & Rodbell, M. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 2577–2581
41. Sontag, J.-M., Thierse, D., Rouot, B., Aunis, D. & Bader, M.-F. (1991) Biochem. J. **274**, 339–347
42. Kleppisch, T., Ahnert-Hilger, G., Gollasch, M., Spicher, K., Hescheler, J., Schultz, G. & Rosenthal, W. (1992) Pfluegers Arch., in the press
43. Carroll, A., Rhoads, A. & Wagner, P. (1990) J. Neurochem. **55**, 930–936
44. Winslow, J. W., Van Amsterdam, J. R. & Neer, E. J. (1986) J. Biol. Chem. **261**, 7571–7579
45. Knight, D. E. & Baker, P. F. (1983) FEBS Lett. **160**, 98–100
46. Peppers, S. C. & Holz, R. W. (1986) J. Biol. Chem. **261**, 14665–14670
47. Gomperts, B. (1986) Trends Biochem. Sci. **11**, 290–292
48. Melancon, P., Glick, B., Malhotra, V., Weidman, P., Serafini, T., Orci, L. & Rothman, J. E. (1989) Soc. Gen. Physiol. Ser. **44**, 175–186
49. Orci, L., Malhorta, V., Amherdt, M., Serafini, T. & Rothman, J. E. (1989) Cell **56**, 357–368

Received 18 March 1991/23 December 1991; accepted 14 January 1992