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

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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.

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(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 $\mu$M 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 $\alpha$-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 $\mu$M. 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 $\mu$M were applied. Activation of exocytosis by p[NH]ppG was most prominent with free $Ca^{2+}$ concentrations

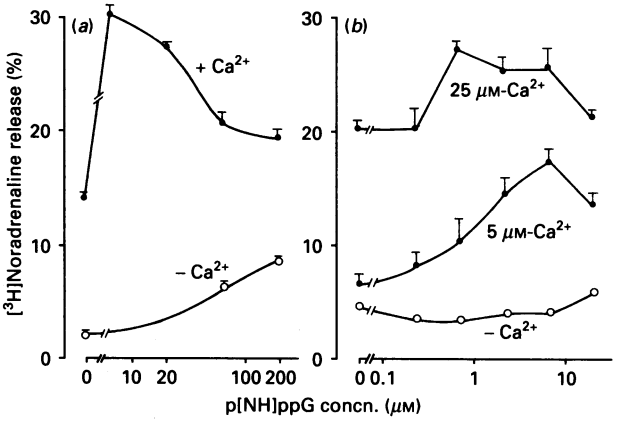


**Fig. 1. Dose–response curve for effects of p[NH]ppG on $Ca^{2+}$-stimulated exocytosis from alphatoxin-permeabilized bovine adrenal chromaffin cells**

(*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 $\mu$M (●) 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 $\mu$M. Cells were stimulated with 5 or 25 $\mu$M 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 ($\mu$M) | [$^3$H]Noradrenaline release (%) | | |
|---|---|---|---|
| | Control | p[NH]ppG (10 $\mu$M) | GTP[S] (10 $\mu$M) |
| 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 $\mu$M 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. ($\mu$M) | [$^3$H]Noradrenaline release (%) | |
|---|---|---|
| | No $Ca^{2+}$ | $Ca^{2+}$ (24 $\mu$M) |
| 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 |

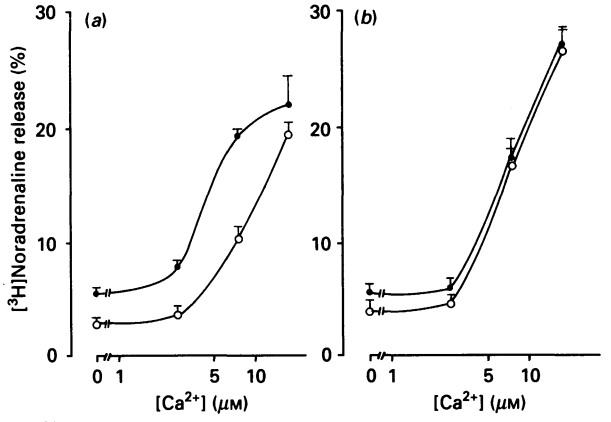


**Fig. 2. Effect of pertussis toxin on the p[NH]ppG-induced stimulation of $Ca^{2+}$-dependent exocytosis from alphatoxin-permeabilized adrenal chromaffin cells**

Adrenal chromaffin cells were loaded for 3 h with [$^3$H]noradrenaline in either the absence (*a*) or the presence (*b*) of 5 $\mu$g of pertussis toxin/ml. The subsequent washing and permeabilization with alphatoxin in either the presence or the absence of 20 $\mu$M-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 $\mu$M-p[NH]ppG. Values are means ± S.D. of three determinations.

around 5 $\mu$M. Effects started at 0.5 $\mu$M- and were maximal (6-fold stimulation of $Ca^{2+}$-dependent exocytosis) at 7 $\mu$M-p[NH]ppG. On using a higher $Ca^{2+}$ concentration (25 $\mu$M), 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 $\mu$M-p[NH]ppG or 20 $\mu$M-GTP[S] for 30 min. The buffer was exchanged for fresh buffer containing 15 $\mu$M 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 $\mu$M 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 $\mu$M) plus $Ca^{2+}$ | 31.3±1.7 | 13.7±1.3 |
| GTP[S] (20 $\mu$M) 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 $\mu$M 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 $\mu$M) plus $Ca^{2+}$ | 12.9±1.0 |
| p[NH]ppG (20 $\mu$M) plus $Ca^{2+}$ | 12.8±1.0 |
| p[NH]ppG (2 $\mu$M) 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 ($\alpha_{common}$ 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 $\alpha_{common}$ 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-

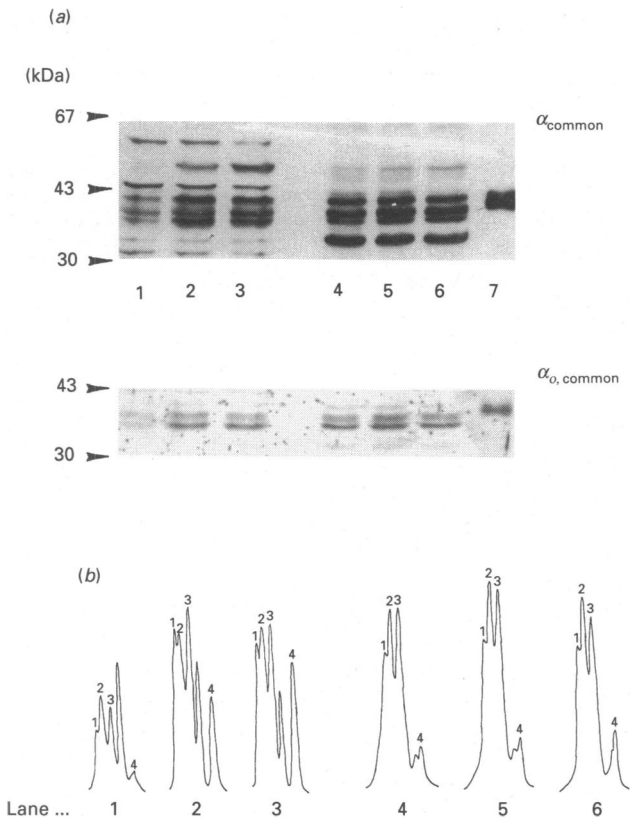


**Fig. 3. p[NH]ppG and GTP[S] provoke the release of G-protein α-subunits from SLO-permeabilized cells**

(*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 $3 \times 10^6$ 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 $\alpha_{common}$ antibody. From the cell extract only half of the material was loaded on the gel. The $\alpha_{common}$ 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 $\alpha_{common}$ antibody reacted with proteins of unknown identity at 48 kDa and in the 34 kDa region. The $\alpha_{common}$ 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 $\alpha_{common}$ 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 $\alpha_{common}$ 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 $\alpha_{common}$ 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 $\alpha_{common}$ 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 $\alpha$-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 $\alpha$-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 $\alpha$-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 $\alpha$-subunits from SLO-permeabilized cells may be responsible for the failure of p[NH]ppG to enhance $Ca^{2+}$-induced exocytosis. The activated $\alpha$-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 $\alpha$-subunits. Under the latter conditions up to 30% of the cellular $\alpha$-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 $\alpha$-subunit of $G_s$, was more pronounced than the loss of the $\alpha$-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 $\alpha$-subunits against proteolytic attack, as shown for the $\alpha$-subunits of $G_o$ and $G_i$ from bovine brain [44]. In this case GTP analogues would increase the detectable amounts of $\alpha$-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 $\alpha$-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 $\alpha$-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 $\alpha$-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 $\alpha$-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.

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