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Expression of I_2-Imidazoline Sites in Rat Prostate

EFFECT OF CASTRATION AND AGING

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ABSTRACT. Clonidine, idazoxan, and related imidazoline adrenergic drugs bind to non-adrenergic sites in brain and several peripheral tissues. These sites, termed imidazoline receptors, appear to exist in two major subclasses, I_1 sites labeled by clonidine and I_2 sites labeled by idazoxan. In this study, we investigated whether rat prostate expresses imidazoline receptors and, if so, whether their expression can be regulated by circulating testosterone. Studies in rat ventral prostate membrane revealed that [^{3}H]idazoxan, but not [^{3}H]*p*-aminoclonidine, bound to non-adrenergic sites. The binding of [^{3}H]idazoxan was saturable (B_{max}: 941 ± 105 fmol/mg protein) and high affinity (K_D: 16.4 ± 2.3 nM). The rank order of the inhibition of binding by imidazoline ligands was cirazoline > clonidine > UK 14,304 > guanabenz, indicating an I_2 subclass of imidazoline receptors. Bilateral orchiectomy increased the number of binding sites (B_{max}) for [^{3}H]idazoxan without changing the affinity (K_D). Testosterone replacement, while completely restoring the plasma testosterone levels, only partially reversed the increase in B_{max}. In contrast, the binding of [^{3}H]idazoxan to prostate membranes of rats in different age groups (4, 7, and 16 months) revealed a progressive decrease in the B_{max} without any change in K_D. We conclude that the rat prostate expresses the I_2 subclass of imidazoline receptors and that the expression is regulated by circulating testosterone. BIOCHEM PHARMACOL 51;4:455–459, 1996.

KEY WORDS. imidazoline receptors; prostate; castration; aging; agmatine

We reported recently that the canine prostate gland expresses a non-adrenergic binding site for clonidine and allied drugs, the imidazoline receptor [1]. The receptor is of the I_1 subclass [2, 3] with high affinity for clonidine and is localized to the epithelial portion of the gland. However, it is not known if the prostate expresses the I_2 subclass of imidazoline receptors [4] and if agmatine, an endogenous ligand for imidazoline receptors [5], binds to these sites.

In the prostate, the expression of a number of receptors, as well as growth-related molecules, is dependent upon circulating testosterone. Thus, castration results in a reduction in serum testosterone paralleled by a general involution of the prostate [6], and initiates apoptosis of epithelial cells in the ventral prostate. In addition, castration reduces the expression of several proteins, including prostate steroid binding protein, and increases the expression of others, including testosterone-repressed prostate message and transforming growth factor-β [7–9]. Castration also alters the expression of some neurotransmitter-related receptors. Thus, peripheral benzodiazepine receptors are up-regulated, whereas the number of muscarinic cholinergic receptors [10, 11] is reduced. It is believed that some of the age-dependent changes in prostate constituents also reflect a senescent decline of testosterone synthesis. It is not known whether prostatic imidazoline receptors are regulated by testosterone and aging.

Therefore, in the present study we investigated the binding of [^{3}H]idazoxan (I_2 selective ligand) and [^{3}H]PAC‖ (I_1 ligand) to rat prostate membranes and the effects of castration and aging on the expression of imidazoline receptors.

MATERIALS AND METHODS

Animals and Surgical Procedures

Studies were performed on 57 male Sprague–Dawley rats weighing 290–360 g. Bilateral orchiectomy was performed in 42 rats, while 15 rats underwent a sham procedure. All surgeries were performed aseptically under metofane anesthesia. Three days (N = 20) or seven days (N = 10) after surgery, the animals were killed by CO_2 gas, and the ventral prostates were removed rapidly and placed immediately in ice-cold sucrose

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‖ *Abbreviation:* PAC, *p*-aminoclonidine.

(0.32 M)-HEPES (5 mM) buffer (pH 7.4) containing 0.1 mM EDTA. Prostates from sham animals were used individually. Since prostates of castrated animals were very small, it was necessary to pool them to obtain enough membranes for binding studies. For aging studies, 6 rats from groups of 4, 7, and 16 months of age were used.

Testosterone Replacement

Testosterone (1 mg testosterone propionate in 0.2 mL corn oil, s.c.) was administered to 6 rats immediately after surgery and on each of the 2 days following surgery while 6 castrated rats received only corn oil on the same schedule. The ventral prostate was taken out and processed as above on day 3 following surgery and testosterone replacement. Testosterone was measured by radioimmunoassay as previously described [12].

Membrane Preparation

The prostates were freed of excess fat and connective tissue, washed in the above buffer, blotted dry, and weighed. Ventral prostates were homogenized in the above buffer using a Teflon–glass homogenizer and centrifuged at 1000 *g* for 10 min at 4°. The supernatant was centrifuged at 12,000 *g* for 20 min, and the resulting pellet was resuspended in 20 mL of 50 mM Tris–HCl buffer containing 5 mM EDTA (pH 7.7), using a polytron (setting 4) for 10 sec, and centrifuged. The pellet was resuspended and re-centrifuged two more times. The membranes were stored at −70° until use.

Ligand Binding Assays

The binding of [^{3}H]ligands to membranes was performed as described earlier [4, 5]. Briefly, the final washed pellet was suspended in fresh Tris–HCl buffer (pH 7.7) to provide approximately 100 μg protein per assay tube. Binding assays were performed in Tris–HCl buffer (pH 7.7) using 1 nM [^{3}H]PAC, or 5 nM [^{3}H]idazoxan. For saturation binding assays, the resuspended membrane was incubated with different concentrations of [^{3}H]idazoxan (0.5 to 200 nM) in triplicate for 30 min at 25°. Non-specific binding was defined by a 10 μM concentration of unlabeled ligand, and epinephrine (10 μM) was included in all incubations to block any binding to α_2-adrenergic receptors. Following incubation at 25° for 30 min, the reaction was terminated by rapid vacuum filtration over Whatman GF/B filters and washed with 10 mL of ice-cold buffer. Filters were suspended in scintillation fluid, and radioactivity was counted in a liquid scintillation counter. The binding data were analyzed using LIGAND (Elsevier Biosoft), a parametric nonlinear regression analysis. Membrane protein was assayed by the Coomassie blue method (Pierce) using bovine serum albumin as standard. Statistical analysis was carried out using Student's *t*-test (see Tables 1 and 2) or one-way ANOVA (see Fig. 4).

RESULTS

In pilot experiments, it was observed that [^{3}H]idazoxan but not [^{3}H]PAC specifically bound to non-adrenergic sites in

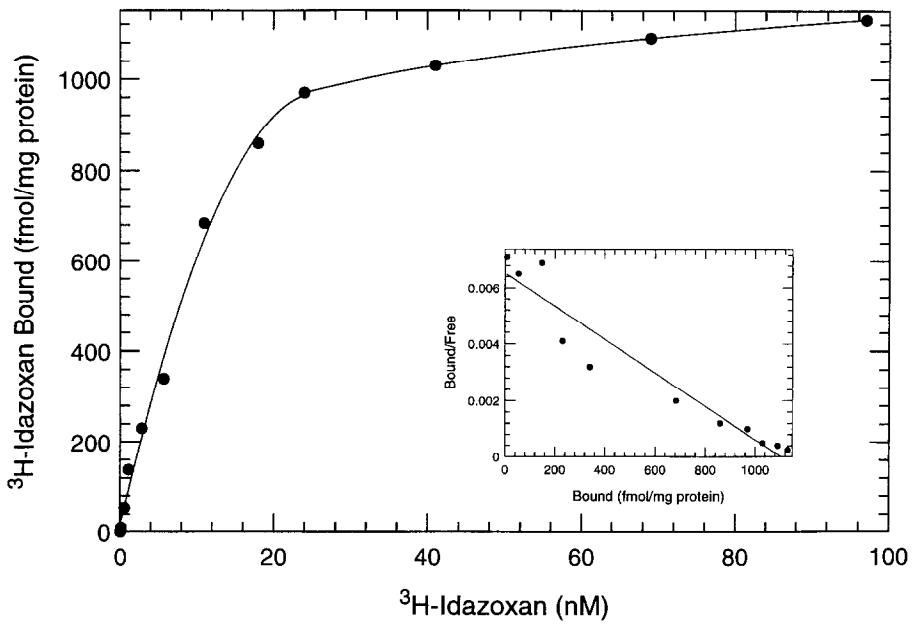

FIG. 1. Saturation binding of [^{3}H]idazoxan to membranes of rat ventral prostate. Membranes (about 100 μg protein) were incubated with various concentrations of [^{3}H]idazoxan in the presence of 10 μM epinephrine; non-specific binding was defined by 10 μM idazoxan. Values are from one representative experiment repeated with three different cell membrane preparations. Scatchard analysis of the specific binding is shown in the inset. The kinetic constants given in the text are from all three experiments analyzed by LIGAND.

membranes of the rat prostate. Therefore, all subsequent experiments utilized [^{3}H]idazoxan as the ligand.

The binding of [^{3}H]idazoxan to prostate membranes was specific, high affinity, and saturable (Fig. 1). Scatchard analysis resulted in a single site fit with a K_D of 16.4 ± 2.3 nM and a B_{max} of 941 ± 105 fmol/mg protein (Fig. 1 inset).

The pharmacological specificity of this binding site was analyzed by examining the relative potencies of imidazoline agents to inhibit the binding of [^{3}H]idazoxan (5 nM) to prostate membranes. The agents included cirazoline, clonidine, guanabenz, UK 14,304 [5-bromo-*N*-(4,5-dihydro-1*H* imidazol-2-yl)-6-quinoxalinamine] and agmatine, an endogenous ligand [5]. The rank ordering of displacement is shown in Fig. 2. Cirazoline was the most potent agent (K_i of 35 ± 4 nM) followed by clonidine (140 ± 24 nM) and UK 14,304 (205 ± 34 nM) with guanabenz the least potent (>10 μM). Agmatine inhibited [^{3}H]idazoxan binding with a K_i of 1 ± 0.8 μM. The pseudo-Hill slope values for cirazoline (0.51 ± 0.05), clonidine (0.72 ± 0.06), UK 14,304 (0.68 ± 0.05), and agmatine (0.71 ± 0.04) were less than unity, indicating that prostate membranes contain multiple binding sites or interactive forms of binding sites as reported in other tissues [13]. The results indicate that the rat prostate expresses a form of the I_2 class of imidazoline receptors (idazoxan preferring) that differ from the I_2 site expressed in rabbit tissues and in bovine adrenal chromaffin, all of which have a higher affinity for synthetic guanidinium compounds [4, 14, 15].

The effects of bilateral orchiectomy on the expression of I_2 sites in the prostates of rats castrated 3 or 7 days previously were compared with sham-operated controls. As expected (Table 1), castration decreased the weight of the ventral prostate by approximately 50% at 3 days and by over 80% at 7 days, did not change the body weight (Table 1), and reduced plasma testosterone concentration below detectable levels (Table 1).

Castration, while not altering the affinity (K_D) of [^{3}H]idazoxan to prostate membranes at 3 days (Fig. 3) (15.5 ± 1.4 vs 16.3 ± 1.8), almost doubled the numbers of binding sites (B_{max}) (from 986 ± 176 to 1911 ± 145 fmol/mg protein, Fig. 3). The B_{max} was even higher in one experiment in which binding was measured in ventral prostate membranes pooled from 10 animals 7 days following the procedure (2928 fmol/mg protein).

We examined the effect of testosterone replacement (1 mg/day, s.c., × 3) on the augmented expression of I_2 sites resulting from castration. Testosterone replacement increased prostate weight to over 60% of control without affecting body weight. It also resulted in the recovery of plasma testosterone concentrations to levels similar to those in sham-operated controls (Table 1). Testosterone replacement only partially reduced the elevated B_{max} (1506 ± 399), which was significantly different from the sham control group but not from the castrated animals (Fig. 4).

To assess whether aging, which, like castration, reduces the androgen environment of the prostate, influences the expression of imidazoline receptors, we measured the binding of [^{3}H]idazoxan to prostate membranes in rats of 4, 7, and 16 months of age. As shown in Table 2, there was a progressive decrease in the number of [^{3}H]idazoxan binding sites in older rats (915 to 450 fmol/mg protein) without a change in affinity (K_D). Thus, the changes in the numbers of I_2 imidazoline sites in the rat prostate which occur during aging are opposite in direction from those produced by castration.

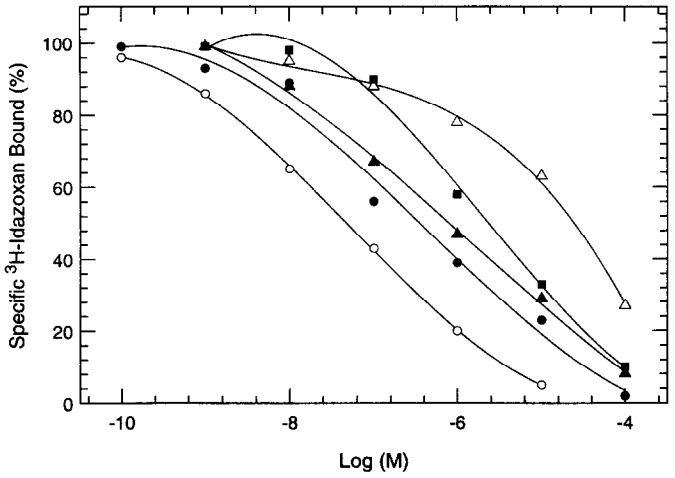

FIG. 2. Inhibition of [^{3}H]idazoxan binding to prostate membranes by various adrenergic agents. Membranes (50–100 μg protein) were incubated with 5 nM [^{3}H]idazoxan in the presence of various concentrations of cirazoline (○—○), clonidine (●—●), UK 14,304 (▲—▲), agmatine (■—■) and guanabenz (△—△). Epinephrine (10 μM) was present in all the incubations, and non-specific binding was defined by 10 μM idazoxan. Specific binding was about 70% of the total binding and varied between 3000 and 3500 dpm. Values are from a representative experiment; the K_i values were calculated from two (guanabenz and UK 14,304) or three (cirazoline, clonidine, and agmatine) experiments by LIGAND.

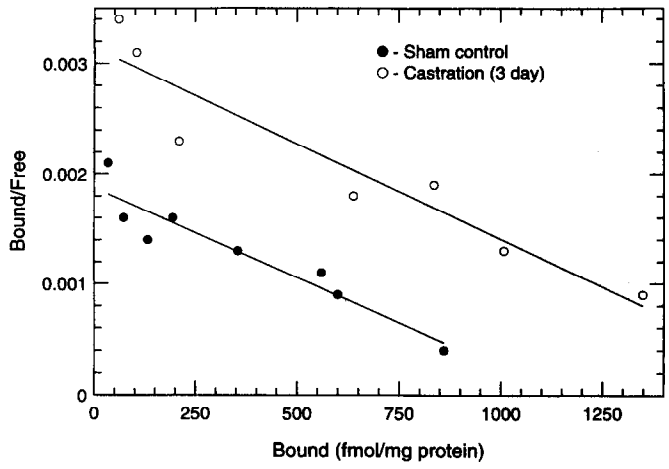

FIG. 3. Saturation binding of [^{3}H]idazoxan to rat prostate membranes following castration. The ventral prostates were pooled from 4 rats in castrated animals, while those of the sham animals were used individually. Membranes were incubated with increasing concentrations of [^{3}H]idazoxan in the presence of epinephrine (10 μM); non-specific binding was defined by 10 μM idazoxan. Values are from one representative experiment; kinetic constants given in the text were obtained from five experiments.

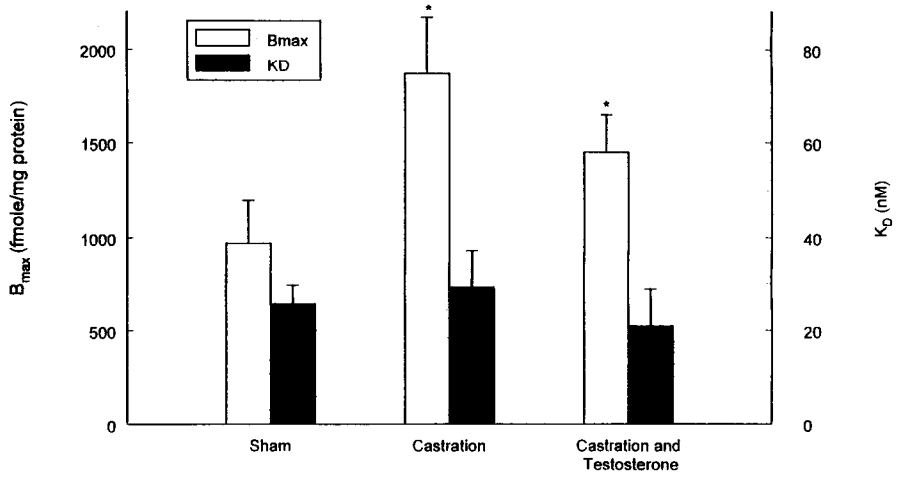

FIG. 4. Effects of castration (3 days) and testosterone treatment on the kinetics of [^{3}H]idazoxan binding to rat prostate membranes. Values are from three experiments with prostate membranes pooled from 2 animals; each assay was performed in triplicate.
Key: *$P < 0.001$ compared with sham control.

DISCUSSION

In the present study, we observed that membranes of the rat prostate express imidazoline receptors. The fact that membranes saturably bound [^{3}H]idazoxan but not [^{3}H]PAC with high affinity, and that binding of [^{3}H]idazoxan was displaced by other ligands in the order of cirazoline > clonidine > UK 14,304 > guanabenz indicates that the imidazoline receptor is of the I_2 subclass [2] and differs from the imidazoline receptor sites in dog [1] and human (Felsen D, unpublished data) prostates which are of the I_1 subclass. The reason for the species difference is not known. However, it is of interest that humans and dogs are the only two species in which age-dependent benign prostatic hyperplasia occurs [16].

The present study has also demonstrated that castration led, within 3 days, to an increase in the numbers but not the affinity of the prostatic I_2 sites, with no change in the K_D at 3 days. These results are, in part, comparable to changes in the peripheral benzodiazepine receptor in rabbit prostate, which 3 weeks after castration doubled in density with a decrease in affinity (K_D) [10]. The highest concentration of the peripheral benzodiazepine receptors is found in the mitochondrial outer membrane [17], similar to the localization of I_2 imidazoline sites in mitochondrial membranes in many tissues including liver, adrenal medulla, and brain cells [4, 14]. Although subcellular localization of the I_2 imidazoline site in the prostate was not investigated in this study, it is highly probable that this site is expressed in mitochondria in the prostate.

TABLE 1. Effect of castration on rat body and ventral prostate weights and plasma testosterone levels

	Prostate weight (mg)	Body weight (g)	Testosterone (ng/mL)
Sham (15)	279.1 ± 15.4	335 ± 7	3.18 ± 0.53
Cast-3 day (20)	141.8 ± 7.2	323 ± 7	ND
Cast-7 day (10)	32.1 ± 2.9	327 ± 3	ND
Cast 3 + T (6)	443.7 ± 33.1	321 ± 6	4.72 ± 0.91

Ventral prostates were removed from rats, and membranes were prepared for binding studies. Plasma testosterone (T) was measured in these rats by radioimmunoassay. Values are means ± SEM from the number of rats specified in parentheses. ND = not detected.

In the present studies, the number of I_2 sites was lower in older animals. These results are similar to those found by Yazawa and Honda [18], who examined the M_3-muscarinic cholinoceptor. However, Kondo *et al.* [19] reported that while cholinergic binding in the prostate is unchanged with age, there are more α_1 and α_2 receptors in prostates from older animals. Testosterone was not measured in the aging rats in this study. Previous reports comparing young and old rats demonstrated that there is a decline in serum testosterone with aging [20, 21]. Thus, despite the fact that aging and castration are both associated with lowered serum testosterone, in the present study, the B_{max} of I_2 imidazoline sites decreased with aging, whereas it increased with castration. It is not clear why the results with the two groups are different. However, the precipitous decline in serum testosterone following castration does not mirror the slow decline of testosterone with aging. The fact that testosterone replacement failed to reverse the increased expression of I_2 sites suggests that there may be differences in the mode of regulation by endogenous versus exogenous testosterone.

The role of transmitters and their receptors has not been studied extensively with regard to prostate function. Stimulation of α_1-adrenergic receptors by clonidine has been shown to contract the smooth muscle of the prostate and thereby increase secretion [22]. There are no reports on the effect of imidazoline/α_2-adrenergic agonists on either prostatic contraction or growth.

In conclusion, we have demonstrated, for the first time, the presence of I_2 imidazoline sites in the rat prostate. The fact that the expression of these receptors is regulated by both testosterone and aging suggests a physiological role for these

TABLE 2. Effect of age on [^{3}H]Idazoxan binding to rat prostate membranes

Age (months)	K_d (nM)	B_{max} (fmol/mg protein)
4	21.3 ± 1.5	915 ± 45
7	18.3 ± 2.4	655 ± 24
16	19.8 ± 1.9	450 ± 21

Ventral prostates were removed from rats, and membranes were prepared as described in Materials and Methods. Saturation binding of [^{3}H]idazoxan was performed in these membranes, and the binding kinetics were calculated using Scatchard analysis. Values are means ± SEM from 6 animals.

sites in the prostate. The function of I_2 imidazoline sites in the prostate remains to be elucidated, but may be connected to hormone-related growth of the prostate or to prostatic secretion.

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