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Allosteric Modulation of A_{2A} Adenosine Receptors by Amiloride Analogues and Sodium Ions

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ABSTRACT. Allosteric regulation of rat A_{2A} adenosine receptors by amiloride, amiloride analogues, and sodium ions was studied by investigating their ability to influence the dissociation of [^{3}H]4-{2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*][1,3,5]triazin-5-yl-amino]ethyl}phenol ([^{3}H]ZM241385) from receptors in rat striatal membranes. Both amiloride and its analogues accelerated the dissociation, the analogues being more potent than amiloride itself. In contrast, sodium ions decreased the rate of [^{3}H]ZM241385 dissociation in a concentration-dependent manner, and this rate was not influenced by guanosine triphosphate, *N*-ethylmaleimide, suramin, or the selective A_{2A} adenosine receptor antagonist, 5-amino-2-(2-furyl)-7(2-phenylethyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (SCH58261). The effect of competition between the amiloride analogue 5-(*N*,*N*-hexamethylene)amiloride (HMA) and sodium ions on [^{3}H]ZM241385 dissociation was also explored. The addition of sodium ions resulted in a concentration-dependent rightward shift of the HMA response curve. The slopes of the HMA concentration–response curves in the presence and absence of sodium ions were not significantly different, which suggests that sodium ions and amiloride analogues act at a common allosteric site on the A_{2A} adenosine receptor. There was a lack of correlation between the displacement of ligand binding and the allosteric potencies of the amiloride analogues. BIOCHEM PHARMACOL **60**;5:669–676, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. A_{2A} adenosine receptor; allosteric modulation; amiloride; sodium ion; [^{3}H]ZM241385

The A_{2A} adenosine receptor is a member of the superfamily of G protein-coupled receptors, which is involved in many important physiological processes such as cardiovascular homeostasis and brain function related to dopaminergic pathways [1]. Agonists and antagonists of A_{2A} adenosine receptors may have important therapeutic applications. A_{2A} adenosine receptor agonists are thought to be useful in the treatment of schizophrenia [2] and Huntington's disease [3]. Parkinson's disease, on the other hand, may be treated with antagonists of A_{2A} adenosine receptors [4].

Several G protein-coupled receptors have been shown to be regulated allosterically. For example, a variety of compounds are allosteric modulators of ligand binding at muscarinic receptors [5–9]. The flexible nature of these interactions, together with the potential for subtype selectivity, makes allosteric sites attractive for therapeutic intervention [10]. The potential usefulness of such a site for drug design is emphasized by the success of the benzodiazepines, which act at an allosteric site on γ-aminobutyric acid (GABA) receptors, enhancing the response to GABA [11].

Although allosteric regulation of muscarinic receptors has been well documented, it has been studied only to a limited extent at other G protein-coupled receptors. Allosteric enhancement of agonist binding at the A_1 adenosine receptor has been demonstrated for a series of 2-amino-3-benzoylthiophene derivatives [12, 13]. Furthermore, ligand binding to α_2-adrenergic and D_2 dopamine receptors is regulated by both sodium ions [14–17] and amiloride [15, 18]. Amiloride interacts with a number of cation-binding proteins [19], suggesting that the regulation of these receptors by amiloride and cations might be associated. We have demonstrated that ligand binding to A_1 adenosine receptors is modulated allosterically by sodium ions, but not by amiloride analogues [20, 21]. Recently, we also found that both amiloride and sodium ions exerted some effects on A_{2A} adenosine receptors [22], but the mechanism of action was unclear.

In this study, the allosteric regulation of A_{2A} adenosine receptors by amiloride analogues (Fig. 1) and sodium ions was addressed more extensively by investigating their ability to influence the dissociation of a newly introduced, commercially available radiolabeled antagonist, [^{3}H]ZM241385,† from receptors in rat striatal membranes.

MATERIALS AND METHODS

Materials

[^{3}H]ZM241385 (17 Ci/mmol) was from Tocris Cookson Ltd. GTP was from Aldrich. Bovine serum albumin was

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† *Abbreviations:* HMA, 5-(*N*,*N*-hexamethylene)amiloride; MIBA, 5-(*N*-methyl-*N*-isobutyl)amiloride; MGCMA, 5-(*N*-methyl-*N*-guanidinocarbonyl-methyl)amiloride; CGS15943, 5-amino-9-chloro-2-(2-furyl)-1,2,4-triazolo[1,5-*c*]quinazoline; ZM241385: 4-{2-[7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-*a*]1,3,5]triazin-5-yl-amino]ethyl}phenol; and BCA, bicinchoninic acid.

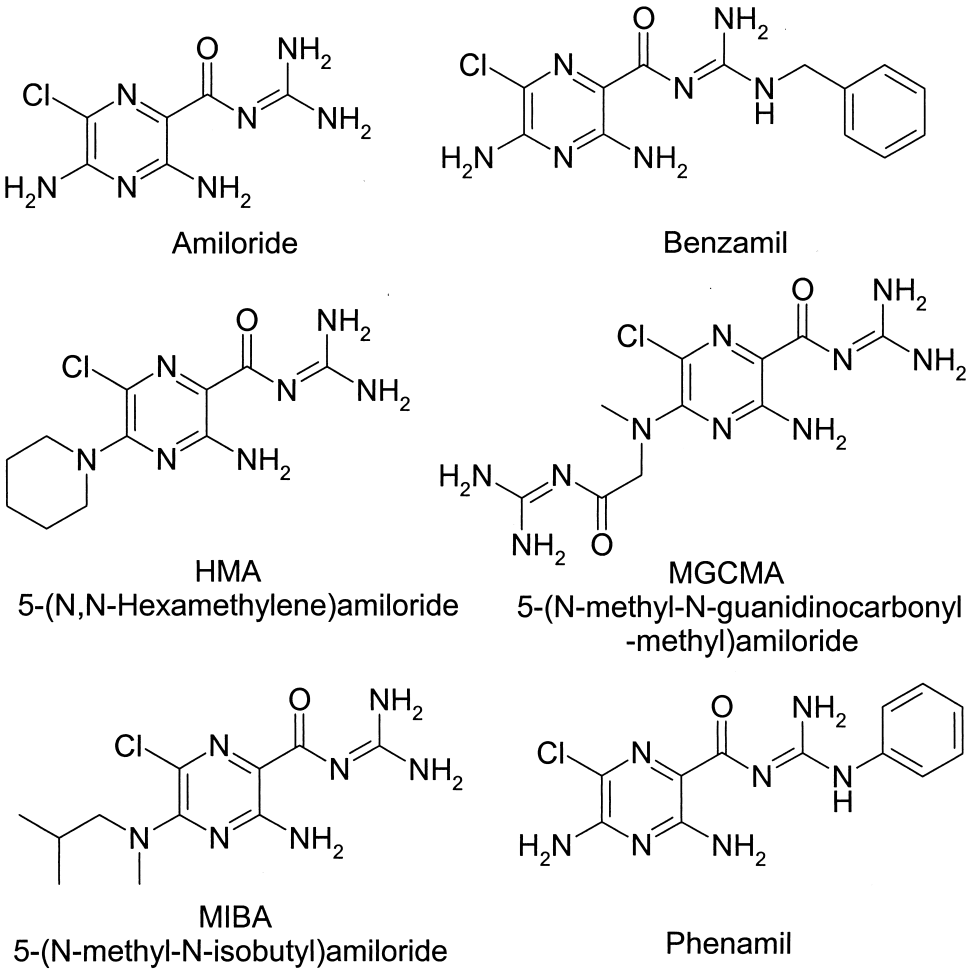

FIG. 1. Chemical structures of amiloride and its analogues examined in this report.

purchased from Sigma. Adenosine deaminase was obtained from Boehringer Mannheim. BCA and BCA protein assay reagent were from Pierce Chemical Co. Amiloride was from Merck. Benzamil, HMA, MIBA, and phenamil were from RBI. CGS15943 was a gift from Ciba-Geigy. All other chemicals were from standard commercial sources and of analytical grade.

Membrane Preparation

Striatal tissue from Wistar rats was homogenized in ice-cold 50 mM Tris–HCl buffer, pH 7.4, using an electric homogenizer. The homogenate was centrifuged at 50,000 *g* for 10 min at 4°, and the pellet was washed in fresh buffer. The final pellet was stored at −80° until the binding experiments. Since adenosine was found to be present in the binding assays with rat striatal membranes, a pretreatment with adenosine deaminase (2 units/mL) was performed. Protein concentrations were measured by the BCA method [23].

Radioligand Binding Assays

For saturation experiments, membranes (60 μg of protein) were incubated with increasing concentrations (0.125–3.0 nM) of radioligand in duplicate, in a final volume of 0.4 mL of Tris–HCl buffer, at 25° for 120 min. Non-specific binding was defined as that retained on the filter and membranes in the presence of 1 μM CGS15943. Where appropriate, amiloride analogues or NaCl were added to analyze their effects on the binding process. Binding reactions were terminated by filtration through Whatman GF/B glass fiber filters under reduced pressure using an MT-24 cell harvester (Brandell). Filters were washed three times with ice-cold buffer and placed in scintillation vials with 5 mL scintillation fluid, and bound radioactivity was determined by using a liquid scintillation counter.

For displacement experiments, membranes (60 μg of protein) were incubated with 1.0 nM of [^{3}H]ZM241385 in duplicate, together with increasing concentrations of the competing compounds, in a final volume of 0.4 mL Tris–HCl buffer at 25° for 120 min.

Allosteric interactions were detected by radioligand dissociation assays. Dissociation of [^{3}H]ZM241385 was measured as follows. Membranes (60 μg) were preincubated with 1.0 nM [^{3}H]ZM241385 in a total volume of 0.4 mL Tris–HCl buffer for 120 min. The dissociation was then initiated by the addition of 1 μM CGS15943 with or without allosteric modulators. Amiloride analogues were dissolved in DMSO, with the final DMSO concentration

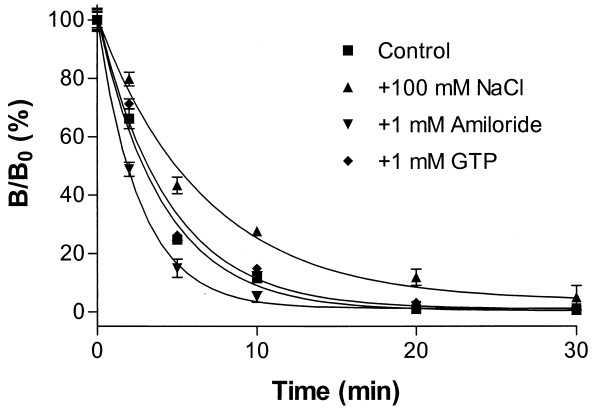

FIG. 2. Dissociation of [^{3}H]ZM241385 at 25° in the absence or presence of various potential allosteric modifiers. Membranes were first pre-equilibrated with [^{3}H]ZM241385; then, the dissociation was started by mixing with CGS15943 (1 μM) and the various test compounds. *Data points*, expressed as the percentage of specific [^{3}H]ZM241385 binding in the absence of CGS15943 (B/B_0), after equilibration; *curves*, computer-generated single exponential best fits derived by non-linear regression analysis of the data points. The data points are from three independent experiments performed in duplicate.

being ≤1.0%, and the appropriate vehicle was added in control experiments. The time–course of dissociation of total binding was measured by rapid filtration at appropriate time intervals. Non-specific binding was measured after a 120-min incubation in the presence of 1 μM CGS15943.

Statistical Analysis

Binding parameters were estimated by GraphPad Prism software (GraphPad) IC_{50} values obtained from competition curves were converted to K_i values by using the Cheng–Prusoff equation [24]. Data were expressed as mean ± standard error for the number of experiments indicated. Data from experiments measuring the effect of HMA on [^{3}H]ZM241385 dissociation, in the presence of sodium ions, were subjected to Schild analysis [25].

RESULTS

Dissociation of [^{3}H]ZM241385 from Rat Striatal Membranes: Effects of Amiloride and Sodium Ions

The kinetics of the dissociation of [^{3}H]ZM241385 from A_{2A} adenosine receptors was determined after the addition of an excess of the competing ligand CGS15943. In the initial experiments, the dissociation of [^{3}H]ZM241385 was followed over a 30-min time–course, and the data obtained under these conditions (Fig. 2) reflected a mean off-rate of 0.24 ± 0.02 min^{-1} (mean ± standard error, N = 3).

Next, the dissociation of [^{3}H]ZM241385 was measured in the presence of 1 mM amiloride or 100 mM NaCl during a 30-min time–course at 25° (Fig. 2). Amiloride increased the dissociation rate of [^{3}H]ZM241385, whereas sodium ions decreased it. By comparison, GTP (1 mM), *N*-ethylmaleimide (1 mM), suramin (1 mM), and the selective A_{2A} receptor antagonist 5-amino-2-(2-furyl)-7(2-phenylethyl)pyrazolo[4,3-*e*]-1,2,4-triazolo[1,5-*c*]pyrimidine (0.1 mM) did not significantly influence the rate of dissociation. The dissociation rates (k_{-1}) in the presence of 100 and 1000 mM NaCl and 1 mM amiloride were 0.15 ± 0.02, 0.11 ± 0.01, and 0.32 ± 0.04 min^{-1}, respectively, significantly different from the k_{-1} value of 0.24 ± 0.02 min^{-1} in the absence of allosteric modulators ($P < 0.05$).

To investigate whether the anion or the cation contributed to the effect of the salt, we performed similar experiments with choline chloride and sodium nitrite. Sodium nitrite behaved like NaCl, whereas 100 mM of choline chloride did not show any effect on [^{3}H]ZM241385 dissociation (data not shown). Thus, sodium rather than chloride ions were responsible for the allosteric effect.

The rate of [^{3}H]ZM241385 dissociation at 25° was very rapid in the presence of amiloride analogues. Specific [^{3}H]ZM241385 binding was almost completely lost within 1 min in the presence of one of the amiloride analogues, HMA (0.3 mM, data not shown). Hence, further experiments with amiloride analogues were performed at 4°. To further study the allosteric effects of sodium ions, we kept the incubation temperature at 25°.

Concentration Dependence of the Allosteric Effects of Amiloride and Amiloride Analogues

Figure 3 demonstrates the influence of increasing concentrations of amiloride and its analogues on the dissociation of [^{3}H]ZM241385 in the presence of 1 μM CGS15943. Dissociation was allowed to proceed for 120 min before the

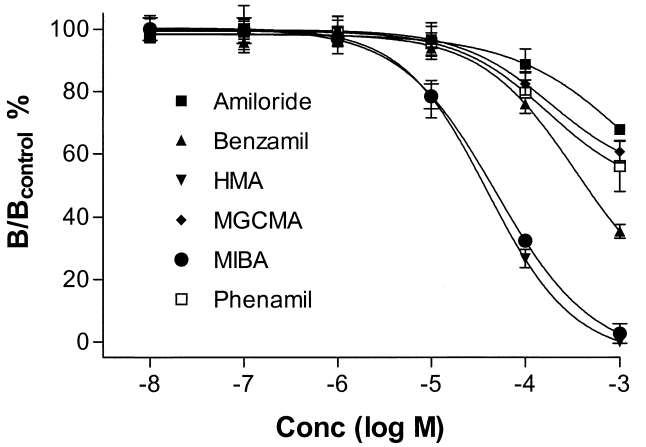

FIG. 3. Concentration dependence of amiloride and its analogues for acceleration of [^{3}H]ZM241385 dissociation from A_{2A} adenosine receptors. Membranes were first pre-equilibrated with [^{3}H]ZM241385, then the dissociation was induced by 1 μM CGS15943 in the absence and presence of increasing concentrations of amiloride and its analogues and the reaction terminated after 120 min. The results are expressed as the ratio of specific [^{3}H]ZM241385 binding in the presence of 1 μM CGS15943 plus various concentrations of amilorides (B) over that in the presence of 1 μM CGS15943 alone ($B_{control}$). The data points are from three independent experiments performed in triplicate.

reaction was terminated by filtration. Low concentrations of amiloride and amiloride analogues (<1 μM) did not modify the dissociation of [^{3}H]ZM241385. However, at higher concentrations (>1 μM), amiloride and its analogues accelerated the dissociation rate in a concentration-dependent manner. The amiloride analogues were more potent in accelerating the dissociation than amiloride itself. The order of their allosteric potencies was HMA > MIBA > benzamil > phenamil ≥ MGCMA ≥ amiloride. It should be noted that EC_{50} values obtained from the curves in Fig. 3 depend on the time point of measurement and do not necessarily reflect concentrations for half-maximum occupancy of the allosteric binding site.

Time–Course of [^{3}H]ZM241385 Dissociation in the Presence of Amiloride Analogues

To determine the affinities of amiloride and amiloride analogues for the antagonist-occupied A_{2A} adenosine receptor, their effects on [^{3}H]ZM241385 dissociation were examined. The experiment was again carried out at 4°. Amiloride increased the dissociation rate in a concentration-dependent manner (Fig. 4A). A more pronounced effect on the [^{3}H]ZM241385 dissociation rate was found for the amiloride analogue HMA (Fig. 4B). As for amiloride, the dissociation curves remained monoexponential in the presence of HMA. HMA (1.0 mM) produced an 11.6 ± 1.7-fold (N = 3) enhancement of the off-rate compared with the 1.21 ± 0.15-fold (N = 3) enhancement by the same concentration of amiloride. The dissociation rate (k_1) in the absence of amiloride was 0.007 ± 0.002 min^{-1}. The k_{-1} values in the presence of 1.0 mM amiloride analogues were 0.008 ± 0.002 (amiloride), 0.017 ± 0.002 (benzamil), 0.080 ± 0.005 (HMA), 0.008 ± 0.001 (MGCMA), 0.04 ± 0.005 (MIBA), and 0.013 ± 0.002 (phenamil) min^{-1}. The rank order of allosteric potencies was the same as that obtained from the concentration–effect curves in Fig. 3.

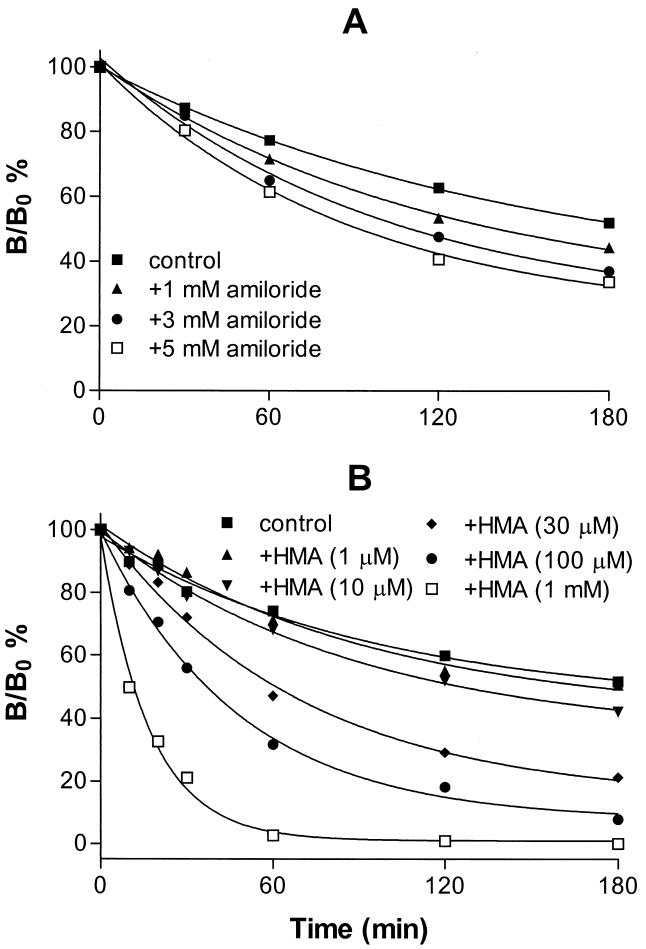

FIG. 4. Dissociation of [^{3}H]ZM241385 at 4° in the absence and presence of various concentrations of amiloride (A) or HMA (B). After the pre-equilibration of [^{3}H]ZM241385 with membranes, the dissociation was started by addition of 1 μM CGS15943 mixed with various concentrations of amiloride and HMA. The data points are from a representative experiment performed in triplicate. The mean k_{-1} values calculated from three independent experiments are listed in the text.

Concentration Dependence of the Allosteric Effects of Sodium Ions

To obtain a measure of the effect of increasing concentrations of sodium ions on the dissociation rates, the amount of [^{3}H]ZM241385 remaining bound after 8 min was determined for different concentrations of sodium ions. In contrast to the effects of amiloride analogues, sodium ions decreased the dissociation rate in a concentration-dependent manner (Fig. 5).

Effect of Sodium Ions on the Acceleration of the [^{3}H]ZM241385 Dissociation Rate Produced by HMA

As described above, sodium ions decreased the rate of [^{3}H]ZM241385 dissociation, whereas amiloride analogues accelerated the dissociation. This experiment was performed to investigate whether the two modulators act at a common site to produce these effects. The addition of sodium ions resulted in a concentration-dependent rightward shift of the HMA concentration–effect curve (Fig. 6A). The slopes of the HMA curves in the absence and presence of 10, 50, and 100 mM sodium ions were 1.0 ± 0.18, 1.0 ± 0.15, 0.97 ± 0.11, and 1.0 ± 0.18, respectively, values which were not significantly different ($P > 0.05$). To calculate dose–ratio values, EC_{50} values of HMA in the presence of various concentrations of NaCl were determined. Schild analysis yielded a linear plot (Fig. 6B). The estimated K_B value for NaCl was 48 ± 13 mM. The slope was 1.0 ± 0.1 (mean ± standard error from analysis of pooled dose–ratio data), which suggests that HMA and sodium ions act at a common allosteric site on A_{2A} adenosine receptors. However, the slope of the dose–response curve in the presence of 1 M sodium ions was significantly different from the other curves, indicating that a high concentration of sodium ions does not interact with the amiloride binding site in a purely competitive manner.

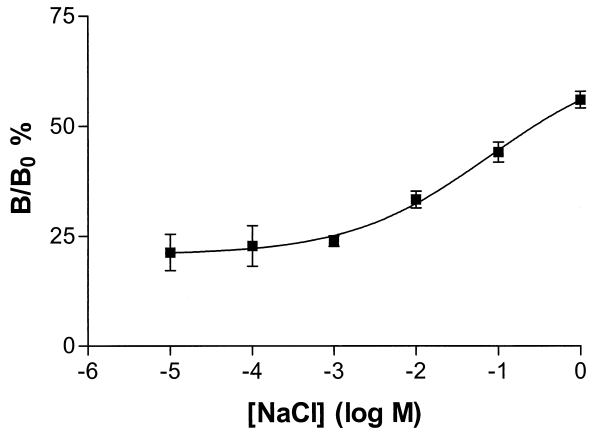

FIG. 5. Concentration–response curve for the slowing of dissociation of [^{3}H]ZM241385 by sodium ions. [^{3}H]ZM241385 (1 nM) was pre-associated with 60 μg of rat striatal membranes for 2 hr at 25° without additions (total binding) or in the presence of 1 μM CGS15943 (non-specific binding). At the end of the preincubation period, 1 μM CGS15943 was added simultaneously with vehicle or various concentrations of NaCl. The incubations were terminated after an additional 8 min. The results are expressed as the ratio of specific [^{3}H]ZM241385 binding at 8 min (*B*) over that at time 0 (B_0), time 0 representing the time when the association of [^{3}H]ZM241385 binding reached equilibrium and CGS15943 was not yet added to dissociate the binding. The data points are from three independent experiments performed in triplicate.

Displacement of Amiloride Analogues for [^{3}H]ZM241385 Binding to A_{2A} Adenosine Receptors from Rat Striatal Membranes

Figure 7 shows that the displacement curve of amiloride for [^{3}H]ZM241385 equilibrium binding to striatal A_{2A} adenosine receptors was steep and adequately described by a one-site model (K_i = 9.7 ± 1.1 μM, N = 3). The pseudo-Hill coefficient (n_H) was 1.0 ± 0.1. The affinity of amiloride for A_{2A} adenosine receptors was greater than that of MGCMA (K_i = 89 ± 13 μM), but lower than that of benzamil (K_i = 2.2 ± 0.3 μM), phenamil (K_i = 2.6 ± 0.4 μM), MIBA (K_i = 3.0 ± 0.2 μM), and HMA (K_i = 3.3 ± 0.5 μM) (N = 3 for each compound). In order to be consistent with the dissociation kinetic experiment, the displacement experiment was repeated at 4°. The K_i values were 10.8 ± 1.0, 1.8 ± 0.2, and 179 ± 42 μM for amiloride, benzamil, and MGCMA, respectively. This result is consistent with that from the displacement experiments at 25°.

Association of [^{3}H]ZM241385 to Rat Striatal Membranes

Association of [^{3}H]ZM241385 was measured in the absence and presence of 100 and 1000 mM NaCl at 25° to determine whether ionic strength affected the binding process (Fig. 8). The association rate constants (k_1) in the absence and the presence of 100 and 1000 mM NaCl were 0.29 ± 0.03, 0.35 ± 0.03, and 0.52 ± 0.05 min^{-1} nM^{-1}, respectively. Hence, NaCl significantly increased the association rate of [^{3}H]ZM241385 ($P < 0.05$).

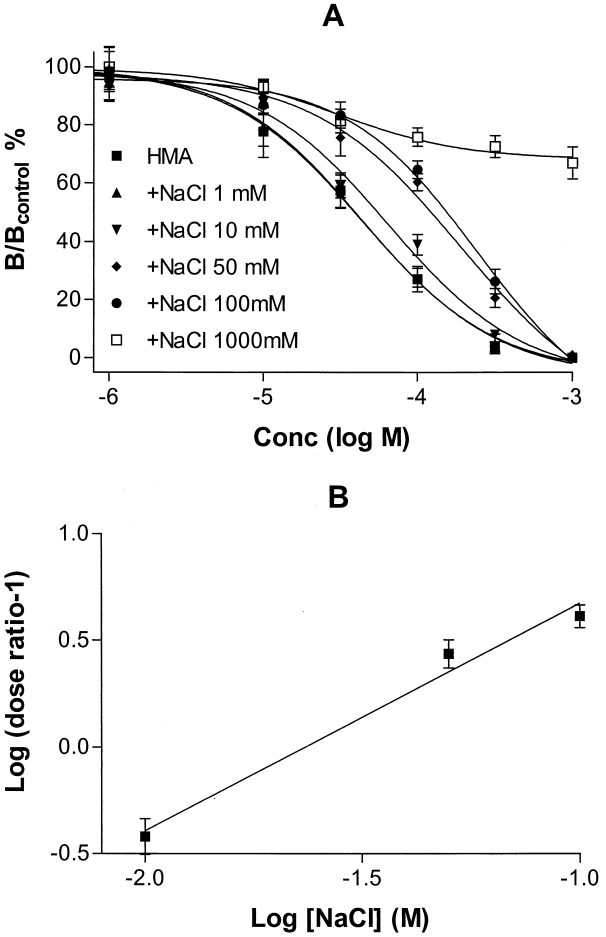

FIG. 6. Acceleration of [^{3}H]ZM241385 dissociation from A_{2A} adenosine receptors produced by HMA in the presence of NaCl. (A) HMA concentration dependence curve for increasing the [^{3}H]ZM241385 dissociation in the presence of a range of concentrations of NaCl. Membranes were first pre-equilibrated with [^{3}H]ZM241385, then the dissociation was started by mixing with CGS15943 (1 μM) and various concentrations of HMA in the absence or presence of different concentrations of NaCl. The incubations were terminated after an additional 2 hr. The results are expressed as the ratio of specific [^{3}H]ZM241385 binding in the presence of 1 μM CGS15943 plus different concentrations of HMA with or without various concentrations of NaCl (*B*) over that in the presence of 1 μM CGS15943 alone ($B_{control}$). The data points are from three experiments performed in triplicate. (B) Schild plot of data from A (10–100 mM NaCl).

Saturation Binding of [^{3}H]ZM241385 to A_{2A} Adenosine Receptors in the Presence of NaCl

Equilibrium [^{3}H]ZM241385 saturation binding experiments were performed in the presence of 100 and 1000 mM NaCl (Fig. 9). Sodium ions increased the affinity of the radioligand. The K_d values of [^{3}H]ZM241385 in the absence and the presence of 100 and 1000 mM sodium ions were 0.47 ± 0.03, 0.36 ± 0.04, and 0.25 ± 0.04 nM, respectively. The corresponding B_{max} values were 703 ± 33, 706 ± 23, and 681 ± 49 fmol/mg protein. The K_d values calculated from the saturation binding experiments are consistent with, although not identical to, those calculated from the kinetic experiments (corresponding K_d values were 0.83, 0.43, and

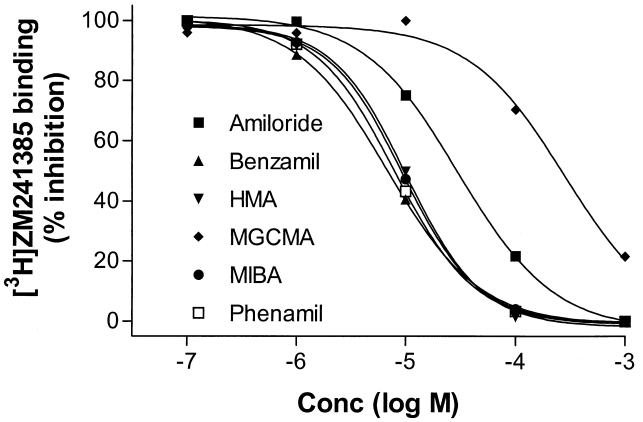

FIG. 7. Competition of amiloride and its analogues with [^{3}H]ZM241385 for binding to rat striatal membranes. Membranes were incubated at 25° for 120 min with [^{3}H]ZM241385 (1 nM) and increasing concentrations of amiloride and amiloride analogues. The data points are from a representative experiment performed in duplicate. The mean K_i values calculated from three independent experiments are listed in the text.

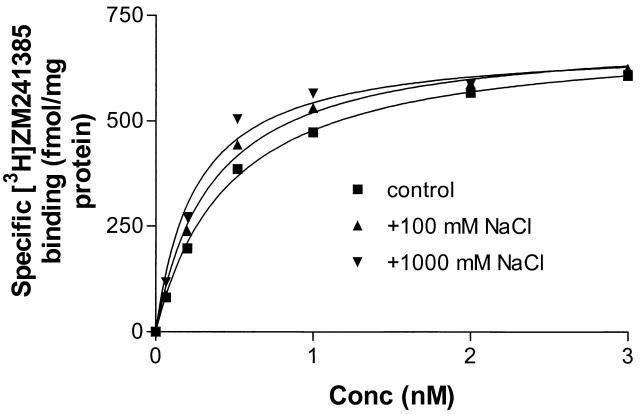

FIG. 9. Saturation binding of [^{3}H]ZM241385 to A_{2A} adenosine receptors in the absence or presence of different concentrations of NaCl. Membranes (60 μg of protein) were incubated with increasing concentrations (0.125–3.0 nM) of radioligand, in a final volume of 0.4 mL of Tris–HCl buffer, at 25° for 120 min. Non-specific binding was defined as that retained on the filter and membranes in the presence of 1 μM CGS15943. The data points are from a representative experiment performed in duplicate. The K_d and B_{max} values listed in the text are from three independent experiments. Binding parameters were obtained from non-linear regression analysis of the saturation curve using a single-site binding isotherm.

0.21 nM). Thus, sodium ions significantly increased the affinity of [^{3}H]ZM241385 binding to A_{2A} adenosine receptors ($P < 0.05$), but did not influence the B_{max} value.

DISCUSSION

In the present study, it was demonstrated that A_{2A} adenosine receptors are modulated allosterically by amiloride analogues and sodium ions. The allosteric nature of the interaction was shown by the increase or decrease in the dissociation rate of the antagonist [^{3}H]ZM241385 from A_{2A} adenosine receptors. Although allosteric modulation is known for other receptors, the allosteric regulation of A_{2A} adenosine receptors has not been reported. The present experiments demonstrated that sodium ions decreased the dissociation of [^{3}H]ZM241385 from A_{2A} adenosine receptors, which is different from their effects on α_2-adrenergic receptors: sodium ions significantly increased the rate of dissociation of the antagonist [^{3}H]yohimbine from α_2-adrenergic receptors [26, 27]. Amiloride affects ligand binding to several G protein-coupled receptors [17, 21]. Moreover, functional antagonism between sodium ions and amiloride analogues has been reported [28, 29]. In this study, it was found that all the amilorides accelerated the dissociation rate of [^{3}H]ZM241385, and that the amiloride analogues were more potent in accelerating the rate of dissociation than amiloride itself.

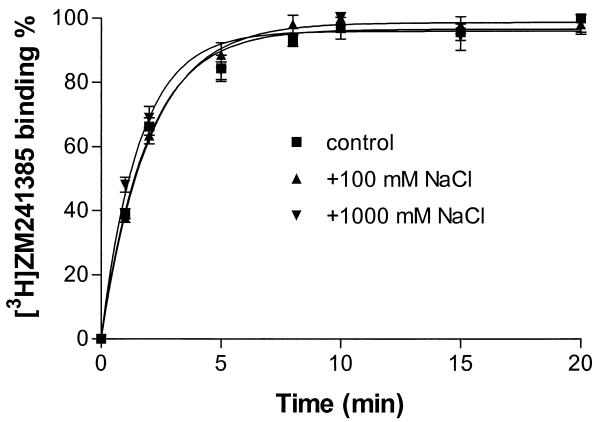

FIG. 8. Association of [^{3}H]ZM241385 (1 nM) to A_{2A} adenosine receptors in the absence or presence of 100 and 1000 mM NaCl. The data points are from three independent experiments performed in triplicate. The k_i values calculated from these experiments are listed in the text.

The effects of the variation in the structures of the amilorides on the [^{3}H]ZM241385 dissociation rate is striking. HMA, MGCMA, and MIBA differ from amiloride in the structure of their 5-amino substituent. Amiloride (1 mM) itself caused only a 1.2-fold increase in the dissociation rate, and the presence of the *N*-methyl-*N*-guanidinocarbonyl-methyl group in MGCMA (1 mM) increased the dissociation rate only a further 1.2-fold. However, the more lipophilic analogues MIBA and HMA (1 mM) caused a 10-fold increase in the effects caused by amiloride. The presence of a benzyl or phenyl moiety on the guanidino group as in benzamil or phenamil (1 mM) only slightly increased dissociation.

The magnitude of the effects on the [^{3}H]ZM241385 dissociation rate was not correlated with the affinity of the amiloride analogues in displacing [^{3}H]ZM241385 binding. Thus, HMA and phenamil, which had similar affinities in a displacement experiment, had the highest and lowest effects on the [^{3}H]ZM241385 dissociation rate, respectively. In a competition binding experiment, MGCMA was less potent than amiloride, but it proved more potent than amiloride in accelerating the rate of dissociation of

[^{3}H]ZM241385. The results show that there are distinct structural requirements for allosteric modulation of A_{2A} adenosine receptor binding and that these requirements differ from those for competitive A_{2A} antagonist activity.

In contrast to the modulation of Na^+/H^+ exchange, the terminal guanidino-substituted derivative benzamil accelerated the dissociation rate with a higher potency than the parent compound [30]. This suggests that allosteric regulation of A_{2A} adenosine receptors is not a reflection of the ability of the receptor to modulate Na^+/H^+ exchange. Similar findings have been reported for amiloride analogues at the D_2 dopamine [31] and the α_2-adrenergic receptor [17].

It has been demonstrated that amiloride interacts with α_1-, α_2-, and β-adrenergic receptors. Only α_2-adrenergic receptors, but not α_1- or β-adrenergic receptors, have an allosteric site to which amiloride binds [32]. Interaction of amiloride and its analogues with A_1 adenosine receptors has also been reported [33, 34]. Amiloride displaced ligand binding to A_1 adenosine receptors with a K_i value in the low micromolar range. The order of potencies of amiloride analogues to displace a radiolabeled antagonist, [^{3}H]DPCPX (8-cyclopentyl-1,3-dipropylxanthine), from A_1 adenosine receptors [33] was similar to that for A_{2A} adenosine receptors, as demonstrated in the present study. In contrast to the effects of amiloride and its analogues on A_{2A} adenosine receptors, the dissociation of [^{3}H]DPCPX from A_1 adenosine receptors was unaltered in the presence of amiloride or its analogues in a concentration exceeding the K_i value 10-fold, which suggests a purely competitive mode of interaction with the A_1 adenosine receptor [21].

It has been proposed that allosteric modulation by hydrophobic compounds could be due to non-specific disruption of the membrane lipid environment [35]. Therefore, it was important to determine whether the compounds used in the current study acted at a specific site. The interaction between amiloride analogues and sodium ions was therefore exploited. Sodium ions both inhibited the effect of HMA and shifted the HMA dose–response curve to the right in a concentration-dependent manner (Fig. 6). This observation cannot be accounted for by a non-specific membrane effect and indeed suggests that the modulators are acting at a specific site on the A_{2A} adenosine receptors. A high concentration of sodium ions (1 M) also shifted the curve to the right, but not in a parallel manner. Apparently, lower concentrations of sodium ions and amiloride analogue act at a common site on A_{2A} adenosine receptors, whereas a higher concentration (1 M) of sodium ions may also act at another site. The effects of sodium ions and amilorides as demonstrated in this study are different from their allosteric effects on the α_2-adrenergic receptor [26], in which sodium ions and amiloride do not act at a common allosteric site.

In summary, we demonstrated that A_{2A} adenosine receptors are regulated allosterically by sodium ions and amiloride analogues in a characteristic, unprecedented way. The allosteric site demonstrated in the present study may provide the basis for some form of modulation at this receptor, and may distinguish it from A_1 adenosine receptors. Eventually, new chemical entities may be discovered that specifically target this site, increasing the options for selective intervention at A_{2A} adenosine receptors.

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