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POSTSYNAPTIC LOCALIZATION OF α_2-ADRENERGIC RECEPTORS IN RAT SUBMANDIBULAR GLAND[1]

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Abstract

Norepinephrine is known to inhibit its own release from presynaptic nerve terminals through α_2-adrenergic receptors, which presumably have a presynaptic localization. α_2-Adrenergic receptors (as determined by [^{3}H]clonidine binding) appear in rat submandibular gland membranes following reserpine treatment. These α_2 receptors seem to be localized postsynaptically, based on the following evidence. (*1*) Partial destruction of the presynaptic nerve terminals with 6-hydroxydopamine did not decrease the density of α_2-adrenergic receptors following subsequent reserpine administration. (*2*) Duct ligation, which results in atrophy of the gland, markedly decreased the density of the receptors following subsequent reserpine administration. (*3*) Surgical denervation resulted in the appearance of high levels of α_2-adrenergic receptors. (*4*) The changes in α_2 receptors paralleled the changes in postsynaptic β-adrenergic receptor binding (as determined by [^{3}H]dihydroalprenolol). While these results establish the existence of postsynaptic α_2-adrenergic receptors in an innervated tissue, the concomitant presence of a low density of presynaptic α_2 receptors has not been eliminated.

Several decades ago, Ahlquist (1948) proposed that the cellular effects of catecholamines could be divided into two groups, which he designated as α and β, according to the potencies of various analogs in interacting with the proposed receptors. Subsequently, Lands et al. (1967) suggested that β receptors could be divided further into two groups, β_1 and β_2. More recently, α receptors also have been subclassified into α_1 and α_2 subtypes. Initially, this subclassification was based on the presumed anatomic localization of the receptors. α_1-Adrenergic receptors were designated as the receptors mediating the classical postsynaptic action of norepinephrine, while the receptors which mediate the presynaptic inhibition of norepinephrine release were called α_2 receptors (Langer, 1977; Starke, 1977). However, it has since become apparent that some α receptors, such as those on human platelets (Hoffman et al., 1979) and adipocytes (Burns et al., 1981), which have the pharmacologic characteristic of α_2 receptors are not presynaptic receptors. Thus, a classification based on function has been developed: α_1 receptors mediate excitatory functions, while α_2 receptors are inhibitory (Berthelson and Pettinger, 1977).

While this definition of α_1 and α_2 receptors appears reasonable, the localization of α_2 receptors in innervated tissues still remains to be answered. Functional studies, such as those involving the release of [^{3}H]norepinephrine, indicate a presynaptic localization for the α_2 receptor (Langer, 1977; Starke, 1977). On the other hand, binding studies have indicated that most, if not all, of the α_2 receptors may be postsynaptic (U'Prichard et al., 1979, 1980). In these studies, the density of α_2 receptors is determined following chemical or surgical denervation. If the α_2 receptors labeled in these binding studies are presynaptic, it would be expected that their density would decrease. However, this does not appear to happen; it is thought, therefore, that these α_2 receptors are postsynaptic. The objection to this approach is the possibility that presynaptic membrane fragments containing the α_2 receptors may adhere to the postsynaptic cell. Recently, we have found in the rat submandibular gland that α_2 receptor sites (as determined by [^{3}H]clonidine binding), which are absent in control tissue, appear following reserpine administration (Bylund and Martinez, 1980a). This system eliminates the possibility of [^{3}H]-clonidine binding to α_2 receptors on presynaptic membranes following chemical or surgical denervation. Preliminary studies using this experimental design have indicated the possibility of postsynaptic α_2 receptors in this tissue (Bylund and Martinez, 1980b; Pimoule et al., 1980). The results reported here provide definitive evidence for the postsynaptic localization of α_2 receptors in innervated tissues.

Materials and Methods

Materials. [^{3}H]Dihydroalprenolol (DHA) (47 to 58 Ci/mmol), [^{3}H]clonidine (22.2 Ci/mmol), and [^{3}H]norepi-

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nephrine (30.6 Ci/mmol) were purchased from New England Nuclear. Reserpine and 6-hydroxydopamine hydrobromide were obtained from Sigma Chemical Co. The electrochemical detector used for the determination of norepinephrine levels was purchased from Bioanalytical Systems.

Animals. Male Sprague-Dawley rats (175 to 225 gm) were subjected to one or more of the following treatments. (*1*) Catecholamines were depleted by seven daily injections of 0.5 mg/kg of reserpine (intraperitoneally). (*2*) A partial chemical sympathectomy (Thoenen and Tranzer, 1968) was produced by two intravenous injections of 6-hydroxydopamine (34 mg/kg, free base, dissolved in a solution of 0.1% ascorbic acid and 0.95% saline) within a 16- to 24-hr period followed, a week later, by two additional injections (68 mg/kg). (*3*) The main excretory duct was ligated unilaterally. (*4*) Rats with unilateral superior cervical ganglionectomy were obtained from Zivic-Miller Laboratories, Inc., Allison Park, PA. Rats were sacrificed by decapitation 2 weeks after the first 6-hydroxydopamine injection or duct ligation, 1 week after the ganglionectomy, or 1 day after the final reserpine injection. For rats receiving a combination of treatments, reserpine administration was started 1 week after the first 6-hydroxydopamine injection or duct ligation. In the experiments using ligated and ganglionectomized animals, the contralateral glands were used as controls for these procedures. Uninjected animals were used as controls for reserpine and 6-hydroxydopamine treatments.

Radioligand binding assays. The rats were anesthetized with phenobarbital (5 mg/kg), and the submandibular glands were removed rapidly and separated from the adjoining sublingual glands. Glands were weighed, cooled in ice cold 0.9% saline, and homogenized in 50 mM Tris-HCl, pH 8.0 (Tissumizer, setting 80, 30 sec). The homogenate was centrifuged for 10 min at 49,000 × *g*, resuspended in buffer, and centrifuged again. The pellet, a crude particulate fraction, then was resuspended, generally at 100 vol, and used for the adrenergic receptor binding assays. α_2-Adrenergic and β-adrenergic receptors were assayed as previously described (Bylund and Martinez, 1980a; Bylund and Snyder, 1976).

Norepinephrine assay. Submandibular glands were removed rapidly and frozen with tongs cooled with dry ice. The glands were weighed and then stored at −70°C until assayed. The norepinephrine content of the gland was determined as previously described (Felice et al., 1978). Briefly, the glands were homogenized in 50 mM $HClO_4$ and centrifuged. The supernatant was neutralized and alumina was added. The alumina then was washed and 50 mM $HClO_4$ was added to elute the absorbed catecholamines. The catecholamines were separated by high pressure liquid chromatography using a C_{18} reverse phase column and quantitated using an electrochemical detector.

Norepinephrine uptake. Rats were injected (intravenously, tail vein) with 50 μCi of [^{3}H]norepinephrine (diluted in saline) and sacrificed 30 min later. The salivary glands were removed and homogenized in 0.04 N $HClO_4$. The homogenate was treated with alumina as indicated above for the determination of norepinephrine levels except that the radioactivity of the $HClO_4$ eluate was determined by scintillation spectroscopy at 25% efficiency.

Results

In our first set of experiments, we compared the effects of reserpine and 6-hydroxydopamine administration on [^{3}H]clonidine binding to a particulate fraction of rat submandibular gland (Table I). Since there is a decrease in gland weight following reserpine administration, B_{max} data are presented as the density of binding sites (picomoles per gm of tissue and femtomoles per mg of protein) and as the number of binding sites (picomoles per gland). We also studied [^{3}H]dihydroalprenolol binding to postsynaptic β-adrenergic receptors so that the effect of the various procedures on α_2- and β-adrenergic receptors could be compared. One week of reserpine administration, which reduced glandular norepinephrine over 90%

TABLE I

Effect of 6-hydroxydopamine and reserpine administration on adrenergic receptor binding in rat submandibular gland

Submandibular glands were removed from control and treated rats, and a crude particulate fraction was prepared by homogenization and centrifugation. B_{max} and K_D values were determined from saturation experiments using [^{3}H]clonidine and [^{3}H]DHA to label α_2- and β-adrenergic receptors, respectively. Rats were killed 2 weeks after the start of 6-hydroxydopamine treatment and 1 week after the start of reserpine treatment.

^{3}H-Ligand	Control	6-Hydroxydopamine	Reserpine	6-Hydroxydopamine and Reserpine
[^{3}H]Clonidine, *n*	5	11	3	7
K_D, nM	N.D.[a]	N.D.	2.8 ± 0.5	2.4 ± 0.2
B_{max}				
Picomoles per gm of tissue	0.6 ± 0.2	1.4 ± 0.2	4.5 ± 0.3	4.7 ± 0.4
Femtomoles per mg of protein	11 ± 3	24 ± 4	83 ± 12	82 ± 8.9
Picomoles per gland	0.15 ± 0.05	0.31 ± 0.06	0.75 ± 0.18	0.67 ± 0.7
[^{3}H]Dihydroalprenolol, *n*	14	15	6	6
K_D, nM	0.79 ± 0.10	0.69 ± 0.04	0.70 ± 0.05	0.70 ± 0.07
B_{max}				
Picomoles per gm of tissue	11.7 ± 1.0	14.0 ± 0.9	21.5 ± 1.2	24.6 ± 1.0
Femtomoles per mg of protein	232 ± 23	282 ± 22	563 ± 97	619 ± 84
Picomoles per gland	2.7 ± 0.2	3.1 ± 0.2	3.5 ± 0.2	3.5 ± 0.2

[a] A meaningful K_D could not be determined due to the low level of specific binding (Bylund and Martinez, 1980a, b). The B_{max} was estimated from saturation experiments by assuming a K_D of 2.0 nM.

(from 1.62 ± 0.14 μg/gm to 0.12 ± 0.02 μg/gm), resulted in a large increase in α_2 receptor binding, as we have reported previously (Bylund and Martinez, 1980a, b), as well as approximately doubling the density of β receptors. The administration of 6-hydroxydopamine resulted in an apparent partial sympathectomy of the submandibular gland as indicated by a 70% decrease in the amount of norepinephrine in the glands (to 0.49 ± 0.03 μg/gm). Following 6-hydroxydopamine administration, the density of both α_2- and β-adrenergic receptors was increased only slightly (Table I). If the α_2 receptors labeled by [^{3}H]clonidine have a presynaptic localization, then reserpine administration following the partial sympathectomy with 6-hydroxydopamine should cause the appearance of significantly fewer α_2 receptors than reserpine administration alone. Since the density of α_2 receptors after the combination of treatments was essentially identical to that observed with reserpine alone, it would appear that these receptors could not have a presynaptic localization.

In our second series of experiments, we determined the effect of a postsynaptic modification on receptor binding using a procedure that does not alter presynaptic function. This was done by ligating the main excretory duct of the gland which results in a 65% reduction in gland weight but no loss in amount of norepinephrine in the gland (Anden et al., 1966). Two weeks after ligation, the number of α_2-adrenergic receptors remained at the essentially undetectable levels of the control contralateral gland, while the β-adrenergic receptor levels decreased dramatically (Table II). Reserpine administration, during the 2nd week after duct ligation, increased the number of α_2 receptors in the ligated gland to only 15% of the number of the receptors in the contralateral (unligated) gland. These data indicate a postsynaptic localization for the α_2-adrenergic receptors.

For our third series of experiments, the effect of unilateral superior cervical ganglionectomy was studied. This procedure resulted in a relatively complete denervation as indicated by a decrease of 93% in the amount of [^{3}H]norepinephrine accumulated by the gland (25,900 ± 3,200 dpm/gm of tissue in the denervated gland versus 370,000 ± 74,000 dpm/gm in the contralateral gland, n = 7). Both α_2- and β-adrenergic receptors increased markedly 1 week after surgical denervation (Table III), indicating that α_2 receptors could not be located on adrenergic nerve terminals.

Discussion

The rat submandibular gland provides an excellent model system for the study of the localization of autonomic receptors since the postsynaptic and presynaptic components can be lesioned selectively. Control submandibular glands lack detectable α_2-adrenergic receptors,

TABLE III

Effect of unilateral superior cervical ganglionectomy on adrenergic receptor binding in rat submandibular gland

The right (control) and left (denervated) submandibular glands were removed 1 week after unilateral superior cervical ganglionectomy, and a crude particulate fraction was prepared by homogenization and centrifugation. B_{max} and K_D values were determined from saturation experiments using [^{3}H]clonidine and [^{3}H]DHA to label α_2- and β-adrenergic receptors, respectively. [^{3}H]DHA data are taken from Bylund et al. (1981).

^{3}H-Ligand	Control Gland	Denervated Gland
[^{3}H]Clonidine, n	6	6
K_D, nM	N.D.[a]	2.4 ± 0.3
B_{max}		
Picomoles per gm of tissue	1.6 ± 0.4	7.6 ± 0.9
Femtomoles per mg of protein	29 ± 7	122 ± 13
Picomoles per gland	0.3 ± 0.1	1.3 ± 0.2
[^{3}H]Dihydroalprenolol, n	6	6
K_D, nM	0.60 ± 0.03	0.55 ± 0.06
B_{max}		
Picomoles per gm of tissue	12.7 ± 1.8	20.8 ± 2.2
Femtomoles per mg of protein	180 ± 12	282 ± 17
Picomoles per gland	2.3 ± 0.2	3.9 ± 0.3

[a] N.D., not determined.

TABLE II

Effect of unilateral ligation of the main excretory duct of the submandibular gland and of reserpine administration on adrenergic receptor binding

Submandibular glands were removed from control and treated rats, and a crude particulate fraction was prepared by homogenization and centrifugation. B_{max} and K_D values were determined from saturation experiments using [^{3}H]DHA to label α_2- and β-adrenergic receptors, respectively. Rats were killed 2 weeks after ligation of the excretory duct and 1 week after the start of reserpine treatment.

^{3}H-Ligand	Control	Ligation	Reserpine	Reserpine + Ligation
[^{3}H]Clonidine, n	3	3	5	4
K_D, nM	N.D.[a]	N.D.	2.9 ± 0.2	N.D.
B_{max}				
Picomoles per gm of tissue	0.5 ± 0.1	0.2 ± 0.1	4.1 ± 0.4	1.5 ± 0.3
Femtomoles per mg of protein	9 ± 2	11 ± 5	95 ± 8	60 ± 4
Picomoles per gland	0.13 ± 0.03	0.02 ± 0.01	0.82 ± 0.06	0.12 ± 0.03
[^{3}H]Dihydroalprenolol, n	4	4	6	3
K_D, nM	0.94 ± 0.05	0.64 ± 0.16	0.79 ± 0.07	0.62 ± 0.12
B_{max}				
Picomoles per gm of tissue	11.0 ± 1.1	2.6 ± 0.8	18.0 ± 1.5	4.0 ± 0.9
Femtomoles per mg of protein	205 ± 22	111 ± 25	610 ± 130	264 ± 34
Picomoles per gland	2.8 ± 0.2	0.2 ± 1	3.3 ± 0.2	0.3 ± 0.1

[a] N.D., not determined.

but, in glands from reserpine-treated animals, the level of these receptors is comparable to that of the postsynaptic α_1 receptor. These α_2-adrenergic receptors have the expected pharmacologic specificity since yohimbine (an α_2 antagonist) is 80-fold more potent than prazosin (an α_1 antagonist) in inhibiting [^{3}H]clonidine binding (Bylund and Martinez, 1980a) and prazosin is 14,000 times more potent at α_1 receptors (labeled by [^{3}H]WB4101) than α_2 receptors in this tissue. A major objection to previous studies on the localization of receptors by binding studies is that the observed binding still could be presynaptic even after chemical or surgical denervation. For example, Arnett and Davis (1979), in reference to the rat submandibular gland, state that "the possibility that some presynaptic membrane persists after degeneration of the nerve terminal cannot be excluded." Furthermore, several groups have presented evidence that receptor binding sites in membranes from lesioned neurons may persist for 21 to 48 days following the lesion (Olney and deGubareff, 1978; Fields et al., 1978). In the present study, the lack of α_2 receptors in the control submandibular gland overcomes this objection.

The data in Tables I and III, which show an increase in α_2 receptor binding following a partial chemical sympathectomy and reserpine administration or following surgical denervation, indicate that the observed binding cannot be localized on adrenergic nerve terminals and, therefore, must be either postsynaptic or presynaptic on non-adrenergic nerves. The studies with ligated glands (Table II) indicate that these receptors are not localized on either cholinergic or adrenergic nerve terminals since previous studies have shown that these nerve tracts are unaltered by ligation (Anden et al., 1966; Ohlin and Perec, 1967) but apparently are localized on the acinar cells since these cells undergo atrophy following duct ligation. Furthermore, the alterations in α_2 receptor binding with the various procedures paralleled the alterations in the binding of [^{3}H]dihydroalprenolol to postsynaptic β_1-adrenergic receptors. In agreement with this conclusion, a recent preliminary report has suggested a postsynaptic location of α_2 receptors in vascular smooth muscle (Hamilton and Reid, 1980).

Our results on the β-adrenergic receptor binding following chemical and surgical denervation are in agreement with previous studies (Arnett and Davis, 1979; Pointon and Banerjee, 1979). Similarly, an increase in α-adrenergic binding following denervation has been reported also (Arnett and Davis, 1979; Pointon and Banerjee, 1979). However, the non-selective α drug [^{3}H]dihydroergocryptine was used in these studies and since the relative contribution of α_1 and α_2 receptors to the total binding was not determined, it is not possible to compare their results with ours.

The design of our experiments is partially dependent on the lack of significant binding of [^{3}H]clonidine in the control submandibular gland using our experimental conditions. It is possible that, under other experimental conditions, the binding of [^{3}H]clonidine would be observed. However, neither changes in pH (7.0 to 8.0), buffer (sodium potassium phosphate, glycylglycine), guanosine 5′-triphosphate (1 to 100 μM), nor ion concentration (0 to 10 mM Mg_2Cl, 0 to 100 mM NaCl, or 0 to 100 mM KCl) improved the binding (D. B. Bylund and J. R. Martinez, submitted for publication). We also have been unable to observe any significant binding of *p*-[^{3}H]aminoclonidine. Another possibility would be that all of the receptors in the control gland are in the "low affinity" state, which would not be labeled by agonists and that the various treatments convert them to the "high affinity" state which would be labeled by agonists. If this were true, then the binding of an antagonist, such as [^{3}H]-yohimbine, which should bind to both states of the receptor, should be observed. However, we have been unable to demonstrate [^{3}H]yohimbine binding from either control or reserpine-treated animal glands under any of the experimental conditions mentioned above. Finally, the submandibular gland has a relatively high norepinephrine content which could prevent the binding of α_2 radioligands if the particulate fraction used for the assays were not washed sufficiently. However, [^{3}H]clonidine binding was not observed after repeated washing at 4°C with intermediate incubations at 37°C. In addition, binding of α_1 and β antagonist ligands is readily observable in our standard tissue preparation, which indicates that sufficient norepinephrine has been removed to allow at least [^{3}H]yohimbine binding to α_2 receptors if they were present.

These studies do not provide information about the functional significance of these α_2 receptors. The appearance of these receptors probably is not just an experimental artifact, since high levels of α_2 receptors are found in glands from immature rats (D. B. Bylund, unpublished observation). One possibility is that the receptors may be negatively coupled to adenylate cyclase as is the case in the adipocyte and platelet (Burns et al., 1981; Grant and Scrutton, 1979). However, in preliminary studies, we have not been able to demonstrate an inhibitory effect on cyclic adenosine 3′:5′-monophosphate accumulation (D. B. Bylund and L. R. Forte, unpublished observations). We also have been unable to show any α_2 receptor-mediated effect on K^+ release from submandibular gland slices (J. R. Martinez, unpublished observation). On the other hand, the α_2-adrenergic inhibition of [^{3}H]norepinephrine release has been demonstrated in submandibular gland slices, and the receptors mediating this function were reported to be localized presynaptically (Filinger et al., 1978). If this is the case, then the α_2 receptors which mediate the inhibition of norepinephrine release either (*1*) are not labeled by [^{3}H]clonidine or (*2*) are not sufficiently dense to be detected by the present binding assay techniques. On the other hand, it recently has been suggested that the α-adrenergic inhibition of norepinephrine release is mediated trans-synaptically (Manukhin and Volina, 1979). According to this hypothesis, the activation of postsynaptic α_2 receptors results in the release of a factor (possibly prostaglandin E; Hedquist, 1974) which then diffuses across the synaptic gap and acts presynaptically. Thus, postsynaptic α_2 receptors could mediate presynaptic function.

In conclusion, these studies demonstrate the postsynaptic localization in submandibular gland of the α_2-adrenergic receptors which appear following reserpine treatment or surgical denervation. Thus, at least some innervated, as well as noninnervated, tissues contain α_2-adren-

ergic receptors which do not have a presynaptic localization.

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