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Allosteric Interactions between the Antagonist Prazosin and Amiloride Analogs at the Human α_{1A}-Adrenergic Receptor[1]

RAY A. LEPPIK,[2] ANITA MYNETT,[2] SEBASTIAN LAZARENO, and NIGEL J. M. BIRDSALL

National Institute for Medical Research, The Ridgeway, Mill Hill, London (R.A.L., A.M., N.J.M.B.); and Medical Research Council Collaborative Centre, London, United Kingdom (S.L.)

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ABSTRACT

It has been demonstrated previously that amilorides can interact with a well defined allosteric site on the human α_{2A}-adrenergic receptor. In this study, the question was explored as to whether the human α_{1A}-adrenergic receptor also possesses an equivalent allosteric site. The six amilorides examined strongly increased the dissociation rate of the antagonist [^{3}H]prazosin from the α_{1A}-adrenergic receptor in a concentration-dependent manner. With the parent amiloride, the dissociation data were well fitted by an equation derived from the ternary complex allosteric model, compatible with amiloride acting at a defined allosteric site on the α_{1A}-adrenergic receptor. In contrast, the dissociation data for [^{3}H]prazosin in the presence of the amiloride analogs were not compatible with the equation derived from a one-allosteric-site model, but could be fitted well by an equation derived from a two-allosteric-site model. However, certain individual parameters could not be resolved. The observed dissociation rate constants increased steeply with increasing amiloride analog concentration, and in some cases the data could be fitted with a logistic equation. The slope factors calculated from such fits were 1.2 to 2.1. It is concluded that the structure-binding relationships of the amilorides at the α_{1A}- and α_{2A}-adrenergic receptors are different. The interactions of the five amiloride analogs, but not the parent amiloride, with the α_{1A}-adrenergic receptor are compatible with the presence of two (but not one) allosteric sites, and is thus more complex than that found for the α_{2A}-adrenergic receptor.

Apart from the field of muscarinic acetylcholine receptors, the investigation of allosterism among G protein-coupled receptors has been relatively little explored, despite the potential of allosteric sites as alternative targets for the development of subtype selective drugs (Birdsall et al., 1999). Thus, it is not known whether allosteric interactions are a general characteristic of this family of receptors. This is in contrast to the ligand-gated ion channel receptors, where multiple allosteric sites are known to exist (Galzi and Changeux, 1994). For example, it has been shown that benzodiazepines, barbiturates, and anesthetic steroids can act allosterically to enhance agonist responses at γ-aminobutyric acid$_A$ receptors (for reviews, see Macdonald and Olsen, 1994; Smith and Olsen, 1995).

One therapeutically important group of G protein-coupled receptors is the adrenergic receptors. Within this group, allosterism has only been explored for the α_2-adrenergic receptor subtypes. It has been shown that amilorides act allosterically at the α_{2A}-subtype (Nunnari et al., 1987; Leppik et al., 1998a), and possibly also at the α_{2B}-subtype (Wilson et al., 1991). At the α_{2A}-adrenergic receptor, binding of amilorides to the allosteric site increased the dissociation rate of antagonists such as yohimbine, from 2-fold for amiloride to ~150-fold for both 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EPA) and 5-(*N*,*N*-hexamethylene)-amiloride (HMA) (Leppik et al., 1998a). Analysis of the data revealed that the amilorides exert strong negative cooperativities on the binding of the antagonists examined. Competition experiments indicated that the amilorides were acting via a common allosteric site, with no evidence for the presence of a second allosteric site (Leppik et al., 1998a).

It is not known whether an allosteric site exists on the α_1- or β-adrenergic receptor subtypes, other members of the adrenergic receptor family. For the muscarinic receptor family, the relatively strong sequence identity, both within the putative transmembrane domains (containing the primary binding site) and in the extracellular loops (74 and 53%, respectively, between the human M_1 and M_2 receptors), means that it is not surprising that a comparable allosteric site exists on all five muscarinic receptor subtypes (Ellis et al., 1991). In contrast, there is a lower sequence identity between the human α_{1A}- and α_{2A}-adrenergic receptors in the comparable regions, being 44 and 33%, respectively, in the transmembrane domains and extracellular loops. Therefore,

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ABBREVIATIONS: EPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; HMA, 5-(*N*,*N*-hexamethylene)-amiloride; CHO, Chinese hamster ovary; DMA, 5-(*N*,*N*-dimethyl)-amiloride; BZA, benzamil; MBA, 5-(*N*-methyl-*N*-isobutyl)-amiloride.

there can be no a priori assumption that α_1-adrenergic receptors have an allosteric site with a pharmacology similar to that described for the α_{2A}-adrenergic receptor. In this study, we examine the effect of amilorides on the binding of the antagonist [^{3}H]prazosin at one of the α_1-adrenergic receptor subtypes, the human α_{1A}-adrenergic receptor.

Experimental Procedures

Materials. [^{3}H]Prazosin (70–87 Ci/mmol) was from DuPont/NEN, Hounslow, Middlesex, UK. The amiloride analogs, phentolamine HCl, Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham, and polyethylenimine were from Sigma Chemical Co., Poole, Dorset, UK. Other tissue culture reagents were from Gibco BRL, Paisley, UK. Aqueous stock solutions (10 mM) of the amilorides in HEPES buffer were prepared fresh as required, as described previously (Leppik et al., 1998a).

Cell Culture and Membrane Preparation. The clonal Chinese hamster ovary (CHO)-K1 cell line stably expressing the human α_{1A}-adrenergic receptor (Ford et al., 1997) was generously provided by Dr. Richard Eglen of Roche Bioscience (Palo Alto, CA). The cell line was grown in Dulbecco's modified Eagle's medium nutrient mixture F-12 Ham supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 I.U./ml penicillin, and 50 μg/ml streptomycin, at 37° in 5% CO_2. The initial selection agent G418 was absent during routine cell culture. Membranes were prepared as described previously (Leppik et al., 1998a). Briefly, near-confluent cells were harvested in cold buffer 1 (20 mM Na-HEPES, pH 7.4, 10 mM EDTA), then homogenized and centrifuged. The pellet was resuspended in buffer 2 (20 mM Na-HEPES, pH 7.4, 0.1 mM EDTA), recentrifuged, again resuspended in buffer 2, then stored at −70°C. Protein concentrations were determined by the method of Bradford (1976), with BSA as the standard.

Radioligand-Binding Assays. For saturation experiments, membranes (2 μg of protein) were incubated with increasing concentrations (0.04–5 nM) of [^{3}H]prazosin 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 20° for 120 min. Nonspecific binding was defined as the binding retained on the filter and membranes in the presence of 10 μM phentolamine. Where appropriate, amiloride analogs were added to both total and nonspecific binding-assay tubes to control for effects on binding. The use of organic solvents to dissolve the amilorides was avoided, due to the previously observed effects of the organic solvents tested on the equilibrium binding of antagonists to the α_{2A}-adrenergic receptor (Leppik et al., 1998a). Bound and free ligand were separated by rapid filtration under vacuum through GF/B glass fiber filters (Whatman; Maidstone, Kent, UK), pretreated with 0.1% polyethylenimine, by 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) added, the filters soaked overnight, and then counted. For competition experiments, membranes (5 μg of protein) were incubated with ~0.5 nM [^{3}H]prazosin in duplicate, together with increasing concentrations of competing agent, in a final volume of 1 ml of assay buffer, at 20° for 60 min.

For dissociation kinetic studies, membranes (100 μg protein/ml) were first pre-equilibrated with [^{3}H]prazosin (~2 nM) in assay buffer at room temperature for 1 h. To commence the dissociation, aliquots (100 μl) of the membrane suspension were quickly added with vortexing to pairs of tubes pre-equilibrated at 20°, each tube containing assay buffer (900 μl) supplemented with phentolamine (11.1 μM) and various concentrations of the amiloride(s) to be tested. Additions were timed so that 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 at all time points, phentolamine (10 μM) was included in a second batch of membranes plus radioligand, then the experiment repeated. The filtrations and counting were performed as described 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 fitted by nonlinear regression analyses with 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 article. Logistic fits of the effects of amiloride, DMA, BZA and HMA on the k_{obs} of [^{3}H]prazosin dissociation were fitted using the GraphPad Prism curve-fitting software (GraphPad Software, San Diego, CA).

Competition experiment data were fitted to a one-site equation as described previously (Leppik et al., 1998a). The derived apparent affinity constant was converted to the affinity constant K_1 with the Cheng-Prusoff correction (Cheng and Prusoff, 1973). In the analyses, the slopes were 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 amiloride analog, the equations used are given in the Appendix (eqs. 8 and 9). In some instances the calculated observed dissociation rates for given amiloride analog concentrations were fitted to a logistic equation (eq. 13). For the effect of competition between two amilorides on radioligand dissociation, the equation used was that derived in a previous study (Leppik et al., 1998a).

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 paired t test was used. In the text and in the tables, all relevant differences or fold increases in dissociation rate are significant at the 1% level.

Results

Characterization of Antagonist Binding at α_{1A}-Adrenergic Receptor. Initially, the equilibrium-binding properties of [^{3}H]prazosin at the human α_{1A}-adrenergic receptor permanently expressed in a CHO-K1 cell line (Ford et al., 1997) were characterized. The nonspecific binding was defined as the residual binding measured in the presence of 10 μM phentolamine. The saturation curve for the specific binding of [^{3}H]prazosin was compatible with the presence of a uniform population of binding sites. The log affinity value (log K_L) calculated for the [^{3}H]prazosin binding (10.02 ± 0.04; five experiments) was in good agreement with that previously reported (9.92 ± 0.01) (Ford et al., 1997). However, the B_{max} estimate from these experiments was 16.0 ± 0.9 pmol/mg protein (five experiments), ~10-fold higher than that previously reported for this cell line (Ford et al., 1997). The higher B_{max} estimate found in the current study may be a reflection of the different growth and assay conditions used [D. Daniels, personal communication (Roche Bioscience, Palo Alto, CA)].

The log affinity values for phentolamine and for (±)-niguldipine also were determined in equilibrium competition experiments with [^{3}H]prazosin. The values obtained (8.49 ± 0.08, three experiments, and 8.6 ± 0.6, two experiments) agree with values previously reported for the cloned α_{1A}-adrenergic receptor (Ford et al., 1994).

Effect of Amiloride Analogs on Equilibrium Binding of [^{3}H]Prazosin. It has been reported previously that amiloride decreased [^{3}H]prazosin affinity, but not the B_{max}, in

equilibrium-binding studies with the α_1-adrenergic receptor in rat renal cortical membranes, and this effect was attributed to competition between the prazosin and the amiloride (Howard et al., 1987). However, this result is equally compatible with the ternary complex allosteric model (Fig. 1). It was important to also establish that, in CHO membranes containing the human α_{1A}-adrenergic receptor, there was again no significant change in B_{max} with representative amilorides. Amiloride and HMA (Fig. 2) were chosen because they were found to have the smallest and largest effects, respectively, on the [^{3}H]prazosin dissociation rate (see below). Both amiloride and HMA decreased the observed affinity constant for [^{3}H]prazosin. At concentrations up to the highest practical concentration that either could be tested, no significant change in B_{max} was found ($P > .1$; Student's paired t test) (Table 1). These results are compatible with either a simple competitive or an allosteric model for the effect of the amilorides on [^{3}H]prazosin binding.

The affinities of the amilorides at the unoccupied α_{1A}-adrenergic receptor were determined in inhibition studies with [^{3}H]prazosin at 20°C. The data were initially fitted with an equation containing a slope factor, but the derived slope factors were normally found to be within the range 0.9 to 1.1, so the data were refitted to a simple one-site model (Table 2). As expected, the log affinities of amiloride and HMA thus obtained [4.97 ± 0.09 (n = 3) and 5.98 ± 0.07 (n = 3), respectively] were in good agreement with values estimated from the data in Table 1 [5.09 ± 0.08 (n = 6) and 5.85 ± 0.08 (n = 6) respectively, assuming either a competitive interaction or an allosteric interaction with high negative cooperativity]. In view of the fact that complex interactions of high concentrations of amilorides on [^{3}H]prazosin kinetics were observed (see next section), any additional effects of high concentrations of HMA, 5-(*N*,*N*-dimethyl)-amiloride (DMA), or amiloride on [^{3}H]prazosin equilibrium binding were investigated. Inhibition curves, performed in the presence of a high concentration of [^{3}H]prazosin (3–5 nM; 30–50 times its K_d) were shifted to the right by the factor predicted for a competitive interaction (data not shown). The slope factors for these inhibition curves were again not significantly different from 1, indicating no detectable additional effects of high micromolar-low millimolar concentrations of these ligands on equilibrium [^{3}H]prazosin binding, which might indicate formation of X_2R.

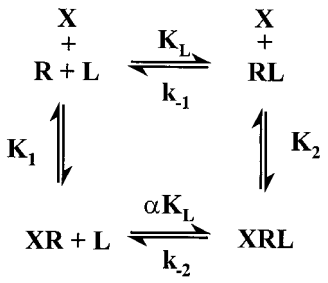
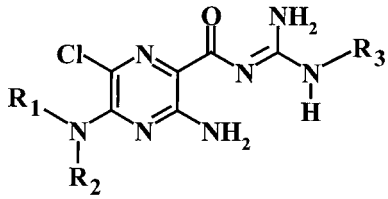


Fig. 1. Schematic representation of the ternary complex allosteric model. In this scheme, radioligand L and allosteric agent X bind to two separate sites on the receptor R. K_L and K_1 are the affinity constants for L and X, respectively, binding to R; K_2 is the affinity of X for RL and α ($= K_2/K_1$) the cooperativity factor between X and L. k_{-1} and k_{-2} are the rate constants for L dissociating from RL and XRL, respectively.

Effect of Individual Amilorides on [^{3}H]Prazosin Dissociation. The dissociation of [^{3}H]prazosin alone from the human α_{1A}-adrenergic receptor was found to be monoexponential, with a dissociation rate of 0.021 min^{-1} ($t_{1/2}$ = 33 min) at 20°C (Table 3). All of the amilorides were found to strongly increase the [^{3}H]prazosin dissociation rate in a concentration-dependent manner (Figs. 3 and 4; Table 3). For all the amiloride analog concentrations investigated, the dissociation curves were monoexponential.

With the parent amiloride, the data could be fitted to the one-allosteric-site equation (eq. 9), derived from the ternary complex allosteric model (Fig. 1). The simultaneous analysis



Amiloride analog	Abbreviation	R_1	R_2	R_3
Amiloride	-	H	H	H
Benzamil	BZA	H	H	CH_2Ph
5-(N,N-dimethyl)-amiloride	DMA	CH_3	CH_3	H
5-(N-ethyl-N-isopropyl)-amiloride	EPA	CH_3CH_2	$(CH_3)_2CH$	H
5-(N-methyl-N-isobutyl)-amiloride	MBA	CH_3	$(CH_3)_2CHCH_2$	H
5-(N,N-hexamethylene)-amiloride	HMA	$CH_2CH_2CH_2CH_2CH_2CH_2$		H

Fig. 2. Structural formulae of amiloride and of the analogs examined in this study.

of all of the data from an individual experiment gave an excellent fit (Fig. 3; Table 4), with the maximum increase in the [^{3}H]prazosin dissociation rate caused by amiloride (k_{-2}/k_{-1}) calculated to be ~20-fold at 20°C, and the log affinity of amiloride at the prazosin-occupied receptor to be 2.1, equivalent to a dissociation constant ($1/K_2$) of 8.5 mM. The dissociation data were not significantly better fitted with the two-allosteric-site equation (eq. 8) ($P > .1$). Thus, the amiloride data are compatible with amiloride acting at a single allosteric site to modulate [^{3}H]prazosin dissociation, with no evidence for amiloride acting at a second allosteric site at the concentrations tested.

For the other amilorides examined, the situation was found to be more complex. For DMA [and also for benzamil (BZA)], the dissociation data could be moderately well fitted (Fig. 4A) with the one-allosteric-site equation (eq. 9), for DMA concentrations up to 4 mM (the solubility limit). However, deviations between the data points and the theoretical lines were observed at 0.4 and 1 mM DMA, and neither of the parameter estimates, k_{-2} and log K_2, converged to stable values on successive iterations. This indicated that a one-allosteric-site model was not appropriate. The fit of the data to the two-allosteric-site equation (eq. 8) gave an excellent and significantly better fit ($P < .01$; Fig. 4B), but again some parameters, including the estimated maximum off-rate, k_{-3}, could not be defined. For the three remaining amilorides, HMA, EPA, and MBA, the deviation from the one-allosteric-site model was more marked. With HMA for example, the one-allosteric-site fit was obviously not valid (Fig. 4C), whereas the two-allosteric site fit was excellent (Fig. 4D). Thus, the data for HMA are also compatible with the interaction of HMA with two allosteric sites. As with DMA, however, neither fit defined all parameters. Comparable results also were found with EPA and MBA (data not shown).

The two-site model (as well as the one-site model) predicts that the dissociation rate asymptotes as [X] $\rightarrow \infty$. When the [^{3}H]prazosin dissociation rates in the presence of the amilorides (k_{obs}) were plotted versus amiloride analog concentration [X] (Fig. 5), there were no signs of a maximal plateau being approached for the five amiloride analogs. The gradient progressively increased with [X]; doubling of the concentration of the amiloride analog resulted in more than a doubling of the increase in dissociation rate. This behavior is not in accord with the scheme shown in Fig. 1 and in eq. 10, which predicts that a doubling in concentration of allosteric ligand will never result in more than a doubling of the change in dissociation rate. The data provide evidence of a complex interaction despite the experiments only monitoring the "tail" of a dose-response curve. Only the data for amiloride itself indicated that a plateau for k_{obs} was being approached. This lack of evidence of an approach to a maximum dissociation rate for the five analogs could rationalize why the fit of the data by the two-allosteric-site model (eq. 8) would not give defined parameters.

All data sets were fitted with the polynomial equation $k_{obs} = a + b \cdot [X] + c \cdot [X]^2$ to estimate the initial gradient b (Table 5). This parameter represents the sensitivity of the [^{3}H]prazosin dissociation rate to low concentrations of the amilorides, and is equal to the product of K_2 and $(k_{-2} - k_{-1})$ according to the one- or two-site models (eq. 12). The initial gradient derived for amiloride (43 ± 3) was in good agreement with that calculated from the parameters given in Table 4, i.e., $K_2 \cdot (k_{-2} - k_{-1}) = 48$.

Despite the apparent lack of evidence of the approach to a plateau of the k_{obs} values of the amiloride analogs in Fig. 5, the precision of some data sets of k_{obs} versus concentration allowed the fitting of the data to a logistic equation derived

TABLE 1

Effect of amiloride or HMA on the binding of [^{3}H]prazosin to the human α_{1A}-adrenergic receptor

Values are means ± S.E. from three saturation-binding assays, in which membranes containing the α_{1A}-adrenergic receptor were incubated at 20°C for 120 min with increasing concentrations of [^{3}H]prazosin (0.04–5 nM) in a final volume of 1 ml of assay buffer. LogK_{obs} is the observed log affinity constant of the [^{3}H]prazosin in the presence of amiloride analog at the α_{1A}-adrenergic receptor. In the absence of amiloride analog, logK_{obs} = logK_L (Fig. 1).

Amiloride	Amiloride Conc.	Log K_{obs}	B_{max}
	μM		*pmol/mg protein*
None	0	10.07 ± 0.06	17.0 ± 0.9
Amiloride	10	9.72 ± 0.13	17.1 ± 1.9
	100	8.95 ± 0.06	18.5 ± 0.5
HMA	1	9.88 ± 0.06	16.4 ± 0.9
	10	9.04 ± 0.03	15.9 ± 1.1

TABLE 2

Affinity of the amiloride compounds at the α_{1A}-adrenergic receptor

The log affinities were determined in equilibrium inhibition experiments versus [^{3}H]prazosin, performed at 20°C for 60 min. The data were fitted with a one-site equation (Leppik et al., 1998), with the slope factor set at 1, then the derived log apparent affinity constants were converted to the log affinity constants logK_1 with the Cheng-Prusoff correction (Cheng and Prusoff, 1973). $K_d = 1/K_1$. Values are means ± S.E. from three experiments.

Amiloride Analog	Log K_1	K_d
		μM
Amiloride	4.97 ± 0.09	11 ± 2
EPA	5.58 ± 0.04	2.7 ± 0.3
HMA	5.98 ± 0.07	1.1 ± 0.2
DMA	6.09 ± 0.02	0.82 ± 0.03
BZA	6.12 ± 0.07	0.79 ± 0.14
MBA	6.32 ± 0.06	0.49 ± 0.07

TABLE 3

Effect of amiloride analogs on the [^{3}H]prazosin dissociation rate constant

The experiments were performed at 20°C as described in the legend of Fig. 3, in the presence of the stated concentrations of the amiloride analogs. Values (min^{-1}) are means ± S.E. of three to six experiments. In the absence of amiloride analog, the [^{3}H]prazosin dissociation rate was 0.0212 ± 0.0004 min^{-1} (n = 19). The fold increase is the dissociation rate observed in the presence of the amiloride divided by the rate in its absence.

Amiloride	n	100 μM	Fold Increase in Rate	300 μM	Fold Increase in Rate	1 mM	Fold Increase in Rate
		k_{obs} (min^{-1})		k_{obs} (min^{-1})		k_{obs} (min^{-1})	
Amiloride	5	0.026 ± 0.001	1.23 ± 0.04	0.033 ± 0.002	1.57 ± 0.08	0.059 ± 0.001	2.79 ± 0.07
DMA	6	0.032 ± 0.001	1.50 ± 0.06	0.051 ± 0.002	2.42 ± 0.09	0.134 ± 0.006	6.3 ± 0.3
BZA	3	0.035 ± 0.001	1.65 ± 0.05	0.065 ± 0.001	3.07 ± 0.06	0.218 ± 0.010	10.3 ± 0.5
EPA	3	0.047 ± 0.001	2.22 ± 0.08	0.133 ± 0.008	6.30 ± 0.42		
MBA	3	0.051 ± 0.001	2.39 ± 0.08	0.175 ± 0.009	8.25 ± 0.46		
HMA	3	0.116 ± 0.002	5.46 ± 0.15	0.622 ± 0.054	29.4 ± 2.6		

from eq. 10, by adding a slope factor n to give eq. 13. This equation has one fewer parameter than the two-site model. Estimates of the maximal off-rates (k_{max}) of [^{3}H]prazosin in the presence of amiloride, DMA, HMA, and BZA could be obtained (Fig. 6, inset). These ranged from 1.9 to 3.7 min^{-1} for the latter three compounds, a 100- to 200-fold increase in off-rate. The estimated slope factors for DMA, BZA, and HMA were all >1, varying from 1.2 (BZA) to 2.1 (HMA). As expected, the fit of the amiloride k_{obs} data to eq. 13 gave a slope factor of ~1, and a maximal dissociation rate (0.35 ± 0.21 min^{-1}) similar to that found for the direct fit of the dissociation data to the one-allosteric site model (0.43 ± 0.09 min^{-1}).

Competitive Interactions between Amiloride and DMA as Detected by Their Effects on [^{3}H]Prazosin Dissociation. To further explore the allosteric interactions of amiloride, the effect of competition between it and DMA on the [^{3}H]prazosin dissociation rate was examined. The concentrations of DMA were chosen such that its effect alone on the dissociation rate of [^{3}H]prazosin could be reasonably well fitted to a one-site model. The dissociation data obtained were well fitted by the appropriate one-allosteric-site equation (eq. 9; Leppik et al., 1998a) (Fig. 7). From the fit, the parameter estimates which related to amiloride were defined, and were not significantly different ($P > .05$) from those obtained for the effect of amiloride alone on [^{3}H]prazosin dissociation (Table 4). However, the DMA parameter estimates again were not defined, as found with DMA alone. The effects of DMA and amiloride on [^{3}H]prazosin dissociation rate were additive, indicating that the concentrations of these ligands were insufficiently high to detect experimentally a significant modulation of the dissociation enhancing effects of one ligand by the other.

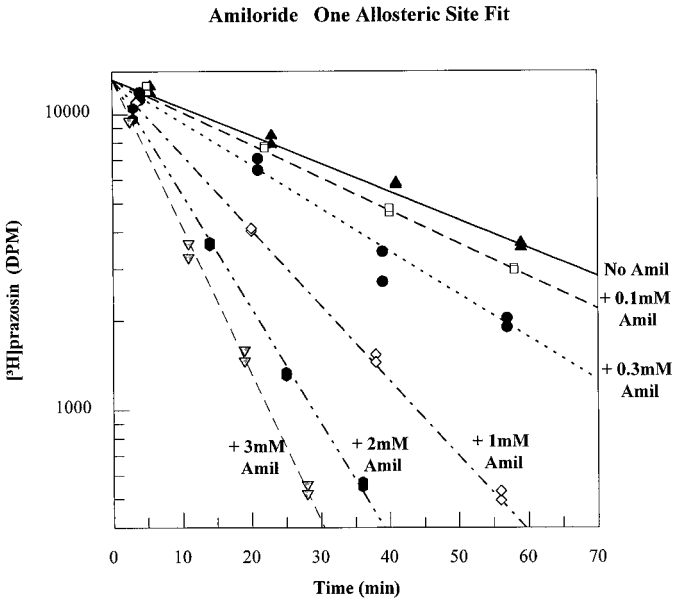


Fig. 3. Dissociation of [^{3}H]prazosin at 20°C in the absence or presence of various concentrations of amiloride. Membranes were first pre-equilibrated with [^{3}H]prazosin, then the dissociation experiment initiated by mixing aliquots with phentolamine and various concentrations of amiloride. Individual data points from one experiment are shown. The lines represent the simultaneous fit of the one allosteric site equation (eq. 9), with time and amiloride concentration as independent variables. The results of five experiments are summarized in Table 3.

Discussion

To further investigate the question as to whether α-adrenergic receptors in general possess allosteric sites, we chose to examine the effect of amilorides on the binding of the antagonist [^{3}H]prazosin at one of the α_1-adrenergic receptors, the α_{1A} subtype. Equilibrium binding studies with the cloned human α_{1A}-adrenergic receptor provided evidence that the amilorides were interacting competitively or with high negative cooperativity with [^{3}H]prazosin (Table 2). Of the six analogs examined, amiloride had the lowest affinity (K_d = 11 μM), whereas the dissociation constants of the other amilorides clustered around 1 μM (Table 2). The dissociation constants calculated for amiloride, EPA, and BZA (Table 2) were 3- to 25-fold lower than those reported previously for α_1-adrenergic receptors on rat renal cortical membranes or on the Madin-Darby canine kidney cell line (Howard et al., 1987). These differences may be due to species variation or to different experimental conditions.

No correlation between affinity and size of the 5-*N*-alkyl side chain was found. This is in contrast to the α_{2A}-adrenergic receptor, where the affinities increased progressively with increase in size of the 5-*N*-alkyl group (Leppik et al., 1998a). The structure-binding relationships of the amilorides at the unliganded α_{1A}- and α_{2A}-receptors are clearly different, suggesting that their effects on binding are not due to a simple membrane perturbation.

As found for the α_{2A}-receptor, none of the inhibition curves obtained in the current study, even those carried out with a high concentration of [^{3}H]prazosin, deviated from a simple binding isotherm. In addition [^{3}H]prazosin binding in the presence of high concentrations of the amilorides did not differ from nonspecific binding. This is compatible with either competition of the ligands at the primary binding site of the α_{1A}-receptor, or allosterism with high negative cooperativity (Ehlert, 1988). Hence, to explore potential allosteric interactions between the amilorides and prazosin, kinetic rather than equilibrium studies were required (Lee and El-Fakahany, 1991; Leppik et al., 1994; Lazareno and Birdsall, 1995).

All of the amilorides examined strongly increased the [^{3}H]prazosin dissociation rate (Table 3), indicative of an allosteric modulation of the [^{3}H]prazosin binding. For all ligands and concentrations the dissociation curves were monoexponential. At any given concentration of the amilorides, the magnitude of the increases in rate is dependent on the size of the 5-*N*-alkyl side chain (Table 3), in a manner similar but not identical with that found at the α_{2A}-adrenergic receptor. However, such a rank order of dissociation rates is not necessarily a reflection of the rank order of the affinities of the allosteric ligands at the allosteric site of an occupied receptor, as has been demonstrated for the antagonist-occupied α_{2A}-receptor (Leppik et al., 1998a). Thus, quantitative analyses of the effects of several concentrations of amiloride analogs on [^{3}H]prazosin dissociation are required to derive the estimates and rank orders of allosteric ligand affinities at the antagonist-occupied α_{1A}-receptor.

Such an analysis proved feasible with the parent amiloride, where the data were well fitted with the equation derived from the simple allosteric model (Fig. 3), indicating that amiloride indeed acts at an allosteric site to modulate [^{3}H]prazosin dissociation. The observed cooperativity be-

tween amiloride and prazosin is strongly negative (Table 4), compatible with the competition data. For the radiolabeled antagonists examined, amiloride has a comparable affinity at both the antagonist-occupied human α_{1A}- and α_{2A}-adrenergic receptors (Leppik et al., 1998a). However, the maximum fold increase in the antagonist dissociation rate caused by the binding of amiloride is 10-fold higher for the α_{1A}-adrenergic

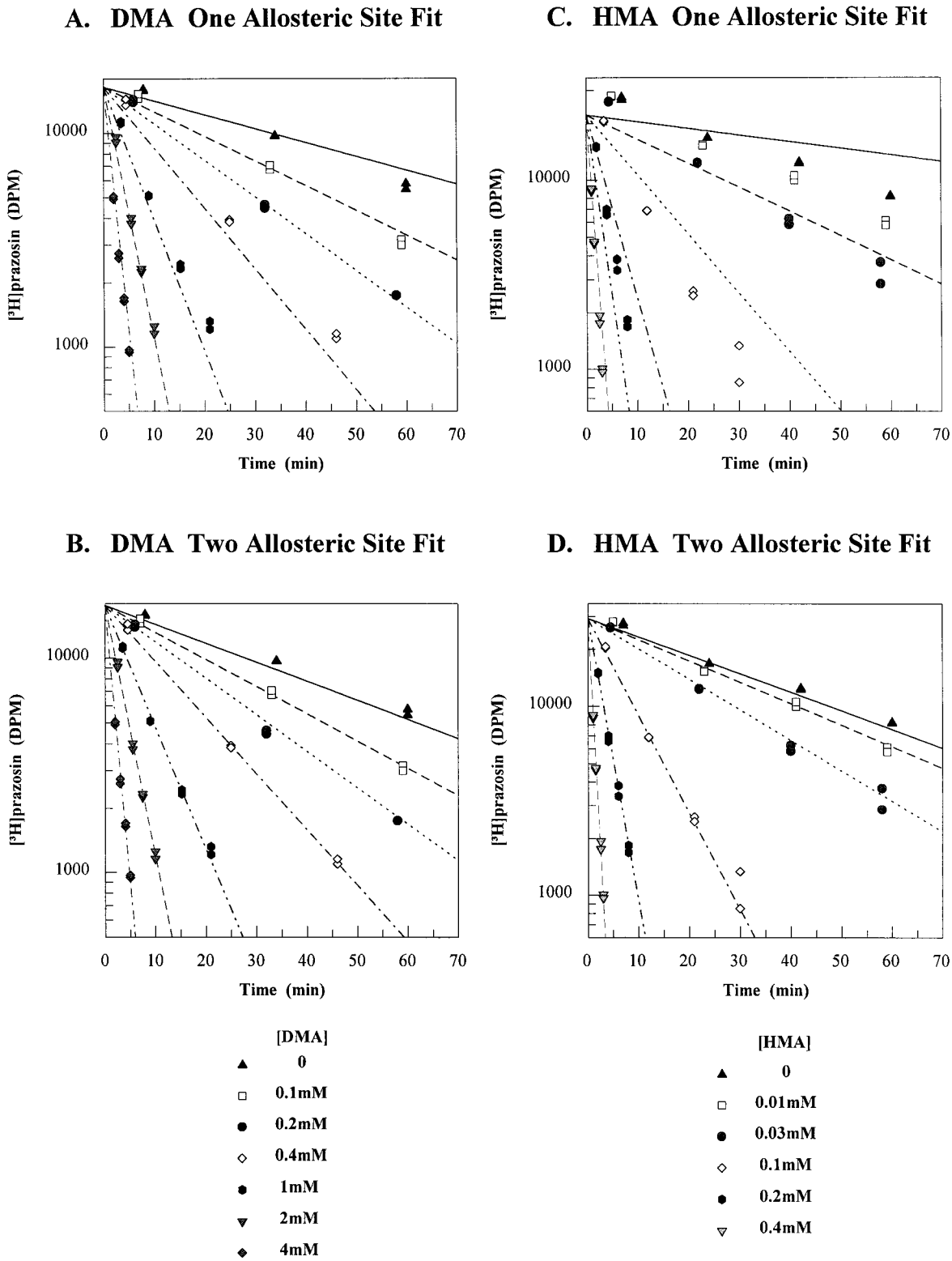


Fig. 4. Dissociation of [^{3}H]prazosin at 20°C in the absence or presence of various concentrations of DMA or HMA. The experiments were performed as described in the legend to Fig. 3. Individual data points from one experiment are shown. The data were fitted simultaneously to either the one-allosteric-site equation (A and C) or the two-allosteric-site equation (B and D) (eq. 9 and 8, respectively), with time and amiloride analog concentration as independent variables. The results of six (DMA) and three (HMA) experiments are summarized in Table 3.

receptor than that observed at the α_{2A}-adrenergic receptor (Leppik et al., 1998a).

The dissociation data for HMA (and the other amiloride analogs examined) are compatible with it modulating [^{3}H]prazosin dissociation via two allosteric sites (Fig. 4D), but not one site (Fig. 4C). Despite the excellent fit to the two-site model, estimates of parameters describing the allosteric interaction were not obtained. The explanation was evident when the observed dissociation rates (k_{obs}) of [^{3}H]prazosin in the presence of the amiloride analogs were plotted against analog concentration (Fig. 5). The k_{obs} values increased monotonically in an upwardly concave manner in the limited concentration range imposed either by the insolubility of the analogs or by our inability to measure high dissociation rates (>1 min^{-1}). There was no apparent indication of a plateau being approached. This is in contrast to amiloride itself (Fig. 5), and to the situation found for the effect of the amilorides on [^{3}H]yohimbine dissociation from the α_{2A}-adrenergic receptor, where the approach to a k_{obs} plateau is evident (data not shown).

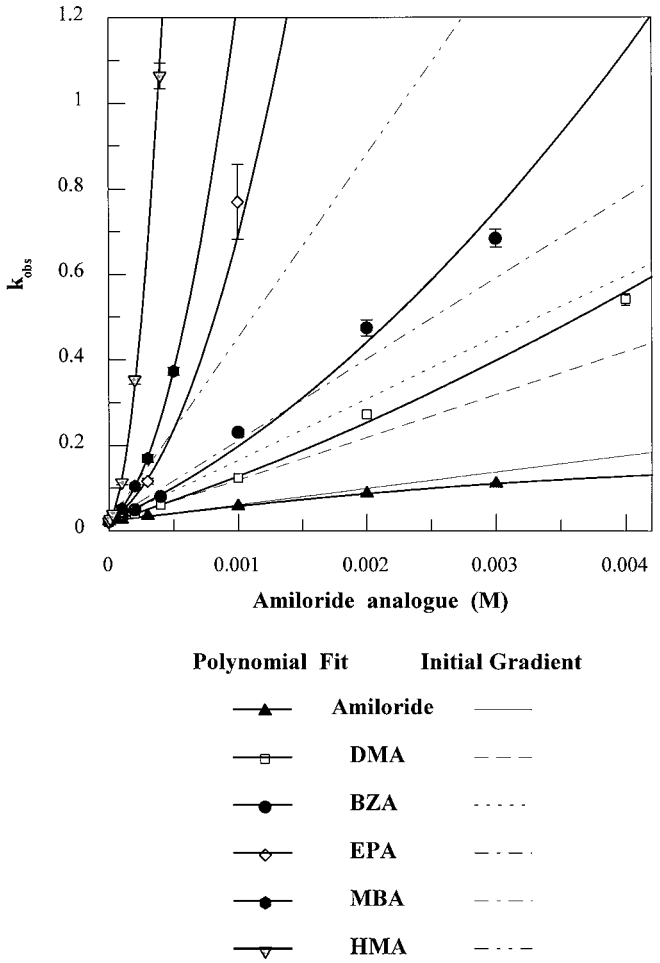


Fig. 5. The concentration dependence of the amilorides on the observed dissociation rate (k_{obs}) of [^{3}H]prazosin. The experiments were performed as described in the legend to Fig. 3. For each amiloride analog concentration, the k_{obs} was calculated with a single exponential decay equation. The derived k_{obs} values, together with their calculated errors, were fitted to the polynomial equation $k_{obs} = a + b \cdot [X] + c \cdot [X]^2$, with b being the initial gradient, and X being the amiloride analog concentration. The straight lines portray the initial gradients for each analog. The initial gradients for EPA and MBA are superimposable, so are shown with the same line style. The results of two to six experiments are summarized in Table 5.

It was however possible to fit some of the k_{obs} data to a logistic equation (eq. 13) (Fig. 6). The calculated slope factors ranged from 1.2 to 2.1, reflecting the complex apparently positive cooperative interactions evident even in the tail of the dose response curves (Figs. 4 and 5). The slope factors were essentially independent of the uncertainty of the k_{max} values associated with the considerable extrapolation. Any interpretation of the k_{max} values obtained should be treated with caution, because eq. 13 is a logistic equation and not directly derived from a model.

Analyses of the effect of the amilorides on the k_{obs} of [^{3}H]prazosin (Fig. 5) gave estimates of the initial gradients for all six amilorides (Table 5). This, from the one- or two-site models (see Appendix), is $K_2 \cdot (k_{-2} - k_{-1})$ and is a measure of the sensitivity of the antagonist dissociation kinetics to low concentrations of the amilorides. With amiloride, where estimates of the individual parameters are obtainable (Table 4), the calculated product is in agreement with the initial gradient. The product $K_2 \cdot (k_{-2} - k_{-1})$ also was calculated for the modulation of [^{3}H]yohimbine dissociation by amilorides at the α_{2A}-adrenergic receptor (Leppik et al., 1998a) (Table 5). To enable a more direct comparison of the values for the two receptor subtypes, the initial gradient values were normalized by dividing by k_{-1}. The patterns of the normalized values differ between the two adrenergic receptor subtypes, the estimates being ~20-fold larger at the α_{1A}-adrenergic receptor for amiloride and BZA, ~5-fold larger for DMA, approximately the same for MBA and EPA, and ~2-fold lower at the α_{1A}-adrenergic receptor for HMA. With amiloride, the ~20-fold difference reflects in large part the difference in antagonist dissociation rates from the amiloride-occupied receptors, given that the amiloride affinities at both antagonist-occupied receptor subtypes are comparable (Table 4; Leppik et al., 1998a).

Multiple allosteric sites for different ligands are well known with ion channel receptors (Galzi and Changeux, 1994), and with G protein-coupled receptors the obvious example of two distinct allosteric sites is the modulation of agonist binding by both an allosteric ligand and a G protein. For the α_{2A}-adrenergic receptor, it also has been shown that

TABLE 4

Effect of amiloride on [^{3}H]prazosin dissociation in the absence and presence of DMA

The experiments were performed at 20°C, as described in the legend of Fig. 3. k_{-1} and k_{-2} are the dissociation constants for the dissociation of [^{3}H]prazosin from either the unoccupied receptor or from the receptor occupied by amiloride, and logK_2 is the log affinity of amiloride at the prazosin-occupied receptor (Fig. 1). The logα_{obs} is the difference between logK_2 and the log affinity of the amiloride at the unoccupied receptor (logK_1; Table 2). Values are means ± S.E. of n experiments.

Amilorides	n	k_{-1}	k_{-2}	Fold Increase (k_{-2}/k_{-1})	LogK_2	logα_{obs}
		(min^{-1})	*(min^{-1})*			
Amiloride	4	0.020 ± 0.001	0.43 ± 0.09	22 ± 5	2.07 ± 0.12	−2.90 ± 0.12
Amiloride + DMA	3	0.021 ± 0.001	0.49 ± 0.06	24 ± 4	1.96 ± 0.06	−3.01 ± 0.06

TABLE 5

Comparison of the initial gradients for the concentration dependence of the effect of amilorides on the dissociation of [^{3}H]prazosin or [^{3}H]yohimbine from the α_{1A}- or α_{2A}-adrenergic receptors, respectively

For the α_{1A}-adrenergic receptor, the initial gradient estimates were derived as described in the legend of Fig. 5, and are expressed as means ± S.E. of n experiments. For the α_{2A}-adrenergic receptor, the values were calculated with published data (Leppik et al., 1998). To enable comparison, each estimate was divided by the antagonist dissociation rate (k_{-1}) in the absence of amiloride analog (0.0212 ± 0.0004 min^{-1} for [^{3}H]prazosin/α_{1A}-adrenergic receptor, 0.034 ± 0.001 min^{-1} for [^{3}H]yohimbine/α_{2A}-adrenergic receptor). The k_{-2} is the antagonist dissociation rate from the amiloride analog-occupied receptor, and K_2 is the affinity of the amiloride analog at the antagonist-occupied receptor.

Amiloride	n	α_{1A}-Adrenergic Receptor		α_{2A}-Adrenergic Receptor	
		Initial Gradient $K_2 * (k_{-2} - k_{-1})$	Affinity * Fold Increase $K_2 * (k_{-2} - k_{-1})/k_{-1}$	Initial Gradient $K_2 * (k_{-2} - k_{-1})$	Affinity * Fold Increase $K_2 * (k_{-2} - k_{-1})/k_{-1}$
Amiloride	4	43 ± 3	2,100	3.4	101
DMA	6	102 ± 5	4,900	34	1,010
BZA	2	141 ± 3	6,700	11	310
EPA	2	186 ± 3	8,800	270	7,900
MBA	2	187 ± 2	8,900	400	12,000
HMA	3	393 ± 20	19,000	1,100	32,000

Na$^+$ and amilorides act via different sites to modulate [^{3}H]yohimbine binding (Horstman et al., 1990). However, there are few reports suggesting two allosteric sites for the same ligand on G protein-coupled receptors. In one such report, the biphasic effect of gallamine on [^{3}H]quinuclidinyl-benzilate dissociation from muscarinic receptors at low, but not high, ionic strength was attributed to gallamine acting via two allosteric sites (Ellis and Seidenberg, 1989). However, none of the studies attempted to fit data to a model, to support and quantitate the postulated mechanism.

An alternative explanation of the results reported herein is that receptor dimerization (if it occurs for these receptors) may be modulated by the amilorides. However, as the data are compatible with the simpler model with two allosteric sites on a monomeric receptor, we have not considered a more complex dimerization model that involves 13 molecular species and 12 equilibrium constants.

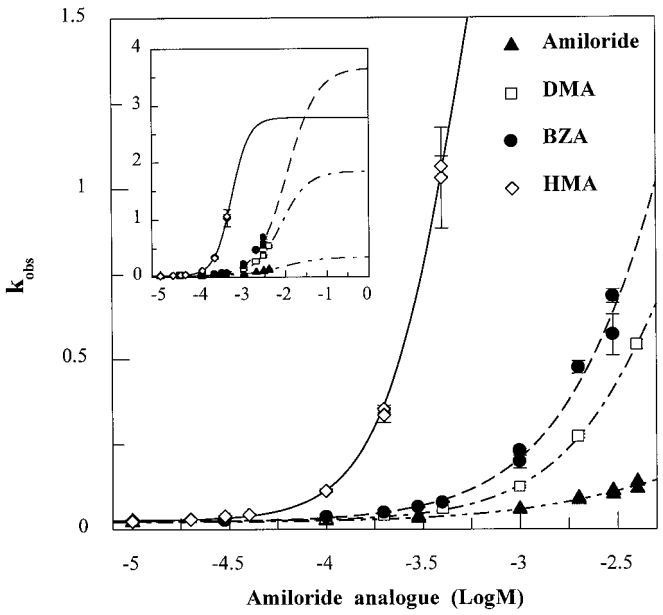


Fig. 6. Logistic fits of the effects of amiloride, DMA, BZA, and HMA on the k_{obs} of [^{3}H]prazosin dissociation at 20°C. Data from an individual experiment (DMA) or from combined experiments (four, two, or three experiments, respectively, for amiloride, BZA, or HMA) were fitted to eq. 13. The estimated slope factor, log affinity at the [^{3}H]prazosin-occupied receptor (log K_2), and maximum dissociation rate from the occupied receptor (k_{max}) for amiloride were 0.95 ± 0.17, 2.07 ± 0.47, and 0.35 ± 0.21 min^{-1}, respectively. The corresponding values for the analogs were DMA, 1.34 ± 0.14, 2.10 ± 0.22, and 1.85 ± 0.22; BZA, 1.20 ± 0.11, 1.94 ± 0.61, and 3.7 ± 4.9; and HMA, 2.06 ± 0.24, 3.29 ± 0.14, and 2.8 ± 1.1, respectively. The best-fit theoretical curves are shown on the main figure and the inset.

Studies on the effect of competition between two allosteric ligands on antagonist dissociation is a useful technique for exploring further the interactions that are occurring (Ellis and Seidenberg, 1992; Waelbroeck, 1994; Proska and Tucek, 1995; Leppik et al., 1998a). When the effect of competition between amiloride and DMA on [^{3}H]prazosin dissociation was examined, the data obtained were well fitted by the appropriate one-allosteric-site equation (Fig. 7). The parameter estimates from the fit that relate to amiloride were defined, and agreed with those obtained for the effect of amiloride alone on [^{3}H]prazosin dissociation (Table 4). However the effects of amiloride and DMA were additive, suggesting that the concentrations of amiloride and DMA were both too low relative to their respective K_2 values for one ligand to affect the dose-effect curve of the other. This experiment did however indicate that DMA was not causing a nonspecific perturbation of either the receptor or the membrane and supports the conclusion that all the kinetic effects observed are receptor-specific.

In summary, the results from the current study are compatible with amiloride, in the concentration range tested, interacting with a single, defined, allosteric site on the human α_{1A}-adrenergic receptor to modulate binding of the antagonist [^{3}H]prazosin, with no evidence for interaction at a second allosteric site. In contrast the data for the amiloride analogs examined are compatible with interaction at two sites to modulate antagonist binding.

Acknowledgments

The gift by Dr. Richard Eglen of Roche Bioscience of the CHO cell line expressing the human α_{1A}-adrenergic receptor is gratefully acknowledged.

Appendix

Radioligand Dissociation in the Presence of an Allosteric Agent That Can Interact with Two Allosteric Sites. The dissociation of a radioligand L from a receptor R in the presence of an allosteric agent X that can interact with two

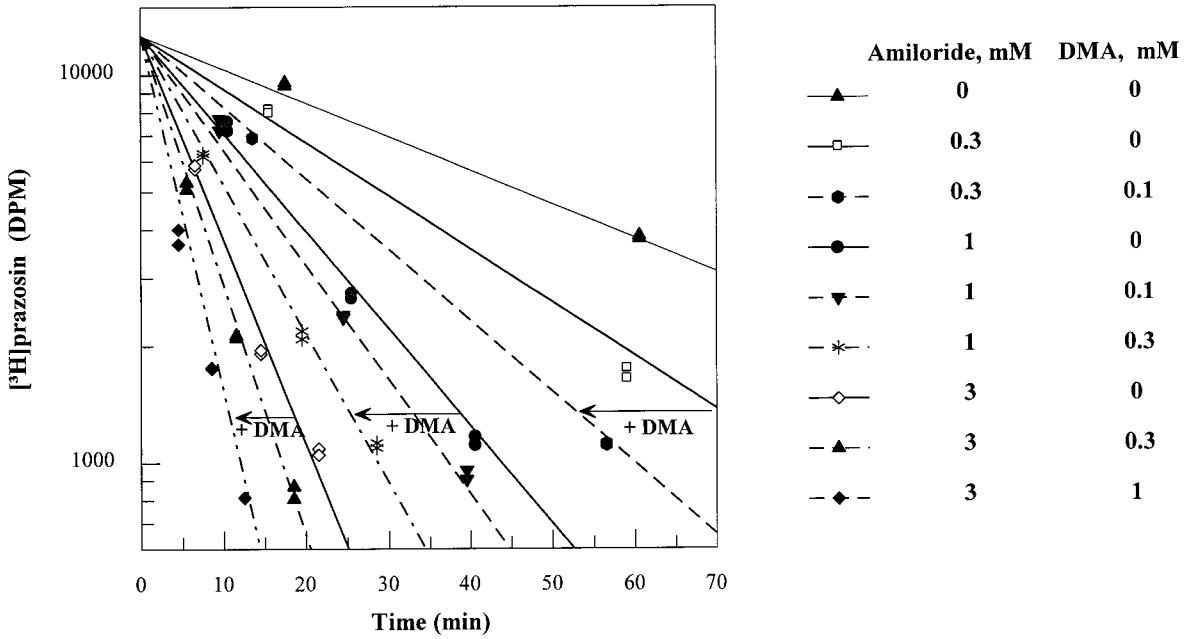
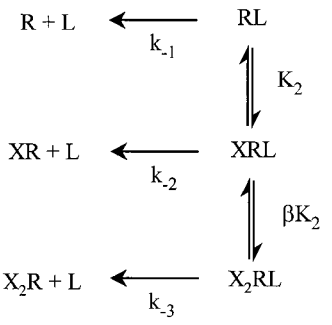


Fig. 7. Effect of DMA on the modulation by amiloride of [³H]prazosin dissociation at 20°C. The experiments were performed as described in the legend to Fig. 3. Individual data points from one experiment are shown. The lines represent the simultaneous fit of the data to the equation derived in a previous study (eq. 9; Leppik et al., 1998), with time, and amiloride and DMA concentrations as independent variables. The results of three experiments are summarized in Table 4.

allosteric sites can be represented by eq. 1:



(1)

In this representation, the individual allosteric sites are not distinguished because analysis of dissociation data would not allow the interactions of X at each site to be characterized. Thus, in eq. 1, K_2 is the effective affinity constant for the binding of the first molecule of the allosteric agent X to RL; β is the homotropic cooperativity factor associated with the binding of the second molecule of X to XRL, which is equivalent to a scaling factor describing how tightly the second molecule of X binds relative to the first; k_{-1} is the dissociation rate constant for the dissociation of L from RL; and k_{-2} and k_{-3} are the effective dissociation rate constants for the dissociation of L from XRL and X_2RL, respectively.

In the dissociation experiments, radioligand L is first preequilibrated with the receptor R. Then, at time zero, the allosteric agent X is added. If X has fast binding kinetics, equilibrium would be rapid, and the proportions of RL, XRL, and X_2RL would remain constant during the dissociation. The proportion, p, of radioligand bound as XRL is as follows:

$$p = \frac{K_2 \cdot [X]}{1 + K_2 \cdot [X] + \beta \cdot (K_2 \cdot [X])^2} \quad (2)$$

the proportion, q, of radioligand bound as X_2RL is as follows:

$$q = \frac{\beta \cdot (K_2 \cdot [X])^2}{1 + K_2 \cdot [X] + \beta \cdot (K_2 \cdot [X])^2} \quad (3)$$

and the proportion of radioligand bound as RL is as follows:

$$1 - p - q = \frac{1}{1 + K_2 \cdot [X] + \beta \cdot (K_2 \cdot [X])^2} \quad (4)$$

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

$$\frac{dB}{dt} = -k_{-1} \cdot [RL] - k_{-2} \cdot [XRL] - k_{-3} \cdot [X_2RL] \quad (5)$$

Substituting eq. 2–4 into eq. 5:

$$\frac{dB}{dt} = -\{k_{-1} \cdot (1 - p - q) + k_{-2} \cdot p + k_{-3} \cdot q\} \cdot B \quad (6)$$

Expanding and rearranging eq. 6:

$$\frac{dB}{dt} = -\left\{\frac{k_{-1} + k_{-2} \cdot K_2 \cdot [X] + k_{-3} \cdot \beta \cdot (K_2 \cdot [X])^2}{1 + K_2 \cdot [X] + \beta \cdot (K_2 \cdot [X])^2}\right\} \cdot B \quad (7)$$

Integrating:

$$B_t = B_0 \cdot e^{-\left\{\frac{k_{-1} + k_{-2} \cdot K_2 \cdot [X] + k_{-3} \cdot \beta \cdot (K_2 \cdot [X])^2}{1 + K_2 \cdot [X] + \beta \cdot (K_2 \cdot [X])^2}\right\} \cdot t} \quad (8)$$

where B_t is the total radioligand L bound at time t, and B_0 the total radioligand bound at time zero. In the fitting of the data to eq. 8, t and [X] were independent variables, and the equation was recast in terms of log K_2, log β and log [X] (Hulme and Birdsall, 1992).

In the presence of only one allosteric site, one reverts back to the ternary complex allosteric model (Fig. 1). For this, eq.

8 reduces to the equation derived by Lazareno and Birdsall (1995):

$$B_t = B_o \cdot e^{-\left\{\frac{k_{-1} + k_{-2} \cdot K_2 \cdot [X]}{1 + K_2 \cdot [X]}\right\} \cdot t} \quad (9)$$

In the fitting of the data to eq. 9, t and [X] were independent variables, and the equation was again recast in terms of log K_2 and log [X].

With these models, it is of value to examine the effect of amiloride analog concentration [X] on the observed dissociation rate (k_{obs}) of the radioligand. For the one-allosteric-site model:

$$k_{obs} = \frac{k_{-1} + k_{-2} \cdot K_2 \cdot [X]}{1 + K_2 \cdot [X]} \quad (10)$$

with $k_{obs} \rightarrow k_{-2}$ as [X] $\rightarrow \infty$.

Similarly, for the two-allosteric-site model:

$$k_{obs} = \frac{k_{-1} + k_{-2} \cdot K_2 \cdot [X] + k_{-3} \cdot \beta \cdot (K_2 \cdot [X])^2}{1 + K_2 \cdot [X] + \beta \cdot (K_2 \cdot [X])^2} \quad (11)$$

with $k_{obs} \rightarrow k_{-3}$ as [X] $\rightarrow \infty$.

In both cases, the initial gradient is given by:

$$\left(\frac{dk_{obs}}{d[X]}\right)_{[X] \rightarrow 0} = (k_{-2} - k_{-1}) \cdot K_2 \quad (12)$$

In those cases where the two-allosteric-site eq. 8 gave a better fit of the data than the one-allosteric-site eq. 9, a modified version of the one-allosteric-site equation for k_{obs} (eq. 10) also was tested, in which a slope factor n was added to eq. 10 to give eq. 13, with k_{max} being the off rate when [X] $\rightarrow \infty$:

$$k_{obs} = \frac{k_{-1} + k_{max} \cdot (K_2 \cdot [X])^n}{1 + (K_2 \cdot [X])^n} \quad (13)$$

References

Birdsall NJM, Farries T, Gharagozloo P, Kobayashi S, Lazareno S and Sugimoto M (1999) Subtype selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: Functional studies. *Mol Pharmacol* **55:**778–786.

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.

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 (IC_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Ehlert FJ (1988) Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol Pharmacol* **33:**187–194.

Ellis J, Huyler J and Brann MR (1991) Allosteric regulation of cloned m1–m5 muscarinic receptor subtypes. *Biochem Pharmacol* **42:**1927–1932.

Ellis J and Seidenberg M (1989) Gallamine exerts biphasic allosteric effects at muscarinic receptors. *Mol Pharmacol* **35:**173–176.

Ellis J and Seidenberg M (1992) Two allosteric modulators interact at a common site on cardiac muscarinic receptors. *Mol Pharmacol* **42:**638–641.

Ford APDW, Daniels DV, Chang DJ, Gever JR, Jasper JR, Lesnick JD and Clarke DE (1997) Pharmacological pleiotropism of the human recombinant α_{1A}-adrenoceptor: Implications for α_1-adrenoceptor classification. *Br J Pharmacol* **121:**1127–1135.

Ford APDW, Williams TJ, Blue DR and Clarke DE (1994) α_1-Adrenoceptor classification: Sharpening Occam's razor. *Trends Pharmacol Sci* **15:**167–170.

Galzi J-L and Changeux J-P (1994) Neurotransmitter-gated ion channels as unconventional allosteric proteins. *Curr Opin Struct Biol* **4:**554–565.

Horstman DA, Brandon S, Wilson AL, Guyer CA, Cragoe EJ Jr and Limbird LE (1990) An aspartate conserved among G-protein receptors confers allosteric regulation of α_2-adrenergic receptors by sodium. *J Biol Chem* **265:**21590–21595.

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.

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 (1991) Allosteric antagonists of the muscarinic acetylcholine receptor. *Biochem Pharmacol* **42:**199–205.

Leppik RA, Lazareno S, Mynett A and Birdsall NJM (1998a) Characterization of the allosteric interactions between antagonists and amiloride analogues at the human α_{2A}-adrenergic receptor. *Mol Pharmacol* **53:**916–925.

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.

Leppik RA, Mynett A and Birdsall NJM (1998b) Allosteric modulation of [^{3}H]prazosin binding at the human α_{1A} adrenoceptor. *Naunyn-Smiedeberg's Arch Pharmacol* **358:**604.

Macdonald RL and Olsen RW (1994) $GABA_A$ receptor channels. *Annu Rev Neurosci* **17:**569–602.

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.

Proska J and Tucek S (1995) Competition between positive and negative allosteric effectors on muscarinic receptors. *Mol Pharmacol* **48:**696–702.

Smith GB and Olsen RW (1995) Functional domains of $GABA_A$ receptors. *Trends Pharmacol Sci* **16:**162–168.

Waelbroeck M (1994) Identification of drugs competing with d-tubocurarine for an allosteric site on cardiac muscarinic receptors. *Mol Pharmacol* **46:**685–692.

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, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK. E-mail: rleppik@nimr.mrc.ac.uk