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N-ETHYLMALEIMIDE AFFECTS AGONIST BINDING TO A_1 ADENOSINE RECEPTORS DIFFERENTLY IN THE PRESENCE THAN IN THE ABSENCE OF LIGAND

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SUMMARY: The effect of sulphydryl reagents (N-ethylmaleimide -NEM- , 4-hydroxymercuriobenzoate -HMB- and 5-5'-dithio-bis-2-nitrobenzoate -DTNB-) on agonist and antagonist binding to A_1 adenosine receptors from pig brain was studied. The action of the mercurial agent HMB was found to be strong and seemed to be nonspecific. The effects of either NEM or DTNB were milder and more specific. The characterization of the agonist binding in membranes pretreated with moderate concentrations of DTNB and NEM led to reduced affinities for both high- and low-affinity sites without marked modifications of maximal binding or of proportion of affinity states. These results for NEM are surprising since the compound is usually used to mimick the effects of Gpp(NH)p, i.e. to shift high-affinity states to low-affinity states. It was found that this Gpp(NH)p-like effect of NEM is only possible when the compound is included in the assay medium. Similarly, Gpp(NH)p produces the uncoupling of the receptor molecule from G protein if included in the assay medium. Thus, membranes pretreated with Gpp(NH)p exhibited both affinity states and with similar equilibrium binding parameter values to those of the crude membranes.

In basis of the coupling to G proteins the A_1 adenosine receptors display two affinity states for the agonist binding. Thus the uncoupled receptor displays low-affinity for the agonists whereas the receptor linked to the G protein displays high-affinity characteristics (1, 2). In contrast, the binding of antagonists takes place with the same affinity for both the coupled and the uncoupled receptor (3-5). As in other receptor systems, the uncoupling of the receptor can be achieved by using guanine nucleotides or analogues (1, 6-9). Besides, in previous reports working with pig brain cortical membranes we have demonstrated that the co-existence of the two affinity forms for agonists requires the integrity of the membrane (9, 10).

Chemical modification of amino acid residues is a useful technique to study the ligand recognition site(s) of receptors. Klotz et al (11) and Garritsen et al. (12), using a histidine-specific reagent provided evidence of the existence of two histidine residues involved in ligand binding and a free carboxyl group that is present in the vicinity of the recognition site. Sulphydryl groups are important for receptor-G protein coupling (3, 4, 6) whereas they have no direct role in the binding of ligands (11). N-ethylmaleimide (NEM) has been used to shift the A_1 adenosine receptors from a high-affinity state to a low-affinity state for agonists (3, 4, 6). Since this effect is similar to that exerted by guanine nucleotides or analogues, it is assumed that NEM produces the uncoupling of A_1 adenosine receptors from receptor-G protein complexes. Thus NEM is an alternative compound which is used to mimic the effects of the non-hydrolizable guanine nucleotide analogue Gpp(NH)p.

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The aim of the present study is to analyze the effect of different sulphydryl reagents upon the A_1 adenosine receptor of pig brain cortical membranes. The Gpp(NH)p-like effect caused by NEM has been further investigated and it is shown that the compound displays very different effects when used in the pretreatment of membranes or when incorporated into the incubation assay medium.

MATERIALS AND METHODS

Materials: [Adenine-2,8-3H,ethyl-2-3H]-PIA ([^{3}H] R-PIA) (54.0 Ci/mmol) and 8-Cyclopentyl-1,3-[3H]dipropylxanthine ([^{3}H] DPCPX) (109.2 Ci/mmol) were purchased from New England Nuclear Research Products (Boston). N6-(R)-(phenylisopropyl)adenosine (R-PIA), adenosine deaminasa and Gpp-(NH)p were obtained from Boehringer Mannheim; 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) was purchased from Research Biochemicals Inc.; N-ethylmaleimide (NEM), crystallized bovine serum albumin and 50 % polyethylenimine were from Sigma; 5,5`-dithio-bis-(2-nitrobenzoate) (DTNB) was from Merck and Sodium 4-Hydroxymercuribenzoate (HMB) from Ega-Chemie.
Pig brain cortical membranes were prepared as described elsewhere (9). Buffer used was 50 mM Tris-HCl buffer pH 7.4 which, in the last wash (30 min at 25ºC), contained 0.2 UI/ml adenosine deaminase.

Chemical treatment of membranes with sulphydryl group-specific reagents: Membranes (1 mg/ml) were resuspended with 50 mM Tris-HCl buffer, pH 7.4. Then the indicated amounts of sulphydryl-contaning solutions were added and, unless otherwise stated, after 1 hour at 25ºC the suspension was centrifuged at 105,000 x g for 30 minutes at 4ºC. The pellet was resuspended in the same volume of 50 mM Tris-HCl buffer and was washed once more as described above. Control experiments were done without the sulphydryl reagents.

Radioligand binding experiments and protein determination: Agonist and antagonist binding to pig brain cortical membranes and protein determination were measured as described previously (9, 10, 13, 14).

Analysis of equilibrium binding isotherms: In isotherm binding analysis a total binding was fitted to the previously developed equations (9) corresponding to the sum of specific plus nonspecific binding. The individual saturation isotherms were analyzed by non-linear regression using the RENZFITTER program (Elsevier Biosoft) or other available programs (See 9). Five replicates of each point were performed. Goodness of fit was tested according to the reduced c^2 or SD values given by the programs. Modified F test was used to analyze whether the fit to the two-states model significantly improved the fit to the one-state model (See 9).

RESULTS

Characterization of agonist and antagonist binding in membranes pretreated with sulphydryl reagents: As usual (9, 14, 21), control membranes displayed two affinity states for the agonist whose K_d and R_H values are indicated in table 1. HMB indiscriminately reduced the binding as deduced by the disappearance of high-affinity binding sites and the reduction in both R_L and affinity of the low-affinity sites (table 1). The effect of DTNB was more specific since the total maximum binding did not decrease. Besides, DTNB led to significant reductions in ligand affinity for both affinity-states. The effects caused by pretreatment of membranes with NEM were also a decrease in agonist affinity to either low-affinity or high-affinity receptors. These results for NEM are surprising since NEM is generally used to mimick Gpp(NH)p effects. It is described that these two compounds are capable of shifting high-affinity centers to low-affinity centers. Thus the well known conversion of high- to low-affinity states mediated by Gpp(NH)p in these membranes (9) is only possible if the compound is present in the binding assay medium but not if the membranes are pretreated with it (table1). Interestingly, the pretreatment with both Gpp(NH)p and NEM did not produce any modification in the equilibrium binding parameters with respect to control membranes. Thus, it is

Table 1

EFFECT OF SULPHYDRYL REAGENTS ON [^{3}H]R-PIA EQUILIBRIUM BINDING PARAMETERS. The isotherms of binding to pretreated membranes were determined as indicated in Methods. Data points corresponding to quintuplicates were adjusted to a one-site or to a two-site model by a nonlinear regression program using the equations described in Methods. Values are mean±SD given by the program. R : maximum binding. K_d : dissociation equilibrium constant. (n.d. not detected).

	High-affinity		Low-affinity		R_H+R_L
Reagent (mM)	R_H (pmol/mg prot)	K_{dH} (nM)	R_L (pmol/mg prot)	K_{dL} (nM)	(pmol/mg prot)
None	0.12±0.09	0.1±0.1	0.63±0.08	3.5±1.1	0.7±0.1
HMB (0.1)	n.d.	n.d.	0.34±0.05	9.2±3.1	0.34±0.05
DTNB (1.0)	0.12±0.01	2.8±0.5	1.3±0.5	61±4.	1.3±0.5
NEM (0.6)	0.08±0.02	0.5±0.2	0.73±0.01	10.3±0.1	0.81±0.02
Gpp(NH)p (0.1)	0.16±0.06	0.05±0.06	0.63±0.05	2.5±0.6	0.8±0.1
Gpp(NH)p (0.1) plus	0.15±0.09	0.06±0.08	0.64±0.09	3±1.0	0.8±0.1

evident that Gpp(NH)p protects adenosine receptors against the loss of affinity caused by NEM. Similar experiments were performed with the antagonist for which only a single affinity state is present in control membranes (table 2). The affinity for the antagonist was not modified by the action of either DTNB, HMB or NEM. However, the maximum binding increased with DTNB and NEM, and slightly decreased by 0.1 mM HMB action. In experiments performed with membranes pretreated with Gpp(NH)p and NEM it was found that the increase of maximum binding induced by NEM was not prevented by Gpp(NH)p.

Table 2

EFFECT OF SULPHYDRYL REAGENTS ON [^{3}H]DPCPX EQUILIBRIUM BINDING PARAMETERS. The isotherms of binding to pretreated membranes were determined as indicated in Methods. Data points corresponding to quintuplicates were adjusted to a one-site model by a nonlinear regression program. Values are mean±SD given by the program. R : maximum binding. K_d : dissociation equilibrium constant.

Reagent (mM)	R (pmol/mg prot)	K_d (nM)
None	0.74±0.01	0.45±0.02
HMB (0.1)	0.62±0.02	0.37±0.04
DTNB (1)	1.06±0.01	0.45±0.02
NEM (0.6)	0.95±0.01	0.45±0.02
Gpp(NH)p (0.1)	0.74±0.08	0.45±0.02
Gpp(NH)p (0.1) plus NEM (0.6)	1.10±0.04	0.50±0.07

Table 3
AGONIST AND ANTAGONIST EQUILIBRIUM BINDING PARAMETERS IN ABSENCE AND IN PRESENCE OF NEM IN THE INCUBATION ASSAY MEDIUM. Values±SD (given by the nonlinear regression program). R : maximum binding; K_d : dissociation equilibrium constant. (n.d. not detected).

	without NEM		with NEM	
Ligand	R (pmol/mg prot)	K_d (nM)	R (pmol/mg prot)	K_d (nM)
[^{3}H]R-PIA				
High-affinity	0.19±0.05	0.14±0.08	n.d.	n.d.
Low-affinity	0.46±0.04	3.1±0.7	0.69±0.02	4.3±0.3
[3H]DPCPX	0.88±0.01	0.70±0.04	1.01±0.01	0.72±0.03

Effect of the presence of NEM in the binding medium: The saturation isotherm for [^{3}H]R-PIA was carried out in presence of NEM in the assay medium. The results appear in table 3 where the disappearance of the high-affinity component is confirmed, whereas the maximum binding, which now coincides with R_L, was not affected. A similar experiment carried out in presence of 500 µM DTNB also led to the characterization of a single affinity state of K_d=4.4±0.7 nM. When the isotherm was performed with the antagonist [^{3}H]DPCPX in presence of NEM, it appeared that the maximum binding was slightly increased (15 %) while the affinity for the single antagonist binding site remained unchanged (table 3). Thus the antagonist binding was similar when the membranes were pretreated with NEM or when NEM was added to the incubation buffer.

DISCUSSION

The results presented indicate that the inhibitory effect of HMB seems to be nonspecific since by analyzing the modification of equilibrium parameters of agonist binding, both the high-affinity and the low-affinity components were affected. Also, in contrast with the increase of antagonist binding induced by DTNB or NEM, HMB caused inhibition of antagonist binding. This mercurial compound would lead to changes in the membrane structure which in turn may lead to alterations of ligand binding.

The preincubation of membranes with Gpp(NH)p does not produce any effect on agonist binding (table 1). This indicates that the shift of affinity for agonist binding, which is always evident when Gpp(NH)p is present in the incubation assay medium (9), is either reversible or only possible in the presence of the agonist. For the Gpp(NH)p-like effect produced by NEM it was evident that this compound can shift its affinity for agonists when it is present in the binding medium (table 3). In contrast, after the pretreatment of membranes with NEM, the two affinity states are detected but the affinity for the agonist decreases. Interestingly these effects are totally absent when the pretreatment is performed in the presence of Gpp(NH)p, showing that both substances interact with the same proteic domain. Thus, in our case, the shift of high-affinity to low-affinity sites by NEM (or even by DTNB as indicated in Results), is only possible in the presence of [^{3}H]R-PIA. These results contrast with those

reported by Garritsen et al . (12) who, in calf brain membranes, related no differences in adding NEM directly to the assay buffer and in pretreating the membranes with the compound. Taking our results into account, a fair assumption would be that there is one(or more) SH group susceptible of modification by NEM near the Gpp(NH)p binding site; thus the alteration of such a group is prevented by Gpp(NH)p by steric hindrance. The modification of this group in absence of Gpp(NH)p is possible by NEM and also by DTNB. Such a group must be very important for the coupling of the receptor to G protein since its modification in the presence of the agonist shifts the high-affinity sites to the low-affinity ones, thus giving rise to the Gpp(NH)p-like effect. The change of events when R-PIA is present in the assays (i.e. the conversion of high- to low-affinity centers) strongly supports the view that the agonist produces conformational changes in the receptor molecule. In contrast, since antagonists lacked these influences it can be assumed that the conformational changes induced by antagonists are limited.

Contrasting with the results found with agonists, the affinity of A_1 adenosine receptors for antagonists is not modified by guanine nucleotides or analogues. On the other hand, the presence of NEM increases the maximum number of binding sites for the antagonist (table 2) independently of the presence of GppNHp in the medium. This is a trend which is not always found (1, 2, 8, 15, 16)but which seems to depend on the conditions of receptor isolation and of assay. As also found here, in a recent paper, Klotz et al (17) demonstrated an NEM-induced increase in [^{3}H]DPCPX binding which was not caused by an affinity change but rather by an increased maximum binding. A number of hypotheses have been offered to explain the increase of antagonist binding in presence of guanine nucletides, or sulphydryl reagents (NEM) none of which is, at present, supported by experimental data. A remote possibility is the competitive interaction with endogenous adenosine. Another possibility is that one state of the receptor is preferentially stabilized by antagonists (18,19). This seems not to be the case, since as demonstrated here and also by Klotz et al. (17) when using GppNHp or NEM, no changes in kinetic parameters required for the stabilization of the receptor in a guanine-insensitive state were found. Klotz et al (17) have suggested that [^{3}H]DPCPX binding is inhibited when receptors are coupled to G protein; therefore uncoupling of A_1 receptors from G protein by GTP or by NEM results in an increased antagonist binding; also these authors advanced the hypothesis that antagonists may bind to, e.g., RG_i but not RG_0(or viceversa), whereas agonists bind to both coupled complexes. However, this contrast with the fact that competition of agonists for [^{3}H]DPCPX binding shows that the radioligand labels both G protein-coupled and -uncoupled receptors (20). Moreover, we have reported here that agonists and antagonists show the same maximum binding (0.7-0.8 pmols/mg protein). On the other hand, we would like to emphasize a recent report in which we show that the agonist R-PIA, at concentrations higher than 20 nM, is able to shift high-affinity states to low-affinity states (21). This conduct of the agonist, which may be related with desensitization mechanisms, opens the possibility that the two-affinity model considered in all papers at present is not suitable since it cannot explain the agonist-induced interconversion among affinity states. Suitable models for explaining agonist-antagonist binding behaviour of A_1 adenosine receptors should be sought which could explain apparent increases in maximum binding not based on the apperance of spare receptors or on the existence of various interacting G proteins.

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