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Chronic Treatment With Dexmedetomidine Desensitizes α_2-Adrenergic Signal Transduction

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REID, K., Y. HAYASHI, J. HSU, P. A. MAGUIRE, B. C. RABIN, T.-Z. GUO AND M. MAZE. *Chronic treatment with dexmedetomidine desensitizes α_2-adrenergic signal transduction.* PHARMACOL BIOCHEM BEHAV **57**(1/2) 63–71, 1997.—Tolerance to the hypnotic response was induced in rats by chronically infusing dexmedetomidine, a novel α_2-adrenergic agonist. The α_2-adrenocepter affinity for dexmedtomidine and *para*-iodoclonidine was significantly reduced in tolerant rats, while B_{max} was uncharged. The ability of pertussis toxin (PTX) to ribosylate guanine nucleotide regulatory proteins (G proteins) ex vivo was reduced in tolerant rats; the quantity of PTX-sensitive G proteins was unchanged. Forskolin-stimulated adenylyl cyclase was less sensitive to inhibition by dexmedetomidine in the tolerant rats; however, acute intraperitoneal injection of dexmedetomidine still reduced cyclic adenosine monophosphate levels in tolerant rats. Both the decrease in ribosylation and the lower α_2-adrenoceptor binding affinity may reflect a decrease in the ability of the G protein to couple to the α_2 adrenoceptors in the loecus coeruleus of tolerant rats. In this state, the α_2 adrenoceptors are less capable of transducing the effector response (inhibition of adenylyl cyclase). © 1997 Elsevier Science Inc.

Adrenergic receptor α_2 Agonist Dexmedetomidine Tolerance Locus coeruleus
Pertussis toxin–sensitive G proteins Adenylyl cyclase cAMP

RATS become desensitized to the hypnotic action of an α_2 agonist following chronic treatment with an α_2 agonist (27). This state of tolerance is due to a pharmacodynamic, not pharmacokinetic, mechanism, because the attenuated anesthetic response is evident even when the α_2 agonist is delivered directly into the locus coeruleus (LC) (27), the site of α_2 hypnotic action (7).

The development of tolerance is a widespread biologic phenomenon in which responsiveness decreases with continuing exposure and has been particularly well defined for adrenergic responses transduced by β and α_1 receptor–effector mechanisms (5). Characterization of the molecular mechanisms involved in the development of tolerance to α_2 adrenoceptor–mediated effects in the CNS first requires that one define the transduction components involved for these responses. Recently we demonstrated that α_2 adrenoceptors in the LC mediate the hypnotic-anesthetic action of α_2 agonists (7). This precise anatomic localization allowed us to perturb various molecular components, in a site-specific manner, to define the steps in the signal tranduction pathway for the hypnotic-anesthetic action. The initial postreceptor components involved in the hypnotic action of α_2 agonists are a pertussis toxin-sensitive guanine nucleotide regulatory protein (G protein) (8) coupled, in an inhibitory fashion, to adenylyl cyclase (6). The subsequent decrease in cyclic adenosine monophosphate (cAMP) and cAMP-dependent protein kinase activity probably causes various species of ion channels (21) to be mostly present in the dephosphorylated state which alters ion translocation and membrane conductance, and decreases neuronal firing (31).

In this study we addressed the steps in the transduction pathway that are altered in the tolerant rats. We now report

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on the changes in α_2 adrenoceptor binding, pertussis toxin-sensitive G proteins, and adenylyl cyclase in the LC of rats made tolerant to the hypnotic action of the α_2 agonist dexmedetomidine.

METHODS

Development of Tolerance

Rats were made tolerant to the anesthetic action of an α_2 agonist, dexmedetomidine, as previously described (27). Briefly, rats were administered dexmedetomidine chronically using Alzet osmotic minipumps (Model 2002 or 1007D; Alza, Palo Alto, CA) which discharge their contents at a mean pumping rate of 0.48 ± 0.02 μl/h. The pumps were inserted subcutaneously during halothane anesthesia in the dorsal thoracic region and loaded to deliver 3 or 5 mg/kg per h for 7 or 14 days. In the initial experiments control animals were also implanted with the osmotic pumps containing only the vehicle. This group did not differ in behavioral response from sham-operated control animals; therefore, we used the latter. Plasma dexmedetomidine levels were assayed as previously described (29) with modifications to improve the signal–noise ratio and reproducibility. Blood was sampled on days 2 and 7 in six rats.

Loss of Righting Reflex

Hypnotic response to dexmedetomidine, 100 mg/kg, IP, was defined by the loss of the rat's righting reflex (LORR), and its duration was measured in minutes and referred to as sleep-time. 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. Hypnotic response was only tested in the 14-day treatment group and was performed between 1000 and 1800 h as described previously (27).

α_2-Receptor Binding

Animals infused chronically with dexmedetomidine 5 mg/kg per h for 7 or 14 days were decapitated following 30 s exposure to CO_2. The LC was removed from each side of the freshly harvested brain using the punch technique. Punches from 2-mm brain slices at the dorsal-ventral location of the LC were obtained. LCs from three rats were pooled, which yielded sufficient tissue for one assay (36 data points). Brains were sliced fresh and the LCs removed over an ice-cold glass plate using an 0.8-mm-bore glass pipette. Unless otherwise specified, all further manipulations were performed at 4°C. The LC's were homogenized rapidly in 20 vol. of ice-cold Tris–EDTA buffer (Tris-HCl, 50 mM; EDTA, 0.8 mM; pH 7.5 at 4°C) 5 × 5 s at 60% maximum power. Following centrifugation at 500 × *g* for 5 min, the supernatants were collected and centrifuged at 30,000 × *g* for 20 min. Pellets were resuspended in Tris–EDTA buffer and recentrifuged as above. Pellets were then stored at −80°C in 5 vol. of ice-cold phosphate buffer (potassium phosphate, 50 mM; pH 7.65 at 25°C) until radioligand binding assay was performed (stored no longer than 3 weeks).

The radiolabeled ligand binding method was adapted from Baron and Siegel (1). Frozen membrane aliquots were thawed and resuspended in 1.5 ml of Tris–$MgCl_2$ buffer (Tris-HCl, 50 mM; $MgCl_2$, 10 mM; pH 7.6 at 25°C; referred to as the wash buffer), and incubated for 15 min at 37°C to remove any residual endogenous cathecolamines. Following centrifugation at 30,000 × *g* for 20 min twice in the wash buffer, the final pellet was resuspended in 0.8 ml of ice-cold Tris–$MgCl_2$ / EGTA buffer (Tris-HCl, 50 mM; $MgCl_2$, 10 mM; EGTA, 1 mM; pH 7.6 at 25°C; referred to as the incubation buffer). Each assay tube (final vol.: 50 μl) contained 0.5–1.0 nM of *para* (*p*)-[^{125}I]iodoclonidine, "cold" displacing ligand (*p*-iodoclonidine or dexmedetomidine) in concentrations ranging from 10^{-11} to 10^{-6} M and 20 μl of membrane in incubation buffer. Nonspecific binding was defined using phentolamine, 10 μM. Binding was initiated by addition of the membranes and was incubated at 25°C for 90 min at which time equilibrium was established. Binding was terminated by addition of ice-cold wash buffer, followed by filtration through pretreated (0.1% polyethyleneimine) Whatman GF/B filters (Maidstone, Kent, UK) using the Hoefer filtration unit. The filters were washed three times with cold wash buffer, and remaining radioactivity on the filters was determined using a Packard Auto-Gamma Counter. The displacement constants (K_i's) of dexmedetomidine and iodoclonidine were determined. Also, the receptor density (B_{max}) was calculated. Calculation of both K_i and B_{max} was performed using LIGAND (Biosoft) (20). B_{max} is expressed as fentomoles per LC-punch and K_i's in nanomoles. Data were analyzed statistically by the unpaired *t*-test with $p < 0.05$ considered significantly different.

G Protein Function and Amount

ADP ribosylation. Three LC's, harvested as described above, were disrupted by aspirating repeatedly through a 27-ga hypodermic needle in 0.5 ml ice-cold homogenizing solution containing $MgCl_2$, 6 mM; EDTA, 1 mM; benzamidine, 3 mM ; dithiothreitol (DTT), 1 mM; soybean trypsin inhibitor, 1 μg/ml; 5% sucrose in a Tris-HCl buffer, 50 mM, pH 8.0. The samples were centrifuged at 12,200 × *g* for 15 min at 4°C. The pellet was resuspended in 70 μl ribosylation buffer containing thymidine, 10 mM; iproniazide-phosphate, 10 mM; $MgCl_2$, 5 mM; DTT, 2.8 mM; adenosine triphosphate (ATP), 2.5 mM; guanosine triphosphate (GTP), 2.0 mM; soybean trypsin inhibitor, 0.8 μg/ml; 4% sucrose in a Tris–HCl buffer, 50 mM; 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 of a Tris buffer [containing 50 mM Tris–HCl, pH 6.8, 1,mM DTT, and 2% (wt./vol.) sodium dodecyl sulfate (SDS)] and divided into three aliquots (30 μl each) for storage at −20°C for up to 4 days.

For the ADP-ribosylation experiments, identical amounts of total protein from each sample were pipetted into microcentrifuge tubes and diluted to a volume of 80 μl with the ribosylation buffer. Pertussis toxin (PTX) was activated in DTT, 50 mM; 1 h at room temperature. Then 2 μg of the activated PTX was added to the sample and ^{32}P-NAD (30 Ci/mmol; New England Nuclear) 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 sample was centrifuged at 12,200 × *g* for 15 min at 4°C and the pellet was neutralized by adding 20 μl of NaOH, 0.4 M, and 30 μl of Tris buffer. Samples were heated at 75°C for 5 min, after which 20 μl of *N*-ethylmaleimide, 100 mM, was added and the samples were left at room temperature for 15 min. To these samples was added 50 μl of a Tris–HCl buffer, 50 mM; 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-mer-

captoethanol, 1% SDS, 0.002% (wt./vol.) bromophenolblue, 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 of protein, were subjected to one-dimensional SDS–polyacrylamide gel electrophoresis (PAGE) (MINI-PROTEAN; BioRad) in gradient gels from 4–20% polyacrylamide. The amount of protein chosen in the assay was on the linear part of the standard curve previously perfomed in our lab (data not shown). Gels were dried and autoradiographed. G protein bands were analyzed with a densitometer (Ultroscan XL Enhanced Laser Densitometer; Pharmacia-LKB). Data are expressed as a percentage of control [absorbance units (AU)], which was considered to be a 100% signal. Densitometry data were analyzed by two-tailed unpaired *t*-test. Data were considered to be statistically significantly different when the $p < 0.05$.

Immunoblotting

For the immunoblotting studies, samples in Tris buffer were treated with *N*-ethylmaleimide and 7 μg of protein was subjected to SDS-PAGE, as described above. The amount of protein chosen in the assay was on the linear part of the standard curve for each subtype previously perfomed in our lab (data not shown). Proteins in the resulting gel were transferred to nitrocellulose paper by high-voltage Mini Trans-Blot electrophoresis (BioRad). Prestained molecular-weight standards (mol. wt. 18,500–106,000 Da) were used to confirm adequate transfer. Immunolabeling of the G proteins on the nitrocellulose paper was performed using rabbit polyclonal antisera (Dupont-NEN) directed against the α-subunit of G_o, $G_{i1,2}$ (both 1:1000), and G_{i3} (1:300) and ^{125}I-labeled goat anti-rabbit immunoglobulin (Ig)G (1000 cpm/μl; New England Nuclear). Both the blocking and incubation buffers contained 5% nonfat dry milk (NFDM; BioRad), 0.005% Tween-20, 0.02% Na-azide, 2 mM $CaCl_2$, 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. Data are expressed as a percentage of control (AU), which was considered to be a 100% signal. Densitometry data were analyzed by two-tailed unpaired *t*-test. Data were considered to be statistically significantly different when $p < 0.05$.

cAMP Content

Two different methods were used to determine cAMP levels.

Protein Kinase Method. Animals were decapitated following 30 s exposure to CO_2, 20 min after an acute IP injection of dexmedetomidine (250 μg/kg) or saline. The tissues were prepared according to Gilman ([11]). Briefly, the LC was removed from each side of the freshly harvested brain using the punch technique. Punches from 2-mm brain slices at the dorsal-ventral location of the LC were obtained. Brains were sliced fresh and the LCs removed over an ice-cold glass plate using an 0.8-mm-bore glass pipette. All further manipulations were performed at 4°C. Two or four LC punches per sample (i.e., from both sides of one or two rats) were sonicated in 0.3 ml of ice-cold 5% trichloracetic acid. The disrupted tissue was centrifuged at 12,000 × *g* at 4°C for 20 min. The supernatant was transferred to tubes containing 25 μl of HCl 1 M and extracted with 0.75 ml ether, three times. The ether phase was discarded and the aqueous solution evaporated under a stream of N_2 at 70°C. The extract was stored at −20°C overnight. On the following day, the extract was dissolved in 110 μl of sodium acetate buffer, 100 mM, pH 4.5, at 4°C. Samples were divided into two aliquots (50 μl each). Into each aliquot, 25 μl of ^{3}H-cAMP was added, resulting in a final concentration of 5 nM/tube. Binding was initiated following addition of 25 μl of protein kinase (5 μg; Sigma cat. no. P5511) and was maintained for 2 h at 4°C. The final incubation volume was 100 μl and the final concentration of the sodium acetate buffer was 50 mM.

The incubation was terminated by adding 0.5 ml of ice-cold potassium phosphate buffer, 20 mM, pH 6. The samples were rapidly filtered through Whatman GF/B filters prewetted with the potassium phosphate buffer. Filters were washed three times. The ^{3}H-cAMP content retained on the filter was determined by liquid scintillation spectroscopy. Nonspecific binding, determined by adding 2 mM cAMP to the assay, was subtracted from each value. The linear range was 1–32 pmol/assay tube. The cAMP levels were correlated to amount of protein (picomole cAMP per milligram of protein), determined by the method of Lowry (18). Data were analyzed by ANOVA and posthoc Fisher test, and were considered to be statistically significantly different when $p < 0.05$.

Enzyme Immunoassay Method. Two LC's per sample were harvested and extracted following identical IP injections exactly as described before, except halothane was used instead of CO_2 to anesthetize the animal before decapitation. The extracts were stored at −20°C overnight after extraction and then dissolved in assay buffer, provided by the Amersham kit (cyclic AMP Biotrak enzymeimmunoassay, cat. no. RPN 225; Amersham Corp., Arlington Heights, IL) which contained 50 mM acetate buffer, pH 5.8, 0.02% bovine serum albumin, and 0.005% thimerosal. Briefly, the method is based on competition between cAMP in the sample and a fixed quantity of peroxidase-labelled cAMP for a limited (fixed) amount of binding sites on an antibody which is specific for cAMP. The bound antibody–peroxidase–cAMP "complex" is then firmly attached to a second antibody, which is precoated in the wells of a microtiter plate. All of the nonbound cAMP is then washed away with a washing procedure. The amount of peroxidase-labelled cAMP bound to the antibody is determined by adding a substrate called tetramethylbenzidine–hydrogen peroxide single-pot substrate (TMB) and finally by stopping this substrate–peroxidase reaction by adding H_2SO_4. The resulting color change is read in a microtiterplate spectrophotometer at 450 nm. The more cAMP that is present in the unknowned sample, the less peroxidase-labelled cAMP binds to the antibody. The assay was linear between 100 and 3200 fmol cAMP/well. The amount of tissue was adjusted to work witin this linear range. The cAMP levels were correlated to amount of protein (picomole cAMP per milligram protein), determined by the method of Lowry (18). Data were analyzed by ANOVA and posthoc Fisher test. Data were considered to be statistically significantly different when $p < 0.05$.

Adenylyl Cyclase Activity

LCs from eight rats per group were harvested, pooled, and homogenized in 80 mM Tris–maleate/2 mM $MgSO_4$/2 mM isobutylmethylxantine/0.2 mM EGTA buffer, pH 7.4 (referred to as buffer A). The homogenate was centrifuged at 30,000 × *g* for 20 min at 4°C and washed, and the resuspended pellet was incubated for 15 min at 37°C. The pellet was washed and resuspended in a final volume of 1.4 ml buffer A, and sonicated twice for 5 s on ice. To 100 μl of this membrane preparation, 25 μl 200 mM creatine-phosphate (final concentration = 20 mM), 25 μl 2 mg/ml creatine kinase (final concentration = 0.2 mM), and 25 μl 0.5 mM GTP (final concentration = 50 μM) were added.

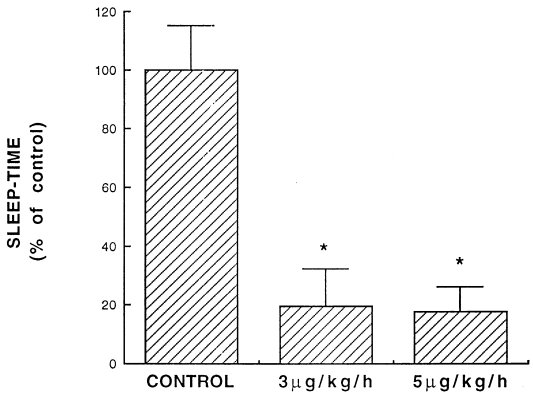


FIG. 1. Hypnotic response to dexmedetomidine in rats treated chronically with dexmedetomidine. Rats (n = 7–9) were implanted with osmotic minipumps set to deliver 3 or 5 μg/kg per h. The control group were sham-operated naive rats. On the 14th day, rats were administered dexmedetomidine, 100 μg/kg, IP, and the duration of LORR was measured and referred to as sleep-time as described in Methods. Data are expressed with respect to the control group, which was considered to be 100% responsive (mean ± SEM). Data were analyzed by analysis of variance and posthoc Fisher test where appropriate. *$p < 0.05$.

Seven different incubation media were prepared as follows: (a) ATP/buffer A/40% DMSO in buffer A ("basal"); (b) ATP/buffer A/forskolin in 40% DMSO–buffer A ("max"); and (c) and (d) ATP/Dex/forskolin in 40% DMSO–buffer A (Dex 10^{-12}–10^{-6} M).

In each medium the final concentrations of ATP and forskolin were 0.5 and 0.1 mM, respectively. Both the membrane preparation and incubation media were preincubated separately for 10 min at 30°C.

The incubation (final vol. = 250 μl) was initiated by adding 75 μl of each incubation medium to the membrane preparation and terminated after 5 min by adding 250 μl 100 mM sodium-acetate, pH 4.0, and thereafter boiling the samples for 3 min. The reaction mixture was pelleted and the supernatant was stored at −80°C. The cAMP content was assayed by the enzyme immunoassay (EIA) method described above. The assays were performed in duplicate. Data were analyzed by ANOVA and posthoc Fisher test. Data were considered to be statistically significantly different when p was < 0.05.

RESULTS

Plasma Dexmedetomidine

The osmotic minipump maintained a stable plasma level of dexmedetomidine ranging between 5.6 ± 0.6 nM and 4.8 ± 0.9 nM on days 2 and 7, respectively, during infusion of 5 μg/kg per h.

Behavioral Studies

Tolerance to the hypnotic properties of dexmedetomidine was evident after 14 days of dexmedetomidine infusion for 3 or 5 mg/kg per h (Fig. 1); this is similar to the findings which we reported previously following the 7-day exposure (27).

Receptor Binding Studies

As noted before, p-[^{125}I]iodoclonidine binds with high affinity to α_2 adrenoceptors. The B_{max} for this binding site was unaffected by chronic dexmedetomidine treatment for either 7 or 14 days (Table 1). However, the displacement curves (Fig. 2a and b) show that the affinity of the α_2-adrenergic receptor was significantly reduced in the tolerant animals (14 days). The calculated K_i values tended to be higher for both p-iodoclonidine and dexmedetomidine in the 7-day-tolerant rats and were significantly greater in rats after 14 days of dexmedetomidine treatment (Table 1).

G Proteins

Using antibodies against the α subunits of three species of PTX-sensitive G proteins ($G_{o\alpha}$, $G_{i\alpha 1,2}$, and $G_{i\alpha 3}$), we could detect no significant change in the amount of G proteins present in the LC in the tolerant animals after either 7 or 14 days of treatment (Table 2). In contrast, the function of PTX-sensitive G proteins was altered in the tolerant animals. After 14 days of dexmedetomidine treatment there was a significant reduction in the ability of PTX to ribosylate G proteins (Fig. 3A and B). A similar trend was present following 7 days of treatment, but this did not achieve statistical significance (Fig. 3).

Adenylyl Cyclase

Forskolin-stimulated adenylyl cyclase activity was less sensitive to inhibition by dexmedetomidine in the 7-day-tolerant rats (Fig. 4). The EC_{50} value for the control was 5.8×10^{-11} M (95% confidence limits of 3.9×10^{-12} to 8.5×10^{-10}) vs. 1.5×10^{-8} M (1.8×10^{-9} to 1.2×10^{-7} M), which represents

TABLE 1

COMPETITIVE RADIOLABELED LIGAND BINDING ASSAYS ON LOCUS COERULEUS MEMBRANE PREPARED FROM CONTROL 7- AND 14-DAY-TREATED RATS

Exposure Time	Control B_{max} (fmol/LC)	Tolerant B_{max} (fmol/LC)	Control K_i PIC (nM)	Tolerant K_i PIC (nM)	Control K_i DEX (nM)	Tolerant K_i DEX (nM)
7 days	2.89 ± 1.85	6.34 ± 3.07	0.79 ± 0.40	1.19 ± 0.50	0.13 ± 0.03	0.20 ± 0.09
14 days	3.27 ± 0.70	4.80 ± 0.78	0.63 ± 0.21	2.76 ± 0.48*	0.11 ± 0.03	0.48 ± 0.07*

Rats were treated with dexmedetomidine, 5 μg/kg per h or vehicle for 7 or 14 days via an osmotic pump. Rats were sacrificed, LC's from three rats were pooled, and membranes were prepared for radiolabeled ligand binding and displacement studies as described in Methods. PIC = para-iodoclonidine, DEX = dexmedetomidine. B_{max} is expressed as fentomoles per LC-punch (since the protein concentrations did not vary), and K_i in nanomoles (mean ± SEM). p-[^{125}I]iodoclonidine was used as the radiolabeled ligand and the B_{max} was calculated using p-iodoclonidine as the self-competitive ligand. Data were analyzed by using LIGAND analysis and Students t-test. *$p < 0.005$ (n = 3–4 assays).

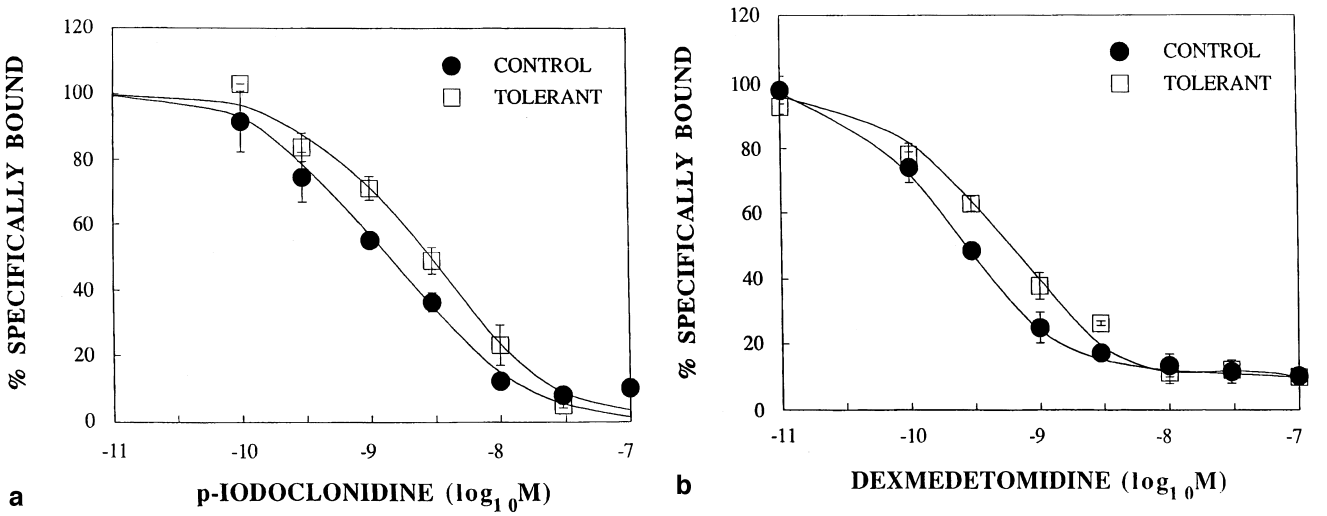


FIG. 2. (a) Displacement of *p*-[^{125}I]iodoclonidine by *p*-iodoclonidine. Radiolabeled ligand binding assays using LC membrane preparations from control and 14-day-tolerant rats were performed as described in Methods (n = 3 assays/point). (b) Displacement of *p*-[^{125}I]iodoclonidine by dexmedetomidine. Radiolabeled ligand binding assays using LC membrane preparations from control and 14-day-treated rats were performed as described in Methods (n = 3 assays/point).

a 250-fold decrease in sensitivity. Basal adenylyl cyclase activity was unaltered.

cAMP Content

At day 14, using both the protein kinase and EIA method, we demonstrated a significant decrease in the cAMP content in the LC following an acute IP injection of dexmedetomidine (250 μg/kg) (Fig. 5a and b) in both tolerant and naive states. While the basal cAMP level tended to be lower in the tolerant state, these data were not significantly different from the control.

DISCUSSION

After 14 days of dexmedetomidine treatment, rats become hyporesponsive to the hypnotic effects of this α_2 agonist. These data are qualitatively similar to those we previously reported in rats treated for 7 days (27). There was no change in α_2-adrenoceptor density; however, the affinity of the receptor for α_2 agonists was diminished in tolerant animals after 14 days of dexmedetomidine treatment. There was no significant alteration in the immunoreactive amount of various species of pertussis toxin-sensitive G proteins in the tolerant rats. However, the degree to which these G proteins could be ribosylated by in vitro pertussis toxin was significantly reduced in the tolerant animals. Forskolin-stimulated adenylyl cyclase was less responsive to the inhibitory effect of dexmedetomidine in the tolerant rats. Nevertheless, a supramaximal dose of dexmedetomidine was still able to reduce cAMP content. We interpret these data to indicate that prolonged treatment with the α_2 agonist may be capable of dissociating the α subunit of the pertussis toxin-sensitive G protein from its βγ dimer, resulting in conversion of the α_2 adrenoceptor to a lower-affinity state. The dysfunctional receptor–G protein complex is less capable of inhibiting adenylyl cyclase. An α_2 agonist signal can still be transduced in the tolerant rats, albeit at a very much higher dose of dexmedetomidine, which is characteristic of a desensitized state.

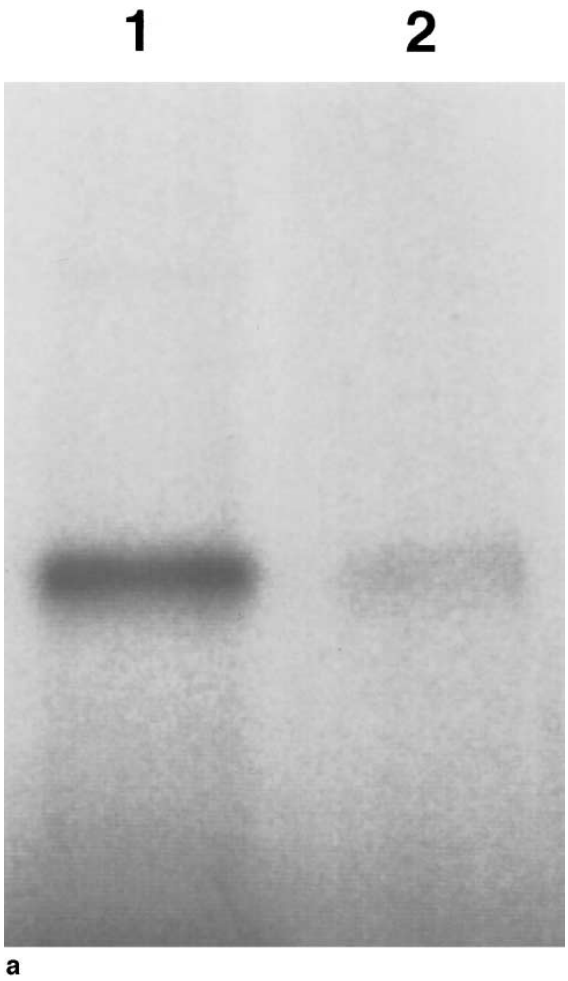
TABLE 2

QUANTIATION OF PTX-SENSITIVE α SUBUNITS ON LOCUS COERULEUS MEMBRANE PREPARED FROM 7- AND 14-DAY TREATED RATS

Exposure Time	G_0 (% control)	$G_{i\alpha 1,2}$ (% control)	$G_{i\alpha 3}$ (% control)
7 days	89 ± 14	102 ± 20	89 ± 24
14 days	98 ± 9.5	124 ± 19	90 ± 13

Rats were treated with dexmedetomidine, 3 μ/kg per h, or vehicle for 7 or 14 days via an osmotic pump. Rats were sacrificed, membrane protein from the LC was harvested, and α subunits of the G proteins were tested by immunoblotting as described in Methods. Data presented here were normalized by the densitometry as a percentage of the relevant control absorbance values (AU), which was considered to be 100%. Data are presented as mean ± SEM. Data were analyzed by ANOVA and posthoc Fisher test where appropriated. *$p < 0.05$ (n = 6–9).

p-[^{125}I]Iodoclonidine exhibits high affinity for α_2 adrenoceptors (10) and has the highest specific activity of any available α_2-adrenergic ligand. This latter feature was particularly useful in our study, because the LC is a small structure. Although *p*[^{125}I]iodoclonidine does exhibit some binding to the imidazoline-preferring receptor, this class of receptor is not thought to be present in the LC because of its functional insensitivity to rilmenidine (4). Using both dexmedetomidine, the α_2 agonist used to develop tolerance, and *p*-iodoclonidine, the "cold" analog of the radiolabeled ligand, we were able to demonstrate a significant decrease in affinity of both ligands for the α_2 adrenoceptors in the LC of 14-day-tolerant rats. Qualitatively similar data were seen in the 7-day-tolerant rats, although this did not achieve statistical significance. It is notable that there was no downregulation in receptor density (B_{max}). Although receptor downregulation is a cornerstone in the development of tolerance for the other adrenergic systems, this does not occur for many cell types that express α_2A adrenoceptors, including platelets (19), fat cells (26), or a cell culture line



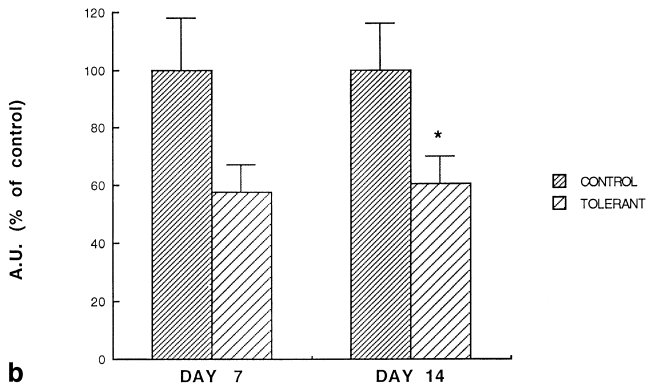


FIG. 3. (a) Representative autoradiogram of ^{32}P-labeled α subunit of pertussis toxin-sensitive G proteins from naive (lane 1) or tolerant (lane 2) animals. LC's from rats were extracted and treated with PTX in the presence of ^{32}P-labeled ADP. The same amount of protein was applied and separated on SDS-PAGE (see Methods). (b) In vitro ribosylation of G proteins in 7- and 14-day-tolerant rats. Rats were treated with dexmedetomidine, 3 μg/kg per h, or vehicle for 7 or 14 days via an osmotic pump. Rats were sacrificed and membranes prepared from the LC and ribosylated in vitro with ^{32}P-labeled NAD in the presence of activated PTX, 2 μg. The samples were subjected to SDS-PAGE electrophoresis and autoradiography was performed with subsequent densitometry analysis as described in Methods. Data (mean ± SEM) presented here were normalized by expressing the densitometry as a percentage of the relevant control absorbance values (AU), which was considered to be 100%. Data were analyzed by ANOVA and post-hoc Fisher test where appropriate. *$p < 0.05$ (n = 7–16).

(16), which are chronically exposed to an α_2 agonist. However, downregulation does occur in NG 108-15 chronically exposed to epinephrine (32); it is notable that in this cell line only α_2B adrenoceptors are present. It has recently been suggested that only α_2A adrenoceptors are present in the LC (30). It may be inferred from these earlier studies that downregulation occurs for some but not all α_2-adrenoceptor subtypes. Receptor downregulation is thought to be a consequence of phosphorylation through either cAMP-dependent protein kinase or β-adrenergic receptor kinase (βARK) (2). The human platelet α_2-adrenergic receptor has been shown to be a substrate for βARK (3), but in contrast to the β_2 adrenoceptor, the region of the receptor undergoing phosphorylation is located in the third intracellular loop and not in the carboxy-terminal tail of the protein (25). Furthermore, the α_2-adrenergic receptors lack consensus sequences for cAMP-dependent, PKA-mediated phosphorylation.

α_2-adrenoceptors are coupled to pertussis toxin-sensitive G proteins (14). With the α_2-adrenergic receptor binding site unoccupied, the G protein exists with GDP tightly bound to its α subunit, promoting a high-affinity binding state in the receptor. When an agonist molecule occupies the receptor-binding site, the receptor is thought to undergo a conformational change that promotes the dissociation of GDP from the α subunit of the G protein, whereby coupling to the receptor can occur. GTP instantaneously occupies the site formerly occupied by GDP, causing the receptor to lower its affinity for the bound agonist, which now dissociates. Also, the GTP-bound α subunit dissociates from the βγ dimer, allowing it to activate the effector molecule. Termination of signal transmission results from the hydrolysis of bound GTP through the intrinsic hydrolase activity of the α subunit. Based on the k_{cat} values of the hydrolase activity, the lifetime of the activated α subunit is several seconds, which allows for considerable amplification of the signal (15). An alternative theory proposes that the free βγ dimer and not the GTP-bound α subunit is the mechanism for propagating the signal to the effector mechanism (17). In either event, if the amount of G protein is decreased or if the receptor is not capable of coupling to the G protein, the agonist signal cannot be propagated into a biologic response.

Of the pertussis toxin-sensitive G proteins, $G_{\alpha o}$ is the major G protein in the mammalian brain, where it constitutes up to 1% of membrane protein. The $G_{\alpha i}$ family is composed of at least three closely related α subunits (αi1–3) that are encoded by different genes although the functional significance of this microheterogeneity is not known (22). Other PTX-sensitive G proteins (e.g., $G_{\alpha k}$) are less well characterized and were not investigated in this study because of the lack of appropriate reagents. Western blot studies did not reveal any change in the amount of the most prevalent species of pertussis toxin-sensitive G proteins, G_o and G_{i1-3}. These data corroborate the

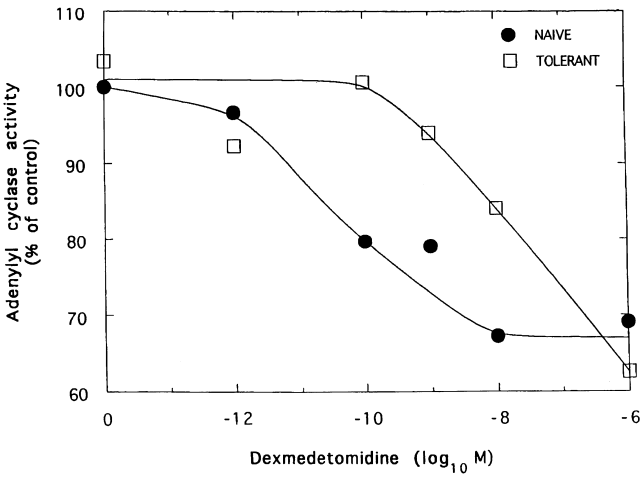


FIG. 4. Inhibition of forskolin-stimulated adenylyl cyclase by dexmedetomidine, 10^{-12}–10^{-6} M, in LC membrane preparations from control and tolerant rats. Rats (n = 8/group) were implanted with osmotic minipumps set to deliver 5 μg/kg per h for 7 days. Locus coeruleus were harvested and membranes prepared and analyzed for adenylyl cyclase on the same day, as described in Methods. Adenylyl cyclase was stimulated with forskolin, 100 μM. Each point represents the mean of two measurements from one LC membrane preparation originating from eight rats. Data were analyzed by ANOVA and posthoc Fisher test where appropriate. *$p < 0.05$ compared with the naive membranes performed without dexmedetomidine.

in vivo studies of Nestler et al. (23) but are at variance with studies of α_2 adrenoceptors in transfected cells (16) and for other inhibitory neurotransmitter receptors in which both an increase (24) and a decrease (12) in G_i have been noted. Because we did not analyze all the species of PTX-sensitive G proteins, there may be a reduction in the amount of PTX-sensitive G proteins in the LC of tolerant rats which may have gone undetected in our study.

One method of testing whether the G protein is coupled to the receptor is to determine whether the α subunit is associated with its βγ dimer. Because ADP ribosylation can occur only if the G protein is in the fully associated trimeric state (14), the extent to which the α subunit is ribosylated by pertussis toxin indirectly measures the degree to which the α_2 adrenoceptor is able to couple to the G protein. Our data show that ADP ribosylation of G proteins by pertussis toxin ex vivo was significantly reduced in the LC of 14-day-tolerant animals. This is probably not due to a lesser amount of substrate available, as Western blots revealed no difference in the major species of PTX-sensitive G proteins. Our data suggest that in the tolerant animals, pertussis toxin-sensitive G protein is not amenable to ribosylation, possibly because it is not in the trimeric, and fully associated, form. Recently, endogenous ribosylase activity has been demonstrated (9). The mode of regulation of ribosylase activity has not been reported, and thus its relationship to the development of tolerance is not known. It is possible that an increase in endogenous ribosylase activity may ribosylate the G proteins in vivo, rendering it less ribosylatable ex vivo. Functionally, an in vivo ribosylated G protein would not be capable of propagating the signal from the receptor to the effector.

Whatever the mechanism for the dysfunctional coupling between receptor and effector, our data clearly demonstrate that forskolin-stimulated adenylyl cyclase in the LC of tolerant animals is more than two orders of magnitude less sensitive to inhibition by the α_2 agonist compared with the naive rats. Both basal and forskolin-stimulated adenylyl cyclase activity was unaltered, which differs from the findings by Nestler et al. (23), who showed a modest (±15%) increase in forskolin-stimulated adenylyl cyclase activity in the LC following a 2-week period of treatment with the α_2-adrenergic agonist clonidine. Unlike dexmedetomidine, clonidine is a partial agonist with lower selectivity for the α_2 vs. α_1 adrenoceptor and is

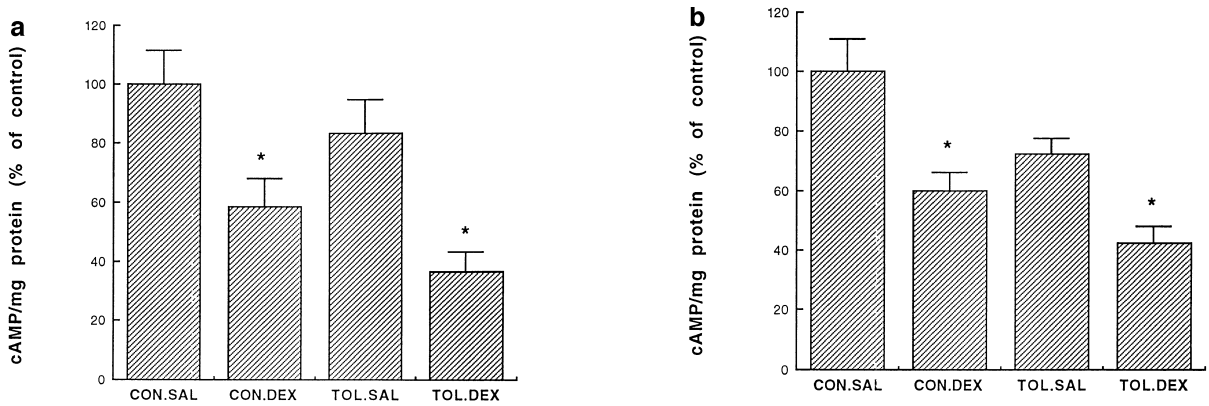


FIG. 5. (a) cAMP content in the LC of 14-day-tolerant rats. Rats were treated with dexmedetomidine, 5 μg/kg per h, or vehicle for 14 days via an osmotic pump. Following an acute IP injection of dexmedetomidine (250 μg/kg), rats were sacrificed and LC's were harvested and treated as described in Methods (protein kinase method). Data (picomoles cAMP per milligrams protein), are expressed as a percentage of control, which was considered to be 100% responsive (mean ± SEM). Data were analyzed by ANOVA and posthoc Fisher test where appropriate. *$p < 0.05$ (n = 4–13). (b) cAMP content in the LC of 14-day-tolerant rats. Rats were treated with dexmedetomidine, 5 μg/kg per h, or vehicle for 14 days via an osmotic pump. After an acute IP injection of dexmedetomidine (250 μg/kg), rats were sacrificed and LC's were harvested and treated as described in Methods (enzyme immunoassay method). Data (picomoles cAMP per milligram protein) are expressed as a percentage of control, which was considered to be 100% responsive (mean ± SEM). Data were analyzed by ANOVA and posthoc Fisher test where appropriate. *$p < 0.05$ (n = 8–15). CON SAL: naive animal injected with saline; CON DEX: naive animal injected with dexmedetomidine; TOL SAL: tolerant animals injected with saline; TOL DEX: naive animal injected with dexmedetomidine.

less potent. It is not clear to what extent these differences contribute to the disparity in adenylyl cyclase results in the two studies. It is noteworthy that cAMP has been shown to upregulate transcription of the α_2-C10 receptor gene in cultured cells, constituting a biologically meaningful feedback loop between adenylyl cyclase activity and inhibitory receptor function (28). Figure 4 shows that even in the tolerant state, dexmedetomidine inhibits adenylyl cyclase to the same degree as was noted for naive animals, but at a much higher dose. These data are consistent with the finding that cAMP content in the LC can be significantly reduced after a large dose of dexmedetomidine, IP, in tolerant animals (Fig. 5a and b). There is a tendency toward lower basal cAMP content in the tolerant animals. This may be due to retained agonist, although this explanation appears unlikely, because the tissue is extensively washed. We previously demonstrated that such a large dose of dexmedetomidine results in a hypnotic response, albeit significantly lower than in naive animals (27).

Whether a cause-and-effect relationship exists between the lowered binding affinity of the α_2 adrenoceptor, the probable uncoupling of the G protein, the decrease in inhibition of adenylyl cyclase, and functional α_2 hyporesponsiveness in behavior has not yet been firmly established. The α_2 hyporesponsiveness is already present in the 7-day-tolerant animals (27) at a time when there are no significant changes in either the receptor or G protein function. In both cases, these cohorts display a qualitatively similar strong trend which may achieve statistical significance with a larger number of animals in each cohort. Also, the significant changes we saw in adenylyl cyclase activity was definitely present at day 7; therefore, we did not perform a second adenylyl cyclase activity experiment at day 14. Finally, we recently reported that tolerance develops to the hypnotic properties of α_2 agonists when relatively few receptors ($\pm$20%) are disrupted (13). This suggests that the receptor reserve is quite small, and therefore, pharmacologically meaningful alterations may be evident before there are statistically significant differences in the biochemical parameters of signal transduction.

In conclusion, the tolerance that develops to the hypnotic properties of dexmedetomidine, an α_2 agonist, may be due to a dysfunctional G protein that does not permit the signal to be transduced from the receptor to the effector pathway in the LC. The nature of the mechanism involved in creating a dysfunctional G protein remains unidentified.

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