A Reassessment of Guanine Nucleotide Effects on Catecholamine Secretion from Permeabilized Adrenal Chromaffin Cells*

(Received for publication, September 14, 1988)

Marie-France Bader, Jean-Marie Sontag‡, Danièle Thiersé, and Dominique Aunis
From the Groupe de Neurobiologie Structurale et Fonctionnelle, Institut National de la Santé et de la Recherche Médicale Unité U44, Centre de Neurochimie du Centre National de la Recherche Scientifique, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France

The role of guanine nucleotides in catecholamine secretion was investigated in α-toxin-permeabilized chromaffin cells. The stable GTP analogues, GTP-γ-S (guanosine 5′-(γ-thio)triphosphate) and GMP-PNP (guanosine 5′-(β,γ-imido)triphosphate), potentiated calcium-evoked catecholamine release in a dose-dependent manner. This effect was reversed by GDP-β-S (guanosine 5′-(β-thio)diphosphate) indicating that a GTP-binding protein plays a modulatory role in the calcium-dependent secretory process in chromaffin cells. Calcium and the phosphorylating nucleotide ATP were both necessary for secretion, even in the presence of GTP analogues, suggesting that the activation of a GTP- regulatory protein alone does not trigger exocytosis in these cells. TPA (12-0-tetradecanoylphorbol-13-acetate), a direct activator of protein kinase C, was found to mimic the effects of the GTP analogues, inducing a dose-dependent potentiation of the calcium-evoked release in α-toxin-permeabilized cells. Treatment of the permeabilized cells with sphingosine, a potent inhibitor of protein kinase C, completely abolished the stimulatory effects of both TPA and GTP-γ-S. Moreover, long term incubation of chromaffin cells with TPA, a treatment which depletes cells of protein kinase C activity, suppressed the stimulatory effects of GTP-γ-S. Protein kinase C is activated when it becomes membrane-bound in the presence of calcium and diacylglycerol; here, GTP-γ-S was found to enhance the calcium-induced translocation of protein kinase C to membranes in α-toxin-permeabilized cells. These results suggest that guanine nucleotides modulate secretion by activating protein kinase C-linked events in chromaffin cells. Furthermore, the potentiation of calcium-induced secretion in α-toxin-permeabilized cells following activation of protein kinase C either directly with TPA or indirectly with GTP analogues provides additional support for the concept that protein kinase C may exert a positive control directly on the intracellular exocytotic machinery.

One approach to elucidate the intracellular mechanisms underlying exocytosis in secretory cells involves permeabilization of the plasma membrane, which permits manipulation of the intracellular environment. A number of procedures for membrane permeabilization have been developed recently. In these systems, cells release their secretory products by exocytosis simply in response to an elevation in calcium to micromolar concentrations (1-5). From such studies, a possible regulatory function of GTP analogues has been proposed in exocytosis of many different cell types but the manner in which this control is expressed varies. To date, two separate mechanisms have emerged. In platelets (6) and mast cells (7), GTP and GTP analogues act synergistically with calcium, significantly reducing the quantity of this divalent cation required to induce secretion. In other systems such as rabbit neutrophils (8) or insulinoma RINm 5F cells (9), GTP analogues appear to both potentiate the response to calcium and activate exocytotic secretion independently of calcium.

For several experimental reasons, adrenal medullary chromaffin cells provide another excellent tool to study the molecular mechanisms underlying release of secretory products (10, 11). Postreceptor events controlling exocytosis have been extensively studied in these cells as they have proven amenable to cell permeabilization techniques (12-15). Unexpectedly, certain results reported for chromaffin cells contradict data obtained from other secretory systems. In bovine chromaffin cells permeabilized by exposure to high voltage electric fields, guanine nucleotides inhibit or potentiate calcium-evoked exocytosis, depending on the GTP analogue used (16), whereas in cells permeabilized with detergents, GTP analogues have no effect on calcium-induced secretion but slightly enhance calcium-independent catecholamine release (17). Since permeabilization with a high voltage discharge can only be used for cells in suspension (2), this contradiction may reflect structural and functional differences between freshly isolated cells and cells maintained in culture. On the other hand, treatment of chromaffin cells with detergents induces a prompt leakage of cytoplasmic proteins (18) suggesting that the lack of effect of GTP analogues on calcium-evoked secretion may be due to the release of some GTP-binding proteins or target proteins controlled by them.

Previously we have described the use of α-toxin from Staphylococcus aureus as a tool for selectively permeabilizing the plasma membrane of chromaffin cell (19). The lesions generated by the toxin have a dimension which permits flux of ions and small metabolites but prevents the escape of larger molecules like cytoplasmic proteins (19-24). Thus, α-toxin preserves the general cytoplasmic organization (25) and is certainly advantageous to study the requirements of exocytosis for ions, nucleotides, and modulators of similar size. We therefore have undertaken a detailed characterization of the effects of guanine nucleotides on the exocytotic process using α-toxin-permeabilized chromaffin cells. We demonstrate that GTP analogues potentiate calcium-dependent catecholamine secretion but do not trigger secretion in the absence of cal-

* This work was supported in part by Research Grants 85-061 and 88-065 from the French Direction des Recherches, Etudes, & Techniques. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

‡ Supported by an Association pour la Recherche contre le Cancer fellowship.
Intracellular Regulation of Secretion in Chromaffin Cells

1

EXPERIMENTAL PROCEDURES

Materials—[3H]Noradrenaline (16 Ci/mmol) was supplied by Amersham (Les Ulis, France). α-Toxin was from Institut Pasteur Production (Paris, France). 12-O-Tetradecanoylphorbol-13-acetate (TPA) and forskolin were purchased from Sigma. Digitonin was from Merck (Darmstadt, West Germany). All nucleotides were purchased from Boehringer GmbH (Mannheim, West Germany). Sphingosine was a generous gift from Dr. N. Neskevic (Centre de Neurochimie, Strasbourg, France).

Culture of Chromaffin Cells—Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase and purified on self-generating Percoll gradients (19, 26). They were suspended in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum containing cytosine arabinoside (10 μM), fluorodeoxyuridine (10 μM), streptomycin (50 μg/ml), and penicillin (50 units/ml). Cells were grown on 24 multiple 16-mm Costar plates (Cambridge, MA) at a density of 2.5 × 10⁶ cells/well and maintained at 37 °C in a humidified atmosphere containing 5% CO₂, 95% air. Release experiments were carried out at 37 °C on 5–7-day-old cultures.

[3H]Noradrenaline Release from Permeabilized Cells—Chromaffin cells were loaded for 30 min with [3H]noradrenaline, washed four times with Locke’s solution (19), two times with calcium-free Locke’s solution (containing 1 mM EGTA) and once with calcium-free KG medium (150 mM glutamate, potassium salt, 10 mM PIPES, 5 mM nitrophenetic acid, 0.5 mM EGTA, 5 mM MgATP, 4.5 mM magnesium acetate, 0.2% bovine serum albumin, pH 7.0). Washing intervals were set constant at 10 min. Cells were subsequently permeabilized for 30 min with 40 units/ml α-toxin in 200 μl of calcium-free KG medium. Extracellular fluids were then removed and cells were stimulated with 200 μl of KG medium containing various amounts of calcium chloride to yield the indicated free Ca²⁺ concentrations and magnesium acetate to maintain a final free Mg²⁺ concentration of 1 mM. Release of [3H]noradrenaline is expressed as the percentage of total radioactivity present in the cells prior to calcium-induced stimulation. The amount of [3H]noradrenaline released during permeabilization remained close to 5% of the total radioactivity present in the cells prior to permeabilization.

S. aureus α-Toxin—S. aureus α-toxin was purified by ammonium sulfate precipitation at 80% saturation and stored as a suspension in an 80% saturated ammonium sulfate solution at 4 °C. The hemolytic activity of the toxin solutions was determined with sheep erythrocytes (19). Cultured chromaffin cells (2.5 × 10⁶ cells) were added to the culture medium containing 40 units/ml a-toxin. The cell solutions were sonicated twice for 20 s to ensure complete permeabilization and homogenized in 0.6 ml of ice-cold buffer composed of 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM diithiothreitol, 2 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptin, 20 μg/ml aprotinin, 20 μg/ml pepstatin. The cell solutions were sonicated twice for 5 s at setting 9 on Ultrasonic Cell Disruptor (MSE Scientific Instruments, Sussex, England). The homogenate (0.1 ml) was centrifuged at 100,000 × g for 20 min, the supernatant was removed, and the membrane fraction was resuspended in 0.3 ml of homogenizing solution. Triton X-100 was added to homogenates and membrane fractions to give a final concentration of 0.1%. A 40-μl sample of the homogenate (total cellular protein kinase C) or of the membrane fractions (membrane-bound protein kinase C) was added to the protein kinase C assay medium (27) containing 1.75 mM CaCl₂, 16 μg/ml phosphatidylserine, and 100 μM diacylglycerol to reduce the effect of Triton X-100 on protein kinase C activity (27). Protein kinase C was assayed by the protocol described by Castagna et al. (28). Protein kinase C activity defined as Ca²⁺- and phosphatidylserine/TPA-dependent activity was expressed in terms of picomoles of [32P]incorporated into proteins from [γ-32P]ATP.

Calcium Concentration—The exact free calcium concentration in KG medium was calculated as described (29) using the stability constants given by Sillen and Martell (30). The free calcium concentration was also measured using a selective calcium minielectrode operating with a neutral carrier incorporated into a polyvinyl chloride membrane (31).

Presentation of Data—All experiments described were carried out on at least two different cell preparations. In the figures which are representative of a typical experiment, data are given as the mean of triplicate determinations on the same cell preparation ± S.E.M.

RESULTS

Effects of Guanine Nucleotides on Secretion from α-Toxin-permeabilized Chromaffin Cells—Fig. 1 shows the effect of GTP-γ-S, a nonhydrolyzable GTP analogue, on catecholamine secretion from α-toxin-permeabilized chromaffin cells. GTP-γ-S promoted a dose-dependent rise in calcium-evoked secretion; for example, the enhanced release induced by 50 μM GTP-γ-S was 70% greater than that induced by calcium alone. Similarly, GMP-PNP potentiated calcium-evoked release but was less effective than GTP-γ-S at 200 μM GMP-PNP increased the calcium-induced release by only 60% (data not shown). Thus, GTP-γ-S was relatively more potent, an observation that was also reported for permeabilized platelets (6) and mast cells (7). Stimulation of secretion was specific for nonhydrolyzable guanine nucleotides since we could not reproduce these effects using GTP. In addition, neither GTP-γ-S (Fig. 1) nor GMP-PNP (data not shown) were able to trigger secretion in the absence of calcium, at any of the tested concentrations (Table I).

As expected, the effect of GTP-γ-S could be inhibited by the stable GDP analogue GDP-β-S (Fig. 2). Simultaneous

![Fig. 1. Effect of guanine nucleotides on calcium-evoked catecholamine release from α-toxin-permeabilized chromaffin cells.](image-url)
TABLE I

Nucleotide dependence of calcium-induced catecholamine secretion from α-toxin-permeabilized chromaffin cells

Chromaffin cells were permeabilized for 30 min with α-toxin in a MgATP- and calcium-free KG medium in order to deplete cells of their cytosolic ATP. Cells were then stimulated for 10 min with KG medium in the presence or absence of 20 μM free calcium and with the indicated concentrations of MgATP and GTP-γ-S. Under these conditions, calcium-dependent [3H]noradrenaline release is observed only when MgATP is present during the stimulation period. Guanine nucleotides are unable to induce catecholamine secretion in the absence of exogenous ATP. ND = not determined.

| Nucleotide conditions | 0 Ca** | 20 μM Ca** |
|-----------------------|--------|------------|
| MgATP μM | GTP-γ-S μM | % release | % release |
| 0 | 0 | 6.3 ± 0.7 | 5.8 ± 1.0 |
| 0 | 100 | ND | 6.1 ± 1.2 |
| 5 | 0 | 5.6 ± 0.6 | 18.4 ± 0.8 |
| 5 | 100 | 5.4 ± 0.5 | 24.6 ± 1.2 |

Fig. 2. Reversal by GDP-β-S of the stimulatory effect of GTP-γ-S on calcium-evoked release. Chromaffin cells were permeabilized for 30 min with 40 units/ml α-toxin in calcium-free KG medium. Cells were then stimulated with KG medium containing 100 μM free calcium and the indicated concentrations of GTP-γ-S in the presence (squares) or absence (circles) of 500 μM GDP-β-S. Results are expressed relative to the response obtained with 100 μM free calcium in the absence of GTP-γ-S (21.4 ± 0.5% [3H]noradrenaline (3H-NA) cell content). The basal calcium-independent release (0.7 ± 0.2% in the absence of GDP-β-S and 2.1 ± 0.3% in the presence of 500 μM GDP-β-S) was not significantly modified over the range of GTP-γ-S concentrations tested and was not subtracted.

Incubation of 500 μM GDP-β-S with GTP-γ-S completely blocked the secretory response induced by 20 μM GTP-γ-S and significantly reduced the effect of higher GTP-γ-S concentrations (50 μM GTP-γ-S enhanced secretion by 15% in these conditions compared to 60% in the absence of GDP-β-S). However, the inhibitory GDP analogue alone had no effect on calcium-dependent release from α-toxin-permeabilized cells, even at concentrations as high as 1 mM (data not shown).

In order to further characterize guanine nucleotides activity, we examined the effects of GTP-γ-S on the calcium dose-response curve for secretion. The presence of 50 μM GTP-γ-S during the stimulation period produced a leftward shift in the calcium activation curve for secretion since the relative stimulatory effect was more pronounced at low calcium concentrations (Fig. 3). This observation can be interpreted as a guanine nucleotides-mediated increase in the apparent affinity of exocytosis for calcium. A particular feature of the use of α-toxin as a permeabilizing agent compared to high voltage discharge or detergent lies in the possibility of inducing the release of high levels of catecholamines at higher calcium concentrations. However, free calcium levels of 100 μM induce a maximal secretory response, and the addition of more calcium has no further effect on the extent of secretion (Fig. 3).

In contrast, the combined presence of 50 μM GTP-γ-S and 100 μM free calcium during the stimulation period increased the level of the secretory response (Fig. 3), indicating that guanine nucleotides are also able to enhance the maximal effects of the Ca**+-activated processes.

MgATP Dependence of Calcium-induced Release from α-Toxin-permeabilized Cells—In the preceding experiments, MgATP was present during the permeabilization and the stimulation period. We have previously shown that MgATP is essential for calcium-stimulated release from α-toxin-permeabilized chromaffin cells and cannot be replaced by other nucleotides (19). This requirement for ATP is observable only when cells are depleted of their cytoplasmic ATP by permeabilizing in ATP-free medium. In view of a recent report showing that calcium combined with nonhydrolyzable analogues of GTP causes exocytic secretion from permeabilized mast cells (7), we examined whether GTP-γ-S supported calcium-induced release from α-toxin-treated cells depleted of endogenous ATP prior to stimulation. Cells were first permeabilized in calcium- and ATP-free medium and subsequently stimulated with permeabilizing medium containing 100 μM free calcium. As shown in Table I, catecholamine release did not occur when ATP was absent during stimulation even in the presence of 100 μM GTP-γ-S. Thus, in contrast to mast cells, guanine nucleotides cannot synergize with calcium to trigger secretion in the absence of phosphorylating nucleotides in permeabilized chromaffin cells.

Fig. 3. Effect of GTP-γ-S on the calcium dose response curve for secretion. Chromaffin cells were permeabilized with α-toxin for 30 min in calcium-free KG medium. Extracellular fluids were subsequently removed and cells were stimulated for 10 min with KG medium containing the indicated free calcium concentrations, in the absence (squares) or presence (circles) of 50 μM GTP-γ-S. Inset: noradrenaline release obtained in the presence of GTP-γ-S and expressed relative to the response obtained from control cells. The presence of GTP-γ-S causes an increase in the extent of noradrenaline release for each calcium concentration tested. [3H]-NA, [3H]noradrenaline.
**Table II**

**Effect of inositol trisphosphate (IP3) on calcium-evoked secretion from α-toxin-permeabilized chromaffin cells**

Cells were treated for 30 min with α-toxin in calcium-free KG medium. Following removal of extracellular fluids, cells were stimulated for 10 min with KG medium containing the indicated free calcium concentrations, in the absence or presence of IP3. IP3 at concentrations up to 100 μM did not increase the extent of secretion at any of the calcium concentrations tested. Moreover, if guanine nucleotide concentrations were increased in the calcium-free medium, the extent of secretion was modified by the presence of IP3. The extent of secretion observed at any calcium concentration was identical to that observed in the calcium-free medium, which is known to release calcium from intracellular stores.

**Table III**

**Effect of NTA concentration on the secretory response to GTP-γ-S and TPA**

Chromaffin cells labeled with [3H]noradrenaline were permeabilized for 30 min with α-toxin in calcium-free KG medium. Cells were then stimulated for 10 min with KG medium containing either 5 or 15 mM NTA and the appropriate amount of CaCl2 to give the indicated free calcium concentration, in the presence or absence of 200 nM TPA or 100 μM GTP-γ-S. Increasing NTA concentration does not alter the ability of TPA and GTP-γ-S to enhance the release of catecholamines when the calcium concentration is buffered at 20 μM by NTA.
modulate secretion by activating protein kinase C, we examined the effect of GTP-γ-S and TPA on α-toxin-permeabilized chromaffin cells preincubated with sphingosine in order to block protein kinase C activity. As shown in Fig. 7A, treatment of permeabilized cells with sphingosine completely suppressed the stimulatory effects of GTP-γ-S and TPA on calcium-dependent secretion. In many cell types, chronic exposure to active phorbol esters results in down-regulation of protein kinase C with a loss of enzyme activity (42-44). A similar decrease in protein kinase C activity was found in chromaffin cells incubated with TPA: for example, 24 h incubation with 1 μM TPA decreased total cellular protein kinase C activity from 140 to 44 pmol/min/mg protein and

Fig. 4. Effect of TPA and sphingosine on secretion from α-toxin-permeabilized chromaffin cells. A, cells were exposed to 40 units/ml α-toxin in calcium-free KG medium and then incubated for 10 min in calcium-free KG medium containing various concentrations of TPA. The medium was subsequently removed and cells were stimulated with KG medium containing 20 μM free calcium (closed symbols). Basal release (open symbols) was determined in calcium-free KG medium. B, α-toxin-permeabilized chromaffin cells were incubated for 10 min in calcium-free KG medium containing the indicated concentrations of sphingosine. Cells were subsequently stimulated with KG medium containing 20 or 100 μM free calcium (closed symbols) in the absence of sphingosine. Basal release was determined in either calcium-free KG medium (open squares) or MgATP-free KG medium containing 20 μM free calcium (open circles). Following 10 min stimulation, extracellular fluids were collected and [3H]noradrenaline ([H]NA) was assayed in the solutions and remaining cells. [3H]Noradrenaline release is expressed as a percentage of the total radioactivity present in the cells prior to stimulation.

Fig. 5. Calcium dose-response curve for catecholamine secretion in the presence of TPA. Chromaffin cells labeled with [3H]noradrenaline ([H]NA) were permeabilized with α-toxin in calcium-free KG medium and subsequently stimulated with KG medium containing the indicated free calcium concentrations in the absence (circles) or presence (squares) of 100 nM TPA. The inset shows noradrenaline release obtained in the presence of TPA and expressed relative to the response obtained from control cells. TPA mimics the effect of guanine nucleotides on the calcium activation curve for catecholamine release.

Fig. 6. Effect of a submaximal concentration of TPA on the GTP-γ-S dose-response curve for secretion. Chromaffin cells labeled with [3H]noradrenaline ([H]NA) were permeabilized for 30 min with 40 units/ml α-toxin in calcium-free KG medium and subsequently stimulated with KG medium containing 20 μM free calcium and the indicated GTP-γ-S concentrations, in the presence (squares) or absence (circles) of 50 nM TPA. TPA increased the secretory response obtained at submaximal concentrations of GTP-γ-S but was unable to enhance further the response measured at 50 μM GTP-γ-S.
membrane-bound protein kinase C activity from 180 to 53 pmol/min/mg protein. Therefore we examined the effects of GTP-γ-S and TPA on calcium-evoked secretion from chromaffin cells treated 24 h with TPA prior to permeabilization with α-toxin. As illustrated in Fig. 7B, calcium-evoked catecholamine release was not modified but neither GTP-γ-S, nor TPA were able to potentiate the secretory response from cells pretreated with TPA. Taken together, these findings suggest that guanine nucleotides may modulate secretion by a mechanism involving the activation of protein kinase C. In support of the specificity of this effect, neither sphingosine nor long term incubation with TPA affected the response to forskolin, a well established adenylate-cyclase activator, which enhances catecholamine release from intact cells (45, 46) and also from α-toxin-permeabilized cells (Fig. 7).

**Effect of Guanine Nucleotides on Membrane-associated Protein Kinase C Activity in α-Toxin-permeabilized Chromaffin Cells—** Activation of protein kinase C is known to be associated with a translocation of the enzyme from a soluble to a membrane-bound compartment. Indeed, TPA produced a large increase in membrane-bound protein kinase C activity in α-toxin-permeabilized cells (Table IV), which represented a translocation of approximately 85% of the total cellular protein kinase C activity. To confirm that guanine nucleotides may modulate secretion by activating protein kinase C, we examined the effect of GTP-γ-S on the membrane-associated protein kinase C activity. The fraction of protein kinase C that was particulate under resting conditions, i.e. in the absence of calcium (Table IV), represented 26.8% (control) and 24.8% in the presence of calcium. Results are expressed relative to the response obtained from control cells (open columns), stimulated with KG medium containing solely 100 μM free calcium. Results are expressed relative to the response obtained from control cells (Control) of 100 μM spingosine. Extracellular fluids were subsequently removed and cells were stimulated for 10 min with KG medium containing the indicated concentrations of calcium, in the presence of 50 μM GTP-γ-S or 100 nM TPA. Cells were then immediately scraped in ice-cold homogenizing buffer, and the suspension was sonicated and centrifuged to separate membrane from soluble fractions. Data show membrane-bound protein kinase C activity. The results are from three experiments on three different cell preparations with four wells/group in each experiment.

| Experiments | Protein kinase C activity | Increase in membrane-bound activity |
|-------------|---------------------------|-----------------------------------|
|             | pmol/min/mg protein       | %                                 |
| **Experiment 1** |                           |                                   |
| 0Ca²⁺       | 210 ± 5                   | 100                               |
| 4Ca²⁺       | 322 ± 2                   | 153                               |
| 4Ca²⁺ + GTP-γ-S | 368 ± 12                | 175                               |
| 4Ca²⁺ + TPA | 790 ± 29                  | 237                               |
| **Experiment 2** |                           |                                   |
| 0Ca²⁺       | 71 ± 5                    | 100                               |
| 20Ca²⁺      | 100 ± 4                   | 741                               |
| 20Ca²⁺ + GTP-γ-S | 128 ± 8                | 175                               |
| 20Ca²⁺ + TPA | 349 ± 20                  | 491                               |
| **Experiment 3** |                           |                                   |
| 0Ca²⁺       | 240 ± 24                  | 100                               |
| 20Ca²⁺      | 380 ± 18                  | 158                               |
| 20Ca²⁺ + GTP-γ-S | 382 ± 33                | 180                               |
| 20Ca²⁺ + TPA | 682 ± 65                  | 284                               |

*Fig. 7. Effect of sphingosine and long term treatment with TPA on GTP-γ-S, TPA-, and forskolin-induced activation of catecholamine secretion. A, chromaffin cells were permeabilized for 30 min with 40 units/ml α-toxin and then incubated for 10 min in calcium-free KG medium in the presence (Sphingosine) or absence (Control) of 100 μM spingosine. Extracellular fluids were subsequently removed and cells were stimulated for 10 min with KG medium containing either 200 nM TPA (+ TPA) or 100 μM GTP-γ-S (+ GTP-γ-S) or 1 μM forskolin (+ Forsk) in the presence (open columns) or absence (filled columns) of 100 μM free calcium. Results are expressed relative to the response obtained from control cells (Control) of 1 μM TPA. Cells released 20.9 ± 1.5% (control) and 26.8 ± 3.2% (TPA) of their [3H]noradrenaline during the 24-h preincubation period. Cells were then permeabilized and subsequently stimulated for 10 min with KG medium containing either 100 μM GTP-γ-S (+ GTP-γ-S) or 200 nM TPA (+ TPA) or 1 μM forskolin (+ Forsk) in the presence (open columns) or absence (filled columns) of 100 μM free calcium. [3H]Noradrenaline release during stimulation periods was calculated as the percentage of the total radioactivity present in the cells prior to stimulation. Results are expressed relative to the response obtained from control cells (Control) of 1 μM TPA. Cells released 20.9 ± 1.5% (control) and 26.8 ± 3.2% (TPA) of their [3H]noradrenaline during the 24-h preincubation period. Cells were then permeabilized and subsequently stimulated for 10 min with KG medium containing either 100 μM GTP-γ-S (+ GTP-γ-S) or 200 nM TPA (+ TPA) or 1 μM forskolin (+ Forsk) in the presence (open columns) or absence (filled columns) of 100 μM free calcium. [3H]Noradrenaline release during stimulation periods was calculated as the percentage of the total radioactivity present in the cells prior to stimulation. Results are expressed relative to the response obtained from control cells (Control) of 1 μM TPA. Cells released 20.9 ± 1.5% (control) and 26.8 ± 3.2% (TPA) of their [3H]noradrenaline during the 24-h preincubation period. Cells were then permeabilized and subsequently stimulated for 10 min with KG medium containing either 100 μM GTP-γ-S (+ GTP-γ-S) or 200 nM TPA (+ TPA) or 1 μM forskolin (+ Forsk) in the presence (open columns) or absence (filled columns) of 100 μM free calcium.
stimulation of α-toxin-permeabilized cells for 10 min with 20 μM Ca\(^{2+}\) increased the membrane-bound protein kinase C activity. The presence of 50 μM GTP-γ-S during the stimulation period caused a moderate but significant increase in the shift of protein kinase C to membranes in the presence of calcium (Table IV). This shift was reproducible from one chromaffin cell preparation to the other (Table IV; n = 3). Thus, guanine nucleotides promote the association of protein kinase C with chromaffin cell membranes, an observation which is consistent with the effect of guanine nucleotides on catecholamine release being mediated by protein kinase C activation.

The apparent discrepancy between the similar enhancement of catecholamine release caused by GTP-γ-S and TPA and the relative small shift of protein kinase C to membranes induced by GTP-γ-S compared to TPA may suggest that either (i) guanine nucleotides do not entirely modulate secretion by activating protein kinase C or (ii) there is not a complete linear relationship between the amount of membrane-bound protein kinase C and catecholamine secretion. Support for the latter hypothesis comes from recent observations on permeabilized chromaffin cells (60), indicating that a shift in membrane-bound protein kinase C from 20 to 60% did not cause an additional increase in subsequent calcium-dependent secretion.

**DISCUSSION**

The experiments presented here were designed to determine whether GTP analogues can modulate calcium-dependent exocytosis in adrenal medullary chromaffin cells and to determine the cellular mechanisms underlying this control. In platelets (6) and mast cells (7) guanine nucleotides synergize with calcium to trigger the exocytotic reaction. However, the results concerning adrenal chromaffin cells are conflicting. Exposure of electrically permeable bovine and chicken chromaffin cells to guanine nucleotides has different effects: calcium-dependent exocytosis in bovine cells is inhibited by GTP-γ-S but activated by GMP-PNP, whereas in chicken cells is stimulated by both molecules (16). In detergent-permeabilized bovine chromaffin cells, guanine nucleotides cause a small increase in the calcium-independent basal release and the effects of guanine nucleotides and calcium on catecholamine secretion are subadditive (17).

A simple interpretation cannot be easily offered to the various observations obtained with electropermeabilized cells. Concerning the effect of GTP analogues in detergent-treated cells, one plausible explanation may be the leakage of cytoplasmic proteins induced by permeabilization with digitonin (47-49). We have recently presented evidence that one or more of the released cytoplasmic proteins are responsible for the loss of calcium-evoked secretory activity observed following digitonin treatment (18). In view of these observations, we suggest that the inability of guanine nucleotides to increase calcium-dependent secretion in digitonin-permeated chromaffin cells is due to the loss of some GTP-binding proteins and/or proteins controlled by them. Additional support for this idea comes from the observation that phorbol esters only affect secretion when cells are preincubated with these compounds prior to digitonin permeabilization, a treatment which results in the retention of protein kinase C by favoring the membrane-bound form (47, 49).

We have previously characterized catecholamine release from α-toxin-permeabilized chromaffin cells (19) and observed that the minimum free calcium concentration required to trigger secretion is 4 μM. In contrast, detergent-treated cells and electropermeabilized cells show a greater sensitivity for calcium since secretion occurs at 0.5 μM calcium (4, 13). The particularity of α-toxin-treated cells is that the extent of secretion increases in presence of high levels of calcium reaching a maximal release of 40% of the total catecholamines at 100 μM free calcium (25). A similar level of secretion is never observed in detergent-treated cells (25) or in electropermeabilized cells (50) since increasing the calcium concentration above 20 μM results in a progressive inhibition of the secretory response in these permeable cells (25, 50). Interestingly, α-toxin- and digitonin-permeabilized cells release comparable amounts of catecholamines at 20 μM free calcium (25), a cytosolic calcium concentration which is close to the value measured in intact cells following nicotinic stimulation (51, 52).

The integrity of the cytoplasmic composition is better preserved following α-toxin treatment (19, 25). Moreover, α-toxin-permeabilized cells are able to secrete catecholamines over long period of times (60 min) compared to digitonin-permeabilized cells which lose their responsiveness to calcium within 10 min (25). Thus, by using the α-toxin experimental model, we expected to be in the position of identifying with better precision the actual effects of GTP analogues on the overall calcium-dependent exocytotic process. Our data give evidence that in α-toxin-permeabilized chromaffin cells GTP analogues are not able to trigger exocytosis by themselves but potentiate calcium-evoked secretion. In addition, the effects of GTP analogues on secretion seem to be linked to protein kinase C-mediated events, suggesting that guanine nucleotides may act on catecholamine secretion through a putative GTP-binding protein (Gp) which may control phospholipase C activity, thereby generating diacylglycerol and activating protein kinase C (53). These results do not exclude the possibility that other GTP-binding proteins may also be essential components of the transducing machinery related to the secretory process. Indeed the presence of three substrates for pertussis toxin which specifically ADP-ribosylates GTP-binding proteins, has been described recently in both plasma and secretory granule membranes of chromaffin cells (54).

It is of interest to mention that GTP analogues cannot substitute for MgATP to support calcium-evoked exocytosis implying that chromaffin cells do not seem to possess a GTP-binding protein (Gp) that directly stimulates exocytosis as recently described in mast cells (7, 55) and neutrophils (8). Thus it appears that the specific requirements of the exocytic release varies from cell to cell rendering pertinent the question of whether an unique mechanism controls the secretory process. In certain cells containing different granule populations, each type of granule can be released independently following a rise in intracellular calcium concentration which supports the view that more than one secretory control mechanism exists, the nature of which depends on the secretory products to be released. For example, platelets are able to release selectively serotonin or β-glucosaminidase from distinct granules (56), suggesting that the exocytotic process for each granule population is regulated independently. In neutrophils, secretion from two distinct granule populations is modulated differently by calcium (57), but only one class of secretory granules release their contents in response to protein kinase C activation (38). Taken together these observations reinforce the concept that a wide range of control mechanisms underlie the secretory process.

The activation of protein kinase C by phorbol esters has been of central importance in implicating this enzyme in various cellular events. Here we show that the major effect of the activation of protein kinase C either directly with TPA or indirectly with GTP analogues is to increase the extent of
the calcium-dependent catecholamine release. Since both TPA and GTP-γ-S have no effect on exocytosis in the absence of calcium it is likely that protein kinase C may be involved either as a modulator of the calcium-dependent processes or as an integral part of the machinery controlling exocytosis. In order to further define the involvement of protein kinase C in the secretory process, specific antagonists and inhibitors are necessary to inhibit the enzyme. Hannun et al. (39) have previously shown that sphingosine is a potent inhibitor of purified protein kinase C and specifically inhibits the protein kinase C-induced phosphorylation in platelets (39). The mode of inhibition is competitive with respect to phosphor esters, which activate the kinase by interacting at the same site as the natural activator diacylglycerol. Sphingosine has been used to investigate the role of protein kinase C in differentiation of HL-60 cells (40) and to demonstrate that in neutrophils, protein kinase C participates in the secretion of specific granule proteins but not in that of azurophilic granules (38). Sphingosine does not apparently affect other important intracellular signals, including calcium levels (41), calmodulin-dependent phosphorylation (39), and cyclic AMP-dependent processes (41).

Recently, Burgoyne et al. (58) showed that sphingosine inhibits calcium-activated exocytosis in digitonin-permeabilized cells and concluded that endogenous activation of protein kinase C is a major requirement for secretion in chromaffin cells. In contrast, Holz and Senter (59) reported that mild proteolysis with trypsin inhibited the TPA-induced enhancement of secretion without affecting the calcium-evoked release from digitonin-treated cells, implying that protein kinase C activation is not necessary for secretion. In the present study, we have reduced protein kinase C activity in chromaffin cells by using two different approaches: treatment with sphingosine and chronic exposure to active phospholipases which results in down-regulation of protein kinase C with a loss of enzyme activity. Both treatments remove the effect of TPA on secretion without affecting the calcium-dependent secretory response in α-toxin-permeabilized cells. Thus the activation of protein kinase C may not be an obligatory step in the machinery of exocytosis in permeabilized cells. Consistent with this conclusion is the finding that H7 and sphingosine which reduce by 80% the protein kinase C activity in electropermeabilized chromaffin cells have little effect on the calcium-dependent release (50).

To conclude, we propose that the secretory response from α-toxin-permeabilized chromaffin cells consists of a primary response, which is not affected by sphingosine or by long term treatment with TPA, and a secondary response triggered by TPA or guanine nucleotides and inhibited by sphingosine or by long term treatment with TPA. Both primary and secondary responses require calcium and MgATP. Our data suggest that the secondary response could be a protein kinase C-dependent event requiring the activation of the enzyme by a mechanism implicating a GTP-binding protein.

Acknowledgments—We express our sincere gratitude to Dr. Nenad Neskovic for the gift of sphingosine and to Dr. Bruno Rouot and Prof. Allan Schneider for stimulating discussions. We wish to thank Dr. Nancy Grant for revising the manuscript, Marie-Odile Revel and Dr. Anant N. Malviya for protein kinase C measurements, and Françoise Hert for typing the manuscript.

REFERENCES

1. Gomperts, B. D., and Fernandez, J. M. (1985) Trends Biochem. Sci. 10, 414–417
2. Knight, D. E., and Scrutton, M. C. (1986) Biochem. J. 234, 497–506
3. Brooks, J. C., and Trenl, S. (1983) J. Neurochem. 40, 468–473
4. Dunn, L. A., and Holz, R. W. (1983) J. Biol. Chem. 258, 4989–4993
5. Wilson, S. P., and Kirchner, N. (1983) J. Biol. Chem. 258, 4994–4999
6. Hanlam, R. J., and Davidson, M. L. (1984) FEBS Lett. 174, 90–95
7. Howell, T. W., Cockcroft, S., and Gomperts, B. D. (1987) J. Cell Biol. 105, 191–197
8. Barrowman, M. M., Cockcroft, S., and Gomperts, B. D. (1986) Nature 319, 504–507
9. Vallar, L., Biren, T. J., and Wolhime, C. B. (1987) J. Biol. Chem. 262, 5499–5506
10. Livett, B. G. (1984) in Cell Biology of the Secretory Process (Cantin, M., ed) pp. 310–358, Karger, Basel
11. Trifaro, J. M. (1982) Trends Pharmacol. Sci. 3, 389–392
12. Lee, S. A., and Holz, R. W. (1986) J. Biol. Chem. 261, 10789–10796
13. Baker, P. F., and Knight, D. E. (1981) Philos. Trans. R. Soc. Lond. B Biol. Sci. 296, 85–103
14. Perrin, D., Langley, O. K., and Aunis, D. (1987) Nature 326, 498–501
15. Sontag, J. M., Aunis, D., and Bader, M. F. (1988) Eur. J. Cell Biol. 48, 316–321
16. Knight, D. E., and Baker, P. F. (1985) FEBS Lett. 189, 345–349
17. Bittner, M. A., Holz, R. W., and Neubig, R. R. (1986) J. Biol. Chem. 261, 10182–10188
18. Sarafian, T., Aunis, D., and Bader, M. F. (1987) J. Biol. Chem. 262, 16071–16075
19. Bader, M. P., Thiersé, D., Aunis, D., Ahnert-Hilger, G., and Gratzl, M. (1986) J. Biol. Chem. 261, 5777–5783
20. Thelestam, M., and Molby, R. (1979) Biochim. Biophys. Acta 557, 156–169
21. Fuaas, R., Bhakdi, S., Szapegoet, A., Tranum-Jensen, J., Kranz, T., and Wellensiek, H. J. (1981) J. Biol. Chem. 256, 83–94
22. McEwen, B. F., and Arion, W. J. (1985) J. Cell Biol. 100, 1922–1929
23. Ahnert-Hilger, G., Bhakdi, S., and Gratzl, M. (1985) J. Biol. Chem. 260, 12730–12734
24. Hofman, R. J. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 1624–1628
25. Grant, N. J., Aunis, D., and Bader, M. F. (1987) Neuroscience 23, 1143–1155
26. Bader, M. F., Trifaro, J. M., Langley, O. K., Thiersé, D., and Aunis, D. (1986) J. Cell Biol. 102, 636–646
27. Marnoudi, A., Labourdette, G., Marsel, M., Huang, F. L., Huang, K. P., Vincendon, G., and Malviya, A. N. (1989) J. Biol. Chem. 264, 1172–1179
28. Castagna, M., Takai, Y., Kibbuchi, K., Sano, K., Kikkawa, Y., and Nishizuka, Y. (1982) J. Biol. Chem. 257, 7847–7851
29. Flodgaard, H., and Fleron, P. (1974) J. Biol. Chem. 249, 3455–3474
30. Silten, J. O., and Martell, A. E. (1971) Stability Constants of Metal Ion Complexes, Suppl. 1, The Chemical Society, London
31. Simon, W., Amann, D., Oehme, M., and Morf, W. E. (1978) Ann. N. Y. Acad. Sci. 307, 52–70
32. Paris, S., and Poyysegger, J. (1987) J. Biol. Chem. 262, 1970–1976
33. Cockcroft, S., and Gomperts, B. D. (1985) Nature 314, 334–336
34. Banno, Y., Nagao, S., Kataoka, T., Nagata, K., U, M., and Nozawa, Y. (1987) Biochem. Biophys. Res. Commun. 146, 861–869
35. Martin, T. F. J., Laces, D. O., Bajjalieh, S. M., and Kowalchyk, J. A. (1986) J. Biol. Chem. 261, 2918–2927
36. Stoehr, S. J., Smolen, J. E., Holz, R. W., and Agranoff, B. (1986) J. Neurochem. 48, 637–640
37. Kao, L. S. (1988) J. Neurochem. 51, 221–227
38. Wilson, K., Rice, W. G., Kinkade, J. M., Merrill, A. H., Arnold, R. R., and Lambeth, J. D. (1987) Arch. Biochem. Biophys. 259, 204–214
39. Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., and Bell, R. M. (1986) J. Biol. Chem. 261, 12604–12606
40. Merrill, A. H., Jr., Sereni, A. M., Stevens, V. L., Hannun, Y. A., Bell, R. M., and Kinkade, J. M. (1986) J. Biol. Chem. 261, 12610–12615
41. Wilson, E., Olof, M. C., Bell, R. M., Merrill, A. H., Jr., and Lambeth, J. D. (1983) J. Biol. Chem. 261, 12616–12623
42. Rodriguez-Pena, A., and Rozengurt, E. (1984) Biochem. Biophys. Res. Commun. 120, 1053–1059
Intracellular Regulation of Secretion in Chromaffin Cells

43. Hii, C. S. T., Jones, P. M., Persaud, S. J., and Howell, S. L. (1987) *Biochem. J.* **246**, 489-493
44. Hepler, J. R., Earp, H. S., and Harden, T. K. (1988) *J. Biol. Chem.* **263**, 7610-7619
45. Morita, K., Toshihito, D., Kitayama, S., Koyama, Y., and Tsujimoto, A. (1987) *J. Neurochem.* **48**, 243-247
46. Marriott, D., Adams, M., and Boarder, M. R. (1988) *J. Neurochem.* **59**, 616-623
47. Pocotte, S. L., Frye, R. A., Senter, D. R., Terbush, D. R., Lee, S. A., and Holz, R. W. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 930-934
48. Kelner, K. L., Morita, K., Rossen, J. S., and Pollard, H. B. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 2998-3002
49. TerBush, D. R., and Holz, R. W. (1986) *J. Biol. Chem.* **261**, 17099-17106
50. Knight, S. E., Sugden, D., and Baker, P. F. (1988) *J. Membr. Biol.* **104**, 21-34
51. Kao, L.-S., and Schneider, A. S. (1986) *J. Biol. Chem.* **261**, 4881-4888
52. Artaelejo, C. R., Garcia, A. G., and Aunis, D. (1987) *J. Biol. Chem.* **262**, 915-926
53. Cockcroft, S. (1987) *Trends Biochem. Sci.* **12**, 75-78
54. Toutant, M., Aunis, D., Bockaert, J., Homburger, V., and Rouot, B. (1987) *FEBS Lett.* **215**, 339-344
55. Cockcroft, S., Howell, T. W., and Gomperts, B. D. (1987) *J. Cell Biol.* **105**, 2745-2750
56. Knight, D. E., Hallam, T. J., and Scrutton, M. C. (1982) *Nature* **296**, 256-257
57. Lew, P. D., Monod, A., Waldvogel, F. A., Dewald, B., Baggioinini, M., and Pozzan, T. (1986) *J. Cell Biol.* **102**, 2197-2203
58. Burgoyne, R. D., Morgan, A., and O’Sullivan, A. J. (1988) *FEBS Lett.* **238**, 151-155
59. Holz, R. W., and Senter, R. A. (1988) *Cell. Mol. Neurobiol.* **8**, 115-128
60. TerBush, D. R., Bittner, M. A., and Holz, R. W. (1988) *J. Biol. Chem.* **263**, 18873-18879