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INTERACTION OF AMILORIDE AND ITS ANALOGUES WITH ADENOSINE A_1 RECEPTORS IN CALF BRAIN

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Abstract—Amiloride, a potassium sparing diuretic, is known to interact with a number of ion transport systems, receptors and enzymes. Here, we report on the interaction between this drug and the adenosine A_1 receptor as present in calf brain membranes. Adenosine A_1 receptors are characterized by a subnanomolar affinity for the antagonist [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]DPCPX) and the agonist [^{3}H]N^6-R-1-phenyl-2-propyladenosine ([^{3}H]PIA). Amiloride displaces both agonist and antagonist binding with a K_i value in the low micromolar range. This inhibition is counteracted by NaCl and protons, in contrast to the binding of [^{3}H]PIA and [^{3}H]DPCPX. The results suggest that amiloride interacts with the adenosine A_1 receptor at a site distinct from the ligand binding site. In order to elucidate the role of one of the ion transport systems known to be inhibited by amiloride, eight amiloride analogues with different sensitivities for these systems were tested. The potency and order of potency of these compounds towards adenosine A_1 receptors excludes the involvement of the epithelial Na^+ channel, Na^+/H^+ exchanger or Na^+/Ca^{2+} exchanger.

Adenosine, a metabolite of ATP, is ubiquitously present in mammalian tissues and has a variety of physiological effects. In general, it appears to represent a negative feedback mediator which serves to restore the balance between metabolic supply and demand. Most of its effects are mediated through specific membrane-bound receptors, which are subdivided into two types, A_1 and A_2, based on their negative and positive coupling to adenylate cyclase [1]. The A_1 receptor has been well characterized by pharmacological and biochemical methods [2] in which radioligand binding methods have played an important role. Signal transduction mechanisms other than inhibition of adenylate cyclase, such as modulation of calcium homeostasis and activation of potassium channels, have been reported for the A_1 receptor. It cannot yet be excluded that still more systems are involved in the mechanism of action of adenosine [3].

Amiloride, a potassium-sparing diuretic, has been used as a tool in the investigation of several Na^+ transport systems [4]. An effect of amiloride in a biological system is often considered as proof for the involvement of one of these transport systems. This drug, a substituted pyrazinoyl guanidine, inhibits the Na^+ transporters with different affinities, ranging from *ca.* 0.1 μM for the epithelial sodium channel to *ca.* 10 μM for Na^+/H^+ exchange and *ca.* 1000 μM for Na^+/Ca^{2+} exchange. Moreover, the selectivity and potency of amiloride analogues bearing substituents either on the 5-amino nitrogen or on a terminal guanidino nitrogen atom vary considerably for each ion transport system [4]. The structure–activity relationships for these derivatives can be used to elucidate which transport system is involved.

Recently, it has become clear that amiloride affects, apart from ion transport systems, a multitude of receptors and enzymes, including the α- and β-adrenergic receptors, the muscarinic receptors, the atrial natriuretic factor receptor, adenylate cyclase and guanine nucleotide-binding proteins (G proteins) [5–10]. It has been postulated that some of the receptors are involved in Na^+/H^+ exchange [6, 9, 11]. This may well hold for the adenosine A_1 receptor as well.

In this paper, we present our findings on the interaction between the selective A_1 receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX‡), the selective A_1 receptor agonist N^6-R-1-phenyl-2-propyladenosine (PIA), and amiloride at the adenosine A_1 receptor. The amiloride–A_1 receptor interaction and its regulation by GTP, NaCl and pH were investigated and compared with the characteristics of classic A_1 receptor ligands.

Furthermore, we investigated the effect of amiloride analogues [4] that are reported to be selective for Na^+/H^+ exchange on [^{3}H]DPCPX and [^{3}H]PIA binding (5-(*N*-butyl-*N*-methyl)amiloride (MBA), 5-(*N*-isobutyl-*N*-methyl)amiloride (MIBA), 5-(*N*,*N*-hexamethylene)amiloride (HMA) and 5[*N*-(guanidinocarbonylmethyl) - *N* - methyl]amiloride (MGCMA). Moreover, the equilibrium inhibition constants at the A_1 receptor of the epithelial sodium channel inhibitors benzamil, phenamil, 3′,4′-dichlorobenzamil (DCB) and the Na^+/Ca^{2+} ex-

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‡ Abbreviations: B_{max}, maximal binding capacity; CBDMB, 5-(*N*-4-chlorobenzyl)-2′,4′-dimethylbenzamil; CholCl, choline chloride; DCB, 3′,4′-dichlorobenzamil; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; HMA, 5-*N*,*N*-hexamethylene)amiloride; K_d, equilibrium dissociation constant; K_i, equilibrium inhibition constant; MBA, 5-(*N*-butyl-*N*-methyl)amiloride; MGCMA, 5-[*N*-(guanidinocarbonylmethyl)-*N*-methyl]amiloride; MIBA, 5-(*N*-isobutyl-*N*-methyl)amiloride; PIA, N^6-R-1-phenyl-2-propyladenosine.

Table 1. Characteristics of specific [^{3}H]DPCPX and [^{3}H]PIA binding to calf brain membranes

Addition	[^{3}H]DPCPX		[^{3}H]PIA	
	K_d ± SE (pM)	B_{max} ± SE (fmol/mg)	K_d ± SE (pM)	B_{max} ± SE (fmol/mg)
None	74 ± 3	643 ± 10	130 ± 4	604 ± 7
NaCl (145 mM)	70 ± 3	811 ± 12	132 ± 8	559 ± 12
CholCl (145 mM)	95 ± 5	804 ± 14	115 ± 8	613 ± 12

Saturation experiments in the presence of various additions were performed in parallel. The data were analysed as described in Materials and Methods.

change inhibitor 5-(*N*-4-chlorobenzyl)-2′,4′-dimethyl-benzamil (CBDMB) were determined. The aim of this structure–activity study was to ascertain whether one of the Na^+ transport systems, presumably Na^+/H^+ exchange, is involved in the interaction between amiloride and adenosine A_1 receptors, as has been suggested or questioned for other receptors.

MATERIALS AND METHODS

Drugs. [^{3}H]DPCPX (sp. act. 95 or 107 Ci/mmol) and [^{3}H]PIA (sp. act. 40 Ci/mmol) were purchased from Amersham (Buckinghamshire, U.K.). GTP was obtained from Aldrich (Brussels, Belgium), unlabelled PIA from Boehringer (Mannheim, F.R.G.). Amiloride was kindly donated by Merck Sharp and Dohme (Haarlem, The Netherlands, USP grade), MBA by Dr G. Schmalzing (Max Planck Institute, Frankfurt, F.R.G.). The other amiloride derivatives were synthesized as described previously [12]. Stock solutions of the drugs were made in DMSO and kept at −20°. Prior to use the compounds were diluted with the assay buffer. The final DMSO concentration never exceeded 1%. This DMSO concentration did not affect radioligand binding. All other chemicals were obtained from standard commercial sources and were of analytical grade. Solutions were made in distilled water.

Measurement of [^{3}H]*DPCPX and* [^{3}H]*PIA binding.* Calf brain membranes were prepared as described by Van Galen *et al.* [13]. Protein concentrations were measured with the bicinchoninic acid method with bovine serum albumin as standard [14]. The experiments were performed in 20 mM HEPES, buffered to pH 7.4 at room temperature with 10 mM Tris (HEP20). The binding assay was initiated by addition of 100 μL membrane suspension (10–15 μg or 30 μg protein for [^{3}H]DPCPX and [^{3}H]PIA, respectively) to 300 μL assay buffer containing test agents and [^{3}H]DPCPX or [^{3}H]PIA (*ca.* 0.12 or 0.3 nM in displacement studies and 0–1.5 or 0–2 nM in saturation studies, respectively).

In some experiments the pH was varied. As preliminary experiments had indicated that monovalent cations, including Tris, affect the potency of amiloride, the cationic composition of the buffer was kept constant. A fixed amount of Tris and varying amounts of HCl were added, finally resulting in a buffer with 20 mM HEPES, 50 mM Tris and 0–45 mM HCl. The pH was verified after the experiment.

After incubation for 60 min ([^{3}H]DPCPX) or 120 min ([^{3}H]PIA) at 25°, the binding reaction was terminated by addition of 1 mL ice-cold HEP20 and rapid vacuum filtration over prewashed Whatman GF/B or double GF/C filters (this gave identical results). Use of one Whatman GF/C filter resulted in considerably lower B_{max} values.

The filters were washed three times with ice-cold 2 mL HEP20 and subsequently dried. The radioactivity retained on the filters was counted in a LKB Rackbeta 1214 liquid scintillation spectrometer. Specific binding was calculated by subtraction of nonspecific binding, determined in the presence of 10 μM PIA ([^{3}H]DPCPX) or 5 μM 8-phenyltheophylline ([^{3}H]PIA), from total binding. Experiments were performed in duplicate for at least three times with similar results.

Data analysis. Data from displacement and saturation curves were analysed as described previously [15] by a computer-program based on the law of mass action [16]. Statistically significant resolution of two components was tested in a partial F-test. Values calculated from 3–9 experiments are provided with their approximated SE. Pseudo Hill coefficients were determined from a Hill-plot with linear regression analysis.

RESULTS

[^{3}H]DPCPX bound to calf brain membranes with a K_d value of 74 ± 3 pM and a maximal binding capacity of 643 ± 10 fmol/mg protein. [^{3}H]PIA bound to calf brain membranes with a K_d value of 130 ± 4 pM and a B_{max} of 604 ± 7 fmol/mg protein (Table 1).

Specific [^{3}H]DPCPX binding was displaced by PIA. Analysis of the data revealed that a two-binding site model fits the data better than a one-site model ($P < 0.01$). As expected for an agonist of a G protein-coupled receptor, the displacement curve was shifted to the right in the presence of 1 mM GTP (Fig. 1, Table 2).

Amiloride inhibited [^{3}H]DPCPX binding with a K_i value of 2.0 ± 0.2 μM and a pseudo-Hill coefficient close to unity (Fig. 2, Table 3). Addition of GTP hardly affected this K_i value (Fig. 2, Table 3). Moreover, a virtually identical K_i value and pseudo-Hill coefficient were observed for displacement of [^{3}H]PIA binding by amiloride (Table 3). These data indicate that amiloride is not an A_1 agonist.

As amiloride displays Na^+/H^+ exchange inhibiting properties in a similar concentration range [17], we

Table 2. Inhibition of [^{3}H]DPCPX binding by PIA under various conditions

Addition	K_H ± SE (nM)	K_L ± SE (nM)	R_H (%)
None	0.17 ± 0.02	6.4 ± 2.1	81
CholCl (145 mM)	0.23 ± 0.04	9 ± 4	79
NaCl (145 mM)	0.15 ± 0.02	6.8 ± 1.9	74
GTP (1 mM)	0.28 ± 0.09	8.5 ± 1.6	40

The K_i values were determined in parallel experiments and analysed as described in Materials and Methods. The inhibition by PIA was significantly better described by a two-binding site model ($P < 0.01$). K_H and K_L are the K_i values for the high- and low-affinity state, respectively. R_H represents the fraction of receptors that are in the high-affinity state.

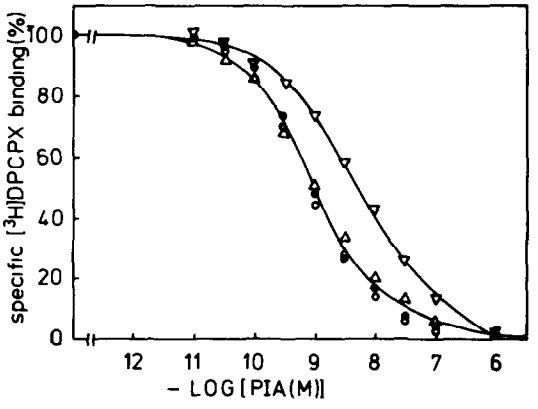


Fig. 1. Displacement of [^{3}H]DPCPX binding by PIA under various conditions: control (●); with 145 mM CholCl (○); with 145 mM NaCl (△); with 1 mM GTP (▽). The data points are from a representative experiment and expressed as percentage of the specific binding in the absence of PIA under the same conditions. The K_i values calculated from three experiments are listed in Table 2.

Table 3. The K_i values for the displacement of specific [^{3}H]DPCPX and [^{3}H]PIA binding by amiloride under various conditions

Addition	K_i ± SE (μM) [^{3}H]DPCPX	[^{3}H]PIA
None	2.0 ± 0.2	2.4 ± 0.1
NaCl (145 mM)	20 ± 1	22 ± 1
CholCl (145 mM)	4.5 ± 0.4	5.2 ± 0.3
GTP (1 mM)	3.0 ± 0.3	ND
Tris/HCl to pH 6.6	9.3 ± 0.7	9.5 ± 0.6
Tris/HCl to pH 7.3	3.4 ± 0.2	4.5 ± 0.2
Tris/HCl to pH 8.4	1.3 ± 0.1	1.7 ± 0.1

The K_i values were determined in parallel experiments and analysed as described in Materials and Methods.
ND, not determined.

investigated the effects of the Na^+ and H^+ concentration on A_1 receptor binding and its inhibition by amiloride. Addition of NaCl (145 mM) or CholCl (145 mM) to the assay enhanced the maximal binding capacity of [^{3}H]DPCPX by 25%. The nonspecific binding was not affected. The affinity of the radioligand was slightly decreased in the presence of CholCl, but unaltered by NaCl (Table 1). In saturation experiments with [^{3}H]PIA as radioligand, no significant effects of 145 mM NaCl or CholCl on the affinity of [^{3}H]PIA were observed, whereas the binding capacity was slightly diminished by NaCl (Table 1). Both NaCl and CholCl lacked effect on the displacement of [^{3}H]DPCPX binding by PIA (Fig. 1, Table 2).

In contrast, addition of 145 mM NaCl resulted in a parallel shift of the curves of inhibition of [^{3}H]DPCPX and [^{3}H]PIA binding by amiloride to the right, corresponding to a 10-fold decrease in the affinity of amiloride for the [^{3}H]DPCPX and [^{3}H]PIA binding sites (Fig. 2, Table 3). The same concentration CholCl gave a 2-fold decrease in affinity. The inhibition curves were virtually identical irrespective of the radioligand that was used. Therefore only the inhibition curves of [^{3}H]DPCPX are shown. A similar pattern was observed when solubilized receptors were used (data not shown).

Next, we examined the influence of the pH on specific [^{3}H]DPCPX binding, [^{3}H]PIA binding, and on their displacement by amiloride. As apparent from Fig. 3, [^{3}H]DPCPX binding was not influenced

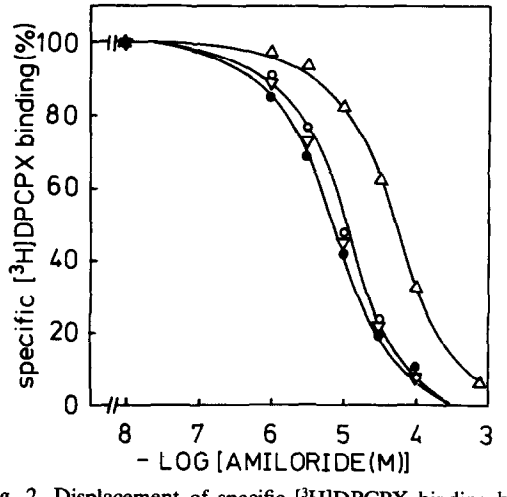


Fig. 2. Displacement of specific [^{3}H]DPCPX binding by amiloride under various conditions: control (●); with 145 mM CholCl (○); with 145 mM NaCl (△); with 1 mM GPT (▽). The data points are from a representative experiment and expressed as percentage of the specific binding in the absence of amiloride under the same conditions. The curves were virtually identical when [^{3}H]PIA was used as radioligand. The K_i values calculated from three experiments are listed in Table 3.

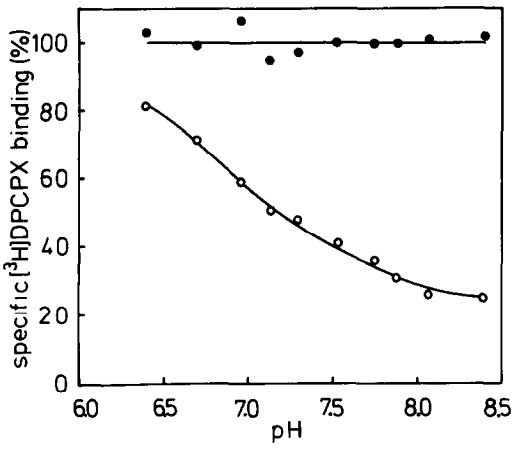


Fig. 3. Effect of pH on the displacement of specific [^{3}H]DPCPX binding by 10 μM amiloride. The data points are from a representative experiment. (●) Control; (○) 10 μM amiloride. The experiment was repeated twice with virtually identical results.

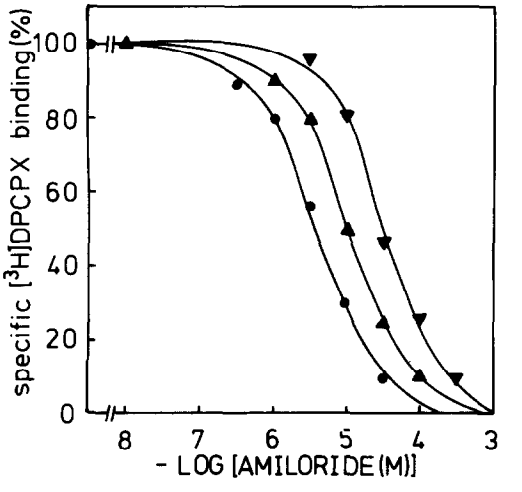


Fig. 4. Displacement of specific [^{3}H]DPCPX binding by amiloride at pH 8.4 (●), 7.3 (▲) and 6.6 (▼). The data points are from a representative experiment and expressed as percentage of the specific binding under the same conditions. The curves were virtually identical when [^{3}H]PIA was used as radioligand. The K_i values calculated from three experiments are listed in Table 3.

by changing the pH from 6.6 to 8.4. [^{3}H]PIA binding was unaltered as well (data not shown). The inhibition by 10 μM amiloride is clearly enhanced from 20% at pH 6.6 to 75% at pH 8.4. Evaluation of this effect over a range of amiloride concentrations showed an increase in K_i value with decreasing pH (Fig. 4, Table 3). A similar phenomenon was observed with the displacement of [^{3}H]PIA by amiloride (Table 3).

In order to verify whether Na^+/H^+ exchange is involved in the inhibitory effect of amiloride on A_1 receptor binding, we tested eight amiloride analogues which are selective for the Na^+/H^+ exchanger, the epithelial Na^+ channel or the Na^+/Ca^{2+} exchanger. The structures and K_i values of these drugs are listed in Table 4. The amiloride analogues tested displaced [^{3}H]DPCPX and [^{3}H]PIA binding with K_i values ranging from 0.07 to 22 μM [^{3}H]DPCPX binding is displaced in the following order of potency: MBA > MIBA > HMA > Benzamil > CBDMB ≥ Phenamil = DCB ≥ Amiloride >> MGCMA. The order of potency for inhibition of [^{3}H]PIA binding differs slightly, due to the relatively small differences in affinity between CBDMB, phenamil, DCB and amiloride. The compounds were almost equally effective in displacing agonist and antagonist binding. It is evident that the lipophilic 5-amino-substituted derivatives are more potent than amiloride. The hydrophilic 5-(*N*-guanidinocarbonylmethyl)-substituted derivative is less potent, whereas the guanidino-substituted analogues are equally effective or slightly more active than amiloride.

DISCUSSION

Characterization of [^{3}H]DPCPX binding in calf brain membranes

Adenosine, A_1 receptors in calf brain membranes are characterized by a subnanomolar affinity for [^{3}H]DPCPX. The affinity of the ligand in our experiments is virtually identical to that reported by Lohse *et al.* [18], although the maximal binding capacity in our preparation is lower.

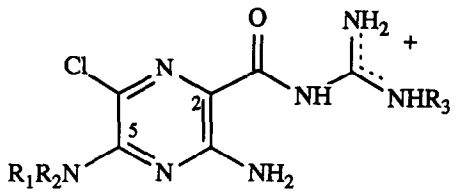
A_1 agonists, such as PIA, differentiate between two binding states. Eighty-one per cent of the receptors in our calf brain preparation is in the high-affinity state in the absence of GTP with a 30-fold difference in affinity between the states. These values are in good agreement with those published by others [18, 19] and confirm the previously observed species differences between rat and calf [20]. The high-affinity state can be largely converted to the low-affinity state by GTP, without the affinity for these states being affected. Complete conversion does not occur even after addition of 1 mM GTP, in agreement with some other reports on this subject [19, 21]. The K_d value of [^{3}H]PIA of 0.130 ± 0.004 nM, as calculated from saturation experiments, is virtually identical to the K_i value of 0.17 ± 0.02 nM of the high-affinity state of the receptor, as calculated from displacement experiments. This implies that [^{3}H]PIA binds to the high-affinity state only in the concentration range used.

The effect of amiloride on adenosine A_1 ligand binding: analogies to Na^+/H^+ exchange

Amiloride, a rather nonspecific Na^+ transport inhibitor, displaces the A_1 receptor agonist [^{3}H]PIA as well as the antagonist [^{3}H]DPCPX from their binding sites in the low micromolar concentration range. The K_i value of the compound of about 2 μM is comparable to that of the well-known adenosine receptor antagonist theophylline [18]. The pseudo-Hill coefficient of the displacement curve, that approximates unity, suggests that amiloride interacts with a single binding site. Furthermore, the virtually identical K_i values of amiloride for inhibition of [^{3}H]PIA binding, [^{3}H]DPCPX binding in the absence

Table 4. K_i values of amiloride derivatives

General structure of the compounds



Na^+/H^+ exchange inhibitors: 5-amino-substituted amiloride derivatives (R_3 = H)

Drug	R_1	R_2	$K_i \pm$ SE (μM) [^{3}H]DPCPX	[^{3}H]PIA
Amiloride	H	H	2.0 ± 0.2	2.4 ± 0.1
MGCMA	CH_2—CO—N=$C(NH_2)_2$	CH_3	22 ± 1	16 ± 1
HMA	—$(CH_2)_6$—		0.41 ± 0.03	0.50 ± 0.03
MBA	$(CH_2)_3CH_3$	CH_3	0.070 ± 0.004	0.092 ± 0.010
MIBA	$CH_2CH(CH_3)_2$	CH_3	0.16 ± 0.01	0.20 ± 0.01

Na^+ channel inhibitors: 2-guanidino-substituted amiloride derivatives (R_1 = R_2 = H)

	R_3		
Benzamil	$C_6H_5CH_2$	0.65 ± 0.04	0.85 ± 0.03
Phenamil	C_6H_5	1.5 ± 0.1	2.3 ± 0.1
DCB	*m,p*-$Cl_2C_6H_3CH_2$	1.6 ± 0.1	2.7 ± 0.2
Na^+/Ca^{2+} inhibitor: CBDMB (R_1 = H)			
R_2 =	*p*-$ClC_6H_4CH_2$	1.2 ± 0.1	4.0 ± 0.4
R_3 =	*o,p*-$(CH_3)_2C_6H_3CH_2$		

Values were calculated from the combined data of 4–8 experiments.

of GTP, and [^{3}H]DPCPX binding in the presence of GTP (when 100, 81 and 40% of the receptors are in the high-affinity state, respectively) imply that amiloride does not differentiate between the high- and low-affinity conformation of the receptor. Therefore, it is very unlikely that amiloride is an A_1 receptor agonist.

The next question is: is amiloride an A_1 antagonist or is a different mechanism of action responsible for the interference with A_1 receptor binding? Besides the effects on the A_1 receptor, amiloride displays Na^+/H^+ exchange inhibiting properties in the low micromolar range. This blockade is antagonized by Na^+ ions and protons [4]. Being interested in the possible involvement of Na^+/H^+ exchange, we investigated the role of Na^+ and H^+.

Physiological concentrations of NaCl attenuate the inhibitory effect of amiloride, its K_i value increases 10-fold in the presence of NaCl. CholCl (145 mM) can only partially mimic this effect, suggesting that, although an increase in ionic strength of the buffer is not without effect, Na^+ counteracts the inhibition by amiloride in a more specific manner. In a solubilized receptor preparation the antagonism between amiloride and NaCl remains intact (data not shown), indicating that the integrity of the membrane environment of the receptor is of minor importance.

In contrast, the equilibrium dissociation constants of [^{3}H]DPCPX and [^{3}H]PIA are not affected by NaCl. NaCl increases the maximal binding capacity of [^{3}H]DPCPX through a less specific mechanism, as CholCl has an identical effect. The maximal binding capacity of [^{3}H]PIA is hardly affected by 145 mM CholCl or NaCl. These results do not clarify the conflicting reports on the effect of NaCl and other salts on adenosine receptor binding [21–24]. However, it is evident that NaCl modulates the A_1 receptor–amiloride interaction in a different manner than the A_1 receptor–adenosine or A_1 receptor–xanthine interaction. This suggests that the binding domains of these compounds at the A_1 receptor are different.

The influence of pH changes on A_1 receptor binding and on its displacement by amiloride supports the idea that amiloride does not interact with the same domain at the receptor as A_1 receptor-selective ligands. The specific binding of A_1 receptor ligands is not altered between pH 6.6 and 8.4, whereas the inhibitory effect of amiloride is markedly attenuated at lower pH values. The effects of amiloride, a weak base with a pK_a of 8.8, are ascribed to the protonated form [4]. As 99.4, 97 and 72% of the drug is protonated at pH 6.6, 7.3 and 8.4, respectively, differences in protonation of the drug cannot account for our results. Even if, contrary to all other effects of amiloride, the unprotonated form would interact with the A_1 receptor, a different ratio between the K_i values at pH 6.6 and 8.4 would be expected.

Our results can readily be explained by the presence of a group on the A_1 receptor with a pK_a in the

Table 5. Affinity of amiloride analogues for Na^+/H^+ exchange, the epithelial sodium channel and Na^+/Ca^{2+} exchange*

Drug	Na^+/H^+ exchange	Na^+ channel	Na^+/Ca^{2+} exchange
Amiloride	84	0.35	1100
MGCMA	1.36	>300	1570
HMA	0.16	>400	100
MBA	0.24	>300	ND
MIBA	0.44	>300	129
Benzamil	>1000	0.38	100
Phenamil	500	0.020	200
DCB	>400	0.85	30
CBDMB	>500	>400	7.3

Structures are presented in Table 4. Data, in μM, are taken from Ref. 4.
ND, not determined.
* The affinities of the drugs were measured in the presence of 140 mM NaCl [27]. In Na^+/H^+ exchange experiments at low external Na^+ concentrations an equilibrium inhibition constant for amiloride of 7 μM has been reported [17].

physiological range (*ca.* 7.6), which is involved in the interaction with amiloride. Such a group was found to be present at the external ion transport site of the Na^+/H^+ exchanger [25] and has also been identified in the binding site of an amiloride derivative on the kidney Na^+/H^+ exchanger [26]. This group seems not to be involved in the binding of the A_1 receptor antagonist DPCPX.

The differential modulation by Na^+ and H^+ indicates that it is unlikely that amiloride is a classic A_1 antagonist.

Effect of amiloride analogues on A_1 receptor binding: structure–activity relationship

It has been postulated that G protein-coupled receptors are linked to Na^+/H^+ exchange [6, 11]. Nunnari *et al.* [9] even suggested that Na^+/H^+ exchange activity may be an intrinsic property of the α_2-adrenoceptor. The above-described results with respect to the A_1 receptor, namely a K_i value of amiloride in the low micromolar range and attenuation of the inhibitory effect by Na^+ and H^+ point to a possible link with Na^+/H^+ exchange. In such a complex amiloride might affect receptor binding indirectly via the Na^+/H^+ exchange system. To test this hypothesis, we investigated the effect of various substituents on the affinity of amiloride analogues for A_1 receptors.

Specific [^{3}H]DPCPX binding is inhibited by amiloride and its derivatives in the following order of potency: MBA > MIBA > HMA > benzamil > CBDMB $\geqslant$ phenamil = DCB $\geqslant$ amiloride >> MGCMA. The absolute values for inhibition of [^{3}H]PIA binding are similar. It is evident that alkylation of the 5-amino group results in a profound increase in the affinity for the A_1 receptor. Hydrophilic substitution at this site on the other hand results in a lower affinity for the A_1 receptor. This is not in agreement with the effects on Na^+/H^+ exchange, where MGCMA is more potent than amiloride (Table 5) [27]. Contrary to the expectations, the analogues having substituents on a terminal guanidino nitrogen atom are equally or more potent than the parent compound. Benzamil is three times more potent than amiloride, whereas phenamil and DCB have similar affinities of about 1.5 μM for the [^{3}H]DPCPX binding site. These compounds have a very low affinity (> 400 μM) for the Na^+/H^+ exchange system and high affinity (0.020–0.085 μM) for the epithelial Na^+ channel (Table 5). The rather selective Na^+/Ca^{2+} exchange inhibitor CBDMB is even more potent in inhibiting [^{3}H]DPCPX binding (1.2 μM) than in inhibiting Na^+/Ca^{2+} exchange (7 μM) [4].

In fact, this structure–activity profile does not resemble those for any of the ion transport systems affected by amiloride (Table 5) [4]. Therefore, the interaction between amiloride derivatives and the A_1 receptor cannot be mediated by any of these, including the Na^+/H^+ exchange system.

Are G_i-coupled receptors linked to Na^+/H^+ exchange?

It has been reported that the α_1-, α_2- and β-adrenergic, as well as the muscarinic receptors are inhibited by amiloride and its analogues [6, 7, 9]. Conceivably, this interference was considered to be related to an interaction with one of the Na^+ transport systems. Limbird and coworkers proposed in several papers [9, 11, 28, 29] that G_i-coupled receptors might be linked to Na^+/H^+ exchange. Even after withdrawal of the supporting functional results [29], the hypothesis was maintained by this group. However, the structure–activity relationships that Howard *et al.* [7] supplied for the α-adrenoceptors, with benzamil being more potent than amiloride, strongly argue against coupling of Na^+/H^+ exchange to α_1- or α_2-adrenoceptors.

The results presented in the present paper exclude a direct link of Na^+/H^+ exchange to the A_1 receptor. Even if stimulation of one of the receptors mentioned would affect the influx of Na^+ or the efflux of protons, this is probably not related to inhibition of receptor binding by amiloride derivatives. Further investigations will have to clarify the functional implications, if any, of the above-described interaction of amiloride with A_1 receptor binding.

Interestingly, Anand-Srivastava [10] reported recently that amiloride attenuates inhibitory hormone responses in rat pituitary membranes. A

most intriguing effect demonstrated was the interaction of amiloride with the G_i protein. Blockade of G_i, however, cannot be an explanation for the displacement of [^{3}H]DPCPX binding by amiloride, which is independent of the coupling of the A_1 receptor to G_i, as indicated by the lack of effect of GTP. Accordingly, the effects of amiloride on the A_1 receptor and on G_i seem to be distinct.

In summary, amiloride interacts with the A_1 receptor in calf brain membranes with an affinity in the low micromolar range. Na^+ and H^+ attenuate the inhibitory effect of amiloride. The target site of the drug seems to be distinct from the specific A_1 ligand binding site. Several analogues of amiloride are more potent than amiloride itself with MBA (K_i value 70 nM) being the most potent. The relationship between the structure of amiloride analogues and their affinity for A_1 receptor differs from the structure–activity relationships for any of the ion transport systems known to be affected by amiloride. We therefore conclude that Na^+/H^+ exchange is not directly involved in the interaction between A_1 receptors and amiloride.

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