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ACCELERATED COMMUNICATION

The Effects of Pertussis Toxin on Autoreceptor-Mediated Inhibition of Dopamine Synthesis in the Rat Striatum

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SUMMARY

Activation of synthesis-modulating dopamine autoreceptors by dopamine or its agonists has been shown to inhibit dopamine synthesis in the rat striatum. However, systemic administration of the direct-acting dopamine agonist apomorphine failed to inhibit dopamine synthesis in striata from rats that had received local unilateral administration of pertussis toxin. Apomorphine did reduce dopamine synthesis by >50% in sham injected control rats as well as in the striata opposite to the side of pertussis toxin injection. Examination of G proteins in striatal tissue revealed that 61% of the G proteins were ADP-ribosylated *in vivo* by direct pertussis toxin injection. These data suggest that guanine nucleotide regulatory proteins mediate the effects of activation of striatal synthesis-modulating dopamine autoreceptors.

G proteins are known to participate in the signaling of receptor-mediated alterations of a variety of intracellular processes including modulation of adenylate cyclase activity, phospholipid turnover, ion channel activity, and buffering of intracellular calcium stores (1–4). The binding of various types of neurotransmitter ligands to their receptors is sensitive to guanosine triphosphate, suggesting that G proteins are associated with many different types of neurotransmitter receptors (4, 5). A number of distinct G proteins have been identified, although the specific intracellular systems that they affect, and the way in which they affect these systems, is poorly understood. Pertussis toxin (islet-activating protein) has proven useful as a biochemical probe to examine the function of two specific subtypes of G proteins, G_i and G_o, because it inactivates these trimeric proteins by ADP-ribosylation of their α subunits (6, 7). In this regard, receptor-mediated alterations in intracellular events have been shown to be inhibited by pertussis toxin in systems in which the receptor is linked to the effector systems by G proteins (3, 8).

DA receptors in the central nervous system have been pharmacologically categorized into D1 and D2 subtypes (9). Recently, pertussis toxin administration has been shown to block DA agonist-induced inhibition of the firing of DA cells in the substantia nigra, an effect thought to be mediated by somatodendritic autoreceptors of the D2 type (10). In addition to these receptors, nigrostriatal neurons also possess synthesis-modulating nerve terminal autoreceptors, which act to decrease DA synthesis when stimulated (11). These synthesis-modulating receptors are also thought to be of the D2 type (12, 13). Both synthesis- and impulse-modulating autoreceptors are likely to participate in the homeostatic regulation of DA neurons; however, it is not yet clear whether these receptors are a single protein or multiple proteins. Characterization of the signal transduction system of synthesis-modulating autoreceptors may provide a clue as to the nature of similarities and/or differences between synthesis- and impulse-modulating autoreceptors. Thus, we have examined the role of G proteins in the function of synthesis-modulating DA autoreceptors by investigating the effects of pertussis toxin on apomorphine-induced inhibition of DA synthesis in the rat striatum.

Methods

Male Sprague Dawley rats (250–300 g; Camm) were used in all experiments. Rats were housed three or four per cage and given free access to food and water at all times.

Pertussis toxin injections. Rats were anesthetized with chloral hydrate (400 mg/kg intraperitoneally) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Pertussis toxin (1.0 μg/2 μl)

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ABBREVIATIONS: G protein, guanine nucleotide regulatory protein; DA, 3,4-dihydroxyphenylethylamine; DOPA, dihydroxyphenylalanine; AAAD, L-aromatic amino acid decarboxylase; GBL, γ-butyrolactone; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; EC, electrochemistry.

or an equal volume of vehicle (2 μl of 0.1 M phosphate-buffered saline) was injected (0.2 μl/min) unilaterally into the striatum [coordinates: AP= +0.2; L= +2.8; V = −6.0 according to the atlas of Paxinos and Watson (14)]. All rats were used for experimentation 2 days after surgery.

Drug administration. The ability of apomorphine to antagonize GBL-induced increases in DOPA accumulation after AAAD inhibition was used to determine the functional state of synthesis-modulating autoreceptors (15). Rats that had received intrastriatal pertussis toxin or vehicle 2 days before were administered either apomorphine (1 mg/kg subcutaneously) or vehicle (0.9% saline) 40 min before sacrifice, followed in some cases by GBL (750 mg/kg intraperitoneally at −35 min) and in some cases by *m*-hydroxybenzylhydrazine (NSD-1015, an AAAD inhibitor, 100 mg/kg intraperitoneally at −30 min). After drug pretreatment, rats were sacrificed by decapitation and brains were rapidly removed onto ice. A 2-mm thick coronal slice through the striatum surrounding the injection site was made using a brain mold, and a punch (i.d. = 1.6 mm, approximately 0.3 mg of protein/punch) of tissue was taken from the side of injection (ipsilateral) and the opposite striatum (contralateral). The samples were then frozen at −70° until prepared for HPLC analysis.

Tissue preparation-DOPA. Frozen tissue punches were sonicated in 450 μl of 0.1 M perchloric acid containing 10% sodium metabisulfite and dihydroxybenzylamine as an internal standard. After sonication, a 350-μl aliquot was brought to pH 8.4 by the addition of 3 M Tris base. This solution was then poured over miniature alumina columns and elution was assisted by centrifugation. After a wash with distilled water, catechols were eluted with 150 μl of 0.1 M oxalic acid and stored at −70° for no more than 24 hr until assayed. The remaining aliquot (100 μl) was used for protein determination according to the method of Lowry *et al.* (16).

HPLC-EC. The assay for catechols was performed essentially as described by Michaud *et al.* (17). Briefly, 25 μl of prepared sample was injected onto a 5-μm C-8 reverse phase HPLC column, where separation was accomplished with a mobile phase of 4–6% methanol in 0.1 M monobasic sodium phosphate buffer, containing 0.2 mM octylsulfonic acid and 0.1 mM EDTA, brought to pH 3.3 to 3.6 by the addition of phosphoric acid, and pumped through the column at a flow rate of 0.5 to 0.7 ml/min. An LC-3 EC transducer (Bioanalytical Systems, W. Lafayette, IN) with a glassy carbon electrode (+0.7 V versus Ag/AgCl reference) was used for signal detection. Peak heights were determined and the amount of DOPA present was calculated with reference to the internal standard.

Measurement of ADP-ribosylation. Punches of ipsilateral and contralateral striatum from six rats were sonicated in 500 μl of ice-cold homogenization buffer [50 mM Tris, pH 8, 6 mM $MgCl_2$, 1 mM EDTA, 3 mM benzamidine, 1 mM dithiothreitol, 5% (w/v) sucrose, and 1 mg/ml soybean trypsin inhibitor] and subsequently centrifuged at 10,000 × *g* at 4° for 10 min. Pellets were resuspended (by sonication) in 500 μl of ice-cold assay buffer [100 mM Tris, pH 8, 10 mM thymidine, 10 mM isoniazid, 5 mM $MgCl_2$, 2.8 mM dithiothreitol, 2.4 mM benzamidine, 0.8 mM EDTA, 2.5 mM ATP, 2.0 mM GTP, 4% (w/v) sucrose, 0.8 mg/ml soybean trypsin inhibitor, and 0.5% (v/v) Triton X-100]. Aliquots of resuspended pellets (extracts) were subjected to ADP-ribosylation as described previously (18). Briefly, the reaction was carried out in duplicate in 1.5-ml microfuge tubes for 1 hr at room temperature in a final volume of 100 μl under the following conditions: 80 μl of extract were incubated with 1 μg of purified, activated pertussis toxin and 10 μM [^{32}P]NAD (30 Ci/mmol). Reactions were terminated by the addition of 0.75 ml of ice-cold homogenization buffer, and the samples were centrifuged at 10,000 × *g* for 10 min. The pellets were resuspended by vigorous vortexing in 30 ml of a solution containing 40 mM Tris, pH 6.8, 1 mM dithiothreitol, and 2% (w/v) SDS. The samples were incubated in a 75° bath for 5 min, after which time 20 μl of 100 mM *N*-ethylmaleimide were added, and the samples were incubated for an additional 15 min at room temperature. Fifty microliters of a solution containing 40 mM Tris, pH 7.8, 1% (w/v) SDS, 50% (v/v) glycerol, and 6% β-mercaptoethanol were added and the samples were boiled for 2 min. The samples were subjected to one-dimensional SDS-polyacrylamide gel electrophoresis as described by Laemmli (19) with 9% (w/v) acrylamide/0.25% (w/v) bisacrylamide in the resolving gels. The resulting gels were dried and autoradiographed. Individual G protein bands were excised from dried gels and counted using a liquid scintillation counter.

Materials. Purified pertussis toxin was obtained from List Biochemical Laboratories (Campbell, CA). Apomorphine HCl, *m*-hydroxybenzylhydrazine (NSD-1015), and soybean trypsin inhibitor were purchased from Sigma Chemical Company (St. Louis, MO). GBL was obtained from Matheson, Coleman, and Bell (Norwood, OH); [^{32}P]NAD was purchased from New England Nuclear (Boston, MA). All other reagents were purchased from commercial sources and were of the highest purity obtainable.

Statistics. Studies assessing the effects of apomorphine on DOPA accumulation were analyzed using an analysis of variance with a post hoc Dunnetts test. ADP-ribosylation studies were analyzed using the Students' *t* test.

Results

In agreement with previous studies, apomorphine reduced the GBL-induced increase in DOPA accumulation in striata from control rats injected intrastriatally with vehicle and in striata taken from the uninjected side of pertussis-toxin treated rats (Fig. 1). Basal DOPA accumulation in rats administered NSD-1015 but not treated with GBL was 10.6 ± 1.0 ng/mg of protein (n = 8). DOPA accumulation in rats pretreated with GBL and NSD-1015 was 29.6 ± 5.4 ng/mg of protein (n = 7) 2 days after sham injection, and 29.0 ± 2.75 ng/mg of protein (n = 7) in the contralateral uninjected striata. DOPA accumulation in rats treated with both NSD-1015 and GBL was significantly attenuated by pretreatment with apomorphine (sham: 12.3 ± 0.5 ng/mg of protein, n = 6; and contralateral: 8.8 ± 1.2 ng/mg of protein, n = 6, $p \leq 0.05$). Pertussis toxin treatment alone (measured after NSD-1015 alone or in combination with GBL) did not significantly alter levels of striatal DOPA compared with rats that did not receive pertussis toxin (12.2 ± 1.6 ng/mg of protein after NSD-1015, n = 6; and 39.2 ± 5.1 ng/mg of protein after NSD-1015 and GBL, n = 7; Fig. 1). However, pertussis toxin treatment did abolish the ability of apomorphine

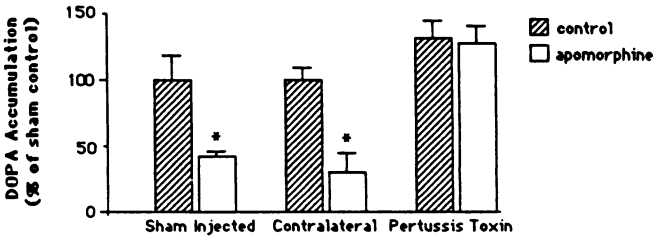

Fig. 1. The effects of pertussis toxin pretreatment on apomorphine-induced decrease in DOPA accumulation in the rat striatum. Rats were injected unilaterally in the striatum with either pertussis toxin (1 μg/2 μl) or 0.1 M phosphate-buffered saline (sham) 2 days before experimentation. On the experimental day, rats were treated with either apomorphine (1 mg/kg subcutaneously at −40 min) or 0.9% saline as well as GBL (750 mg/kg intraperitoneally at −35 min) and NSD-1015 (100 mg/kg intraperitoneally 30 min before sacrifice). DOPA accumulation was measured by HPLC-EC in punches from the pertussis toxin-injected (ipsilateral) and noninjected (contralateral) striata as well as sham injected control rats. Basal DOPA accumulation in rats treated with NSD-1015 was 10.6 ± 1.0 ng/mg of protein/30 min (n = 8). There were seven rats in each control group, six in the sham injected apomorphine-treated and contralateral apomorphine-treated groups, and eight in the pertussis toxin-injected and apomorphine-treated group. *, $p \leq 0.05$.

to significantly reduce DOPA accumulation (37.6 ± 5.4 ng/mg of protein, $n = 8$; Fig. 1).

In order to confirm the effectiveness of *in vivo* pertussis toxin treatment, the effect of such treatment on the ability to ADP-ribosylate G proteins *in vitro* was studied. It was found that pertussis toxin injection dramatically reduced the amount of G protein able to be ADP-ribosylated *in vitro* versus the contralateral noninjected striatum (Fig. 2). As can be seen in the figure, both the 39-kDa (G_o) and the 41-kDa (G_i) bands (24) were significantly reduced by *in vivo* pertussis toxin treatment (37% and 44%, respectively). Total *in vitro* ADP-ribosylation of both G_i and G_o was decreased by 61% (13.8 pmol/mg of protein, contralateral, versus 5.4 pmol/mg of protein, ipsilateral, $p \leq 0.05$). Individually, G_i was reduced from 3.6 to 1.6 pmol/mg of protein ($p \leq 0.05$) and G_o was reduced from 10.2 to 3.8 pmol/mg protein ($p \leq 0.05$).

Discussion

The ability of DA agonists to antagonize GBL-induced increases in DOPA accumulation after inhibition of AAAD has been well characterized as an *in vivo* model for the study of synthesis-modulating DA autoreceptors (15). GBL eliminates drug effects that are mediated via somatodendritic or postsynaptic DA receptors, by inhibiting impulse flow in dopaminergic neurons (20). The rate of DA synthesis after cessation of impulse flow can then be determined *in vivo* by administering an inhibitor of AAAD, in order to block the conversion of DOPA to DA. The short term accumulation of DOPA then reflects *in vivo* tyrosine hydroxylation (21). The elimination of impulse flow in nigrostriatal DA neurons produces an increase in the tyrosine hydroxylation in their terminals. Decreased synaptic levels of DA, produced by the GBL-induced diminution in impulse-dependent release, leads to a disinhibition of events normally under tonic autoreceptor control, thus accounting for the increase in tyrosine hydroxylation observed after cessation of impulse flow. Therefore, the apomorphine-induced inhibition in DOPA accumulation observed herein in both sham injected control rats and in the uninjected striata of the pertussis toxin-treated rats confirms pervious studies, which have shown that apomorphine has activated striatal autoreceptors responsible for synthesis inhibition (11).

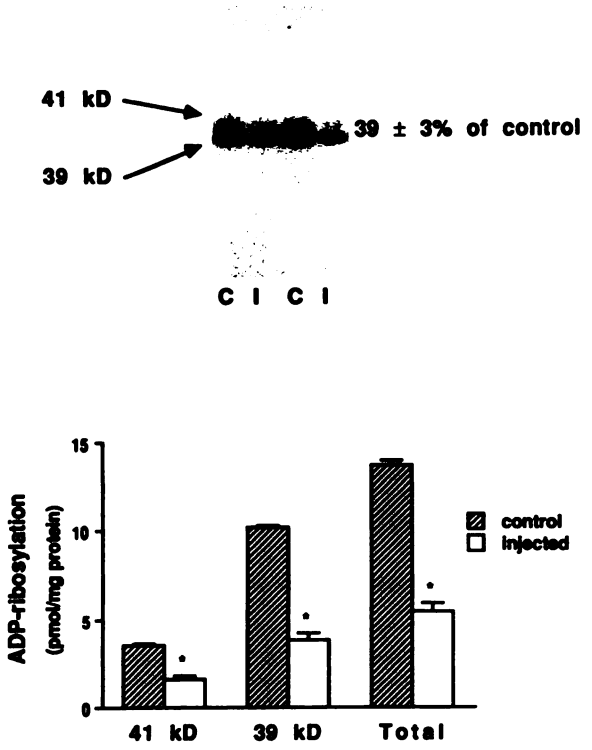

Fig. 2. The effect of *in vivo* pertussis toxin treatment on *in vitro* G protein ADP-ribosylation. Six rats were injected with pertussis toxin (1 μg/2 μl) 2 days before sacrifice. Punches of striatum ipsilateral (*I*, injected) and contralateral (*C*, control) to the side of injection were removed and subjected to ADP-ribosylation with [^{32}P]NAD, SDS-polyacrylamide gel electrophoresis, and autoradiography. The two bands of approximately 39 and 41 kDa, which correspond to the M_r of the α subunits of G_o and G_i, respectively (24), were excised from gels and quantitated using a liquid scintillation counter. Data are expressed as pmoles of ADP-ribose incorporated per milligram of total protein, and *error bars* represent the standard error of the mean. *, $p \leq 0.05$.

Pertussis toxin treatment alone did not significantly alter levels of striatal DOPA, compared with rats that did not receive pertussis toxin, in contrast to what might have been predicted because of the blockade by the toxin of the function of synthesis-modulating autoreceptors. A trend toward an increase in DOPA accumulation was observed in both vehicle and apomorphine groups that had been pretreated with pertussis toxin; however, the groups were not statistically different. Similar to our findings, Innis and Aghajanian (10) have found no effect of pertussis toxin on baseline firing rate in the substantia nigra, although pertussis toxin effectively blocks the somatodendritic DA autoreceptor found in this region. The most likely possibility for this lack of effect of pertussis toxin is that adapative changes have taken place in these neurons in the 2 days following pertussis toxin treatment, which have attenuated any increase in synthesis that might have occurred due to blockade of autoreceptor function. In this regard, although it has been shown that acute transection of the nigrostriatal pathway increases DA synthesis (21), Nyback and Sedvall (22) have shown that DA synthesis is not different from control 24 hr after nigrostriatal transection. These data suggest that a mechanism exists to compensate for the loss in synaptic DA and that it can be observed within 24 hr. Alternative explanations for the lack of effect of pertussis toxin are that the toxin has actions that compensate for the blockade of DA autoreceptors or that the ADP-ribosylation of G proteins in these regions must be complete for significant alterations in the activity of these neurons to be discerned.

In order to verify that the intrastriatal pertussis toxin injections resulted in the ADP-ribosylation of G proteins *in vivo*, we determined the level of remaining G proteins that could be ADP-ribosylated *in vitro* in the presence of purified pertussis toxin and [^{32}P]NAD. The reduced levels of *in vitro* ADP-ribosylation observed in this study are interpreted to suggest that equivalent increases in *in vivo* ADP-ribosylation had been induced by pertussis toxin. The reduction (61%) in *in vitro* ADP-ribosylation we observed is comparable with the results

of Innis *et al.* (23), who showed that direct intraraphe injection of pertussis toxin reduced *in vitro* ADP-ribosylation in the dorsal raphe nucleus by 50%.

Our results combined with those of Innis and Aghajanian (10) suggest that activation of either impulse- or synthesis-modulating DA autoreceptors results in a G protein-mediated event. However, the identity of the second messenger system coupled to the G protein, and whether different intracellular events are triggered by different types of autoreceptors are questions remaining to be answered. An intriguing possibility by which synthesis- and impulse-modulating autoreceptors could both be linked to G proteins, yet produce different functional consequences, may involve either linkage to different G proteins or the coupling of both receptors to the same G protein followed by a divergence in intracellular signaling mechanisms. Activation of the impulse-modulating receptor has been shown to produce a hyperpolarization of the cell body, an effect that is mediated by an increase in potassium conductance (24). The G protein activated by the impulse-modulating autoreceptor might, therefore, be directly coupled to a potassium channel, as has been shown for γ-aminobutyric acid and serotonin receptors in the hippocampus (1). The G protein activated by the synthesis-modulating autoreceptor might mediate the inhibition of adenylate cyclase or alter phosphoinositide turnover, other intracellular events shown to occur after D2 receptor activation (4).

G proteins are the fundamental components in the transduction mechanism of many receptors and, because of their diversity and the numerous intracellular events they trigger, may provide a means by which similar, pharmacologically defined receptors subserve different functions. Further definition of the intracellular events related to specific DA autoreceptor activation may yield insight into the mechanism by which this receptor can subserve multiple functions.

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