Alpha-2 Adrenergic Receptors in the Bovine Retina

Presence of Only the Alpha-2D Subtype

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Purpose. To identify and characterize the alpha-2 adrenergic receptor subtypes present in the bovine neurosensory retina.

Methods. Radioligand saturation and inhibition binding assays were performed with the antagonist radioligands [³H]RX821002 and [³H]rauwolscine.

Results. [³H]RX821002 bound to a single class of receptors with the characteristics of an alpha-2 adrenergic receptor with an affinity (K_D) of 0.16 nM and a receptor density (B_{max}) of 1500 fmol/mg protein. Correlation of the affinities (pK_i values) for nine antagonists in the bovine neurosensory retina with the alpha-2D receptor of the bovine pineal gave a correlation coefficient of 0.99. The correlation coefficients for the alpha-2A (0.84), alpha-2B (0.36), and alpha-2C (0.39) subtypes were much lower. The presence of a minor population of alpha-2B or alpha-2C receptors was excluded.

Conclusions. A high density of alpha-2D adrenergic receptors is present in the bovine neurosensory retina. Neither the alpha-2B nor the alpha-2C subtype is detectable. Invest Ophthalmol Vis Sci. 1995;36:1885–1892.

Norepinephrine, an important neuroregulator, controls many physiological functions in the eye. The actions of norepinephrine are mediated by a family of receptors collectively known as adrenergic receptors. Numerous agents have been developed that interact with adrenergic receptors, many of which are used for diagnostic and therapeutic purposes in ophthalmology. Three major types or subfamilies of adrenergic receptors have been identified: alpha-1, alpha-2, and beta.[1] Within each of these three main types of adrenergic receptors, three or more subtypes have been defined. Based on pharmacologic characteristics, the alpha-2 adrenergic receptor subfamily of receptors consists of alpha-2A, -2B, -2C, and -2D subtypes.[2,3] Each of these four pharmacologic subtypes has been cloned from multiple species.[3] Sequence analyses of the alpha-2 subtypes indicate, however, that alpha-2A and alpha-2D are actually species orthologues rather than

two distinct subtypes. Alpha-2A receptors have been identified in the human platelet, the human HT29 cell line[1] and the pig,[4] whereas alpha-2D receptors have been identified in the bovine pineal gland[5] and in the rat[6,7] and mouse.[8]

Adrenergic drugs are effective ocular hypotensive agents. Epinephrine (an alpha-1, alpha-2, and beta adrenergic agonist) has been used since the turn of the century to lower intraocular pressure, but beta-adrenergic antagonists are the most commonly used drugs for the medical treatment of glaucoma. Alpha-2 adrenergic agonists are also approved for use in reducing intraocular pressure, although their mechanism of action is unclear.[9] The newer agents, which are more selective for the alpha-2 than the alpha-1 receptor type, may produce different effects on aqueous humor dynamics.[10,11] The development of subtype-selective alpha-2 adrenergic agents for topical application is considered desirable to reduce systemic and ocular side effects. Although most side effects involve the anterior segment, there have been some reports of decreased posterior choroidal circulation and epinephrine maculopathy.[12] An understanding of the distribution of alpha-2 receptor subtypes in the eye would be useful in designing new drugs with greater effectiveness and fewer adverse effects.

Alpha-2 adrenergic receptors have been identified in some ocular tissues, including the rabbit iris–ciliary body by radioligand binding studies[13,14] and the rabbit retina using the inhibition of cyclic adenosine monophosphate production as the assay.[15] The bovine retina contains alpha-2 adrenergic receptors, as demonstrated by radioligand binding studies.[16,17] However, the characteristics of the alpha-2 adrenergic receptor subtype present in the bovine retina, particularly with regard to the possibility that there may be more than one subtype, has not been reported. In this study, we evaluated the characteristics of the alpha-2 adrenergic receptors in the bovine neurosensory retina. On the basis of receptor-binding experiments using [3H]-RX821002 and [3H]rauwolscine as radioligands, we concluded that the alpha-2 adrenergic receptors of the bovine neurosensory retina are alpha-2D and that neither the alpha-2B nor the alpha-2C subtype was detectable.

METHODS

Drugs and Chemicals

[3H]RX821002 (specific activity 55 Ci/mmol) was obtained from Amersham International (London, UK), and [3H]rauwolscine (specific activity 77.9 Ci/mmol) was obtained from DuPont NEN Research Products (Boston, MA). Rauwolscine, WB 4101, and spiroxatrine were purchased from Research Biochemicals, Inc. (Natick, MA). Oxymetazoline was obtained from Sigma (St. Louis, MO). The following were generous gifts: prazosin from Pfizer, Inc. (Groton, CT); ARC-239 from Boehringer–Ingelheim (Ridgefield, CT); SK&F 104078 from SmithKline and Beecham (Swedeland, PA); and phentolamine from Ciba–Geigy Corp. (Suffern, NY). Prazosin was prepared in methanol, spiroxatrine in 80% DSMO/20% 1 M HCl, SK&F 104078 in 50% EtOH, and all other drugs in 5 mM HCl. Drugs were prepared as 5- or 10-mM stock solutions and diluted in 5 mM HCl.

Tissue Preparation

Bovine eyes (Pel-Freez Biologicals, Rogers, AR) were thawed and dissected on ice. The neurosensory retina was suspended in 25 ml ice-cold 50 mM Tris-HCl, pH 8, and homogenized with a Tissumiser (model TR-10; Tekmar, Cincinnati, OH). The homogenate was filtered through a 53-μm nylon mesh and centrifuged at 1,400 rpm for 10 minutes. The supernatant was transferred to another tube, recentrifuged at 20,000 rpm for 10 minutes, and frozen at −80°C. On the day of the assay, the pellet was brought to volume in 25 mM glycylglycine, pH 7.4. The study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Radioligand Binding Assays

Saturation and competition binding experiments were performed as described.[2,18] Briefly, saturation experiments were performed using two sets of duplicate tubes that contained 970 μl of membrane suspension and 20 μl of the appropriate concentration of the radioligand ([3H]RX821002 or [3H]rauwolscine). One set contained 10 μl of 100 μM (−)-norepinephrine to determine nonspecific binding. Specific binding was calculated as the difference between total and nonspecific binding. After a 30-minute incubation period at room temperature, the suspensions were filtered through GF/B glass fiber filter strips (Whatman, Clifton, NJ) that had been soaked overnight in 0.1% polyethylenimine, using a 48-sample manifold (Brandel Cell Harvester; Biomedical Research and Development, Gaithersburg, MD). The tubes and filters were washed twice with 5 ml ice-cold 50 mM Tris-HCl (pH 8.0), and the radioactivity on the filter was determined by liquid scintillation spectroscopy. The K_D and B_{max} values were calculated from nonlinear regression of bound versus free ligand concentration. K_D values are geometric means, and B_{max} values are arithmetic means. Protein concentrations were determined by the method of Bradford[19] with bovine serum albumin used as the standard.

For inhibition experiments, 20 μl of a fixed concentration of radioligand [3H]RX821002 (approximately 0.2 nM near the K_D concentration) and various concentrations of unlabeled drug (10 μl) were added to duplicate tubes containing 970 μl of the membrane suspension. Assays were then performed as described for saturation experiments. Competition experiments were performed using a low concentration (0.1 nM) of [3H]rauwolscine in a final volume of 3 ml. In some of these experiments, membranes from Chinese hamster ovary cells stably transfected with the alpha-2C adrenergic receptor[20] were added to the neurosensory retina membranes to simulate the presence of two subtypes in the assay. Competition binding data were analyzed with the GraphPAD Inplot program (San Diego, CA) to determine IC_{50} assuming a one-site model, and pseudo Hill slope was determined from fitting the data to the four-parameter logistic equation. IC_{50} values were converted to K_i values by the method of Cheng and Prusoff[21] and are presented as geometric means.

RESULTS

[3H]RX821002 has proven to be a useful radioligand for labeling alpha-2 adrenergic receptors in a variety of tissues and species.[22–25] [3H]RX821002 has similar, high affinities for all four pharmacologic subtypes of the alpha-2 adrenergic receptor.[24–27] [3H]-
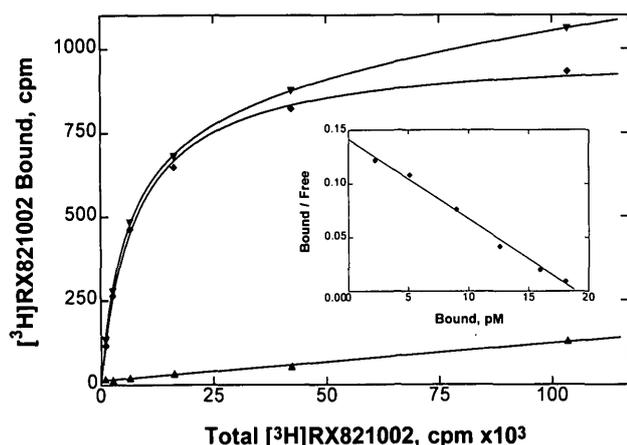
FIGURE 1. Saturation of [³H]RX821002 binding in the bovine neurosensory retina. Various concentrations of [³H]-RX821002 (total [³H]RX821002) were incubated with membranes prepared from the bovine neurosensory retina. The specific binding (♦) was calculated as the difference between the total binding (▲) and the nonspecific binding (▼) as determined by 10^{-4} M norepinephrine. The inset is the linear Rosenthal transformation[28] of the saturation binding data plotted as bound [³H]RX821002 divided by free [³H]RX821002 versus bound [³H]RX821002 converted to units of picomolar. For this experiment the K_D as calculated by nonlinear regression analysis was 0.13 nM, and the B_{max} was 1515 fmol/mg protein. The mean values for four experiments are given in the Results section.

Rauwolscine, another commonly used radioligand for studying alpha-2 adrenergic receptor, is generally not a good radioligand for labeling the alpha-2D receptor subtype because of its relative low-binding affinity and associated high-nonspecific binding. It is, however, useful for detecting a minor population of alpha-2B or alpha-2C receptors in a tissue containing mostly alpha-2D adrenergic receptors. The results of saturation experiments in the bovine neurosensory retina indicate that [³H]RX821002 is an excellent ligand in this tissue. As shown in Figure 1, binding is saturable, of high affinity (K_D of approximately 0.2 nM) and density (B_{max} of approximately 1.5 pmol/mg protein), and the nonspecific binding is low (5% at the K_D concentration). When the data are transformed by the Rosenthal procedure,[28] they fall on a straight line, indicating that a single class of binding sites is being labeled. The mean values for four saturation experiments are a K_D of 0.163 ± 0.018 nM and a B_{max} of 1470 ± 70 fmol/mg protein.

The pharmacologic characteristics of the alpha-2 adrenergic receptor in the bovine neurosensory retina were assessed by competition radioligand binding studies (Fig. 2). The affinities of eight alpha-2 adrenergic compounds were determined by inhibition of [³H]RX821002 binding in membrane preparations. Table 1 shows the K_i values with pseudo Hill slopes

for these compounds, which are a subset of the compounds we used previously to characterize the alpha-2A, -2B, -2C, and -2D subtypes.[24,25,29] The pseudo Hill slopes were all near 1, consistent with the conclusion that only a single subtype of alpha-2 adrenergic receptor is present in the bovine neurosensory retina. The affinities of the drugs are similar for the bovine neurosensory retina and the bovine pineal gland, but clearly they are different from the alpha-2A, -2B, and -2C subtypes, as determined previously using the human clones transfected into COS cells. For example, the alpha-2 receptors from bovine neurosensory retina and pineal gland have a 6- to 20-fold higher affinity (lower K_i values) for phentolamine than the three human clones. By contrast, the affinities of the neurosensory retina, pineal, and alpha-2D (rat clone transfected into COS cells) receptors for rauwolscine and prazosin are significantly less than the alpha-2A, -2B, and -2C adrenergic receptor subtypes.

Rather than compare affinities for two tissues or receptor subtypes one drug at a time, it is often helpful to consider all the drugs in a single, simultaneous comparison by correlation analysis. In this procedure, the K_i values are first converted to their negative logarithms (pK_i values) so that drugs of widely differing affinities can be compared on a single graph, with the values for one subtype plotted on the x-axis and the values for the other subtype plotted on the y-axis. Figure 3 presents the correlation of the pK_i values for the bovine neurosensory retina with our published
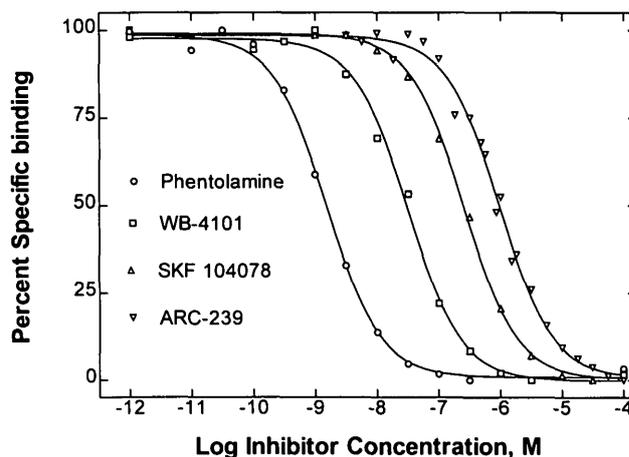

Log Inhibitor Concentration, M

FIGURE 2. Inhibition of [³H]RX821002 binding by alpha-2 adrenergic antagonists. Various concentrations of the antagonists, as indicated on the abscissa, were incubated with approximately 0.3 nM [³H]RX821002. The percent specific binding in the absence of any inhibiting compound is 100% and is indicated by the data points at −12 inhibitor concentration. Norepinephrine at 10^{-4} M was included in all experiments to determine nonspecific binding and is included in the curves as the −4 data points. The data given are from a single experiment. Mean K_i values for three to six experiments are given in Table 1.

TABLE 1. Affinities of Drugs for α-2 Adrenergic Receptors

| Drug | Bovine Neurosensory Retina | | | | Bovine† Pineal | Human Clones‡ | | | Rat Clone† 2D |
| | K_i (nmol/l) | | | n§ | Slope‖ | | 2A | 2B | 2C | |
						K_i (nmol/l)				
RX821002*	0.16	±	0.02	5		0.33	0.29	1.05	0.37	0.54
Phentolamine	0.62	±	0.05	4	0.94	0.71	4.1	6.8	13	4.9
WB4101	9.2	±	1.3	4	0.98	13.2	0.91	4.3	0.40	46
Rauwolscine	12.1	±	1.3	6	1.09	9.5	0.32	0.37	0.13	19.2
Spiroxatrine	12.5	±	2.6	4	1.10	10.5	5.5	0.19	0.13	70
Oxymetazoline	25	±	2	3	0.82	35	3.3	754	31	17
SKF 104078	120	±	10	4	0.97	153	40	70	55	248
ARC-239	345	±	15	3	0.99	352	131	2.7	3.8	749
Prazosin	3303	±	334	3	1.24	1370	302	31	11	890

* K_D values from saturation experiments.
† Data from reference 25.
‡ Data from references 24 and 29.
§ Number of experiments.
‖ Pseudo Hill coefficient.

data for the alpha-2D receptor of the bovine pineal gland.[25] The correlation between the bovine neurosensory retina and the bovine pineal is excellent (correlation coefficient, r, of 0.99), and the correlation line is nearly indistinguishable from the line of identity (dashed line, Fig. 3), indicating that the pharmacologic characteristics of the two receptors are identical. These results indicate that the bovine neurosensory retina receptor is similar to the alpha-2D receptor
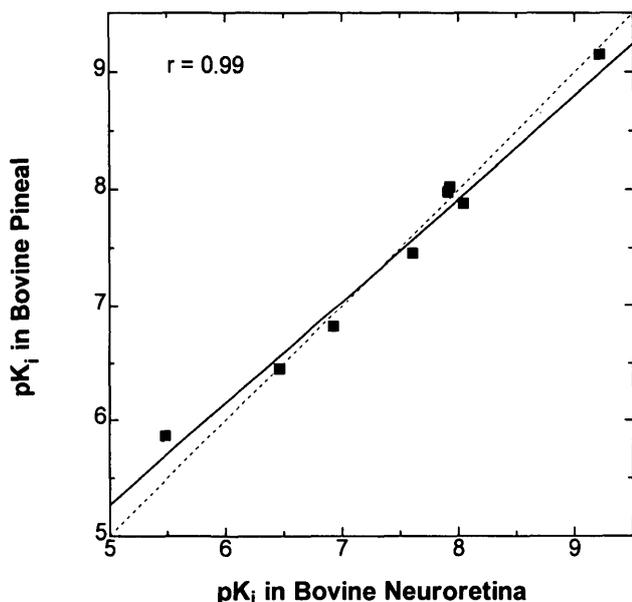
of the bovine pineal receptor and, thus, can be classified reasonably as alpha-2D.

We also used correlation analyses to compare the bovine neurosensory retina data with our published data for the human alpha-2A, -2B, and -2C clones,[24,29] as well as the rat alpha-2D clone[25] (Fig. 4). For the alpha-2A, -2B, and -2C subtypes of the alpha-2 adrenergic receptor, the correlation coefficients are poor (r = 0.36 to 0.80; Table 2), indicating that the character-

FIGURE 3. Correlation of antagonist affinities for alpha-2 adrenergic receptors in the bovine neurosensory retina and the bovine pineal gland. The affinities (pK_i values) are derived from Table 1. The slope is given in Table 2. The solid line represents the least squares correlation line, and the dashed line represents the line of identity.
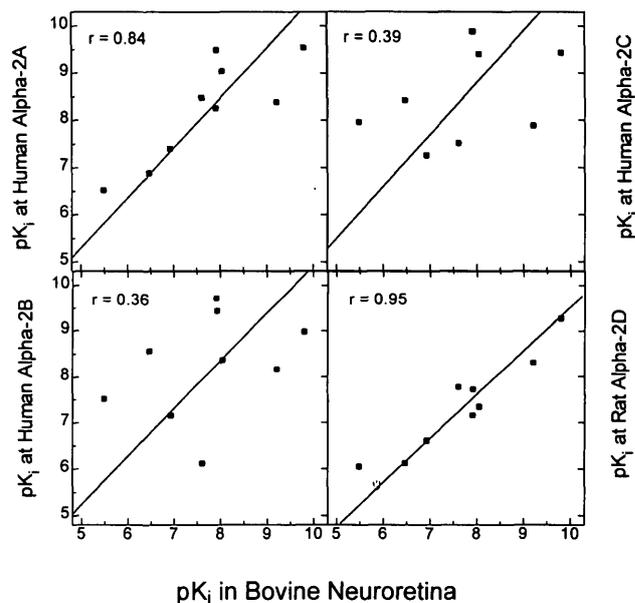

FIGURE 4. Correlation of antagonist affinities between the bovine neurosensory retina and COS cells transfected with the human alpha-2A, -2B, and -2C and the rat alpha-2D adrenergic receptors expressed in COS cells. The correlation coefficients and slopes are summarized in Table 2. The solid lines represent the least squares correlation lines.

TABLE 2. Correlation of pK_i Values for the Bovine Neurosensory Retina With Those of the α-2A, -2B, -2C, and -2D Subtypes

	Correlation Coefficient (r)	Slope
Bovine pineal (α-2D)	0.99	0.89
Human α-2A clone	0.84	0.69
Human α-2B clone	0.36	0.31
Human α-2C clone	0.39	0.31
Rat α-2D clone	0.95	0.75

istics of these receptors are different from the bovine neurosensory retina. By contrast, the rat alpha-2D pK_i values are similar to the bovine neurosensory retina ($r = 0.95$).

Saturation experiments with [³H]rauwolscine yielded inconsistent results at higher radioligand concentrations, probably because of the higher nonspecific binding and lower affinity of rauwolscine for the alpha-2D subtype than for [³H]RX821002. At lower concentrations, the results were inconsistent but indicated the possible presence of a minor population of alpha-2C receptors, which have a high affinity for rauwolscine. Furthermore, the Y79 cell, a human retinoblastoma cell line, appears to contain only the alpha-2C subtype.[30] Thus, we postulated that there might be a low density of alpha-2C receptors in the bovine neurosensory retina. To test this possibility, we performed inhibition experiments with a low concentration of [³H]rauwolscine. These experiments also used rauwolscine as the competing ligand because it has the greatest selectivity for alpha-2C over alpha-2D. The data from these experiments ($K_i = 9.9 \pm 1.6$ nM, slope $= 0.84$, $n = 3$) did not fit a two-site model significantly better than a one-site model, which is inconsistent with the hypothesis of the presence of some alpha-2C adrenergic receptors.

Recent data from our laboratory indicate that [³H]rauwolscine has a higher affinity for the alpha-2C subtype in phosphate buffer than in our normal glycylglycine assay buffer (Deupree, unpublished data, 1994). In contrast, the alpha-2A and -2B subtypes have higher affinity in glycylglycine buffer than in phosphate buffer. Thus, the use of phosphate buffer should increase the sensitivity of the assay to detect the presence of a small amount of the alpha-2C subtype. The data for the inhibition of [³H]rauwolscine binding by rauwolscine in phosphate buffer ($K_i = 15$ nM, slope $= 0.97$, $n = 3$) similarly did not fit a two-site model significantly better than a one-site model. When these data are transformed and plotted as bound versus bound $\times$ inhibitor concentration,[31] a straight line results (Fig. 5), which is also inconsistent with the presence of some alpha-2C adrenergic recep-
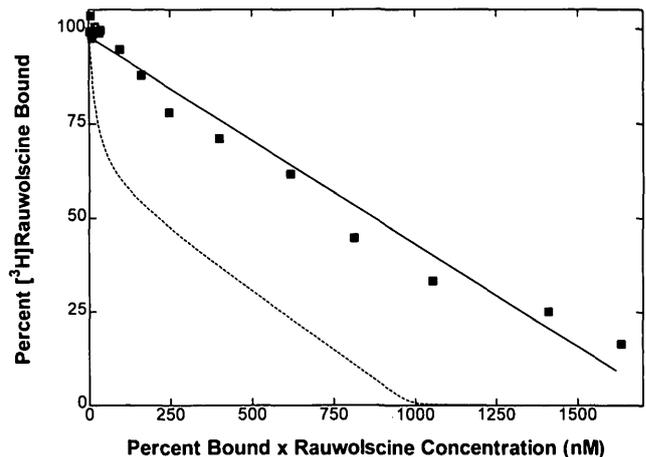

FIGURE 5. Inhibition of [³H]rauwolscine binding by rauwolscine in sodium phosphate buffer. The concentration of [³H]rauwolscine was 0.1 nM, and the final assay volume was 3.0 ml. The inhibition data (mean of two experiments) have been transformed to bound versus bound $\times$ inhibitor concentration as described.[31] The solid line represents the linear regression line of the transformed data. The dashed line represents the expected curve based on 2% of the receptors being alpha-2C ($K_i = 0.2$ nM) and 98% alpha-2D ($K_i = 16$ nM).

tors. The dotted line in Figure 5 is the expected data for 2% of the alpha-2C subtype and 98% of the alpha-2D subtype. Thus, if there are alpha-2C receptors in the bovine neurosensory retina, they represent <2% of the total density. To determine experimentally if a low percentage of alpha-2C would be detectable as indicated by the theoretical analysis above, an inhibition assay was performed with membranes from neurosensory retina tissue "spiked" with CHO cell membranes expressing only the alpha-2C subtype. These data are presented in Figure 6. The straight line is for unspiked neurosensory retina membranes and is essentially identical to that shown in Figure 5. The curved line is for neurosensory retina membranes spiked with CHO membranes such that 4% of the total alpha-2 receptors in the assay are alpha-2C and the other 96% are alpha-2D. These data indicate that a low percentage of alpha-2C receptors in the presence of a high percentage of alpha-2D receptor is easily detectable in this asasy system.

We also considered the possibility that a low density of alpha-2B adrenergic receptors might be present in the neurosensory retina. ARC-239 has a more than 100-fold selectivity for the alpha-2B (2.7 nM) than for the alpha-2D ($K_i = 345$nM) subtype. The inhibition curves for ARC-239 against [³H]RX821002 modeled as a single site ($n_H = 0.99$; Table 1; Fig. 2). Because [³H]RX821002 has 6.5-fold lower affinity for the alpha-2B (1.05 nM) than the alpha-2D subtype (0.16 nM), it is not a good radioligand for detecting a small contribution of the alpha-2B subtype in the presence
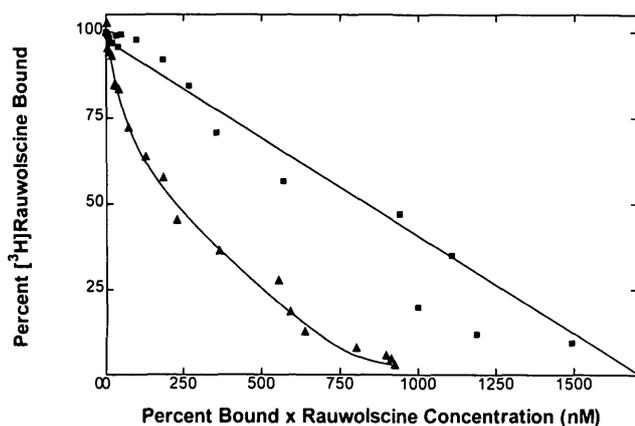
FIGURE 6. Inhibition of [³H]rauwolscine binding to "spiked" tissue by rauwolscine in sodium phosphate buffer. The details of the assay are as in Figure 5 for the "unspiked" experiment (■). For the spiked experiment (▲), sufficient membranes from Chinese hamster ovary cells transfected with the alpha-2C receptor[20] were added to the neurosensory retina membranes so that the concentration of alpha-2C receptors was 4% of the total, as determined by separate saturation experiments with [³H]rauwolscine.

of predominately alpha-2D receptors. Therefore, we repeated the ARC-239 inhibition experiments using [³H]rauwolscine, which has a 40-fold higher affinity for alpha-2B (0.4 nM) than alpha-2D (16 nM). The data for the inhibition of [³H]rauwolscine binding by ARC-239 in glycylglycine buffer (K_i = 970 nM, slope = 1.3, n = 2) did not fit a two-site model significantly better than a one-site model. When these data are transformed and plotted as Bound versus Bound × Inhibitor Concentration, a straight line results (Fig. 7), which is inconsistent with the presence of some alpha-2B adrenergic receptors. The dotted line in Figure 7, as in Figure 5, represents the expected data for 2% of the alpha-2B subtype and 98% of the alpha-2D subtype. Thus, we found no evidence for the presence of the alpha-2B subtype.

DISCUSSION

Whereas beta adrenergic receptors have been characterized in multiple ocular tissues and in multiple species, the alpha-2 adrenergic receptor has received much less attention. There have been several autoradiographic studies of alpha-2 adrenergic receptor localization in ocular tissues, but none of these have addressed the issue of alpha-2 subtypes.[32-34] Our saturation experiments show that [³H]RX821002 is an excellent ligand for labeling alpha-2 adrenergic receptors in the bovine neurosensory retina with high affinity and a remarkably high receptor density. The competition radioligand binding studies show that the pK_i values for the bovine neurosensory retina are
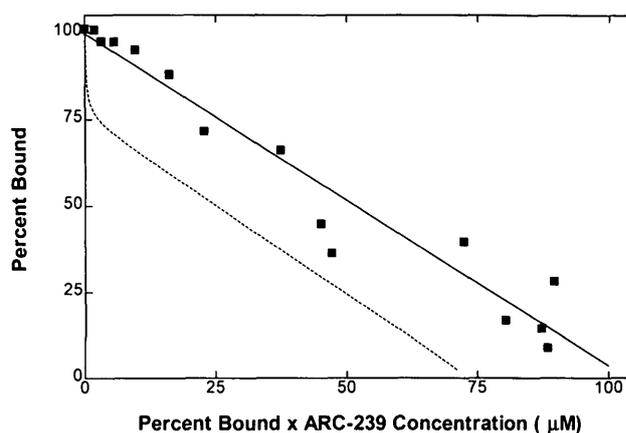

FIGURE 7. Inhibition of [³H]rauwolscine binding by ARC-239 in glycylglycine buffer. The concentration of [³H]-rauwolscine was 0.10 nM, and the final assay volume was 3 ml. The inhibition data (mean of two experiments) have been transformed to bound versus bound × inhibitor concentration as described.[31] The solid line represents the linear regression line of the transformed data. The dashed line represents the expected curve, based on 2% of the receptors being alpha-2B and 98% alpha-2D, assuming K_i values for ARC-239 of 2.7 nM and 970 nM for the alpha-2B and alpha-2D subtypes, respectively, and K_D values for [³H]rauwolscine of 0.4 nM and 16 nM for the alpha-2B and -2D subtypes, respectively.

closely correlated with pK_i values from bovine pineal, which is the prototypical tissue for alpha-2D adrenergic receptor subtype.[5]

The alpha-2 receptors of the bovine retina have been characterized recently by radioligand binding using several antagonist radioligands, although no B_{max} values were given.[17] This study investigated the alpha-2 adrenergic receptor subtypes in human and bovine frontal cortex and bovine retina, and the authors found that these three tissues had a low-affinity receptor for the alpha-2 adrenergic antagonist prazosin. They concluded from these data that the alpha-2 subtype present in the bovine neurosensory retina is alpha-2A. The strength of this conclusion is limited by the use of a single drug (prazosin), instead of the 5 to 10 generally used, and by not considering the possibility of the pharmacologic alpha-2D subtype, a species orthologue of the alpha-2A subtype. The alpha-2A adrenergic receptor is present in humans, rabbits, and pigs, whereas alpha-2D subtype is present in the bovine, rat, mouse, and guinea pig. Because alpha-2A and -2D are species orthologues and thus are mutually exclusive in the same species, the alpha-2 adrenergic receptor subtype in bovine neurosensory retina would be expected to be alpha-2D rather than alpha-2A.

Our data indicate that fewer than 2% to 4% of the total alpha-2 receptors in the neurosensory retina are of the alpha-2C subtype. It is unusual to be able

to exclude the presence of a given alpha-2 subtype to such a low level. Two factors make it possible in this system—a high density of total alpha-2 adrenergic receptors and a radioligand highly selective for the subtype excluded. The approximate 100-fold selectivity of [^{3}H]rauwolscine for alpha-2C over alpha-2D, coupled with our assay conditions (0.1 nM [^{3}H]rauwolscine), indicates that if there were 2% alpha-2C and 98% alpha-2D, approximately 40% of the binding in the absence of a competing ligand would be to the alpha-2C. By inhibiting the binding with a subtype-selective drug (rauwolscine), the presence of the alpha-2C subtype would be obvious (dotted line, Fig. 5; curved line, Fig. 6). Similar experiments in which [^{3}H]rauwolscine was inhibited by ARC-239 excluded the presence of the alpha-2B subtype. Based on these data, we concluded that the alpha-2D receptor is the only alpha-2 adrenergic subtype present in the bovine neurosensory retina. It should be noted, however, that if either (or both) the alpha-2C or alpha-2B subtype were present, our experiments would not easily have distinguished between them, and additional experimental strategies would have been needed to determine how much of these other two subtypes was present.

The usual range of B_{max} values for alpha-2 receptors in tissues is on the order of 50 to 500 fmol/mg protein. The very high density of alpha-2D receptors in the neurosensory retina (1500 fmol/mg protein) suggests that these receptors are actually in the retina, not only in the retinal vessels. What function the alpha-2D receptors have in the retina and whether they play a role in the development of cystoid macular edema after long-term application of epinephrine remains to be investigated. The effects of alpha-2 agonists on the bovine neurosensory retina remain unknown, although our data support the idea that at least some of the adrenergic modulation of the retina may occur through the alpha-2D receptor in bovine eyes.

Key Words

adrenergic antagonist, alpha-2 adrenergic, catecholamines, receptor subtypes, receptors, retinal

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