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Pertussis Toxin-Sensitive GTP-Binding Proteins Characterized in Synaptosomal Fractions of Embryonic Avian Cerebral Cortex

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Abstract

Pertussis toxin (PTX)-sensitive GTP-binding proteins (G proteins) are essential intermediaries subserving neuronal signal transduction pathways that regulate excitation-secretion coupling. Despite this established role, relatively little is known regarding the identity, subcellular distribution, and relative abundance of this class of G proteins in synaptic nerve endings. Here, sucrose density gradient centrifugation was combined with 1- and 2-dimensional gel electrophoresis to characterize PTX-sensitive G protein α subunits in synaptosomal fractions of embryonic (day 12) chick cerebral cortical homogenates. These findings demonstrate multiple isoforms of M_r 40–41 kDa $G_{i\alpha}$ and $G_{o\alpha}$ subunits that can be identified on the basis of PTX-catalyzed ADP-ribosylation and immunoblot analysis. *COMP BIOCHEM PHYSIOL* 119B;1:201–211, 1998.

Keywords

G protein; pertussis toxin; neuron; synapse

INTRODUCTION

Pertussis toxin (PTX), a bacterial exotoxin secreted by virulent strains of *Bordetella pertussis*, is a unique pharmacological probe for analysis of signal transduction pathways mediated by heterotrimeric guanyl nucleotide-binding proteins [G proteins; (9,40,53)]. PTX blocks diverse G protein-mediated phenomena, including inhibition of adenylyl cyclase, stimulation of phospholipases A_2 and C, modulation of ion channel gating, and regulation of stimulus-secretion coupling. This broad spectrum of physiological antagonism results from PTX-catalyzed ADP-ribosylation, a covalent modification of G protein α subunits that prevents their activation by cell surface receptors. Notably, the specificity with which PTX targets G proteins has allowed a direct assessment of the role that these signal transducing elements play in synaptic plasticity, differentiation, and gene expression.

PTX is one member of a family of structurally related “A–B” toxins that also includes cholera and diphtheria toxins (16,48). The S_1 subunit of PTX is the active (A) protomer which catalyzes mono-ADP-ribosylation of G proteins through formation of a thioglycosidic bond between ADP-ribose (derived from NAD^+) and the sulfhydryl moiety of a consensus cysteine residue located four amino acids from the C-terminus of G protein α subunits. It is

this catalytic action of the S_1 subunit that blocks activation of G proteins by cell surface receptors (38).

In vertebrate nervous systems it is the α subunits of G_i and G_o proteins that serve as substrates for PTX (5,28,29,42,46). These M_r 39–41 kDa α subunits ($G_{i\alpha}$ and $G_{o\alpha}$, respectively) are structurally homologous, yet functionally distinct guanyl nucleotide-binding proteins with intrinsic GTP-ase activity. To date, three distinct subtypes of $G_{i\alpha}$ and multiple subtypes of $G_{o\alpha}$ have been described. All are members of a large family of high molecular weight G proteins that includes transducin (G_t , found in retinal photoreceptors) and the PTX-insensitive G proteins G_s , and G_{olf} , and G_z (31,34). In neurons, the PTX-sensitive G proteins are essential components subserving signalling pathways that regulate excitation-secretion coupling (1,12,21,22,55). For example, PTX was reported to block alpha-2 adrenergic, GABA-B, and opiate receptor-mediated inhibition of calcium channel function (21), as well as the stimulatory effect of acetylcholine on potassium channel function (41). Such effects correlate positively with the ability of PTX to block inhibitory effects of noradrenaline, GABA, and enkephalin on the Ca^{2+} -dependent exocytosis of noradrenaline (1), substance P (13,22), and glutamate (12) from synaptic nerve endings. In general, the importance of the $G_{i\alpha}$ and $G_{o\alpha}$ PTX substrates to processes regulating synaptic function has remained somewhat enigmatic due, in part, to uncertainties concerning their subcellular localization in nerve terminals. Here is reported the characterization of PTX-sensitive $G_{i\alpha}$ and $G_{o\alpha}$ proteins in synaptic nerve endings of embryonic (day 12) chick cerebral cortex. These findings should provide a foundation for future studies directed at determining the developmental regulation of G protein expression as it relates to synaptic function in the central nervous system.

MATERIALS AND METHODS

Preparation of Cerebral Cortical Homogenates

Freshly dissected cortices obtained from 12-day-old chick embryos were suspended in ice-cold Buffer A containing (in mM): 100 Tris HCl (pH 7.8), 1.2 $MgCl_2$, 0.2 EDTA, 1.5 EGTA, 16 dithiothreitol, and (in mg/ml) 5.0 α -glucose, 0.1 leupeptin, 0.1 soybean trypsin inhibitor. Tissue was homogenized on ice in a Wheaton C homogenizer, and the homogenate was centrifuged (20 min, $200 \times g$; $4^\circ C$) to obtain a P_1 pellet. The supernatant was then recentrifuged (20 min, $100,000 \times g$; $4^\circ C$) to obtain a P_2 pellet and a soluble fraction (cytosol). P_1 and P_2 preparations were washed twice in ice-cold Buffer A. The pellets were then resuspended in Buffer A containing the indicated concentration of detergent (typically 0.1% Lubrol). Proteins were solubilized by a second round of homogenization to yield a final preparation containing 6–9 mg protein/ml, as determined by the method of Peterson (45). For experiments examining the electrophoretic mobility of G proteins (Figs. 1–3) or characteristics of the ADP-ribosylation reaction (Figs. 4, 5–7, and 8), particulate material in detergent-solubilized homogenates was removed by centrifugation (15 min, $3,600 \times g$; $4^\circ C$) prior to initiating the ADP-ribosylation reaction. In contrast, quantitative ADP-ribosylation and immunoblotting of subcellular fractions (Table 2; Fig. 6) was assessed using homogenates that were not precleared by centrifugation, thereby avoiding removal of less readily soluble proteins that might serve as substrates for PTX.

Subcellular Fractionation

Discontinuous density gradient centrifugation of cerebral cortical homogenates was performed according to Whittaker *et al.* (54). All procedures were conducted at $4^\circ C$. Cortices were homogenized in 0.32 M sucrose (10% w/v), centrifuged (10 min, $1,000 \times g$), and the pellet discarded. The supernatant (S_1) was centrifuged (20 min, $10,000 \times g$), the resulting supernatant (S_2) discarded, and the pellet (synaptosomal fraction) collected. The

synaptosomal fraction was suspended in lysis buffer (10 mM Tris HCl, pH 7.8; 1 mM EDTA), equilibrated for 1 hr, and centrifuged (20 min, $10,000 \times g$). The pellet was discarded and the supernatant was collected to obtain Fraction A. Fraction A was layered on a sucrose step gradient (0.4–1.2 M, 0.2 M increments) and centrifuged (2 hr, $53,000 \times g$). Gradient fractions (4 ml each) were collected, diluted twofold, and recentrifuged (60 min, $200,000 \times g$). The resulting pellets were then re-suspended in buffer A.

Preactivation of PTX

PTX (List Biochemicals) was preactivated (30 min, 21°C) in distilled H_2O containing (in mM): 100 Tris HCl (pH 7.8), 25 dithiothreitol, and 1 ATP. The activated PTX was stored at -20°C in 50% glycerol. ATP minimizes direct effects of guanyl nucleotides on the enzymatic activity of PTX by saturating the nucleotide recognition site on the toxin. (33,35,39).

ADP-Ribosylation of G Proteins

Table 1 lists the composition of Solutions 1–3 used in the ADP-ribosylation reaction. Preactivated PTX was added to ice-cold Solution 2 prior to initiating the ADP-ribosylation reaction. The reaction was then initiated by adding Solution 2 to substrate proteins to yield 50 μl of Solution 3 containing PTX (final concentration 2.4 $\mu\text{g}/\text{ml}$, unless otherwise noted), and (in mM): 85 Tris HCl (pH 7.8), 10 dithiothreitol, 10 thymidine, 10 isoniazide, 6 MgCl_2 , 3 ATP, 0.9 EGTA and (in μM): 100 GTP, 120 EDTA, 5 NAD and 0.5 [^{32}P]NAD (New England Nuclear NEG-023, 0.7–1.3 $\mu\text{Ci}/\text{assay}$, final specific activity 1.9–4.7 Ci/mmol). Typically, the ADP-ribosylation reaction was allowed to proceed for 1 hr at room temperature (21°C).

Pretreatment of Homogenates with Guanyl Nucleotides

Homogenates solubilized in Lubrol and suspended in Buffer A were treated for 30 min with guanyl nucleotides at the indicated temperatures. The ADP-ribosylation reaction was then initiated by adding Solution 2 to the sample proteins. Although no attempt was made to vary the duration of pre-treatment, it is recognized that the rate at which guanyl nucleotides associate with G proteins is dependent on the subtype of α subunit, the temperature, and the concentration of added MgCl_2 . (8,15). GTP, GTP- γ -S, Gpp(NH)p, and Gpp(CH_2)p (lithium salts) were from Boehringer-Mannheim.

Quantification of PTX-Catalyzed ADP-Ribosylation

PTX-catalyzed incorporation of [^{32}P]ADP-ribose was determined by liquid scintillation counting of trichloroacetic acid (TCA)-precipitated proteins. After incubation with PTX in sealed 10×75 mm borosilicate glass culture tubes, the reaction was terminated by adding 1 ml of 0.03% (w/v) sodium deoxycholate solution. Protein and carrier detergent were precipitated with 100 μl of 72% TCA (w/v), vortexed, and centrifuged ($3,600 \times g$, 15 min). The supernatant was aspirated carefully and the pellet was resuspended in 1 ml 0.1 N NaOH. After vortexing, solubilized proteins were re-precipitated with 100 μl 72% TCA, and centrifuged a second time ($3,600 \times g$, 15 min). The supernatant was aspirated and the pellet resuspended in 0.5 ml of a solution containing two parts dH_2O , one part 10% SDS (w/v), and one part 0.8 N NaOH. ^{32}P -incorporation was measured by liquid scintillation counting. With this protocol $\geq 90\%$ of [^{32}P]ADP-ribose incorporation was associated with proteins of M_r 40–41 kDa, as confirmed by scintillation counting of SDS-PAGE gel slices.

SDS-PAGE of Ribosylated Proteins

Ribosylated proteins were diluted 1:1 in sample buffer containing 187.5 mM Tris HCl (pH 6.8), 5.0% (v/v) 2-mercaptoethanol and (in % w/v): 30% sucrose, 3.0% SDS, and .003%

bromophenol blue. Following denaturation of the samples (100°C, 5 min), discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (32). Polyacrylamide stacking (4% T) and resolving (10% T) gels contained bisacrylamide (2.5% C), and were run at constant current (35 mA/gel, 3.5 hr) in a water-cooled vertical electrophoresis unit. Proteins were visualized by Coomassie blue (14) or silver staining (37). ¹⁴C-methylated reference standards (Amersham) included (in kDa): phosphorylase b, 92.5; bovine serum albumin, 69.0; ovalbumin, 46.0; carbonic anhydrase, 30.0; and lysozyme, 14.3 M_r was determined by a plot of R_f vs. log molecular weight. Gels were dried and used to expose Kodak X-Omat AR film (0.5–14 days, –70°C) with Cronex intensifying screens.

Analysis by Isoelectric Focusing

Isoelectric focusing (IEF) was performed according to the Ames and Nikaido (2) variation of the O'Farrell (44) procedure. Ribosylated proteins were diluted 1:2 in sample buffer containing 9.5 M urea, 5% (v/v) 2-mercaptoethanol, and (in w/v) 1.6% LKB ampholine pH 5–7, 0.4% LKB ampholine pH 3.5–10.0, 8% NP-40, and 1% SDS. Samples were applied to the basic end of IEF tube gels (4% T, 2.5% C polyacrylamide, 1.5 mm diameter), and electrophoresed (500 V, 16 hr) without prefocusing. The IEF pH gradient was determined by measuring the pH of slices obtained from blank tube gels run in parallel with each set of samples, and was linear between pH 4.5 and 6.5. Gels were equilibrated in 2% SDS (w/v), 10% glycerol, 5% 2-mercaptoethanol, and 62.5 mM Tris HCl (pH 6.8) prior to electrophoresis in the second dimension by discontinuous SDS-PAGE.

Immunoblot Analysis of Pertussis Toxin Substrates

Primary antisera tested included affinity-purified preparations of AS/7 (18), directed against the C-terminus deca-peptide sequence (KENLKDCGLF) of mammalian transducin α , and GO/1 (19) directed against the C-terminus sequence (ANNLRGCGLY) of mammalian G_o α . AS/7, but not GO/1, cross-reacts with mammalian G_i α 1 and 2. GO/1 recognizes G_o α subunits purified from bovine brain, and exhibits weak cross-reactivity with mammalian G_i α 3. Immunoblotting was performed as described by Goldsmith *et al.* (18). Following SDS-PAGE, proteins were electro-transferred (30 V, 16 hr) onto nitrocellulose membranes (0.45 μ m). Electrophoresis was confirmed by noting transfer onto the nitrocellulose of prestained molecular weight markers, or by silver staining of gels subsequent to transfer. Nitrocellulose membranes were exposed to a 500-fold dilution of the primary antiserum overnight, followed by a 2-hr incubation in HRP-conjugated, goat anti-rabbit secondary antiserum (Kirkegaard and Perry). Immunoreactivity was visualized using 4-chloro-naphthol dye solution as substrate for peroxidase.

Immunoprecipitation of G Proteins

Lubrol-solubilized P₂ membranes (10 μ g protein in 50 μ l Buffer A) were radiolabeled by PTX-catalyzed ADP-ribosylation. The radiolabeled proteins were added to 130 μ l NMT buffer containing (in mM): 150 NaCl, 10 MgCl₂, and 20 Tris HCl (pH 7.8). Affinity-purified G protein antiserum (final concentration 1–80 μ g/ml) was then added, and the mixture was allowed to equilibrate overnight at 4°C. Immune complexes were specifically adsorbed by incubation (4 hr, 4°C) with Protein G Sepharose CL4B (Pharmacia LKB, equilibrated in NMT-buffer). Adsorbed immune complexes were pelleted by centrifugation (3,600 $\times$ g, 5 min), washed twice in NMT-buffer, resuspended in scintillant, and counted.

RESULTS

PTX-Catalyzed ADP-Ribosylation of Cerebral Cortical G Proteins

We sought to identify assay conditions under which PTX catalyzed the selective incorporation of [32 P]ADP-ribose in $G_{O\alpha}$ and $G_{i\alpha}$ substrates. Figure 1 illustrates the protocol with which such selectivity was achieved. Cerebral cortical homogenates were fractionated by differential centrifugation, whereupon ADP-ribosylation of crude synaptosomal (P_2) preparations was assessed by SDS-PAGE/autoradiography. As illustrated in lanes 1 and 2 of Fig. 1, specific labeling (defined as that requiring PTX) was resolved as a faint 40/41 kDa doublet (lower arrow), whereas non-specific labeling of a 115 kDa substrate was commonly observed (upper arrow). Comparison of lanes 1 and 3 illustrates that the intensity of specific labeling was dramatically increased by solubilization of the P_2 preparations in 0.1% Lubrol prior to initiating the ADP-ribosylation reaction. In contrast, labeling of the 115 kDa substrate remained unaffected (cf., lanes 2 and 4). Under these conditions of detergent solubilization, the unidentified substrate for non-specific labeling remained in the particulate fraction and was readily eliminated from the assay by centrifugation (cf., lanes 3 and 5; see Materials and Methods). Lubrol-solubilization and centrifugation therefore provide a means by which specific labeling is optimized in this assay (cf., lanes 1 and 5).

Detection of ADP-Ribosylated G_i and G_o

SDS-PAGE combined with autoradiography and immunoblotting was performed to assess whether the M_r 40/41 kDa PTX substrates in P_2 homogenates were in fact α subunits of G_i and G_o . As illustrated in Fig. 2 ribosylated proteins were resolved on 10% gels as a clearly defined doublet (left), and then transferred to nitrocellulose for immunoblot analysis (right). Primary antisera tested included AS/7 and GO/1, exhibiting specificity for $G_{i\alpha}$ and $G_{o\alpha}$, respectively. When nitrocellulose blots were probed with these antisera, three forms of immunoreactivity were detected. AS/7 labeled a 40/41 kDa doublet ($G_{i\alpha}$ -like immunoreactivity), whereas GO/1 labeled a single 40 kDa band ($G_{o\alpha}$ -like) that apparently co-migrated with the smaller form of $G_{i\alpha}$. Auto-radiograms prepared from both blots revealed radiolabeled proteins migrating as a doublet with electrophoretic mobility identical to that of the immunoreactive labeling (data not shown). This was to be expected since these antisera recognize not only native, but also ribosylated forms of α subunits.

Multiple Isoforms of G_i and G_o

Two-dimensional gel electrophoresis combining isoelectric focusing and SDS-PAGE was performed to test whether the doublets observed on autoradiograms of 1-D gels result from radiolabeling of three distinct α subunits, as suggested by the immunoblot analysis. Figure 3 illustrates an autoradiogram prepared from a 2-D gel demonstrating not only three, but four or more distinct substrates for PTX-catalyzed ADP-ribosylation in these P_2 homogenates. The predominant substrate is a relatively acidic protein (pI 5.4) of M_r 40 kDa and constitutes ca. 55% of total substrate, as determined by densitometry. Also evident are three more basic proteins, each accounting for ca. 15% of total labeling. Two share a similar pI (5.8) but differ in M_r (40 vs 41 kDa), whereas a third protein corresponds to pI 6.0 and M_r 41 kDa. The tendency of these proteins to aggregate (and streak) when loaded on IEF tube gels in sufficient concentration to allow immunological detection precluded immunoblot analysis of the 2-D gels. However, the overall pattern of radiolabeling suggests that the predominant 40 kDa acidic substrate is most likely $G_{o\alpha}$, whereas the three more basic 40/41 kDa substrates may correspond to isoforms of $G_{i\alpha}$ (see Discussion).

Quantification of PTX-Catalyzed ADP-Ribosylation

Since G proteins constitute the predominant substrate for PTX in these cortical membranes, quantitative measurements of ADP-ribosylation were made possible by scintillation counting of TCA-precipitated samples. To optimize this assay, a systematic analysis was performed examining the time-course, temperature-dependence, and detergent-sensitivity of the ADP-ribosylation reaction. As illustrated in Fig. 4A, specific incorporation (defined as [32 P]ADP-ribose incorporation observed in the presence of PTX minus that in its absence) approached an equilibrium value (45 pmol/mg pro) within 120 min following addition of PTX to the reaction mix. Incorporation was linearly related to protein concentration (4A, inset), and as illustrated in Fig. 4B, increasing concentrations of PTX accelerated the rate but not the extent of labeling. In P_1 homogenates (4A), this equilibrium value was ca. 25 times that observed in the absence of added PTX. In contrast, in P_2 homogenates (4B), a 40–60-fold stimulation was observed, reflecting the differential enrichment of substrate in these two preparations (see below Table 2A).

PTX-catalyzed ADP-ribosylation also exhibited significant temperature-dependence. As illustrated in Fig. 5A, the rate but not the extent of specific incorporation was decreased by lowering the reaction temperature. $T_{1/2}$ (the time required to reach one-half the equilibrium value of incorporation) was 9, 13, and 24 min at 37, 21, and 4°C, respectively. Lower incubation temperatures are preferred since thermal denaturation of the substrate was readily observed (see below).

A similar quantitative analysis was performed to characterize in greater detail the detergent-sensitivity of the ADP-ribosylation reaction. As illustrated in Fig. 5B, homogenates were assayed for PTX-catalyzed [32 P]ADP-ribose incorporation under conditions of non-ionic (Lubrol, Tween-20, Triton X-100, and NP-40), zwitterionic (CHAPS), or anionic (deoxycholate) detergent solubilization. Consistent with a previous report that detergent-solubilization directly stimulates the enzymatic activity of PTX (39), a 9.7-, 12.8-, and 13.6-fold stimulation of specific incorporation relative to control (i.e., no detergent) was observed following solubilization in 0.1% Lubrol, NP-40, and Triton X-100, respectively. CHAPS, Tween-20, and deoxycholate were less effective in descending order. Increasing the concentration of all six detergents to 1% resulted in a generalized inhibition of the ADP-ribosylation reaction (data not shown).

Subcellular Distribution of G_i and G_o

The relative abundance of PTX substrates in various subcellular fractions was assessed using cerebral cortical homogenates fractionated by differential centrifugation. As summarized in Table 2A, ca. 62% of the total pool of cerebral cortical PTX substrate resides in the crude synaptosomal fraction. To more accurately assess the subcellular distribution of $G_{i\alpha}$ and $G_{o\alpha}$, we performed sucrose density gradient centrifugation of an enriched synaptosomal lysate prepared according to Whittaker *et al.* (54). As summarized in Table 2B, ADP-ribosylation of the individual step gradient fractions demonstrated that significant quantities of PTX substrate reside in fractions 1–5 (0.4–1.2 M sucrose), with fractions 1 and 2 containing ca. 57% of the total. Fractionation in this manner allowed full recovery of $G_{i\alpha}$ and $G_{o\alpha}$: ca. 96% of the applied substrate retained its activity in the subsequent ADP-ribosylation assay. The accuracy with which this assay distinguishes amongst the individual fractions was confirmed by SDS-PAGE/immunoblot analysis. As illustrated in Fig. 6, a wide-spread but differential distribution of PTX substrates was noted, visualized by AS/7 and GO/1 immunostaining. Note that the overall pattern of immunostaining (Fig. 6) is in agreement with the pattern of [32 P]ADP-ribose incorporation observed in the individual gradient fractions (Table 2).

Guanyl Nucleotide-G Protein Interactions Analyzed by ADP-Ribosylation

Guanyl nucleotides regulate the conformational state of G proteins, thereby determining the efficiency with which α subunits serve as substrates for PTX. To assess whether this ADP-ribosylation assay provides an analytical tool with which to analyze these interactions, two conformation-dependent processes were examined: stabilization of G proteins by GTP, and their activation by GTP- γ -S. Figure 7 summarizes experiments that demonstrate a role for guanyl nucleotides in protecting G proteins against thermal denaturation. As illustrated in Fig. 7A, homogenates preincubated at 4 or 21°C prior to addition of PTX retained their ability to be ADP-ribosylated irrespective of whether the preincubate contained added GTP. In contrast, incorporation of [32 P]ADP-ribose was nearly eliminated by preincubation at 37°C in buffer to which no GTP was added. Notably, thermal denaturation was blocked by including 100 μ M GTP in the preincubate, whereas 100 μ M GTP- γ -S provided only partial protection.

Figure 7B illustrates that the stabilizing action of GTP was mediated by a high affinity binding site (EC_{50} , 8 μ M) that also recognized GDP- β -S. Also tested were GTP- γ -S, Gpp(NH)p, and Gpp(CH₂)p, analogs of GTP that activate G proteins. Compared to GTP and GDP- β -S, these analogs exhibited ca. 25-fold greater potency in the assay (EC_{50} for GTP- γ -S, 0.3 μ M). This observation is as expected since the affinity of the nucleotide binding site for activating nucleotides is known to exceed that for GTP (24). Note also that the efficacy of these activating analogs (defined as maximal protective action) was consistently less than that measured for GTP: saturating concentrations (10 μ M) of GTP- γ -S, Gpp(NH)p, and Gpp(CH₂)p were, respectively, 31, 35, and 58% as effective as a saturating concentration of GTP (300 mM). As shown below, the limited efficacy of these analogs is only apparent: their full protective action is masked by their activating function.

Receptor-Site Analysis of the Guanyl Nucleotide Binding Domain

G proteins no longer serve as efficient substrates for PTX when pretreated with activating nucleotides such as GTP- γ -S (35,36). As illustrated in Fig. 8A, this interaction between receptor (G protein) and ligand (GTP- γ -S) was quantitated by establishing the dose-dependence of the response (inhibition of ADP-ribosylation). Notably, the threshold (0.03 μ M) and IC_{50} (0.3 μ M) values for GTP- γ -S-induced inhibition of ADP-ribosylation matched the threshold and EC_{50} values for protection by GTP- γ -S against denaturation (cf., Figs 7B and 8A). This overlapping concentration-dependence appears to allow GTP- γ -S to protect G proteins against thermal denaturation while simultaneously inducing activation of their α subunits. This observation may explain why the efficacy of GTP- γ -S in the denaturation assay did not match that of GTP.

The IC_{50} for GTP- γ -S-induced inhibition of ADP-ribosylation deduced from Fig. 8A exceeds by ca. 10-fold the apparent K_d for [35 S]GTP- γ -S binding to purified G_i or G_o (24). Although this most likely reflects nonequilibrium binding of the nucleotide to its receptor under our assay conditions, we sought additional confirmation that GTP- γ -S acts via a single high affinity binding site. Concentration-response data presented in Fig. 8A was modeled according to the Hill equation ($B/B_{max} = [(S)^n / (IC_{50}^n + (S)^n)]$ where B is [32 P]ADP-ribose incorporation, S is nucleotide concentration, η is the Hill coefficient, and IC_{50} is the concentration (300 nM) of GTP- γ -S that reduced to half-maximal the nucleotide-sensitive [32 P]ADP-ribose incorporation. As illustrated in Fig. 8B, a Hill plot revealed that for GTP- γ -S, the binding interaction exhibited a Hill coefficient of 1.02. Moreover, as illustrated in Fig. 8C, a plot of fractional response (B/B_{max}) vs. nucleotide concentration confirmed that for GTP- γ -S, experimentally-derived values for inhibition of ADP-ribosylation match calculated values predicted by the Hill equation assuming a η of 1.02.

DISCUSSION

The ADP-ribosylation of cerebral cortical homogenates reported here demonstrates an enrichment of PTX substrates in synaptosomal preparations, with significant labeling observed in vesicular and plasma membrane density gradient fractions. The substrate specificity of this labeling was confirmed by two-dimensional gel electrophoretic analysis which revealed selective ADP-ribosylation of four distinct G protein α subunits. Although definitive characterization of these substrates will require primary amino acid sequence information, they are provisionally identified as isoforms of $G_{i\alpha}$ (possibly subtypes 1–3) and $G_{o\alpha}$ on the basis of M_r , pI, and cross-reactivity with antisera specific for mammalian α subunits. It is noteworthy that mammalian cerebral cortex also contains multiple substrates for PTX, including $G_{i\alpha}$ 1 and 2 (4,27) and novel forms of $G_{o\alpha}$ (19,23,26,47). Although the distribution of these isoforms within nerve terminals remains largely unexplored, PTX substrates are found in synaptic plasma membranes (7,11,49–51) and secretory vesicles (43,51).

Limitations of the ADP-Ribosylation Assay

Accurate measurement of PTX substrates in cellular lysates is complicated by uncertainties regarding the extent to which substrate and/or co-factor availability limits the ADP-ribosylation reaction. In this assay, unfractionated synaptosomes contained 75–100 pmol substrate/mg protein, accounting for ca. two-thirds of the total substrate available for ADP-ribosylation in the cortex. Assuming a M_r of 40 kDa for α subunits, PTX substrate accounts for 0.3–0.4% (w/w) of cellular protein. Although this value is in agreement with previous estimates indicating an unexpectedly high concentration of G proteins in the brain, it may still represent an underestimate. ADP-ribosylation of purified α subunits by PTX requires addition of $\beta\gamma$ dimers to the assay mixture (10,30,42). Such a requirement for $\beta\gamma$ might render α subunits unavailable for ADP-ribosylation in cellular lysates (25), thus preventing a direct measurement of the total substrate pool. An additional potential source of error in the assay results from limitations of co-factor availability. Endogenous NAD in cellular lysates may dilute the specific activity of radiolabeled NAD, whereas NAD-glycohydrolases may decrease the concentration of added tracer. We have attempted to minimize these complications by washing membranes with homogenization buffer, and by including the glycohydrolase inhibitor isoniazide (17) in the reaction mixture.

Nucleotide Regulation of ADP-Ribosylation

We have illustrated the applicability of ADP-ribosylation for studies directed at analysis of the guanyl nucleotide binding site central to G protein activation. Pretreatment of cortical homogenates with nucleotides (GTP- γ -S, Gpp(NH)p, Gpp(CH₂)p) that activate G proteins rendered α subunits insensitive to subsequent ADP-ribosylation by preactivated PTX. In contrast, activating and non-activating nucleotides (GTP- γ -S, GDP- β -S) also protected against thermal denaturation of the substrate proteins. A likely explanation for these findings is that guanyl nucleotides influence substrate availability by regulating the conformational state of G proteins (6,35,36,52). Our concentration-response and receptor-site analyses support this conclusion and indicate that a single nucleotide recognition site on the α subunit confers not only thermal stability, but also susceptibility to activation. The half-saturating concentrations of GTP- γ -S required for protection against denaturation matched those required for inhibition of ADP-ribosylation, and binding data for activation by GTP- γ -S was well fit assuming a Hill co-efficient of 1.0. GDP- β -S apparently binds to this site but does not induce the conformational switch necessary to convert the heterotrimer from PTX-sensitive to insensitive, as is observed with activating nucleotides.

CONCLUSION

In summary, these findings establish the usefulness and practicality of an ADP-ribosylation assay that is an effective means by which to characterize and quantitate various subtypes of pertussis toxin substrates in embryonic avian cerebral cortex. The PTX-catalyzed incorporation of [³²P]ADP-ribose is shown to be specific for multiple subtypes of G_{iα} and G_{oα} subunits, and these α-subunits are found to exhibit a subcellular distribution within synaptosomes that is consistent with their established regulatory role in the control of ion channel function and excitation-secretion coupling.

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FIG. 1.

Optimization of PTX-catalyzed ADP-ribosylation. Cerebral cortical proteins were resolved by SDS-PAGE, and an autoradiogram of the dried gel was intentionally overexposed to illustrate the method by which selective labeling was achieved. In a P_2 homogenate not solubilized with Lubrol, specific incorporation appeared as a faint doublet (lower arrow, lane 1) running at ca. 40 kDa, whereas non-specific labeling appeared as a single band running at ca. 115 kDa (upper arrow, lanes 1,2). The PTX-independent nonspecific labeling most likely reflects the activity of endogenous poly ADP-ribosyltransferases. Note that when the homogenate was solubilized in 0.1% Lubrol, specific labeling was markedly enhanced (lane 3), whereas non-specific labeling was unaffected (lane 4). High speed centrifugation (airfuge) of the solubilized preparation to remove the 115 kDa substrate eliminated nonspecific labeling (lanes 5 and 6). All ADP-ribosylation reactions were run in parallel, and each lane on the gel received 40 μ g of membrane protein.

FIG. 2.

SDS-PAGE combined with autoradiography and immunoblotting identifies G_i and G_o proteins serving as substrates for PTX-catalyzed ADP-ribosylation. (Left) Autoradiogram of a 10% gel illustrating the relative electrophoretic mobility of ribosylated G proteins in a P_2 homogenate (20 μ g protein). [14 C]-methylated molecular weight standards (lane 1) served as reference markers for estimating M_r . Incorporation of [32 P]ADP-ribose appeared as a M_r 40 and 41 kDa doublet (lane 2). (Right) Immunoblot of ribosylated proteins (100 μ g/lane) performed with antisera that distinguish between $G_{i\alpha}$ (AS/7) and $G_{o\alpha}$ (GO/1). P_2 homogenate proteins were resolved by SDS-PAGE and transferred to nitrocellulose for immunostaining. Note that GO/1 immunoreactivity appeared as a single 40 kDa band (lane 1), whereas AS/7 immunoreactivity appeared as a 40 and 41 kDa doublet (lane 2).

FIG. 3.

Two-dimensional analysis of ribosylated G proteins by combined IEF/SDS-PAGE/autoradiography. A P_2 homogenate ($1 \mu\text{g}$) was applied to the basic end of a 4% polyacrylamide tube gel for IEF (500 V, 16 hr) in the first dimension (arrow 1). Separation in the second dimension (arrow 2) was by discontinuous SDS-PAGE (10% resolving gel). Illustrated is an autoradiogram of the 2-D gel demonstrating four distinct substrates for PTX-catalyzed ADP-ribosylation. Note that the predominant substrate is a relatively acidic protein (pI 5.4) of M_r 40 kDa. Note also three more basic proteins, two of which share similar pIs (5.8) but which differ in M_r (40 vs. 41 kDa). A third protein corresponds to pI 6.0 and M_r 41 kDa.

FIG. 4.

(A) PTX-catalyzed ADP-ribosylation is saturable with time and is a linear function of protein concentration. Illustrated is the time course of [32 P]ADP-ribose incorporation in a P_1 homogenate ($90 \mu\text{g protein/assay}$, 21°C). Squares and triangles denote specific ($+120 \text{ ng PTX/assay}$) and non-specific ($-PTX$) incorporation, respectively. Inset illustrates the protein concentration-dependence of ADP-ribosylation (100 min incubation). A linear regression line was fit to the data ($r^2 = 0.987$). (B) Increasing concentrations of PTX (30–240 ng/50 μl assay) accelerate the rate but not the extent of ADP-ribosylation (P_2 homogenate; $80 \mu\text{g protein per assay}$). Numerical values in A and B are expressed as the mean value for each determination ($n = 3$). The standard error of the mean was $\leq 5\%$ for each determination in this and in all subsequent figures.

FIG. 5.

(A) Temperature-dependence of ADP-Ribosylation. Illustrated is the time course of [32 P]ADP-ribose incorporation in P_1 homogenates incubated at 4, 21, or 37°C as indicated in the inset (68 μ g protein/assay). (B) ADP-ribosylation is stimulated by detergent-solubilization. All six detergents were tested at a final concentration of 0.1% (a concentration that exceeds the CMC for all four non-ionic detergents, but not that for deoxycholate or CHAPS). [32 P]ADP-ribose incorporation was determined after solubilization and centrifugation to eliminate unsolubilized proteins (P_1 homogenate, 40 μ g protein, and 120 ng PTX per assay, 21°C). Each data point is the average value of three individual assay determinations.

FIG. 6.

Subcellular distribution of M_r 40/41 kDa $G_{i\alpha}$ and $G_{o\alpha}$ -like immunoreactivity in cerebral cortical synaptosomes. P_2 lysates were layered on sucrose step gradients (0.4–1.2 M, 0.2 M increments) as illustrated, fractionated by discontinuous sucrose density gradient centrifugation, and the fractions assayed for AS/7 (left) or GO/1 (right) immunoreactivity by combined SDS-PAGE/immunoblotting (200 μ g protein/lane; arrows indicate the direction of electrophoresis). This is the same preparation as analyzed in Table 2B. Morphological characterization of this type of lysate by Whittaker et al. (54) demonstrated that the 0.4 M gradient fraction is enriched in synaptosomes, whereas the 0.6 M fraction contains predominantly microsomes.

FIG. 7.

Guanyl nucleotides regulate G protein stability. (A) Thermal denaturation of G proteins. P₂ homogenates (80 μ g protein/assay) were preincubated for 30 min at 4, 21, or 37°C in buffer containing no added nucleotides and either 100 μ M GTP or GTP- γ -S. ADP-ribosylation was then initiated by addition of PTX (120 ng/assay, reaction run for 75 min at 21°C). Specific incorporation is expressed relative to control (defined as incorporation measured without the preincubation step). Note that in the absence of added nucleotide, raising the preincubation temperature to 37°C reduced [³²P]ADP-ribose incorporation to 10% of control, and that GTP blocked this effect. (B) Guanyl nucleotides differentially protect against denaturation. ADP-ribosylation was measured as described in part A following preincubation for 30 min

at 37°C in homogenization buffer containing 0.01–300 μ M of GTP, GDP- β -S, Gpp(CH₂)p, Gpp(NH)p, or GTP- γ -S (86 μ g protein, 120 ng PTX/assay, P₂ homogenate, 75 min, 21°C). Each data point is the average value of three individual assay determinations.

FIG. 8.

Receptor-site analysis of guanyl nucleotide-G protein interactions. (A) Activating nucleotides inhibit ADP-ribosylation. Homogenates were pretreated (30 min, 21°C) with 0.01–300 μM of the indicated nucleotide prior to addition of PTX to the assay (120 ng PTX/assay, P₂ homogenate, 80 μg protein, reaction run 75 min at 21°C). (B) Hill plot of experimentally-derived values for GTP- γ -S-induced inhibition of ADP-ribosylation. Solid line was obtained by linear regression analysis of the raw data ($R^2 = 0.989$). (C) Concentration-response relationship for GTP- γ -S-induced inhibition of ADP-ribosylation. Filled circles indicate experimentally-derived values for fractional response vs. nucleotide concentration. Solid curve was calculated according to the Hill equation (see text). Note that the x-axis in Figs 8B and C refers to the concentration of GTP- γ -S added to each assay. Each data point is the average value of three individual assay determinations.

The chemical composition of solutions included in the ADP-ribosylation assay. Sample protein was suspended in homogenization buffer, and PTX was preactivated as described in Materials and Methods. All solutions were prepared on ice immediately prior to initiating the ADP-ribosylation reaction

TABLE 1

Solution 1		Solution 2		Solution 3	
Ingredient	Concentration (mM)	Ingredient	Volume (μl)	Ingredient	Volume (μl)
MgCl ₂	60	Solution 1	50	Solution 2	20
NAD	0.05	PTX (60 μg/ml)	20	Sample Protein	30
ATP	30	Tris HCl (100 mM)	110		
Isoniazide	120	[³² P] NAD (1 μCi)	20		
Thymidine	100				
GTP	1.0				
Tris HCl (pH 7.8)	13				
EDTA	0.2				
EGTA	1.5				
Dithiothreitol	16				

TABLE 2

(A) Quantification of PTX-catalyzed ADP-ribosylation in soluble and particulate fractions of chick cerebral cortex. Homogenates were fractionated as described in Materials and Methods, solubilized in 0.1% lubrol, and assayed for incorporation of [32 P]ADP-ribose by scintillation counting. The relative distribution of protein in this homogenate was P₁ (37.9%), P₂ (29.7%), soluble protein (32.4%). (B) Subcellular distribution of PTX substrates as analyzed by discontinuous sucrose density gradient centrifugation of ADP-ribosylated chick cerebral cortical P₂ homogenates. Homogenates (7.5 mg protein) were lysed, fractionated on sucrose step gradients (0.4–1.2 M, 0.2 M increments), solubilized in 0.1% Lubrol, and assayed for PTX-catalyzed ADP-ribosylation by liquid scintillation counting. The relative distribution of protein in this preparation was 0.4M (24.7%), 0.6M (15.2%), 0.8M (18.7%), 1.0M (14.4%), 1.2M (7.8%), pellet (19.2%). This is the same preparation as illustrated in Fig. 8

A. PTX-catalyzed incorporation of [32 P]ADP-ribose in embryonic chick cerebral cortical homogenates		
Fraction	[32 P]ADP-ribose (pmol/mg protein)	[32 P]ADP-ribose incorporation (% total pmol in all 3 fractions)
P ₁ (200 × g)	30	33.7
P ₂ (100,000 × g)	70	61.6
Soluble protein	5	4.7

B. The subcellular distribution of ribosylated G proteins as analyzed by discontinuous sucrose density gradient centrifugation of cerebral cortical P ₂ homogenates		
Gradient fraction (Molarity)	[32 P]ADP-ribose (pmol/mg pro)	[32 P]ADP-ribose incorporation (% total P ₂ substrate activity)
0.4	100	34
0.6	108	22
0.8	84	21
1.0	63	12
1.2	24	2
Pellet	20	5
% of total P ₂ substrate activity recovered:		96