Modification of Chromaffin Cells with Pertussis Toxin or N-Ethylmaleimide Lowers Cytoskeletal F-actin and Enhances Ca\(^{2+}\)-dependent Secretion*

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In an attempt to identify proteins involved in the secretory response, bovine chromaffin cells were modified with N-ethylmaleimide (NEM). NEM concentrations less than 30 μM enhanced norepinephrine secretion evoked by nicotine or by K\(^{+}\) depolarization and increased Ca\(^{2+}\)-dependent secretion from digitonin-permeabilized cells. Higher concentrations of NEM inhibited secretion. The protein modified by NEM which was responsible for the enhancement of secretory activity appeared to rapidly diffuse out of the digitonin-permeabilized cells. When proteins which diffuse from control digitonin-permeabilized cells were incubated with pertussis toxin and [\(^{32}\)P]NAD, several proteins were ADP-ribosylated. However, when proteins from cells preincubated with 30 μM NEM were incubated with pertussis toxin and [\(^{32}\)P]NAD, these GTP-binding proteins (G-proteins) were not ADP-ribosylated, which suggests that they were modified in the cell by NEM. Stimulation of norepinephrine secretion by NEM was not additive with that caused by pertussis toxin. Modification of chromaffin cells with pertussis toxin or with 30 μM NEM caused a 40–50% decrease in the amount of cytoskeletal F-actin. This decrease in cytoskeletal F-actin may account for the increase in secretory activity.

Stimulation of chromaffin cells by nicotine or K\(^{+}\) depolarization results in an increase in intracellular Ca\(^{2+}\) which triggers catecholamine secretion. The plasma membranes of these cells can be permeabilized by exposure to intense electrical fields (1), to pore-forming toxins (2), or to low concentrations of detergents such as digitonin (3–5). Norepinephrine (NE) secretion by these permeabilized cells is both ATP- and Ca\(^{2+}\)-dependent. An advantage of permeabilization with digitonin is that proteins can diffuse into and out of the cell (5–8). Incubation of digitonin-permeabilized chromaffin cells results in a progressive loss of proteins and a decrease in secretory activity (7). As the addition of these leaked proteins prevents this loss of secretory activity, cytosolic proteins appear to be required for secretion (7, 9). One approach to identify proteins involved in the secretory response is to add either specific proteins (9–11) or fractions from cytosolic extracts to these depleted cells and look for a restoration of secretion. An alternate approach to identify proteins involved in the secretory response is to use chemical modification.

There have been several reports on the effects of N-ethylmaleimide (NEM) on catecholamine secretion by bovine chromaffin cells. Ferris et al. (12) found that while incubation of perfused adrenal medulla with 50 μM NEM enhanced acetylcholine-stimulated catecholamine release, incubation with 100 μM NEM inhibited secreted. Similarly, Tachikawa et al. (13) found that while 100 μM NEM enhanced acetylcholine-stimulated release from isolated chromaffin cells, 500 μM NEM inhibited secretion. It is not known whether these effects result from an alteration of Ca\(^{2+}\) influx into these cells or of some processes that occur after Ca\(^{2+}\) influx. Wilson and Kirshner (4) and Frye andHolz (14) have reported that NEM inhibits catecholamine release from digitonin-permeabilized chromaffin cells. However, the results shown by Frye and Holz suggest that low concentrations of NEM might enhance secretion by digitonin-permeabilized cells.

Because NEM can modify pertussis toxin-sensitive G-proteins (15–17) and because pertussis toxin modification of chromaffin cells increases both nicotine-stimulated catecholamine secretion by intact cells (18–21) and Ca\(^{2+}\)-dependent catecholamine secretion by permeabilized cells (20), we examined the possibility that the enhancement of secretion caused by low concentrations of NEM might result from the modification of a pertussis toxin-sensitive G-protein. We also examined the effects of pertussis toxin and NEM on the level of cytoskeletal F-actin in chromaffin cells. We found that both pertussis toxin and low concentrations of NEM cause a 40–50% decrease in cytoskeletal F-actin. This decrease in F-actin may account for the increase in secretory activity.

MATERIALS AND METHODS

Cell Culture—Chromaffin cells were isolated from bovine adrenal glands essentially as described by Greenberg and Zinder (22) and purified on self-generating Percoll (Pharmacia LKB Biotechnology Inc.) gradients (3). The cells were suspended in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal calf serum, 100 units/ml penicillin, 100 units/ml streptomycin, 40 μg/ml gentamycin, 10 μM cytosine arabinoside, and 10 μM 5-fluoro-2’-deoxycytidine (3) and grown on 24- or 48-well plates. The cells were plated at 2.5 × 10⁴ cells/cm² and used 3–8 days after plating. Approximately 24 h prior to a secretion experiment, the culture medium was replaced by DMEM containing 10% fetal calf serum, 100 units/ml penicillin, and 100 units/ml streptomycin.

NEM and Pertussis Toxin Modifications of Chromaffin Cells—For

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1 The abbreviations used are: NE, norepinephrine; ECTA, [ethyl-enehial(oxyethylenenitrilo)tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NEM, N-ethylmaleimide; PIPES, piperoxine-N,N'-bis(2-ethanesulfonic acid); TPA, 12-O-tetradecanoylophosphoril-13-acetate; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin; PBS phosphate-buffered saline.
NEM modification, the cells were incubated for 20 min in 145 mM NaCl, 5.6 mM KCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 15 mM HEPES, pH 7.4, 5.6 mM glucose, and 0.5 mM sodium ascorbate (PSS) in the presence of various concentrations of NEM. For pertussis toxin modification, the cells were incubated for 4 h in culture media containing various concentrations of pertussis toxin (List Biological Laboratories).

[³H]NE secretion by intact cells—Chromaffin cells cultured on 48-well plates were labeled by a 1.5-h incubation at 37 °C with 125 μl of 0.25 μM [³H]NE (10 Ci/mmol) in DMEM supplemented with 0.5 mM sodium ascorbate. The cells were washed with PSS and then incubated in DMEM supplemented with 10% fetal bovine serum for 2-4 h at 37 °C. All subsequent steps were performed at room temperature. The cells were incubated for 20 min in 125 μl of PSS. It was during this 20-min incubation that the cells were treated with NEM. The cells were then stimulated by the addition of 10 μM nicotine in PSS or by K⁺ depolarization (56 mM KCl, 95 mM NaCl, 2.2 mM CaCl₂, 0.5 mM MgCl₂, 15 mM HEPES, pH 7.4, 5.6 mM glucose, and 0.5 mM sodium ascorbate). After 10 min, the release medium was removed and placed on ice until centrifugation at 12,000 × g for 4 min. The supernatant from this centrifugation was removed and counted. The cells attached to the wells were solubilized with 125 μl of 5 mM PIPES or 1 Triton X-100. This lysate was combined with the pellet of the above centrifugation and counted. [³H]NE release is expressed as a percent calculated by multiplying the amount of [³H]NE in the supernatant by 100 and dividing by the total [³H]NE recovered. Three wells were used for all determinations, and release values reported as the mean ± SD.

[³H]NE secretion by digitonin-permeabilized cells—Cells prelabeled with [³H]NE were incubated for 20 min in PSS in the presence and in the absence of NEM. The cells were then permeabilized with 10 μM digitonin in 125 μl of KCl buffer (139 mM potassium glutamate, 2 mM MgCl₂, 2 mM MEATP, 5 mM EDTA, and 20 mM PIPES, pH 6.6) or KG buffer containing various concentrations of CaCl₂. The free Ca²⁺ concentrations were calculated according to Fabiato and Fabiato (23). Unless stated otherwise, the free Ca²⁺ concentration was 10 μM. After 15 min, the permeabilization buffer was removed, and the NE released was determined. In some experiments, the cells were first permeabilized by a 6-min incubation in KG buffer containing 10 or 15 μM digitonin. These permeabilization buffers were removed and [³H]NE secretion measured in KG buffer or in KG buffer containing 10 μM free Ca²⁺. In other experiments the digitonin-permeabilized cells were washed with KG buffer for various lengths of time prior to measuring [³H]NE secretion in KG buffer containing 10 μM free Ca²⁺.

Isolation of released cytosolic proteins from digitonin-permeabilized cells—For these protein isolations, the cells were plated on 6-well plates at a density of 4 × 10⁶ cells/cm² (7, 9). Control, NEM-modified, and pertussis toxin-modified cells were incubated for 15 min in KG buffer containing 15 μM digitonin. The supernatants were removed, dialyzed, and concentrated (9).

ADP-ribosylation of released proteins by pertussis toxin—Proteins released from digitonin-permeabilized chromaffin cells were ADP-ribosylated with pertussis toxin described by Brothers et al. (24). 60 μg of released proteins were incubated with 5 μg of pertussis toxin in 100 μl of 100 mM Tris, pH 8.0, 10 mM thymidine, 1 mM ATP, 100 μM GTP, 10 mM diethiothreitol, 2.5 mM MgCl₂, 1 mM EDTA, 0.5 mM L-a-dimyristoyl phosphatidylcholine, and 10 μM [³²P]NAD. After 30 min at 30 °C, the reaction was terminated by the addition of 2% sodium dodecyl sulfate and 20 μl/ml BSA. After precipitation with 10% trichloroacetic acid, the proteins were washed twice with ethyl ether, dried, and electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel. ADP-ribosylated proteins were visualized by autoradiography.

Assay for Cytoskeletal F-actin using Rhodamine-Phalloidin—This assay is based on that of Howard and Oresajo (25). The chromaffin cells were cultured on 24-well plates at a density of 2.5 × 10⁶ cells/cm². Control cells, cells pretreated with 1 μg/ml pertussis toxin, and cells pretreated with 30 μM NEM were fixed with 300 μl of 3.7% formaldehyde in PBS (150 mM NaCl and 20 mM NaF, pH 7.2) for 15 min. The fixed cells were permeabilized with 300 μl 0.2% Triton X-100 in PBS for 5 min. The cells were then incubated with 300 μl of 1.85 μM rhodamine-phalloidin (Molecular Probes) in PBS for 15 min (5). The cells were washed three times with PBS, and the samples dissolved in 1 ml of 0.1 N NaOH. Fluorescence was measured using an SLM 8000 fluorimeter. Each sample was performed in duplicate. In some experiments, the cells were stimulated with 10 μM nicotine prior to fixing with formaldehyde.

Fluorescence microscopy—Chromaffin cells were cultured on collagen-coated glass coverslips. Control cells, cells pretreated with 1 μg/ml pertussis toxin, and cells pretreated with 30 μM NEM were fixed with 3.7% formaldehyde in PBS for 15 min, permeabilized with 0.2% Triton X-100 in PBS for 5 min, and blocked by a 30-min incubation in PBS containing 10 mg/ml BSA and 10 mM Tris, pH 7.2 (26). The cells were then incubated with 0.165 μM rhodamine-phalloidin in PBS for 10 min, washed three times with PBS, and mounted in PBS. The stained cells were observed by fluorescent light microscopy.

RESULTS

Effect of NEM on norepinephrine secretion—The effect of NEM on norepinephrine secretion by intact chromaffin cells is shown in Fig. 1. The cells were incubated in a physiological saline solution (PSS) containing various concentrations of NEM. After 20 min, these solutions were removed, and the cells stimulated by the addition of nicotine or by K⁺ depolarization. While preincubation in NEM had little or no effect on basal release, low concentrations of NEM enhanced secretion induced by either nicotine or K⁺ depolarization; 30 μM NEM gave about maximum stimulation. Preincubation with higher NEM concentrations inhibited secretion. This confirms the results of Ferris et al. (12) with perfused adrenal medulla and those of Tachikawa et al. (13) with isolated chromaffin cells. The time courses of NE release in 10 μM nicotine from control cells and from cells pretreated with 30 μM NEM are shown in Fig. 2. During the first 3 min after the addition of nicotine, the rate of NE release from NEM-modified cells was about twice that from control cells. After 3 min, the rate of release from NEM-modified cells was comparable to that from control cells.

Digitonin-permeabilized cells were used to determine whether the effects of NEM result from modification of proteins which affect secretion distal to Ca²⁺ influx. Chromaffin cells were incubated for 20 min in PSS containing different concentrations of NEM. The cells were then permeabilized in KG buffer containing 10 μM digitonin either in the absence of Ca²⁺ or in the presence of 10 μM free Ca²⁺ (Fig. 3). Preincubation with low concentrations of NEM increased Ca²⁺-dependent NE secretion, and preincubation with high concentrations of NEM inhibited secretion. As observed with release from intact cells, pretreatment with 30 μM NEM gave about maximum stimulation. The Ca²⁺ dependencies of NE release from digitonin-permeabilized control cells and from digitonin-permeabilized cells preincubated with 30 μM NEM are shown in Fig. 4. For both control and NEM-modified

![Fig. 1. Effect of NEM pretreatment on nicotine and K⁺-stimulated NE release.](http://www.jbc.org/)
cells, 10 μM free Ca\(^{2+}\) gave nearly maximum secretion, and 1 μM free Ca\(^{2+}\) gave about half-maximum stimulation. Thus, the effects of NEM on NE secretion by chromaffin cells do not result solely from changes in Ca\(^{2+}\) influx or from a change in Ca\(^{2+}\) sensitivity.

**Comparison of Stimulation of Secretion by NEM and Pertussis Toxin**—The effects of low concentrations of NEM on chromaffin cells described above are similar to those reported for pertussis toxin modification (21). Incubation of chromaffin cells with pertussis toxin increases both nicotine- and K\(^+\)-stimulated catecholamine secretions (18-21), and pretreatment with pertussis toxin increases Ca\(^{2+}\)-dependent secretion from permeabilized chromaffin cells (21). As shown in Fig. 5, the increase in secretory activity caused by NEM was not additive with that caused by pertussis toxin. Ca\(^{2+}\)-dependent secretion from cells pretreated with pertussis toxin and then with 30 μM NEM was the same as that from cells treated only with NEM. Sontag et al. (21) reported that NEM inhibited the pertussis toxin-dependent enhancement of NE secretion. However, the lowest NEM concentration they show is about 40 μM. When concentrations of NEM greater than 30 μM were used, the stimulating effect of NEM was also lost (Figs. 1 and 3).

**Loss of NEM Enhancement of Ca\(^{2+}\)-dependent Secretion**—Pertussis toxin stimulation of Ca\(^{2+}\)-dependent secretion by permeabilized chromaffin cells is observed only when secretion is measured immediately after permeabilization. Once the cells are permeabilized by digitonin or streptolysin-O, this enhancement of Ca\(^{2+}\)-dependent secretion by pertussis toxin is rapidly lost (21). To determine whether the enhancement of secretion caused by NEM is also rapidly lost, Ca\(^{2+}\)-dependent NE secretion from digitonin-permeabilized cells was examined using three different protocols (Table I). When Ca\(^{2+}\) and digitonin were added simultaneously, cells pretreated with 30 μM NEM gave about 50% more Ca\(^{2+}\)-dependent NE release than did cells not treated with NEM (Table I). (The results shown in Figs. 3 and 4 were obtained using this protocol.) However, when the cells were first permeabilized by a 6-min incubation in 10 μM digitonin in the absence of Ca\(^{2+}\), and then secretion measured in buffers which did not contain digitonin, Ca\(^{2+}\)-dependent release from cells treated with 30 μM NEM was only about 25% greater than that from cells not treated with NEM. When a 5-min wash in absence of
TABLE I

| Protocol used for permeabilization and release | % norepinephrine released in 10 μM Ca^2+ |
|---------------------------------------------|--------------------------------------|
| Control cells                               | 19.5 ± 0.5                            |
| NEM-modified cells                          | 28.9 ± 1.0                            |

Release measured after permeabilization:

| Release measured after permeabilization | % norepinephrine released in 10 μM Ca^2+ |
|----------------------------------------|--------------------------------------|
| No Ca^2+                                | 20.7 ± 0.9                            |
| 0.5 μM Ca^2+                            | 25.5 ± 1.2                            |
| 1.0 μM Ca^2+                            | 16.4 ± 0.8                            |
| 2.0 μM Ca^2+                            | 17.8 ± 0.4                            |

* Release measured during a 15-min incubation in KG buffer adjusted to give 10 μM free Ca^2+ containing 10 μM digitonin.

† Cells were permeabilized by a 6-min incubation in KG buffer containing 10 μM digitonin, and then release was measured during a 15-min incubation in KG buffer adjusted to give 10 μM free Ca^2+.

‡ Cells were permeabilized by a 6-min incubation in KG buffer containing 10 μM digitonin, washed for 5 min in KG buffer, and then release was measured during a 15-min incubation in KG buffer adjusted to give 10 μM free Ca^2+.

TABLE II

**Effect of NEM-modified cytosolic proteins**

The cells were permeabilized by a 6-min incubation in 15 μM digitonin and then incubated in the absence of Ca^2+ for 15 min in the presence of the proteins indicated. These incubation solutions were removed, and the amount of [3H]NE secretion was measured in the presence and absence of Ca^2+. The amount of [3H]NE released was measured 15 min after the addition of release media. Immediately after permeabilization NE secretion in 10 μM Ca^2+ was 22.5 ± 0.8%.

| Proteins present during 15-min incubation | % norepinephrine released in 10 μM Ca^2+ |
|------------------------------------------|--------------------------------------|
| No Ca^2+                                 | 1.7 ± 0.1                            |
| 2 mg/ml BSA                              | 11.1 ± 0.5                           |
| 2 mg/ml proteins from control cells†    | 1.9 ± 0.5                            |
| 2 mg/ml proteins from cells incubated in 30 μM NEM‡ | 16.5 ± 1.7                           |
| 2 mg/ml proteins from cells incubated in 15 μM digitonin§ | 2.3 ± 0.3                            |
| 2 mg/ml proteins from cells incubated in 15 μM digitonin§ | 22.9 ± 1.9                           |

* Proteins which leaked from control cells during a 15-min incubation in 15 μM digitonin.

† Proteins which leaked from NEM-modified cells during a 15-min incubation in 15 μM digitonin.

Ca^2+ was added between permeabilization and measurement of secretion. Ca^2+-dependent release from cells treated with 30 μM NEM was only slightly greater than that from cells not treated with NEM. Thus, once the cells were permeabilized, the enhancement of Ca^2+-dependent secretion caused by preincubation with NEM, like that caused by pertussis toxin, was rapidly lost.

Incubation of digitonin-permeabilized cells in the absence of Ca^2+ results in a progressive decrease in Ca^2+-dependent secretion and a loss of cytosolic proteins (7, 9). When the proteins released during this incubation are collected, concentrated, and added back to the incubation media, this loss in secretory activity is reduced (7, 9). Thus, proteins which readily diffuse from digitonin-permeabilized cells are required for Ca^2+-dependent secretion. The ability of proteins which leak from NEM-modified cells to prevent this loss of secretory activity was compared to that of proteins which leak from control cells (Table II). Untreated cells were permeabilized by a 6-min incubation in 15 μM digitonin, and then incubated for 15 min in KG buffer containing 2 mg/ml BSA or 2 mg/ml proteins which leaked from control cells or 2 mg/ml proteins which leaked from cells pretreated with 30 μM NEM. Ca^2+-dependent secretion from cells incubated for 15 min in BSA was about 50% of that observed when secretion was measured immediately after permeabilization. While proteins from both control and NEM-modified cells reduced the loss of Ca^2+-dependent secretion (Table II), Ca^2+-dependent secretion from cells incubated in proteins from NEM-modified cells was about 35% greater than that from cells incubated in proteins from control cells.

 Pretreatment of chromaffin cells with 100 μM NEM causes an 80–90% inhibition in Ca^2+-dependent secretion (Fig. 3). To determine whether this inhibition resulted from the modification of a cytosolic protein, cells were treated with 100 μM NEM, permeabilized by a 6-min incubation in 15 μM digitonin, and then incubated for 15 min in KG buffer containing 2 mg/ml BSA or 2 mg/ml control cytosolic extract. This incubation with unmodified proteins did not give any increase in Ca^2+-dependent secretion (data not shown). This suggests that the inhibition of secretion caused by 100 μM NEM is at least in part due to the modification of a protein or proteins which do not readily diffuse from the permeabilized cell. In contrast, the protein(s) modified by low concentrations of NEM which stimulate Ca^2+-dependent secretion appears to rapidly diffuse from the permeabilized cell (Tables I and II).

**ADP-ribosylation of Leaked Proteins**—To determine whether NEM modified a pertussis toxin-sensitive G-protein, chromaffin cells were treated with pertussis toxin or with NEM and then permeabilized with digitonin. The proteins which leaked from the cells were collected and examined for the presence of proteins which could be ADP-ribosylated when incubated with [32P]NAD and pertussis toxin (Fig. 6). When proteins from control cells were used, pertussis toxin ADP-ribosylated two proteins with molecular masses of about 40 and 41 kDa (Fig. 6, lane B), confirming the result of Sonntag et al. (21). A protein with a molecular mass of 38 kDa was ADP-ribosylated even when pertussis toxin was not added to the reaction mixture (Fig. 6, lane A). A similar size protein is ADP-ribosylated when chromaffin cell membranes are incubated with [32P]NAD in the absence of pertussis toxin (27). When proteins from either pertussis toxin or NEM-modified cells were used, [32P] incorporation into the 40- and 41-kDa proteins was inhibited. This suggests that these proteins were modified in the intact cell by pertussis toxin and by NEM. The concentrations of NEM and pertussis toxin needed to modify the 40- and 41-kDa proteins in the cell were similar to those which stimulate secretion. When proteins from cells treated with 100 μM NEM were used, there was also a decrease in the ADP-ribosylation of the 38-kDa protein. As the concentration of ADP-ribosylation of released proteins by pertussis toxin. Proteins which leaked from digitonin-permeabilized control cells, NEM-modified cells, and pertussis toxin-modified cells were incubated for 30 min with pertussis toxin and [32P]NAD. [32P]-labeled proteins were detected by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels and autoradiography. Lane A, proteins from control cells incubated with [32P]NAD but no pertussis toxin. Samples in lanes B-I were incubated with [32P]NAD and pertussis toxin: lane B, proteins from control cells; lane C, proteins from cells pretreated with 10 ng/ml pertussis toxin; lane D, proteins from cells pretreated with 100 ng/ml pertussis toxin; lane E, proteins from cells pretreated with 1 μg/ml pertussis toxin; lane F, proteins from cells pretreated with 1 μM NEM; lane G, proteins from cells pretreated with 10 μM NEM; lane H, proteins from cells pretreated with 30 μM NEM; lane I, proteins from cells pretreated with 100 μM NEM.
centration of NEM needed to modify this 38-kDa protein was greater than that required to stimulate NE release, it seems unlikely that its modification by NEM is responsible for the increase in secretory activity.

Stimulation by NEM Does Not Result from Activation of Protein Kinase C, cAMP-dependent Protein Kinase, or Phospholipase A2—Ca²⁺-dependent secretion in permeabilized chromaffin cells can be increased by the activation of protein kinase C or by the addition of cAMP or arachidonic acid. However, the enhancement of secretion caused by pertussis toxin does not appear to result from the activation of protein kinase C or from the release of either cAMP or arachidonic acid (18, 21).

A 20-min preincubation of chromaffin cells with the phorbol ester, TPA, increased Ca²⁺-dependent secretion by digitonin-permeabilized cells, but this stimulation by TPA was additive with that caused by preincubation with 30 μM NEM (Fig. 7). When cells which had been treated for 24 h with 1 μM TPA to down-regulate protein kinase C were used (28), NEM still caused a 50% increase in Ca²⁺-dependent NE release (data not shown). We also found that the enhancement of Ca²⁺-dependent secretion by NEM was not inhibited by 0.5 μM staurosporine, a potent inhibitor of both protein kinase C (29, 30) and cAMP-dependent protein kinase (31), or by 30 μM PKI, a peptide inhibitor of cAMP-dependent protein kinase (32), or by 100 μM quinacrine, an inhibitor of phospholipase A₂ (33, 34). NEM concentrations less than 50 μM have little or no effect on arachidonic acid production (14). These results suggest that stimulation of secretion by NEM, like that caused by pertussis toxin, does not result from activation of protein kinase C, cAMP-dependent protein kinase, or phospholipase A₂.

Effect of NEM and Pertussis Toxin on F-actin—Stimulation of neutrophils with the chemotactic peptide formylmethionylleucyl-phenylalanine (fMet-Leu-Phe) causes an increase in actin polymerization. Pretreatment with pertussis toxin inhibits this increase in F-actin (35, 36). We used rhodamine-phalloidin to determine whether modification of chromaffin cells with 30 μM NEM or with pertussis toxin alters actin polymerization. As shown in Fig. 8, prior to stimulation with nicotine both pertussis toxin-modified and NEM-modified chromaffin cells contained about half as much cytoskeletal F-actin as did control cells. Measurement of G-actin using the deoxyribonuclease I assay (37) also showed that unstimulated pertussis toxin-modified and NEM-modified cells contained about half as much F-actin as did control cells (data not shown). As has been observed by others (38–40), stimulation of control cells with nicotine caused a rapid decrease in F-actin content (Fig. 8). Stimulation of pertussis toxin-modified and NEM-modified cells with nicotine caused only very slight decreases in F-actin. After a 30-s stimulation of pertussis toxin-modified and NEM-modified cells with nicotine, the F-actin content of pertussis toxin-modified and NEM-modified cells began to increase, and after 5 min, the amounts of F-actin present in all three types of cells were comparable to those of pertussis toxin-modified and NEM-modified cells as did control cells. Measurement of G-actin using the deoxyribonuclease I assay (37) also showed that unstimulated pertussis toxin-modified and NEM-modified cells contained about half as much F-actin as did unstimulated control cells.

![Fig. 7. Effect of NEM on TPA enhancement of Ca²⁺-dependent NE release.](image)

![Fig. 8. Effect of pertussis toxin and NEM on cytoskeletal F-actin.](image)

**TABLE III**

| TPA added during 24-h incubation | Additions to 20 min incubation in PSS | Relative F-actin content % |
|-------------------------------|--------------------------------------|---------------------------|
| None                          | None                                 | 100                       |
| None                          | 30 μM NEM                            | 58 ± 4                    |
| None                          | 30 μM quinacrine                     | 99 ± 1                    |
| None                          | 30 μM quinacrine and 30 μM NEM      | 59 ± 2                    |
| None                          | 200 nM TPA                          | 98 ± 3                    |
| None                          | 30 μM forskolin                      | 100 ± 1                   |
| None                          | 1 μg/ml pertussis toxin*             | 59 ± 1                    |
| 1 μM                          | 1 μM TPA                             | 98 ± 2                    |
| 1 μM                          | 1 μM TPA and 30 μM NEM              | 58 ± 2                    |
| 1 μM                          | 1 μM TPA and 1 μg/ml pertussis toxin* | 59 ± 1                    |

*These cells were incubated with pertussis toxin for 4 h in culture media prior to determining cytoskeletal F-actin levels.
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actin depolymerization by NEM (Table III).

To determine whether this decrease in actin polymerization required external Ca++, chromaffin cells were incubated for 20 min in normal PSS or in a similar buffer which contained 5 mM EGTA instead of CaCl2 and both in the presence and absence of 30 μM NEM. After these incubations, the amounts of F-actin in these cells were determined using rhodamine-phalloidin (Table IV). Even in the absence of external Ca++, NEM still caused a large decrease in F-actin content. Since the incubation with pertussis toxin lasted 4 h, a similar experiment with pertussis toxin was not performed.

Fluorescence Microscopy—The effect of pretreatment with 30 μM NEM or with 1 μg/ml pertussis toxin on the distribution of F-actin within chromaffin cells was examined using fluorescence microscopy (Fig. 9). F-actin was visualized by labeling with rhodamine-phalloidin. As has been observed by others (38, 40, 41), in control cells there was a continuous fluorescent ring near the periphery of the cell (Fig. 9, panels a and b). In NEM-modified cells (Fig. 9, panels c and d) and in pertussis toxin-modified cells (Fig. 9, panels e and f) this ring was thinner and less fluorescent. Also, the actin filaments in these modified cells appeared patchy. This patchy appearance is similar to that reported by Vitale et al. (40) for nicotine-stimulated control cells. Thus, both NEM and pertussis toxin appear to decrease the amount of cortical F-actin and alter its distribution.

DISCUSSION

The results presented here demonstrate that treatment of intact chromaffin cells with NEM results in the modification of proteins which affect steps in the secretory response which occur after Ca++ influx. The effect of low NEM concentrations on NE secretion by chromaffin cells is very similar to that of pertussis toxin. Incubation of intact chromaffin cells with 30 μM NEM or with 0.1-1 μg/ml pertussis toxin results in about a 50% increase in secretion induced by nicotine or K+ depolarization (18-21), and both treatments enhance Ca++-dependent secretion from permeabilized cells. The proteins responsible for these increases rapidly leak out of digitonin-permeabilized cells. The enhancement of secretion by NEM was not additive with that caused by pertussis toxin, and the effects of both NEM and pertussis toxin appear to be independent of the activation of protein kinase C, cAMP-dependent protein kinase, or phospholipase A2.

Sontag et al. (21) showed that when proteins which leak from digitonin-permeabilized cells are incubated with pertussis toxin and [32P]NAD, several G-proteins are ADP-ribosylated. We confirmed this observation and showed that when proteins from pertussis toxin-modified cells were used, these proteins no longer incorporated 32P, indicating that they had been ADP-ribosylated in the cell by pertussis toxin. Similarly when proteins from NEM-modified cells were used, these G-proteins did not incorporate 32P when incubated with pertussis toxin and [32P]NAD, which is consistent with their having been modified in the cell by NEM. Pertussis toxin modifies a cysteine residue located four amino acids from the carboxyl termini of the α-subunits of both Gs and Go (15). After reaction with NEM, these G-proteins cannot be ADP-ribosylated by pertussis toxin. This inhibition by NEM results either from the modification of the same cysteine modified by pertussis toxin (15) or alternatively from the modification of other cysteine residues in the α-subunits (42). Modification by pertussis toxin or NEM uncouples G-proteins from their receptors (15-17). The dose dependence for the enhancement of secretion by NEM (Figs. 1 and 3) was similar to that for the inhibition of ADP-ribosylation of the leaked G-proteins by pertussis toxin (Fig. 6). Consistent with NEM modifying the α-subunits of G-proteins is the observation that incubation with 30 μM [14C]NEM resulted in the modification of 41-kDa protein(s) which rapidly leaked from the permeabilized cells (data not shown). However, it is possible that NEM did not directly modify these pertussis toxin-sensitive G-proteins, but rather that its effect on these proteins was indirect; for example NEM may have modified some other protein which induced ADP-ribosylation of these G-proteins by an endogenous ADP-ribosyltransferase.

Modification of chromaffin cells with a low concentration of NEM or with pertussis toxin caused a 40-50% decrease in cytoskeletal F-actin. These depolymerizations do not result from increases in Ca++ influx, as incubation with NEM in the absence of extracellular Ca++ caused a large decrease in F-actin (Table IV), and pertussis toxin modification does not increase Ca++ influx (15-21). The decreases in F-actin caused by NEM and by pertussis toxin, like the increases in secretory activity, do not appear to result from an activation of protein kinase C or from increases in the level of either cAMP or arachidonic acid.

The effect of pertussis toxin on actin polymerization in chromaffin cells is different from its effect on actin polymerization in neutrophils. While pertussis toxin modification decreased the F-actin content of unstimulated chromaffin cells, it has no effect on the amount of F-actin in resting neutrophils, rather it inhibits the increase in F-actin caused by stimulation with fMet-Leu-Phe (36, 37). The mechanism by which pertussis toxin alters actin polymerization in neutrophils is not known (43).

Chromaffin cells contain a mesh work of actin filaments just below their plasma membranes. It has been proposed that

![Fig. 9. Localization of F-actin by fluorescence microscopy.](https://www.jbc.org/)

Control cells (panels a and b), cells pretreated with 30 μM NEM (panels c and d), and cells pretreated with 1 μg/ml pertussis toxin (panels e and f) were fixed, permeabilized, and stained with rhodamine-phalloidin.
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this mesh work acts as a barrier which inhibits the movement of secretory granules to the plasma membrane (39, 40). Stimulation of chromaffin cells with nicotine or K+ depolarization results in a rapid depolymerization of these cortical actin filaments. This depolymerization is consistent with these filaments acting as a barrier to granule movement (38-41). Other observations in support of this hypothesis are that cytochalasin D and deoxyribonuclease I, which depolymerize actin filaments, increase Ca2+-dependent secretion (44, 45) from permeabilized chromaffin cells, and that phallolidin, which stabilizes actin filaments, partially inhibits secretion (44).

If the cortical actin filaments form a barrier which prevents the granules from moving to the plasma membrane, then the decreases in cytoskeletal F-actin caused by NEM and pertussis toxin could account for the increased secretory activities of these modified cells. Depolymerization of the cortical actin filaments by either NEM or pertussis toxin would allow a larger fraction of the secretory granules to move close to the plasma membrane in the resting cells. When these modified cells are stimulated by nicotine or K+ depolarization, a larger number of vesicles would be available for fusion, resulting in an increase in the initial rate of exocytosis. As shown in Fig. 2, the biggest difference in the rates of NE release from control and NEM-modified cells occurred during the first minute of stimulation. After 1 min, the rate of NE release from both control cells and NEM-modified cells decreased. The changes in F-actin content paralleled these changes in the rates of NE release. During the first 30 s of stimulation by nicotine, there was a rapid decrease in the amount of F-actin in control cells. After 1 min, the amount of F-actin began to increase back toward resting levels. It was during the first minute of stimulation that the rate of release from control cells was most rapid. The actin repolymerization, which begins after about 1 min of stimulation, could slow the movement of the granules to the plasma membrane and, thereby, reduce the rate of secretion. The initial F-actin contents of NEM-modified and pertussis toxin-modified cells were 40-50% that of control cells. During the first 30 s of stimulation by nicotine, there was little change in the amounts of F-actin in these modified cells. However, after about 1 min, the amounts of F-actin began to increase, and after 5 min, they were comparable to that of control cells. After 3 min of stimulation by nicotine, the rate of NE release from NEM-modified cells was comparable to that from control cells. In general, the changes in F-actin content seemed to precede the changes in the rates of secretion. This is consistent with depolymerization of the cortical actin filaments allowing the granules to approach the plasma membrane but not inducing them to fuse. The observation that NEM and pertussis toxin modifications have little effect on basal release indicates that depolymerization of the cortical actin is not sufficient to induce secretion. Pertussis toxin does increase basal release from intact cells, but this increase in basal release, 10-20% NE released during a 4-h incubation, is much less than the increase in evoked release, 10% during a 10-min incubation (18, 21, 27).

While a decrease in F-actin may account for the increased secretory activities of NEM-modified and pertussis toxin-modified cells, the mechanism by which NEM and pertussis toxin modifications cause a decrease in F-actin is not known. The effects of pertussis toxin on actin depolymerization and NE release by chromaffin cells are unusual. Usually pertussis toxin modification blocks the effect of an agonist by inhibiting the interaction of the G-protein with its receptor. In contrast, the effects of pertussis toxin on actin depolymerization and NE release appear to occur in the absence of any agonist. This suggests that this particular G-protein(s) may function independent of cell surface receptors (21, 27). There are a number of reports with different types of cells which suggest that a group of GTP-binding proteins (referred to as G,s) have roles in secretion which are independent of second messengers (46).

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