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CHEMICAL MODIFICATION OF ADENOSINE A_1 RECEPTORS

IMPLICATIONS FOR THE INTERACTION WITH R-PIA, DPCPX AND AMILORIDE

ANJA GARRITSEN, AD P. IJZERMAN,* MARGOT W. BEUKERS and WILLEM SOUDIJN
Center for Bio-Pharmaceutical Sciences, Division of Medicinal Chemistry, P.O. Box 9502, 2300 RA Leiden, The Netherlands

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Abstract—Amiloride, a potassium sparing diuretic, inhibits the specific binding of [^{3}H]8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and [^{3}H]N^6-R-1-phenyl-2-propyladenosine (PIA) to adenosine A_1 receptors in calf brain. This interaction is different from the agonist–receptor or the antagonist–receptor interaction as Na^+ and H^+ counteract the inhibitory effect of amiloride whereas these ions hardly affect the binding of the classic A_1 receptor ligands. In the present study, the effects of protein modifiers on the equilibrium inhibition constant of amiloride are compared with effects of these reagents on the affinities of DPCPX and PIA. It is demonstrated that the affinities of amiloride and [^{3}H]DPCPX are changed after treatment with a carboxyl-modifying reagent but unaffected by modification of histidyl, arginyl and cystein residues. The maximal binding capacity of [^{3}H]DPCPX is enhanced by sulfhydryl modification, whereas the number of [^{3}H]DPCPX binding sites is reduced by treatment with a histidine-modifying reagent. The histidyl residues of the [^{3}H]DPCPX binding site can be partially protected against modification by 300 μM amiloride, present during treatment of the membranes. An equivalent concentration of 8-phenyltheophylline results in complete protection. The apparent affinity of PIA is altered by modification of histidyl, carboxyl, arginyl and cystein residues. In the latter two cases, uncoupling of the G protein seems to be the major reason for the decrease in affinity of PIA. The results suggest that amiloride is an A_1 antagonist with binding characteristics that differ from the classic A_1 antagonists such as DPCPX.

Adenosine is a universal modulator of physiological processes. Most of its effects are mediated via membrane-bound receptors, which are subdivided into two classes, A_1 and A_2, based on their inhibitory and stimulatory effect on adenylate cyclase, respectively. In general, adenosine serves to restore the balance between energy consumption and supply in the cell [1].

In the accompanying paper [2], we demonstrated that amiloride, a potassium sparing diuretic (Fig. 1A), displaces adenosine A_1 receptor ligands from their binding sites. The inhibitory effect of amiloride occurs in the low micromolar range, is independent of coupling to a G protein, and is attenuated by physiological concentrations Na^+ and H^+. This marked influence of pH and NaCl concentration, which is absent in the interaction between the A_1 receptor and 8-cyclopentyl-1,3-dipropylxanthine (DPCPX‡, Fig. 1B), a well known A_1 receptor antagonist, or N^6-R-1-phenyl-2-propyladenosine (PIA, Fig. 1C), an agonist, suggests that amiloride inhibits specific binding via a site distinct from the specific adenosine receptor ligand binding site [2]. Thus, our interest was directed towards the characteristics of the site of interaction of amiloride.

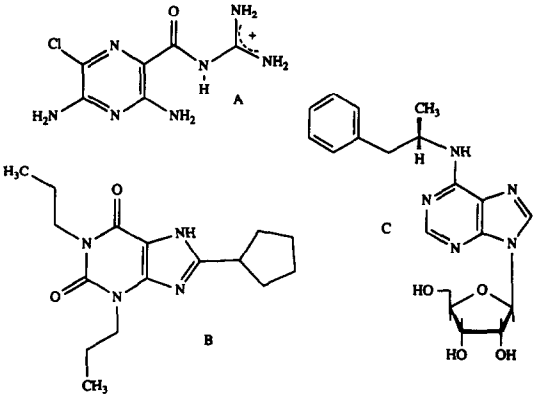

Fig. 1. The chemical structures of amiloride (A), DPCPX (B) and PIA (C).

The most widely accepted action of amiloride is the inhibition of Na^+ transport systems [3]. A structure–activity relationship study with eight amiloride analogues, however, revealed that none of these systems is directly involved in the interaction of amiloride with the A_1 receptor [2]. It is, however, conceivable that the amiloride binding site at the A_1 receptor will

* To whom correspondence should be addressed.

‡ Abbreviations: B_{max}, maximal binding capacity; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DEP, diethylpyrocarbonate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; IC_{50} half-maximal inhibiting concentration; K_d, equilibrium dissociation constant; K_i, equilibrium inhibition constant; NEM, *N*-ethylmaleimide; n_H, pseudo-Hill coefficient; PGO, phenylglyoxal; PIA, N^6-R-1-phenyl-2-propyladenosine.

have chemical features in common with these Na^+ transport proteins.

Some of the Na^+ transport systems have been characterized by chemical modification of amino acid residues [4–8]. From these studies it appears that both histidyl and carboxyl groups are involved in Na^+/H^+ exchange. In the Na^+-transport through epithelia arginyl, carboxyl and histidyl groups are involved.

Recently, Klotz *et al.* [9] characterized the binding site of selective A_1 receptor ligands by means of chemical modification of amino acid residues. They provide evidence that histidyl residues are essential in the A_1 ligand binding site. Distinct histidyl residues would be involved in agonist and antagonist binding. Although several other modifiers affected agonist or antagonist binding, these effects could be ascribed to modification of a G protein or amino acid residues at a site removed from the A_1 ligand binding site.

The aim of this study is to further characterize the site of interaction of amiloride on the A_1 receptor by treatment with group-specific reagents. This may elucidate which amino acids are involved in the interaction between amiloride on the A_1 receptor, and whether these are identical to those involved in A_1 receptor binding [9] or the ion transport systems [4–8].

MATERIALS AND METHODS

Drugs

[^{3}H]DPCPX (sp. act. 107 Ci/mmol) and [^{3}H]PIA (sp. act. 40 Ci/mmol) were purchased from Amersham (Buckinghamshire, U.K.), PIA from Boehringer (Mannheim, F.R.G.) and *N*-ethylmaleimide from the Sigma Chemical Co. (St Louis, MO, U.S.A.). GTP, phenylglyoxal (PGO) and diethylpyrocarbonate (DEP) were from Aldrich (Brussels, Belgium). *N,N'*-Dicyclohexylcarbodiimide (DCCD) was from Merck (Darmstadt, F.R.G.). Amiloride was kindly donated by Merck Sharp and Dohme (Haarlem, The Netherlands, USP grade). 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.* [10]. Protein concentrations were measured with the bicinchoninic acid method with bovine serum albumin as standard [11]. 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 (*ca.* 0.12 nM in displacement studies and 0–1.5 nM in saturation studies) or [^{3}H]PIA (*ca.* 0.3 nM).

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 filters or double GF/C filters (this gave identical results). The filters were washed three times with 2 mL ice-cold 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.

Treatment with group-specific reagents

Modification of sulfhydryl groups. Sulfhydryl groups were alkylated with *N*-ethylmaleimide (NEM) [12]. NEM, dissolved in assay buffer, was added directly to the incubation mixture. Preliminary experiments indicated that this way of treatment yields results that are identical to those obtained when membranes are incubated with NEM prior to the binding assay. Unless otherwise indicated the final concentration in the assay was 1 mM.

Modification of arginyl groups. Arginyl groups were modified by treatment with phenylglyoxal (PGO) [13]. PGO was dissolved in DMSO in a concentration of 0.4 M. Unless otherwise indicated 10 μL/mL of this solution was added to the membrane suspension giving a final concentration of 4 mM. After preincubation for 30 min at 37°, the suspension was cooled, diluted 10-fold and used in a binding assay. From fluorescence spectrometry measurements it was concluded that amiloride did not react with PGO in this concentration (data not shown).

Modification of histidyl groups. Histidyl groups were modified by treatment with the histidine-specific reagent diethylpyrocarbonate (DEP) [14]. As DEP is very susceptible to hydrolysis, the effective concentration of the stock solution was regularly checked by measuring the modification of 10 mM histidine in assay buffer spectrophotometrically [14]. Prior to use, DEP was mixed with ethanol to 200 times the required concentration. The reaction was initiated by addition of this solution (5 μL/mL) to the membrane suspension in HEP20, pH 7.4. After treatment for 15 min at room temperature, the membrane suspension was used in a binding assay. As the half-life of DEP is very short in aqueous solutions, hydrolysis of DEP terminated the reaction [14].

Protection of [^{3}H]DPCPX binding against histidine modification was investigated as follows: before the experiment the membrane suspension was centrifuged for 5 min at 10,000 *g* in a Sigma 202M table centrifuge. The pellet was resuspended and the membranes (*ca.* 1 mg/mL) were incubated at room temperature without ligand (control), with 300 μM amiloride or with 375 nM 8-phenyltheophylline. In all the incubations 1% DMSO was present. After 60 min 5 μL/mL DEP was added to a final concentration of 2 mM. The samples were incubated for 15 min at room temperature and centrifuged for 10 min at 10,000 *g*. The pellet was washed three times by resuspension in 1 mL ice-cold HEP20 (pH 7.4 at 0°) and centrifugation. Finally, the membranes were resuspended in HEP20 (pH 7.4 at 25°) in a concentration of *ca.* 0.1 mg/mL protein. This suspension was used in a binding assay.

Modification of carboxyl groups. Carboxyl groups were modified by treatment with *N,N'*-dicyclohexylcarbodiimide (DCCD) [6, 15]. DCCD solutions of 200 times the required concentration were made in ethanol. The treatment was initiated by addition of 5 μL/mL of this solution to the membrane suspension in HEP20. Unless otherwise indicated the final concentration was 1 mM. After preincubation for 30 min at room temperature, the suspension was used in a binding assay. It has been reported that DCCD does not react with amiloride [6].

Data analysis

Data from dispacement and saturation curves were analysed as described previously [16] by a computer program based on the law of mass action [17]. Statistically significant resolution of two components was tested in a partial F-test. K_d and K_i values calculated from at least three experiments are provided with their approximated SE. IC_{50} values and pseudo-Hill coefficients were determined from a Hill-plot by linear regression analysis.

RESULTS

Effect of chemical modifications on antagonist binding

The effects of chemical modifications on the binding parameters of [^{3}H]DPCPX in calf brain membranes are presented in Table 1 and Fig. 2. The binding parameters of [^{3}H]DPCPX under control conditions were described in the accompanying paper [2].

In the presence of 1 mM NEM, specific [^{3}H]DPCPX binding was slightly increased to 112 ± 4% of the control (N = 5) due to an increase in binding capacity without a change in affinity of the radioligand (Table 1). Treatment of membranes for 30 min at 37° with 4 mM PGO had no significant effect on the specific binding of a subsaturating concentration [^{3}H]DPCPX (107 ± 4% versus control (N = 6)) indicating that affinity and binding capacity of the radioligand were unaltered. Treatment of membranes for 15 min at room temperature with DEP decreased specific [^{3}H]DPCPX binding in a concentration-dependent manner (Fig. 3). Treatment for 60 min at 4° gave identical results. Interestingly, the effect of DEP was markedly enhanced if 145 mM NaCl was present during the preincubation (Fig. 3). The reduction in specific [^{3}H]DPCPX binding by DEP treatment was mainly due to a decrease in maximal binding capacity, whereas the reagent had only a marginal effect on the K_d value of [^{3}H]DPCPX (Table 1). Finally, treatment of membranes for 30 min at room temperature with DCCD decreased [^{3}H]DPCPX binding in a concentration-dependent manner as well (Fig. 4). NaCl (145 mM) diminished the effect of high, but not that of low (<1 mM) concentrations of DCCD. DCCD decreased the specific [^{3}H]DPCPX binding only at

Table 1. Effect of group-specific reagents on the binding parameters of [^{3}H]DPCPX in calf brain membranes

Reagent	K_d ± SE (pM)	B_{max} ± SE (fmol/mg)
None	74 ± 3	643 ± 10
NEM (1 mM)	71 ± 3	720 ± 10
DEP (1 mM)	83 ± 4	504 ± 7
DCCD (1 mM)	210 ± 17	614 ± 19

Saturation isotherms were determined after treatment of the membranes with reagents as described in Materials and Methods. Values were calculated from the combined data of 3–4 experiments. PGO had no effect on the binding of a subsaturating concentration [^{3}H]DPCPX indicating that neither affinity nor maximal binding capacity was affected.

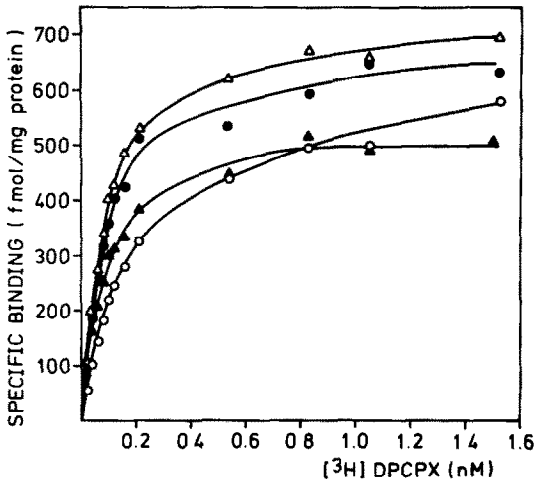

Fig. 2. The influence of modifications on [^{3}H]DPCPX binding. Saturation isotherms before (●) and after treatment with NEM (△), DEP (▲) or DCCD (○) were determined in a parallel experiment. Data points are from a representative experiment that was repeated twice with similar results.

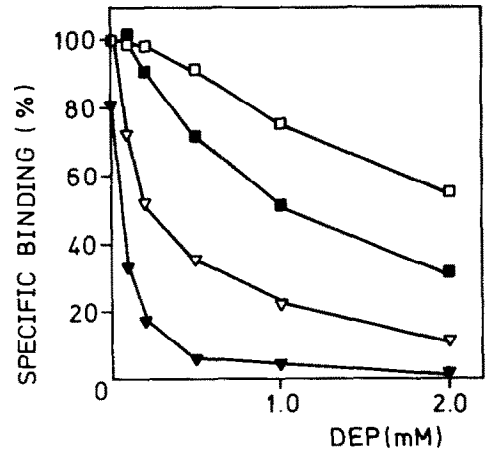

Fig. 3. Concentration-dependency of the modification by DEP; influence of NaCl. Calf brain membranes were treated with DEP for 15 min at room temperature in the presence (■, ▼) or absence (□, ▽) of 145 mM NaCl. [^{3}H]DPCPX (■, □) and [^{3}H]PIA (▼, ▽) binding was measured as described in Materials and Methods. Data points are from a representative experiment that was repeated twice with similar results.

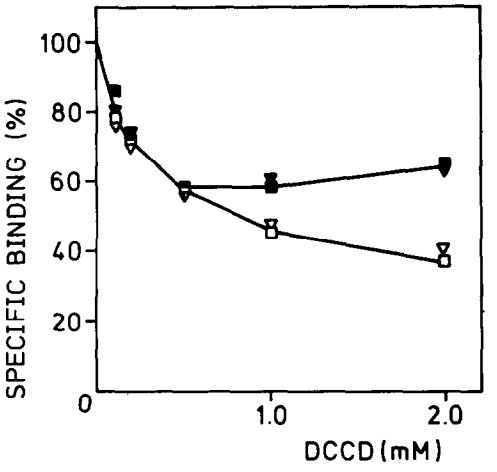

Fig. 4. Concentration-dependency of the modification by DCCD; influence of NaCl. Calf brain membranes were treated with DCCD for 30 min at room temperature in the presence (■, ▼) or absence (□, ▽) of 145 mM NaCl. [^{3}H]DPCPX (■, □) and [^{3}H]PIA (▼, ▽) binding was measured as described in Materials and Methods. Data points are from a representative experiment that was repeated twice with similar results.

subsaturating concentrations of the radioligand (Fig. 2): the affinity of the radioligand was lowered from 74 to 210 pM, whereas the maximal binding capacity was unchanged (Table 1).

Effect of chemical modifications on agonist binding

Two approaches were used to study the effects of modifications on the binding of agonists. First, [^{3}H]DPCPX binding was displaced by unlabelled PIA. This method was preferentially used, as the advantage is that a differentiation can be made between effects on the number of A_1 receptors, effects on the ratio between the high- and low-affinity state, and on the affinities of these states. Secondly, specific [^{3}H]PIA binding was measured to provide additional evidence. The binding parameters of [^{3}H]PIA under control conditions were described in the accompanying paper [2].

An overview of the effects of the modifiers on the affinity of unlabelled PIA is given in Table 2 and the four panels of Fig. 5. The effect of uncoupling of the G protein from the A_1 receptor by addition of GTP is included in Table 2 for comparison with the effects of NEM, PGO, DEP and DCCD.

In the presence of NEM, the apparent affinity of PIA was markedly reduced. Specific [^{3}H]PIA binding was reduced as well (not shown). This effect was more pronounced with 1 mM than with 0.1 mM NEM. The apparent IC_{50} value of PIA increased 10-fold in the presence of 1 mM NEM. Data analysis showed that the fraction of high-affinity binding sites was decreased and that the K_H value was increased.

Treatment with 4 mM PGO gave qualitatively similar effects: much higher PIA concentrations were needed to displace [^{3}H]DPCPX binding. This was related to a decrease in the fraction of high-affinity sites, combined with a slight decrease in the affinity. In accordance, specific [^{3}H]PIA binding was reduced.

Specific binding of [^{3}H]PIA was lower after treatment with 1 mM DEP. As shown in Fig. 3, [^{3}H]PIA binding was more sensitive to modification by DEP than [^{3}H]DPCPX binding and NaCl enhanced the influence of DEP. Consistent with the decrease in [^{3}H]PIA binding, the apparent affinity of unlabelled PIA for displacement of [^{3}H]DPCPX binding was markedly reduced (Fig. 5, Table 2). Analysis of the curves revealed that the affinities of the high- and low-affinity states were both decreased, whereas the ratio of the states remained similar to the control value.

Treatment with DCCD resulted in a decrease in [^{3}H]PIA binding, which was identical to the decrease in [^{3}H]DPCPX binding after the same treatment (Fig. 4). The IC_{50} value of PIA (Table 2) was approximately two-fold higher after DCCD treatment. The affinities of both high- and low-affinity state were reduced and the fraction of high-affinity sites was unchanged.

Uncoupling of G proteins from the receptor by 1 mM GTP resulted in a partial shift from high- to low-affinity state; the affinity of the states was unaltered.

Effect of chemical modifications on the displacement of [^{3}H]DPCPX binding by amiloride

A major aim of this study was to identify factors that affect the binding of amiloride in a different manner than the binding of adenosine receptor agon-

Table 2. The effects of group-specific reagents on the displacement of [^{3}H]DPCPX binding by PIA

Reagent	Conc.	IC_{50} ± SE (nM)	n_H ± SE	K_H ± SE (nM)	K_L ± SE (nM)	R_H (%)
None	—	0.78 ± 0.13	0.72 ± 0.04	0.17 ± 0.02	6.4 ± 2.1	81
NEM	1 mM	9.9 ± 2.9	0.74 ± 0.03	0.64 ± 0.32	8.5 ± 1.6	30
DEP	1 mM	7.9 ± 2.1	0.66 ± 0.01	1.7 ± 0.4	50 ± 40	75
DCCD	1 mM	1.4 ± 0.3	0.66 ± 0.04	0.56 ± 0.06	84 ± 54	84
PGO	4 mM	5.1 ± 4.3	0.62 ± 0.06	0.38 ± 0.18	13 ± 6	52
GTP	1 mM	5.7 ± 2.4	0.65 ± 0.06	0.28 ± 0.09	8.5 ± 1.6	40

Displacement curves were determined after treatment of the calf brain membranes with reagents as described in Materials and Methods. The inhibition of 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. Values were calculated from the combined data of 3–7 experiments.

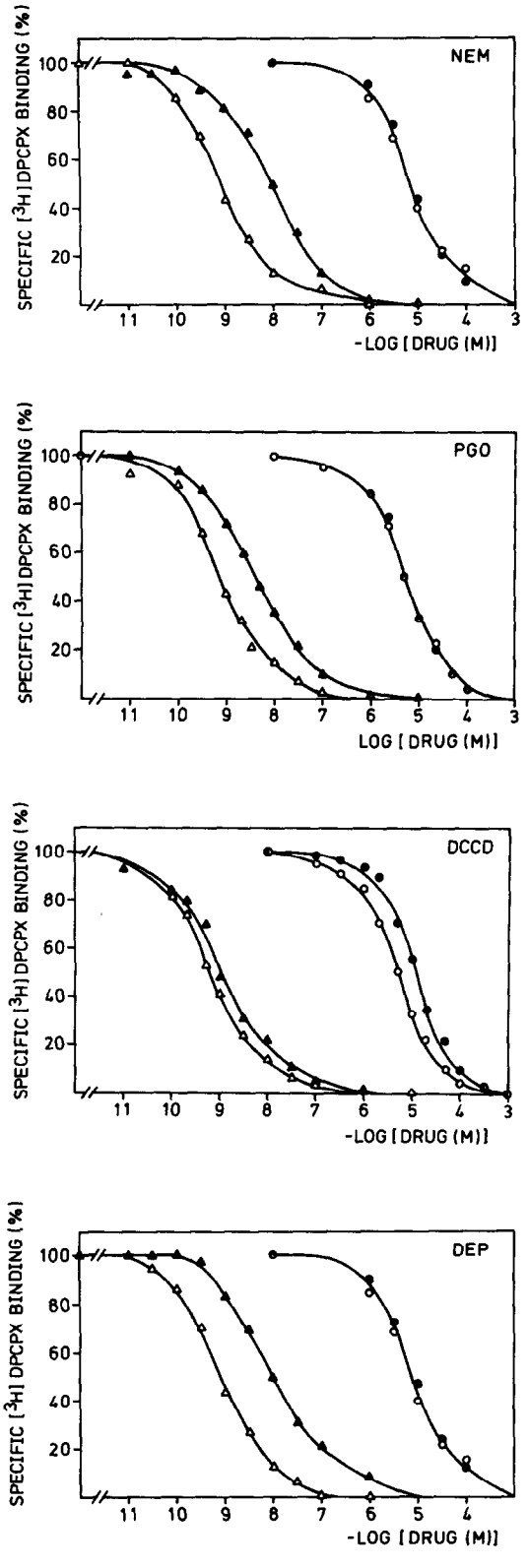

Fig. 5. Influence of NEM, PGO, DCCD and DEP on the displacement of [^{3}H]DPCPX binding by amiloride (○, ●) and PIA (△, ▲). Data points are from a representative experiment that was repeated twice with similar results. Open symbols represent the untreated, closed symbols the treated calf brain membrane preparation.

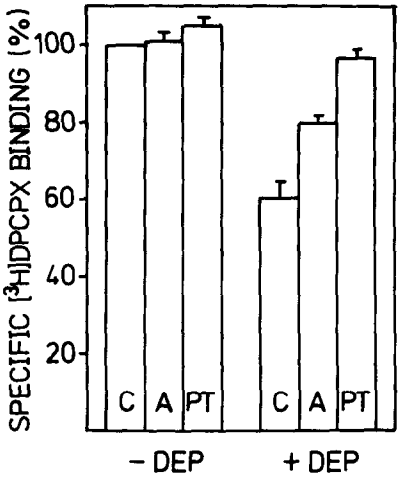

Fig. 6. Protection of [^{3}H]DPCPX binding against DEP modification of 300 μM amiloride (A) or 375 nM 8-phenyltheophylline (PT). C: control experiment with 1% DMSO only. Data points are means ± SE of three experiments and are presented as the percentage specific [^{3}H]DPCPX binding versus control (C, −DEP).

ists and antagonists. As evident from Fig. 5, treatment of the calf brain membranes with NEM, PGO or DEP did not influence the displacement curve of amiloride. The K_i values were 2.2 ± 0.2, 2.2 ± 0.2 and 2.3 ± 0.1 μM for NEM, PGO and DEP, respectively, compared to 2.0 ± 0.2 μM for the control. A similar lack of effect was observed in the presence of GTP (K_i value 3.0 ± 0.2 μM). The only modifier that had a distinct effect on the affinity of amiloride was DCCD. Treatment with this reagent increased the apparent K_i value to 5.6 ± 0.4 μM.

As reported above, the number of [^{3}H]DPCPX binding sites was reduced after treatment with DEP. Obviously, such a decrease in binding capacity cannot be detected with an unlabelled ligand. In order to assess whether amiloride binds to the same site as DPCPX, a protection experiment was performed in which 300 μM amiloride (150 × K_i value) was present during the treatment with DEP. The binding site was protected against modification by DEP, though not completely. In contrast, a classic competitive antagonist 8-phenyltheophylline prevented modification by DEP completely in an equivalent relative concentration (150 × K_i = 375 nM). Increasing the concentration of both ligands to 400 times the K_i value did not enhance the protecting effect of amiloride (data not shown). The fact that a control, that was preincubated with amiloride, but not treated with DEP (Fig. 6; −DEP, A), was identical to the control value (−DEP, C) demonstrates that amiloride had been completely removed by the wash procedure used.

DISCUSSION

The use of group-specific reagents in protein chemistry is commonly practiced. This method can also

be used for the characterization of membrane bound proteins such as receptors. In the present study several reagents were used in an attempt to differentiate between the interaction with the adenosine A_1 receptor of an A_1 agonist (PIA), an A_1 antagonist (DPCPX) and the structurally unrelated diuretic amiloride (for chemical structures see Fig. 1). The modifying reagents were selected on the basis of their ability to modify the A_1 receptor, coupling to G proteins, Na^+/H^+ exchange or epithelial Na^+ transport. In Table 3, a resumé of the effects of protein modifiers is presented.

Sulfhydryl groups are known to be involved in receptor-G protein coupling [9, 18]. Treatment with NEM abolishes the coupling between G protein and receptor. This would not affect the interaction with antagonists, but would largely reduce high-affinity agonist binding. In this paper, we demonstrate that the affinity of [^{3}H]DPCPX is unaltered after NEM treatment. The maximal binding capacity of the radioligand is slightly enhanced. Such an increase in antagonist binding is sometimes seen in the presence of GTP as well [19, 20]. It may be consequent to the removal of tightly bound endogenous adenosine which has not been removed by the pretreatment with adenosine deaminase. Alternatively, a separate state of the receptor which is preferentially labelled by antagonists may be stabilized [19, 21].

Table 3. The effect of group specific reagents on the affinity of [^{3}H]DPCPX, PIA and amiloride

Modifier (residue)	[^{3}H]DPCPX K_d	[^{3}H]DPCPX B_{max}	PIA IC_{50}	Amiloride IC_{50}
NEM (sulfhydryl)	=	↑	↑↑	=
PGO (arginyl)	=	=	↑↑	=
DEP (histidyl)	=	↓	↑↑	=
DCCD (carboxyl)	↑	=	↑	↑

As far as the agonist PIA is concerned, we indeed observed a 10-fold decrease in the apparent affinity of PIA due to a reduction of the fraction of high-affinity sites from 81 to 30% (Table 2). This effect is similar to that observed in the presence of GTP. A slight increase in the K_H value of PIA is also observed.

Treatment with NEM does not alter the K_i value of amiloride. The lack of effect in this case indicates that: (i) sulfhydryl groups are not involved in the interaction between amiloride and the A_1 receptor; and (ii) amiloride does not differentiate between the high- and low-affinity state of the receptor. The former has also been observed in the interaction between amiloride and the epithelial sodium channel [22]. The latter supports our finding that uncoupling of the G protein by addition of GTP does not influence the K_i value of amiloride.

PGO, an arginine modifier [13], seems to influence receptor–G protein coupling as well. A pattern very similar to that of NEM treatment is observed: [^{3}H]DPCPX binding is not affected, the apparent affinity of PIA is largely reduced, the fraction of high-affinity sites is reduced, and the K_i value of amiloride is identical to the control. The effect of PGO on agonist binding is observed only at 37° and not at room temperature or at lower concentrations. This may explain the discrepancy with an earlier study [9]. Our results suggest that apart from sulfhydryl groups, arginine residues are involved in receptor–G protein coupling.

The effects of DEP, a histidyl-modifying reagent, are evidently more complex than those of NEM and PGO. Consistent with the observations of Klotz *et al.* [9], [^{3}H]PIA binding is more susceptible to modification by DEP than [^{3}H]DPCPX binding. Further analysis of these effects shows that DEP causes a decrease in the total number of [^{3}H]DPCPX binding sites as well as a decrease in the affinity of PIA for both the high- and low-affinity state of the receptor. The more pronounced effect on agonist binding (Fig. 3) can thus be explained by a decrease in affinity of PIA in addition to the decrease in the number of binding sites. As the fraction of high-affinity sites is not altered, uncoupling of a G protein does not seem to be the cause of the higher IC_{50} value of PIA.

The differential sensitivity of [^{3}H]PIA and [^{3}H]DPCPX binding to modification by DEP may be explained by the existence of two distinct histidyl residues [9]. Modification of the histidyl residue involved in antagonist binding gives rise to the observed decrease in maximal binding capacity, whereas the affinity of the remaining binding sites for the agonist PIA is largely reduced by modification of a second histidyl residue involved in agonist binding only.

A novel finding in the present study is that the presence of NaCl during DEP treatment augments the effect of DEP on both agonist and antagonist binding. An analogous effect has been observed in studies on the modification of the Na^+/H^+ exchanger by DEP [7]. A conformational change of the A_1 receptor induced by NaCl might facilitate the exposure of the histidyl residues to the reagent.

Modification by DEP does not affect the affinity of amiloride. In the accompanying paper, we showed that the affinity of amiloride is largely reduced at low pH values [2]. It was calculated from pH studies between pH 6.6 and 8.4 that amiloride interacts with a group with a pK_a of *ca.* 7.6 [2]. This is likely to be a histidyl residue, as histidine is the only amino acid with a pK_a in the physiological pH range. An explanation for the disparity between modification and pH studies is that the modified binding sites fail to bind [^{3}H]DPCPX, and can therefore no longer be detected, whereas the affinity of amiloride for remaining sites is unaltered. This would imply that the same histidine residue is involved in [^{3}H]DPCPX binding and in the interaction with amiloride. To verify this possibility the capability of amiloride to protect the [^{3}H]DPCPX binding site against DEP modification was assessed. The presence of 300 μM amiloride during DEP treatment partially prevents the decrease in [^{3}H]DPCPX binding. This amiloride

concentration is 150 times the K_i value in calf brain membranes and accordingly sufficient to block all A_1 receptors. An equivalent relative concentration of 8-phenyltheophylline completely abolishes the effect of DEP. The partial protection of amiloride could not be converted to a complete protection by increasing the concentration of the diuretic. This difference in protection capability suggests that the recognition site of amiloride overlaps the [^{3}H]DPCPX binding site only partially.

Interestingly, [^{3}H]DPCPX binding is constant between pH 6.6 and 8.4 [2]. Extending this pH range to pH 5.2, virtually identical [^{3}H]DPCPX binding was observed at all pH values (data not shown). Apparently, the degree of protonation of a histidyl residue, essential in [^{3}H]DPCPX binding, is not of major influence on the binding of the radioligand, contrary to its effects on the affinity of amiloride.

DCCD is the only modifier that has any effect on the equilibrium inhibition constant of amiloride. [^{3}H]PIA and [^{3}H]DPCPX binding are decreased to the same extent, both in the absence and presence of NaCl. It is remarkable that addition of NaCl attenuates the effect of DCCD only at higher concentrations of the reagent. This may be caused by a reduction in the accessibility of the lipophilic reagent to the membrane proteins in a buffer with higher ionic strength. The decrease in [^{3}H]DPCPX binding is due to a three-fold decrease in affinity of the radioligand, while the binding capacity remains unaltered. A similar reduction in affinity is observed for PIA and amiloride. This suggests that the modified carboxyl group is not an essential point of interaction (in this case all specific binding would be abolished), but close enough to the binding site to change its conformation.

In summary (Table 3), the apparent affinity of the A_1 agonist PIA is largely diminished after treatment with NEM, PGO or DEP, whereas DCCD has a less marked effect. The affinity of the antagonist DPCPX is unaltered after treatment with NEM, PGO or DEP. Treatment with DCCD results in a three-fold decrease in affinity of the radioligand. The number of [^{3}H]DPCPX binding sites is slightly increased in the presence of NEM and decreased after treatment with DEP. The presence of 8-phenyltheophylline during the DEP treatment abolishes the effect of the reagent. These results indicate that: (i) cystein and arginyl residues are important in receptor–G protein coupling; (ii) a carboxyl group is located in the vicinity of the adenosine recognition site; and (iii) histidine is important in the binding site of A_1 receptor ligands. It is difficult to reconcile the importance of histidine with a lack of effect of pH changes between pH 5.2 and 8.4.

Comparing the effects of the modifiers on the receptor interaction with agonist, antagonist and amiloride, it appears that amiloride is not an A_1 receptor agonist. The effect of the modifications on the affinity of [^{3}H]DPCPX and amiloride are, however, very much alike. Evidently, a decrease in binding capacity, as observed for [^{3}H]DPCPX, will not be detected for an unlabelled compound. However, the reduction in [^{3}H]DPCPX binding by DEP treatment can be partially prevented by amiloride, suggesting that the amiloride recognition site overlaps the binding site of a classic A_1 receptor antagonist. This incomplete protection, in contrast to the complete protection by 8-phenyltheophylline, supports the idea that amiloride is an adenosine A_1 receptor antagonist with a behaviour different from classic A_1 antagonists such as DPCPX.

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