0026-895X/98/050916-10$3.00/0

MOLECULAR PHARMACOLOGY, **53:**916–925 (1998).

Characterization of the Allosteric Interactions between Antagonists and Amiloride Analogues at the Human α_{2A}-Adrenergic Receptor

RAY A. LEPPIK, SEBASTIAN LAZARENO, ANITA MYNETT, and NIGEL J. M. BIRDSALL

National Institute for Medical Research, London NW7 1AA, UK (R.A.L., A.M., N.J.M.B.) and Medical Research Council Collaborative Centre, London NW7 1AD, UK (S.L.)

Received October 10, 1997; Accepted January 29, 1998

This paper is available online at http://www.molpharm.org

ABSTRACT

The purpose of this study was to determine whether there is a well-defined allosteric site on the human α_{2A}-adrenergic receptor. To explore this question, we examined the effects of amiloride analogues on the dissociation of [^{3}H]yohimbine, [^{3}H]rauwolscine, and [^{3}H]RX821002. The dissociation data fitted well to an equation derived from the ternary complex allosteric model with amiloride analogue concentration and time as two independent variables. The estimated maximal increase in the [^{3}H]yohimbine dissociation rate caused by the 5-*N*-alkyl amilorides varied from 2-fold for the parent amiloride to 140- and 160-fold for 5-(*N*,*N*-hexamethylene)-amiloride and 5-(*N*-ethyl-*N*-isopropyl)-amiloride, respectively. The calculated log affinities at the yohimbine-occupied receptor ranged from 1.75 for 5-(*N*-ethyl-*N*-isopropyl)-amiloride to 2.5 for 5-(*N*,*N*-hexamethylene)-amiloride. The increase in affinity found at the yohimbine-occupied receptor was not correlated with increase in size of the 5-*N*-alkyl side chain, in contrast to the situation found at the unoccupied receptor. The effect of competition between two amilorides on yohimbine dissociation also was explored. The data obtained were well fitted by the equation derived from the relevant model, with the off-rate increases caused by 5-(*N*,*N*-hexamethylene)-amiloride being either decreased or increased by the competing amiloride analogue in line with predictions, and the parameters derived from the fits were in good agreement with those obtained in the above dissociation assays. Thus, the data are compatible with the amilorides competing at the one allosteric site on the α_{2A}-adrenergic receptor and rules out the possibility that the amilorides are acting in a nonspecific fashion.

Within many subtypes of G protein-coupled receptors, molecular modeling studies predict that there is a high degree of amino acid identity around the primary neurotransmitter or hormone binding site (Trumpp-Kallmeyer *et al*., 1992). Thus, the development of drugs with subtype selectivity can be a daunting task, and within the muscarinic receptor field, for example, it has proved to be very difficult to design antagonists selective for one subtype over the other subtypes and so far impossible to discover subtype-selective agonists (Caulfield and Birdsall, 1998; Tucek and Proska, 1995). Within the adrenergic field, a limited number of compounds are known with 10–100-fold higher affinity for certain subtypes (Bylund *et al*., 1994). However, also in this field, there remains a need for the development of further and more subtype-selective drugs (Ruffolo *et al*., 1995).

It thus would be desirable to have an alternative target for the development of such drugs, and such an alternative could be an allosteric site. The potential usefulness of such a site for drug design is emphasized by the success of the benzodiazepines, which act at an allosteric site on the γ-aminobutyric $acid_A$ receptors, enhancing the response to γ-aminobutyric acid (Barker *et al*., 1986).

A schematic representation of allosterism is shown in Fig. 1. In this model, the binding of an allosteric agent X to the allosteric site modulates the binding of a ligand L at the primary binding site. Depending on the magnitude and direction of the changes in the equilibrium and kinetic binding constants, the affinity of L at the primary site will either increase ($\alpha > 1$, positive cooperativity), decrease ($\alpha < 1$, negative cooperativity), or remain unchanged ($\alpha = 1$, neutral cooperativity). This opens the possibility for the modulation of the action of the natural agonist of a receptor in a subtype-specific manner if, for example, the agonist activity is enhanced at one subtype (positive cooperativity) but inhibited at other subtypes (negative cooperativity) (Lazareno and Birdsall, 1995).

This work was supported by a ROPA Research Grant (R.A.L., A.M.).

ABBREVIATIONS: DMA, 5-(*N*,*N*-dimethyl)-amiloride; CHO, Chinese hamster ovary; BZA, benzamil; EPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; HMA, 5-(*N*,*N*-hexamethylene)-amiloride; MBA, 5-(*N*-methyl-*N*-isobutyl)-amiloride; DMF, *N*,*N*-dimethylformamide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Allosteric interactions have been best characterized with the muscarinic family (Stockton *et al*., 1983; Lee and El-Fakahany, 1991a; Leppik *et al*., 1994; Lazareno and Birdsall, 1995; Tucek and Proska, 1995), and it is of interest to note that many of the somewhat subtype-selective compounds discovered to date have also been reported to act at the allosteric site. These include the m2 selective compounds gallamine (Stockton *et al*., 1983), himbacine (Lee and El-Fakahany, 1990; Matsui *et al*., 1995), and methoctramine (Lee and El-Fakahany, 1991b; Matsui *et al*., 1995), thus highlighting the potential value of the allosteric site in generating subtype selectivity. In regard to the enhancement of agonist activity, it has recently been found that the alkaloid brucine allosterically enhances the action of acetylcholine at only the m1 muscarinic receptor subtype, whereas *N*-chloromethyl-brucine allosterically enhances acetylcholine action at only the m3 muscarinic receptor subtype (Birdsall *et al*., 1997).

Although allosterism has been best characterized with the muscarinic receptors, it has been studied only to a limited extent at other G protein-coupled receptors. Therefore, it is not known whether allosterism is a general phenomenon of this class of receptors and hence of general value in drug discovery. The adrenergic receptors are another important therapeutic area, and it has been reported that amiloride analogues increase antagonist dissociation rates from the α_2-adrenergic (Howard *et al*., 1987, Jagadeesh *et al*., 1990), α_{2A}-adrenergic (Nunnari *et al*., 1987), and α_{2B}-adrenergic (Wilson *et al*., 1991) receptors. It was postulated that the amilorides were acting via an allosteric site. However, all of the above workers reported that the amilorides caused a decrease in the number of antagonist binding sites (B_{max}), a finding that is not compatible with the allosteric model.

Our work was undertaken to explore whether the modulation by amiloride analogues (Fig. 2) of antagonist binding at the human α_{2A}-adrenergic receptor is compatible with the ternary complex allosteric model of Fig. 1. A preliminary account of some of these data has been published previously (Leppik *et al*., 1997).

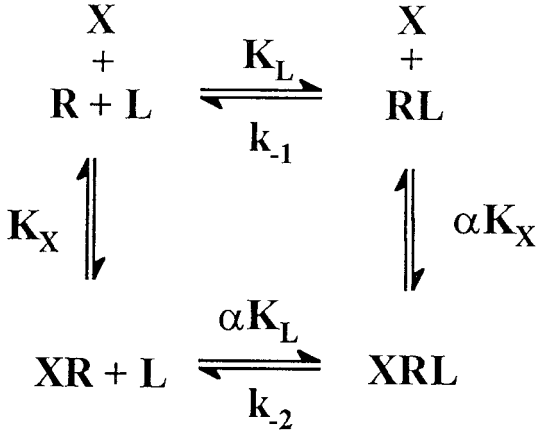

Fig. 1. Schematic representation of the ternary complex allosteric model. In this scheme, radioligand L and allosteric agent X bind to two separate sites on the receptor R. K_L and K_X are the affinity constants for L and X, respectively; α is the cooperativity factor of X with L; and k_{-1} and k_{-2} are the dissociation rate constants for L dissociating from RL and XRL, respectively.

Experimental Procedures

Materials. [^{3}H]Yohimbine (70–90 Ci/mmol) and [^{3}H]rauwolscine (70–90 Ci/mmol) were from DuPont NEN (Hounslow, Middlesex, UK). [^{3}H]RX821002 (40–70 Ci/mmol) was from Amersham International (Little Chalfont, Buckinghamshire, UK). Amiloride HCl, DMA HCl, HMA, and phentolamine HCl were from Sigma Chemical (Poole, Dorset, UK). BZA, EPA, and MBA were from RBI (Poole, Dorset, UK). Tissue culture reagents were from GIBCO BRL (Paisley, UK).

Fresh stock solutions (10 mM) of the amilorides were used. When present as HCl salts, the amilorides were dissolved in water with warming; then, 0.5 M Na-HEPES buffer, pH 7.4 (1:25, v/v), was added. Otherwise, the amilorides were dissolved with warming in 20 or 40 mM HCl, with the pH adjusted to ~7 with Na-HEPES salt, and 0.5 M Na-HEPES buffer, pH 7.4, added to give a final solution containing 10 mM amiloride analogue and 20 mM HEPES, pH 7.4.

Cell culture and membrane preparation. The CHO cell line stably expressing the human α_{2A}-adrenergic receptor (Kurose and Lefkowitz, 1994) was generously provided by Professor Robert J. Lefkowitz (Howard Hughes Medical Institute, Duke University Medical Center, Durham, NC). The cell line was grown in α-minimum essential medium supplemented with 10% newborn calf serum, 2 mM L-glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin, at 37° in 5% CO_2. For membrane preparations, the cell line was grown in 24.5 × 24.5-cm^2 plates until almost confluent; then, the cell layer was washed twice with phosphate-buffered saline, harvested in cold buffer 1 (20 mM Na-HEPES, pH 7.4, 10 mM EDTA), homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY) (setting 6, 10 sec), and centrifuged at 40,000 × *g* for 15 min. The pellet was resuspended by vortexing in buffer 2 (20 mM Na-HEPES, pH 7.4, 0.1 mM EDTA) and then recentrifuged. The pellet was again resuspended in buffer 2 by passage through a 23-gauge needle and then a 27-gauge needle, diluted to 1 mg of protein/ml, and then stored at −70°. Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

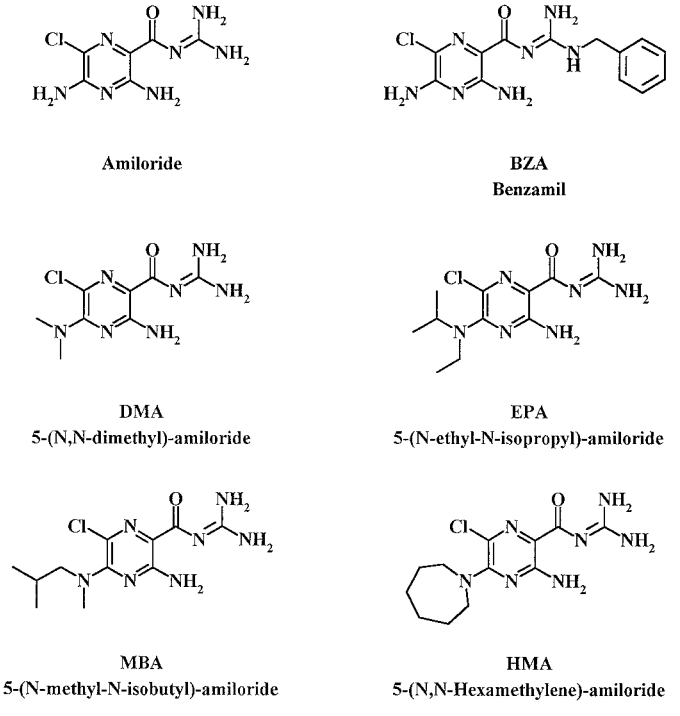

Fig. 2. Structural formulae of amiloride and of its analogues examined in this report. BZA contains a benzyl substituent attached to the guanidinium moiety of amiloride, whereas the other analogues contain 5-*N*-alkyl substituents.

Radioligand binding assays. For saturation experiments, membranes (10 μg of protein) were incubated with increasing concentrations (0.1–10 nM) of radioligand in duplicate, in a final volume of 1 ml of assay buffer (20 mM Na-HEPES, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$), at 30° for 60 min or 20° for 120 min, or with increasing concentrations (0.2–50 nM) of radioligand in duplicate, in a final volume of 0.4 ml of assay buffer at 30° for 60 min. Nonspecific binding was defined as the binding retained on the filter and membranes in the presence of 20 μM phentolamine. Where appropriate, amiloride analogues or solvents were added to both total and nonspecific binding assay tubes to control for effects on binding. Bound and free ligands were separated by rapid filtration under vacuum through GF/B glass-fiber filters (Whatman, Maidstone, Kent, UK), using a Brandell cell harvester (Semat, St. Albans, Hertfordshire, UK). The filters were washed three times with cold 20 mM sodium phosphate buffer, pH 7.4, transferred to scintillation vials, scintillation cocktail (Beckman, Palo Alto, CA) was added, and the filters were soaked overnight and counted.

For competition experiments, membranes (10 μg of protein) were incubated with approximately the K_d concentration of radioligand in duplicate, together with increasing concentrations of competing agent, in a final volume of 1 ml of assay buffer at 30° for 60 min or 20° for 120 min.

For dissociation kinetic studies, membranes (200 μg of protein/ml) were first preequilibrated with radioligand (5 nM) in assay buffer at room temperature for 2 hr. To commence the dissociation, aliquots (50 μl) of the membrane suspension were quickly added with vortexing to pairs of tubes preequilibrated at either 20° or 30°, with each tube containing assay buffer (950 μl) supplemented with phentolamine (21 μM) and various concentrations of the amiloride or amilorides to be tested. Additions were timed so the contents of all the tubes in the dissociation assay were filtered at the same time and had been preincubated with the radioligand for the same time (Hulme and Birdsall, 1992). To determine nonspecific binding, phentolamine (20 μM) was included in a second batch of membranes plus radioligand; then, the experiment repeated. The filtrations and counting were performed as above, except that filters were washed only twice, but with larger volumes of wash buffer, to keep harvesting time to a minimum but nevertheless reduce nonspecific binding.

Data analysis. Data were all fitted by nonlinear regression analyses, using the Grafit curve-fitting software (Erithacus Software, Staines, Middlesex, UK). This procedure allows the use of two or more independent variables (e.g., time and concentration), which was necessary for many of the analyses reported in this report.

Competition experiment data were fitted to the one-site equation:

$$[RL] = \frac{([RL_0] - NS)}{1 + 10^{n*(\log K_{app} + \log[C])}} + NS \quad (1)$$

where [RL] is the concentration of bound radioligand L in the presence of the competing agent C, $[RL_0]$ is the concentration of bound radioligand L in the absence of C, NS is the nonspecific binding, n is the slope factor, and K_{app} is the apparent affinity constant. The latter was converted to the affinity constant K_i using the Cheng-Prusoff correction (1973). In most analyses, n was constrained to 1 because the inhibition curves did not deviate significantly from a simple binding isotherm.

Data from dissociation experiments performed in the absence of added amilorides were fitted to a single exponential decay equation. For data obtained from radioligand dissociation experiments performed in the presence of one or two amiloride analogues, the equations used are given in the Appendix (eqs. 10 and 9, respectively).

For the statistical comparison of the goodness of fit of data to two separate equations, the F test of the Grafit software was used. For statistical comparison of two sets of data, a Student's unpaired or paired *t* test was used.

Results

The equilibrium antagonist binding properties of the α_{2A}-adrenergic receptor. As a necessary prelude to the allosteric binding studies, the equilibrium binding properties of three ^{3}H-labeled antagonists at the human α_{2A}-adrenergic receptor, permanently expressed in a CHO cell line (Kurose and Lefkowitz, 1994), were characterized. The nonspecific binding was defined as the residual binding measured in the presence of 20 μM phentolamine. In all instances, the saturation curves for the specific binding of the ^{3}H-antagonists were compatible with the presence of a uniform population of binding sites. The experiments were carried out at both 20° and 30°, the two temperatures used in the dissociation studies. The K_d values calculated for the antagonists (0.7–3 nM, Table 1) were comparable with those reported in previous studies (Fraser *et al*., 1989; Halme *et al*., 1995). [^{3}H]RX821002 had a 2–3-fold higher affinity than [^{3}H]rauwolscine or [^{3}H]yohimbine, and all three radioligands had a 50–100% higher affinity at 20° than at 30°. The B_{max} estimates in these experiments ranged between 16 and 18 pmol/mg of protein and were independent of radioligand or assay temperature.

Previous workers had dissolved amiloride compounds in organic solvents (Howard *et al*., 1987; Nunnari *et al*., 1987; Jagadeesh *et al*., 1990), so the effect of three common solvents, ethanol, DMSO, and DMF, on the affinities of the antagonists [^{3}H]yohimbine, [^{3}H]rauwolscine, and [^{3}H]RX821002 were examined. All three solvents, at 1% concentration, decreased antagonist affinities 1.3–5-fold at 30° without changing the B_{max} value, with DMF producing the greatest decrease in affinity and [^{3}H]rauwolscine being the most sensitive radioligand (data not shown). Consequently, these solvents were not used in the current study. As an alternative, it was found that the HCl salts of the amiloride analogues were soluble at 10 mM concentrations in Na-HEPES buffer, whereas the parent amilorides could first be dissolved in dilute HCl and then the pH adjusted with Na-HEPES salt to give 10 mM stock solutions.

TABLE 1

Affinity and B_{max} values for the binding of three antagonists at the human α_{2A}-adrenergic receptor

Values are mean ± range/2 from two saturation binding assays, which were incubated at either 30° for 60 min or 20° for 120 min. The membranes were from a CHO cell line permanently expressing the cloned human α_{2A}-adrenergic receptor (Kurose and Lefkowitz, 1994). The K_L value (=1/K_d) is the affinity of the antagonists at the α_{2A}-adrenergic receptor (Fig. 1).

Ligand	30°			20°		
	K_d	logK_L	B_{max}	K_d	logK_L	B_{max}
	nM		*pmol/mg protein*	*nM*		*pmol/mg protein*
Yohimbine	3.1 ± 0.2	8.50 ± 0.02	18 ± 3	2.1 ± 0.1	8.69 ± 0.03	18 ± 2
Rauwolscine	2.5 ± 0.2	8.60 ± 0.04	17 ± 3	1.8 ± 0.2	8.74 ± 0.04	18 ± 2
RX821002	1.4 ± 0.1	8.86 ± 0.02	16 ± 2	0.72 ± 0.05	9.14 ± 0.03	17 ± 2

Effect of amiloride analogues on the equilibrium binding of antagonists. Because it had been reported that 0.1 and 0.3 mM amiloride decreased both antagonist affinity and B_{max} in equilibrium binding studies (Howard *et al*., 1987; Nunnari *et al*., 1987; Jagadeesh *et al*., 1990), the effect of amiloride on [^{3}H]yohimbine and [^{3}H]rauwolscine binding was reexamined. The affinities of the radioligands were decreased in the presence of 0.1 and 0.3 mM amiloride (Fig. 3, Table 2). In contrast to the previous studies, however, the calculated B_{max} values for either radioligand (Table 2) were not significantly affected ($p > 0.1$, Student's paired t test) by the presence of the amiloride.

The affinities of the amilorides at the unoccupied α_{2A} receptor were determined in competition studies with [^{3}H]yohimbine as radioligand. The data were initially fitted with an equation containing a slope factor, but the derived slope factors were normally found to lie between 0.8 and 1.0. Because analysis showed that the deviations of the slopes from unity were not significant ($p > 0.1$) for all the inhibition curves, the data were refitted to a simple one-site model (Table 3). The log affinity values calculated at 20° and 30° were very similar and essentially identical to the log affinity values determined at 30° using either [^{3}H]rauwolscine or [^{3}H]RX821002 as alternative radioligands (data not shown). With [^{3}H]rauwolscine or [^{3}H]RX821002, the slope factors were within the range of 1.0 ± 0.1 (data not shown).

With the amilorides, the effect of increasing hydrophobic substitution at the 5-position of the pyrazine ring (Fig. 2) was to increase the inhibitory potency of the amiloride analogues, with HMA being >100-fold more potent than the parent amiloride (Table 3). The benzyl substituent in benzamil, on the other hand, resulted in only a ~10-fold increase in potency.

Antagonist dissociation at the α_{2A}-adrenergic receptor. The dissociation data for all three radioligands at 20° and 30° fitted well to a monoexponential function. At 20°, RX821002, despite having the highest affinity, had a faster off-rate than either yohimbine or rauwolscine (0.083 ± 0.002 versus 0.034 ± 0.001 and 0.023 ± 0.001 min^{-1}, respectively; see Table 4). At 30°, the dissociation rates were ~3.5-fold (yohimbine and rauwolscine) or 4.5-fold (RX821002) faster than those at 20° (data not shown).

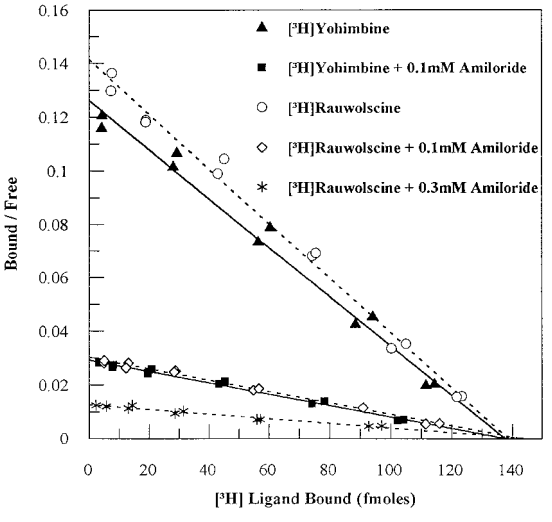

Fig. 3. Scatchard plots of [^{3}H]yohimbine and [^{3}H]rauwolscine binding in the absence and presence of amiloride. Individual *data points* are shown. Membranes were incubated with increasing concentrations of radioligand at 30° for 60 min in the absence or presence of the stated concentrations of amiloride. Nonspecific binding was measured in the presence of 20 μM phentolamine.

TABLE 2

Effect of amiloride on the binding of [^{3}H]yohimbine or [^{3}H]rauwolscine to the human α_{2A}-adrenergic receptor

Values are mean ± standard error from three saturation binding assays, in which membranes containing the α_{2A}-adrenergic receptor were incubated at 30° for 60 min with increasing concentrations of radioligand (0.2–50 nM) in a final volume of 0.4 ml of assay buffer. The K_d value (=1/K_L, Fig. 1) is the dissociation constant of the antagonists in the absence or presence of amiloride at the α_{2A}-adrenergic receptor.

Ligand	Amiloride	K_d	B_{max}
	mM	*nM*	*pmol/mg protein*
Yohimbine	0	2.8 ± 0.1	14.4 ± 0.4
	0.1	13.3 ± 1.0	14.5 ± 0.5
Rauwolscine	0	2.4 ± 0.1	14.0 ± 0.1
	0.1	11.5 ± 0.2	14.0 ± 0.1
	0.3	29.1 ± 1.0	13.8 ± 0.3

Effect of individual amilorides on antagonist dissociation. To determine the affinities of the amilorides at the antagonist-occupied α_{2A} receptor, their affects on [^{3}H]yohimbine dissociation were examined. [^{3}H]Yohimbine was chosen as the main radioligand for this study because it had a slower dissociation rate than [^{3}H]RX821002 (above) and a lower nonspecific binding (as defined by 20 μM phentolamine) than [^{3}H]rauwolscine. Amiloride, in a concentration-dependent manner, increased the dissociation rate (Fig. 4a). The dissociation curves remained monoexponential, and hence the data could be fitted to eq. 10 (Appendix). The simultaneous analysis of all the data gave an excellent fit, with the maximum increase in the [^{3}H]yohimbine dissociation rate caused by amiloride (k_{-2}/k_{-1}) calculated to be 2.0-fold at 20° and the log affinity of amiloride at the yohimbine-occupied receptor to be 2.12 ± 0.02, equivalent to a dissociation constant ($1/\alpha K_X$) of 7.7 ± 0.4 mM (Table 4). The estimates of k_{-1} agreed well with the dissociation constant derived from studies on [^{3}H]yohimbine dissociation alone (Table 4).

More pronounced effects on the [^{3}H]yohimbine dissociation rate were found for the 5-*N*-alkyl-substituted amilorides. This is illustrated for EPA (Fig. 4b). As found for amiloride, the dissociation curves remained monoexponential in the presence of EPA, but 3 mM EPA produced a 20 ± 2-fold (three experiments) enhancement of the off-rate compared with the 1.20 ± 0.01-fold (four experiments) enhancement by the same concentration of amiloride. Because the affinity estimate for EPA at the yohimbine-occupied receptor was found to be lower than for the other amilorides (Table 4), it was

TABLE 3

Affinity of the amiloride compounds at the α_{2A}-adrenergic receptor

The log affinities were determined in competition equilibrium experiments versus [^{3}H]yohimbine, performed at either 30° for 60 min or 20° for 120 min. The data were fitted with eq. 1, with the slope factor set at 1; then, the derived log apparent affinity constants were converted to the log affinity constants logK_i using the Cheng-Prusoff correction. Values are mean ± standard error from n experiments.

Amiloride analogue	30°		20°	
	n	logK_i	n	logK_i
Amiloride	3	4.54 ± 0.03	2	4.52 ± 0.03
BZA	2	5.50 ± 0.05	2	5.45 ± 0.09
DMA	4	5.64 ± 0.03	2	5.44 ± 0.01
EPA	3	5.78 ± 0.06	2	5.78 ± 0.05
MBA	4	6.27 ± 0.05	2	6.25 ± 0.01
HMA	6	6.78 ± 0.07	2	6.68 ± 0.01

difficult to estimate the maximum off-rate, k_{-2}, and hence $\log\alpha K_X$, with precision; the solubility of EPA precluded the use of higher concentrations. To obtain better defined estimates, the data from three experiments were converted to B_t/B_o values, pooled, and then fitted. From the fit, the estimated maximal increase in off-rate was 155 ± 22-fold, and the log affinity at the yohimbine-occupied receptor 1.75 ± 0.07, equivalent to $1/\alpha K_X$ of 18 ± 3 mM. In this case only, the errors quoted in Table 4 are the standard errors from the fit by the Grafit software, and not the standard error of the mean of individual experiments.

In the case of HMA, the fitting of the data from individual experiments was not a problem because the estimated log affinity of HMA at the yohimbine-occupied receptor was higher (2.47 ± 0.02, or $1/\alpha K_X$ = 3.4 ± 0.2 mM) than that for EPA. The calculated maximal increase in yohimbine dissociation rate caused by HMA was found to be 138 ± 15-fold.

The smallest effect on off-rate was found with BZA, with only a slight change evident in the dissociation rate on the addition of 1 mM BZA. Even 3 mM BZA resulted in only a ~20% increase in the [^{3}H]yohimbine dissociation rate. As a result of these small changes, the affinity of BZA at the yohimbine-occupied receptor could not be determined by this type of experiment.

The effect of selected amilorides on the dissociation of the two other radiolabeled antagonists, [^{3}H]rauwolscine and [^{3}H]RX821002, was investigated (Table 4). HMA and DMA produced comparable effects on the dissociation of these antagonists, although HMA produced a 3-fold smaller increase in k_{-2} for [^{3}H]rauwolscine dissociation.

From the data generated in these experiments and those summarized in Table 3, the cooperativities between the binding of the amilorides and the antagonists could be estimated. In all instances, these interactions were characterized by

TABLE 4

Effect of amiloride analogues on antagonist dissociation from the α_{2A}-adrenergic receptor

The experiments were performed at 20°, as described in the legend of Fig. 4. k_{-1} and k_{-2} are the dissociation constants for the dissociation of the antagonist from the unoccupied and the amiloride-occupied receptor, respectively (Fig. 1). The $\log\alpha_{obs}$ is the difference between the log affinity of the amiloride analogue at the antagonist-occupied receptor ($\log\alpha K_X$) and the log affinity at the unoccupied receptor ($\log K_i$, Table 3). Values are mean ± standard error of n experiments.

Antagonist	Amiloride analogue	n	k_{-1}	k_{-2}	Fold increase	$\log\alpha K_X$	$\log\alpha_{obs}$
			min^{-1}		k_{-2}/k_{-1}		
Yohimbine		14	0.034 ± 0.001				
	Amiloride	2	0.030 ± 0.001	0.060 ± 0.002	2.02 ± 0.01	2.12 ± 0.02	−2.40 ± 0.02
	DMA	3	0.035 ± 0.002	0.184 ± 0.005	5.3 ± 0.2	2.36 ± 0.03	−3.08 ± 0.03
	EPA[a]	3	0.031 ± 0.001	4.8 ± 0.6	155 ± 22	1.75 ± 0.07	−4.02 ± 0.09
	MBA	2	0.032 ± 0.001	3.3 ± 0.1	101 ± 2	2.09 ± 0.03	−4.15 ± 0.03
	HMA	4	0.028 ± 0.003	3.7 ± 0.1	138 ± 15	2.47 ± 0.02	−4.21 ± 0.02
Rauwolscine		5	0.023 ± 0.001				
	DMA	2	0.021 ± 0.001	0.135 ± 0.003	6.30 ± 0.05	2.27 ± 0.05	−3.17 ± 0.05
	HMA	3	0.025 ± 0.004	1.33 ± 0.03	57 ± 10	2.63 ± 0.03	−4.05 ± 0.03
RX821002		3	0.083 ± 0.002				
	DMA	2	0.076 ± 0.001	0.54 ± 0.05	7.1 ± 0.8	2.07 ± 0.07	−3.37 ± 0.07

[a] The DPM from three experiments were converted to B_t/B_o; then, the combined data fitted. The error values quoted are the standard errors from the fit.

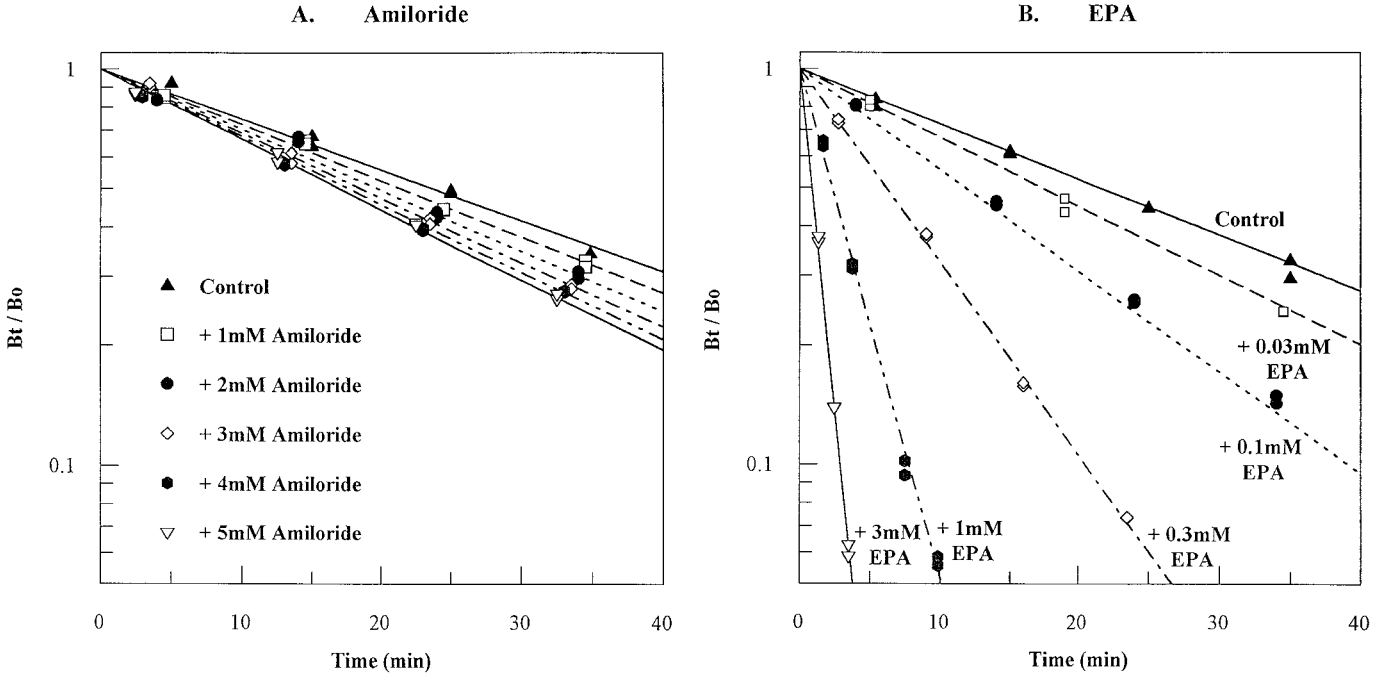

Fig. 4. Dissociation of [^{3}H]yohimbine at 20° in the absence or presence of various concentrations of amiloride or EPA. Membranes were first preequilibrated with [^{3}H]yohimbine; then, the dissociation commenced by mixing with phentolamine and various concentrations of the amiloride to be tested. Individual *data points* are shown. The data from each experiment were simultaneously fitted to eq. 10 (see Appendix), with time and amiloride analogue concentration as independent variables.

very large negative cooperativities (Table 4), values varying from 4×10^{-3} to 6×10^{-5}.

Competitive interactions between amiloride analogues as detected by their effects on [^{3}H]yohimbine dissociation. Despite the excellent agreement between the experimental data and the predictions of the ternary complex allosteric model, the high concentrations of amilorides required to show kinetic effects raised the possibility that the kinetic effects could be the consequence of nonspecific perturbations of the structure of the receptor or membrane, which serendipitously fitted the model.

The differential effects of amilorides on the dissociation of ^{3}H-antagonists allows a more critical examination of the interactions that are occurring. If the effects are nonspecific, they should be additive, but if there is a defined allosteric site, the competitive effects of two amilorides at that site will give quantitatively predictable effects on ^{3}H-antagonist dissociation. The conditions for the experiments described below were decided by simulation using eq. 9 from the Appendix.

The effect of the presence of either amiloride or DMA on the large increase in [^{3}H]yohimbine dissociation rate caused by HMA was examined (Fig. 5, a and b). It was found that amiloride reduced the off-rate increase caused by 0.1 or 0.3 mM HMA (Fig. 5a), as predicted by the simulations. In contrast, DMA was found to further increase the off-rate increase caused by 0.03 or 0.1 mM HMA but to decrease slightly the effect of 0.3 mM HMA (Fig. 5b), again as predicted. When all of the data of each experiment were fitted simultaneously to eq. 9, the values calculated (Table 5) agreed well with those values derived from [^{3}H]yohimbine dissociation experiments performed with only one amiloride present (Table 4).

Because the above competition dissociation experiments gave good estimates of the affinities ($\log\alpha K_X$ and $\log\beta K_Y$) of each amiloride analogue at the yohimbine-occupied receptor, as well as good estimates of the [^{3}H]yohimbine dissociation rate from the receptor occupied by either one of the amiloride analogues (k_{-2} and k_{-3}), the effect of competition between BZA and HMA was examined. The aim was to determine

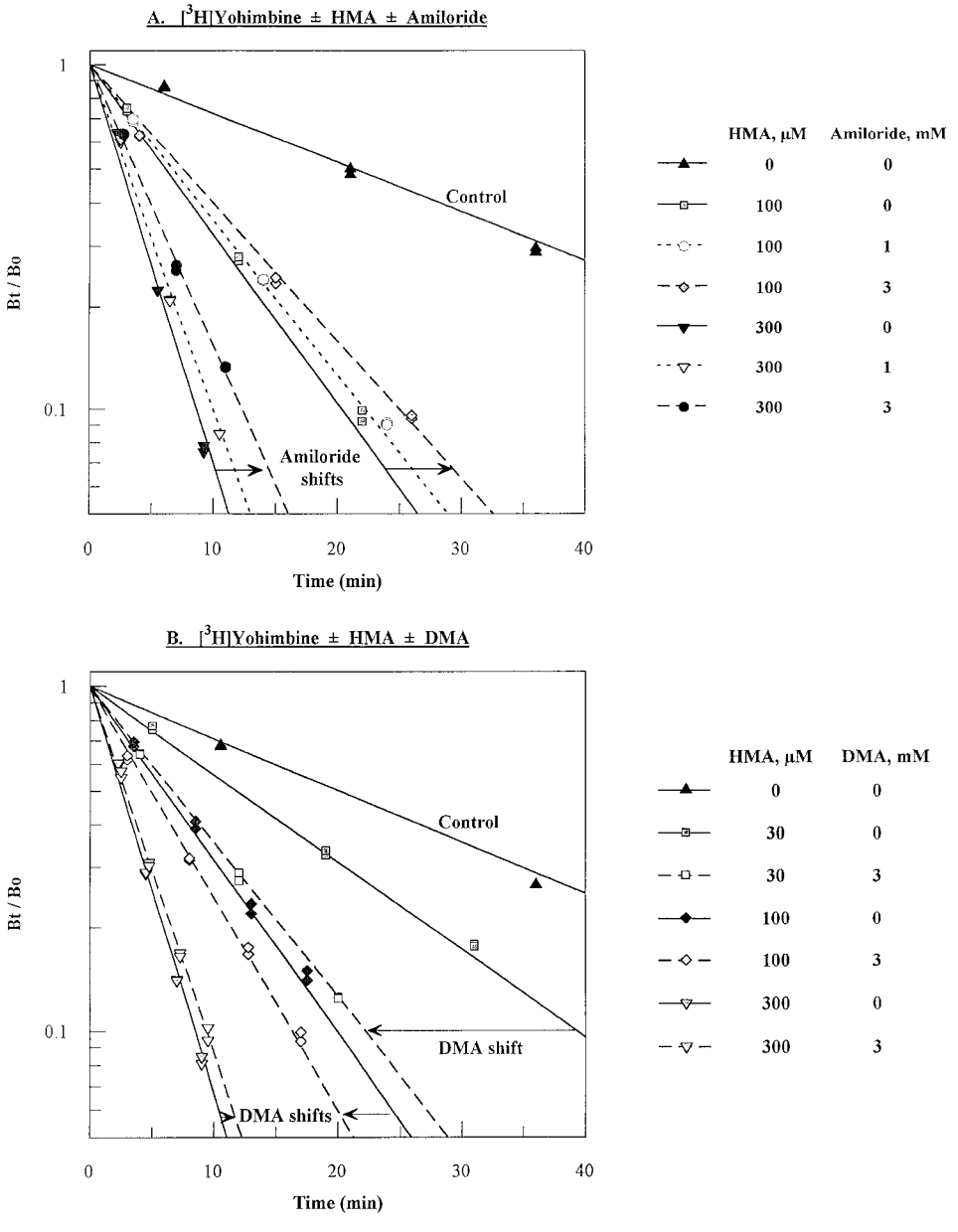

Fig. 5. Dissociation of [^{3}H]yohimbine at 20° in the absence or presence of various concentrations of HMA with and without amiloride or HMA with and without DMA. Membranes were first preequilibrated with [^{3}H]yohimbine, then the dissociation commenced by mixing with phentolamine and various concentrations of the amilorides to be tested. Individual *data points* are shown. The data from each experiment were simultaneously fitted to eq. 9 (see Appendix), with time and amiloride analogue concentrations as independent variables.

whether the observed slight effect of BZA on yohimbine off-rate was due to a low affinity or small kinetic effects of BZA at the yohimbine-occupied receptor. BZA was found, in fact, to be more potent than amiloride in reversing the dissociation rate increase caused by HMA (Figs. 5a and 6). Simultaneous fitting of all the data in Fig. 6 revealed that BZA had the highest affinity of the amilorides tested at the yohimbine-occupied receptor ($\log\beta K_Y = 3.07 \pm 0.05$, $1/\beta K_Y = 0.87 \pm 0.10$ mM) and a very slight effect on the yohimbine dissociation rate from the BZA-occupied receptor [fold increase (k_{-3}/k_{-1}) $= 1.43 \pm 0.05$] (Table 5).

Discussion

We explored whether there is a well-defined allosteric site on the human α_{2A} adrenergic receptor, at which amiloride analogues could act to modulate antagonist binding to the primary binding site. This question has been investigated qualitatively in previous studies (Howard *et al*., 1987; Nunnari *et al*., 1987; Jagadeesh *et al*., 1990). However, no specific model was suggested for these data, and they do not fit the ternary complex allosteric model (Fig. 1) because the parent amiloride apparently decreased the B_{max} of the ^{3}H-antagonist used to label the receptors. Our studies used cloned human α_{2A}-adrenergic receptors expressed in a CHO cell line. In membrane preparations from this line, radiolabeled antagonists were found to bind to a uniform population of sites, which were expressed at a high density and displayed the appropriate pharmacology (Table 1, and data not shown). In our experiments, it was found that amiloride, at the concentrations used by others, did not significantly affect the calculated B_{max} values for either [^{3}H]rauwolscine or [^{3}H]yohimbine binding (Fig. 3, Table 2). The reason for the different result is not known.

Allosteric interactions between two ligands at a receptor can be explored and quantified by both equilibrium and kinetic binding studies (Stockton *et al*., 1983; Lee and El-Fakahany, 1991a; Leppik *et al*., 1994; Lazareno and Birdsall, 1995). Analyses of equilibrium binding data allow the estimation of both ligand affinities K_L and K_X and the cooperativity factor α (Fig. 1). However, a major limitation of equilibrium studies is that the analysis is difficult with high negative cooperativities (<0.01), the situation in the current study, because it then is not technically feasible to distinguish between the competition of two ligands at the one site and strong negative cooperativity between two sites.

In the kinetic approach, the effect of a test ligand on the dissociation rate of a radioligand is examined. Analysis of data from these experiments allows the estimation of the affinity of an allosteric ligand at the allosteric site (αK_X, Fig. 1) when the primary site is occupied by the radioligand. In the current study, all of the amilorides tested were found to increase the rate of [^{3}H]yohimbine dissociation from the α_{2A} receptor (Fig. 4, Table 4). For each amiloride analogue concentration tested, the dissociation is monoexponential. The data from each experiment (except for those with BZA, which had a very small effect on dissociation rate) could be well fitted (Fig. 4) by eq. 10 (Appendix), showing that the data are compatible with the ternary complex allosteric model. From the fits, the calculated maximal increases of the amilorides on [^{3}H]yohimbine dissociation ranged up to 160-fold. With such large increases in off-rate, the experiments are feasible technically only if the dissociation rate of the amilorides from the yohimbine-occupied receptor is very rapid, so as soon as the washing begins of the membranes trapped on the filters with cold buffer, the amiloride analogue rapidly dissociates, leaving only yohimbine occupying the receptor. The facts that the data are well-fitted and that the individual dissociation curves intersect at approximately the same point at zero time support this scenario.

The effect of the variation in the structures of the amilorides on the [^{3}H]yohimbine dissociation rate is striking. Amiloride itself causes only a 2-fold increase in dissociation rate, and the addition of two methyl groups to the 5-amino function, to give DMA (Fig. 2), increases the yohimbine dissociation rate only a further 3-fold (Table 4). However, the addition of an additional three or four carbons, to give MBA, EPA, or HMA, causes a 50–80-fold increase in the effect caused by amiloride. The position of substitution also is important because the addition of a benzyl moiety to the other side of the molecule, to give BZA, slightly reduces the effect (from 2.0- to 1.4-fold, Tables 4 and 5). It can be speculated that within the allosteric site, the pocket around the 5-amino function is only large enough to accommodate a dimethylamino function, and larger groups cause a change in receptor conformation, leading to the dramatic increase in the yohimbine dissociation rate. The guanidinium function, on the other hand, may occupy a pocket large enough to accommodate a benzyl functionality, so the addition of such a grouping to the amiloride structure has only a minor effect on the yohimbine dissociation rate.

Because the increase caused by BZA is so slight, it could be quantified only in a competition dissociation assay (Fig. 6, Table 5). In these assays, the facts that the fits are so good, that the off-rate increases caused by HMA are either decreased or increased by the competing amiloride analogue in line with predictions, and that the parameters derived from the fits (Table 5) are in good agreement with those derived from dissociation assays performed with individual amilo-

TABLE 5

Effect of amiloride competition on yohimbine dissociation

The experiments were performed at 20°, as described in the legends of Figs. 5 and 6. k_{-1}, k_{-2}, and k_{-3} are the dissociation constants for the dissociation of [^{3}H]yohimbine from either the unoccupied receptor or from the receptor occupied by amiloride X or Y, respectively, and $\log\alpha K_X$ and $\log\beta K_Y$ are the log affinities of amiloride X and amiloride Y, respectively, at the yohimbine-occupied receptor (Appendix). The $\log\beta_{obs}$ is the difference between $\log\beta K_Y$ and the affinity of the amiloride analogue at the unoccupied receptor ($\log K_i$, Table 3). Values are mean ± standard error of n experiments.

Amiloride X	Amiloride Y	n	k_{-1}	Amiloride X		Amiloride Y		
				k_{-2}	$\log\alpha K_X$	k_{-3}	$\log\beta K_Y$	$\log\beta_{obs}$
			min^{-1}	min^{-1}		min^{-1}		
HMA	Amiloride	3	0.030 ± 0.001	4.1 ± 0.4	2.34 ± 0.04	0.053 ± 0.008	2.34 ± 0.05	−2.18 ± 0.06
HMA	DMA	2	0.031 ± 0.003	3.8 ± 0.6	2.37 ± 0.08	0.185 ± 0.007	2.26 ± 0.02	−3.18 ± 0.06
HMA	BZA	2	0.030 ± 0.001	4.2 ± 0.5	2.37 ± 0.04	0.043 ± 0.001	3.07 ± 0.05	−2.39 ± 0.05

rides (Table 4) show that the data are compatible with the amilorides acting at the same site and rule out the possibility that the amilorides are acting in a nonspecific fashion (e.g., by perturbation of the membrane).

The calculated log affinity values of the 5-*N*-alkyl amilorides at the yohimbine-occupied receptor were found to range from 1.75 ($1/\alpha K_X$ = 17.6 mM) for EPA to 2.47 ($1/\alpha K_X$ = 3.4 mM) for HMA (Table 4). Thus, the spread in affinities of the 5-*N*-alkyl amilorides at the yohimbine-occupied receptor (5-fold) is far smaller than the range in affinities at the unoccupied receptor (145-fold) (Table 3). In addition, the increase in affinity of the amiloride analogues at the [^{3}H]yohimbine-occupied receptor is not correlated with the increase in size of the 5-*N*-alkyl side chains, again in contrast to the situation with the unoccupied receptor (Table 3). This suggests either that the presence of an antagonist such as yohimbine at the primary binding site has a marked effect on the conformation of the allosteric site or that, for the unoccupied receptor, the amilorides are capable of binding at both the primary and allosteric binding sites. In the latter case, the affinity values derived from competition studies may reflect, partially or totally, binding at the primary site. The mathematics of equilibrium competition between [^{3}H]yohimbine and the amilorides indicate that these two possibilities cannot be distinguished. This situation is in contrast to that found with the D_2 dopamine receptor, where "steep" competition curves between MBA and [^{3}H]spiperone were better fitted with an equation derived from a model allowing MBA to interact with both the allosteric site and the primary site, with the MBA/MBA allosteric interaction exhibiting strong positive cooperativity (Hoare and Strange, 1996).

It also should be noted that the apparent association rate constant of [^{3}H]yohimbine to the receptor/amiloride analogue complex ($\alpha K_L \times k_{-2}$) is approximately constant (0.8–2.5 $\times$ 10^5 M^{-1} min^{-1}) and ~100-fold slower than its association rate constant to the unliganded receptor ($K_L \times k_{-1}$).

The magnitude of the effects on the yohimbine dissociation rate is not correlated with the affinity of the amilorides at the yohimbine-occupied receptor. Thus, HMA and EPA, which have the largest effects on the yohimbine off-rate, have the highest and lowest affinities, respectively, of the 5-*N*-alkyl amilorides at the yohimbine-occupied receptor (Table 4). BZA, on the other hand, has the smallest off-rate effect but the highest affinity of the amilorides tested (Table 5). Thus, it is incorrect to use the magnitude of the effect on antagonist dissociation rate as a measure of the relative affinities of amiloride analogues at the liganded α_{2A}-adrenergic receptor, as has been done in some previous studies.

The observed log cooperativity value ($\log\alpha_{obs}$) is the difference between the log affinities of the amiloride analogue at the unoccupied and the antagonist occupied receptor. The calculated α_{obs} values for the yohimbine/amiloride analogue interactions (Table 4) show a 65-fold variation. If the amilorides can only interact with the allosteric site, then the α_{obs} value would correspond to the α depicted in Fig. 1.

In principle, the magnitude of an allosteric interaction is dependent on both of the ligands involved. The modulation by DMA and HMA of [^{3}H]rauwolscine, [^{3}H]RX821002, and [^{3}H]yohimbine dissociation (Table 4) showed only small variations in the observed cooperativities between the amilorides and antagonists and small differences in the increases in dissociation rates caused by the amilorides This is different from the situation found for the M_2 muscarinic receptor in rat heart, where variation in antagonist structure produced a 10-fold variation in the negative cooperativity with gallamine (Stockton *et al.*, 1983).

Thus, the results obtained in the current work are compatible with the existence of a discrete allosteric site on the α_{2A}-adrenergic receptor, to which the amiloride analogues can bind and allosterically modulate antagonist binding at the primary site. The very different structure-binding relationships displayed by the amilorides at the unoccupied versus the antagonist-occupied receptor could argue either that the amilorides also interact with the primary binding site at the α_{2A}-adrenergic receptor or that antagonist occupation at the primary site induces a substantial conformational change in the allosteric site that decreases the affinity of the amilorides by occluding a specific hydrophobic interaction.

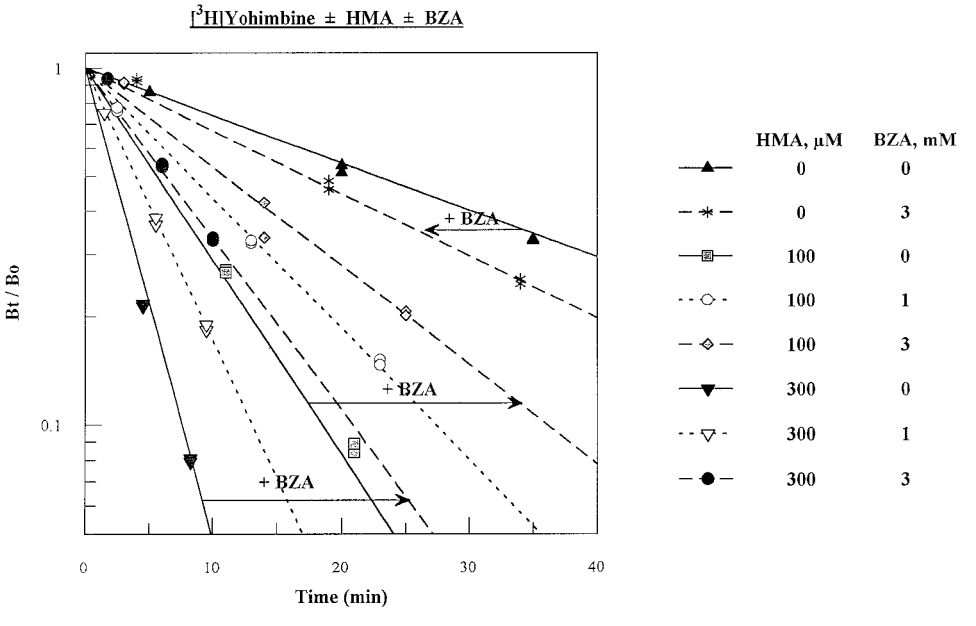

Fig. 6. Dissociation of [^{3}H]yohimbine at 20° in the absence or presence of various concentrations of HMA with and without BZA. The experiments were performed as described in the legend of Fig. 5. Individual data points are shown. The data from each experiment were simultaneously fitted to eq. 9 (see Appendix), with time and HMA and BZA concentrations as independent variables.

Appendix

Radioligand Dissociation in the Presence of Allosteric Agents

$$\begin{array}{ccc} XR + L & \underset{k_{-2}}{\overset{\alpha K_L}{\rightleftharpoons}} & XRL \\ K_X \uparrow\downarrow & & \uparrow\downarrow \alpha K_X \\ R + L & \underset{k_{-1}}{\overset{K_L}{\rightleftharpoons}} & RL \\ K_Y \uparrow\downarrow & & \uparrow\downarrow \beta K_Y \\ YR + L & \underset{k_{-3}}{\overset{\beta K_L}{\rightleftharpoons}} & YRL \end{array} \qquad (2)$$

In eq. 2, K_L, K_X, and K_Y are the affinity constants for the radioligand L and the allosteric agents X and Y for the receptor; k_{-1}, k_{-2}, and k_{-3} are the dissociation rate constants for the dissociation of L from RL, XRL, and YRL, respectively; and α and β are the allosteric cooperativity factors of X and Y, respectively, with L. In the scheme, X and Y compete for the same allosteric site, which is distinct from the radioligand binding site.

In the dissociation experiments, radioligand L is first preequilibrated with the receptor R. Then, at time 0, the allosteric agents X and Y are added. If X and Y have fast binding kinetics, equilibrium would be rapid, and the proportions of RL, XRL and YRL would remain constant during the dissociation. The proportion, p, of radioligand bound as XRL is:

$$p = \frac{[X]\cdot\alpha\cdot K_X}{1 + [X]\cdot\alpha\cdot K_X + [Y]\cdot\beta\cdot K_Y} \qquad (3)$$

The proportion, q, of radioligand bound as YRL is:

$$q = \frac{[Y]\cdot\beta\cdot K_Y}{1 + [X]\cdot\alpha\cdot K_X + [Y]\cdot\beta\cdot K_Y} \qquad (4)$$

and the proportion of radioligand bound as RL is:

$$1 - p - q = \frac{1}{1 + [X]\cdot\alpha\cdot K_X + [Y]\cdot\beta\cdot K_Y} \qquad (5)$$

If B is the total concentration of bound radioligand L, then:

$$\frac{dB}{dt} = -k_{-1}\cdot[RL] - k_{-2}\cdot[XRL] - k_{-3}\cdot[YRL] \qquad (6)$$

Substituting eqs. 3–5 into eq. 6:

$$\frac{dB}{dt} = -\{k_{-1}\cdot(1 - p - q) + k_{-2}\cdot p + k_{-3}\cdot q\}\cdot B \qquad (7)$$

Expanding and rearranging eq. 7:

$$\frac{dB}{dt} = -\left\{\frac{k_{-1} + k_{-2}\cdot[X]\cdot\alpha\cdot K_X + k_{-3}\cdot[Y]\cdot\beta\cdot K_Y}{1 + [X]\cdot\alpha\cdot K_X + [Y]\cdot\beta\cdot K_Y}\right\}\cdot B \qquad (8)$$

Integrating:

$$B_t = B_o\cdot e^{-\left\{\frac{k_{-1}+k_{-2}\cdot[X]\cdot\alpha\cdot K_X+k_{-3}\cdot[Y]\cdot\beta\cdot K_Y}{1+[X]\cdot\alpha\cdot K_X+[Y]\cdot\beta\cdot K_Y}\right\}\cdot t} \qquad (9)$$

where B_t is the total radioligand L bound at time t, and B_0 is the total radioligand bound at time 0. In the fitting of the data with eq. 9, t, [X] and [Y] were independent variables, and the equation was recast in terms of log affinity constants, log concentrations, and log cooperativity factors (Hulme and Birdsall, 1992).

In the presence of only one allosteric ligand, X, one reverts back to the ternary complex allosteric model (Fig. 1). For this, eq. 9 reduces to the equation derived by Lazareno and Birdsall (1995):

$$B_t = B_o\cdot e^{-\left\{\frac{k_{-1}+k_{-2}\cdot[X]\cdot\alpha\cdot K_X}{1+[X]\cdot\alpha\cdot K_X}\right\}\cdot t} \qquad (10)$$

In the fitting of the data with eq. 10, t and [X] were independent variables, and the equation was again recast in terms of log affinity constant, log concentration, and log cooperativity factor (Hulme and Birdsall, 1992).

Acknowledgments

We are grateful to Professor Robert Lefkowitz for provision of the CHO cell line expressing the human α_{2A}-adrenergic gene.

References

Barker JL, Harrison NL, and Mariani AP (1986) Benzodiazepine pharmacology of mammalian CNS neurons. *Life Sci* **39:**1959–1968.

Birdsall NJM, Farries T, Gharagozloo P, Kobayashi S, Kuonen D, Lazareno S, Popham A, and Sugimoto M (1997) Selective allosteric enhancement of the binding and actions of acetylcholine at muscarinic receptor subtypes. *Life Sci* **60:**1047–1052.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:**248–254.

Bylund DB, Eikenberg DC, Hieble JP, Langer SZ, Lefkowitz RJ, Minneman KP, Molinoff PB, Ruffolo RR Jr, and Trendelenburg U (1994) International Union of Pharmacology. IV. Nomenclature of adrenoceptors. *Pharmacol Rev* **46:**121–136.

Caulfield MP and Birdsall NJM (1998) International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev*, in press.

Cheng Y-C and Prusoff WH (1973) Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Fraser CM, Arakawa S, McCombie WR, and Venter JC (1989) Cloning, sequence analysis, and permanent expression of a human α_2-adrenergic receptor in Chinese hamster ovary cells: evidence for independent pathways of receptor coupling to adenylate cyclase attenuation and activation. *J Biol Chem* **264:**11754–11761.

Halme M, Sjöholm B, Savola J-M, and Scheinin M (1995) Recombinant human α_2-adrenoceptor subtypes: comparison of [^{3}H]rauwolscine, [^{3}H]atipamezole and [^{3}H]RX821002 as radioligands. *Biochim Biophys Acta* **1266:**207–214.

Hoare SRJ and Strange PG (1996) Regulation of D_2 dopamine receptors by amiloride and amiloride analogs. *Mol Pharmacol* **50:**1295–1308.

Howard MJ, Hughes RJ, Motulsky HJ, Mullen MD, and Insel PA (1987) Interactions of amiloride with α- and β-adrenergic receptors: amiloride reveals an allosteric site on α_2-adrenergic receptors. *Mol Pharmacol* **32:**53–58.

Hulme EC and Birdsall NJM (1992) Strategies and tactics in receptor-binding studies, in *Receptor-Ligand Interactions: A Practical Approach* (Hulme EC, ed) pp 63–176, IRL Press, Oxford, UK.

Jagadeesh G, Cragoe EJ Jr, and Deth RC (1990) Modulation of bovine aortic *alpha*-2 receptors by Na^+, 5′-guanylylimidodiphosphate, amiloride and ethylisopropylamiloride: evidence for receptor-G protein precoupling. *J Pharmacol Exp Ther* **252:**1184–1196.

Kurose H and Lefkowitz RJ (1994) Differential desensitization and phosphorylation of three cloned and transfected α_2-adrenergic receptor subtypes. *J Biol Chem* **269:**10093–10099.

Lazareno S and Birdsall NJM (1995) Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. *Mol Pharmacol* **48:**362–378.

Lee NH and El-Fakahany EE (1990) The allosteric binding profile of himbacine: a comparison with other cardioselective muscarinic antagonists. *Eur J Pharmacol* **179:**225–229.

Lee NH and El-Fakahany EE (1991a) Allosteric antagonists of the muscarinic acetylcholine receptor. *Biochem Pharmacol* **42:**199–205.

Lee NH and El-Fakahany EE (1991b) Allosteric interactions at the m1, m2 and m3 muscarinic receptor subtypes. *J Pharmacol Exp Ther* **256:**468–479.

Leppik RA, Lazareno S, Mynett A, and Birdsall NJM (1997) The allosteric interac-

tions of antagonists with amiloride analogues at the human α_{2A}-adrenoceptor. *Br J Pharmacol* **122:**69P.

Leppik RA, Miller RC, Eck M, and Paquet J-L (1994) Role of acidic amino acids in the allosteric modulation by gallamine of antagonist binding at the m2 muscarinic acetylcholine receptor. *Mol Pharmacol* **45:**983–990.

Matsui H, Lazareno S, and Birdsall NJM (1995) Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. *Mol Pharmacol* **47:**88–98.

Nunnari JM, Repaske MG, Brandon S, Cragoe EJ Jr, and Limbird LE (1987) Regulation of porcine brain α_2-adrenergic receptors by Na^+, H^+ and inhibitors of Na^+/H^+ exchange. *J Biol Chem* **262:**12387–12392.

Ruffolo RR Jr, Bondinell W, and Hieble JP (1995) α- and β-Adrenoceptors: from the gene to the clinic. 2. Structure-activity relationships and therapeutic applications. *J Med Chem* **38:**3681–3716.

Stockton JM, Birdsall NJM, Burgen ASV, and Hulme EC (1983) Modification of the binding properties of muscarinic receptors by gallamine. *Mol Pharmacol* **23:**551–557.

Trumpp-Kallmeyer S, Hoflack J, Bruinvels A, and Hibert M (1992) Modeling of G-protein-coupled receptors: application to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors. *J Med Chem* **35:**3448–3462.

Tucek S and Proska J (1995) Allosteric modulation of muscarinic acetylcholine receptors. *Trends Pharmacol Sci* **16:**205–212.

Wilson AL, Seibert K, Brandon S, Cragoe EJ Jr, and Limbird LE (1991) Monovalent cation and amiloride analog modulation of adrenergic ligand binding to the unglycosylated α_{2b}-adrenergic receptor subtype. *Mol Pharmacol* **39:**481–486.

Send reprint requests to: Dr. Ray Leppik, Department of Physical Biochemistry, NIMR, The Ridgeway, Mill Hill, London NW7 1AA, United Kingdom. E-mail: r.leppik@nimr.mrc.ac.uk