0026-895X/00/051091-09$3.00/0
MOLECULAR PHARMACOLOGY
Copyright © 2000 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 58:1091–1099, 2000

Vol. 58, No. 5
191/861610
Printed in U.S.A.

Agonist Binding and Function at the Human α_{2A}-Adrenoceptor: Allosteric Modulation by Amilorides

RAY A. LEPPIK and NIGEL J. M. BIRDSALL

National Institute for Medical Research, London, United Kingdom

Received April 3, 2000; accepted August 14, 2000

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

ABSTRACT

It has been found previously that amilorides act via an allosteric site on the α_{2A}-adrenergic receptor to strongly inhibit antagonist binding. In this study, allosteric modulation of agonist binding and function at the α_{2A}-adrenergic receptor was explored. The dissociation rate of the agonist [^{3}H]UK14304 from α_{2A}-receptors was decreased by the amilorides in a concentration-dependent manner. This contrasts with the increases in ^{3}H-antagonist dissociation rate found previously. The agonist-amiloride analog interaction data could be fitted to equations derived from the ternary complex allosteric model. The calculated log affinities of the amilorides at the [^{3}H]UK14304-occupied receptor increased with the size of the 5-*N*-alkyl side chain and ranged from 2.4 for amiloride to 4.2 for 5-(*N,N*-hexamethylene)-amiloride. The calculated negative cooperativities cover a narrow range, in sharp contrast to the broad range found for antagonist-amiloride analog interactions. The effects of the amilorides on the agonist actions of UK14304, epinephrine, and norepinephrine were explored using a [^{35}S]GTPγS functional assay, and the parameters calculated for the cooperativities and affinities of the UK14304-amiloride analog interactions, using the equation derived from the ternary complex allosteric model, were in good agreement with those derived from the kinetic studies. Therefore both the binding and functional data provide further support for the existence of a well defined allosteric site on the human α_{2A}-adrenergic receptor. The binding mode of the amilorides at the agonist-occupied and antagonist-occupied receptor differs markedly but, within each group, the structure of either the agonist or the antagonist examined has only a slight effect on the allosteric interactions.

Currently used drugs that bind to G protein-coupled receptors produce their therapeutic effects either by mimicking (in the case of agonists) or blocking (in the case of antagonists) the action of the endogenous signaling molecule, by competing with it at the same primary binding site on the receptor. The effect of such drugs is to either generate continuous stimulation or chronic blockade of receptor function at all the receptor molecules. In certain therapeutic indications, it may be more desirable to have an alternative approach, that of controlling the activity of the endogenous agonist at a given receptor.

This regulation of receptor function can be accomplished if a ligand operates by an allosteric mechanism, i.e., the ligand binds to a different site on the receptor from the endogenous agonist, to modulate agonist binding and function. The simplest scheme is shown in Fig. 1 (solid lines). The two parameters that describe the action of an allosteric agent X are its affinity for the receptor (K_X) and its cooperativity (α) with a primary binding site ligand L, which may be the endogenous agonist. The cooperativity factor α can be greater than, less than, or, in a special case, equal to 1: this corresponds to positive, negative, or neutral cooperative interactions, respectively.

One example of such a potential therapeutic area is the use of an allosteric muscarinic enhancer ($\alpha > 1$ with L being acetylcholine) in the early stages of Alzheimer's disease, to alleviate the cognitive deficits that are thought to be caused by the localized degeneration of cholinergic nerve terminals and the associated acetylcholine deficit (Giacobini, 1990; Ehlert et al., 1994). In fact, M_2 muscarinic receptors were the first G protein-coupled receptor at which allosterism was demonstrated, when the antagonism by gallamine of the negative inotropic effect of muscarinic agonists in the heart was investigated (Clark and Mitchelson, 1976). This work was confirmed and extended by Stockton et al. (1983). From a therapeutic viewpoint, however, it is necessary to be able to enhance acetylcholine function in a subtype-selective manner, and this has recently been achieved with brucine and related compounds (Birdsall et al., 1997, 1999; Lazareno et al., 1999).

There have been relatively few reports on the detailed characterization of the effects of allosteric ligands on agonist

Financial support: R.A.L. is a recipient of a ROPA research grant. A preliminary report of the work described in this manuscript was presented at the XIIIth International Congress of Pharmacology, 1998 (Leppik et al., 1998b).

ABBREVIATIONS: GTPγS, guanosine-5′-*O*-(3-thio)-triphosphate; CHO, Chinese hamster ovary; BZA, benzamil; DMA, 5-(*N,N*-dimethyl)-amiloride; EPA, 5-(*N*-ethyl-*N*-isopropyl)-amiloride; HMA, 5-(*N,N*-hexamethylene)-amiloride; MBA, 5-(*N*-methyl-*N*-isobutyl)-amiloride.

binding (especially kinetics) and function. Outside of the muscarinic area, studies on adenosine receptors have shown that PD81,723 enhances agonist binding and function at the adenosine A_1 receptor as well as modulating agonist kinetics at the receptor-G protein complex (Bruns and Fergus, 1990; Cohen et al., 1994; Musser et al., 1999).

With adrenergic receptors, it has been demonstrated that amilorides can act allosterically at a single allosteric site on the α_{2A}-adrenergic receptor, and in a more complex manner at the α_{1A}-adrenergic receptor, to inhibit antagonist binding (Nunnari et al., 1987; Leppik et al., 1998a, 2000). However, there have been no studies of agonist-amiloride interactions. In this paper we present a quantitative investigation of the allosteric interactions of amiloride analogs on agonist binding and function at the α_{2A}-adrenergic receptor, including the effects of the amilorides on the actions of the endogenous agonists, epinephrine and norepinephrine.

Experimental Procedures

Materials. [^{3}H]UK14304 (27 Ci/mmol) was from DuPont NEN (Hounslow, Middlesex, UK), and [^{35}S]GTPγS (1050 Ci/mmol) was from Amersham International (Little Chalfont, Buckinghamshire, UK). Amiloride HCl, BZA, DMA HCl, EPA, HMA, MBA, GDP, UK14304, epinephrine, norepinephrine, and phentolamine HCl were from Sigma Chemical Co. (Poole, Dorset, UK). 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). The use of organic solvents to dissolve the amilorides was avoided, because of the previously observed effects of organic solvents on the equilibrium binding of antagonists to the α_{2A}-adrenergic receptor (Leppik et al., 1998a).

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-minimal essential medium supplemented with 10% newborn calf serum, 2 mM L-glutamine, 50 I.U./ml penicillin, and 50 μg/ml streptomycin, at 37°C in 5% CO_2. 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, and then stored at −70°C. Protein concentrations were determined by the method of Bradford (1976), using bovine serum albumin as the standard.

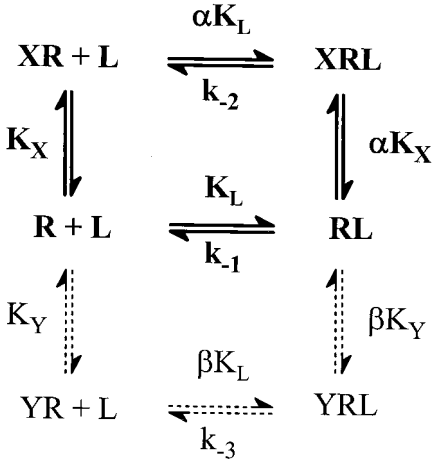

Fig. 1. Schematic representation of the ternary complex allosteric model (solid lines, heavy text) and a representation of the effect of competition between two allosteric agents at the allosteric site on the binding of the radioligand at the primary binding site (whole of figure). In this scheme, the radioligand L and the allosteric agents X and Y bind to the primary and allosteric sites, respectively, on the receptor R. K_L, K_X, and K_Y are the affinity constants for L, X, and Y, respectively, for binding to R, α, and β are the cooperativity factors between X or Y, respectively, and L, and k_{-1}, k_{-2}, and k_{-3} are the rate constants for L dissociating from RL, XRL, and YRL, respectively.

Radioligand Binding Assays. For competition experiments, membranes (20, 25, or 30 μg of protein) were incubated with approximately 6, 0.6, or 0.3 nM [^{3}H]UK14304, respectively, in duplicate, together with increasing concentrations of competing agent, 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°C for 120 min. Nonspecific binding was defined as the binding retained on the filter and membranes in the presence of 20 μM phentolamine. Bound and free ligand 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, and transferred to scintillation vials. Scintillation cocktail (Beckman, Palo Alto, CA) was added, and the filters were soaked overnight and then counted.

In exploratory association experiments, in which sets of tubes containing membranes (20 μg) in assay buffer (950 μl) were placed in a 30°C bath and then [^{3}H]UK14304 (50 μl, 120 nM) was added to individual tubes at varying times, the association of [^{3}H]UK14304 appeared biphasic, with an initial rapid rise, followed by a steady upward rise ("creep") for at least 3 h. A biphasic association was also reported by Neubig et al. (1988), who demonstrated that the fast component was UK14304 concentration dependent, but the slow component was not. However, if in the association assay the membrane suspension was instead kept on ice, and aliquots (100 μl, 20 μg membranes) were only added to tubes containing assay buffer (850 μl) at 30°C 5 min before the [^{3}H]UK14304 (50 μl) addition, the creep was eliminated, with the level of [^{3}H]UK14304 bound remaining constant for between 1 and 6 h, at all the concentrations examined (0.1–10 nM). This would suggest that components in the system responsible for the generation or maintenance of the high-affinity state of the receptor are unstable at 30°C but are stabilized by UK14304 concentrations as low as 0.1 nM.

Because of this instability, membranes were also kept on ice until exposed to agonist in the dissociation assay protocol developed. Thus, aliquots (100 μl) of an ice-cold membrane suspension (25 μg) were added at various times to tubes containing [^{3}H]UK14304 (16.7 nM) in assay buffer (150 μl) at 30°C, each mixture was left 40 min for equilibration, and then an aliquot (200 μl) of this mixture was transferred to a second tube at 30°C containing phentolamine (25 μM) in assay buffer (800 μl) to commence the measurement of the dissociation. Additions were timed so that the contents of the second set of tubes were filtered at the same time. To determine nonspecific binding at all time points, phentolamine (33 μM) was included in a second batch of the tubes containing the radioligand, and then the experiment was repeated. In those experiments in which the effects of the amilorides on the dissociation of [^{3}H]UK14304 were examined, the amilorides were also included in the second set of tubes with the phentolamine. 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. With this protocol, two-site exponential fits of the dissociation data were found to be not significantly better than one-site fits ($P > .1$).

[^{35}S]GTPγS Assays. In each tube of a set of 24 tubes, membranes (10 μg) were incubated at 30°C with GDP (1 μM), [^{35}S]GTPγS (0.05 nM), a given concentration of the amiloride to be tested (where relevant), and an increasing concentration of agonist in duplicate, in a final volume of 1 ml of assay buffer. Sufficient sets of 24 tubes

(normally five to seven sets) were assayed consecutively, to enable the desired concentration range of the amiloride to be tested to be covered. Where the agonist was either epinephrine or norepinephrine, sodium metabisulfite (1 mM) was also included. Sodium metabisulfite had been found in initial tests to be necessary, to minimize a decrease in the [^{35}S]GTPγS bound at concentrations of epinephrine or norepinephrine over 0.1 mM (data not shown). The time course for each set of 24 tubes was started by the addition of the membrane suspension and terminated by filtration after 60 min, as described above.

Data Analysis. Data were 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 paper.

Competition experiment data were fitted to a one-site equation with a slope factor, as described previously (Leppik et al., 1998a). Because direct binding experiments of [^{3}H]UK14304 and the α_{2A}-adrenergic receptor have shown that the binding is complex (Neubig et al., 1988, and *Results*), a simple affinity constant for UK14304 binding at this receptor could not be derived. Thus, in each set of competition experiments between [^{3}H]UK14304 and the amilorides, a competition experiment versus phentolamine was performed as a control. The calculated log IC_{50} from the control experiment and the previously determined log affinity value for phentolamine (7.25; R. Leppik, unpublished data) were used to calculate an apparent log affinity value for the UK14304 at the concentration used, using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The IC_{50} values for the amilorides were then converted to the log affinity constant log K_x using the derived apparent log affinity value for [^{3}H]UK14304 in the Cheng-Prusoff correction.

Data from dissociation experiments performed in the absence of added amilorides were fitted to either the single- or the double-exponential decay equations of the Grafit software. For data obtained from radioligand dissociation experiments performed in the presence of one or two amiloride analogs, the equations used and the method of fitting are as described in a previous study (equation 10 and 9, respectively, Leppik et al., 1998a).

Data from [^{35}S]GTPγS assays performed in the absence of amiloride analogs were fitted by the four parameter logistic equations of the Grafit software. Those assays performed in the presence of a range of amiloride analog concentrations were simultaneously fitted by the equation derived in a previous study (equation 37, Lazareno and Birdsall, 1995). In the fitting of the data, the agonist and amiloride concentrations were independent variables, and the equation was recast in terms of log affinity constant, log EC_{50}, log concentrations, and log cooperativity factor (Hulme and Birdsall, 1992).

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.

Results

Effect of Amiloride Analogs on the Equilibrium Binding of [^{3}H]UK14304. The data from competition experiments between [^{3}H]yohimbine and UK14304 at the human α_{2A}-adrenergic receptor, permanently expressed in a CHO-K1 cell line (Kurose and Lefkowitz, 1994; Leppik et al., 1998a), were compatible with the presence of two high-affinity GTP-sensitive agonist sites and one low-affinity GTP-insensitive agonist site, with log affinities of 10.2 ± 0.2, 8.4 ± 0.1 and 6.59 ±0.02, respectively (K_d values: 0.1, 5.8, and 260 nM, respectively; eight experiments, data not shown). The two high-affinity sites were present in approximately equal proportions and together represented 32 ± 3% of the total binding sites labeled by [^{3}H]yohimbine. The high-affinity binding constants are in reasonable agreement with those previously reported (Neubig et al., 1988), but the proportion of high-affinity sites is somewhat lower, perhaps reflecting the higher B_{max} (20 ± 2 pmol/mg protein) found in the cell membranes from the transfected cells used in the current work. GTP (1 mM) converted both high-affinity states to the low-affinity state of the receptor. Equilibrium [^{3}H]UK14304 binding more directly suggested the presence of two high-affinity agonist binding sites in approximately equal proportions, with K_d values of 0.21 ± 0.02 and 2.2 ± 0.2 nM.

The affinities of the amilorides were determined in competition experiments with the α_2-selective agonist [^{3}H]UK14304 at 30°C. For those three amilorides successfully studied in the [^{35}S]GTPγS assay (below), the corrected log affinity values (log K_x) (see under *Experimental Procedures*; three experiments) calculated for amiloride, DMA, and HMA, 4.60 ± 0.04, 5.49 ± 0.03, and 6.74 ± 0.04, respectively, were in good agreement with those values found previously in competition experiments versus the antagonist [^{3}H]yohimbine (Leppik et al., 1998a). No evidence for residual [^{3}H]UK14304 binding at high amiloride analog concentrations was observed, which would argue against the existence of low negative cooperativity ($0.05 < \alpha < 1$) between the amilorides and [^{3}H]UK14304. For those competition experiments performed with low (0.3 nM) concentrations of [^{3}H]UK14304, the slope factors for both phentolamine and the amilorides were not significantly different from 1, but at higher (6 nM) concentrations of [^{3}H]UK14304, the slope factor was lower (0.75 ± 0.10). The estimated log K_x values are independent of the concentration of [^{3}H]UK14304 used in the binding assay. These results are entirely compatible with either high negative cooperativity or competition of the amilorides with the [^{3}H]UK14304 at primarily one (0.3 nM) or both (6 nM) of the high-affinity UK14304 binding sites.

Modulation by Amilorides of [^{3}H]UK14304 Dissociation from the α_{2A}-Adrenergic Receptor. In the dissociation assay protocol developed, the membrane suspension containing the α_{2A}-adrenergic receptor was kept on ice, and then aliquots were added to tubes containing [^{3}H]UK14304 in assay buffer at 30°C. After 40 min of equilibration, an aliquot of this mixture was transferred to a second tube at 30°C containing phentolamine with or without amiloride analog in assay buffer, to start the dissociation. With this protocol, the dissociation of the [^{3}H]UK14304 from the α_{2A}-adrenergic receptor was found to be monoexponential in the absence or presence of a given concentration of amiloride analog; a double-exponential equation did not give a significantly better fit of the data ($P > .1$). [^{3}H]UK14304 alone had a dissociation rate from the α_{2A}-adrenergic receptor at 30°C of 0.041 ± 0.003 min^{-1} ($t_{1/2}$ = 17 min; n = 6).

When the effects of the amilorides (Fig. 2) on [^{3}H]UK14304 dissociation from the α_{2A}-adrenergic receptor at 30°C were examined, the data suggested that the amilorides were causing a slight decrease in the [^{3}H]UK14304 dissociation rate. However, only the effect of HMA was large enough to be quantitated. When the HMA data were fitted (Fig. 3) by the equation derived from the ternary complex allosteric model (Fig. 1, solid lines) (Lazareno and Birdsall, 1995), the parameters obtained (Table 1) showed that the [^{3}H]UK14304 dissociation rate from the HMA-occupied receptor was ~2.7-fold slower than that from the unoccupied receptor. The log affinity of HMA at the [^{3}H]UK14304-occupied receptor (logαK_x)

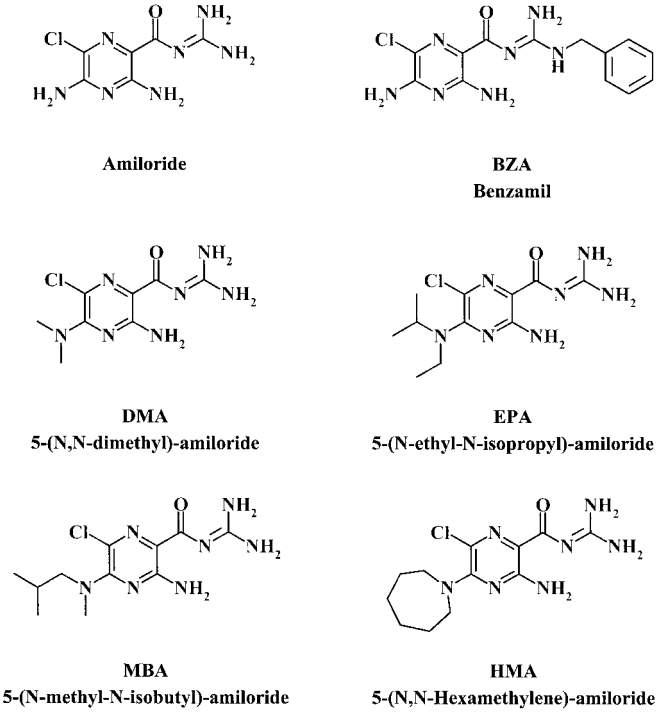

Fig. 2. Structural formulae of amiloride and the analogs examined in this paper.

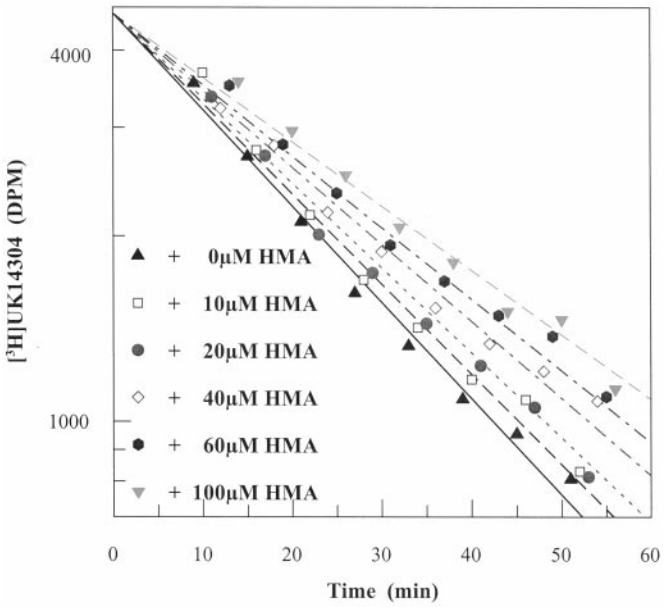

Fig. 3. Dissociation of [^{3}H]UK14304 in the absence or presence of various concentrations of HMA. Aliquots of an ice-cold membrane suspension were added to [^{3}H]UK14304 pre-equilibrated at 30°C and then, after 40 min, a portion was transferred to a second tube at 30°C containing phentolamine and various concentrations of HMA to commence the dissociation measurement. Individual data points from one experiment are shown. The data from each experiment were simultaneously fitted to the equation from the allosteric model (Fig. 1, solid lines) (eq. 10, Leppik et al., 1998), with time and HMA concentration as independent variables. The results are summarized in Table 1.

was 4.24 ($1/\alpha K_X$: 58 μM). As the log affinity of HMA at the unoccupied α_{2A}-adrenergic receptor (log K_x) was 6.74 (see above), the difference between these two log affinity values, the observed log cooperativity factor (logα), was -2.50 ($1/\alpha$ = 320).

Because the other amilorides caused even smaller changes in the [^{3}H]UK14304 dissociation rate, their effects could only be quantitated by their modulation of the HMA effect on [^{3}H]UK14304 dissociation. This was done for amiloride and DMA, the two other amilorides successfully studied in the [^{35}S]GTPγS assay (below). The data were well fitted (Fig. 4) by the dissociation equation derived from the model that allows competition between two allosteric agents at the allosteric site (Fig. 1) (Leppik et al., 1998a). Those parameter estimates derived for HMA agreed well with the ones calculated for HMA alone (Table 1). As anticipated, the [^{3}H]UK14304 dissociation rates from the amiloride-occupied or DMA-occupied receptors were only slightly decreased compared with the dissociation rate of [^{3}H]UK14304 from the unoccupied receptor, the fold decreases being 1.5 and 1.3, respectively (Table 1). The log affinities of amiloride and DMA at the [^{3}H]UK14304-occupied receptor were calculated to be 2.39 and 3.39 ($1/\alpha K_X$: 4100 and 410 μM), respectively (Table 1). Use of the log affinity values for amiloride and DMA at the unoccupied receptor derived above enabled the observed log cooperativities to be calculated as -2.21 and -2.10 ($1/\alpha$ = 160 and 130), respectively (Table 1).

Characterization of Agonist-Stimulated Binding of [^{35}S]GTPγS to the α_{2A}-Adrenergic Receptor. [^{35}S]GTPγS assay conditions were established using norepinephrine. The basal (or nonspecific) level of [^{35}S]GTPγS binding was defined as that level of binding in a given time period in the absence of added agonist. In all assays, norepinephrine (and epinephrine) was used in the presence of 1 mM sodium metabisulfite, to minimize oxidation of the catechol moiety. In the presence of 1 μM GDP, 0.05 nM [^{35}S]GTPγS, and 0.3 mM norepinephrine, the stimulated binding increased over a 90-min time interval (data not shown). Sixty minutes was chosen as the standard incubation time. Under these assay conditions, full agonists

TABLE 1

Effect of amiloride analogs on [^{3}H]UK14304 dissociation from the α_{2A}-adrenergic receptor

The experiments were performed at 30°C, as described in the legend of Fig. 3. The data from each experiment were fitted to the single-exponential equations derived in a previous study (equations 10 and 9, respectively, Leppik et al., 1998). k_{-1}, k_{-2}, and k_{-3} are the dissociation constants for the dissociation of [^{3}H]UK14304 from the unoccupied (k_{-1}), HMA-occupied (k_{-2}), or amiloride- or DMA-occupied (k_{-3}) receptors. The log α_{obs} and log β_{obs} are the calculated differences between the log affinity of the amiloride analog at the agonist-occupied receptor (log αK_X or log βK_Y) and the log affinity at the unoccupied receptor (log K_X, described in the second paragraph under *Results*). Values are means ± S.E. of three experiments.

Amiloride *Y*	k_{-1}	HMA (Amiloride *X*)				Amiloride *Y*			
		k_{-2}	Fold change	log αK_X	log α_{obs}	k_{-3}	Fold change	log βK_Y	log β_{obs}
	min^{-1}	min^{-1}	k_{-2}/k_{-1}			min^{-1}	k_{-3}/k_{-1}		
	0.043 ± 0.005	0.015 ± 0.002	0.37 ± 0.07	4.24 ± 0.05	−2.50 ± 0.05				
Amiloride	0.036 ± 0.001	0.016 ± 0.001	0.45 ± 0.01	4.28 ± 0.03	−2.46 ± 0.03	0.024 ± 0.002	0.67 ± 0.06	2.39 ± 0.05	−2.21 ± 0.05
DMA	0.038 ± 0.001	0.015 ± 0.001	0.41 ± 0.01	4.20 ± 0.03	−2.54 ± 0.03	0.029 ± 0.002	0.77 ± 0.02	3.39 ± 0.12	−2.10 ± 0.12

gave over a 5-fold stimulation over basal of [^{35}S]GTPγS binding to membranes containing the α_{2A}-adrenergic receptor. There was no evidence for constitutive activity; addition of the antagonist phentolamine (1 μM) in the absence of agonists did not cause a decrease in basal level of [^{35}S]GTPγS binding (data not shown).

The effect of a range of α_2-adrenergic receptor agonists on the stimulation of [^{35}S]GTPγS binding to CHO membranes containing the α_{2A}-adrenergic receptor was examined. Norepinephrine, epinephrine, and UK14304 were found to be full agonists, giving over a 5-fold stimulation over basal (Table 2), with the order of potency UK14304 > epinephrine > norepinephrine. Guanabenz, *p*-aminoclonidine, and clonidine were found to be partial agonists, giving approximately half-maximal stimulation (Table 2). Similar results were reported by Jasper et al. (1998) in studies with the human α_{2A}-adrenergic

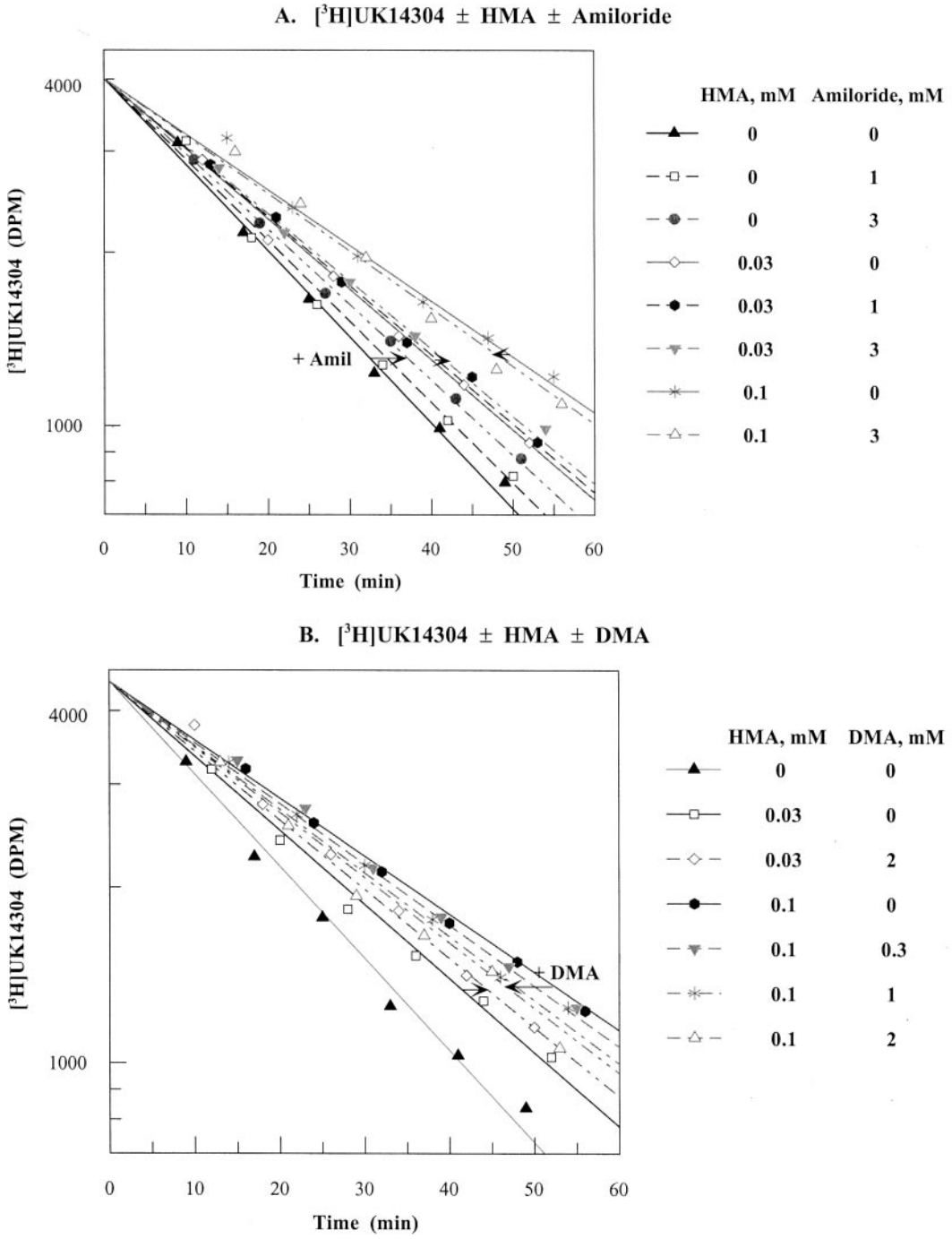

Fig. 4. Effect of either amiloride (A) or DMA (B) on the modulation by HMA of [^{3}H]UK14304 dissociation at 30°C. The experiments were performed as described in the legend to Fig. 3. Individual data points from one experiment are shown per graph. The lines represent the simultaneous fit of the data to the equation derived from the allosteric model that allows for competition between the allosteric agents at the allosteric site (Fig. 1) (eq. 9, Leppik et al., 1998). In the fitting, time, HMA concentration and either amiloride or DMA concentration were independent variables. The arrows show the direction of the effect of amiloride (A) or DMA (B) on the modulation by HMA of the [^{3}H]UK14304 dissociation. The parameters derived from the fits are summarized in Table 1.

TABLE 2

Stimulation by agonists of [^{35}S]GTPγS binding to membranes containing the α_{2A}-adrenergic receptor

Membranes were incubated at 30°C for 60 min with GDP, [^{35}S]GTPγS, sodium metabisulfite (epinephrine and norepinephrine only), and an increasing concentration of agonist in duplicate. The data from each experiment were fitted with a four-parameter logistic equation, to give the log EC_{50}, E_{max}, and basal and slopes for each dose-response curve. Values are means ± S.E. of n experiments.

Agonist	n	log EC_{50}	Stimulation	Slope
			E_{max}/basal	
Guanabenz	2	−7.83 ± 0.08	2.52 ± 0.08	−0.67 ± 0.07
UK14304	4	−7.77 ± 0.02	5.54 ± 0.25	−0.66 ± 0.02
p-Aminoclonidine	3	−7.64 ± 0.02	2.76 ± 0.16	−0.81 ± 0.04
Clonidine	2	−7.52 ± 0.01	2.98 ± 0.35	−0.78 ± 0.03
Epinephrine	3	−6.75 ± 0.12	5.49 ± 0.23	−0.62 ± 0.03
Norepinephrine	6	−6.19 ± 0.06	5.19 ± 0.10	−0.64 ± 0.02

receptor expressed in an HEK 293 cell line. Oxymetazoline and L(−)-norephedrine were also tested and were found to be even less efficacious agonists, giving less than a 2-fold stimulation over basal (data not shown). *p*-Aminoclonidine caused a biphasic stimulation, the level of [^{35}S]GTPγS binding reaching a plateau 2.8 times basal at 10 μM and then showing a second rise at higher *p*-aminoclonidine concentrations. This second rise was not blocked by the antagonist yohimbine (1 μM). The *p*-amino grouping is critical for this second phase, because this second phase is not shown by clonidine. Despite the yohimbine result, the *p*-aminoclonidine still could be acting via the α_{2A}-adrenergic receptor, because CHO membranes lacking the α_{2A}-adrenergic receptor did not display either phase. This phenomenon was not explored further in the current study.

Effect of Amiloride Analogs on the Stimulation of [^{35}S]GTPγS Binding by Agonists. All six of the amiloride analogs tested (Fig. 2) caused a concentration-dependent decrease in the potency of norepinephrine to stimulate [^{35}S]GTPγS binding to CHO membranes containing the α_{2A}-adrenergic receptor (Fig. 5; not shown). However, above a given concentration, all of the amilorides also caused a decrease in the maximal level of norepinephrine-induced [^{35}S]GTPγS binding, and in some cases there was also a variation in the level of basal binding at these higher concentrations. Thus, the equation derived by Lazareno and Birdsall (eq. 37, 1995) from the allosteric model could only be applied to data derived using amiloride analog concentrations below those concentrations that caused changes in maximal and/or basal levels of [^{35}S]GTPγS binding. With amiloride and DMA, a decrease in the maximal stimulation of [^{35}S]GTPγS binding by norepinephrine, epinephrine, or UK14304 was only found at 3 mM amiloride analog concentration. The data obtained with concentrations of amiloride or DMA up to 1 mM were well fitted by the equation from the allosteric model (Lazareno and Birdsall, 1995), for their effect on either norepinephrine-, epinephrine-, or UK14304-stimulated [^{35}S]GTPγS binding (Fig. 5 and Table 3; not shown). The data were significantly ($P < .05$) better fitted by the equation derived from the allosteric model (eq. 37, Lazareno and Birdsall, 1995) than by the equation for the competitive model (eq. 37, $b = 0$, Lazareno and Birdsall, 1995). The analyses by the allosteric model give separate estimates of both the log affinity of the amilorides at the unoccupied receptor (log K_X) and the log cooperativity between the amilorides and the agonists (log α) (Table 3), unlike a dissociation assay that gives only estimates of a single combined parameter, the log affinity of the allosteric ligand at the radioligand-occupied receptor (log αK_X).

In the case of HMA, decreases in the maximal stimulation of [^{35}S]GTPγS binding by norepinephrine, epinephrine, or UK14304 were found with 0.1 mM HMA. The data obtained with HMA concentrations up to 0.03 mM were well fitted (Fig. 5 and Table 3; not shown) by the equation from the allosteric model. With the other amilorides examined, BZA, MBA, and EPA, there was a decrease in the maximal level of norepinephrine-stimulated [^{35}S]GTPγS binding with 0.1, 0.1, and 1 mM amiloride analog, respectively, together with a decrease (BZA and EPA) or an increase (MBA) in basal binding at these amiloride analog concentrations. Below these concentration limits, it was found that the concentration ranges over which these amilorides were causing a decrease in agonist potency were too restricted to enable the data to be fitted other than by an equation derived from a competitive model. The only parameter estimates that could be derived for these three amilorides from the [^{35}S]GTPγS assay were their log affinities at the unoccupied receptor, and these estimates are within 0.2 log unit of the corresponding corrected affinity values estimated from competition studies versus [^{3}H]UK14304 and [^{3}H]yohimbine (Leppik et al., 1998a; data not shown). This competitive-like behavior is compatible with allosterism. The allosteric ternary complex model (Fig. 1), when there is negative cooperativity, behaves as a competitive model until sufficiently high concentrations of X and L are present to enable a significant level of the ternary complex, $X.R.L$, to be formed.

Discussion

Previous studies have shown that the α_{2A}-adrenergic receptor possesses a well defined allosteric site, at which amilorides can act to modulate antagonist binding at the primary binding site (Leppik et al., 1998a). This differs from the situation found for the α_{1A}-adrenergic receptor, for which the data are compatible with two, but not one, allosteric sites at which amilorides can bind and modulate antagonist binding (Leppik et al., 2000). In this paper, we quantitate the behavior of amilorides, acting via the allosteric site on the α_{2A}-adrenergic receptor, to modulate agonist binding and function.

Initially, competition studies were carried out between the potent, α_2-selective agonist [^{3}H]UK14304 and the amilorides at the α_{2A}-adrenergic receptor. The affinities calculated for the amilorides at the unoccupied receptor are in good agreement with those previously determined from competition studies versus the antagonist [^{3}H]yohimbine (Leppik et al., 1998a). This argues that the amilorides do not have additional potent effects on G proteins that disrupt receptor-G

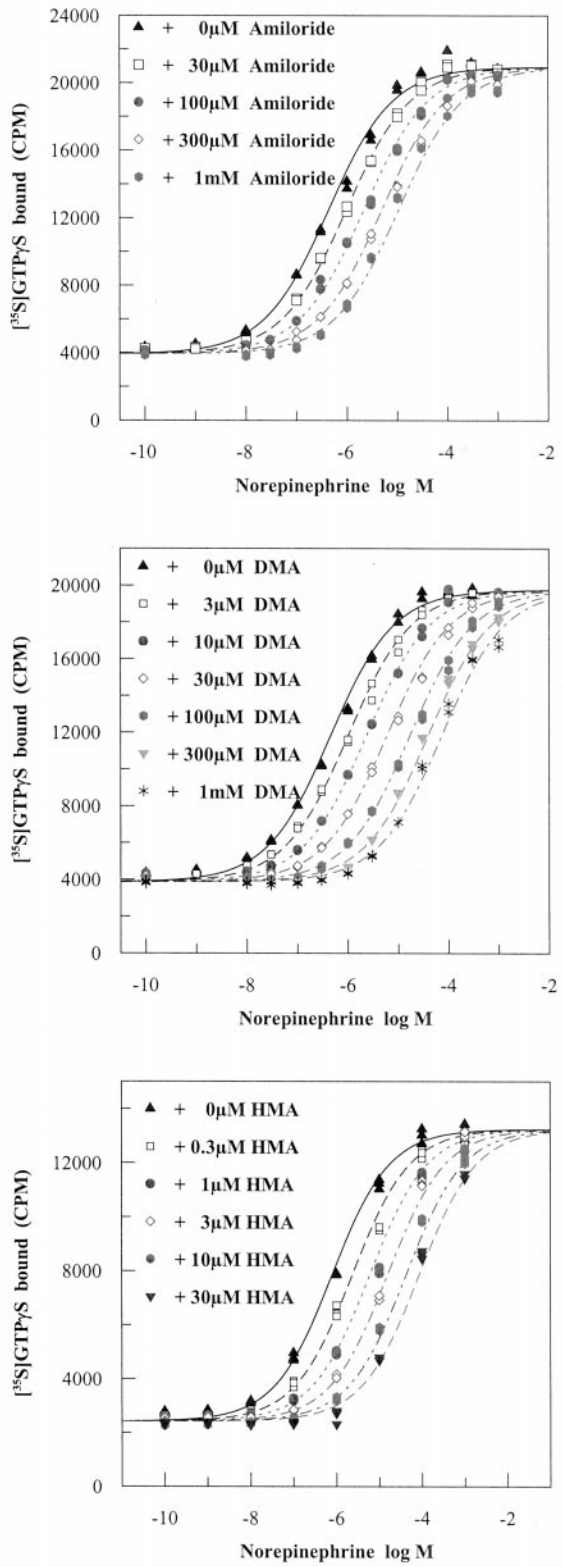

Fig. 5. Effect of amiloride analogs on the stimulation by norepinephrine of [^{35}S]GTPγS binding to membranes containing the α_{2A}-adrenergic receptor. In each set of 24 tubes, membranes were incubated at 30°C for 60 min with GDP, [^{35}S]GTPγS, sodium metabisulfite, a given concentration of amiloride analog, and an increasing concentration of norepinephrine in duplicate. Individual data points from one experiment are shown per graph. The data from each experiment were simultaneously fitted to the equation from the allosteric model (eq. 37, Lazareno and Birdsall, 1995), with time and amiloride analog concentration as independent variables. The results are summarized in Table 3.

protein interactions. These data are compatible with either competition between [^{3}H]UK14304 and the amilorides at the agonist binding site or negative allosteric modulation ($\alpha <$ 0.05) by the amilorides of agonist binding via an allosteric site.

In kinetic studies, the amilorides examined caused modest concentration-dependent decreases in the dissociation rate of the [^{3}H]UK14304 from the α_{2A}-adrenergic receptor. The decreases were so slight that only HMA showed a large enough effect to enable the dissociation data to be fitted by the equation derived from the ternary complex allosteric model (Fig. 3). For the other amilorides, the parameters defining the allosteric interaction could only be derived by analyzing the effect on the [^{3}H]UK14304 dissociation rate of the competition between each amiloride and HMA at the allosteric site. This was done for both amiloride and DMA, the two other amilorides successfully studied in the [^{35}S]GTPγS assay, and the data obtained could be well fitted (Fig. 4) by the relevant equation (Leppik et al., 1998a). The reversal of the kinetic effects of HMA by amiloride and DMA, in a manner compatible with the allosteric model, argues against their interactions being nonspecific in nature.

The [^{3}H]UK14304 dissociation rates from the amiloride-, DMA-, or HMA-occupied receptors were estimated to be 1.5-, 1.3-, or 2.7-fold slower, respectively, than from the unoccupied receptor (Table 1). These slight decreases are in contrast to the larger increases caused by the amiloride analogs (especially HMA) on antagonist dissociation rates (Leppik et al., 1998a). For example, the [^{3}H]yohimbine dissociation rates from the amiloride-, DMA-, or HMA-occupied receptors were 2-, 5-, and 140-fold faster, respectively, than that from the unoccupied receptor (Leppik et al., 1998a).

From the analysis of the kinetic data, the calculated affinities of the amilorides at the [^{3}H]UK14304-occupied receptor were found to correlate with the size of the 5-*N*-alkyl side chain. Amiloride, with only a 5-amino function (Fig. 2), has a log affinity of 2.38 ($1/\alpha K_X$: 4 mM), whereas DMA, which has a 5-dimethylamino function, has a log affinity of 3.39 ($1/\alpha K_X$: 400 μM), and HMA, with a hexamethylene ring on the 5-amino function, has a log affinity of 4.24 ($1/\alpha K_X$: 60 μM; Table 1). These values parallel the rank order of affinities of the amilorides for the unoccupied α_{2A}-adrenergic receptor but are ~100-fold weaker. The differences between the log affinities at the [^{3}H]UK14304-occupied and unoccupied receptors, the log cooperativities, range from −2.2 for amiloride to −2.5 for HMA (Table 1).

The values in Table 1 contrast with those found for the amilorides at antagonist-occupied α_{2A}-adrenergic receptors (Leppik et al., 1998a). There, not only do the log affinities not correlate with the size of the 5-*N*-alkyl side chain but they also are found in a narrow range (2.1–2.5), whereas the observed log cooperativities vary considerably (−2.4 to −4.2). In the current study, it is the log affinities at the [^{3}H]UK14304-occupied receptor that vary (2.4–4.2), whereas the log cooperativities are approximately constant.

The kinetic data suggest that the apparent association rate constants of [^{3}H]UK14304 for the receptor occupied by one of the amilorides ($\alpha.K_L.[k_{-2}$ or $k_{-3}]$) varies only over a narrow range, and is ~500-fold slower than the association rate constant of [^{3}H]UK14304 for the unliganded receptor ($K_L.k_{-1}$); i.e., ($\alpha.[k_{-2}$ or $k_{-3}])/k_{-1}$ = 0.0012 to 0.0061 (Table 1). The magnitude of the decrease of the association rate

TABLE 3

Amiloride analog modulation of the stimulation by agonists of [^{35}S]GTPγS binding to membranes containing the α_{2A}-adrenergic receptor

In each set of 24 tubes, membranes were incubated at 30°C for 60 min with GDP, [^{35}S]GTPγS, sodium metabisulfite (norepinephrine only), a given concentration of amiloride analog, and an increasing concentration of agonist in duplicate. The data from each experiment were simultaneously fitted by the equation derived in a previous study (eq. 37, Lazareno and Birdsall, 1995), with agonist and amiloride concentrations as independent variables, to give the log EC_{50}, log K_X, and log α. The log EC_{50} is the log concentration of agonist needed to give half-maximal stimulation of [^{35}S]GTPγS binding in the absence of amiloride analog, log K_X and log αK_X (the sum of the estimated values of log K_X and log α) are the affinities of the amiloride analogs at the unoccupied and agonist-occupied receptor, respectively, and log α is the cooperativity factor for the interaction between the agonist and the amiloride analog. Values are means ± S.E. of n experiments.

Agonist	Amiloride Analog	n	log EC_{50}	log K_X	log α	log αK_X	Slope
Norepinephrine	Amiloride	4	−6.24 ± 0.07	4.69 ± 0.03	−1.80 ± 0.14	2.90 ± 0.15	0.61 ± 0.03
	DMA	4	−6.17 ± 0.06	5.61 ± 0.04	−2.31 ± 0.07	3.30 ± 0.08	0.65 ± 0.01
	HMA	3	−6.30 ± 0.15	6.87 ± 0.06	−2.61 ± 0.23	4.26 ± 0.17	0.66 ± 0.01
UK14304	Amiloride	3	−7.88 ± 0.03	4.72 ± 0.05	−1.71 ± 0.16	3.01 ± 0.15	0.73 ± 0.01
	DMA	3	−7.96 ± 0.06	5.85 ± 0.04	−2.67 ± 0.27	3.19 ± 0.25	0.68 ± 0.02
	HMA	3	−8.06 ± 0.03	7.03 ± 0.01	−2.66 ± 0.04	4.37 ± 0.05	0.69 ± 0.03

constant is comparable to that found in the antagonist-amiloride analog kinetic studies [(α.[k_{-2} or k_{-3}])/k_{-1} = 0.0030–0.0088 (Leppik et al., 1998a)] and implies that the amilorides examined have similar slowing effects on the association rates of agonists or antagonists but very different effects on the dissociation of either agonists or antagonists from the ternary complex.

The [^{35}S]GTPγS functional assay was chosen because it is a direct measure of the G protein activation caused by receptor-agonist interactions and is performed under the same assay conditions as used in the ligand binding studies reported above. The assay has proved its worth in functional allosteric studies with muscarinic receptors (Lazareno and Birdsall, 1995; Birdsall et al., 1999), and it has also been used recently in studies of agonist activation of recombinant α_2-adrenergic receptor subtypes (Jasper et al., 1998; Peltonen et al., 1998). In the current study, using membranes expressing a high level of the α_{2A}-receptor, norepinephrine, epinephrine, and UK14304 were found to be full agonists, whereas guanabenz, *p*-aminoclonidine, and clonidine are partial agonists (Table 2). For all agonists, the slope factors were less than 1, reflecting a complex situation, possibly the interaction of the α_{2A}-adrenergic receptor with more than one G protein.

When the effects of the amilorides on agonist function were explored, all of the amiloride analogs tested were found to cause concentration-dependent decreases in the potencies of either norepinephrine, epinephrine, or UK14304 to stimulate [^{35}S]GTPγS binding. However, because high concentrations of the amilorides were found to have deleterious effects in the assay with any of the agonists, only the data obtained with amiloride, DMA, or HMA were amenable to analysis by the equation derived from the allosteric model (Lazareno and Birdsall, 1995). For these three amilorides, good fits of the data were obtained (Fig. 5 and Table 3). As predicted by the ternary complex allosteric model, the log affinities of the amilorides at the unoccupied receptor (Table 3) are in good agreement with the values obtained from competition equilibrium experiments versus [^{3}H]UK14304 or ^{3}H-antagonists (Leppik et al., 1998a), whereas the log affinity values at the UK14304-occupied receptor and the cooperativities are in reasonably good agreement with the values obtained from the kinetic studies (Table 1). This suggests that the magnitude of the heterotropic cooperativity between agonist and an amiloride is independent of receptor-G protein coupling, because the functional assays are carried out in the presence of 1 μM GDP, whereas the [^{3}H]UK14304 kinetic studies are carried out at the high-affinity [^{3}H]UK14304-receptor-G protein complex and in the absence of added guanyl nucleotides.

It is of interest to note that, with the [^{35}S]GTPγS assay, the affinities of each particular amiloride at either the UK14304-occupied or norepinephrine-occupied receptors are essentially the same. A similar situation was found with three different antagonists, for which the affinities of the amilorides at the antagonist-occupied receptors were only slightly affected by which antagonist was used (Leppik et al., 1998a). It thus suggests that the binding mode of the amilorides at the agonist-occupied and antagonist-occupied α_{2A}-adrenergic receptor are very different, and that the actual structures of the agonists or antagonists examined have only a marginal effect on that interaction.

The modulation of [^{3}H]UK14304 dissociation by the amilorides is only compatible with the allosteric model (Fig. 1), and the data from the [^{35}S]GTPγS experiments were significantly better fitted by the equation derived from the allosteric model (eq. 37, Lazareno and Birdsall, 1995) than that from a competitive model (eq. 37, b = 0, Lazareno and Birdsall, 1995). The agreement in the values of the parameters derived from the two approaches argues that the amilorides are acting via the one allosteric site to modulate both agonist dissociation and function.

The results cannot exclude the possibility that the amilorides can compete for the agonist binding site at low concentrations and bind at the allosteric site only at much higher concentrations. This is a problem intrinsic to cooperative interactions. The model (Fig. 1) only specifies that a binary complex *XR* is formed and does not specify to which site *X* is binding. Equally the formation of *XRL* only specifies that the two ligands bind simultaneously and does not say to which site *X* or *L* are binding. Any model with *X* capable of binding either to the competitive or allosteric site is formally indistinguishable from the simple allosteric model in equilibrium or kinetic studies. To determine whether the amilorides can bind to the agonist binding site would require alternative approaches.

It has previously been demonstrated that allosteric agents can modulate agonist binding and function at muscarinic receptor subtypes. Thus, gallamine was found to exhibit ~300-fold negative cooperativity on both the function and affinity of acetylcholine at M_2 muscarinic receptors (Stockton et al., 1983; Lazareno and Birdsall, 1995). Recently, compounds related to brucine have been shown to exhibit positive, neutral, or negative cooperativity on acetylcholine binding and function, depending on which brucine analog and which muscarinic receptor subtype were being studied (Birdsall et al., 1997, 1999; Lazareno et al., 1998). It has therefore been possible to discover allosteric enhancers even when the

original allosteric ligands exhibited strong negative cooperativity. The finding of only 60-fold negative cooperativity between amiloride and the endogenous ligand norepinephrine at the α_{2A}-adrenergic receptor means that it is possible that allosteric enhancers at this receptor also await discovery.

It has been demonstrated recently that the benzodiazepines, lorazepam and midazolam, can act as weak agonists of very low intrinsic activity at the three α_1-adrenergic receptor subtypes and that they increase the maximal inositol phosphate response of phenylephrine, clonidine, or epinephrine at one or more subtypes (Waugh et al., 1999). These actions are ascribed to an allosteric mechanism. However, the data from the binding and functional studies are not compatible with the predictions of the ternary complex allosteric model (Fig. 1) and suggest that a more complex mechanism is involved. Thus, the work reported here is the first clear demonstration of allosteric modulation of both agonist binding and function at an adrenergic receptor subtype.

Acknowledgments

We are grateful to Professor Robert Lefkowitz for the provision of the CHO cell line expressing the human α_{2A}-adrenergic gene and to Dr. Lazareno for helpful discussions, particularly with regard to the [^{35}S]GTPγS assays.

References

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.

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.

Bruns RF and Fergus JH (1990) Allosteric enhancement of adenosine A_1 receptor binding and function by 2-amino-3-benzoylthiophenes. *Mol Pharmacol* **38:**939–949.

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.

Clark AL and Mitchelson F (1976) The inhibitory effect of gallamine on muscarinic receptors. *Br J Pharmacol* **58:**323–331.

Cohen F, Lazareno S and Birdsall NJM (1994) Allosteric interactions of PD81,723 at the human adenosine A_1 receptor. *Br J Pharmacol* **112:**51P.

Ehlert FJ, Roeske WR and Yamamura HI (1994) Muscarinic receptors and novel strategies for the treatment of age-related brain disorders. *Life Sci* **55:**2135–2145.

Giacobini E (1990) The cholinergic system in Alzheimer disease. *Prog Brain Res* **84:**321–332.

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.

Jasper JR, Lesnick JD, Chang LK, Yamanishi SS, Chang TK, Hsu SAO, Daunt DA, Bonhaus DW and Eglen RM (1998) Ligand efficacy and potency at recombinant α_2 adrenergic receptors: Agonist-mediated [^{35}S]GTPγS binding. *Biochem Pharmacol* **55:**1035–1043.

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, Birdsall B, Fukazawa T, Gharagozloo P, Hashimoto T, Kuwano H, Popham A, Sugimoto M and Birdsall NJM (1999) Allosteric effects of four stereoisomers of a fused indole ring system with ^{3}H-N-methylscopolamine and acetylcholine at M_1-M_4 muscarinic receptors. *Life Sci* **64:**519–526.

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.

Lazareno S, Gharagozloo P, Kuonen D, Popham A and Birdsall NJM (1998) Subtype-selective positive cooperative interactions between brucine analogues and acetylcholine at muscarinic receptors: Radioligand binding studies. *Mol Pharmacol* **53:**573–589.

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, Mynett A and Birdsall NJM (1998b) Agonist function at the human α_{2A} adrenoceptor: Allosteric modulation by amilorides. *Naunyn-Schmiedeberg's Arch Pharmacol* **358:**R118.

Leppik RA, Mynett A, Lazareno S and Birdsall NJM (2000) Allosteric interactions between the antagonist prazosin and amiloride analogs at the human α_{1A}-adrenergic receptor. *Mol Pharmacol* **57:**436–445.

Musser B, Mudumbi RV, Liu J, Olson RD and Vestal RE (1999) Adenosine A_1 receptor-dependent and -independent effects of the allosteric enhancer PD 81,723. *J Pharmacol Exp Ther* **288:**446–454.

Neubig R, Gantzos R and Thomsen W (1988) Mechanism of agonist and antagonist binding to α_2 adrenergic receptors: Evidence for a precoupled receptor-guanine nucleotide protein complex. *Biochemistry* **27:**2374–2384.

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.

Peltonen JM, Pihlavisto M and Scheinin M (1998) Subtype-specific stimulation of [^{35}S]GTPγS binding by recombinant α_2-adrenoceptors. *Eur J Pharmacol* **355:**275–279.

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.

Waugh DJJ, Gaivin RJ, Damron DS, Murray PA and Perez DM (1999) Binding, partial agonism, and potentiation of 1-adrenergic receptor function by benzodiazepines: A potential site of allosteric modulation. *J Pharmacol Exp Ther* **291:**1164–1171.

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