Effects of S-Nitroso-Cysteine on Proteins That Regulate Exocytosis in PC12 Cells: Inhibitory Effects on Translocation of Synaptophysin and ADP-Ribosylation of GTP-Binding Proteins

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Received June 26, 2000 Accepted August 21, 2000

ABSTRACT—S-Nitroso-cysteine (SNC) inhibits Ca2+ -induced noradrenaline (NA) release from PC12 cells. Since SNC stimulated Ca2+ mobilization from intracellular Ca2+ pools and SNC-induced inhibition of NA release was not washed-out, SNC may modify exocytosis-related proteins that overcome Ca2+ mobilization. In the present study, we investigated the effects of SNC on exocytosis-related proteins in PC12 cells. Ionomycin stimulated NA release and increased the immunoreactivity of synaptophysin in the cytosol fraction. A 25-kDa synaptosome-associated protein (SNAP-25), which localizes to plasma membranes and vesicles, increased in the cytosol fraction after stimulation. The increases in these proteins by ionomycin were inhibited in PC12 cells treated with 0.6 mM SNC. Synaptobrevin and synapsin-1 in the cytosol fraction, and syntaxin and 43 kDa growth-associated protein in the membrane fraction were not affected by ionomycin or SNC. Incubation of each protein with SNC did not affect antibody immunoreactivity. [22P]ADP-ribosylation of GTP-binding proteins (Gα/Gβ) by pertussis toxin, but not Gαs by cholera toxin, was inhibited in SNC-treated PC12 cells and by co-addition of SNC to the assay mixture. These findings suggest that 1) SNC inhibits translocation of vesicles containing synaptophysin and SNAP-25, and 2) SNC reacts with cysteine residues in Gα/Gβ, causing inhibition of ADP-ribosylation by pertussis toxin.

Keywords: S-Nitroso-cysteine, Exocytosis-related protein, Synaptophysin, GTP-binding protein, PC12 cell

Neurotransmitters are released from nerve terminals by Ca2+-dependent exocytotic processes including interactions between synaptic vesicle proteins and presynaptic plasma membrane proteins (1). Enhanced neurotransmitter release is one of the crucial events in the induction of long-term potentiation, a synaptic mechanism that is involved in learning and memory. The importance of nitric oxide (NO) as a neuromodulator became apparent recently, and NO is believed to be a retrograde messenger for long-term potentiation at least in the CA1 region of the hippocampus (2, 3). NO can stimulate release of neurotransmitters and neuropeptides (4, 5). It was reported that NO compounds in the presence of thiol compounds such as l-cysteine (4, 5) and S-nitroso-cysteine (SNC) (6) stimulate noradrenaline (NA) release from rat hippocampus in vitro and in vivo. Lonart and Johnson (7) reported that NO evokes NA release from hippocampal slices via Ca2+-dependent vesicular and Ca2+-independent nonvesicular components. However, Meffert et al. (8) reported that NO stimulates Ca2+-independent synaptic vesicle release from hippocampal synaptosomes, and that NO increases synaptic vesicle docking/fusion reactions (9). The mechanisms by which NO regulates the release of neurotransmitters are not well understood.

PC12 cells, a neuroendocrine cell line derived from rat pheochromocytoma, have been used as a neuron model. Various proteins that participate in exocytosis such as syntaxin, 25-kDa synaptosome-associated protein (SNAP-25), synaptobrevin, synaptophysin, synapsin and 43-kDa neuronal growth-associated protein (GAP-43) are expressed in PC12 cells (10–18), and exocytosis of vesicles in PC12 cells uses the same conserved machinery that functions in synaptic neurotransmission (19, 20). SNC inhibits Ca2+-induced NA release from PC12 cells (21); however, SNC also induced a continuous increase in intracellular free Ca2+ concentration ([Ca2+]i) by Ca2+ mobilization from caffeine-sensitive intracellular Ca2+ pools in PC12 cells (22). These findings suggest that exocytosis-related proteins in PC12 cells may be modified by NO. Exposure of PC12 cells
and dorsal root ganglion neurons to 3-morpholinosydnonime (SIN-1), which decomposes spontaneously to peroxynitrite and NO, inhibited fatty acylation with \(^{3}H\)palmitate by SNAP-25 or GAP-43 (23). Hess et al. (23) suggested that the substrate cysteine thiols were modified. However, the effects of NO compounds on other exocytosis-related proteins in PC12 cells have not been extensively studied. In the present study, we examined the effect of SNC on translocation and immunoreactivity of exocytosis-related proteins in vesicles in PC12 cells. SNC inhibited 1) translocation of synaptophysin and SNAP-25-containing vesicles to cytosol fractions by ionomycin, and 2) ADP-ribosylation of GTP-binding proteins (G proteins) by pertussis toxin in PC12 cells.

MATERIALS AND METHODS

\(^{3}P\)NAD (20–40 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE, USA). Cholera toxin was obtained from List Biological Lab. (Campbell, CA, USA). Pertussis toxin and sodium nitroprusside were obtained from Wako Pure Chemical (Osaka). SNC and 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene (NOC-18) were purchased from Dojindo Lab. (Kumamoto). SIN-1 and S-nitroso-N-acetylpenicillamine were purchased from Biomol (Plymouth Meeting, PA, USA) and Research Biochem. Int. (Natick, MA, USA), respectively. Ionomycin, mastoparan and phorbol myristate acetate were obtained from Sigma (St. Louis, MO, USA). Adenosine 5′-O-(3-thiotriphosphate) (ATPγS) was purchased from Boehringer-Mannheim (Germany).

PC12 cell culture and incubation with stimulants

PC12 cells were grown under 10% CO\(_2\) at 37°C, in collagen-coated dishes in Dulbecco’s modified essential medium supplemented with 5% heat-inactivated fetal bovine serum and horse serum as described previously (24, 25). PC12 cell suspension (50–70 μg protein/tube) were stimulated with ionomycin, mastoparan (wasp venom toxin) or vehicle at 37°C in modified Tyrode’s buffer (20 mM HEPES (pH 7.4), 0.14 M NaCl, 5 mM KCl, 5 mM glucose, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\)) for 10 min (total volume, 200 μl). In some experiments, PC12 cells were incubated for 10 min in the presence or absence of 0.6 mM SNC, and the washed cells were used for experiments. The reaction was terminated by centrifugation at 4,000 × g for 30s at 4°C. Cells were suspended in an ice-cold phosphate buffer (200 mM potassium phosphate (pH 7.4), 0.5 mM benzamidine and phenylmethanesulfonyl fluoride), and homogenized at 4°C with 20 strokes of a glass/teflon homogenizer, and centrifuged at 1,000 × g for 2 min. The resultant supernatant was centrifuged at 40,000 × g for 20 min at 4°C to prepare cytosolic and membrane fractions. When cells were suspended and homogenized with a buffer (150 mM NaCl, 10 mM HEPES (pH 7.4), 1 mM EGTA, 1 mM MgCl\(_2\) and protease inhibitors) as described previously (10, 13), similar findings were obtained.

Immunoblotting analysis

Immunoblotting was conducted as described previously (25) with some modifications. Proteins (20–40 μg/lane) were fractionated by SDS-PAGE and transferred at 4°C to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Anti-synaptophysin antibody (diluted to 1:2500 in phosphate-buffered saline containing 0.1% Tween 20) (Chemicon Int., Inc., Temecula, CA), anti-synaptobrevin antibody (diluted to 1:25) (Chemicon Int.), anti-ADP-ribosylation factor 3 (ARF3) antibody (diluted to 1:250) (Transduction Laboratories, Lexington, KY, USA), anti-rat SNAP-25 antibody (1:500) (Wako Pure Chemical), and anti-rat syntaxin antibody (1:500) (Wako Pure Chemical) were used. Immunoreactivity was assessed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or mouse IgG (diluted to 1:2000) (Amersham, Buckinghamshire, UK) and detected by chemiluminescence using the ECL system (Amersham). Images of the reactive bands were recorded on Kodak X-Omat AR film and analyzed by laser densitometry. Under the conditions of the present study, the densities of bands were linear in proportion to the amount of proteins.

\(^{3}P\)ADP-ribosylation of membrane fraction by pertussis toxin and cholera toxin

ADP-ribosylation assays were conducted as described previously (25). Assay tubes (final volume, 150 μl) contained 40–60 μg of membrane proteins, 100 mM potassium phosphate (pH 7.4), 5 mM MgCl\(_2\), 0.5 mM benzamidine, 0.5 mM phenylmethanesulfonyl fluoride, 10 mM thymidine, 0.2 mM ATP, 0.1 mM NADP, 1 μM \(^{3}P\)NAD (3–5 μCi/tube) and 5 μg of A subunit of cholera toxin or pertussis toxin (toxins were preactivated by incubation with 20 mM dithiothreitol at 30°C for 20 min). In the cholera toxin reactions, 0.1 mM guanosine 5′-O-(3-thiotriphosphate) was added to the assay mixture. After addition of 10% trichloroacetic acid, proteins were precipitated by centrifugation (10,000 × g, 10 min, 4°C) and dissolved in Laemmli buffer (90°C, 5 min) before analysis by SDS-PAGE. Images of the radioreactive bands were analyzed by laser densitometry.

Statistical significance

Data are expressed as mean ± S.E.M. For pairwise comparisons, Student’s two-tailed t-test was used. A probability value of P<0.05 was considered significant.
RESULTS

Effects of SNC on synaptophysin and SNAP-25

PC12 cells have synaptic small vesicles and dense-core vesicles and NA has been localized to dense-core vesicles (10, 12). Centrifugation at 27,000–30,000 × g for 30–35 min of the total homogenates without nuclear can separate free-form vesicles, which remain in the supernatants, from sedimented larger membranes that contain plasma membranes and vesicles bound to membranes and cytoskeleton (10, 13). We prepared the cytosol and membrane fractions from PC12 cells by their method with minor modifications. The cytosol fraction, which corresponds to the supernatant without mitochondria, contained a substantial amount of free vesicles; and the membrane fraction contained vesicles that bound with membranes and a few free vesicles. Several lines of evidence suggest that synaptophysin on vesicles plays a role in exocytotic processes (1). First we measured anti-synaptophysin antibody immunoreactivity in the cytosol fraction of PC12 cells that were stimulated with 10 μM ionomycin or 15 μM mastoparan, which stimulated NA release (21, 24, 25). Synaptophysin immunoreactivity in the cytosol fraction was significantly increased after stimulation (Fig. 1A and Table 1). In the absence of extracellular CaCl2, ionomycin had no effect (data not shown), as previously reported (26). Addition of 0.6 mM SNC significantly inhibited increases in synaptophysin immunoreactivity in the cytosol fraction after stimulation, although SNC alone increased the immunoreactivity slightly but not significantly. It has been proposed that NO reacts with proteins covalently (27), thus SNC may react with synaptophysin, resulting in inhibition of immunoreactivity with the antibody. However, incubation of the cytosol fraction with 0.6 mM SNC for 20 min at 37°C did not affect synaptophysin immunoreactivity (Fig. 1B, lanes 1–3). Thus, SNC appeared to inhibit the translocation of synaptophysin to the cytosol fraction. As reported previously (10), high levels of synaptophysin immunoreactivity were detected in the membrane fraction (lanes 4–6). In some cases, as shown in Fig. 1B, the immunoreactivity was slightly inhibited by 0.6 mM SNC. In other cases, however, the immunoreactivity was not modified by SNC.

SNAP-25 has been suggested to be a presynaptic plasma membrane protein. Recently, it was reported that significant pools of SNAP-25 are localized in synaptic vesicles (17). Shimazaki et al. (28) reported that a 28-kDa protein in PC12 cells, which was cleaved by botulinum toxin A, was recognized by anti-SNAP-25 antibody. In the present experimental conditions, SNAP-25 immunoreactivity was significant in membrane fractions and slight in cytosolic fractions. Previously we reported that SNAP-25 immunoreactivity in the cytosolic fraction increased in PC12 cells treated with 10 μM ionomycin in an extracellular CaCl2-dependent manner; the immunoreactivity in ionomycin-stimulated cells was 414% compared with that in control (non-stimulated) cells (26). Increases of SNAP-25 immuno-

![Figure 1](https://example.com/figure1.png)

**Fig. 1.** Translocation of synaptophysin to the cytosol fraction by ionomycin and mastoparan and its inhibition by SNC. Panel A: Translocation of synaptophysin to the cytosol fraction by stimulants. PC12 cells were first incubated with 0.6 mM SNC or vehicle for 10 min at 37°C. The washed cells were stimulated with 10 μM ionomycin, 15 μM mastoparan or vehicle for 8 min at 37°C. Similar amounts of protein (3–6 μg protein/lane) from cytosol fractions were subjected to SDS-PAGE, and immunoblots were prepared with anti-synaptophysin antibody. Data are typical of 3 independent experiments. Table 1 summarizes the findings. Panel B: Cytosol (15–20 μg protein/lane) and membrane (8–12 μg protein/lane) fractions from control PC12 cells were incubated with the indicated concentrations of SNC for 20 min at 30°C and then subjected to SDS-PAGE. Data are typical of 3 independent experiments.

<table>
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<th>Addition</th>
<th>Synaptophysin immunoreactivity (%)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>10 μM Ionomycin</td>
<td>228 ± 31*</td>
</tr>
<tr>
<td>15 μM Mastoparan</td>
<td>250 ± 28*</td>
</tr>
<tr>
<td>SNC-treated</td>
<td>136 ± 19</td>
</tr>
<tr>
<td>10 μM Ionomycin</td>
<td>144 ± 20*</td>
</tr>
<tr>
<td>15 μM Mastoparan</td>
<td>65 ± 32*</td>
</tr>
</tbody>
</table>

PC12 cells were first incubated with 0.6 mM S-nitroso-cysteine or vehicle for 10 min at 37°C. The washed cells were stimulated with 10 μM Ionomycin, 15 μM Mastoparan or vehicle for 8 min at 37°C. Similar amounts of protein from cytosol fractions were subjected to SDS-PAGE, and immunoblots were prepared with anti-synaptophysin antibody. Values are reported relative to the amount of synaptophysin immunoreactivity in non-stimulated PC12 cells, 100%, and are the mean ± S.E.M. from 3–4 independent experiments. *Significantly different from the vehicle alone (without stimulants). †Significantly different from the values in non-treated cells.
reactivity in the cytosol fraction by ionomycin were inhibited in PC12 cells treated with 0.6 mM SNC; the value was 150 ± 30% (n = 3), which was almost same as that in control cells. Co-incubation of SNC and SNAP-25 in the membrane fraction did not affect immunoreactivity. The present findings suggest that 1) exocytotic stimulation causes the translocation of synaptopophysin- and SNAP-25-containing vesicles to the cytosol fraction, and 2) SNC inhibited the translocation of vesicles by ionomycin.

Effect of SNC on synaptobrevin, synapsin-I and syntaxins

Synaptobrevins, also called vesicle-associated membrane proteins, are a family of 18–20 kDa proteins enriched in synaptic vesicles. Synaptobrevin-2, but not -1, was detected in PC12 cells and a substantial amount of synaptobrevin-2 is associated with vesicles (29). In the present study, however, synaptobrevin immunoreactivity was predominantly detected in membrane fractions and was quite low in cytosol fractions. Synaptobrevin immunoreactivity in membrane fractions was not affected by stimulation with ionomycin or mastoparan (Fig. 2A). Co-addition of SNC also had no effect.

Synapsin-I associates with synaptic vesicles and binds to cytoskeletal proteins including F-actin. Synapsin-I immunoreactivity was detected in both cytosol and membrane fractions, and synapsin-I immunoreactivity in the cytosol (Fig. 2B) and membrane fractions (not shown) was not affected by stimulants or SNC. Incubation of cytosol and membrane fractions with 0.6 mM SNC also did not affect immunoreactivity of synaptobrevin and synapsin-I.

Next we examined the effects of SNC on syntaxins, which are presynaptic plasma membrane proteins. Anti-syntaxin antibody immunoreactive bands were predominantly detected in membrane fractions from PC12 cells. Syntaxin immunoreactivity in membrane fractions from PC12 cells incubated with stimulants or SNC was not affected (Fig. 2C). Incubation of membrane fractions with 0.6 mM SNC did not affect immunoreactivity.

Effects of SNC on Gα/Gβ, GAP-43, G, and ARF

Heterotrimeric G proteins may regulate several steps in the secretory pathways, and treatment with pertussis toxin or cholera toxin modify vesicle budding and formation (30). Next we investigated the effects of SNC on G proteins. Addition of SNC to the reaction mixture for ADP-ribosylation inhibited pertussis toxin-induced [32P]ADP-ribosylation of Gα subunits of Gαi in a concentration-dependent manner (Fig. 3A, lanes 1–3). The ADP-ribosylation in the presence of 0.5 mM SNC was significantly less than that in the absence of SNC (Table 2). To study whether the effect of SNC is reversible, intact PC12 cells were incubated with 0.5 mM SNC for 20 min at 37°C, and then the washed membrane fractions were used for ADP-ribosylation assays (lanes 4–6). The [32P]ADP-ribosylation reaction by pertussis toxin in membranes from 0.5 mM SNC-treated cells was significantly less than that in control membranes from vehicle-treated cells (Table 2), and SNC further inhibited reactions in a concentration-dependent manner. Figure 3B shows the effects of various NO compounds on [32P]ADP-ribosylation reaction by pertussis toxin. Addition of 0.5 mM SNC inhibited ADP-ribosylation (lane 2) compared with vehicle (lane 1), but 0.5 mM NaN3 (lane 3) and S-nitroso-N-acetylpenicillamine (lane 5) had no effect. Addition of 0.5 mM sodium nitroprusside slightly inhibited ADP-ribosylation (lane 4).
Fig. 3. Inhibition of [3H]ADP-ribosylation of G\textsubscript{i}/G\textsubscript{o} by SNC. Panel A: Intact PC12 cells were incubated with vehicle (None, lanes 1 – 3) or 0.5 mM SNC (lanes 4 – 6) for 20 min at 37\textdegree{C}, and then the membrane fractions were prepared. The washed membrane fractions (40 \textmu{g} protein/tube) were used for the ADP-ribosylation assay in the presence of the indicated concentrations of SNC. Data are typical of 3 independent experiments. Table 2 summarizes the findings obtained using 0.5 mM SNC. Panel B: The membrane fraction from control PC12 cells were incubated in ADP-ribosylation assay mixtures with vehicle (lane 1), 0.5 mM SNC (lane 2), NaNO\textsubscript{2} (lane 3), sodium nitroprusside (lane 4, SNAP) or S-nitroso-N-acetylpenicillamine (lane 5, SNAP). Data are typical of 2 independent experiments.

Table 2. Inhibition of pertussis toxin-induced [3H]ADP-ribosylation of Gi/Go by SNC

<table>
<thead>
<tr>
<th>Addition</th>
<th>[3H]ADP-ribosylation (%)</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>SNC (0.5 mM) in vitro</td>
<td>65 \pm 9\textsuperscript{a}</td>
</tr>
<tr>
<td>SNC (0.5 mM) in situ</td>
<td>46 \pm 8\textsuperscript{a}</td>
</tr>
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Membrane fractions from control PC12 cells were used for [3H]ADP-ribosylation assays in the presence or absence of 0.5 mM SNC (in vitro). Intact PC12 cells were incubated with vehicle (None) or 0.5 mM SNC for 20 min at 37\textdegree{C} (in situ). Membrane fractions from control and SNC-treated cells were used for [3H]ADP-ribosylation assays without SNC. Data are presented relative to [3H]ADP-ribosylation in membrane fractions from control cells without SNC, 100%, and are the means \pm S.E.M. from 3 – 4 independent experiments. \textsuperscript{a}Significantly different from vehicle alone (without SNC).

GAP-43 is a neuron-specific protein and has several functions including neurotransmission and regulation of binding of GTP to G\textsubscript{o}, a major component of the neuronal growth cone (31). GAP-43 immunoreactivity was detected slightly in cytosol and significantly in membrane fractions, but immunoreactivity in either fraction was not affected by stimulators or SNC. Incubation of GAP-43 in membrane fractions with 0.6 mM SNC did not affect GAP-43 immunoreactivity (Fig. 4A).

Culture of PC12 cells with cholera toxin for 1 – 2 days inhibited Ca\textsuperscript{2+}-induced NA release and abolished the immunoreactivity of \alpha subunit of G\textsubscript{i}, in the membrane fraction in PC12 cells (32). However, [3H]ADP-ribosylation of \alpha subunits of G\textsubscript{i}, by cholera toxin was not affected by addition of up to 0.6 mM SNC in an assay mixture or by treatment of intact PC12 cells with SNC (data not shown). These findings suggest the selective effect of SNC on G\textsubscript{i}/G\textsubscript{o}, not G\textsubscript{i}.

ARF is a family of small-molecular-weight, monomeric GTP-binding proteins and is involved in intracellular vesicular transport and endocytosis (33). A large portion of ARF exists in the cytosol fraction, and ARF3 localizes to membrane fractions after exocytotic stimulation by ATP\textsubscript{S} in PC12 cells (25). Incubation of cytosol fraction with SNC did not affect the ARF3 immunoreactivity with
antibody (Fig. 4B). ARF3 translocated to membrane fractions after stimulation with 10 μM ionomycin (208 ± 24%, n = 3). The translocation was also detected in SNC-treated PC12 cells (185 ± 15%, n = 3), which was almost same as that in control cells (Fig. 4C).

DISCUSSION

Translocation of synaptophysin and SNAP-25 to cytosol fraction in PC12 cells by stimulation and its inhibition by SNC treatment

SNC inhibited NA release caused by a Ca ionophore and mastoparan from PC12 cells (21). In the present study, we investigated the effect of SNC on the translocation of vesicles after stimulation in PC12 cells. Synaptophysin is a major synaptic vesicle protein. Some studies showed that synaptophysin is localized to small synaptic-like vesicles, not to the large dense-cored vesicles that take up and release [3H]NA in PC12 cells (10, 34). In contrast, James and Richard (15) found that synaptophysin is present on both types of vesicles in PC12 cells. Linstedt and Kelly (13) reported that when PC12 cells were incubated at 37°C for 30 min, some synaptophysin redistributed into the supernatant fraction, which corresponds to the cytosol fraction in our experiments. We found that stimulation with ionomycin, which stimulated NA release in a Ca²⁺-dependent manner, increased the immunoreactivities of synaptophysin and SNAP-25 in the cytosol fraction in PC12 cells (Fig. 1 and Table 1). Mastoparan, which stimulated NA release in the absence of extracellular CaCl₂ (24), also increased the immunoreactivities in the cytosol fraction in PC12 cells. Incubation of each protein with SNC did not affect antibody immunoreactivity. These findings suggest that 1) synaptophysin- and SNAP-25-containing vesicles translocate to cytosol fractions by stimulation, and 2) SNC inhibits their translocation and thus inhibits NA release in PC12 cells.

Although synaptobrevins are present on vesicles in PC12 cells (17, 29), synaptobrevin immunoreactivity in cytosol fractions was quite low in the present study. In addition, the immunoreactivity of synapsin-I in cytosol fractions was not changed by stimulation with ionomycin. The reasons the translocation of synaptobrevins and synapsin-I immunoreactivity in the cytosol fraction after stimulation could not be detected are not clear at present. The protein composition of vesicles may be different in vesicles before and after stimulation in PC12 cells.

Effects of SNC on G₁/G₂ and GAP-43

Pertussis toxin-sensitive G proteins including G₁ have been shown to be involved in the regulation of exocytosis (35). GAP-43 is proposed to be an endogenous adapter for the secretory vesicle-bound G₁ and can thereby regulate Ca²⁺-induced exocytosis in chromaffin cells (35). Antisense GAP-43 inhibited Ca²⁺-induced dopamine release from PC12 cells (14). Hess et al. reported that cysteine residues within G₁ was stably modified by NO (36) and that fatty acylation with [3H]palmitate in cysteine residues of GAP-43 was inhibited by NO (23). In the present study, we found that [3P]ADP-ribosylation of G₁/G₂ in the membrane fraction was inhibited by SNC and this SNC-induced inhibition was not washed out (Fig. 3A). GAP-43 immunoreactivity in both the cytosol and membrane fractions with the antibody was not affected by SNC. We could not detect translocation of GAP-43 or G₁/G₂ immunoreactivity to cytosol fractions in PC12 cells by ionomycin (data not shown). Although our findings suggest that G₁/G₂ may be one of the target proteins of NO, the contribution of this modification by NO to NA release still remained to be determined.

Characteristics of NO-regulated exocytosis in cells

There are several points that require clarification to establish the physiological role of NO on neurotransmission. The first point is that NO showed diverse effects on NA release in different tissues. As mentioned in the introduction, NO compounds stimulate NA release from hippocampal slices and synaptosomes (7, 8). Also we reported that coadministration of NO compounds and thiol compounds such as l-cysteine and dithiothreitol (4, 5) and SNC (6) stimulate NA release from rat hippocampus in vivo and in vitro. Meffert et al. (9) showed that NO compounds increased formation of the synaptobrevin/SNAP-25/syntaxin complex in experiments using recombinant proteins and proposed that this association contributed to Ca²⁺-independent vesicle release by NO compounds. However, SNC inhibited NA release (21) and translocation of synaptophysin- and SNAP-25-containing vesicles to cytosol fractions after exocytotic stimulation in PC12 cells. In several tissues, NO compounds have been shown to inhibit the release of neuropeptides (37, 38). These findings suggest the diverse effects of NO on neurotransmitter release. NO compounds may regulate different proteins in different cells.

The second question is about active species of NO. It was reported that only SNC, not other NO compounds (NOC-18, SIN-1, S-nitrosoglutathione, etc.), inhibits Ca²⁺-induced NA release from PC12 cells (21). Accordingly, only SNC inhibited translocation of synaptophysin-containing vesicles (Table 1) and [3P]ADP-ribosylation of G₁/G₂ (Fig. 3B). Other NO compounds tested and 0.6 mM l-cysteine did not affect translocation of vesicles. Sodium nitroprusside slightly inhibited [3P]ADP-ribosylation of G₁/G₂ by pertussis toxin (Fig. 3B), and the translocation of synaptophysin- and SNAP-25-containing vesicles to cytosol fractions was limited (data not shown). The reason why SNC, but not other NO compounds, inhibits NA release,
translocation of vesicles to cytosol and ADP-ribosylation of G\textsubscript{i}/G\textsubscript{q} in PC12 cells is unclear. In the present study, modification of proteins by SNC, not NO radicals, may occur easily. The involvement of a cyclic GMP-dependent system(s) on inhibition of NA release by SNC was excluded in PC12 cells (21), in accordance with other reports showing stimulation of NA release by NO compounds in a cyclic GMP-independent manner from the hippocampus (4–6, 8).

In summary, SNC inhibited 1) translocation of synaptophysin- and SNAP-25-containing vesicles to cytosol fractions after exocytotic stimulation and 2) ADP-ribosylation of G\textsubscript{i}/G\textsubscript{q} by pertussis toxin in PC12 cells. Numerous cysteines are present in putative NO targets; synaptophysin has four cysteines that make intramolecular disulfide bonds and regulate complex formations with other molecules, and SNAP-25 has four cysteines (1). Which proteins and thiol residues can be modified by SNC remains to be determined.

REFERENCES


