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DOPAMINE D2 RECEPTOR BINDING IN ADRENAL MEDULLA: CHARACTERIZATION USING [^{3}H]SPIPERONE

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Abstract—The possibility that dopamine may function as a neuromodulator or neurotransmitter in the adrenal gland, and not merely serve as a precursor to the catecholamines, has been suggested. If this hypothesis is correct, receptors for dopamine should be identifiable in the adrenal. The present work demonstrates the existence of a high-affinity receptor in adrenal medulla using [^{3}H]spiperone as the radioligand to label the receptors. [^{3}H]Spiperone bound rapidly, reversibly, and with high affinity to bovine adrenal medullary membranes. Scatchard analysis yielded a K_d of 0.09 nM and a B_{max} of 51 fmol/mg protein. In competition binding experiments, dopaminergic antagonists were at least 100 times more potent in displacing [^{3}H]spiperone from its binding sites than adrenergic or serotonergic receptor antagonists. Similarly, agonists at the dopamine receptor more readily competed for [^{3}H]spiperone binding than other receptor agonist drugs tested. Furthermore, D2 selective antagonists and agonists were much more potent than D1 receptor ligands. These results suggest that [^{3}H]spiperone may bind to a high-affinity D2 dopamine receptor in adrenal medulla.

That dopamine acts as a neurotransmitter or possibly a neuromodulator in specific brain regions in the central nervous system, in addition to serving a precursor role for the catecholamines, is now well established. Moreover, dopamine also appears to function in its own right in a wide variety of tissues in the peripheral nervous system [1–5]. One such peripheral tissue which responds to dopamine is the adrenal medulla. Functional studies performed by Artalejo *et al.* [6] showed that dopaminergic agonists inhibit nicotine-evoked catecholamine release from adrenal medullary chromaffin cells, whereas dopamine antagonists enhance amine secretion. As well, it was demonstrated that dopamine could be released from adrenal medullary cells [6]. These observations could imply that dopamine receptors are present on adrenal medullary chromaffin cells.

The present experiments were done to determine whether dopamine receptors could be detected in the adrenal medulla using receptor binding techniques. A high-affinity site with the characteristics of a D2 dopamine receptor could be identified using the radioligand [^{3}H]spiperone.

METHODS

Tissue preparation. Fresh bovine adrenal glands were obtained from the slaughterhouse and immediately placed on ice after removal from the animal. The medulla, which was dissected from the cortex, was homogenized in 5 vol. of 0.32 M sucrose using a glass homogenizer with a motor-driven Teflon pestle. This was followed by centrifugation of the homogenate at 750 *g* for 15 min; the pellet was subsequently discarded and the supernatant fraction retained. To the supernatant was added 50 mM Tris·HCl, pH 7.4, such that the concentration of original tissue in buffer was 1 g/25 ml. After centrifugation at 45,000 *g*/15 min, the supernatant fractions were discarded and the pellets stored at −70° until use. Storage of the pellets for up to 2 months did not result in any appreciable loss in binding of the radioligand.

Before assay, the above pellets were resuspended in cold H_2O with a Brinkmann Polytron at setting no. 7 for 10 sec (1 g original tissue in 25 ml). The suspension was centrifuged at 45,000 *g* for 15 min; the pellet was retained, resuspended in 50 mM Tris·HCl, pH 7.4 (1 g/25 ml) and frozen at −70° for 1 hr. After thawing, the membranes were centrifuged at 45,000 *g* for 15 min. The final pellets were resuspended with a Polytron in incubation buffer (1 g original tissue in 40 ml) consisting of (in mM): Tris, 50; NaCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; 0.1% ascorbic acid and 1 μM pargyline. The buffer was adjusted to pH 7.4 with HCl.

During the course of the tissue preparation, that is, the various centrifugations and other steps discussed above, 1 g original adrenal medullary tissue yielded approximately 10 mg membrane protein.

[^{3}H]*Spiperone binding.* Binding of [^{3}H]spiperone to adrenal medullary membranes was done as previously outlined [7] with modifications as described below. A 2.7-ml aliquot of adrenal medullary membranes (approximately 0.6 mg protein) was incubated for 10 min at 37° with 0.25 nM [^{3}H]spiperone, unless otherwise indicated, in the absence or presence of various drugs; each assay tube contained a final volume of 3 ml. To determine nonspecific bind-

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ing (+)-butaclamol at a concentration of 3×10^{-6} M was used. At the concentration of [^{3}H]spiperone (0.25 nM) routinely used, the nonspecific binding was approximately 10% of the total; the nonspecific binding increased to about 40% of total binding at 1.0 nM [^{3}H]spiperone. The reaction was stopped by filtration through Whatman GF/C glass fiber filters, followed by two washes of 5 ml of cold 50 mM Tris·HCl, pH 7.4. The filters were allowed to air dry for 1 hr and counted in a liquid scintillation counter 6 hr after addition of 7 ml of scintillation fluid.

Subcellular fractionation. Subcellular fractions were prepared as previously described for adrenal medulla [8] with modifications as detailed below. Adrenal medulla (30 g) were homogenized in 8 vol. of 0.32 M sucrose containing 10 mM Tris·HCl, pH 7.4, using a glass homogenizer with a motor-driven Teflon pestle (2000 rpm, 7 strokes). The homogenate was centrifuged at 1000 *g* for 10 min; the supernatant (S_1) was further fractionated while the pellet (P_1) was used only to test for the presence of [^{3}H]spiperone binding sites. The P_2 pellet was obtained by centrifugation of S_1 at 12,000 *g* for 10 min; further fractionation of P_2 was achieved by centrifugation on a discontinuous sucrose gradient. The pellet was resuspended in 9 ml of 0.32 M sucrose and was layered over a gradient consisting of equal layers (9 ml) of 0.8 M and 1.2 M sucrose. The gradient was centrifuged at 80,000 *g* for 75 min using a swinging bucket rotor. After centrifugation the various fractions were collected. Each fraction was diluted in 50 mM Tris·HCl, pH 7.4, and centrifuged at 100,000 *g* for 45 min. The supernatant fractions were discarded and the pellets stored at −70°. They were further processed as detailed in "tissue preparation" before the [^{3}H]spiperone binding assay; the assay was done as described.

Protein determinations. Protein was determined according to Lowry *et al.* [9] using bovine serum albumin as standard.

Data analysis. Equilibrium binding data were analyzed using a computerized method called LIGAND [10]. The IC_{50} values for 50% inhibition of [^{3}H]spiperone binding were estimated by linear regression analysis of log-logit plots. Inhibition constants (K_i) were calculated using the equation, $K_i = IC_{50}/(1 + [^3H]\text{spiperone}/K_d)$. Statistical comparisons were made using Student's *t*-test.

Materials. [^{3}H]Spiperone (spiperone, [benzene ring-^{3}H], 24.8 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Ascorbic acid, 5′-guanylylimidophosphate, guanosine 5′-triphosphate (GTP), trypsin (EC 3.4.21.4, from bovine pancreas type XI), papain (EC 3.4.22.2, papainase from *Papaya* latex type III), pepsin (EC 3.4.23.1, from porcine stomach mucosa), neuraminidase (from *Clostridium perfringens* type V), phospholipase A_2 (EC 3.1.1.4, lecithinase A from bee venom), and phospholipase C (lecithinase C from *Cl. perfringens* type I) were obtained from the Sigma Chemical Co., St. Louis, MO.

RESULTS

Effect of incubation buffer on the binding of [^{3}H]spiperone to an adrenal medullary or adrenal cortical membrane preparation. Experiments were done to determine the optimal incubation buffer for the binding of [^{3}H]spiperone to adrenal medullary membranes. The following buffers were tested: (1) 50 mM Tris, pH 7.4, (2) 50 mM Tris–salt, pH 7.4 [containing (in mM) NaCl, 120; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; 0.1% ascorbic acid and 1 μM pargyline] and (3) 50 mM Tris–120 mM NaCl. From Table 1 it is evident that, for the adrenal medullary preparation, the nonspecific binding defined using 3×10^{-6} M (+)-butaclamol was much lower when the Tris–salt buffer was used; specific binding was similar with either buffer. The Tris–NaCl buffer gave a value for nonspecific binding higher than the Tris–salt buffer, although the specific binding was the same (data not shown).

In the preparation of the adrenal medullary tissue, care was taken to dissect all adrenal cortical tissue from the medulla. To ensure that the [^{3}H]spiperone binding observed to adrenal medullary membranes was not due to contamination from cortical tissue, [^{3}H]spiperone binding was measured to a preparation of cortical membranes. Table 1 shows that the number of specific binding sites is very small in cortical tissue, particularly in the Tris–salt buffer which was routinely used in all other experiments.

Characteristics of [^{3}H]spiperone binding to adrenal medullary membranes. Linearity with increasing membrane concentrations or protein content is depicted in Fig. 1. A protein concentration of approximately 0.6 mg/sample was used in all subsequent assays. No specific binding was observed

Table 1. Effect of incubation buffer on specific and nonspecific binding of [^{3}H]spiperone to an adrenal medullary or adrenal cortical membrane preparation

		[^{3}H]Spiperone binding (fmol/mg protein)	
Tissue	Buffer	Specific	Nonspecific
Medulla	Tris	25.6 ± 3.0	8.4 ± 2.2
	Tris–salt	24.3 ± 2.1	2.0 ± 0.7
Cortex	Tris	6.1 ± 2.6	18.9 ± 1.2
	Tris–salt	2.4 ± 0.8	12.3 ± 3.6

Binding of [^{3}H]spiperone (0.25 nM) to the membrane preparations (0.6 mg protein) was done as described except that for some of the experiments the final pellet was resuspended in 50 mM Tris, pH 7.4, rather than the usual Tris–salt buffer at pH 7.4, as indicated above in the table. Each value represents the mean ± SE of three to four separate experiments, each done in triplicate.

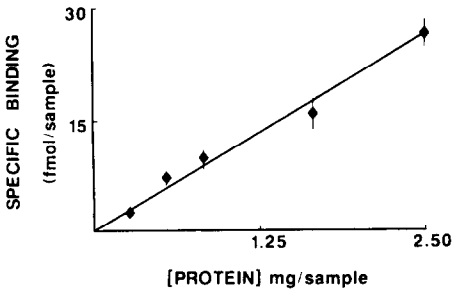

Fig. 1. Effect of increasing membrane concentration on the binding of [^{3}H]spiperone to adrenal medulla. Each symbol represents the mean ± SE of three to four separate experiments each done in triplicate. Where the SE is not depicted, it was less than 5% of the mean.

after treatment of the membranes at 90° for 15 min; this indicates that the receptor site was sensitive to heat and was probably denatured during the course of this treatment.

Equilibrium binding studies are shown in Fig. 2. The saturation isotherm is depicted in the top panel. Scatchard analysis of the saturation curve (B) yielded a K_d of 0.09 nM and a B_{max} of 51 fmol/mg protein. These results are representative of three experiments (N = 3; B_{max} = 43 ± 3.3 fmol/mg protein and K_d = 0.14 ± 0.03 nM).

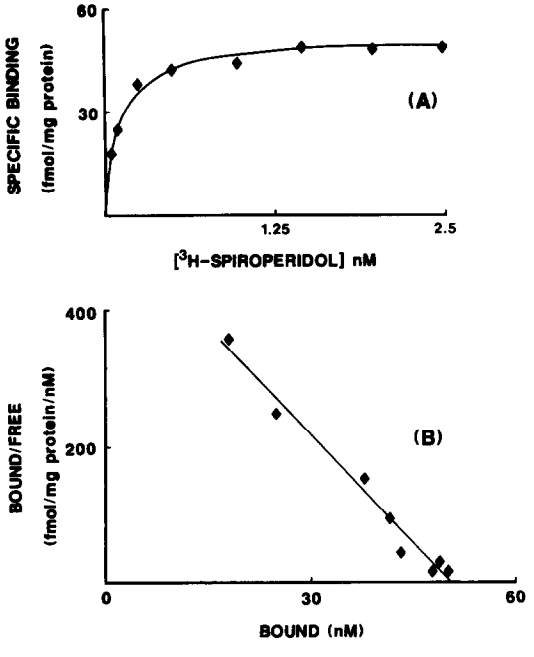

Fig. 2. Characteristics of [^{3}H]spiperone binding to adrenal medullary membranes. (A) Saturation curve of the binding of [^{3}H]spiperone ([^{3}H]spiroperidol; 0.05 to 2.5 nM) to adrenal medullary membranes. Membranes (0.6 mg protein) were incubated with the radioligand in the absence or presence of 3 × 10^{-6} M (+)-butaclamol for 10 min at 37° as described in Methods. Scatchard analysis of the data is depicted in (B). Apparent K_d (nM) and B_{max} (fmol/mg protein) values were determined by a computerized method (LIGAND). The results are representative of three separate experiments, each done in triplicate.

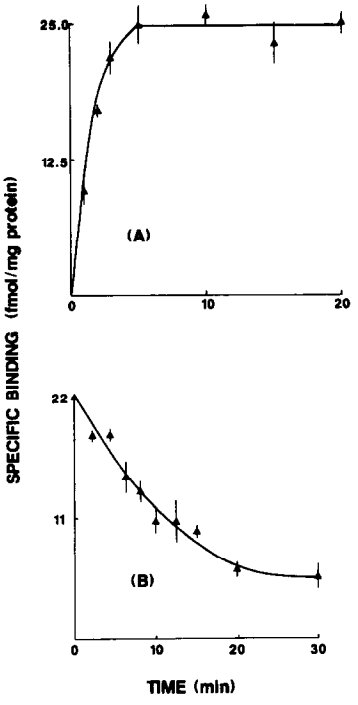

Fig. 3. Association and dissociation kinetics of [^{3}H]spiperone binding to adrenal medullary membranes. Association of the radioligand to the membranes, depicted in (A), was determined from the specific binding at successive incubation times from 1 to 20 min, terminated by rapid filtration. (B) Dissociation of the binding of [^{3}H]spiperone from adrenal medullary membranes was measured by first incubating for 10 min at 37° followed by addition of 3 × 10^{-6} M (+)-butaclamol to the assay tubes; specific binding was determined at successive times thereafter. The symbols represent the mean ± SE of three to five separate experiments, each done in triplicate.

Studies on the kinetics of [^{3}H]spiperone binding are presented in Fig. 3. The half time ($T_{\frac{1}{2}}$) of association was approximately 1.5 min and the binding reached equilibrium within approximately 5 min (Fig. 3A). The $T_{\frac{1}{2}}$ for dissociation was approximately 7 min and the dissociation curve reached a plateau within 20 min (Fig. 3B).

The abilities of various drugs to compete for the binding of [^{3}H]spiperone to its binding site were tested subsequently (Fig. 4 and Table 2). Dopaminergic antagonists were at least 100 times more potent in displacing [^{3}H]spiperone from its binding sites than adrenergic or serotonergic receptor antagonists. Dopaminergic agonists were not as potent as dopaminergic antagonists in competing for [^{3}H]spiperone binding; on the other hand, they were much more potent than other receptor agonist drugs in competing for binding of the radioligand. As well, D2 antagonists and agonists were more potent than the D1 antagonist SCH 23390 and the D1 agonist SKF 38393 in competition binding experiments, suggesting that [^{3}H]spiperone binds to a site with D2 dopamine receptor characteristics in adrenal medullary cells.

The competition curves in the presence of dopamine were biphasic; analysis of the data using

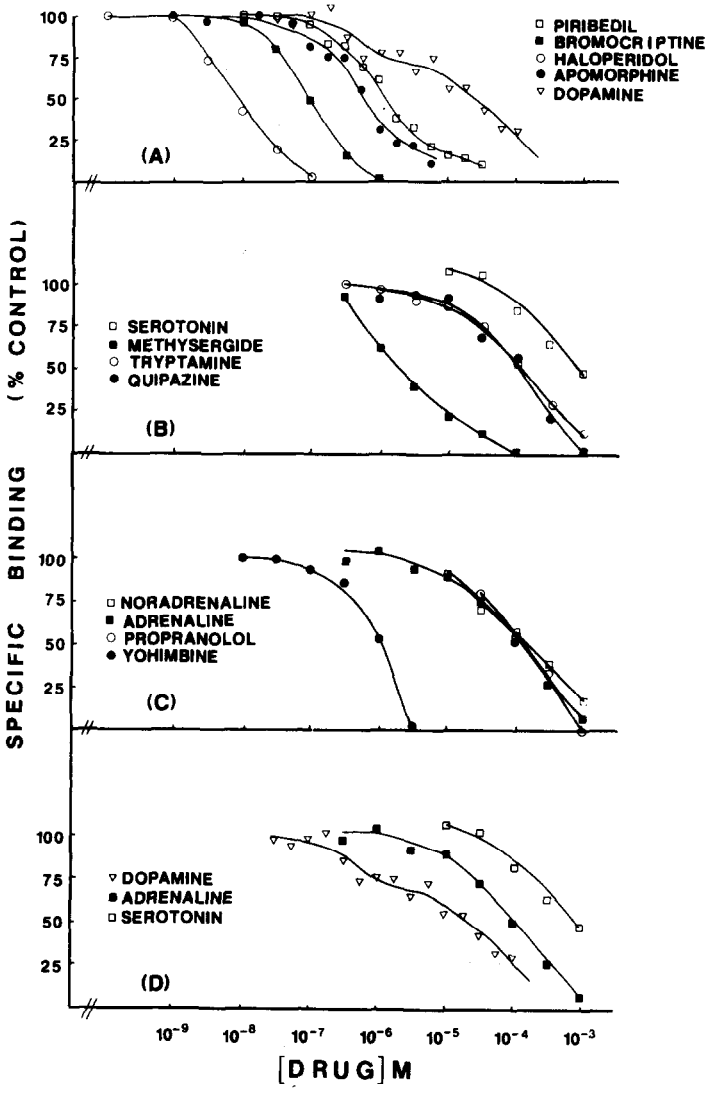

Fig. 4. Competition curves of different groups of drugs for the binding of [^{3}H]spiperone to adrenal medulla. Membranes (0.6 mg protein) were incubated with 0.25 nM [^{3}H]spiperone for 10 min in the presence of the indicated concentrations of drugs. Specific binding is expressed as a percentage of control binding. The curves shown are representative of three to seven separate experiments, each done in triplicate. See the legend of Table 4 for control values.

LIGAND indicated that a model for two binding sites fitted the data significantly better than a model for a single binding site ($F \approx 9.9$; $P < 0.01$). The K_d values for the high (K_H) and the low (K_L) affinity sites were 247 and 17,000 nM, while the $\%R_H = 38$. The effects of guanine nucleotides were tested subsequently on the [^{3}H]spiperone competition curves in the presence of various concentrations of dopamine. However, neither 5'-guanylylimidodiphosphate nor guanosine 5'-triphosphate (GTP), at a concentration of 100 μM, altered the shape of the dopamine competition curve in five separate experiments (results not shown).

Analysis of the competition curves in the presence of the dopamine agonists apomorphine and piribedil using LIGAND indicated that a model for a single site best fitted the data. The competition curves for dopamine and these agents as drawn in Fig. 4 were computer generated.

Subcellular distribution of the [^{3}H]*spiperone binding sites in adrenal medulla.* Subcellular fractionation studies showed that the highest concentration of sites was localized to the interface between the 0.32 M and the 0.8 M sucrose layer (Table 3). High densities of radioligand binding sites were also found in the 0.32 M and 0.8 M sucrose layers themselves, probably due to contamination from the interface between these two layers. These observations suggest that [^{3}H]spiperone binding sites are localized to a specific subcellular fraction and do not simply distribute uniformly throughout the various subcellular components in adrenal medulla.

Table 2. Inhibition of specific [^{3}H]spiperone binding in adrenal medulla by various classes of drugs

Drug	K_i (nM)
Dopaminergic antagonists	
Fluphenazine	0.1 ± 0.01
(+)-Butaclamol	0.9 ± 0.4
Domperidone	1.4 ± 0.3
Haloperidol	2.8 ± 0.9
SCH 23390	1,970 ± 520
Dopaminergic agonists	
Bromocriptine	38 ± 16
Apomorphine	42 ± 9
Piribedil	664 ± 137
SKF 38393	5,523 ± 840
Serotonergic drugs	
Methysergide	256 ± 58
Quipazine	16,300 ± 4,000
Tryptamine	81,000 ± 22,000
Serotonin	93,000 ± 19,000
Adrenergic drugs	
Yohimbine	495 ± 175
Propranolol	19,900 ± 4,950
Adrenaline	15,100 ± 1,700
Noradrenaline	37,300 ± 10,300

Binding of [^{3}H]spiperone (0.25 nM) to adrenal medulla membrane preparations was done as described in Methods. Membranes were incubated with four to fourteen different concentrations of the drugs for 10 min at 37°. The IC_{50} values were determined by log-logit analysis using linear regression analysis to fit the points to the best straight lines. The IC_{50} was then converted to an inhibitory constant (K_i) by using the equation $K_i = IC_{50}/(1 + [^3H]\text{spiperone}/K_d)$. Each value represents the mean ± SE of three to seven separate experiments, each done in triplicate.

Effects of various enzymic treatments on the binding of [^{3}H]spiperone to adrenal medullary membranes. Receptors are generally protein molecules, possibly with carbohydrate moieties attached, embedded in a lipid bilayer. To determine the importance of these various groupings in the binding of the radioligand to the receptor recognition site, the membranes were treated with enzymes which cleave these different molecular entities at various points. Two proteolytic enzymes were tested (Table 4). Trypsin, which acts upon peptide linkages involving the carboxyl groups of arginine and lysine, was found to result in a decrease in binding, whereas pepsin, which acts somewhat preferentially on peptide bonds involving aromatic amino acids such as phenylalanine, tryptophan and tyrosine, had no effect on [^{3}H]spiperone binding at concentrations of the enzyme up to 30 μg/ml. Two types of phospholipases were also tested. Phospholipase A_2, which catalyzes the fatty ester hydrolysis of 1,2-*sn*-diacylphosphoglycerides in the 2 position, markedly inhibited binding, whereas phospholipase C, which catalyzes cleavage of the bond between phosphoric acid and glycerol, had no effect at 30 μg/ml.

DISCUSSION

The adrenal medulla is embryologically derived from the neural crest and shares many properties in common with sympathetic neurons including its ability to synthesize catecholamines from L-tyrosine. In the biosynthetic process, dopamine is produced and while it has generally been considered that this dopamine is used only for the synthesis of catecholamines, evidence obtained from a variety of peripheral nervous system tissues now suggests that dopamine *per se* may have a functional role [1–5]. It has been shown recently that in the adrenal medulla nicotine-stimulated catecholamine secretion can be regulated by dopaminergic agonists and antagonists, and it has also been demonstrated that dopamine can be released from adrenal medullary chromaffin cells [6]. These observations prompted us to determine whether dopamine receptors could be detected in adrenal medulla using receptor binding techniques.

The present experiments demonstrate the presence of high-affinity receptors in adrenal medulla using radiolabeled spiperone. The characteristics of the adrenal medullary [^{3}H]spiperone binding site resembled those of the [^{3}H]spiperone binding site identified in brain and pituitary. Binding of the radioligand to the receptor complex is very rapid (min) and reversible [7, 11], and the affinity of [^{3}H]spiperone for its binding site in adrenal medulla closely matched the affinity previously reported for brain [12–15]. Competition binding experiments

Table 3. Subcellular distribution of [^{3}H]spiperone binding in adrenal medulla

Fraction	Protein concn (mg/fraction)	Specific binding (fmol/fraction)	Specific binding (fmol/mg protein)
P_1 – 1,000 *g* pellet	592 ± 14	0	0
P_2 – 12,000 *g* pellet	230 ± 8	2235 ± 50	9.7 ± 0.6
A – uppermost layer	1.7 ± 0.1	4.9 ± 0.6	2.9 ± 0.5
B – 0.32 M sucrose layer	2.4 ± 0.1	63 ± 6	27.0 ± 3.4
C – interface (0.32–0.8 M)	4.2 ± 0.2	188 ± 18	45.0 ± 6.0
D – 0.8 M sucrose layer	7.6 ± 0.1	190 ± 10	25.0 ± 1.3
E – interface (0.8–1.2 M)	14 ± 1	64 ± 22	4.5 ± 1.7
F – 1.2 M sucrose layer	12 ± 1	70 ± 8	5.8 ± 0.9
G – pellet	198 ± 10	1020 ± 250	5.1 ± 1.5

The binding of [^{3}H]spiperone (0.25 nM) was determined in the various fractions from adrenal medulla, which were isolated using sucrose density gradient centrifugation as described. Each value represents the mean ± SE of three determinations. The results are representative of two other experiments.

Table 4. Effects of various enzymic treatments on the binding of [^{3}H]spiperone to adrenal medullary membranes

Enzyme	Concentration (μg/ml)	Number of experiments	Specific binding (% control)
Control		7	100 ± 6
Trypsin	1.0	5	87 ± 6
	3.0	5	71 ± 5*
	10	4	57 ± 8*
	30	2	58 ± 8*
Pepsin	30	4	97 ± 3
Phospholipase A_2	0.003	3	91 ± 2
	0.01	3	69 ± 2*
	0.03	2	50
	0.10	3	5 ± 3†
Phospholipase C	30	3	105 ± 7
Neuraminidase	30	4	101 ± 5

[^{3}H]Spiperone binding sites were assayed as described using a 0.25 nM concentration of the radioligand. The binding experiment was initiated by addition of membranes to the incubation tubes containing the radioligand, an appropriate concentration of enzyme and buffer or 3×10^{-6} M (+)-butaclamol to define nonspecific binding. Control specific binding was 18.5 ± 0.1 fmol/mg protein (N = 7). Each value represents the mean ± SE of the indicated number of experiments, each of which was done in triplicate.

*† Significance of differences from control: * $P \leq 0.01$, and † $P \leq 0.001$.

further suggested similarity of the adrenal medullary [^{3}H]spiperone binding site with the dopaminergic receptor in brain as the K_i values for the different classes of drugs in adrenal medulla were very similar to those observed in brain [7, 11, 16–19]. Furthermore, this work suggests that [^{3}H]spiperone is binding to a D2 dopamine receptor.

In an attempt to determine whether changes in the receptor environment could influence the binding of the radioligand, the effects of various phospholipases and proteolytic enzymes were examined. Phospholipase A_2, at a concentration of 0.03 μg/ml (= 0.05 μg/mg protein), resulted in a marked decrease in [^{3}H]spiperone binding; these results suggest that the adrenal medulla enzyme is somewhat more sensitive to alterations in membrane lipids induced by phospholipase A_2 than the brain binding site [20]. Phospholipase C, on the other hand, had no effect on [^{3}H]spiperone binding, indicating that cleavage at specific phospholipid sites is important and/or that specific breakdown products of phospholipase A_2 action are involved in its effect on the receptor. Proteolytic enzymes were also tested on [^{3}H]spiperone binding. The most pronounced effect on binding was produced by trypsin, suggesting the presence of arginine or lysine groups near the receptor recognition site.

Previous work by others had shown that a dopaminergic receptor was present in the adrenal cortex using receptor binding techniques [21–23]. This adrenal cortical dopamine receptor is most likely distinct from the adrenal medullary site; evidence for this stems from the very different binding characteristics which the adrenal cortical dopamine receptor exhibits as compared to the medullary receptor. Using [^{3}H]spiperone as the radioligand, Dunn and Bosmann [22] obtained a K_d for binding to the receptor of 6.9 nM and, as well, a much larger B_{max} (173 fmol/mg protein) than that observed in the present work. The K_d for the binding of another dopaminergic radiolabeled antagonist, [^{3}H]sulpiride, was similarly greater (6.2 nM); the dissociation kinetics of the adrenal cortical receptor were also much more rapid than those observed for the receptor in medulla [22]. Furthermore, the present results show that, under the assay conditions used, the binding of [^{3}H]spiperone to adrenal cortical membranes was very low compared to binding of the radioligand to the adrenal medullary membrane preparation.

To conclude, these results suggest that the peripheral [^{3}H]spiperone binding site in adrenal medulla has the characteristics of a dopamine receptor. Many of its properties resemble those of the brain and pituitary dopamine receptors; however, it appears to be distinct from the dopaminergic [^{3}H]spiperone binding site previously described in the adrenal cortex.

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