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Regulation of Autonomic Receptors in Rat Submandibular Gland

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SUMMARY

The binding of receptor specific radioligands to autonomic receptors in the rat submandibular gland was characterized after chronic drug administration and surgical sympathetic denervation. Reserpine administration resulted in an up-regulation of both *alpha*$_2$-adrenergic receptors labeled by [^{3}H]clonidine and *beta*$_1$-adrenergic receptors labeled by [^{3}H]dihydroalprenolol. The increase in *alpha*$_2$-receptors was half-maximal 24 hr after a single injection of reserpine, and was about 10-fold greater than control after seven daily injections. By contrast, the *beta*-adrenergic receptor density was the same as control after 3 days of reserpine administration, but within 7 days was about 2-fold greater than control. Guanethidine or yohimbine administration also resulted in an up-regulation of *alpha*$_2$-adrenergic receptors. Reserpine administration or unilateral superior cervical ganglionectomy increased the density of *alpha*$_1$-adrenergic receptor binding sites 24–80%. Norepinephrine and methoxamine, but not clonidine, caused potassium to be released from submandibular gland slices. Prazosin, but not yohimbine, blocked this response to norepinephrine, indicating that the response was mediated by *alpha*$_1$-adrenergic receptors. Potassium release elicited by *alpha*$_1$-agonists was augmented in slices from animals that received reserpine. Neither drug treatment nor sympathetic denervation altered muscarinic cholinergic receptor binding. The densities of muscarinic and *beta*-adrenergic receptors were found to be 23–51% higher in glands from female rats than in glands from male rats.

INTRODUCTION

The rat salivary gland has become a useful model system for the study of the regulation of autonomic receptors. The usefulness of this system is due to several factors: (a) The amount, rate of secretion, and composition of the saliva are modified by both sympathetic (*alpha*$_1$-, *alpha*$_2$-, and *beta*$_1$-adrenergic) and parasympathetic (muscarinic cholinergic) receptor systems (1). (b) Several biochemical parameters can be measured *in vitro*. These include potassium release (*alpha*$_1$-adrenergic and muscarinic cholinergic), stimulation of adenylate cyclase, and an increase in cyclic AMP levels (*beta*$_1$-adrenergic) and the inhibition of norepinephrine release (*alpha*$_2$-adrenergic) (2–5). (c) All four receptor systems can be readily studied with the radioligand binding technique (6–9). (d) These four receptor systems are regulated by various drugs and surgical procedures which increase or decrease the amount of neurotransmitter which is available at the receptor site (9–18). Several studies have indicated that the adrenergic receptors of the rat submandibular gland are very responsive to changes in the levels of catecholamines. The most remarkable of these is the greater than 10-fold increase in the levels of *alpha*$_2$-adrenergic receptors, as determined by the binding of [^{3}H]clonidine, which is seen after reserpine treatment (9). Following reserpine treatment, the number of *beta*-adrenergic receptors in the submandibular glands approximately doubles (16).

Most previous work has been limited to those changes observed in one or two receptor systems following the administration of one or two drugs at a single dose for a fixed period of time. In this communication we report data for four receptor systems at various times during and after administration of a number of different drugs and after surgical denervation. In addition, we have also studied the effect of *alpha*-adrenergic agents on potassium release and cyclic AMP levels.

MATERIALS AND METHODS

Animal treatment and tissue preparation. Male Sprague-Dawley rats (175–225 g) once or twice daily received i.p. injections of reserpine, yohimbine, guanethidine, isoproterenol, atropine, or physostigmine as is indicated in the legends to Tables 1–12. Approximately 24 hr after the final injection the rats were anesthetized with pentobarbital. The submandibular glands were rapidly removed and separated from the adjoining sublingual glands, weighed, and then cooled in ice-cold 0.9% NaCl solution. For the radioligand binding assays the glands

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were homogenized in 20 volumes of 50 mM Tris-HCl (pH 8.0) (Tissumizer setting 80 for 30 sec). This homogenate was centrifuged for 10 min at 49,000 × *g*, resuspended in buffer, and centrifuged again. The pellet, a crude particulate fraction, was then resuspended in the appropriate buffer as indicated in Table 1 for the various receptor assays. Male Sprague-Dawley rats with unilateral superior cervical ganglionectomy were obtained from Zivic-Miller Laboratories, Inc. (Allison Park, Pa.). Seven days after surgery the animals were killed and the glands and tissues were prepared as indicated above. For the measurement of cyclic AMP levels, four 0.5-mm slices were prepared from each gland using a Stadie-Riggs microtome. For the measurement of potassium release, the submandibular glands were immediately placed in a beaker containing 15–20 ml of incubation medium (see below) which had been previously gassed with a 95% O_2-5% CO_2 mixture for at least 30 min and warmed to 37°. Slices were prepared by cutting the glands with a sharp scalpel blade into pieces approximately 1 mm^3.

Radioligand binding assays. Autonomic receptors were assayed using the radioligands indicated in Table 1. For saturation experiments, total binding was determined with one set of incubation tubes which contained 970 μl of the membrane suspension and 20 μl of increasing concentrations of the radioligand diluted in 5 mN HCl. The final concentrations of the radioligands are given in Table 1. To a parallel set of incubations, 10 μl of an appropriate concentration of an unlabeled drug were added and used to define nonspecific binding. Specific binding was calculated as the difference between total and nonspecific binding. After a 30-min incubation at 23°, the suspensions were filtered through GF/B glass-fiber filter paper (Whatman, Inc., Clifton, N. J.), using a manifold (Brandel Cell Harvester, Biomedical Research and Development, Gaithersburg, Md.) which filters 24 samples simultaneously. The tubes and filter paper were washed twice with 5 ml of ice-cold Tris-HCl, and then the radioactivity retained on the filter paper was determined for each sample by scintillation spectroscopy. The B_{max} and K_D were calculated from a linear regression of the data plotted as bound/free versus bound (19). Since several of the treatments affect the weight of the glands, the results are expressed as both the density (picomoles per gram of tissue and picomoles per gram of protein) and number (picomoles per gland) of binding sites. For the inhibition experiments, a fixed concentration of radioligand was added to the tissue suspension, which contained increasing concentrations of various unlabeled drugs. For each inhibition curve, 10 concentrations of the unlabeled drug were used ranging at least from 100-fold above the IC_{50} to 1000-fold below the IC_{50}. The suspensions were then incubated and filtered as described above for saturation experiments.

Cyclic AMP response to alpha-adrenergic agents. Slices of submandibular glands (20 mg) were incubated in 2 ml of Krebs-Ringer bicarbonate medium (pH 7.4) (95% O_2-5% CO_2) for 15 min at 37° in a Dubnoff shaking incubator. After preincubation, adrenergic drugs or vehicle were added and the incubation was continued for 10 min. Each slice was transferred to 1 ml of 50 mM sodium acetate (pH 4) maintained at 100° for a period of 3 min. The slices were then homogenized and centrifuged at 2000 × *g* for 20 min. The supernatant was removed for cyclic AMP determination by radioimmunoassay (20, 21).

Potassium release. Slices from the glands of three or four animals were thoroughly mixed and then divided into two to four equal portions and rapidly placed into nitrocellulose tubes containing 2 ml of the incubation medium. Each slice system was preincubated in this system for 15 min then washed in fresh medium and placed in 2 ml of new medium for the final incubation. Both the preincubation and the final incubation were carried out in media which were constantly bubbled with the O_2-CO_2 mixture at 37° in a shaking incubator. Various adrenergic drugs were added to the medium at the time of the final incubation, and subsequently aliquots of the medium were removed at timed intervals for the measurement of potassium concentration. At the end of the incubation period the slices were homogenized and the potassium present in the slices was calculated by using the formula previously described (22). The incubation medium had the following composition (in millimolar concentrations): NaCl, 118.5; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 24.5; β-hydroxybutyric acid, 5.0; nicotinamide, 10.0; inosine, 10.0; adenine, 0.5; and glucose, 2.8.

Materials. The radioligands were purchased from New England Nuclear Corporation (Boston, Mass.). The following drugs were kindly donated by the indicated companies: (−)-isoproterenol and (+)-norepinephrine, Ster-

TABLE 1

Standard radioligand assay conditions

Receptor and ^{3}H-ligand[a]	Buffer	pH	Specific activity	Concentration range	Drug for determination of nonspecific binding
			Ci/mmole	*nM*	
Alpha$_1$-adrenergic					
[^{3}H]WB4101	40 mM Tris-HCl	8.0	25	0.3–5.0	0.1 mM (−)-Norepinephrine
[^{3}H]prazosin	40 mM Tris-HCl	8.0	17	0.3–5.0	0.1 mM (−)-Norepinephrine
Alpha$_2$-adrenergic					
[^{3}H]clonidine	40 mM Tris-HCl	7.4	24	0.2–6.0	1.0 μM (−)-Norepinephrine
[^{3}H]PAC	40 mM Tris-HCl	7.4	49	0.1–3.0	1.0 μM (−)-Norepinephrine
[^{3}H]yohimbine	25 mM Glycylglycine	7.4	85	0.1–2.0	10 μM (−)-Norepinephrine
Beta-adrenergic, [^{3}H]DHA	40 mM Tris-HCl	8.0	50	0.1–3.0	0.3 μM (−)-Propranolol
Muscarinic cholinergic, [^{3}H]QNB	40 mM Tris-HCl	8.0	40	0.2–4.0	1.0 μM Atropine

[a] WB4101, (2,6-dimethoxyphenyloxyethyl)aminoethyl-1,4-benzodioxane; PAC, *p*-aminoclonidine; DHA, (−)-dihydroalprenolol; QNB, quinuclidinylbenzilate.

TABLE 2

Time course of the effect of reserpine on alpha$_2$-adrenergic receptors

Reserpine (0.5 mg/kg) was injected daily for 7 days. One day after the last injection, the submandibular glands were removed and the binding of [^{3}H]clonidine was determined.

Reserpine treatment	n	Gland weight	K_D	B_{max}		
				pmoles/g tissue	pmoles/g protein	pmoles/gland
		mg	*nM*			
Days of treatment						
0	9	218 ± 5	—[a]	0.3 ± 0.2	9 ± 2	0.07 ± 0.03
0.13	4	197 ± 10	—	1.6 ± 0.2	29 ± 5	0.33 ± 0.04
0.5	5	194 ± 10	1.8 ± 0.1	2.5 ± 0.5	52 ± 11	0.48 ± 0.09
1	5	256 ± 10	2.0 ± 0.1	3.1 ± 0.7	70 ± 17	0.79 ± 0.19
2	5	212 ± 4	2.3 ± 0.3	3.4 ± 0.4	72 ± 9	0.68 ± 0.06
3	6	230 ± 8	2.4 ± 0.2	4.5 ± 0.9	106 ± 16	1.04 ± 0.22
5	2	185 ± 7	1.8 ± 0.2	5.8 ± 0.8	130 ± 32	1.06 ± 0.10
7	8	150 ± 6	1.9 ± 0.3	6.2 ± 0.9	141 ± 18	0.95 ± 0.17
Days after 1 week of treatment						
2	5	162 ± 5	2.6 ± 0.3	4.0 ± 0.3	80 ± 7	0.64 ± 0.06
3	5	162 ± 5	2.1 ± 0.1	2.3 ± 0.5	45 ± 8	0.36 ± 0.08
5	5	195 ± 13	—	1.1 ± 0.1	21 ± 2	0.23 ± 0.04

[a] At very low levels of specific binding, a reliable K_D could not be determined. The B_{max} was estimated by assuming a K_D of 2.0 nM.

ling-Winthrop Research Institute (Rensselaer, N. Y.); phentolamine, Ciba-Geigy Corporation, Pharmaceuticals Division (Summit, N. J.); prazosin, Pfizer Chemical Division, Pfizer, Inc. (New York, N. Y.); and clonidine, Boehinger Ingelheim (Elmsford, N. Y.). Yohimbine, (−)-norepinephrine, (−)-epinephrine, Tris-HCl, and glycylglycine were purchased from Sigma Chemical Company (St. Louis, Mo.); guanethidine was obtained from Ciba Pharmaceutical Company (Summit, N. J.).

RESULTS

Alpha$_2$-adrenergic receptors. We have previously shown that [^{3}H]clonidine appears to label relevant *alpha$_2$*-adrenergic receptor binding sites in the submandubular gland and that the density of these receptors increases dramatically after 1 week of reserpine treatment (9). The density of receptors in control tissues is very low. Although there is generally a small amount of specific binding, which would amount to about 0.3 pmole/g of tissue, or about 10 pmoles/g of protein, it would be difficult to show whether or not this binding were actually to *alpha$_2$*-receptors since there is not sufficient specific binding for reliable inhibition experiments. We have attempted a number of experimental manipulations in order to increase the binding of [^{3}H]clonidine (changes in buffer and pH; addition of $MgCl_2$), but these have been unsuccessful. We also have been unable to observe binding with [^{3}H]*p*-aminoclonidine or [^{3}H]yohimbine. The submandibular gland has a high norepinephrine content which could prevent the binding of *alpha$_2$*-radioligands, particularly if the fraction used for the assays was not sufficiently washed. However, [^{3}H]clonidine binding was not observed after repeated washings at 4° and incubations at 37°. In addition, since we were able to observe good binding of [^{3}H]DHA,[1] [^{3}H]WB4101, and [^{3}H]prazosin, sufficient norepinephrine has been removed from the particulate fraction to allow the binding to *alpha*- and *beta*-receptors.

The density of *alpha$_2$*-adrenergic receptors in the rat submandibular gland increased rapidly following reserpine treatment and also decreased rapidly once treatment was terminated (Table 2). There was a marked increase in the number and density of receptors as early as 3 hr following a single injection of reserpine. The half-maximal increase was reached after 1 or 2 days of reserpine treatment and reached an apparent plateau after 7 days of drug administration. Two to three days after the last of seven daily doses of reserpine, the density of *alpha$_2$*-receptors had decreased to about one-half that seen after 7 days of reserpine administration and by 4 days after cessation of treatment was approaching control levels. A similar time course was seen regardless of how the data were expressed, and there were no significant changes in K_D at the different time points (Table 2). The effect of reserpine administration is also dose-dependent. Administration of 0.1 mg/kg for 7 days resulted in an increase in the density of *alpha*-adrenergic receptors to about 50% that seen in rats treated for the same time period with 0.5 mg/kg (Table 3). As in the case of glands from control animals, no significant binding of [^{3}H]yohimbine was detected in glands from animals that received reserpine.

The effect of other drugs which might modify the availability of norepinephrine at the *alpha$_2$*-receptors was also studied. The administration of yohimbine, an *alpha$_2$*-antagonist, also resulted in a dose-dependent increase in the density of *alpha$_2$*-adrenergic receptors. Similarly, guanethidine, a drug which depletes norepinephrine stores as does reserpine, also produced a modest increase in the density of *alpha$_2$*-adrenergic receptors. Isoproterenol, a *beta*-adrenergic agonist, was without effect.

The study of receptor regulation by receptor binding studies is more meaningful if the function of those recep-

[1] The abbreviations used are: DHA, dihydroalprenolol; WB4101, (2,6-dimethoxyphenyloxyethyl)aminomethyl-1,4-benzodioxane; QNB, quinuclidinylbenzilate.

TABLE 3

Effect of drug administration on submandibular alpha$_2$-adrenergic receptors

Drug treatments were as follows: reserpine, 0.1 mg/kg daily for 7 days; yohimbine, 1 mg/kg daily for 6 days and 5 mg/kg daily for 7 days; guanethidine, 20 mg/kg daily for 14 days; isoproterenol, 8 mg/kg twice daily for 9 days. Values given are means ± standard error of the mean, which were calculated from [^{3}H]clonidine saturation experiments.

Treatment and dose	n	K_D	B_{max}		
			pmoles/g tissue	pmoles/g protein	pmoles/gland
mg/kg/day		*nM*			
Control, 0	3	—	0.30 ± 0.2	2 ± 1	0.06 ± 0.04
Reserpine, 0.1	4	2.3 ± 0.4	3.4 ± 0.6	64 ± 15	0.71 ± 0.14
Yohimbine					
1	2	—	0.2 ± 0.1	6 ± 3	0.05 ± 0.03
5	2	3.4 ± 0.3	2.9 ± 0.4	65 ± 15	0.44 ± 0.10
Guanethidine, 20	3	—	2.4 ± 0.1	45 ± 3	0.42 ± 0.04
Isoproterenol, 16	4	—	0.4 ± 0.1	7 ± 3	0.15 ± 0.02

tors can also be studied. Two possible functions for *alpha$_2$*-adrenergic receptors could be the inhibition of adenylate cyclase or an effect on potassium release. Accordingly, we studied the ability of *alpha*-agonists and antagonists to modify the accumulation of cyclic AMP. As can be seen from Table 4, yohimbine did not have any effect on the norepinephrine-stimulated accumulation of cyclic AMP in slices from submandibular glands. Furthermore, there were no differences between the slices from control and reserpine-treated animals which would be consonant with the large increase in *alpha$_2$*-receptor binding. Similarly, there was no effect of these agents on potassium release, although *alpha$_1$* agents were very effective (Table 5).

Beta-adrenergic receptors. The radioligand [^{3}H]DHA labels *beta*-adrenergic receptor sites in the rat submandibular gland, which are solely of the *beta$_1$* subtype (16). We and others have previously shown that the administration of reserpine for 7 days increases the density of *beta*-adrenergic receptors in the rat submandibular gland (15–17). The development of the up-regulation of these receptors with time is given in Table 6. In contrast to the *alpha$_2$*-receptors, where the half-maximal increase was seen 1–2 days after drug administration was started, for the *beta*-adrenergic receptors the half-maximal increase was not observed until 4–5 days. After the termination of reserpine treatment the density of receptors decreased back toward control levels. The affinity of [^{3}H]DHA for the receptor did not change (Table 6). Also in contrast to the *alpha$_2$*-adrenergic receptors, a dose of 0.1 mg/kg was as effective as the higher dose of reserpine in increasing the number and density of adrenergic receptors (Table 7).

Guanethidine was also effective in increasing the density of *beta* adrenergic receptors and was as effective as reserpine (Table 7). Isoproterenol is known to increase dramatically the weight of the rat submandibular gland. Thus, following isoproterenol treatment, the density of receptors expressed either as picomoles per gram of tissue or as picomoles per gram of protein decreased, although

TABLE 4

Effect of alpha-adrenergic agents on cyclic AMP levels in submandibular slices

Submandibular slices from control- and reserpine-treated (0.5 mg/kg daily for 7 days) rats were preincubated for 15 min at 37° and then incubated in the presence of adrenergic agents for 10 min. The cyclic AMP content of the slices was determined by radioimmunoassay. Values are means ± standard error of the mean.

Addition	n	Cyclic AMP content	
		Control	Reserpine-treated
		pmoles/mg protein/10 min	
None	16	0.67 ± 0.05	0.76 ± 0.09
Yohimbine, 100 μM	4	1.09 ± 0.07	2.08 ± 0.45
Yohimbine, 10 μM + prazosin, 1.0 μM	4	1.53 ± 0.04	1.25 ± 0.08
Norepinephrine			
1.0 μM	4	1.75 ± 0.1	18.8 ± 0.8
10 μM	8	18.9 ± 1.9	106 ± 6
100 μM	16	42 ± 5	106 ± 8
Isoproterenol, 10 μM	16	40 ± 5	132 ± 10
Epinephrine, 100 μM	12	37 ± 5	78 ± 4
Epinephrine, 100 μM + yohimbine, 10 μM	4	30 ± 7	103 ± 15
Norepinephrine, 100 μM			
+ Yohimbine, 1 μM	12	41 ± 4	85 ± 8
+ Yohimbine, 10 μM	8	45 ± 6	125 ± 13
+ Prazosin, 1 μM	8	29 ± 5	102 ± 9
+ Yohimbine, 100 μM + prazosin, 1 μM	4	64 ± 3	90 ± 7

TABLE 5

Potassium release from submandibular gland slices

Slices were preincubated, washed, and finally incubated in an enriched, oxygenated Krebs-Ringer bicarbonate medium. Drugs were added to the final incubation medium to provide the final concentrations shown, and potassium release was measured after 10 min of incubation. In experiments involving antagonists (prazosin and yohimbine), these were added to the incubation medium 10 min before norepinephrine. Values represent means ± standard error of the mean.

Stimulant	n	% K^+ release (10 min)	
		Control	Reserpine-Treated
None (basal release)	6	6.0 ± 0.9	6.2 ± 1.1
Norepinephrine, 20 μM	6	15.9 ± 1.4	22.6 ± 1.9
Clonidine, 10 μM	3	8.1 ± 1.0	8.9 ± 1.2
Methoxamine, 3 μM	3	15.0 ± 0.9	18.5 ± 0.8
Norepinephrine, 20 μM + prazosin, 10 μM	4	6.8 ± 0.7	6.4 ± 0.9
Norepinephrine, 20 μM + yohimbine 10 μM	4	18.5 ± 1.2	21.7 ± 1.1

TABLE 6

Time course of the effect of reserpine on beta$_1$-adrenergic receptors

Reserpine (0.5 mg/kg) was injected daily for 7 days. One day after the last injection, the submandibular glands were removed and the binding of [^{3}H]DHA was determined.

Reserpine treatment	n	Gland weight	K_D	B_{max}		
				pmoles/g tissue	pmoles/g protein	pmoles/gland
		mg	*nM*			
Days of treatment						
0	6	195 ± 2	0.80 ± 0.05	11.7 ± 1.0	226 ± 49	2.24 ± 0.32
0.5	4	187 ± 9	0.76 ± 0.07	11.5 ± 0.9	254 ± 14	2.18 ± 0.27
2	1	196	0.50	12.6	295	2.45
3	4	227 ± 8	0.90 ± 0.04	13.0 ± 0.9	317 ± 14	2.98 ± 0.28
4	5	176 ± 4	0.77 ± 0.05	16.7 ± 0.6	352 ± 17	2.94 ± 0.08
7	9	174 ± 10	0.71 ± 0.03	20.9 ± 1.1	475 ± 37	3.58 ± 0.23
Days after 1 week of treatment						
2	3	170 ± 9	0.74 ± 0.04	18.5 ± 2.1	373 ± 68	3.11 ± 0.18
3	3	157 ± 11	0.70 ± 0.09	17.1 ± 1.4	339 ± 82	2.72 ± 0.41
5	5	181 ± 7	0.67 ± 0.05	14.9 ± 1.0	272 ± 20	2.70 ± 0.16

the number of receptors per gland remained essentially constant (Table 7). The effect of reserpine and denervtion on *beta*-adrenergic-stimulated adenylate cyclase activity has been reported elsewhere (16).

Alpha$_1$-adrenergic receptors. The *alpha$_1$*-antagonist [^{3}H]WB4101 bound in a saturable and reversible manner with the particulate fraction of rat submandibular glands. The potency of various unlabeled adrenergic drugs in inhibiting the binding of [^{3}H]WB4101 is given in Table 8. The (−)-isomers of both epinephrine and norepinephrine were potent inhibitors of [^{3}H]WB4101 binding, whereas the (+)-isomer of norepinephrine was about 40-fold weaker. Similarly, the nonselective *alpha*-adrenergic antagonist phentolamine was also very potent in inhibiting the binding. The *alpha$_1$*-selective antagonist prazosin, with a K_i of 0.37 nM, was 3000-fold more potent than the *alpha$_2$*-selective antagonist yohimbine. On the other hand, yohimbine was 82 times more potent than prazosin in inhibiting the binding of [^{3}H]clonidine in the same membrane preparation. [^{3}H]Prazosin also appeared to label *alpha$_1$*-receptors in this tissue, and the K_i values for adrenergic drugs in inhibiting its binding were very similar to the K_i values against [^{3}H]WB4101. These data are consistent with the conclusion that both [^{3}H]WB4101 and [^{3}H]prazosin label *alpha$_1$*-adrenergic receptor binding sites. Whether or not [^{3}H]WB4101 or [^{3}H]prazosin labels the identical population of *alpha$_1$*-adrenergic receptors is unclear at the present time. In a series of six saturation experiments in which the tissue suspension (control submandibular gland) was the same for [^{3}H]WB4101 and [^{3}H]prazosin, we found the density of binding sites for [^{3}H]prazosin to be 42 ± 11% ($p < 0.001$) higher than that of [^{3}H]WB4101 (8.69 ± 0.63 versus 6.18 ± 0.35 pmoles/g of tissue). The K_D values in these experiments were 0.43 ± 0.06 nM for [^{3}H]prazosin and 0.33 ± 0.11 nM [^{3}H]WB4101. The significance of this finding is not known, but in rat lung we have also found 30% higher binding of [^{3}H]prazosin as compared with [^{3}H]WB4101.[2]

The *alpha$_1$*-adrenergic receptors of the rat submandibular gland were less reponsive to reserpine and denervation than either the *alpha$_2$*- or the *beta*-adrenergic receptors. After 7 days of reserpine treatment at a dose of 0.5 mg/kg, the density of receptors as determined by [^{3}H]WB4101 binding increased only about 55–75%, while the increase following denervation was 24–80% (Table 9). The number of receptors per gland was not significantly different from the number in the control after either type of treatment.

The release of potassium ion from slices of rat submandibular glands incubated *in vitro* has been used as an experimental approach to investigate specific aspects of the stimulus-secretion coupling mechanism in these glands (3, 22, 23). The response is elicited by both cholinergic and *alpha*-adrenergic agents and is critically dependent on extracellular Ca^{2+}. The release of potassium in

[2] J. Latifpour and D. B. Bylund, unpublished observations.

TABLE 7

Effect of drug administration on submandibular beta-adrenergic receptors

Drug treatments were as follows: isoproterenol, 8 mg/kg twice daily for 9 days; reserpine, 0.1 mg/kg daily for 7 days; guanethidine, 20 mg/kg daily for 14 days. [^{3}H]DHA was the radioligand.

Treatment and dose	n	K_D	B_{max}		
			pmoles/g tissue	pmoles/g protein	pmoles/gland
mg/kg/day		*nM*			
Control, 0	3	0.64 ± 0.06	12.1 ± 1.7	220 ± 15	2.1 ± 0.4
Isoproterenol, 16	4	0.82 ± 0.03	4.6 ± 0.3	80 ± 7	1.8 ± 0.2
Reserpine, 0.1	3	0.58 ± 0.03	21.9 ± 2.7	405 ± 68	4.6 ± 0.7
Guanethidine, 20	2	0.82 ± 0.05	24.0 ± 2.5	475 ± 27	4.1 ± 0.4

TABLE 8

Inhibition by adrenergic drugs of [^{3}H]WB4101, [^{3}H]prazosin, and [^{3}H]clonidine binding in submandibular gland membranes

IC_{50} values were determined from log-probit analyses of inhibition curves using 10 concentrations of the unlabeled drug in duplicate. The concentrations of ^{3}H-ligands were as follows: [^{3}H]WB4101, 0.61 nM; [^{3}H]prazosin, 0.91 nM; [^{3}H] clonidine, 0.70 nM. K_i values were calculated from the equation $K_i = IC_{50}/[1 + (^3\text{H-ligand})/K_D]$.

Drug	[^{3}H]WB4101						[^{3}H]Prazosin control			[^{3}H]Clonidine reserpine-treated		
	Control			Reserpine-treated								
	n	K_i	n_H[a]	*n*	K_i	n_H	*n*	K_i	n_H	*n*	K_i	nH
(−)-Epinephrine	5	64 ± 17	0.71	5	74 ± 12	0.79	3	40 ± 4	1.11	3	2.9 ± 0.7	0.66
(−)-Norepinephrine	5	136 ± 46	0.86	5	207 ± 31	0.64	3	77 ± 5	0.92	7	2.6 ± 0.3	0.93
(+)-Norepinephrine	3	13,500 ± 4000	0.76	3	11,500 ± 1700	0.65	2	2,200 ± 60	1.2	1	184	0.83
Clonidine	3	150 ± 70	0.77	3	150 ± 30	0.77	4	94 ± 8	1.17	4	1.3 ± 0.4	0.46
Phentolamine	3	0.71 ± 0.30	0.62	3	1.38 ± 0.20	0.72	3	0.98 ± 0.02	0.89	3	4.5 ± 0.8	0.81
Prazosin	6	0.22 ± 0.04	0.99	6	0.28 ± 0.06	1.08	3	0.32 ± 0.03	0.94	3	2700 ± 1400	0.75
WB4101	3	0.23 ± 0.06	0.78	3	0.34 ± 0.07	0.73	3	0.17 ± 0.01	0.81	5	600 ± 220	0.56
Yohimbine	4	800 ± 84	0.68	4	660 ± 170	0.73	3	450 ± 60	0.94	5	104 ± 15	0.79

[a] n_H, Hill coefficient.

the submandibular gland slices is induced by the *alpha*$_1$-agonist methoxamine but not by the *alpha*$_2$-agonist clonidine (Table 5). Furthermore, the release of potassium elicited by norepinephrine is blocked by the *alpha*$_1$-antagonist prazosin but not by the *alpha*$_2$-antagonist yohimbine. Slices of glands from animals which had received seven daily injections of reserpine (0.5 mg/kg) were more responsive to *alpha*$_1$-agonists than were glands from control animals (Table 5).

Muscarinic cholinergic receptors. The radioligand [^{3}H]QNB binds saturably and reversibly to the particulate fraction of rat submandibular gland and appears to label muscarinic receptors. There were no changes in the K_D or B_{max} values for [^{3}H]QNB binding following administration of reserpine or guanethidine in the same doses which produced marked changes in adrenergic receptors. Atropine, a muscarinic antagonist, and physostigmine, a cholinesterase inhibitor, also failed to modify the binding of [^{3}H]QNB (Table 10). The binding of [^{3}H]QNB was also unchanged after superior cervical ganglionectomy (Table 11).

Autonomic receptors in glands from female and male rats. There is a species-dependent difference in convoluted granular tubules in salivary glands which is marked in mice, but less so in rats (24, 25). The glands of male rats are also heavier, although there is no difference in the ratio of gland weight to body weight. In order to determine whether autonomic receptor binding might be different in female and male rats, we assayed *beta*-adrenergic and muscarinic cholinergic receptors in glands from 11-week-old animals. The density of binding sites in female rats was 41–51% higher for [^{3}H]DHA binding and 23–30% for [^{3}H]QNB binding (Table 12). However, the number of receptors per gland was not significantly different in glands from male and female rats.

DISCUSSION

A major finding of the present study is that the three adrenergic receptors (*alpha*$_1$, *alpha*$_2$, and *beta*$_1$) of the rat submandibular gland respond differently to the same modulation of sympathetic neurohormonal input. The *alpha*$_2$-adrenergic receptors increased dramatically following a decrease in the functional levels of norepinephrine due to either drug treatment (reserpine, guanethidine, yohimbine) or to surgical denervation. Although the density of *beta*-adrenergic receptors increased less dramaticaly than the density of *alpha*$_2$-adrenergic receptors, reserpine or denervation still caused a 2-fold increase. By contrast, the density of *alpha*$_1$-adrenergic receptors increased only approximately 50%. An additional difference between the *alpha*$_2$- and the *beta*-adrenergic responses to reserpine treatment is the time course with which the up-regulation develops. The *alpha*$_2$-receptors responded very quickly and reached 75%

TABLE 9

Effect of reserpine administration and unilateral superior cervical ganglionectomy on submandibular alpha$_1$*-adrenergic receptors*

Reserpine (0.5 mg/kg) was administered daily for 7 days. [^{3}H]WB4101 was the radioligand.

Treatment	*n*	K_D	B_{max}		
			pmoles/g tissue	pmoles/g protein	pmoles/gland
		nM			
Control	5	0.37 ± 0.04	5.3 ± 0.7	96 ± 10	1.08 ± 0.09
Resperine	5	0.33 ± 0.02	8.2 ± 0.6	170 ± 15	1.33 ± 0.08
p (t-test)		>0.1	<0.05	<0.005	>0.05
Control	3	0.36 ± 0.03	5.4 ± 0.4	75 ± 8	1.10 ± 0.16
Denervated	3	0.47 ± 0.06	6.7 ± 0.6	135 ± 6	1.30 ± 0.26
p (paired *t*-test)		>0.05	<0.05	<0.05	>0.10

TABLE 10

Effect of drug administration on submandibular muscarinic cholinergic receptors

Drug treatments were as follows: reserpine, 0.5 mg/kg daily for 7 days; guanethidine, 20 mg/kg daily for 14 days; atropine, 33 μg/kg three times daily for 4 weeks; physostigmine, 2.5 mg/kg three times daily for 4 weeks. [^{3}H]QNB was the radioligand.

Treatment and dose	n	K_D	B_{max}		
			pmoles/g tissue	pmoles/g protein	pmoles/gland
		nM			
Control, 0	6	0.45 ± 0.08	15.3 ± 0.7	257 ± 10	3.0 ± 0.3
Reserpine, 0.5 mg/kg/day	5	0.36 ± 0.06	15.6 ± 1.0	256 ± 23	2.6 ± 0.2
Guanethidine, 20 mg/kg/day	3	0.67 ± 0.06	15.2 ± 1.7	279 ± 24	3.0 ± 0.3
Control, 0	5	0.36 ± 0.03	10.1 ± 0.4	248 ± 8	2.3 ± 0.3
Atropine, 33 μg/kg 3 ×/day	4	0.44 ± 0.02	10.6 ± 0.6	230 ± 11	2.6 ± 0.1
Physostigmine 2.5 mg/kg 3 ×/day	4	0.40 ± 0.01	10.7 ± 1.0	271 ± 29	2.3 ± 0.2

maximal values at a time (3 days) when the density of the beta-receptors was still the same as in controls. Since these procedures modified the sympathetic system it is not surprising that the density of the muscarinic receptors was unaltered. However, chronic treatment with a cholinesterase inhibitor or a muscarinic antagonist, which has been shown to alter muscarinic receptors in other systems (26–29), failed to change the density of the muscarinic cholinergic receptors in the rat submandibular gland. Similarly, we did not observe any changes in muscarinic binding 1 week after sympathetic denervation, although an increase in [^{3}H]QNB binding appears to occur within 6 weeks after denervation (12).

Although the delineation of the regulation of receptor binding sites using the radioligand binding technique is an important area of study, the significance of this regulation is enhanced by the concurrent study of the function of those receptors. We have recently shown that the human adipocyte contains three different adrenergic receptors, each of which is coupled to a distinct biochemical response (30). Similarly, the rat submandibular gland appears to have three distinct adrenergic receptors. The *alpha*$_1$-adrenergic receptor can be labeled by both [^{3}H]WB4101 and [^{3}H]prazosin, and the effect of receptor stimulation can be studied *in vitro* by the release of potassium, whereas the *beta*$_1$-adrenergic receptor is coupled to adenylate cyclase. The functional significance of the *alpha*$_2$-adrenergic receptors labeled by [^{3}H]clonidine is not known at the present time. Stimulation of *alpha*$_2$-receptors did not appear to modify either potassium release or cyclic AMP levels in glands from adult animals. The finding of a large number of [^{3}H]clonidine binding sites in the submandibular glands of early postnatal rats suggests that these receptors may fulfill a developmental function.[3] Inhibition of norepinephrine release in the submandibular gland is apparently mediated by adrenergic receptors having the pharmacological properties of the *alpha*$_2$-subtype. However, these receptors appear to be presynaptic (5) and apparently are not the same as those postsynaptic receptors (31) which are labeled by the currently available radioligands for *alpha*$_2$-adrenergic receptors.

[3] D. B. Bylund and J. R. Martinez, unpublished observations.

The identification of potassium release as a function for *alpha*$_1$-adrenergic receptors which are labeled by [^{3}H]WB4101 and [^{3}H]prazosin is based on two observations. First is the equivalent pharmacology in the two systems. In the binding assay, both the *alpha*$_2$-agonist clonidine and the *alpha*$_2$-antagonist yohimbine were relatively weak, whereas the *alpha*$_1$-antagonist prazosin was extremely potent. Methoxamine, an *alpha*$_1$-agonist, was effective in stimulating potassium release whereas clonidine was ineffective. Furthermore, prazosin was an effective inhibitor of the release elicited by norepinephrine, but yohimbine was not. Second, both the binding and potassium release were increased in roughly proportional amounts following reserpine treatment. For example, the density of receptor binding sites expressed as picomoles per gram of tissue increased 55% following reserpine treatment, while the release of potassium (in excess of the basal) increased approximately 56% when norepinephrine was used as the secretagogue and 37% when methoxamine was used. However, direct comparisons of this type may not be entirely valid since reserpine may have additional effects on the cell which might modify the release of potassium. For example, the density of muscarinic cholinergic receptors did not change following reserpine treatment, although in a previous study of the glands of animals given reserpine we have shown a slight enhancement of potassium release in response to carbachol, a muscarinic agonist (23). The effect of carbachol in that study was 31% greater in animals treated with reserpine, and the effect of norepinephrine was 75% greater. These data suggest that reserpine may increase potassium release both by increasing *alpha*$_1$-adrenergic receptors and by another mechanism which is independent of the receptor.

The studies on the *beta*-adrenergic receptor reported here are in good agreement with previous reports (15–18). Two of these studies have also included information on *alpha*-adrenergic receptors. Pointon and Banerjee (17) reported a 51% increase in the binding of [^{3}H]dihydroergocryptine to submandibular gland membranes following chronic reserpine treatment. However, they made no attempt to differentiate between the *alpha*$_1$- and *alpha*$_2$-subtypes. If it is assumed that [^{3}H]dihydroergocryptine labels both the *alpha*$_1$- and

TABLE 11

Effect of unilateral superior cervical ganglionectomy on submandibular muscarinic cholinergic receptors

[^{3}H]QNB was the radioligand.

Treatment	*n*	K_D	B_{max}		
			pmoles/g tissue	pmoles/g protein	pmoles/gland
		nM			
Control	6	0.45 ± 0.05	12.3 ± 0.6	267 ± 27	2.18 ± 0.06
Denervated	6	0.39 ± 0.04	10.6 ± 0.9	236 ± 17	1.79 ± 0.15
p		> 0.1	> 0.1	> 0.1	> 0.05

alpha$_2$-receptor sites labeled by [^{3}H]WB4101 and [^{3}H]clonidine in our studies, then their 50% increase can be compared with approximately the 160% increase which we have observed in total *alpha*-adrenergic receptor binding. The quantitative difference in the increase between these two studies may be partially accounted for by the use of female Wistar rats in the study by Pointon and Banerjee (17) and male Sprague-Dawley rats in our study. Arnett and Davis (18) also studied the binding of [^{3}H]dihydroergocryptine to various particulate fractions of rat submandibular glands in both control and denervated rats. On the basis of the K_D values for various adrenergic drugs in inhibiting the binding of the radioligands it was concluded that the *alpha*-receptors labeled by [^{3}H]dihydroergocryptine were of the *alpha*$_2$-subtype. This conclusion is difficult to reconcile with the fact that Arnett and Davis (18) found WB4101 to be equipotent with yohimbine in inhibiting the binding of the radioligand and that prazosin was only 8-fold less potent than these antagonists. By contrast, our results show a greater than 3000-fold specificity of prazosin as compared with yohimbine for the *alpha*$_1$-adrenergic site and an 80-fold specificity of yohimbine over prazosin for the *alpha*$_2$-adrenergic site. In addition, our studies of potassium release clearly indicate the presence of an *alpha*$_1$-adrenergic receptor mediating that function.

The increase in *beta*-adrenergic receptors induced by reserpine administration or by sympathetic denervation does not appear to be related to an increase in the activity of adenylate cyclase (16). However, the formation of cyclic AMP was found to be enhanced after these two types of treatment, as a result of reduced activity in the cyclic nucleotide phosphodiesterase (16). It appears, therefore, that changes in the sympathetic input can result not only in alterations in the number of adrenergic receptors, but also in disturbances in the activity of enzymes involved in the regulation of intracellular mediators of the stimulus secretion coupling mechanism.

The physiological significance of drug-induced alterations in adrenergic receptors of the rat submandibular gland can be partially ascertained by the multiple alterations in secretory activity, both *in vivo*, and *in vitro*, observed after chronic reserpine administration (10, 23). Disturbances in fluid, electrolyte, and macromolecule secretion are accompanied by marked morphological changes and by changes in tissue composition. Some of these abnormalities are likely related to changes in Ca^{2+} homeostasis and in cyclic nucleotide metabolism. A normal sympathetic input is clearly important, therefore, in maintaining the physiological integrity and the secretory function of salivary glands.

TABLE 12

Comparison of [^{3}H]DHA and [^{3}H]QNB binding in glands from female and male rats

[^{3}H]DHA and [^{3}H]QNB saturation experiments were performed using the same glands from 11-week-old animals that were littermates. The mean (± standard error of the mean) body weights (in grams), gland weights (for the pair), and ratio of gland weight to body weight were 229 ± 2, 0.36 ± 0.01, and 0.0016 ± 0.0001 for females and 336 ± 11, 0.49 ± 0.02, and 0.0015 ± 0.0001 for males, respectively.

	n	K_D	B_{max}		
			pmoles/g tissue	pmoles/g protein	pmoles/gland
		nM			
[^{3}H]DHA					
Female	4	0.72 ± 0.10	18.0 ± 2.6	339 ± 22	3.3 ± 0.3
Male	4	0.89 ± 0.10	11.9 ± 1.4	240 ± 22	2.9 ± 0.3
p		>0.1	<0.05	<0.025	>0.1
[^{3}H]QNB					
Female	4	0.33 ± 0.05	14.3 ± 0.5	272 ± 13	2.6 ± 0.1
Male	4	0.41 ± 0.06	11.0 ± 0.7	222 ± 11	2.7 ± 0.1
p		>0.1	<0.005	<0.05	>0.1

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