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Pertussis Toxin-Mediated Ribosylation of G Proteins Blocks the Hypnotic Response to an α_2-Agonist in the Locus Coeruleus of the Rat

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CORREA-SALES, C., K. REID AND M. MAZE. *Pertussis toxin-mediated ribosylation of G proteins blocks the hypnotic response to an α_2-agonist in the locus coeruleus of the rat.* PHARMACOL BIOCHEM BEHAV 43(3) 723-727, 1992.—Biologic responses mediated by adrenoceptors are transduced by a receptor-effector mechanism that involves a guanine nucleotide binding protein (G protein). Recently, we determined that the transduction mechanism for the hypnotic response to dexmedetomidine, a highly selective α_2-agonist, is located in the locus coeruleus (LC) of the rat. In this study, we examined the role of pertussis toxin-sensitive (PTX) G proteins in the LC for the hypnotic response to dexmedetomidine. The LC of rats were stereotactically cannulated and treated with PTX, 0.34 μg, or vehicle. Five days later, the hypnotic response to dexmedetomidine, 7 μg into the LC or 50 $\mu g \cdot kg^{-1}$ IP, was tested. On the following day, the LC was harvested and assayed to determine whether the G proteins had been ribosylated by pretreatment with PTX in vivo. Quantitative immunoblotting of $G_{0\alpha}$, $G_{i\alpha1,2}$, and $G_{i\alpha3}$, the α-subunit of three PTX-sensitive proteins, was also performed. In vivo treatment with PTX into the LC blocked the hypnotic response to LC-administered dexmedetomidine and, to a lesser extent, IP-administered dexmedetomidine. The in vivo PTX treatment effectively ribosylated the G proteins. No alteration in the amount of the different species of PTX-sensitive α-subunit was produced by in vivo PTX treatment. These data suggest a pivotal role for PTX-sensitive G proteins in the LC in the hypnotic response to α_2-agonists in the rat.

G proteins: $G_{0\alpha}$, $G_{i\alpha1,2}$, $G_{i\alpha3}$ Pertussis toxin Locus coeruleus Adrenergic receptor: α_2, dexmedetomidine

THE ability of α_2-adrenoceptors to rapidly stimulate an effector system is transduced by a family of membrane-bound guanine nucleotide binding proteins, or G proteins (6). The G proteins are composed of three polypeptide subunits designated as α, β, and γ (in order of decreasing mass). Bacterial toxins from *Vibrio cholera* or *Bordetella pertussis* can covalently modify the G proteins by the addition of an adenosine phosphate (ADP) ribose group to the α-subunits. Both these toxins contain an enzyme that catalyzes the transfer of ADP ribose from nicotinamide adenine dinucleotide (NAD) to specific acceptor sites on the α-subunit (17). Thus, α-subunits can be classified into four groups according to their sensitivity to ribosylation by one, both, or neither toxin (5).

Previously, we reported that animals pretreated with pertussis toxin (PTX) via the intracerebroventricular route had a blunted hypnotic response to systemically administered dexmedetomidine (3). However, this dose and route of PTX administration induced an abnormal physiological state characterized by hyperexcitability and weight loss that raised the possibility that the altered behavioral response was due to nonspecific factors. Data from recent stereotaxic studies suggest that the hypnotic action of dexmedetomidine is mediated by α_2-adrenoceptors in the locus coeruleus (LC) because delivery of dexmedetomidine directly into the LC induces loss of righting reflex (LORR) in rats that is blocked by an α_2-antagonist (1). De Sarro et al. (2) reported that PTX introduced into the LC of rats alters the behavioral response to both agonists and antagonists of the α_2-adrenoreceptor although their studies did not correlate these effects with ADP ribosylation of G proteins. In the current study, we determined whether ADP ribosylation of PTX-sensitive proteins in the LC altered the hypnotic response to dexmedetomidine.

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METHOD

The experimental protocol was approved by the Animal Care and Use Committee at the Palo Alto Veterans Administration Medical Center. Male Sprague-Dawley rats (Bantin and Kingman, Sunnyvale, CA), originating from the same litter, weighing 250–300 g were used. Rats were stratified into control and treatment groups and were matched for weight.

The left LC was stereotactically cannulated with a 24-ga stainless steel cannula according to the following coordinates: with the bregma as the reference, 1.2 mm lateral, 9.7 mm posterior, and at a depth of 6 mm from the skull (13). The surgical procedure was performed with the rat under halothane anesthesia and the cannula was fixed in position with methylmethacrylate resin. After a recovery period of 2–4 days, a 30-ga stainless steel needle, connected to a polyethylene tubing, was inserted through the cannula and positioned 1 mm beyond its tip. Rats received either PTX (List Biological Laboratories, Campbell, CA) 0.34 μg into the LC or 0.5 μl of a solvent vehicle containing sodium phosphate buffer 6.7×10^{-2} M, pH 7.4 (2). Six days after PTX injection, the groups received either dexmedetomidine (Farmos Research & Development, Turku, Finland) 7.0 μg in 0.2 μl delivered into the LC over 1 min or dexmedetomidine 50 $\mu g \cdot kg^{-1}$ IP (0.3 ml). The 5-day latency period was predicated by pilot studies. For both sets of experiments (dexmedetomidine LC and IP), behavior was tested as described below.

Behavioral Testing

All behavioral tests were performed between 10:00 a.m. and 4:00 p.m. The number of animals for each experiment is listed in the legends to the figures. The hypnotic response was defined by the LORR. The duration of the LORR was assessed as the time from the rat's inability to right itself when placed on its back until the time that it spontaneously reverted, completely, to the prone position. This is referred to as sleep time. The observer was not blinded to the various treatments because the observations (loss and restoration of the rat's righting reflex) are unequivocal end points and are not subject to observer misinterpretation. Behavioral data were analyzed by the χ^2 test. Data were considered statistically significantly different when the p value was <0.05.

Biochemical Testing

One day following the behavioral studies, rats were killed and brains were harvested and immediately frozen on dry ice and stored at −70°C. Twenty-four hours later, LC regions from the injected (ipsilateral) and noninjected (contralateral) sides were recovered separately by punch biopsy (12). The inner diameter of the punch needle was 0.8 mm and the brain slices were 1 mm thick. The LCs from three different rats were pooled into one sample that was then processed for the biochemical assays as follows. Tissue was homogenized in 0.5 ml ice-cold homogenizing solution containing 6 mM $MgCl_2$, 1 mM EDTA, 3 mM benzamidine, 1 mM DTT, 1 μg/ml soybean trypsin inhibitor, and 5% sucrose in a 50 mM Tris-HCl buffer, pH 8.0, by aspirating repeatedly through a 27-ga needle. The samples were centrifuged at 12,200 × g for 15 min at +4°C. The pellet was resuspended in 70 μl ribosylation buffer containing 10 mM thymidine, 10 mM iproniazide phosphate, 5 mM $MgCl_2$, 2.8 mM DTT, 2.5 mM ATP, 2.0 mM GTP, 0.8 $\mu g \cdot ml^{-1}$ soybean trypsin inhibitor, and 4% sucrose in a 50 mM Tris-HCl buffer, pH 8.0. The resuspended pellet was divided into three aliquots for protein determination (5 μl), immunoblotting (20 μl), and ADP ribosylation (remainder). The samples for immunoblotting were further diluted in 70 μl 50 mM Tris-HCl buffer, pH 6.8, containing 1 mM DTT and 2% (w/v) SDS (= buffer A) and divided in three aliquots (30 μl each) for storage at −20°C for up to 4 days.

ADP Ribosylation

Identical amounts of total protein (±178 μg) from each sample were pipetted into microcentrifuge tubes and diluted to a volume if 80 μl with the ribosylation buffer. PTX was activated in 50 mM DTT for 1 h at room temperature. Two micrograms of the activated PTX was added to the sample in the ribosylation buffer and [^{32}P]NAD [30 Ci/mmol, New England Nuclear (NEN), Newton, MA] was added to a final concentration of 10 μM in an incubation volume of 115 μl. Samples were incubated for 1 h at room temperature. The in vitro ribosylation reaction was stopped by adding trichloracetic acid, 10%, after which the samples were placed on ice for 15 min. The samples were centrifuged at 12,200 × g for 15 min at +4°C and the pellets were neutralized by adding 20 μl 0.4 M NaOH and 30 μl buffer A. Samples were heated at 75°C for 5 min, after which 20 μl 100 mM *N*-ethylmaleimide was added and the samples were left at room temperature for 15 min. To these samples was added 50 μl of a 50 mM Tris-HCl buffer, pH 6.8, containing 1% SDS, 50% glycerol, and 6% β-mercaptoethanol. Samples were boiled for 2 min, after which an additional sample buffer (20% glycerol, 2% β-mercaptoethanol, 1% SDS, 0.002% (w/v) bromophenol blue, and 100 mM Tris-HCl buffer, pH 6.8) was added and the samples were heated at 95°C for 5 min.

The samples, containing 10 μg protein, were subjected to one-dimensional SDS-PAGE (MINI-PROTEAN; Bio-Rad Laboratories, Hercules, CA) in gradient gels from 4–20% polyacrylamide. Gels were dried and autoradiographed. G-protein bands were analyzed with a densitometer (Pharmacia-LKB Ultroscan XL Enhanced Laser Densitometer).

Densitometry data were analyzed by two-tailed unpaired t-test with Bonferroni correction for multiple testing. Data were considered statistically significantly different when the p value was <0.05.

Immunoblot Analysis

Samples in buffer A were treated with *N*-ethylmaleimide and 7 μg protein was subjected to SDS-PAGE, as described above. Proteins in the resulting gel were transferred to nitrocellulose paper by high-voltage Mini Trans-Blot electrophoresis (Bio-Rad). Prestained molecular weight standards (M_w 18,500–106,000 D) were used to confirm adequate transfer. Immunolabeling of the G proteins on the nitrocellulose paper was performed using rabbit polyclonal antisera (Du Pont-NEN) directed against the α-subunit of either G_0, $G_{i1,2}$ (both 1:1,000), and G_{i3} (1:300) and [^{125}I]labeled goat antirabbit immunoglobulin (IgG) (1,000 cpm/μl, NEN). Both the blocking and incubation buffers contained 5% nonfat dry milk (NFDM; Bio-Rad), 0.005% Tween-20, 0.02% Na-azide, 2 mM $CaCl_2$, and 80 mM NaCl in a 50 mM Tris-HCl buffer, pH 8.0. Quantification of the autoradiograms of the G proteins was performed by densitometry. Densitometry data were analyzed by two-tailed unpaired t-test with Bonferroni correction for multiple testing. Data were considered statistically significantly different when the p value was <0.05.

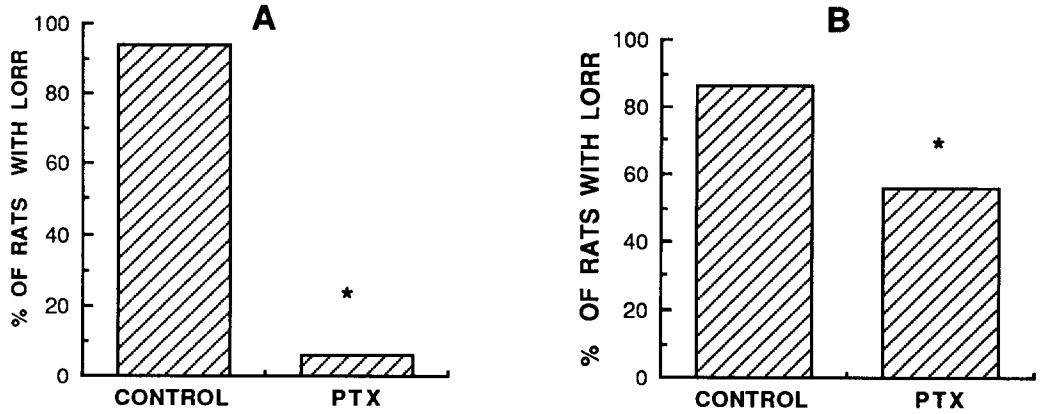

FIG. 1. Effect of pertussis toxin (PTX) on hypnotic response to dexmedetomidine. (A) LC. Rats were treated with PTX 0.34 μg into the LC. Control rats received a similar volume of vehicle alone (0.067 M sodium phosphate buffer, ph 7.4). After a further 6 days, animals were administered dexmedetomidine 7 μg into the LC and the number of rats exhibiting an LORR was determined. *$p < 0.0001$.

(B) IP. Rats ($n = 23$) were treated with PTX 0.34 μg into the LC via an indwelling cannula. Control rats ($n = 22$) received a similar volume of vehicle alone (0.067 M sodium phosphate buffer, ph 7.4). After a further 6 days, animals were administered dexmedetomidine 50 $\mu g \cdot kg^{-1}$ IP and the number of rats exhibiting an LORR was determined. *$p < 0.03$.

RESULTS

Behavioral Experiments

Qualitatively, the behavior of PTX-treated rats was indistinguishable from that exhibited by control rats before administration of dexmedetomidine. In vehicle-treated (control) animals, dexmedetomidine, 7 μg into the LC, induced an LORR in 15 of 16 animals for a duration of 56 ± 9 (mean ± SD) min. Of the 16 rats pretreated with PTX, only 1 lost its righting reflex (duration = 33 min) (Fig. 1A). In separate cohorts of vehicle-treated (control) animals, dexmedetomidine, 50 $\mu g \cdot kg^{-1}$ IP, resulted in LORR in 19 of 22 animals for a duration of 83 ± 35 min. Significantly fewer rats (13 of 23) lost their righting reflex in response to dexmedetomidine, 50 $\mu g \cdot kg^{-1}$ IP, following PTX pretreatment although the duration was similar (96 ± 32 min) (Fig. 1B).

ADP Ribosylation

Dose-response curves comparing increasing protein concentrations with the intensities of the bands detected by [^{32}P]NAD ribosylation suggested that the relationship is linear

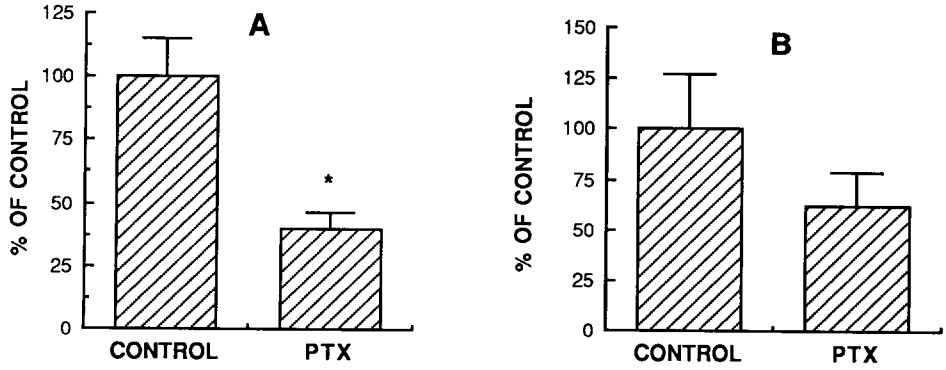

FIG. 2. Effect of in vivo PTX on in vitro ADP ribosylation. Rats were treated with PTX 0.34 μg or vehicle (0.067 M sodium phosphate buffer, ph 7.4) into the LC via an indwelling cannula. After 7 days, rats were killed and membranes prepared from the LC and ribosylated in vitro with [^{32}P] labeled NAD in the presence of activated PTX, 2 μg, and were applied to the SDS-PAGE and autoradiography and subsequent densitometry were performed as described in the Method section. To obtain sufficient protein (6.7 μg/well) for SDS-PAGE, LC from the ipsilateral (A) or contralateral (B) sides of three animals were pooled and because of these logistics experiments were performed on two separate occasions. Data presented here were normalized by expressing the densitometry as a percent of the relevant control value. Data are represented as mean ± SEM (number of samples/experimental group: ipsilateral control = 15, PTX = 14; contralateral control = 7, PTX = 7). *$p < 0.01$.

(R^2 value = 0.944) over a range of 3–30 μg (data not shown). PTX-treated rats had significantly ($p < 0.01$) less [^{32}P]labeled NAD ribosylation compared to vehicle-treated animals by densitometry (Fig. 2A). The LC contralateral to that which was treated with in vivo PTX had a similar profile of ADP ribosylation to that found on the ipsilateral, although there was no statistical difference between vehicle- and PTX-treated cohorts (Fig. 2B).

Immunoblotting

Dose-response curves comparing increasing protein concentrations with the intensities of the bands detected by Western blotting suggested that the relationship is linear up to 10 μg (membrane protein) for each species (data not shown). PTX treatment in vivo did not induce quantitative changes in the species of PTX-sensitive G proteins.

DISCUSSION

These data suggest a pivotal role for PTX-sensitive G proteins in the transduction of the hypnotic response to the α_2-agonist, dexmedetomidine, in the LC of the rat. PTX pretreatment did not alter the quantity of the PTX-sensitive species of α-subunit tested for in the LC. For validation of the autoradiographic studies, we utilized membrane protein rather than purified PTX-sensitive G protein (ribosylation) or the individual species of α-subunit (immunoblotting) for the standard curves. Therefore, we were unable to convert the arbitrary densitometry units into absolute values of either ADP-ribosylated G protein or species of α-subunit.

PTX contains a ribosylase that catalyzes the attachment of ADP ribose to a conserved cysteine residue 4 amino acid from the carboxy terminus of the α-subunit (8). The ADP-ribosylated G protein fails to dissociate when the receptor is activated by its agonist and thereby uncouples the α_2-adrenoceptor from its effector mechanism. Thus, the normal transduction of the biologic response is prevented.

The α-subunit of the G protein has a molecular weight varying between 39–46 kDa and a similar secondary structure irrespective of the source (9). The $G_{0\alpha}$ is the major G protein in the mammalian brain, where it constitutes up to 1% of membrane protein (16). There is extensive homology with the $G_{i\alpha}$ family but the differences are sufficiently great to result in altered kinetics of binding of guanine nucleotides. The $G_{i\alpha}$ family is composed of at least three closely related α-subunits (α_{i1-3}) that are encoded by different genes although the functional significance of this microheterogeneity is not known (11).

It is not clear whether the individual species of G proteins are dedicated to a specific effector mechanism (10). G_i supports receptor-mediated inhibition of adenylate cyclase and is clearly one of the G proteins coupled to the α_2-adrenoceptors (14). G_0 has no obvious role in the regulation of adenylate cyclase and implies a broader role for it in the brain. $G_{0\alpha}$ has been shown to couple receptors to an effector mechanism in which calcium ion translocation is inhibited (4,7) but it has also been linked to an effector mechanism that features potassium conductance (18). Quantitative immunoblotting revealed that none of the species tested was more susceptible to disruption and loss from the LC following in vivo PTX treatment. It should be stressed that we examined the effect of PTX treatment on the amount of the different species of α-subunit at a single time point (i.e., 7 days), and we cannot extrapolate from these data that PTX treatment does not regulate the turnover rates of these species. Therefore, it remains unknown which of the species is more likely to be involved in the transduction of the hypnotic response following activation of the α_2-adrenoceptor. Recent evidence suggests that the G_0 species is less likely than G_i to couple the α_2-adrenoceptor to its effector mechanism in the rat brain (15).

The hypnotic response to IP-administered dexmedetomidine was also attenuated in PTX-treated animals (Fig. 1B) although to a lesser extent than that obtained in the LC-administered cohort (Fig. 1A), a finding also reported by De Sarro et al. (2). A reason for the hypnotic response difference in PTX-treated animals after systemic administration of dexmedetomidine could be the fact that ADP ribosylation of the G proteins in the LC on the contralateral side changed in a qualitatively similar manner (Fig. 2B) to that seen on the ipsilateral side (Fig. 2A) although this did not achieve statistical significance. An alternative reason for the attenuated hypnotic response to dexmedetomidine IP may be a requirement for the participation of both sides of the LC for optimal transduction of the hypnotic response. A similar attenuation to systemically administered dexmedetomidine was seen in rats pretreated with an α_2-antagonist into the LC unilaterally (1).

These studies reveal the importance of PTX-sensitive G proteins in the transmembrane signalling mechanism for the hypnotic response to α_2-agonists in the LC in the rat. Whether a single species of PTX-sensitive G protein exists and what the identity of such a species is needs to be further elucidated.

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