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MOLECULAR PHARMACOLOGY, 42:971-981

Effects of Chemical and Surgical Sympathectomy on Expression of β-Adrenergic Receptors and Guanine Nucleotide-Binding Proteins in Rat Submandibular Glands

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Received May 6, 1992; Accepted September 11, 1992

SUMMARY

Expression of β_1-adrenergic receptors and guanine nucleotide-binding proteins in rat submandibular glands was determined after reserpine administration and sympathetic denervation. Pretreatment of rats with reserpine resulted in up-regulation of the density of β_1-adrenergic receptors and the immunoreactivity of the 64-kDa species of β_1-adrenergic receptor in submandibular membranes, by 2.6 ± 0.3-fold (eight experiments), within 7 days. Steady state levels of β_1-adrenergic receptor mRNA quantified by DNA-excess solution hybridization were 0.15 ± 0.03 amol of β_1-adrenergic receptor mRNA/μg of total cellular RNA (six experiments). β_1-Adrenergic receptor mRNA increased by 50% within 8 hr after pretreatment with reserpine. Maximal levels of 0.37 ± 0.04 amol of β_1-adrenergic receptor mRNA/μg of RNA were attained by 4 days and these levels were sustained for the next 3 days (six experiments). Northern blot hybridization also revealed a 3-fold increase in the 2.5-kilobase β_1-adrenergic receptor mRNA transcript, which was equivalent in magnitude to that determined by solution hybridization. Reserpine pretreatment also affected steady state levels of submandibular guanine nucleotide-binding proteins. Two immunoreactive forms of the α subunit of G_s, migrating as 42 kDa (major) and 50 kDa (minor), were detected in salivary membranes. The immunoreactivity of the 42-kDa species of $G_{s\alpha}$ declined by 50% after 7 days of continuous daily injections of reserpine. In contrast, steady state levels of $G_{i\alpha 2}$ (41 kDa), G_o (39 kDa), and $G_{\beta 2}$ (35 kDa) and their mRNAs in submandibular membranes were unaffected by reserpine pretreatment. The rate of β_1-adrenergic receptor gene transcription assessed by nuclear run-on transcription assay in nuclei of submandibular glands was not altered by reserpine pretreatment. However, reserpine had a dramatic effect on the half-life of β_1-adrenergic receptor mRNA in submandibular glands. The half-life of β_1-adrenergic receptor mRNA in control submandibular glands was 3.5 hr, whereas it increased to 8 hr in reserpine-pretreated glands. Reserpine-promoted stabilization of β_1-adrenergic receptor mRNA provides a mechanism for up-regulation of postjunctional β_1-adrenergic receptors in sympathetically innervated tissues.

G protein-linked signaling pathways are used by cells to respond to a variety of extracellular stimuli and to transduce these signals across the plasma membrane (1, 2). In salivary cells, for example, numerous G protein-linked receptors such as muscarinic, adrenergic, and peptidergic (substance P) receptors are involved in regulating salivary secretion and composition (3, 4). Each of these receptors utilizes a distinct G protein-linked system for transmembrane signaling. The neurotransmitter norepinephrine by itself is capable of activating multiple distinct pathways in salivary cells that include β-AR-mediated stimulation of adenylyl cyclase, α_2-AR-mediated inhibition of adenylyl cyclase, and α_1-AR-mediated stimulation of phospholipase C (5).

This work was supported in part by United States Public Health Service Grant R55-HL48169, the Tennessee Affiliate of the American Heart Association, and The University of Tennessee Physicians Foundation.

Numerous studies conducted *in vitro* have revealed the existence of cross-regulation among several G protein-linked pathways that are involved in transducing the signal of one agonist. Presynaptic activation of β-AR, which are coupled to stimulation of adenylyl cyclase, desensitized and down-regulated the stimulatory β-AR on the one hand and enhanced the signaling of inhibitory receptors on the other (6, 7). Because G proteins can interact with multiple receptors, it is, therefore, not surprising that distinct G protein-linked pathways can be controlled by regulating the levels of their G proteins (8). Persistent activation of β-AR resulted in up-regulation of steady state levels of $G_{i\alpha 2}$ and consequently enhanced the inhibition of adenylyl cyclase by somatostatin, whose receptor is negatively coupled to cyclase via $G_{i\alpha 2}$ (7). Likewise, persistent activation of the inhibitory pathway of adenylyl cyclase increased the expression of β_2-AR and decreased steady state levels of $G_{i\alpha 2}$

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; β-AR, β-adrenergic receptor(s); SSC, standard saline citrate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICYP, iodocyanopindolol; A-R, adenosine receptor(s); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; kb, kilobase(s); α-AR, α-adrenergic receptor(s).

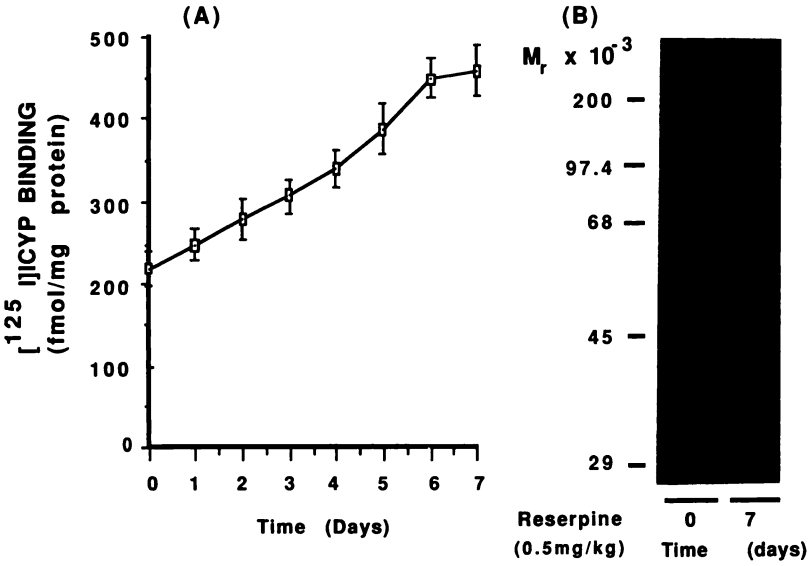

Fig. 1. Effect of reserpine pretreatment on β_1-AR in submandibular membranes. Rats were pretreated with reserpine (0.5 mg/kg/day) or diluent, and submandibular membranes were prepared at the times indicated (A and B). A, Maximal specific binding (B_{max}) of [^{125}I]ICYP, in fmol/mg of submandibular membrane protein, after 0–7 days of reserpine administration. These data are the mean ± standard error of pooled data from eight separate experiments, each in triplicate, that were analyzed simultaneously. The apparent affinity (K_d) of [^{125}I]ICYP for β_1-AR in submandibular membranes prepared from control or reserpine-treated rats was 24 ± 4 pM ($p > 0.05$). B, Equal amounts of membrane protein (100 μg) from submandibular glands of rats pretreated with diluent or reserpine (0.5 mg/kg/day) for 7 days were subjected to immunoblotting and probed using a 1/100 dilution of antiserum (SB-03) directed against sequence 407–419 in human β_1-AR. The intensities of the 64-kDa species of β_1-AR were compared by densitometry. The data represent a typical experiment replicated four times.

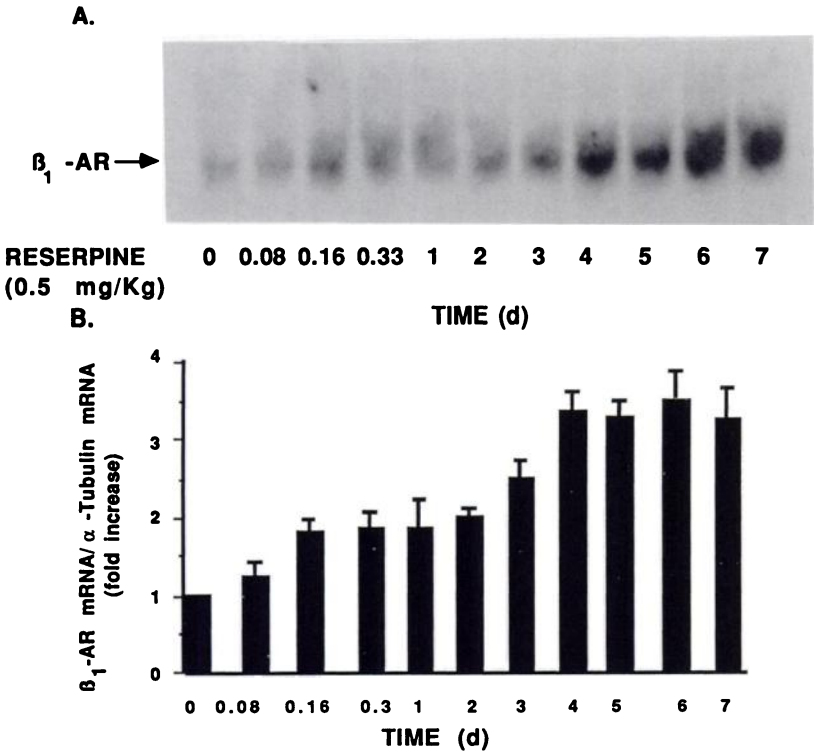

Fig. 2. Time-course of reserpine-induced increase in β_1-AR mRNA, with analysis by Northern blotting. RNA was isolated from submandibular glands of rats pretreated with either solvent or reserpine (0.5 mg/kg/day) for the time periods indicated. A, Total cellular RNA (50 μg) was subjected to RNA blot analysis and probed with ^{32}P-labeled 0.9-kb *Pst*I fragment of rat β_1-AR cDNA, as outlined in Materials and Methods. The autoradiogram is a 2-day exposure with one intensifying screen. The blot was stripped of the first probe and then probed with ^{32}P-labeled 1.1-kb *Pst*I fragment of α-tubulin cDNA. Each lane of the blot was probed by densitometry, and the absorbance of β_1-AR and α-tubulin bands was calculated. B, Average ± standard error fold increase in β_1-AR mRNA relative to α-tubulin mRNA in each lane, as determined from the ratio of their absorbances and expressed in arbitrary units. The mean ± standard error was calculated from three different experiments in which the duration of reserpine treatment was identical.

(9). Thus, there apparently are at least two levels of cross-regulation, one operating at the level of receptors and the other at the level of transducing G proteins, particularly $G_{i\alpha 2}$, which appears to be a key modulator of cross-regulation among distinct classes of G protein-linked receptors (9).

Previous studies conducted *in vivo* in rat submandibular glands also revealed cross-regulation among G protein-coupled receptors. Chemical depletion of presynaptic norepinephrine stores by reserpine resulted in a marked increase in all adrenergic receptors present in salivary glands (5). On the other hand, the densities of muscarinic receptors and of substance P receptors, which interact with different neurotransmitters, were unaltered (5, 10). Thus, the effect of exposure to or depletion of a particular agonist on steady state levels of G protein-linked receptors that interact with this particular agonist appears to be similar both *in vitro* and *in vivo*.

The knowledge accumulating to date from *in vitro* studies has highlighted multiple mechanisms as potential contributors to cross-regulation of distinct receptor pathways (6, 7, 9). However, when excitatory and inhibitory receptors that share the same agonist and effector are activated by the common agonist, signaling from both systems is simultaneously engaged. In this instance, the effect of engaging both of these pathways on steady state expression of their receptors and G proteins is unpredictable. The final outcome may depend on the abundance of receptors and G proteins and the efficacy of their coupling to the effector and to one another. The sympathectomized rat submandibular gland was used to explore the effect

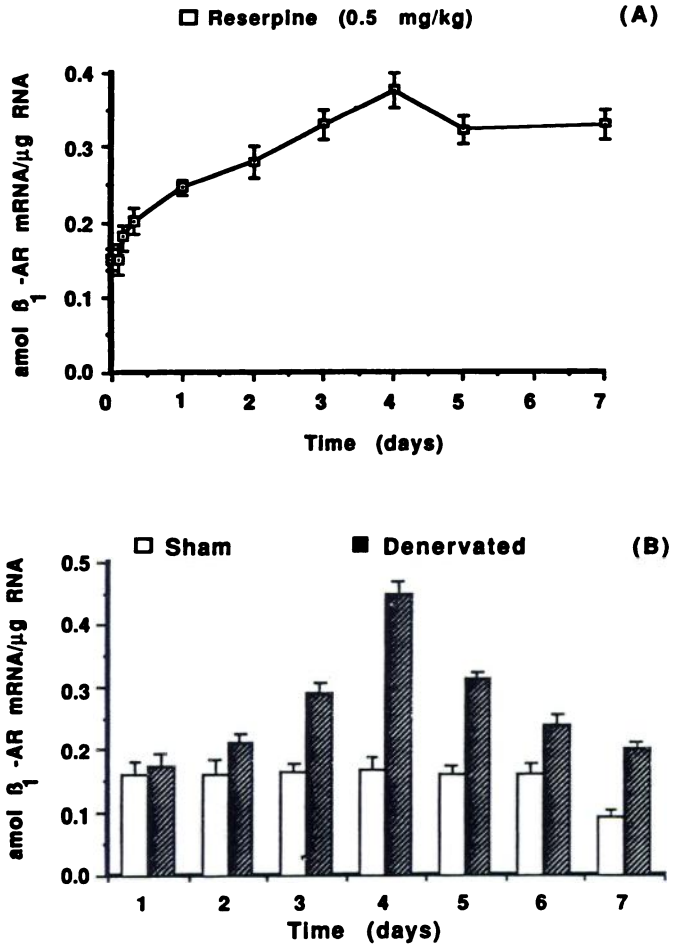

Fig. 3. Time course of reserpine effect on steady state β_1-AR mRNA in submandibular glands, with analysis by DNA-excess solution hybridization. A, ^{32}P-labeled, single-strand β_1-AR probe (100 pg) was incubated with total cellular RNA (100 μg), extracted from submandibular glands of rats pretreated with reserpine (0.5 mg/kg/day), and template DNA (0–48 amol) complementary to the probe. The number of amol of β_1-AR mRNA/μg of RNA was calculated from the amount of ^{32}P incorporated into S1 nuclease-resistant hybrids of probe and RNA. Each time point represents the mean ± standard error of at least six determinations performed in duplicate. B, Unilateral superior cervical ganglionectomy was performed on rats, and RNA was extracted postoperatively from sham-operated or denervated submandibular glands at the times indicated. β_1-AR mRNA in 100 μg of total cellular RNA was measured as outlined above. Each time point represents the mean ± standard error of two determinations performed in triplicate.

of depleting a common agonist on the expression of those receptors and G proteins involved in signaling. In these studies, the effect of depleting norepinephrine, which interacts with β_1- and α_2-AR to stimulate and inhibit adenylyl cyclase, respectively, on the expression of submandibular β-AR and G proteins was determined. The involvement of these changes in cross-regulation of distinct G protein-coupled receptor pathways will be discussed.

Materials and Methods

Animal treatment and the preparation of salivary membranes. Male Sprague-Dawley rats (225–250 g) received a daily intraperitoneal injection of buffer (2% acetic acid, 5% polyethylene glycol in water) or reserpine (0.5 mg/kg) for the duration indicated in the figure legends. Male Sprague-Dawley rats with unilateral superior cervical ganglionectomy were prepared in the laboratory and used as outlined. At the times indicated in the figure legends, submandibular glands were rapidly removed, separated from the adjoining sublingual glands, weighed, and then used for the preparation of membranes or RNA. For the preparation of membranes, salivary glands were suspended in 20 volumes of ice-cold 50 mM Tris·HCl (pH 7.4) containing the protease inhibitors leupeptin (10 μg/ml), aprotinin (10 μg/ml), and phenylmethylsulfonyl fluoride (1 mM). The glands were homogenized with a Brinkman homogenizer at setting 7 for 20 sec, and this homogenate was centrifuged for 10 min at 50,000 × *g*. The pellet was resuspended in the aforementioned solution and centrifuged again. The pellet, consisting of a crude particulate fraction, was then resuspended in 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, with protease inhibitors (10). Protein concentration was determined by the method of Bradford (11) and the membranes were used in the subsequent experiments without delay.

TABLE I

Effect of pretreatment with reserpine on adenylyl cyclase activity in rat submandibular membranes

Rats were injected with 0.5 mg/kg/day reserpine, and submandibular gland membranes were prepared after 0, 1, 4, or 7 days of continuous daily injections of reserpine. Adenylyl cyclase activity was determined in 100 μg of membranes using GTP (0.1 mM) or increasing concentrations of isoproterenol ranging from 1 nM to 1 mM in the presence of 0.1 mM GTP, as outlined in Materials and Methods. The data represent mean ± standard error of three determinations, each performed in triplicate.

Duration of reserpine treatment	Basal activity of adenylyl cyclase	GTP-stimulated adenylyl cyclase	Maximal isoproterenol-stimulated adenylyl cyclase	EC_{50} of isoproterenol
days	*pmol/min/mg*	*pmol/min/mg*	*pmol/min/mg*	*μM*
0	24 ± 5	53 ± 3	110 ± 9	0.15 ± 0.06
1	22 ± 4	58 ± 3	123 ± 7	0.10 ± 0.05
4	19 ± 7	52 ± 4	112 ± 6	0.20 ± 0.06
7	20 ± 5	46 ± 6	104 ± 5	0.22 ± 0.05

Preparation of salivary RNA and Northern blot hybridization. RNA was prepared by suspending the submandibular glands in 4 M guanidine thiocyanate, 2% sarkosyl, 50 mM Tris·HCl (pH 7.5), 10 mM EDTA, 1% mercaptoethanol, followed by homogenization with a Polytron and preparation of RNA by the method of Chirgwin *et al.* (12). Total cellular RNA was quantified spectrophotometrically, aliquoted, and stored at −80° until use. Total cellular RNA was subjected to gel electrophoresis on 1.2% agarose-3% formaldehyde gels (13). The RNA was electroblotted from the gel to nylon membranes overnight in 25 mM sodium phosphate, pH 6.5, followed by UV irradiation to fix the RNA to the blot. The blot was prehybridized in 50% formamide, 5× SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0), 5% SDS, 2× Denhardt's solution, 250 μg/ml sheared salmon sperm DNA, at 42° for 6 hr. After prehybridization, the blot was incubated in prehybridization solution containing [^{32}P]dCTP-radiolabeled probe (2 × 10^6 cpm/ml) for 16 hr at 42°. The blot was washed twice for 5 min in 2× SSC/0.5% SDS at 25° and twice for 15 min in 0.2× SSC/0.5% SDS at 55°, covered with Saran wrap, and subjected to autoradiography (14). To determine minor differences in RNA transfers between the lanes, the blot was then stripped of radioactivity as recommended by the manufacturer and rehybridized with ^{32}P-labeled *Pst*I fragment of α-tubulin cDNA plasmid pT1 (15). The cDNA probes for G proteins and β_1-AR were derived as follows: the β_1-AR probe was a 0.9-kb *Pst*I fragment of the β_1-AR cDNA (16); the $G_{s\alpha}$ probe was a 1.85-kb *Eco*RI fragment of G2 cDNA, which encodes for the larger form of $G_{s\alpha}$; the $G_{i\alpha 2}$ probe was a 2.3-kb *Eco*RI fragment of G18 cDNA; and the G_o probe was a 2.3-kb *Eco*RI fragment of G31 cDNA (17). The G protein cDNAs were provided by Randall R. Reed, Howard Hughes Medical Institute, Johns Hopkins University School of Medicine (Baltimore, MD), and the β_1-AR cDNA was provided by Curtis Machida, Division of Neuroscience, Oregon Regional Primate Research Center (Beaverton, OR).

DNA-excess solution hybridization assays. Preparation of β_1-

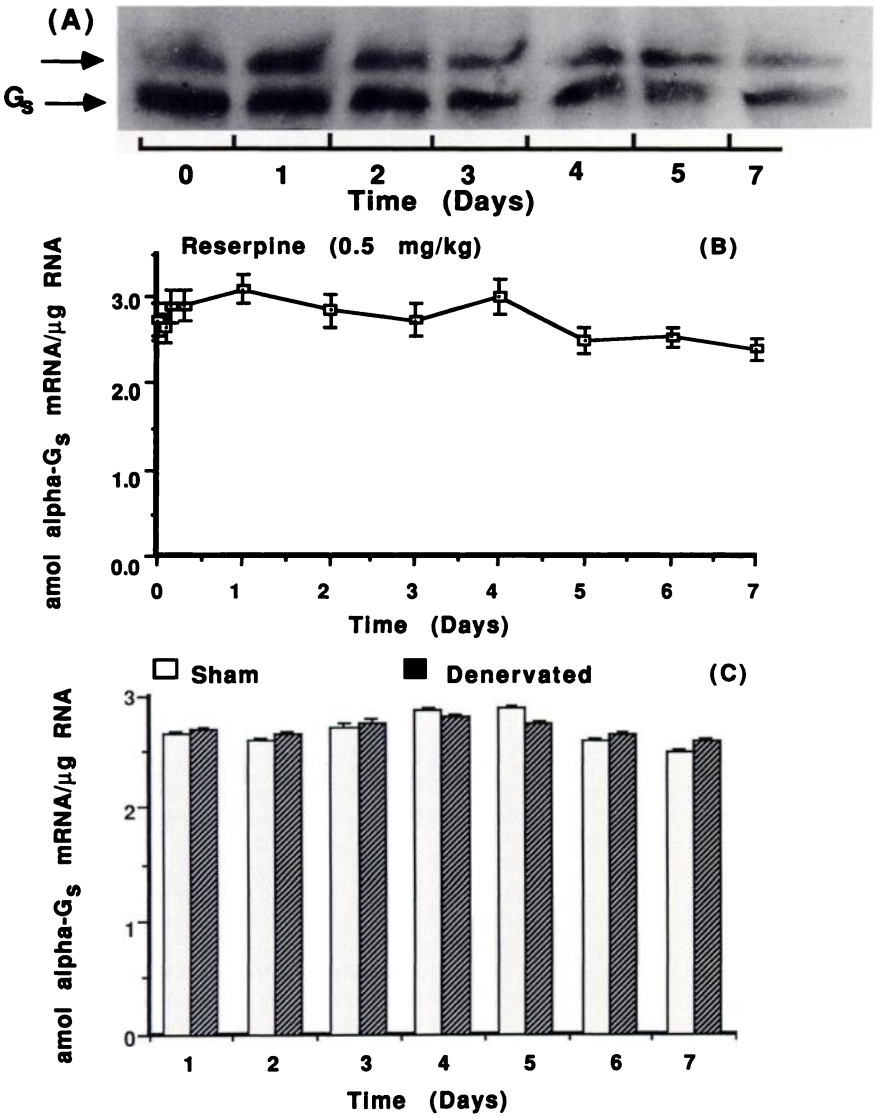

Fig. 4. Effect of sympathectomy on $G_{s\alpha}$ protein and mRNA levels in rat submandibular glands. A and B, Rats were pretreated for the indicated time periods with reserpine (0.5 mg/kg/day) and membranes were prepared from one submandibular gland, whereas RNA was extracted from the other. A, Immunoblot analysis using a 1/200 dilution of the anti-$G_{s\alpha}$ antiserum SB-07 reveals the immune complexes of $G_{s\alpha}$/antibody SB-07 made visible with the second antibody, ^{125}I-labeled goat anti-rabbit IgG. The *arrows* refer to $G_{s\alpha}$ migrating as 50 kDa (*top*) and 42 kDa (*bottom*). B, $G_{s\alpha}$ mRNA levels in 25 μg of total cellular submandibular gland RNA were determined by DNA-excess solution hybridization. Each time point represents the mean ± standard error of four determinations, each performed in duplicate. C, $G_{s\alpha}$ mRNA levels in RNA prepared from submandibular glands after unilateral superior ganglionectomy were determined by DNA-excess solution hybridization. The data represent the mean ± standard error of two determinations, each performed in triplicate.

AR and the other probes specific for each subunit of G proteins was described in previous publications (18, 19). These probes were provided by Craig Malbon, State University of New York at Stony Brook. Uniformly radiolabeled (100 pg) probe was incubated alone, with known amounts of template DNA (used as the standard), and with total cellular RNA (50–150 μg) in 20 mM HEPES (pH 7.0), 0.3 M NaCl, 1 mM EDTA, 100 μg/ml denatured salmon sperm DNA, for 60 hr at 68°. S1 endonuclease buffer (0.28 M NaCl, 4.5 mM $ZnSO_4$, 50 mM sodium acetate, pH 4.5) was then added to each sample and the mixture was incubated for 90 min at 42°. The S1 nuclease-resistant hybrids were precipitated by trichloroacetic acid and collected by vacuum filtration on Whatman GF/C filters (20).

Immunoblot analysis of receptors and G proteins in salivary membranes. Antisera to peptides corresponding to sequences 384–394 of rat $G_{s\alpha}$ (SB-07), 346–355 of rat $G_{i\alpha 2}$ (SB-04), 345–354 of rat G_o (CM-140), 25–39 of $G_{\beta 1}$ (CM-133), 25–39 of $G_{\beta 2}$ (CM-162), and 407–419 of human β_1-AR (SB-03) were generated and used to measure steady state levels of receptor and G proteins on immunoblots (18, 21–22). Antisera denoted CM were kindly provided by Craig Malbon, State University of New York at Stony Brook. Immunoblot analyses were performed on 100 μg of freshly prepared crude membranes from submandibular glands of rats injected with buffer or reserpine. Membranes were solubilized and chemically reduced by incubation in 2× Laemmli sample buffer (23) (0.125 M Tris·HCl, pH 6.8, 4% SDS, 5% sucrose, 0.03% bromophenol blue) containing 20 mM dithiothreitol for 30 min at 37°, followed by alkylation with excess 2-iodoacetamide. The solubilized proteins were subjected to electrophoresis on 11% acrylamide gels containing 0.1% SDS and the separated proteins were transferred electophoretically to nitrocellulose. The nitrocellulose blot was incubated in 10% albumin in phosphate-buffered saline for 30 min at room temperature, rinsed with water, and incubated with rabbit antiserum directed against the various G protein or receptor sequences. The sera were diluted in 0.3% (v/v) Tween 20 in phosphate-buffered saline as indicated in the figure legends and the blot was incubated for 2 hr at 37°. The blot was washed and incubated with goat anti-rabbit antibody conjugated to calf alkaline phosphatase and with 10^6 cpm/ml ^{125}I-labeled goat anti-rabbit IgG, to localize the immunoreactive bands by the chromogenic phosphatase precipitate and to quantitate the immunoreactive bands by autoradiography (24), respectively.

Nuclear run-on transcription assays. Salivary glands were minced with fine scissors and suspended at a concentration of 50 mg of wet weight/ml of ice-cold RNase-free AT buffer (10 mM Tris·HCl, pH 8, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.3 M sucrose, 0.15% Triton X-100) with a sterile glass-Teflon homogenizer (25). The tissue was homogenized with 20 strokes and then layered over 0.5 volume of 0.4 M sucrose in AT buffer. The homogenate was centrifuged at 2500 × *g* for 10 min at 4° and the nuclear pellet was gently resuspended in an equal volume of 0.3 M AT buffer and centrifuged at

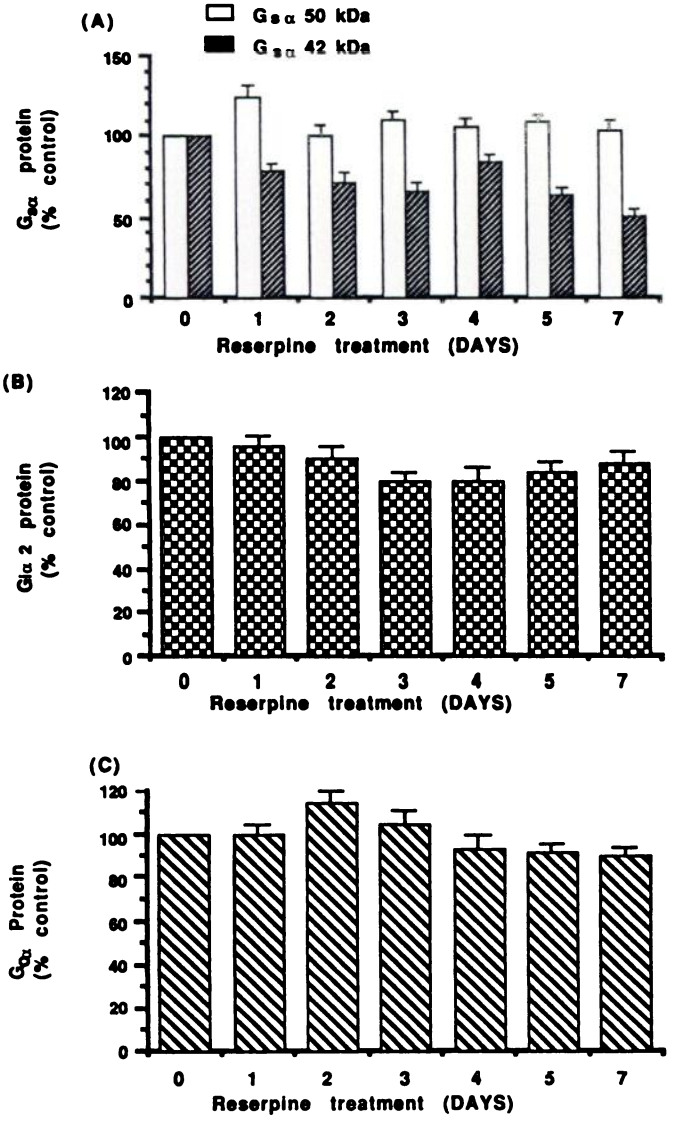

Fig. 5. Measurement of G protein α subunits by quantitative immunoblotting. Autoradiographic intensities of each immunoreactive band in Figs. 4, 6, and 7 were measured by scanning densitometry. The data from an additional two experiments performed in a manner identical to that in Figs. 4, 6, and 7 were similarly scanned. G protein intensities are expressed as percentage of the values obtained from submandibular membranes of rats that were not treated with reserpine. Data are presented as the mean ± standard error of determinations on three groups of submandibular glands obtained from rats treated with reserpine, as indicated in the *abscissa*.

2500 × *g* for 10 min at 4°. The nuclei were resuspended in nucleotide-free transcription reaction mixture composed of 10 mM Tris·HCl, (pH 8.0), 5 mM $MgCl_2$, 0.3 M KCl, 5 mM dithiothreitol, and 20% glycerol and were counted. An equal number of submandibular nuclei (5×10^7/assay) from control and reserpine-treated animals were aliquoted, 10 mM levels of unlabeled GTP, ATP, and CTP and 15 μl of [α-^{32}P]UTP (3000 Ci/mmol) were added, and the mixture was incubated at 30° for 30 min. Newly transcribed RNA was extracted (26) and incubated for 36 hr at 65° with plasmid DNA immobilized on Nytran membranes. After hybridization, each sample was washed two times with 2× SCC for 60 min at 65°. The samples were then treated with DNase-free RNase A (10 μg/ml in 2× SSC) for 30 min at 37°, followed by a wash in 2× SSC at 37° for 60 min. The blots were dried and subjected to autoradiography for 5 days with an intensifying screen.

Adenylyl cyclase assays. Submandibular gland membranes were prepared as outlined earlier, except that phenylmethylsulfonyl fluoride was omitted. Membranes (100 μg of protein) were incubated at 30° in a final volume of 0.1 ml containing 50 mM Tris·HCl (pH 7.4), 6 mM $MgCl_2$, 10 mM creatine phosphate, 1 mM cAMP, 2 mM 2-mercaptoethanol, 1 mg/ml bovine serum albumin, 0.4 mM EGTA, 2 mg/ml creatine kinase, 0.2 mM ATP containing 2 μCi of [α-^{32}P]ATP, and the indicated amounts of guanine nucleotides and isoproterenol. The assay was initiated by the addition of membranes and was terminated after 10 min (27). The cAMP formed was isolated and quantified according to the procedure of Salomon *et al.* (28). Assays were routinely performed in triplicate.

Radioligand binding assays. Submandibular membranes were prepared and suspended in a buffer composed of 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, and protease inhibitors. Binding assays were initiated by adding 10 μg of membranes to six different concentrations of [^{125}I]ICYP ranging from 1 to 100 pM in 200 μl of buffer. These assays were performed at 22° for 60 min, and the amounts of [^{125}I]ICYP bound to cell membranes were quantified after the incubation mixture was filtered on a single Whatman GF/C filter and the filter was washed with ice-cold 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$. Nonspecific binding was defined as [^{125}I]ICYP binding that was insensitive to competition by 1 μM (±)-propranolol (29). All receptor binding parameters were analyzed by the use of a computer operating a nonlinear, least-squares, curve-fitting procedure and the modified LIGAND program (Biosoft-Elsevier, Cambridge, England), to analyze the apparent affinity (K_d), maximal binding (B_{max}), and the Hill coefficient (n_H).

Results

Submandibular glands contain a homogeneous population of β_1-AR that are subject to up-regulation by chemical sympathectomy with reserpine or surgical sympathectomy through ablation of the superior cervical ganglion (5, 30–33). As outlined in Fig. 1A, measurement of β_1-AR by radioligand binding indicates a sustained increase in the density of β_1-AR in submandibular membranes after reserpine administration, from 220 ± 16 fmol/mg of protein to 460 ± 20 fmol/mg of protein, within 7 days (16). β_1-AR expression in submandibular membranes in response to reserpine administration was probed by immunoblotting techniques to provide an additional independent method for assessing the effects of reserpine at the protein level. The β_1-AR-specific antiserum (SB-03) immunoreacted with a 64-kDa protein in submandibular membranes (Fig. 1B). The apparent molecular mass of this protein was identical to that of purified rat β_1-AR (34). Immunoblots of membranes prepared from submandibular glands of rats injected with reserpine for 7 days revealed that the immunoreactivity of the 64-kDa species increased by 2.3 ± 0.2-fold (Fig. 1B). Thus, both radioligand binding and immunoblotting techniques revealed equivalent up-regulation of β_1-AR in salivary membranes of reserpine-treated rats.

The effect of sympathectomy on salivary β_1-AR mRNA levels was determined by Northern blotting and DNA-excess solution hybridization. The latter method, which provides high sensitivity, was used to obtain quantitative estimates of the effect of sympathectomy on salivary β_1-AR mRNA levels. The earliest detectable increase in β_1-AR mRNA levels occurred 8 hr after the administration of reserpine, and this increase was sustained and enlarged for several days after continuous daily injections of reserpine (Fig. 2A). The size of the mRNA transcript that increased after the administration of reserpine was 2.5 kb, which is identical to the size of rat β_1-AR mRNA (16). Maximal β_1-AR mRNA levels were attained within 4 days and were sustained for 7 days, the longest period studied. No appreciable

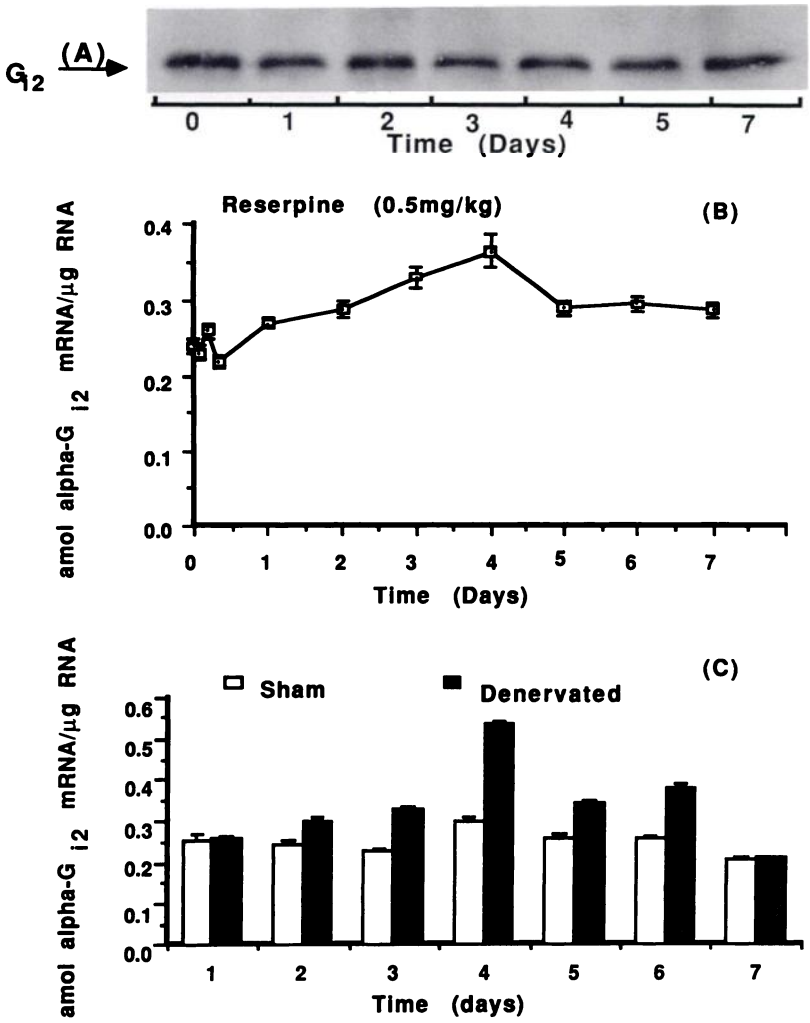

Fig. 6. Effect of sympathectomy on $G_{i\alpha2}$ protein and mRNA levels in rat submandibular glands. Membranes and RNA were prepared as outlined in the legend to Fig. 4. A, Immunoblot analysis of submandibular membranes that were probed using a 1/200 dilution of the anti-$G_{i\alpha2}$ antiserum SB-04. B, $G_{i\alpha2}$ mRNA levels in 50 μg of total cellular submandibular gland RNA were determined by DNA-excess solution hybridization. Each time point represents the mean ± standard error of four determinations, each performed in duplicate. C, $G_{i\alpha2}$ mRNA levels in RNA prepared from submandibular glands after unilateral superior ganglionectomy were determined by DNA-excess solution hybridization. The data represent the mean ± standard error of two determinations, each performed in triplicate.

change in α-tubulin (1.6-kb) mRNA levels occurred throughout the experimental period (data not shown). The magnitude of the increase of β_1-AR mRNA relative to α-tubulin mRNA was computed from densitometric tracing of the autoradiogram (Fig. 2B). The data reveal that β_1-AR mRNA increased by 1.5-fold after 8–12 hr of reserpine administration; a maximal increase of 3-fold was established by day 4 and was sustained for the next 3 days.

The effects of reserpine or surgical denervation on steady state β_1-AR mRNA levels were also measured by the DNA-excess solution hybridization strategy to provide a quantitative assessment of the effects of sympathectomy on the mRNA. Steady state levels of β_1-AR mRNA in submandibular glands of untreated rats were 0.15 ± 0.03 amol of β_1-AR mRNA/μg of total cellular RNA (Fig. 3A). When rats were injected with reserpine, β_1-AR mRNA levels increased by about 60%, to 0.24 ± 0.02 amol/μg of RNA, within 8 hr. These levels steadily increased, and maximal levels of 0.37 ± 0.04 amol/μg of RNA were attained by day 4 and remained essentially unchanged for the next 3 days. Unilateral superior cervical ganglionectomy provides an additional approach to achieve sympathectomy and has an advantage in that the sham-operated gland serves as control for the denervated gland in the same animal. Measurement of β_1-AR mRNA levels in sham and denervated submandibular glands revealed that receptor mRNA levels steadily increased, by about 2–3-fold, after 4 days in the denervated gland (Fig. 3B). These levels eventually decreased to 2-fold above those in the sham-operated gland in the next 3 days, perhaps due to atrophy of the denervated gland. Thus, comparable up-regulation of β_1-AR mRNA levels was obtained whether sympathectomy was achieved chemically or surgically.

The effect of sympathectomy on β-AR-mediated activation of adenylyl cyclase in submandibular membranes and submandibular cAMP content was investigated to determine whether receptor up-regulation was accompanied by significant changes in submandibular adenylyl cyclase activity (Table I). Reserpine pretreatment decreased the basal activity of adenylyl cyclase in submandibular membranes slightly but not significantly, and GTP (10 μM) stimulated this activity by about 2.2-fold. Reserpine pretreatment slightly decreased the GTP-stimulated adenylyl cyclase activity without changing the magnitude of GTP-stimulated adenylyl cyclase activation. β Agonist stimulation of adenylyl cyclase resulted in an additional 2-fold increase in cyclase activity over that already provided by GTP. The maximal isoproterenol-mediated stimulation of adenylyl cyclase decreased slightly in reserpine-treated animals, whereas the fold stimulation over basal remained essentially unchanged. Chemical sympathectomy with reserpine had no effect on the magnitude or half-maximal concentration (EC_{50}) of agonist-mediated stimulation of submandibular adenylyl cyclase. The levels of cAMP in submandibular glands of control rats and of rats injected with reserpine (0.5 mg/kg/day) for 7 days were

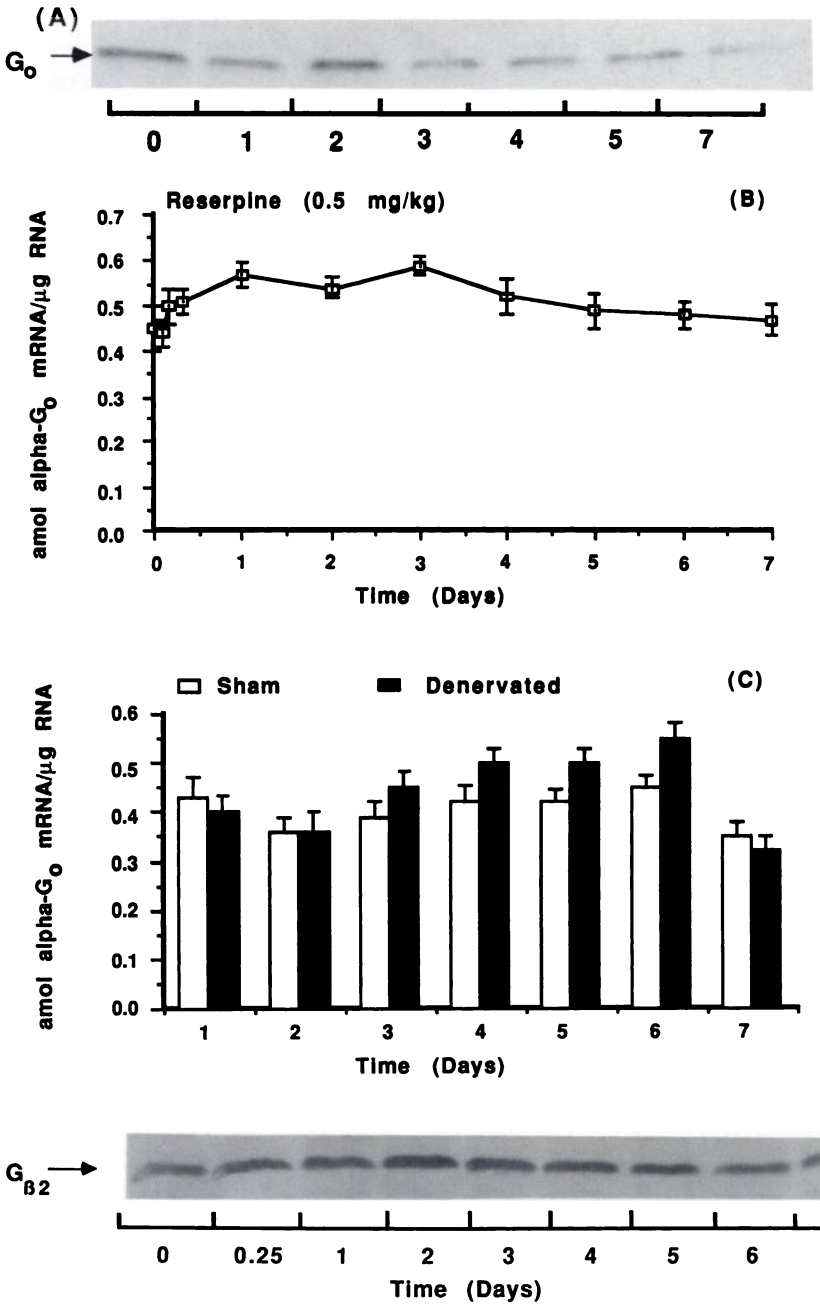

Fig. 7. Effect of sympathectomy on $G_{o\alpha}$ protein and mRNA levels in rat submandibular glands. Membranes and RNA were prepared as outlined in the legend to Fig. 4. A, Immunoblot analysis of submandibular membranes that were probed using a 1/200 dilution of the anti-$G_{i\alpha 2}$ antiserum CM-140. B, G_o mRNA levels in 50 μg of total cellular submandibular gland RNA were determined by DNA-excess solution hybridization. Each time point represents the mean ± standard error of four determinations, each performed in duplicate. C, G_o mRNA levels in RNA prepared from submandibular glands after unilateral superior ganglionectomy were determined by DNA-excess solution hybridization. The data represent the mean ± standard error of two determinations, each performed in triplicate.

Fig. 8. Immunoblot analysis of $G_{\beta 2}$ levels in submandibular glands of rats pretreated with reserpine. Rats were pretreated for the indicated time periods with reserpine (0.5 mg/kg/day). $G_{\beta 2}$ (35 kDa) levels in submandibular membranes were probed by immunoblotting using a 1/100 dilution of the anti-$G_{\beta 2}$ antiserum CM-162.

0.61 ± 0.12 and 0.55 ± 0.15 pmol/mg of protein, respectively (four experiments, $p > 0.05$).

To assess the effect of sympathectomy on the expression of those proteins that transduce receptor signaling to effector activation or inhibition, we measured steady state protein and mRNA levels of various G protein subunits. Two isoforms of the α subunit of G_s, which couples β-AR activation to adenylyl cyclase stimulation, were detected by immunoblotting (Fig. 4A). A major immunoreactive species of $G_{s\alpha}$ of 42 kDa and a minor species of $G_{s\alpha}$ of 50 kDa were detected. In untreated membranes, the abundance of the 42-kDa species was about 2-fold higher than that of the 50-kDa form, as determined by densitometry. After reserpine treatment, the immunoreactivity of the 42-kDa species gradually declined and was reduced to 50% of that in untreated membranes after 7 days (Fig. 5A). The abundance of the higher molecular mass form, migrating as 50 kDa, was unaltered by reserpine administration, and its immunoreactivity level became equal to that of the 42-kDa form after 7 days of continuous reserpine administration (Fig. 4A). The mRNA level of $G_{s\alpha}$ in submandibular glands was 2.7 ± 0.04 amol of $G_{s\alpha}$ mRNA/μg of RNA, which was the highest among the G protein subunit mRNAs (Fig. 4B). $G_{s\alpha}$ mRNA levels were not altered by chemical or surgical sympathectomy (Fig. 4B and 4C).

The levels of the pertussis toxin-sensitive G proteins $G_{i\alpha 2}$ and G_o, which in some cells mediate the inhibition of adenylyl cyclase, were measured by immunoblotting and their mRNA was measured by DNA-excess solution hybridization. The $G_{i\alpha}$ antibody used in this study immunoreacts with $G_{i\alpha 1}$ and $G_{i\alpha 2}$ proteins because its immunogenic sequence is present in both subunits (17). $G_{i\alpha 1}$ mRNA was not detected in rat submandibular glands (data not shown), and only $G_{i\alpha 2}$ levels were determined by immunoblotting (Fig. 6A). The levels of $G_{i\alpha 2}$ (41 kDa)

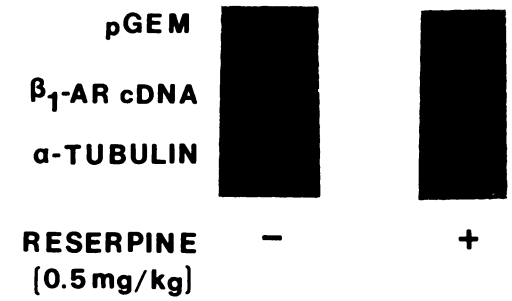

Fig. 9. Effect of pretreatment with reserpine on the transcriptional activity of the β_1-AR gene in submandibular glands. Rats were injected with reserpine (0.5 mg/kg) or diluent for 6 hr. Nuclei were prepared as outlined in Materials and Methods and used without delay. Transcript elongation was allowed to continue in the presence of [α-^{32}P]UTP and unlabeled nucleotides. After elongation, equal amounts of radiolabeled RNA were hybridized either to plasmid (10 μg/slot) harboring the 0.9-kb *Pst*I fragment of rat β_1-AR cDNA (β_1-AR cDNA), to plasmid lacking the receptor cDNA insert (*pGEM*), or to plasmid pT1 containing 1.4-kb α-tubulin cDNA (α-*TUBULIN*). The plasmids harboring β_1-AR cDNA or pGEM-3 were both linearized by *Bam*HI, whereas the pT1 plasmid was linearized with *Pst*I, before immobilization to nylon membranes. Autoradiograms (6-day exposure) were analyzed by scanning densitometry to quantify the relative intensity of each band. These experiments were replicated twice, with similar results.

were not significantly altered by reserpine administration (Fig. 5B). The levels of $G_{i\alpha 2}$ mRNA in reserpine-treated or denervated salivary glands increased by 1.5–2-fold within 4 days of sympathectomy and then returned to control levels by 7 days. During this period, the immunoreactive levels of $G_{i\alpha 2}$ protein did not change, despite this change in their mRNA. The protein (39 kDa) and mRNA levels of G_o, which is the major G protein in brain, were not altered by reserpine pretreatment or surgical sympathectomy (Figs. 5C and 7).

The levels of the β subunits of G proteins, which along with the α and γ subunits form the functional heterotrimeric G protein, were determined next by immunoblotting (Fig. 8). The two molecular forms of the β subunit of G proteins, namely $G_{\beta 1}$ (36 kDa) and $G_{\beta 2}$ (35 kDa), are coexpressed in most tissues. However, the levels of $G_{\beta 1}$ in rat submandibular gland membranes were too low for accurate detection, and only $G_{\beta 2}$ levels were determined. The levels of $G_{\beta 2}$ were not affected after chemical or surgical sympathectomy (Fig. 8).

Reserpine regulation of β_1-AR mRNA levels was explored at the level of transcription by nuclear run-on transcription assays (Fig. 9). These experiments were performed using 50×10^6 freshly prepared nuclei/sample. The nuclei were prepared from submandibular glands of untreated rats and from rats pretreated with reserpine for 6 hr. These determinations did not reveal significant changes in the transcriptional activity of the β_1-AR gene in submandibular glands by reserpine.

One possible mechanism by which steady state β_1-AR mRNA levels may be up-regulated by reserpine was evaluated by investigating the stability of receptor mRNA (Fig. 10). Rats were treated with vehicle or with reserpine (0.5 mg/kg/day) for 6 days and then concurrently with actinomycin D (1.5 mg/kg) for 0–14 hr (35). Salivary glands were excised at 0, 3, 6, and 14 hr after the injection of actinomycin D, and total RNA was prepared. The effect of actinomycin D on β_1-AR mRNA levels in control and reserpine-treated rats was compared by Northern blot analysis (Fig. 10A) and by DNA-excess solution hybridization, coupled with the approach of Rodgers *et al.* (36) to estimate the half-life of receptor mRNA (Fig. 10B). As shown in Fig. 10A, most of the β_1-AR mRNA of control animals degraded within 3 hr of actinomycin D administration. In contrast, the abundance of β_1-AR mRNA in the equimolar amounts of RNA prepared from submandibular glands of reserpine-treated rats was about 2.5-fold higher than the untreated control. Actinomycin D did not substantially change β_1-AR mRNA levels in reserpine-treated animals except after 6 hr; meanwhile, β_1-AR mRNA in the control group was completely degraded by this time. The half-life of β_1-AR mRNA *in vivo* in submandibular glands, as determined by DNA-excess solution hybridization, was about 3.5 ± 1 hr (four experiments); in submandibular glands of reserpine-treated rats, the half-life of β_1-AR mRNA increased to 8 ± 1 hr (Fig. 10B).

Discussion

The induction of postjunctional β_1-AR levels in submandibular glands after surgical or chemical sympathectomy has been recognized for many years (5, 30–33), but the nature of the phenomenon remains poorly understood. One of the basic questions concerning this phenomenon has been the mechanism by which pretreatment with reserpine results in postjunctional changes necessary for the development of receptor regulation. Reserpine pretreatment is known to produce chronic depletion of the endogenous neurotransmitter norepinephrine, which results in the loss of tonic neural stimulation and leads to compensatory changes in postjunctional receptors and effectors (37, 38). Abrupt depletion of stored catecholamines by reserpine can depress the basal activation of the salivary β-AR and consequently reduce the intracellular levels of mediators generated by the receptor signaling pathway. The intracellular mediator generated via the stimulation of β-AR is cAMP, which subsequently increases the activity of cAMP-dependent protein kinases. The abundance or activity of these mediators is regulated, in part, by agonist receptor occupancy (9).

Reserpine pretreatment induced a relatively rapid increase in β_1-AR mRNA levels within 8–12 hr, and these elevated mRNA levels were sustained for the next 7 days. The relative rates of β_1-AR gene transcription were measured after 6 hr from reserpine administration because earlier studies have shown that transcriptional activation of the β-AR gene precedes the observable up-regulation of β-AR mRNA by at least 30 min (18, 39). Nuclear run-on transcription assays did not reveal a significant contribution of a transcriptional mechanism to the early effects of reserpine on β_1-AR mRNA.

The effect of reserpine on β_1-AR mRNA half-life, which was measured after several days of continuous reserpine administration, revealed that reserpine-mediated stabilization of β_1-AR mRNA appeared to be the mechanism by which reserpine up-regulates submandibular β_1-AR mRNA levels. Rat submandibular glands contained high levels of the neurotransmitter norepinephrine, which led to substantial basal β_1-AR activation (5, 33). Catecholamine stores in sympathetically innervated organs are depleted rapidly after the administration of reserpine, and the depletion is almost complete (>95%) within the first day (40). No appreciable changes in submandibular β_1-AR mRNA levels were detected except 8 hr after reserpine administration (Fig. 3; five experiments), suggesting that depletion of the neurotransmitter occurred before the up-regulation of β_1-AR mRNA levels in the submandibular gland. Agonist-mediated down-regulation of β-AR mRNA stability was originally

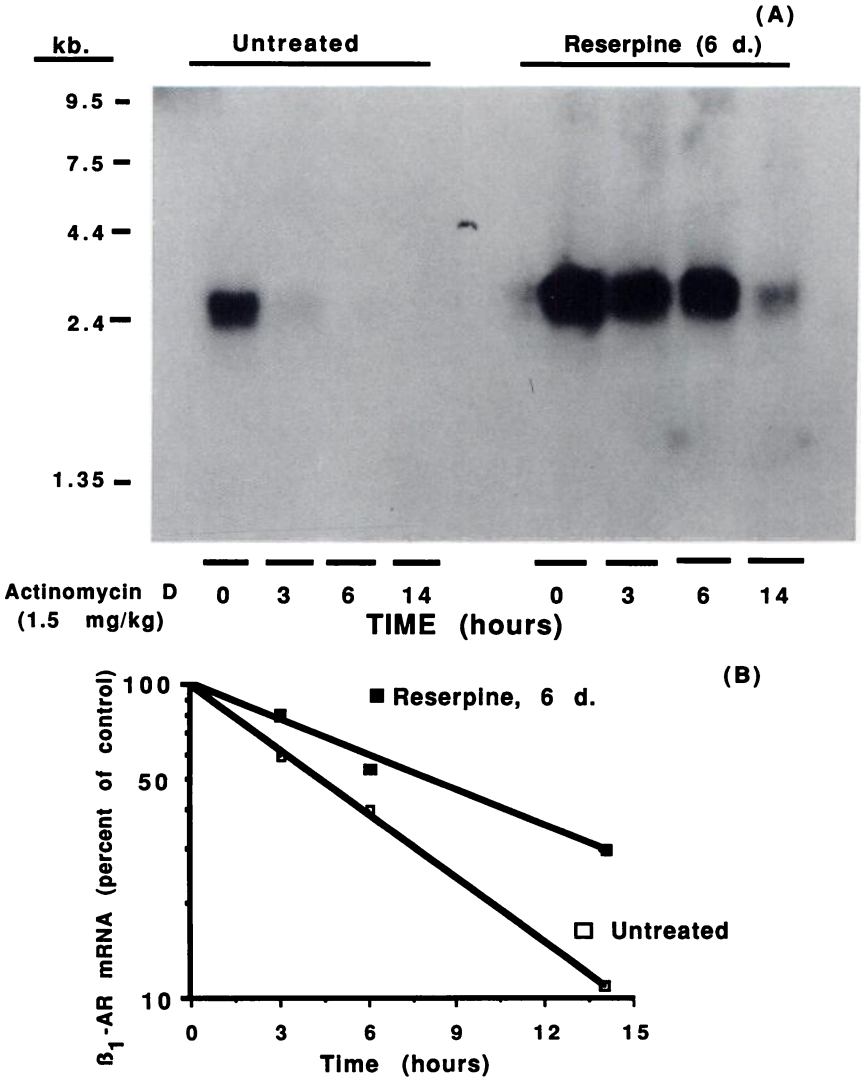

Fig. 10. Effect of reserpine pretreatment on β_1-AR mRNA stability in rat submandibular glands. Rats were pretreated with reserpine (0.5 mg/kg/day) or vehicle for 6 days, and then actinomycin D (1.5 mg/kg) was injected intraperitoneally. Submandibular gland RNA was prepared 0, 3, 6, or 14 hr after the administration of actinomycin D. A, Total cellular RNA (50 μg) was subjected to RNA blot analysis and probed with ^{32}P-labeled 0.9-kb *Pst*I fragment of rat β_1-AR cDNA. Equivalent amounts of transferred RNA were verified by ethidium bromide staining. The blot was hybridized and washed as outlined in Materials and Methods. The autoradiogram is a 5-day exposure with one intensifying screen. B, β_1-AR mRNA levels in 150 μg of RNA were measured by DNA-excess solution hybridization and calculated as amol of β_1-AR mRNA/μg of total cellular RNA. These levels were as follows: vehicle-treated, 0.093; reserpine-treated, 0.26 amol of β_1-AR mRNA/μg of total cellular RNA. The percentage of β_1-AR mRNA remaining after each actinomycin D time period was calculated and plotted on a semilogarithmic scale versus the time period of actinomycin D treatment. The half-life of β_1-AR mRNA was deduced from the 50% value. The data are mean values of four separate experiments, each performed in duplicate.

observed by Hadcock *et al.* (41) in DDT$_1$ MF-2 smooth muscle cells *in vitro*. In these cells, destabilization of β_2-AR mRNA was detected 1 day after agonist exposure, suggesting that the posttranscriptional mechanism is involved in the acute effects of agonists on β-AR mRNA levels (41). Thus, reserpine-mediated depletion of presynaptic norepinephrine and β agonists appears to exert opposing effects on β-AR mRNA stability. These data, taken together with the earlier data demonstrating that reserpine had no effect on the transcriptional activity of the β_1-AR gene, provide strong evidence that the reserpine-promoted increase in submandibular β_1-AR mRNA levels was due to receptor mRNA stabilization during the exposure period.

The effect of reserpine on β_1-AR mRNA stability was exerted without substantial changes in submandibular cAMP levels (5, 33). Agonist-mediated destabilization and, consequently, down-regulation of β-AR mRNA occurs by a process dependent on protein kinase A activity, without necessarily involving substantial changes in cAMP (42). The stabilization of β_1-AR mRNA *in vivo* in response to depletion of prejunctional norepinephrine stores by reserpine may be a reflection of this same mechanism. A decrease in basal adenylyl cyclase activity, intracellular cAMP, and, most importantly, protein kinase A activity would lead to a greater stability of receptor mRNA (42). Indeed, our data revealed that basal adenylyl cyclase activity and cAMP decreased slightly, albeit not significantly, in submandibular membranes of reserpine-treated rats (Table 1).

In vitro studies have also implicated cAMP, which is generated intracellularly upon agonist binding to β-AR, as a *trans*-activator of β-AR gene expression (39). Our data did not reveal a significant contribution of a transcriptional mechanism to the early effects of reserpine on β_1-AR mRNA in rat submandibular glands. This result may be due to many differences between these two systems. First, the levels of cAMP in rat submandibular glands *in vivo* were not altered significantly by reserpine, whereas cAMP levels were substantially elevated in agonist-exposed DDT$_1$ MF-2 cells (6, 39). Thus, transcription of the β_1-AR gene in submandibular glands was not activated because this effect may require significant increases in intracellular cAMP, as suggested by the *in vitro* results (6, 39). Second, there appear to be significant differences among the responses of the three β-AR genes to cAMP. For example, cAMP induced the expression of the β_3-AR gene while it down-regulated β_1-AR mRNA expression in 3T3-F442A fibroblasts (43). The differential effect of cAMP on the β_3-AR gene was mediated by several cAMP response elements in the promoter region of the β_3-AR gene (43). Therefore, cAMP may differen-

tially increase the transcription of the β_2-AR gene in DDT_1 MF-2 cells without having a similar effect on the salivary β_1-AR gene. A cAMP response element in the β_2-AR gene has been localized by DNase footprinting analysis, and its induction of chimeric reporter gene expression has been verified (44). To date, no such studies have been conducted on the β_1-AR gene to determine whether its transcription is modulated by cAMP.

The neurotransmitter norepinephrine interacts with stimulatory (β_1-AR) and inhibitory (α_2-AR) pathways in rat submandibular glands (3). The depletion of norepinephrine by reserpine up-regulated the densities of the stimulatory β_1-AR by 2-fold and of the inhibitory α_2-AR by 20-fold in submandibular glands within 7 days (5, 45). In the control group, the density of β_1-AR in submandibular glands was about 40-fold higher than that of α_2-AR, whereas the density of β_1-AR in the reserpine-treated group was about 3–4-fold higher than that of α_2-AR (5). This phenomenon and its results should provide additional insights concerning cross-regulation between stimulatory and opposing inhibitory pathways that control adenylyl cyclase activity. Depletion of the common neurotransmitter exerted a marked effect on steady state levels of $G_{s\alpha}$ in submandibular membranes. Two molecular forms of $G_{s\alpha}$, of 50 kDa and 42 kDa, were detected in submandibular glands. These two forms are generated by two types of alternative splicing reactions and differ by the insertion of a 15-amino-acid insert in the 50-kDa form of $G_{s\alpha}$ at Asp-71 (46). Both subunits appear to be equipotent in stimulating adenylyl cyclase (1), but the contribution of each subunit to the coupling of β_1-AR to adenylyl cyclase in rat submandibular glands is unknown. Reserpine resulted in a gradual loss of the more abundant 42-kDa form of $G_{s\alpha}$, which culminated in its reduction by 50% within 7 days of continuous daily injections of reserpine. The loss of the low molecular mass form of $G_{s\alpha}$ was accompanied by a small, albeit insignificant, loss of GTP-stimulated adenylyl cyclase activity. The effect of reserpine on β agonist-mediated adenylyl cyclase activity in submandibular membranes did not result in an enhancement of this response, despite the marked increase in β_1-AR density in submandibular membranes (Table 1). The decline in $G_{s\alpha}$ may partially explain the lack of enhanced isoproterenol-mediated stimulation of adenylyl cyclase 7 days after reserpine pretreatment, when β_1-AR levels were up-regulated by at least 2-fold. Other factors, such as impaired receptor-G protein coupling or G protein-effector coupling in reserpine-treated animals, may underlie this phenomenon, particularly because reserpine treatment is associated with significant changes in the ultrastructure of the rat submandibular gland (47). The effect of reserpine on β agonist-mediated adenylyl cyclase activity, however, varies among the different tissues in which an increased density of β_1-AR occurs after the administration of reserpine. In the heart, for example, reserpine pretreatment is associated with β_1-AR up-regulation as well as β agonist-mediated supersensitivity (48). Similarly, in the pineal gland, reserpine pretreatment up-regulates the density of β_1-AR and β agonist-mediated stimulation of pineal adenylyl cyclase activity (49). The effects of reserpine on steady state G protein subunit expression in the myocardium and pineal glands have not been studied but, in light of the effect of reserpine on the sensitivity of adenylyl cyclase to agonist stimulation, a different pattern of G protein subunit regulation may emerge in these tissues.

The pattern of G protein subunit regulation *in vitro* in cells in which a common neurotransmitter activates two receptors oppositely coupled to adenylyl cyclase was recently determined in DDT_1 MF-2 cells (50). DDT_1 MF-2 cells, which have been used extensively as a model system for studying cross-regulation between signal transmission pathways, contain both A_1-R and A_2-R, which are coupled to inhibition and stimulation of adenylyl cyclase, respectively (51). In these cells, persistent activation of A_1- and A_2-R was associated, as expected, with desensitization of A_1- and A_2-R-mediated inhibition and stimulation of adenylyl cyclase activities, respectively (50). The abundance and activities of the G protein subunits $G_{s\alpha}$ and $G_{i\alpha 2}$, which transduce the activation and inhibition of adenylyl cyclase, respectively, were not significantly altered (50). These results are unlike those obtained when either the stimulatory or inhibitory pathways were persistently activated because such an intervention caused marked up- or down-regulation of $G_{i\alpha 2}$, respectively (7, 9). Up- or down-regulation of $G_{i\alpha 2}$ appears to require substantial changes in intracellular cAMP levels. This would occur when adenylyl cyclase is persistently stimulated or inhibited but not when both the stimulatory and inhibitory pathways are simultaneously engaged. cAMP may be involved in the regulation of $G_{i\alpha 2}$ expression by a cAMP response element present in the 5′ noncoding region of the $G_{i\alpha 2}$ gene (52). Regulation of $G_{s\alpha}$, on the other hand, appears to occur largely by posttranscriptional mechanisms, because the $G_{s\alpha}$ gene is devoid of cAMP response elements (46). The mechanism underlying the differential down-regulation of $G_{s\alpha}$ subunits in reserpine-treated animals in currently unknown.

Our results and those obtained earlier by other groups also indicate that reserpine pretreatment seems to affect the expression of various elements involved in signal transduction differently in different tissues. For example, reserpine pretreatment was associated with a decrease in submandibular phosphodiesterase activity (33). Thus, despite the absence of an increase in isoproterenol-mediated stimulation of adenylyl cyclase activity in submandibular membranes prepared from reserpine-treated animals (Table 1), there was a substantial increase in β-agonist-mediated cAMP accumulation in submandibular slices, which appears to be more related to the decrease in phosphodiesterase than to the increase in β-AR (33). Such a phenomenon was not observed *in vitro* or in other sympathetically innervated tissues. Thus, conclusions concerning the effect of certain manipulations on signal transducing subunits that are obtained from *in vitro* studies may not be completely applicable *in vivo*, due to the variability in the mix of receptors, G proteins, and effectors and the repertoire of other specialized proteins in these cells.

Acknowledgments

The author thanks Dariel Gilder and Stanley Lopez for their superb technical assistance. Special thanks go to Barbara J. Kuyper, Department of Health Informatics, for editing the manuscript.

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