Dopaminergic Binding and Inhibitory Effect in the Bovine Adrenal Zona Glomerulosa

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SUMMARY Dopaminergic mechanisms may be involved in the regulation of aldosterone secretion in humans and in the rat. Whether these effects are indirect or are exerted directly at the adrenal level has not yet been resolved. We now report the identification of dopaminergic binding sites in the bovine adrenal zona glomerulosa using [3H]spiperone, a butyrophenone with high affinity for D2 dopamine receptors. Specific [3H]spiperone binding (defined as binding displaceable by 10 μM (+)-butaclamol) reached equilibrium within 20 minutes at 22°C, was reversible, and was heat labile (60°C). Binding was of high affinity and saturable with a $K_A$ of 1.8 ± 0.2 nM and maximal specific binding of 38 ± 8 fmol/mg (means ± SEM; n = 18). [3H]Spiperone binding was unaffected by coincubation with angiotensin II, adrenocorticotropic hormone, or KCl. Binding characteristics, including a dissociation constant at the nanomolar range, greater potency of the D2-agonist LY 171555 relative to the D1-agonist SKF 38393 in inhibiting [3H]spiperone binding, and lack of stimulation of cyclic adenosine 3',5'-monophosphate by dopamine (10⁻⁴ M), were consistent with a predominantly D2-receptor. In vitro studies with collagenase-dispersed adrenal zona glomerulosa cells showed that dopamine (10⁻⁴ M) attenuated angiotensin II-stimulated aldosterone secretion. These observations are consistent with a direct inhibitory effect of dopamine on aldosterone secretion in the adrenal zona glomerulosa.

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KEY WORDS • aldosterone • dopamine • dopamine receptors • adrenal gland

METOCLOPRAMIDE, a dopaminergic antagonist, has been shown to selectively stimulate the secretion of aldosterone and 18-hydroxycorticosterone in humans,1,2 monkeys,3 and rats4,5 in vivo. This effect is independent of changes in established regulators of mineralocorticoid secretion, such as the renin-angiotensin axis, serum potassium, and adrenocorticotropic hormone (ACTH).6-10 The metoclopramide-induced increase in plasma aldosterone is preserved in hypophysectomized or anephric patients1,6 and in hypophysectomized rats.7 Further, the aldosterone response to metoclopramide can be blocked by infusion of dopamine,11,12 but not by dexamethasone,5 saralasin, or converting enzyme inhibitors,5,10 which suggests that a specific dopaminergic mechanism is involved. Disagreement exists as to whether dopamine and metoclopramide directly affect the secretion of aldosterone at the adrenal level. Evidence that these agents modulate aldosterone output by isolated glomerulosa cells in vitro has been equivocal.5,13-18 In sheep, both the ganglionic blocker trimetaphan19 and dexamethasone20 have been observed to block the aldosterone response to metoclopramide, which suggests that ACTH-mediated or neurogenic pathways (or both) could also be involved in the release of aldosterone, at least under certain circumstances. A similar dexamethasone-induced attenuation of the aldosterone response to higher doses of metoclopramide has been also demonstrated in humans.21

If dopamine inhibits the secretion of aldosterone at the adrenal level, its action might be exerted through local dopaminergic receptors. Preliminary evidence suggests that rat22 and calf23 adrenal zona glomerulosa (AZG) contain dopamine binding sites. This study attempted to demonstrate the existence of dopaminergic receptors in membranes from bovine AZG cells.

Materials and Methods

Fresh bovine adrenals were obtained from a local slaughterhouse. The subcapsular layer (outer 0.5 mm) of the bovine adrenals from two to five different animals in each experiment was collected and minced with scissors. Fragments were immediately placed in 1:10 (wt/vol) ice-cold 50 mM Tris buffer (pH 7.4) with 5 mM MgCl₂, 0.1% ascorbic acid, and 10 μM
pargyline and homogenized in a Teflon glass homogenizer by seven strokes (1000 rpm). The homogenate was centrifuged for 10 minutes at 750 g at 4°C. The supernatant was collected and centrifuged for 30 minutes at 45,000 g. The resultant pellet was resuspended in the homogenization buffer and re-centrifuged at 45,000 g for 30 minutes (4°C). Membranes were then resuspended in the same buffer solution (pH 7.4 at 22°C) to yield final protein concentrations of approximately 1.5 to 5 mg/ml.

Membranes (200–700 μg protein) were incubated in triplicate or quadruplicate at 22°C with seven to 12 concentrations of [3H]spiperone (0.4–11 nM; 25 Ci/mmol; New England Nuclear, Boston, MA, USA) for 30 minutes, in the presence or absence of 10 μM (+)-butaclamol. The total incubate volume, 0.5 ml, consisted of 100 μl [3H]spiperone in buffer or 250 μl of (+)-butaclamol or other displacers in buffer, and 150 μl of the membrane preparation. Bound [3H]spiperone was separated from free spiperone by rapid filtration through GF/C glass fiber filters (Whatman, Clifton, NJ, USA) and was followed by two washes with 5 ml of ice-cold Tris buffer solution. The total filtration time (under pressure of −15 atm) was less than 10 seconds. Filters were air dried and subsequently vigorously shaken for 30 minutes in a Beckman beta-counter (Palo Alto, CA, USA) with a counting efficiency of 45%. Specific binding, the difference of [3H]spiperone binding in the absence and presence of 10 μM (+)-butaclamol, was 75 to 85% when the radioligand concentration was in the range of the dissociation constant (Kd; 1–3 nM).

The sources for the different agents used in the binding assays were as follows: dopamine hydrochloride from Astra Pharmaceutical Products, Worcester, MA, USA; (-)-epinephrine, (-)-norepinephrine, serotonin, angiotensin II (ANG II; 1-aspartic, 5 isoleucine), dithiotreitol, and leupeptin from Sigma Chemical, St. Louis, MO, USA; (+)-butaclamol from RBI, Wayland, MA, USA; (+)-sulpiride and (-)-sulpiride from Sigma Chemical, St. Louis, MO, USA; ACTH from Organon, West Orange, NJ, USA; (+)-butaclamol from RBI, Wayland, MA, USA; and metoclopramide from Robbins, Richmond, VA, USA. The SKF 38393 was kindly supplied by Smith Kline & French (Philadelphia, PA, USA), LY 171555 by Eli Lilly (Indianapolis, IN, USA), domperidone by Janssen Pharmaceuticals (Piscataway, NJ, USA), (+)-spiruline and (-)-spiruline by Ravizza (Milan, Italy).

To determine cyclic adenosine 3',5'-monophosphate (cyclic AMP) generation, membranes were prepared as described already, but all steps were performed using a buffer solution containing 50 mM Hepes (pH 7.6), 0.01 M ethylene glycol bis (β-aminoethylther)-N,N,N',N'-tetraacetic acid (EGTA), 0.01 M MgCl₂, 0.01 M KCl, 0.001 M 3-isobutyl-1-methylxanthine and 0.003 M dithiothreitol. The membrane preparation, 40 μl, then was incubated at 37°C in a total volume of 150 μl of the assay buffer, which also contained 0.25 mM adenosine 5'-triphosphate (ATP) and 1 μCi [d-32P]ATP (30 Ci/mmol; New England Nuclear) at final concentrations and 60 μg of creatine phosphokinase with 310 μg of creatine phosphate as previously described.

The AZG cells were prepared from the same tissue fragments used for the binding studies (i.e., the outer subcapsular 0.5 mm of the cortex) using a method adopted from Williams and colleagues for bovine AZG. In brief, tissue was first minced with scissors. Fragments in aliquots of 2.5-g wet weight were preincubated for 50 minutes at 37°C in 12.5 ml of a modified Krebs-Ringer bicarbonate solution with collagenase, 1.85 mg/ml, and DNAse, 0.025 mg/ml. The solution also contained 4% bovine serum albumin; glucose, 2 mg/ml; t-glutamine, 0.2 mg/ml; 1.25% (vol/vol) essential amino acids; and 1.25% (vol/vol) nonessential amino acids. The potassium concentration was 3.7 mmol/L. Dopamine hydrochloride in 0.1% ascorbic acid also was added to the preincubation phase to yield a final concentration of 10⁻⁴ M. At the end of the preincubation phase, the resultant cell dispersion was filtered once through a stainless steel gauze and then washed twice in the Krebs-Ringer buffer solution at room temperature. Cells were then resuspended in the Krebs-Ringer buffer solution to yield 50,000 to 200,000 cells/ml. Dopamine, 50 μl, in 0.1% ascorbic acid or ANG II, or both, was then added to 1.95 ml of the cell suspension. Immediately after 60-minute incubation of triplicate or quadruplicate samples at 37°C, the incubates were frozen and aldosterone (in the total incubate volume) was assayed as previously described.

Results are expressed as means ± SEM. [3H]Spiperone binding data were assessed by using LIGAND, a weighted, nonlinear, least-squares analysis program. Comparisons between Kd and maximal specific binding capacity obtained under different conditions were made by the Student's t test or analysis of variance as necessary.

Results

Binding Studies

Specific binding of [3H]spiperone to membranes from bovine AZG cells increased linearly with protein concentration at a range of 200 to 800 μg per incubate (Figure 1). Equilibrium was reached within 20 minutes, and binding remained stable up to 40 minutes. [3H]Spiperone binding was readily reversible. The addition of 10 μM (+)-butaclamol after 30 minutes of incubation led to rapid dissociation of the ligand from its binding sites. The process was completed within 5 minutes (Figure 2). Based on these studies the reverse rate constant was calculated to be 0.49 min⁻¹.

Specific [3H]Spiperone binding was saturable. The saturation curve and Scatchard plot, based on a typical set of experiments, is shown in Figure 3. The binding data are consistent with a single binding site with a mean Kd of 1.8 ± 0.2 nM and maximal binding capacity of 38 ± 8 fmol/mg (n = 18).

Specific [3H]Spiperone binding was heat labile.
Preincubation of the membrane preparation at 60°C for 10 minutes resulted in complete loss of binding sites. Binding also displayed marked temperature dependence (Figure 4). When incubation temperature was 4°C rather than 22°C, specific [3H]spiperone binding increased by about twofold and remained stable longer, approximately 90% of the maximal binding was still detectable after 120 minutes. At 37°C, maximal specific binding was only about half of that observed at 22°C (see Figure 4). In some experiments, the standard procedure at 22°C was preceded by preincubation of the membrane preparation for 10 minutes at 22°C, with and without various agents. After two washes at 4°C, incubation for 30 minutes was performed as already described. Preincubation of membranes with dopamine (10⁻² or 10⁻³ M) increased subsequent [3H]spiperone binding by 115 ± 25% (n = 4) and raised the binding at 22°C to the range observed at 4°C. Conversely, preincubation or coincubation (i.e., adding agents to the incubation medium) with dithiotreitol (10⁻³ M), a sulfhydryl reducing agent, or leupeptin (0.01–200 μg per incubate), a protease inhibitor, had no consistent effect on [3H]spiperone binding. Preincubation of [3H]spiperone in buffer (in the absence of membranes) at 37°C or 22°C for 30 minutes did not affect subsequent binding to AZG membranes at 4°C or 22°C. However, preincubation of [3H]spiperone in buffer with membranes resulted in decreased ability of the supernatant to rebind to fresh AZG membranes. After a 30-minute incubation at 37°C (with membranes), [3H]spiperone in the supernatant retained only 74.5 ± 3.5% of its original binding activity at 4°C. Following preincubation at 22°C (with membranes), [3H]spiperone retained only 83.3 ± 3.3% of its original binding activity (n = 2).

Results of specificity studies are shown in Figures 5 and 6. Among the agonists, the relative potency, in decreasing order, in displacing [3H]spiperone from its binding sites was LY 171555, a D₂-agonist with an inhibitory concentration, 50% (IC₅₀), of 1.4 ± 0.4 nM (high affinity binding state); SKF 38393, a D₁-agonist with an IC₅₀ of 251 ± 50 nM; dopamine, with an IC₅₀ of 1.78 ± 0.3 × 10⁻⁵ M. Among the antagonists, the order of potency, from most to least, was domperidone (IC₅₀ = 1 ± 0.2 nM; high affinity binding state), butaclamol (IC₅₀ = 40 ± 5 nM) and metoclopramide (IC₅₀ = 40 ± 6 nM), (-)-sulpiride (IC₅₀ = 16 ± 3 μM), (+)-sulpiride (IC₅₀ = 237 ± 20 μM). Notably, the computer-derived best-fitting curves for LY 171555 and domperidone were biphasic, which suggests the existence of both high and low affinity binding states. With LY 171555, however, the high affinity

![Figure 1](http://hyper.ahajournals.org/Downloaded_from)  
**Figure 1.** Specific binding of [3H]spiperone as a function of protein concentrations in adrenal subcapsular membrane preparations. Incubations were performed for 30 minutes with 2 nM [3H]spiperone in the absence and presence of 10 μM (+)-butaclamol.

![Figure 2](http://hyper.ahajournals.org/Downloaded_from)  
**Figure 2.** Association and dissociation curves of specific [3H]spiperone binding to adrenal subcapsular layer. [3H]-Spiperone (2 nM) was added at time zero either in the presence or in the absence of 10 μM (+)-butaclamol. At 30 minutes, after equilibrium had been obtained, 10 μM (+)-butaclamol (arrow) was added to a set of tubes. Points represent the mean of triplicate determinations in one of two such experiments. Maximal specific binding (B_max) in this study was 45 fmol/mg. Inset shows the determination of the first-order reverse (dissociation) rate constant, which was (from the slope of the curve) 0.49 min⁻¹.
fraction was $50 \pm 10\%$ ($n = 3$) of the total binding site population, whereas domperidone displayed high affinity for only $12 \pm 5\%$ ($n = 5$) of the binding sites.

Serotonin, epinephrine, and norepinephrine had no effect on $[^3H]$spiperone binding even with the highest concentrations used ($10^{-4}$ M).

In several experiments, ACTH (2.5 x $10^{-4}$ M), ANG II (2.5 x $10^{-9}$ to 2.5 x $10^{-4}$ M), or potassium (8 mM KCl) was added to the incubation medium and binding studies were repeated in the same membrane preparations with and without these agents. The resulting dissociation constants were 2.2 ± 0.5 ($n = 5$), 2.3 ± 0.6 ($n = 4$), 2.5 ± 0.04 ($n = 3$), and 2.4 ± 0.5 nM ($n = 3$) for control, ANG II, ACTH, and KCl containing preparations, respectively. The maximal binding capacity was also unaffected by coincubation with these agents (maximal specific binding capacity of 43 ± 10, 48 ± 8, 40 ± 10, and 50 ± 13 fmol/mg for control, ANG II, ACTH, and KCl containing preparations, respectively).

**Cyclic AMP Studies**

Although 25 /uH ACTH increased cyclic AMP production by AZG membranes from 74.6 ± 1.4 to 103.9 ± 3.5 pmol/mg/5 min ($p < 0.01$), $10^{-4}$ M dopamine had no effect on basal (73.2 ± 1.2 pmol/mg/5 min) or ACTH-stimulated (104.9 ± 5.4 pmol/mg/5 min) cyclic AMP generation.

Basal aldosterone production (368 ± 40 pg/10^5 cells) increased to 764 ± 56 pg/10^5 cells in the presence of 2.5 x $10^{-4}$ M ANG II ($p < 0.01$). Dopamine ($10^{-4}$ M) had no effect on basal aldosterone production but attenuated the response of AZG cells to ANG II.

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**FIGURE 3.** Scatchard plot (A) and saturation curve (B) of $[^3H]$spiperone binding to bovine adrenal zona glomerulosa membrane preparations. Each point represents the mean of three different experiments run in quadruplicate on the same tissue pooled from three different adrenals. BIF = bound/free radioligands; fm = fmol; $B_{max}$ = maximal specific binding.

**FIGURE 4.** The effect of temperature on the time course of $[^3H]$spiperone (S) binding to adrenal zona glomerulosa membranes. $[^3H]$Spiperone (2 nM) was added to the membrane preparation at each temperature in the presence and absence of 10 /uM (+)-butaclamol. Points represent the mean of three similar experiments (fm = fmol).

**FIGURE 5.** Inhibition of binding of 2.5 nM $[^3H]$spiperone to bovine adrenal zona glomerulosa membranes by the agonists LY 171555, SKF 38393, dopamine, serotonin, (+)-epinephrine, and (+)-norepinephrine. For each agent, three to five experiments at seven different concentrations were run.
Our results demonstrate the existence of specific, saturable, and high affinity binding sites for \(^{3}H\)spiperone in the subcapsular layer of the bovine adrenal cortex. Current classification of dopaminergic receptor includes \(D_1\) dopamine receptors, defined by their association to adenylyl cyclase activation, and \(D_2\) receptors, which do not activate this enzyme. Butyrophenones and ergot derivatives have high affinity for \(D_2\)-receptors, whereas thioxanthenes display high affinity for \(D_1\)-receptors. Dopamine itself exhibits low affinity for \(D_1\)-receptors, low affinity for some \(D_2\)-receptors such as those in the pituitary gland, and a uniformly high affinity only for the more recently defined \(D_1\)-receptors. In this study, dopamine exhibited low affinity for the AZG binding sites as previously described for some pituitary and caudate nucleus membranes. However, dopamine was still a substantially more potent inhibitor of \(^{3}H\)spiperone binding than is SKF 38393, a predominantly \(D_2\)-agonist. That SKF 38393 nevertheless interacted at a considerable potency with the AZG \(^{3}H\)spiperone binding site may suggest either a heterogenous \(D_1D_2\)-receptor population or a cross reactivity of SKF 38393 with \(D_2\)-receptors. Studies in the bovine pituitary and striatum have shown that SKF 38393 not only demonstrates high affinity for \(D_2\) binding sites, but may also function as a partial agonist at classic, well-defined \(D_2\)-receptors in these issues.

Among the antagonists, (-)-sulpiride, known to have a predilection for \(D_2\)-receptors, was a more potent inhibitor than (+)-sulpiride, a predominantly \(D_2\)-antagonist. Of interest is the high affinity of domperidone, a selective \(D_2\)-antagonist, for a small fraction of the AZG binding site population (approximately 10%). This finding may again suggest heterogeneity of the AZG dopamine receptors. Watling and Iversen have similarly observed that the number of high affinity \(^{3}H\)domperidone binding sites in the bovine caudate nucleus or guinea pig striatum was substantially less than the number of receptors specifically labeled by \(^{3}H\)spiperone. These investigators suggested that domperidone labels only a subclass of the entire \(D_2\)-receptor population labeled by \(^{3}H\)spiperone. Clearly, more work is needed with regard to this question in AZG membranes, particularly since it is known that domperidone, as opposed to another predominantly \(D_2\)-antagonist, metoclopramide, does not affect aldosterone secretion in humans.

Finally, a third line of evidence in this study suggesting that the \(^{3}H\)spiperone does not label \(D_2\)-receptors in the AZG was the failure of high concentrations (10^4 M) of dopamine to affect cyclic AMP generation in our membrane preparation under conditions in which adenylyl cyclase could be stimulated by ACTH.
A considerable temperature dependence of specific \([\text{H}]\)spiperone binding to AZG membranes was observed. As a 30-minute preincubation of \([\text{H}]\)spiperone in the assay buffer in the absence of membranes at 22°C or 37°C had no effect on subsequent specific binding to AZG membranes at 22°C, the observed temperature dependence of \([\text{H}]\)spiperone binding did not appear to represent a direct temperature lability of the ligand itself. Further, \([\text{H}]\)spiperone has been used extensively in binding assays at 37°C with similar buffers,32 but to our knowledge, ligand degradation under these circumstances has not been reported. Preincubation of \([\text{H}]\)spiperone with membranes at 22°C or 37°C resulted in approximately 15% and 25% decreases in its binding activity, which suggests some degradation of the ligand by a membrane-contained factor at higher temperatures. However, this decrease accounted for only a fraction of the decreased \([\text{H}]\)spiperone binding observed at 22°C or 37°C.

The protective effect of preincubation with dopamine on subsequent \([\text{H}]\)spiperone binding at 22°C is of interest and might imply that the observed temperature dependence results from binding site rather than ligand degradation. Although the mechanisms underlying the protective effect of dopamine in our assay remain to be elucidated, it is noteworthy that preincubation with dopamine has been previously shown to exert a similar protective effect under different conditions. Preincubation with dopamine resulted in an increase in \([\text{H}]\)dopamine binding to D_2-receptor sites, presumably through clearing of endogenous guanine nucleotides from the guanine nucleotide regulatory factor.40 However, dopamine antagonist binding, with a single reported exception,41 is not affected by guanine nucleotides,42,43 which renders this mechanism an unlikely explanation for the protection exerted by dopamine in our assay in which \([\text{H}]\)spiperone, a D_2-antagonist, was used. With regard to dopamine antagonist binding, preincubation with either dopamine or domperidone inhibited inactivation of \([\text{H}]\)spiperone binding by sulfhydryl alkylating agents, which suggests that receptor occupancy of either agonists or antagonists provides protection against receptor degradation by these agents.43

The failure of dithiotreitol, a sulfhydryl reducing agent, to diminish the observed temperature dependence suggests that decreased binding at higher temperatures does not result from the formation of disulfide bonds. On the other hand, that preincubation with dithiotreitol did not hinder subsequent binding is in agreement with previous observations with pituitary D_2-receptors.43 This finding differs, however, from that in \(\beta\)-adrenergic receptors, in which dithiotreitol inhibited ligand binding, presumably by reducing an essential disulfide bond.44

Finally, the possibility that the temperature dependence of our binding assay is related to tissue contained proteolytic activity, such as impairs the stability of some steroid and acetylcholine receptors,45 was tested by the addition of leupeptin, a protease inhibitor, to the assay medium. Although no protective effect of leupeptin on \([\text{H}]\)spiperone binding was shown, there were other protease inhibitors that might have provided such protection.

The inference that the AZG \([\text{H}]\)spiperone binding site population is pertinent to the dopaminergic inhibition of aldosterone biosynthesis and secretion suffers from the lack of current information regarding the effects of in vivo of dopamine on bovine aldosterone output. Nevertheless, preliminary observations indicate that the binding affinities of the AZG dopamine binding sites are similar in bovine, rat, and human tissues,46 thus suggesting that the easily accessible and relatively abundant bovine adrenal may serve as a useful model for the study of the AZG dopamine receptors. Further, while the effects of dopaminergic agonists and antagonists in living cattle have not been defined, two groups of investigators have demonstrated that dopamine modulates aldosterone output by bovine AZG cells in vitro.13,47 In general, not only is the effective dopamine concentration determined in these studies similar to that found in the present AZG cell experiments, but the doses needed to elicit significant inhibition of aldosterone response are also in agreement with the dopamine concentration required to significantly affect AZG \([\text{H}]\)spiperone binding. McKenna et al.13 reported that 10^{-7} to 10^{-5} M dopamine induced 25 to 45% inhibition of ANG II-stimulated aldosterone biosynthesis in collagenase dispersed bovine AZG cells. DeLean et al.48 failed to show any effect of dopamine on aldosterone in isolated AZG cells in primary culture (72 hours). When AZG cells were cocultured with adrenomedullary cells, however, both dopamine and dopamine-3-sulfate (but not dopamine-4-sulfate) inhibited ANG II-stimulated aldosterone secretion by 50% in a dose-dependent manner (10^{-5}-10^{-4} M),49 which suggests that continuous availability of dopamine is critical for the preservation of AZG responsiveness to this hormone. Of relevance in this regard are the observations that high concentrations of dopamine are found in the adrenal cortex.47,49-51

The observation that high concentrations of dopamine (>10^{-6} M) are required to demonstrate an inhibitory effect on aldosterone secretion by AZG cells in vitro has led investigators to question the specificity of this inhibition.7 The present study suggests that this information should be interpreted in light of the observed low affinity of dopamine to some D_2-receptors in conjunction with the reported high concentrations of dopamine available in the adrenal cortex. Although our results do not conclusively establish the link between the AZG dopaminergic binding sites and dopaminergic inhibition of aldosterone in bovine AZG cells, they nevertheless underscore the need to document preservation of dopaminergic binding sites whenever a dopaminergic effect cannot be shown in AZG cell systems in vitro.
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