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Identification and Characterization of α_{2D}-Adrenergic Receptors in Bovine Pineal Gland

V. SIMONNEAUX,[1] M. EBADI, and D. B. BYLUND

Departments of Pharmacology (V.S., M.E., D.B.B.) and Anesthesiology (D.B.B.), University of Nebraska Medical Center, Omaha, Nebraska 68198-6260

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SUMMARY

The vertebrate mechanisms for generating circadian rhythms, regulating the synthesis of melatonin, and modulating the activities of pineal indole-synthesizing enzymes differ significantly among species. The synthesis of melatonin in mammalian pineal glands is stimulated by β_1-adrenergic receptor agonists and is potentiated by α_1-adrenergic receptor agonists, whereas in the avian pineal gland α_2-adrenergic receptor agonists inhibit its synthesis. By using [^{3}H]rauwolscine, a selective α_2-adrenergic receptor antagonist, we have identified for the first time α_2-adrenergic receptor sites in a mammalian pineal gland. [^{3}H] Rauwolscine bound in a saturable manner to a single class of receptors, with an equilibrium dissociation constant (K_D) of 1.4 nM and a density (B_{max}) of 71 fmol/mg of protein, in crude synaptic membrane preparations from bovine pineal gland. Competition studies carried out with various adrenergic antagonists supported the conclusion that [^{3}H]rauwolscine binding sites were α_2-adrenergic receptors. The bovine pineal α_2-adrenergic receptor appears to represent a pharmacological subtype distinct from the three currently proposed subtypes, i.e., α_{2A} found in a human colonic adenocarcinoma cell line (HT29 cell), α_{2B} found in rat lung, and α_{2C} found in an opossum kidney cell line (OK cell). However, the pharmacologic profile of the pineal α_2 receptor resembles that found in the rat submaxillary gland. We suggest that the bovine pineal receptor may represent a fourth pharmacological subtype, which would be designated as α_{2D}.

The vertebrate pineal glands, by generating circadian rhythms and by functioning as biological clocks, synchronize numerous physiological functions and control many biochemical events. The mammalian pineal glands possess unique features, such as receiving both peripheral and central innervations, being part of the central nervous system but existing outside of the blood-brain barrier, and exhibiting an array of species-specific variations in the control of the synthesis of pineal indoles such as melatonin, 5-methoxytryptamine, and 5-methoxytryptophol (see Refs. 1 and 2 for reviews). In mammalian species, the synthesis of melatonin is primarily but not exclusively subject to adrenergic control. Stimulation of sympathetic neurons at night results in the release of norepinephrine, which interacts with β-adrenergic receptors on pinealocytes and activates the membrane-bound enzyme adenylate cyclase. This results in an increased formation of cyclic AMP, the induction of serotonin *N*-acetyltransferase, and the acetylation of serotonin to *N*-acetylserotonin, which is subsequently methylated to melatonin (see Ref. 3 for a review).

In avian species such as the chicken, norepinephrine acts on α_2-adrenergic receptors to inhibit serotonin *N*-acetyltransferase activity (4, 5), and this appears to be the major adrenergic regulatory pathway. Several studies have provided evidence that α- as well as β-adrenergic receptors are involved in regulating the activity of serotonin *N*-acetyltransferase in mammals (6–8). For example, pretreatment of rats with phenoxybenzamine or phentolamine prevented the rapid light-mediated decrease in the activity of serotonin *N*-acetyltransferase (6). In addition, the administration of clonidine, an α_2 receptor agonist, mimicked the effects of light and reduced the activity of serotonin *N*-acetyltransferase when elevated, and the clonidine-mediated reduction in the activity of serotonin *N*-acetyltransferase was blocked by yohimbine. The possible presence of α_2-adrenergic regulation of melatonin synthesis in the human is suggested by the observation that clonidine administration reduces plasma melatonin levels in a dose-dependent manner (9).

These results suggest that α_2 agonists may regulate melatonin synthesis, and they provide indirect evidence for the presence

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[1]Present address: University of Strasbourg, URA-CNRS 1332, 12 Rue de L'Uneveriste, Strasbourg 67000, France.

ABBREVIATIONS: WB4101, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4-benzodioxane; ARC-239, 2-(2,4-(*O*-methoxyphenyl)-piperazine-1-yl)-ethyl-4,4-dimethyl-1,3-(2*H*,4*H*)-isoquinolindione; SKF 104078, 6-chloro-9-[(3-methyl-2-butenyl)oxy]-3-methyl-1*H*-2,3,4,-tetrahydro-3-benzazapine; 8-OH-DPAT, 8-hydroxy-2-di-*N*-propylamino-tetralin; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; OK, opossum kidney.

of α_2-adrenergic receptors in mammalian pineal gland. In order to provide direct evidence for the presence of these receptors in a mammalian gland, [^{3}H]rauwolscine, a specific α_2 receptor antagonist, was used in radioligand binding assays to label α_2-adrenergic receptors in a membrane preparation of the bovine pineal gland. We now report the identification and initial characterization of α_2-adrenergic receptors in the bovine pineal gland.

Materials and Methods

[^{3}H]Rauwolscine (98 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Chlorpromazine, (−)-norepinephrine, serotonin, and yohimbine were purchased from Sigma Chemical Co. (St. Louis, MO). WB4101 was purchased from Research Biochemicals Inc. (Wayland, MA). The following drugs were kindly donated by the indicated companies: SKF 104078 and SKF 104856, SmithKline Beecham (King of Prussia, PA); prazosin, Pfizer Chemical Division (New York, NY); oxymetazoline, Schering Corporation (Kenilworth, NJ); phentolamine, CIBA-Geigy (Summit, NJ); ARC-239, Thomae GmbH (Biberach, FRG); and BAM 1303, Maruko Pharmaceutical Co. (Nagoya, Japan). Yohimbine and prazosin were solubilized in ethanol, BAM 1303 was dissolved in 80% dimethylsulfoxide/20% 1 mM HCl, and other drugs were dissolved in 5 mM HCl.

Fresh bovine pineal glands were obtained from a local slaughterhouse between 9:00 a.m. and 11:00 a.m. and were kept in ice-cold 0.32 M sucrose solution for a period of not longer than 2 hr thereafter. The glands were dissected free of connective tissue and blood vessels. Only fresh glands were used for saturation studies, because freezing was observed to decrease receptor density. For inhibition experiments, pineal glands were frozen on dry ice and stored at −70° until used. Pineal glands were homogenized in 25 volumes of ice-cold 0.32 M sucrose solution, containing 15 mM potassium phosphate buffer (pH 7.4), for 30 sec with a Tissumizer (Tekmar, Cincinnati, OH) at 85% full speed. Homogenates were centrifuged at 900 × g for 10 min. The supernatant was saved and the pellets were washed in 20 volumes of the same buffer and centrifuged again. The two supernatants were combined and centrifuged at 48,000 × g for 20 min. The crude synaptic membrane preparations were homogenized for 30 sec in 20 volumes of sucrose-potassium phosphate buffer and centrifuged again for 20 min at 48,000 × g. The synaptic membranes were then resuspended and homogenized in 60 volumes of 25 mM glycylglycine buffer (pH 7.6) and were used for receptor binding assays as previously reported for the chicken pineal gland (10).

For α_2-adrenergic receptor saturation experiments, total binding was determined with triplicate tubes containing 970 μl of membrane suspension (about 370 μg of membrane protein) and 20 μl of various concentrations of [^{3}H]rauwolscine (final concentration, approximately 0.05–5 nM) at 22° for 30 min. The nonspecific binding was determined with a parallel set of triplicate tubes that, in addition, contained a final concentration of 100 μM (−)-norepinephrine. Specific binding, calculated as the difference between total and nonspecific binding, was about 70% of total binding. Membrane suspensions were then filtered through a 0.1% polyethylenimine-presoaked Whatman GF/B glass fiber filter strip with a 48-sample cell harvester (Biomedical Research and Development, Gaithersburg, MD). Filters and tubes were washed four times with 5 ml of ice-cold 50 mM Tris·HCl (pH 8.0). Radioactivity trapped on the filters was counted by liquid scintillation photometry with a Beckman LS 2800 scintillation counter at 41% efficiency.

For competition experiments, duplicate tubes containing 970 μl of membrane suspension, 20 μl of [^{3}H]rauwolscine (1.5 nM final concentration), and 10 μl of increasing concentrations of various unlabeled drugs were incubated and processed as for saturation experiments.

For saturation experiments, the K_D and B_{max} values were calculated from computer-assisted nonlinear regression of bound versus free radioligand concentrations. Competition binding data were analyzed using a nonlinear least-squares curve-fitting program (GraphPad Inplot, San Diego, CA) for calculating K_i and Hill slope values. Binding data were also fit to one- or two-binding site models, and the two fits were compared for statistical significance using the F test. All of the values are given as their mean ± standard error. Concentration of protein in the synaptic membrane preparations was determined by the method of Lowry *et al.* (11), with bovine serum albumin as a standard.

Results

[^{3}H]Rauwolscine, a selective α_2-adrenergic receptor antagonist, binds saturably to bovine pineal membranes (Fig. 1). The analyses of these saturation data indicate that [^{3}H]rauwolscine bound with high affinity to pineal membranes, with an equilibrium dissociation constant (K_D) of 1.4 ± 0.1 nM (six experiments). However, the affinity of [^{3}H]rauwolscine for pineal membrane was somewhat lower than its affinity for the human colonic HT29 cell line (0.66 nM) (12) or for the OK cell line (0.07 nM) (13). The density (B_{max}) of pineal α_2-adrenergic binding sites was 71 ± 3 fmol/mg of protein. Nonspecific binding was about 30% of total binding at the K_D value. Linear transformation of the saturation data according to the method of Rosenthal (14) yielded a straight line, indicating that [^{3}H]rauwolscine bound to a single class of receptors over the concentration range used (Fig. 1, *inset*). By comparison, the chicken pineal gland assayed under the same conditions (10) had a 5-fold higher affinity (lower K_D, 0.27 nM) and a 3-fold higher B_{max} (242 fmol/mg protein).

Kinetic studies demonstrated that the binding was rapid and reversible, as expected. At a concentration of [^{3}H]rauwolscine well below the K_D, binding reached steady state by 15–20 min and then was stable until at least 60 min (Fig. 2). An incubation time of 30 min was used for saturation and inhibition assays. The binding was also rapidly reversible, with a $t_{1/2}$ of 5 min and k_{-1} of 0.14 min^{-1}. The kinetic K_D, calculated as k_{-1}/k_{+1}, was 1.2 nM, which is in good agreement with the equilibrium K_D of 1.4 nM.

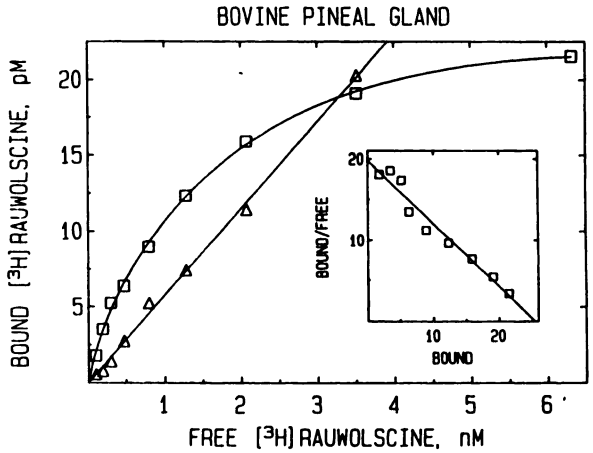

Fig. 1. [^{3}H]Rauwolscine saturation curve. Various concentrations (0.10–6.3 nM) of the selective α_2-adrenergic receptor antagonist [^{3}H]rauwolscine were incubated with membranes from bovine pineal glands (0.35 mg/ml) in the absence (total binding) or presence of 100 μM (−)-norepinephrine (nonspecific binding). Specific binding (□) was calculated as the difference between total binding and nonspecific binding (△). The data for specific binding and free [^{3}H]rauwolscine were fit to a single-site hyperbola by nonlinear regression (GraphPad Inplot) to obtain K_D (1.43 nM) and B_{max} (26.5 pM; 75 fmol/mg of protein). The mean values for six experiments are given in the text. *Inset*, Rosenthal plot of [^{3}H]rauwolscine specific binding (16). This transformation yields a straight line, indicating the existence of a single class of binding sites.

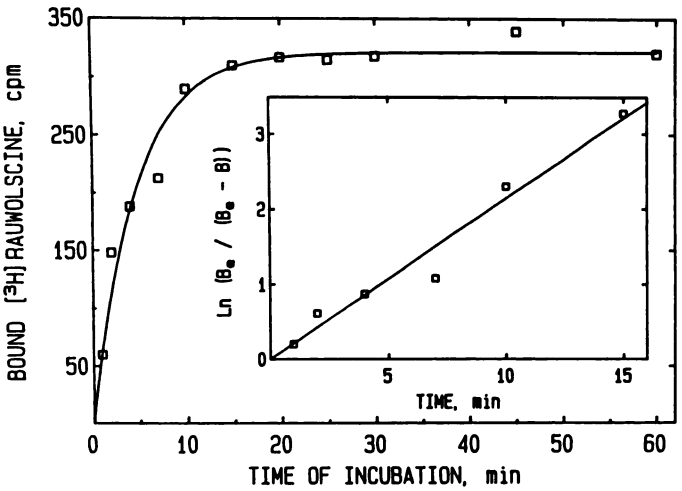

Fig. 2. Association time course for [^{3}H]rauwolscine binding to bovine pineal gland membranes. Incubations were stopped at the times indicated, by filtration. The concentration of [^{3}H]rauwolscine was 0.62 nM. The data shown are from one of three similar experiments. *Inset*, pseudo-first-order association plot of the data. The slope of the line, k_{obs}, was 0.21 min^{-1}. The k_{+1}, calculated as $(k_{obs} - k_{-1})$/free, was 0.11 min^{-1} nM^{-1}.

The results of competition studies (Fig. 3, Table 1) confirmed the α_2-adrenergic nature of [^{3}H]rauwolscine binding, because yohimbine was more potent than prazosin or ARC-239 in inhibiting [^{3}H]rauwolscine binding. A greater potency of prazosin and ARC-239, compared with yohimbine, would be characteristic of binding to an α_1-adrenergic receptor (15). All antagonists interacted at the [^{3}H]rauwolscine binding site in a homogeneous manner, with competition curves fitting best to a one-site model and Hill slopes close to 1.0.

α-Adrenergic agonists such as oxymetazoline and (−)-norepinephrine interacted at [^{3}H]rauwolscine binding sites in a heterogeneous manner, indicating that α_2-adrenergic receptors of bovine pineal gland exist in both high and low affinity states (Fig. 4). (−)-Norepinephrine displayed a biphasic curve ($p < 0.001$) and bound with high affinity to about 35% of α_2-adrenergic receptors. Oxymetazoline also recognized about 35% of α_2-adrenergic receptors in high affinity states, although the significance of a two-site model for its competition curve was low ($p = 0.05$). α_2-Adrenergic receptors are known to couple to guanine nucleotide-binding regulatory proteins, such as G_i. To determine whether the bovine pineal α_2-adrenergic receptor is similarly coupled to a guanine nucleotide-binding protein, the inhibition experiments with (−)-norepinephrine were repeated without and with 100 μM Gpp(NH)p. Gpp(NH)p shifted the inhibition curve 5-fold to the right and increased the Hill slope to 0.74 (three experiments). These results are consistent with the conclusion that the α_2 receptor in the bovine pineal gland is coupled to G_i.

Radioligand binding (12, 16, 17) and functional studies (18), as well as molecular biology techniques (19–22), have established the existence of α_2-adrenergic receptor subtypes (23). The delineation of the specific pharmacological subtype of the α_2-adrenergic receptor in bovine pineal gland was carried out using a variety of adrenergic drugs, by determining their affinities (K_i values) at [^{3}H]rauwolscine binding sites (Table 1). In Table 2, the affinities of these adrenergic drugs for the bovine pineal receptor are compared with their affinities for the known α_2-adrenergic subtypes. Chlorpromazine, prazosin, and ARC-239 had low affinity at the pineal α_2-adrenergic receptor, which is characteristic of the α_{2A} subtype, suggesting that the pineal receptor cannot be reasonably classified as an α_{2B} or α_{2C} receptor. The relative low affinity of [^{3}H]rauwolscine for the pineal receptors (1.5 nM), compared with the OK cell (0.07 nM) (15), and its low affinity for spiroxatine, compared with the high affinity of the α_{2B} and α_{2C} subtypes for this drug (Table 2), support this contention. Several drugs also clearly differentiate the bovine pineal from the α_{2A} subtype and thus militate against designating the pineal receptor as an α_{2A} subtype. For example, WB4101 had an affinity of 7.6 nM in the bovine pineal, which is characteristic of the α_{2B} subtype and 8-fold lower than that of the HT29 cell. BAM 1303 and SKF 104078 were about 25-fold and 15-fold weaker, respectively, on pineal membranes than at the α_{2A} receptor in HT29 cells.

In addition to comparing absolute affinities of the various drugs for the receptor subtypes, it is often useful to compare the ratio of the K_i values for two drugs at each subtype. Table 3 presents such a comparison for six pairs of drugs. Several ratios clearly differentiate the bovine pineal from the α_{2A} receptor of the HT29 cell, including prazosin/SKF 104078 (0.26 versus 10) and prazosin/WB4101 (14 versus 660). Similarly, the bovine pineal receptor can be differentiated by the oxyme-

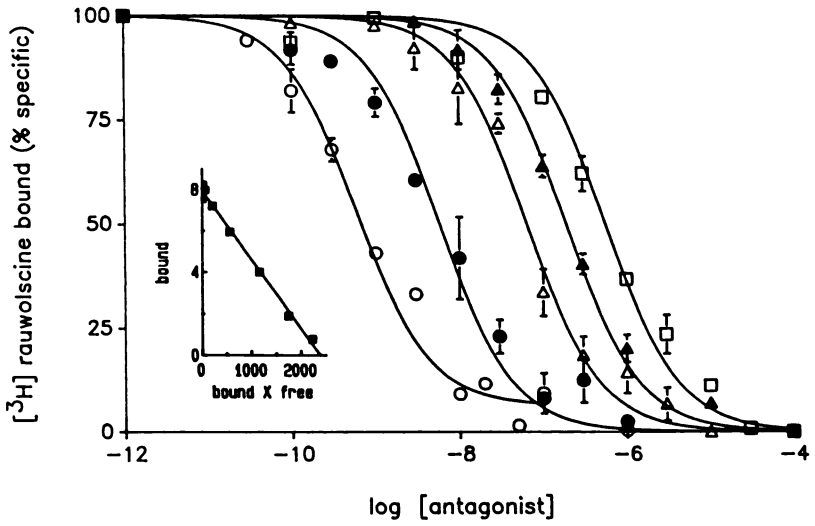

Fig. 3. Inhibition of [^{3}H]rauwolscine binding by α_2-adrenergic antagonists. Pineal membrane suspensions were incubated for 30 min at 22° with 1 nM [^{3}H]rauwolscine and various concentrations, as indicated, of phentolamine (○), yohimbine (●), BAM 1303 (△), prazosin (▲), and chlorpromazine (□). For each concentration of antagonist, [^{3}H] rauwolscine binding is expressed as a percentage of specific binding in the absence of any drug. Results are mean ± standard error (three or four experiments). For all of the antagonists, the data were fit best by a single-site model. Calculated K_i values from these curves are given in Table 1. *Inset*, Inhibition of [^{3}H]rauwolscine binding by the α_2-adrenergic antagonist ARC-239 (■). Inhibition data of one experiment were transformed to the form of bound [^{3}H] rauwolscine versus bound [^{3}H]rauwolscine × ARC-239 concentration, according to the method of Bylund (24). This transformation gives a straight line, indicating that ARC-239 binds to a single class of α_2-adrenergic receptors.

TABLE 1

Inhibition of [^{3}H]rauwolscine binding on bovine pineal membranes by adrenergic receptor agonists or antagonists

Pineal membrane suspensions were incubated with [^{3}H]rauwolscine (approximately 1 nM) and 9–18 concentrations of adrenergic antagonists or agonists for 30 min at 22°. n_H and IC_{50} values were derived from a computer-assisted nonlinear least-squares curve-fitting program; K_i values were calculated using the Cheng-Prusoff correction, using a [^{3}H]rauwolscine K_D value of 2.3 nM (39). Antagonists and serotonin were routinely modeled as one-site binding curves, and only one K_i value is given. Competition curves for agonists fit best to a two-site binding model, in which K_H and K_L are inhibition constants for the high and low affinity sites, respectively, and R_H is the percentage of binding sites in the high affinity state. Results are mean ± standard error of experiments.

	K_i or K_H	K_L	R_H	n_H	n
	nM	nM	%		
Agonists					
Oxymetazoline	1.45 ± 0.32	49 ± 19	34 ± 5	0.63 ± 0.04	3
(−)Norepinephrine	13 ± 3	1780 ± 290	35 ± 1	0.35 ± 0.01	3
Serotonin	4910 ± 40			1.07 ± 0.08	2
Antagonists					
Phentolamine	3.1 ± 0.6			0.76 ± 0.08	9
Rauwolscine	3.4 ± 0.4			0.78 ± 0.08	6
Yohimbine	3.6 ± 0.4			0.84 ± 0.08	3
WB4101	7.6 ± 0.8			0.97 ± 0.15	9
Spiroxatrine	9.3 ± 1.0			0.73 ± 0.05	5
SKF 104856	43 ± 3			1.03 ± 0.09	4
(+)-Mianserin	48 ± 3			0.90 ± 0.18	6
BAM 1303	53 ± 5			0.90 ± 0.07	7
Prazosin	106 ± 10			0.86 ± 0.01	3
ARC-239	149 ± 24			0.99 ± 0.01	3
Chlorpromazine	380 ± 20			0.79 ± 0.05	3
SKF 104078	400 ± 40			1.16 ± 0.23	5
Corynanthine	770 ± 170			0.93 ± 0.11	3
(−)-Mianserin	1000 ± 160			0.77 ± 0.02	4
8-OH-DPAT	1560 ± 140			0.78 ± 0.19	3

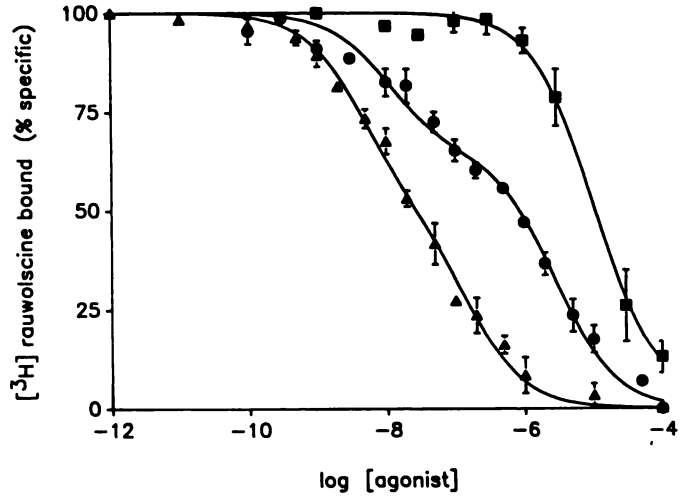

Fig. 4. Inhibition of [^{3}H]rauwolscine binding by various agonists. Pineal membrane suspensions were incubated for 30 min at 22° with 1 nM [^{3}H] rauwolscine and increasing concentrations, as indicated, of oxymetazoline (▲), (−)-norepinephrine (●), and serotonin (■). For each concentration of agonist, radioligand binding is expressed as a percentage of specific binding in the absence of any drug. Results are mean ± standard error (three experiments). For oxymetazoline and (−)-norepinephrine, binding data were fit best by a two-site model. Calculated K_i values are given in Table 1.

tazoline/rauwolscine ratio from the α_{2B} (rat lung; 0.43 versus 160) and the α_{2C} (OK cell; 0.43 versus 1620) receptors.

Another method frequently used to compare affinities of many drugs for various receptor subtypes is correlation of the logarithm of the K_i values. The correlations between the pK_i values of 16 drugs for bovine pineal gland and either the α_{2B}

TABLE 2

Comparison of affinities of various adrenergic compounds for putative α_2-adrenergic receptor subtypes

Values are the negative logarithm of the K_i values (pK_i) for the various drugs. The data for bovine pineal gland are taken from Table 1, and those for rat submaxillary gland are from Michel *et al.* (30). Data for the cloned receptors are from Ref. 19 for human C10 and C4, Ref. 20 for rat C2, Ref. 21 for human C2, and Ref. 22 for rat C4. The data for the other tissues are taken from published (10, 12, 13, 40) and unpublished results from our laboratory.

	pK_i												
Adrenergic compounds	Bovine pineal gland α_{2D}	HT29 cell α_{2A}	Chicken pineal gland α_{2A}	Human C10 clone α_{2A}	Rat lung α_{2B}	Rat C2 clone α_{2B}	Human C2 clone α_{2B}	OK cell α_{2C}	Rat C4 clone	Human C4 clone	Rat submaxillary gland
Oxymetazoline	8.84	9.10	8.49	7.96	7.15	6.21	6.67	7.16	7.47	7.21	8.06
Phentolamine	8.51	8.22	8.25	8.00	8.44			8.01		7.48	8.47
Rauwolscine	8.47	9.36	9.66	8.57	9.35	8.70	9.49	10.37	9.77	9.42	7.73
Yohimbine	8.44	9.19	9.40	8.80	9.00	8.30	8.92	9.73	8.82	9.03	
WB4101	8.12	8.92	9.28	8.11	8.06		7.77	9.57	8.80	9.03	7.22
Spiroxatrine	8.03	7.89			9.47			9.23			6.60
SKF 104856	7.37	7.28			8.20			7.17			
(+)-Mianserin	7.32	8.28	8.15		7.85			8.29			
BAM 1303	7.27	8.60			7.59			9.42			
Prazosin	6.98	6.50	7.02	5.74	8.27	7.57	7.64	7.82	7.70	7.39	6.34
ARC-239	6.83	6.77	6.88		8.80			7.89			
Chlorpromazine	6.42	6.60	7.28		7.66	8.15		7.43	7.55		5.98
8-OH-DPAT	5.81	6.73			6.83			6.93			
SKF 104078	6.40	7.50	7.44	7.07	7.31			7.37		7.48	6.51
Corynanthine	6.11	6.88	7.05	6.15	7.00		7.11	7.55	7.09	7.14	5.81
(−)-Mianserin	6.00	7.34	≥ 7.09		7.34			6.58			

TABLE 3

Comparison of K_i value ratios for various α_2-adrenergic receptor subtypes

The ratio of the K_i values for the indicated drugs in various tissues, cell lines, and cloned receptors were calculated from the data in Table 2. Data for the human platelets and the NG108 cells were taken from Ref. 12. Data for opossum kidney are unpublished results from our laboratory.

Tissue	Oxymetazoline/ rauwolscine	Phentolamine/ rauwolscine	Corynanthine/ phentolamine	Prazosin/ corynanthine	Prazosin/ WB4101	Prazosin/ SKF104078
α_{2D}						
Bovine pineal	0.43	0.91	250	0.13	14	0.26
Rat submaxillary gland	0.47	0.18	460	0.54	8	1.48
α_{2A}						
HT29 cell	2	14	22	2.4	260	10
Chicken pineal	15	26	16	1.1	180	3
Human platelet	3	25	13	2.9	330	13
Human C10	4	4	71	2.5	230	21
α_{2B}						
Rat lung	160	8	26	0.05	0.62	0.11
NC108 cell	165	19	10	0.09	0.58	0.28
Human C2	670			0.30	1.35	
α_{2C}						
OK cell	1620	230	2.9	0.54	56	0.35
Opossum kidney	910	410	0.6	1.80	22	
Human C4	160	87	2.2	0.56	44	1.24
Rat C4	200			0.25	13	

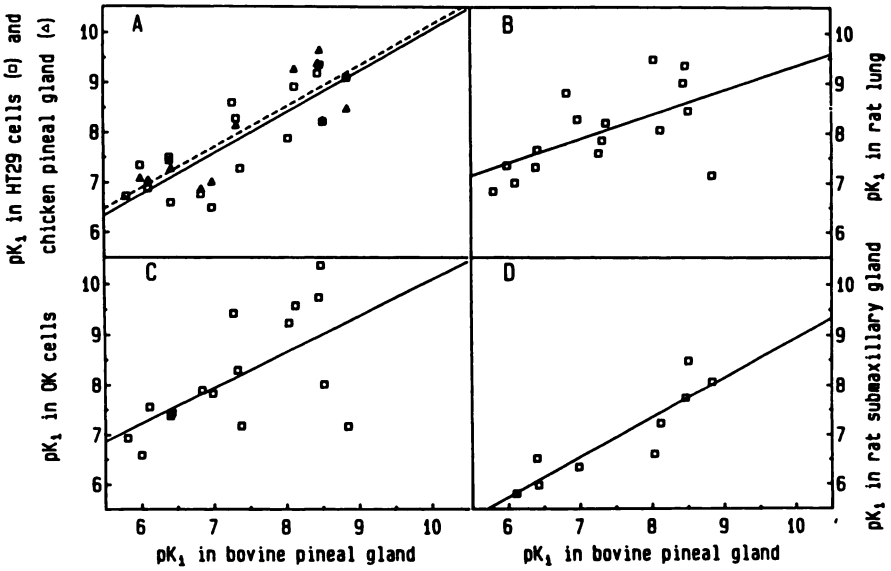

Fig. 5. Correlation between adrenergic drug affinity estimates (pK_i) obtained for the α_2-adrenergic receptors of bovine pineal gland and of various tissues labeled with [^{3}H]rauwolscine. pK_i values are given in Table 2. A, Correlation of drug affinities between bovine pineal gland and human colonic adrenocarcinoma (HT29) cells (□) and between bovine and chicken pineal gland (△). B, Correlation of drug affinities between bovine pineal gland and rat lung. C, Correlation of drug affinities between bovine pineal gland and OK cells. D, Correlation of drug affinities between bovine pineal gland and rat submaxillary gland. Correlation coefficients and slopes are summarized in Table 4.

(rat lung) (Fig. 5B) or the α_{2C} (OK cell) (Fig. 5C) subtype were poor, with correlation coefficients of 0.59 and 0.62, respectively. Better, but not good, correlations were found between bovine pineal and the α_{2A} receptor subtype found in the HT29 cell line (r = 0.82) and the chicken pineal gland (r = 0.84) (Fig. 5A). The correlation coefficients and slopes of the correlation lines are summarized in Table 4.

Based on a comparison of the K_i values of the drugs, ratios of K_i values, and correlation of pK_i values, it appears unreasonable to classify the pineal α_2 receptor as either α_{2A}, α_{2B}, or α_{2C}. We considered the possibility that our results in the pineal gland might be due to the presence of a mixture of α_{2A}- and either α_{2B}- or α_{2C}-adrenergic receptors. However, transformation of ARC-239 inhibition data to the linear form of bound versus bound × drug concentration (24) yielded a straight line (Fig. 3, *inset*). Because ARC-239 clearly differentiates between α_{2A} and both α_{2B} and α_{2C}, it appears that ARC-239 binds to a single subtype of receptor in the bovine pineal. We also considered the possibility that [^{3}H]rauwolscine might be binding to a nonadrenergic site. For example, it had been suggested that under certain conditions [^{3}H]rauwolscine may label serotonin-1A receptors (25). However, previous preliminary work has not provided any evidence for serotonin-1 receptors in the bovine pineal gland (26). Furthermore, the high affinity of phentolamine and the low affinity of serotonin and 8-OH-DPAT for the bovine pineal gland effectively eliminate the possibility that the [^{3}H]rauwolscine site in the bovine pineal gland is a serotonin receptor.

TABLE 4

Correlations of pK_i values

Correlation analyses were done on the data from Table 2. The correlation coefficient is r, and the number of pairs of data points in the analysis is n.

Tissue/cell line	r	Slope	n
Bovine pineal versus HT29 cell (α_{2A})	0.82	0.83	16
Bovine pineal versus chicken pineal (α_{2A})	0.84	0.82	12
Bovine pineal versus rat lung (α_{2B})	0.59	0.49	16
Bovine pineal versus OK cell (α_{2C})	0.62	0.72	16
Bovine pineal versus rat submaxillary gland	0.90	0.80	9
HT29 cell (α_{2A}) versus chicken pineal (α_{2A})	0.95	0.89	12

Discussion

The results of this study demonstrate the presence of α_2-adrenergic receptors in the bovine pineal gland and suggest that the bovine pineal α_2-adrenergic receptor cannot be easily classified as either an α_{2A}, α_{2B}, or α_{2C} pharmacological subtype. This conclusion is based on a comparison of the K_i values, a comparison of the ratio of K_i values, and the correlations of pK_i values for the bovine pineal receptor with the prototype tissues for the three subtypes.

In addition to the three pharmacological subtypes, three α_2-adrenergic receptor subtypes have now been identified by molecular cloning techniques. The cloning of the first α_2 receptor was based on the receptor from human platelet. The human gene encoding this receptor is found on chromosome 10 and, thus, has been identified as the α_2-C10 receptor (27). The pharmacological characteristics of this cloned receptor are identical to those of the α_{2A} receptor, as defined by the HT29 cell and the human platelet. The rat non-glycosylated (RNG) α_2 receptor cloned from the rat (20) and the homologous clone from the human (21, 28) appear to correspond to the α_{2B} receptor, as defined by the neonatal rat lung and the NG108 cell line. The human gene is on chromosome 2 and is identified as α_2-C2. A third subtype, α_2-C4, has been cloned from human (19) and rat (22, 29). It may or may not be the same as the α_{2C} subtype defined by the OK cell. Tables 2 and 3 compare the pharmacological characteristics of these cloned receptors with the prototypic tissues/cell lines for the α_{2A}, α_{2B}, and α_{2C} subtypes, as well as with the bovine pineal receptor. As expected, the ratios in Table 3 for human C10 are very similar to those for the other α_{2A} tissues/cell lines. Similarly, human and rat C2 appear to have the same characteristics as the α_{2B} tissue/cell line. The human and rat C4 clones appear to be generally similar to the α_{2C} subtype. However, the pharmacology of the bovine pineal gland receptor is different from all of these receptors.

Recently, Michel *et al.* (30) have characterized an α_2-adrenergic receptor in rat submaxillary gland that also appears to be different from the previously characterized subtypes. This receptor appears to differ from both the receptor in the rabbit spleen (α_{2A}) and the receptor in the rat kidney (α_{2B}) (30). In order to determine the relationship, if any, between this rat submaxillary gland receptor and the bovine pineal receptor, we correlated the pK_i values for the two tissues (Fig. 5D; Table 4). A good correlation ($r = 0.90$) was found between the pharmacological characteristics of α_2-adrenergic receptors identified in the two tissues. As shown in Table 4, the correlation coefficient for the HT29 cell and chicken pineal gland receptors (both α_{2A} subtype) is 0.95, whereas for the bovine and chicken pineal gland receptors (different subtypes) it is 0.84. A correlation coefficient of 0.90 for the bovine pineal and rat submaxillary gland receptors falls between the two other correlation coefficients, suggesting that these two tissues could express the same subtype. This possibility is strengthened by the fact that the correlation between the bovine pineal and rat submaxillary gland involves data from different laboratories and may be influenced by differences in assay conditions or other variables, whereas for the other correlations all the data are from the same laboratory. Studies are underway in our laboratory to determine whether the bovine pineal and rat submaxillary gland express the same α_2 receptor subtype.

Thus, it appears that the α_2-adrenergic receptor of the bovine pineal gland may be either 1) a new receptor subtype or 2) a species variant of an existing subtype, having significantly different pharmacological characteristics. In either case, it appears to be a new pharmacological subtype. In order to be consistent with the currently accepted pharmacological classification scheme, we would suggest that this receptor subtype be tentatively identified as the α_{2D}-adrenergic receptor.

Ruffolo and co-workers (31–33) have presented evidence that pre- and postjunctional α_2-adrenoceptors may be differentiated by SKF 104078 in functional assays. Although Connaughton and Docherty (34, 35) have been unable to confirm these findings, other laboratories have confirmed that SKF 104078 does appear to be a postjunctional-selective α_2 antagonist at peripheral α_2-adrenergic receptors (36, 37). Binding studies to date have not found SKF 104078 to differentiate among the pharmacologically defined α_{2A}, α_{2B}, and α_{2C} (Table 2) or between the cloned human C-10 and C-4 α_2 receptors (19). The low affinity of SKF 104078 ($K_i = 400$ nM) for bovine pineal α_2-adrenergic receptors suggests that the α_{2D} receptor may represent a pharmacological presynaptic receptor. If this is indeed the case, it is worth emphasizing that all α_{2D} receptors would not need to be localized presynaptically and all presynaptic receptors would not need to be of the pharmacological α_{2D} subtype.

The physiological relevance of α_2-adrenergic receptors in the synthesis of melatonin has been studied in avian pineal gland. In chicken pineal gland, for example, the adrenergic inhibition of serotonin *N*-acetyltransferase activity is mediated by α_2-adrenergic receptors (4, 5). The molecular mechanisms of the rapid decrease of serotonin *N*-acetyltransferase activity in the mammalian pineal gland at the onset of light is still unknown. Alphs and co-workers (6, 8) observed that in rat the intraperitoneal administration of clonidine (an α_2-adrenergic agonist) induced a rapid decrease in pineal serotonin *N*-acetyltransferase activity, mimicking the effect of light. Moreover, this clonidine-mediated reduction in enzymatic activity was blocked by yohimbine. These results suggest the possible presence of functional α_2-adrenergic receptors in mammalian pineal glands. This is consistent with an earlier report of regulation of norepinephrine release in the rat pineal gland mediated by presynaptic α- (presumably α_2-adrenergic receptors (38). The reduction of plasma melatonin levels in the human by clonidine (9) similarly suggests the presence of α_2 receptors in the human pineal gland. Although the precise functions of α_2-adrenergic receptors in the bovine pineal gland are not clear, this adds an additional potential dimension to an already complex mechanism through which mammals synthesize melatonin and other pineal indoles. Similarly, it is possible that the function of α_2-adrenergic receptors in the bovine pineal gland is not associated with the synthesis of melatonin and may possess an unknown function, yet to be delineated.

In summary, the major findings of this study are 1) the identification of α_2-adrenergic receptor in a mammalian pineal gland and 2) the suggestion of a fourth putative pharmacological α_2 subtype, the α_{2D}-adrenergic receptor.

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Send reprint requests to: Dr. David B. Bylund, Department of Pharmacology, University of Nebraska Medical Center, 600 South 42nd Street, Omaha, NE 68198-6260.