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Apparent heterogeneity of cardiac A_1 adenosine receptors as revealed by radioligand binding experiments on *N*-ethylmaleimide-treated membranes

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Summary

While G protein-coupled receptors are often studied by analyzing antagonist radioligand: “cold” agonist inhibition curves using an independent site model, it is now clear that K_L and K_H values determined in these analyses are not reliable estimates of the affinities of the agonists for “free” and G protein-coupled forms of the receptor. Thus, such experiments cannot he used to contrast the characteristics of a given type of receptor in different tissues, i.e., to probe for the existence of receptor subtypes. Since treatment with N-ethylmaleimide treatment blocks receptor: G_i/G_o protein interactions, such analyses on N-ethylmaleimide-pretreated membranes should allow direct assessment of the affinities of competing ligands for the free receptor or for multiple receptor subtypes.

As A_1 adenosine receptors couple to G_i, and perhaps to G_o, we have performed A_1 adenosine receptor radioligand “competition” studies first on control, then on N-ethylmaleimide-pretreated bovine cardiac and cerebral cortical membranes. Results of experiments with the antagonist radioligand [^{3}H]xanthine amine congener appeared to be confounded by ligand binding to A_2 adenosine receptors present in the cardiac membrane preparations. Further experiments utilized the A_1-specific radioligand [^{3}H]1,3-dipropyl-8-cyclopentylxanthine. These experiments confirmed once more that the K_L values determined by computer analysis of “competition” curves performed on control membranes are not reliable estimates of the affinities of the competing ligand for free receptors. Furthermore the results supported the hypothesis that similar analyses on NEM-treated membranes provide reliable estimates of the affinity(s) of competing ligands for free receptors. Lastly, the results suggest that cardiac membranes contain two subtypes of A_1 adenosine receptors that are differentiated by 5′-modified but not N^6-modified adenosine analogs. One of these receptor subtypes appears to be the same as the A_1 receptor detected in cortical membranes.

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Keywords

A_1 adenosine receptor; N-ethylmaleimide pretreatment; Receptor subtypes

Introduction

Effects of exogenous adenosine on cardiac function were first reported almost 60 years ago (Drury and Szent-Györgi 1929). Several years ago we reported that the A_1 adenosine receptor agonist radioligand [^{3}H]R-phenylisopropyladenosine ([^{3}H]R-PIA) binds to adenosine receptors in membranes prepared from newborn chick ventricle and that R-PIA causes the inhibition of isoproterenol-stimulated adenlyate cyclase activity in the same preparations (Hosey et al. 1984). These results suggested that the cardiac adenosine receptor is similar, if not identical to, the well characterized A_1 receptor present in the CNS. This conclusion is supported by subsequent ligand binding studies using a variety of [^{125}I]-labeled agonist radioligands in rat and bovine myocardial membranes (Linden et al. 1985; Lohse et al. 1985). The complete characterization of the cardiac adenosine receptor(s) using radioligand binding methodologies has been difficult because of the relatively low B_{max} values of the agonist radioligands in cardiac membranes (Linden et al. 1985; Hosey et al. 1984; Lohse et al. 1985). More recently three antagonist radioligands ([^{3}H]xanthine amine congener ([^{3}H]XAC) (Jacobson et al. 1986), [^{3}H]8-cyclopentyl-1,3-dipropylxanthine ([^{3}H]CPX) (Bruns et al. 1987; Lohse et al. 1987) and [^{125}I]3-(4-amino)phenethyl-l-propyl-8-cyclopentylxanthine ([^{125}I]BW-A844U) (Patel et al. 1988) which bind to A_1 receptors in the CNS with high affinities became available. This prompted us to perform antagonist radioligand: “cold” ligand competition experiments which we analyzed according to an independent site model with the computer program LIGAND (Munson and Rodbard 1980). Further extensive studies on the analysis of ligand binding to A_1 receptors in bovine brain membranes (Leung et al. 1990) showed that this analysis is inappropriate and that much more complex analyses are necessary in order to adequately describe the behavior of the system. Because of the complexity of these analyses, and the relative paucity of A_1 adenosine receptors in the heart, we felt that this approach was not practical for probing for possible differences between cardiac and brain A_1 adenosine receptors. We therefore performed radioligand “competition” experiments on N-ethylmaleimide (NEM)-treated membranes in which all A_1receptor: G protein interactions appear to be blocked (Leung et al. 1990). This effect of NEM to block high affinity agonist binding to many types of receptors that couple to G_i or G_o appears to be due to a modification of the alpha subunits of these G proteins (Asano and Ogasawara 1986). Under these conditions we postulate that analyses based on the independent site model give valid estimates of the affinities of the various compounds for the free, uncoupled receptors. All of these experiments including the initital experiments on nonpretreated membranes are reported herein in the order in which they evolved. The results show how radioligand “competition experiments” on receptors in NEM-treated membranes can be used for the characterization of receptors that couple to G_i/G_o type G proteins. Furthermore, the results suggest that cardiac membranes may contain a subtype of A_1 adenosine receptor that is not detected in cortical membranes.

Materials and methods

Materials

[^{3}H]XAC (118 Ci/mmol) was from DuPont NEN (Boston, Mass., USA). [^{3}H]CPX (93.8 Ci/mmol) was from Amersham (Arlington Heights, IL, USA). N^6-(3-iodo-4-aminobenzyl)adenosine (IABA) was a gift from Dr. J. Linden (Department of Physiology, University of Virginia School of Medicine, Charlottesville, VA, USA). 5′-N-ethylcarboxamide adenosine (NECA) and 5′-N-cyclopropylcarboxamide adenosine (NCCA) were gifts from Abbott Laboratories (North Chicago, IL., USA). R- and S-PIA were from Boehringer Mannheim (Indianapolis, IN, USA); CHA and CPX were from Research Biochemicals Inc. (Natick, Mass, USA); bacitracin was from Upjohn (Kalamazoo, Mi., USA). Other chemicals were from Sigma (St Louis, Mo., USA). Bovine tissues were obtained from a local slaughter house.

Preparation of bovine cardiac membranes

Bovine cardiac membranes were prepared by a modification of the method of Flockerzi et al. 1983. 200–300 g of bovine ventricle was suspended in 0.3 M sucrose containing 10 mM imidazole HC1 (pH 7.0), 5 mM $MgSO_4$, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 100 μM phenylmethylsulphonyl floride (MSF), minced, and homogenized using a Polytron at setting 7 for 1 min. The homogenate was adjusted to 0.4 M sucrose and centrifuged at 28,000 g for 30 min. The supernatant was collected and diluted 1.5 fold with 150 mM NaCl, 20 mM Tris HC1 (pH 7.0, 25 °C) and 0.1 mM EDTA and centrifuged at 44,000 g for 30 min. The pellet was collected and suspended in 1 M sucrose containing 5 mM $MgSO_4$, 0.1 mM EDTA and 10 mM imidazole HCl (pH 7.0). The membrane suspension was overlaid with 0.3 M sucrose containing the same buffer and centrifuged in a SW40 swinging bucket for 90 min at 178,000 g. The material at the 0.3 M and 1.0 M sucrose interface was collected, diluted 20 fold with 150 mM NaCl containing 10 mM Tris HCl (pH 7.0) and 1 mM EDTA. This suspension was then centrifuged for 30 min at 100,000 g in a TI40 rotor. The final pellet was suspended in 10 mM Tris HCl (pH 7.5) containing 0.1 mM EDTA and stored in liquid nitrogen until use.

Preparation of bovine cerebral cortical membranes

Cerebral cortex from bovine brain was suspended in 3–4 volumes of ice-cold 10% sucrose containing 20 mM Tris HCl (pH 7.0, 25 °C), 1 mM EDTA and 100 μM PMSF and homogenized with a Polytron homogenizer at setting 5 for 15 sec. The homogenate was centrifuged at 400 g for 10 min at 4 °c. The supernatant was removed and the pellet was resuspended in the same solution and recentrifuged for 10 min at 400 g. The supernatants from the two centrifugations were combined and centrifuged at 6500 g for 20 min. The pellet was resuspended in the 20 mM Tris buffer (pH 7.5, 25 °C) containing 1 mM EDTA and after 30 min recentrifuged at 6500 g for 20 min. The pellet was resuspended in the same buffer at a protein concentration of 10 mg/ml and stored in liquid nitrogen until use. Protein concentrations were determined by the method of Bradford (1976) using human gamma globulin as the standard.

Ligand binding assays

Ligand binding assays were performed in a volume of 50 to 500 μl (37 °C, 2 h) containing 20 mM Tris HCl (pH 7.5), 2 mM EDTA, 3 mM $MgSO_4$, 5 U/ml adenosine deaminase, 0,1 mM benzamidine, 0.1 mg/ml bacitracin, 0.01 mg/ml soybean trypsin inhibitor, radioligand and other additions as indicated. Saturation isotherms were analyzed by least squares fits of Scatchard plots or by the nonlinear curve fitting program LIGAND (Munson and Rodbard 1980). Inhibition curves were analyzed by LIGAND. In all cases the data summarized in the tables are the result of simultaneously analyzing three identical experiments (on different membrane preparations). The SEMs given are estimates calculated by the computer program and are not meant to be used in statistical tests. The two site model was accepted over the one site model when the F value based on the "extra sum of squares" principle (Munson and Rodbard 1980) was significant at the $P<0.05$ level. NEM-pretreated membranes were prepared by incubating membranes for 10 min (37 °C) with 3 mM NEM + 0.1 mM Gpp(NH)p (same conditions listed above) followed by the addition of 3 mM DTT. The membranes were subsequently diluted and inhibition experiments in the presence of 0.1 mM Gpp(NH)p were performed. Bound and free ligand were separated by filtration through Whatman GF/A filters. Filter papers for experiments using [^{3}H]XAC were presoaked in 0.3% polyethylenimine for 0.5 h. Filtration was performed on a modified cell harvester (Brandel, Gaithersburg, Md., USA). Filters were washed 3 times with 5 ml aliquots of ice-cold buffer (5 mM glycylglycine pH 7.5, 1 mM $MgSO_4$). Theophylline (5 mM) was used to define non-specific binding.

Results

Binding of [^{3}H]XAC to adenosine receptors in cardiac and cortical membranes

Our initial experiments were performed with [^{3}H]XAC. The binding of [^{3}H]XAC to adenosine receptors in both cardiac and cortical membranes required 2 h to reach equilibrium (data not shown). [^{3}H]XAC bound to adenosine receptors in bovine cardiac membranes with high affinity and good specificity. The K_d calculated from linear Scatchard plots was 0.14±0.05 nM (mean±SEM, $n = 9$), the B_{max} was 139±19 fmol/mg. In twelve similar experiments on cerebral cortical membranes the K_d and B_{max} values calculated from linear Scatchard plots were 0.10±0.02 nM and 581±48 fmol/mg, respectively.

Inhibition of [^{3}H]XAC binding by adenosine receptor agonists and antagonists

Table 1 summarizes the results of our initial series of experiments in which the affinities of a series of agonists and antagonists for adenosine receptors in cardiac and cerebral cortical membranes were estimated based on their ability to inhibit [^{3}H]XAC binding. Panel A of Fig. 1 shows representative inhibition curves for R-PIA and NECA. As expected, the inhibition curves of the antagonists theophylline and XAC were monophasic and single K_d values were calculated. All the agonist inhibition curves were better fit by a a two site model ($P < 0.05$) and two K_d values for each compound were calculated. (We have designated these two K_d values as K_H and K_L as is common in the literature. This nomenclature is used because these K_d values are often interpreted as measures of the affinities of the agonists for coupled (K_H) and uncoupled (K_L) states of the receptor. As discussed below, this is not necessarily true). Note that the N^6-modified analogs appeared to differentiate between the

cardiac and cortical receptors (K_Ls for sites in cardiac membranes > K_{LS} in cortical membranes) while the 5′-modified analogs (NECA and NCCA) behaved similarly in the two preparations.

Inhibition of [^{3}H]XAC binding by adenosine receptor agonists after NEM-pretreatment

The analyses presented in Table 1 are based on a model in which the antagonist binds to all species binding sites with equal affinity and the multiple “sites” for competing ligands are due to multiple independent, noninteracting sites. Extensive work from our laboratory subsequently performed on bovine brain membranes showed that antagonists recognize different states of the receptor with different affinities and that much more complex analyses must be performed in order to characterize the system (Leung et al. 1990). Furthermore, we realized that even if we could perform similar experiments on cardiac membranes, (which we probably could not do because of the paucity of receptors in the cardiac membranes) it was questionable if this approach would be useful for probing for differences between cardiac and brain A_1 adenosine receptors. We therefore decided to interrupt all receptor: G protein interactions so that the system would be simplified and could be legitimately analyzed using the independent site model. We use NEM-treatment to uncouple all receptors as we had previously shown that this treatment, but not the addition of 0.1 mM (Gpp(NH)p alone, accomplishes this goal (Leung et al. 1990). As we were interested in the behaviors of N^6- and 5′-modified adenosines we studied two N^6-substituted analogs, R-PIA and IABA, and two 5′-substituted compounds, NECA and NCCA. Representative inhibition curves for R-PIA and NECA performed under these conditions are shown in panel B of Fig. 1. The analyses of these experiments are summarized in Table 2. In all cases the analyses of the inhibition curves performed on cortical membranes indicated the presence of a single site. The inhibition curves performed on cardiac membranes were better fit by a two site model. Note that we denote the two K_ds as K_1 and K_2, not K_H and K_L, as these values are interpreted to be measures of the affinities of the agonists for separate binding sites. (It is obvious that the estimates of the lower affinity K_d values are very imprecise. This is partially due to the relatively high nonspecific binding of [^{3}H]XAC as the error estimates in the experiments using [^{3}H]CPX were much lower (see below), and to the error model used in these analyses. We have used the error model used in the original paper by Munson and Rodbard (1980), i.e., constant percent error in the dependent variable. While addition of a constant error term usually reduced inordinately large standard error estimates, the means were little affected and we have used the constant percent error model throughout). Several points should be noted. Firstly, the apparent higher affinity K_d values for the N^6-substituted analogs were similar to the single values determined in the cortical membranes. Secondly, the percentage of sites with the higher affinity K_d was much higher for the N^6-analogs as compared to the 5′-analogs. Lastly, the lower affinity site for the 5′-analogs appeared to correspond to the single site present in the cortical membranes, i.e., the site in the cardiac membranes detected by the 5′-analogs with higher affinity was not detected in the cortical membranes.

Inhibition of [^{3}H]CPX binding by adenosine receptor agonists after NEM-pretreatment

The presence of two sites in NEM-pretreated cardiac membranes could be explained by the presence of A_2 receptors in these membranes if these receptors have sufficient affinity for

[^{3}H]XAC to be detected under the conditions employed. That this could be the case was suggested by the report that [^{3}H]XAC binds to A_2 adenosine receptors in rabbit striatum (Ji et al. 1991). Inhibition experiments with [^{3}H]XAC and CPX (an antagonist with reportedly greater A_1-selectivity (Lohse et al. 1987) in control membranes modeled to a single site in cortical membranes (K_d 0.09 ± 0.02 nM, $n = 3$) while those in cardiac membranes modeled to two sites (K_1 0.15 ± 0.04 nM (89% of total sites); K_2 2045 ± 1439 nM, $n = 3$). These results suggested that the cardiac membranes may contain a small population of A_2 adenosine receptors that, under the experimental conditions employed, bind [^{3}H]XAC with high affinity and adenosine receptor agonists with low affinity. We therefore performed a final series of experiments on NEM-pretreated membranes using [^{3}H]CPX. Representative inhibition curves for R-PIA and NECA are shown in Panel C of Fig. 1. The analyses of these experiments are summarized in Table 3. Under these conditions the estimates of the lower affinity K_d values for the 5′-modififed analogs were more precise and more closely approximated those in the cortical membranes (Table 2). The lower affinity site detected by the N^6-modified analogs was either absent (IABA) or still present albeit with a greatly reduced K_d (R-PIA). Most importantly, this last series of experiments confirmed the presence of a site in the cardiac membranes that bind 5′ analogs with high affinity.

Discussion

Previous work from this and other laboratories has suggested that the adenosine receptors in the heart have ligand binding characteristics similar (Linden et al. 1985; Hosey et al. 1984, Lohse et al. 1985) but perhaps not identical to (Leung et al. 1988) the A_1 receptor found in the CNS. The experiments reported herein were performed to further compare and contrast the A_1 receptors present in bovince cardiac and cerebral cortical membranes.

Equilibrium binding studies showed that [^{3}H]XAC binds to adenosine receptors in cardiac membranes and cerebral cortical membranes with high affinity and good specificity. Although the number of sites in the cardiac membranes was only about 25% of that in the cerebral cortical membranes, the specific binding at about the K_d was more than twice nonspecific binding. Under the conditions employed the total and nonspecific cpm were in the range of 600–1000 and 200–400, respectively. It thus appeared that this ligand would be useful for the characterization of cardiac adenosine receptors. The K_ds for [^{3}H]XAC binding to adenosine receptors in cardiac and cortical membranes were similar. (It should be noted that these experiments were performed before the utility of [^{3}H]CPX for studying cardiac A_1 adenosine receptors was reported (Liang 1989; Lohse et al. 1987; Tawfik-Schlieper et al. 1989).

Previous reports on neuronal A_1 receptors have suggested that agonists bind to these receptors with multiple affinities while antagonists bind to all receptors with a single affinity (Bruns et al. 1987; Jacobson et al. 1986; Lohse et al. 1984; Yeung and Green 1983). Similar results are obtained with other receptors that couple to their effectors via G proteins (reviewed in Leung et al. 1990). While mechanistically the multiple agonist affinity states are believed to be due to the agonist differentiating between G-protein coupled and “free” receptors such that some form of a ternary complex model (Wreggett and DeLean 1984) is operational, computer programs such as LIGAND (Munson and Rodbard 1980) which are

based on an independent site model are commonly used to analyze inhibition curves generated in studies on these receptors. The results in Table 1 summarize data obtained with this approach using the antagonist radioligand[^{3}H]XAC. As expected the antagonists inhibited [^{3}H]XAC with a single apparent affinity while the inhibition curves of the agonists were better fit by a two site model. These data suggested that the cardiac and cortical A_1 receptors differ in that the K_L values of the N^6-analogs but not 5′-modified analogs are significantly larger in the heart as compared to the brain.

After performing the experiments and subsequent analyses summarized in Table 1 we became concerned about the appropriateness of the model used in these analyses. Work from other laboratories had shown that analyses with the independent site model does not always give valid estimates of the affinities of the competing ligand for the coupled and uncoupled forms of the receptor proposed to exist with the ternary complex model (Wreggett and DeLean 1984; Abramson et al. 1987). This led us to further studies on bovine cortical A_1 receptors which have been reported elsewhere (Leung and Green 1989; Leung et al. 1990). These studies suggested that: (1) the K_L determined from experiments such as those summarized in Table 1 is not the K_d of the agonist for the free receptor, (2) agonists form two types of high affinity complexes with receptors in cortical membranes (this observation was equally well explained by the presence of more than one type of receptor, or by the presence of one type of receptor that couples to more than one type of G-protein), and (3) antagonist ligands differentiate between free and coupled receptors, the K_d for the binding to free receptors being more favorable. Furthermore, these experiments suggested that the affinities of unlabeled compounds for the free A_1 receptor can be determined from inhibition curves of antagonist radioligand binding to receptors in NEM-pretreated membranes. We therefore performed a second series of inhibition experiments using [^{3}H]XAC and NEM-pretreated membranes (Table 2). A high concentration of Gpp(NH)p was also included in an effort to assure that all coupling of A_1 receptors was blocked. Under these conditions all of the curves for agonist inhibition of ligand binding to receptors in cortical membranes were monophasic. The apparent K_d values, which we interpret as estimates of the affinities of the agonists for free cortical A_1 receptors, were all greater than the K_L values determined in the untreated membranes (Table 1). It should also be noted that these results suggested the presence of a single type of A_1 adenosine receptor, they do not eliminate the possibility that the cortical membranes contain multiple subtypes of A_1 adenosine receptors that recognize the compounds studied with similar affinities.

Agonist inhibition of [^{3}H]XAC binding to NEM-pretreated cardiac membranes remained complex. The majority of the binding (79%-90%) of the compounds with N^6-substitutions (R-PIA and IABA) bound with apparent K_d values similar to those determined in the NEM-pretreated cortical membranes. Interestingly, it was the lower affinity component of the binding of the 5′-modified analogs (NECA and NCCA) that more closely approximated the K_d values determined in the NEM-pretreated cortical membranes.

The significance of the quantitatively minor site in cardiac membranes identified by the N^6-modified analogs was unclear. As [^{3}H]XAC had been demonstrated to bind to A_2 receptor in rabbit striatum (Ji et al. 1991), one possibility was that the low concentration of [^{3}H]XAC used binds to an A_2 receptor present in the cardiac membranes preparations. This would not

necessarily imply that cardiac cells per se contain A_2 adenosine receptors as they could easily arise from membranes from other cell types in the "cardiac" membrane preparations (Schrader and Londos 1983; Schutz et al. 1986). [^{3}H]XAC/CPX inhibition curves suggested that this may be the case in that a minor proportion of the sites identified by [^{3}H]XAC in the cardiac membranes was inhibited only by high concentrations of CPX. We therefore performed a final series of experiments with [^{3}H]CPX, and two N^6- and two 5′-modified analogs (Table 3). Under these conditions one N^6-analog, IABA, exhibited a single K_d, while the second, R-PIA, still exhibited an apparent second minor low affinity site. It is unclear whether or not this low affinity site is "real" or is an artifact of the analysis. More importantly, the K_ds of the 5′-modified analogs for the higher affinity site were unchanged while the apparent K_ds for binding to the lower affinity site decreased to more closely approximate those determined in the cortical membranes. Thus, while it appears that the data obtained with [^{3}H]XAC and [^{3}H]CPX differed quantitatively, perhaps due to [^{3}H]XAC binding to a minor population of A_2 receptors in the cardiac membranes preparations, the results obtained with the two antagonist radioligands did not differ qualitatively. These results therefore suggest that the cortical and cardiac membranes both contain an A_1 receptor subtype that binds N^6 but not 5′-substituted analogs with high affinity, and that the cardiac membranes but not the cortical membranes contain an additional A_1 receptor subtype that binds both N^6 and 5′-substituted analogs with high affinity. The estimated affinities of the 5′-analogs for the putative A_1 subtypes in cardiac membranes are calculated using a single K_d for [^{3}H]CPX. While these values would be differentially affected if, in fact, [^{3}H]CPX binds to the putative receptor subtypes with different affinities, Scatchard plots of [^{3}H]CPX binding to NEM-pretreated cardiac membranes were linear suggesting that [^{3}H]CPX does not significantly differentiate between the putative receptor subtypes.

It should be noted that the utility of using NEM treatment to block receptor: G (specifically G_i/G_o) protein interactions so that ligand binding to free, uncoupled receptors can be unambiguously studied is predicated on the assumption that NEM does not modify the receptor itself so as to modify its ligand binding characteristics. This assumption appears to be valid for A_1 adenosine receptors as NEM does not affect the binding of [^{3}H]CPX to A_1 receptors purified from rat testes (Nakata 1990) or chicken brain (R. D. Green, unpublished data). While it could be argued that we have not tested the effect of NEM on purified cardiac A_1 receptors, such an effect would require that the cardiac receptor differ from that in the brain, a requirement in agreement with the proposed existence of subtypes of cardiac A_1 receptors.

Acknowledgments

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Abbreviations

[^{125}I]ABA	[^{125}I]N^6-p-aminobenzyl)adenosine
[^{3}H]CPX	[^{3}H]8-cyclopentyl-1,3-dipropylxanthine
[^{3}H]R-PIA	[^{3}H]N^6-R-phenylisopropyladenosine

[^{3}H]XAC	[^{3}H]xanthine amine congener
CHA	N^6-cyclohexyladenosine
EDTA	ethylenediaminetetraacetic acid
[^{125}I]BW-A844U	[^{125}I]3-(4-amino)phenethyl-1-propyl-8-cyclopentylxanthine
NCCA	5′-N-cyclopropylcarboxamide adenosine
NECA	5′-N-ethylcarboxamide adenosine
PMSF	phenylmethylsulphonyl fluoride
NEM	N-ethylmaleimide

References

Abramson SN, McGonigle P, Molinoff PB. Evaluation of models for analysis of radioligand binding data. Mol Pharmacol. 1987; 31:103–111. [PubMed: 3027522]

Asano T, Ogasawara N. Uncoupling of γ-aminobutyric acid B receptors from GTP-binding proteins by N-ethylmaleimide: Effect of ethylmaleimide on purified GTP-binding proteins. Mol Pharmacol. 1986; 29:244–249. [PubMed: 3005832]

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 1976; 72:248–254. [PubMed: 942051]

Bruns RF, Fergus JH, Badger EW, Bristol JA, Santay LA, Hartman JD, Hays SJ, Huang CC. Binding of the A_1-selective adenosine antagonist 8-cyclopentyl-l,3-dipropylxanthine to rat brain membranes. Naunyn-Schmiedeberg's Pharmacol. 1987; 335:59–63.

Drury AN, Szent-Györgi A. The physiological activity of adenine compounds with special reference to their actions upon the mammalian heart. J Physiol (Lond). 1929; 68:213–237. [PubMed: 16994064]

Flockerzi V, Mewes R, Ruth P, Hofmann F. Phosphorylation of purified bovine cardiac sarcolemmma and potassium-stimulated calcium uptake. Eur J Biochem. 1983; 135:131–142. [PubMed: 6309517]

Hosey MM, McMahon KK, Green RD. Inhibitory adenosine receptors in the heart: Characterization by ligand binding studies and effects of β-adrenergic receptor stimulated adenylate cyclase and membrane protein phosphorylation. J Mol Cell Cardiol. 1984; 16:931–941. [PubMed: 6151002]

Jacobson KA, Ukena D, Kirk KL, Daly JW. (^{3}H]-Xanthine amine congener of l,3-dipropyl-8-phenyIxanthine: an antagonist radioligand for adenosine receptors. Proc Natl Acad Sci USA. 1986; 83:4089–4093. [PubMed: 3012550]

Ji X-D, Stiles GL, Jacobson KA. [^{3}H]XAC (xanthine amine congener) is a radioligand for A_2-adenosine receptors in rabbit striatum. Neurochem Internat. 1991; 18:207–213.

Leung E, Kwatra MM, Hosey MM, Green RG. Characterization of cardiac A_1-adenosine receptors by ligand binding and photoaffinity labelling. J Pharmacol Exp Ther. 1988; 244:1150–1156. [PubMed: 3252029]

Leung E, Green RD. Density gradient profiles of A_1 adenosine receptors labeled by agonist and antagonist radioligands before and after detergent solubilization. Mol Pharmacol. 1989; 36:412–419. [PubMed: 2506430]

Leung E, Jacobson KA, Green RD. Analysis of agonist: antagonist interactions at A_1 adenosine receptors. Mol Pharmacol. 1990; 38:72–83. [PubMed: 2115114]

Liang BT. Characterization of the adenosine receptor in cultured embryonic chick atrial myocytes: Coupling to modulation of contractility and adenylate cyclase activity and identification by direct ligand binding. J Pharmacol Exp Ther. 1989; 249:775–784. [PubMed: 2732946]

Linden J, Patel A, Sadek S. [^{125}I]Aminobenzyladenosine, a new radioligand with improved specific binding to adenosine receptors in the heart. Circ Res. 1985; 56:279–284. [PubMed: 2982516]

Lohse MJ, Lenschow V, Schwabe U. Two affinity states of R_i adenosine receptors in brain membranes. Naunyn-Schmiedeberg's Arch Pharmacol. 1984; 26:1–9.

Lohse MJ, Ukena D, Schwabe U. Demonstration of R_i-type adenosine receptors in bovine myocardium by ligand binding. Naunyn-Schmiedeberg's Arch Pharmacol. 1985; 328:310–316. [PubMed: 2984586]

Lohse MJ, Klotz K-N, Lindenborn-Fotinos J, Reddington M, Schwabe U, Olsson RA. 8-cyclopentyl-l, 3-dipropylxanthine (DPCPX)-a selective high affinity agonist radioligand for A_1 adenosine receptors. Naunyn-Schmiedeberg's Arch Pharmacol. 1987; 336:204–210. [PubMed: 2825043]

Munson PJ, Rodbard D. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem. 1980; 107:220–239. [PubMed: 6254391]

Nakata H. A_1 receptor of rat testes membranes. J Biol Chem. 1990; 265:671–677. [PubMed: 2104829]

Patel A, Craig RH, Daluge SM, Linden J. ^{125}I-BW-A844U, an antagonist radioligand with high affintiy and selectivity for adenosine A_1 receptors, and ^{125}I-Azido-BW-A844U, a photoaffinity label. Mol Pharmacol. 1988; 33:585–591. [PubMed: 3380075]

Schrader J, Londos C. Is there a highly purified cardiac sarcolemma preparation? Naunyn-Schmiedeberg's Arch Pharmacol. 1983; 322:R14. (abstr).

Schutz W, Freissmuth M, Hausleithner V, Tuisl E. Cardiac sarcolemmal purity is essential for the verification of adenylate cyclase inhibition via A_1-adenosine receptors. Naunyn-Schmiedeberg's Arch Pharmacol. 1986; 333:156–162. [PubMed: 3018595]

Tawfik-Schlieper H, Klotz K-N, Kreye VAW, Schwabe U. Characterization of the K^+-channel-coupled adenosine receptor in guinea pig atria. Naunyn-Schmiedeberg's Arch Pharmacol. 1989; 340:684–688. [PubMed: 2615858]

Wreggett KA, De Lean A. The ternary complex model: Its properties and application to ligand interactions with the D_2-dopamine receptor of the anterior pituitary gland. Mol Pharmacol. 1984; 26:214–227. [PubMed: 6237254]

Yeung S-MH, Green RD. Agonist and antagonist affinities for inhibitory adenosine receptors are reciprocally affected by 5′-guanylyl-imidodiphosphate and N-ethylmaleimide. J Biol Chem. 1983; 258:2334–2339. [PubMed: 6296141]

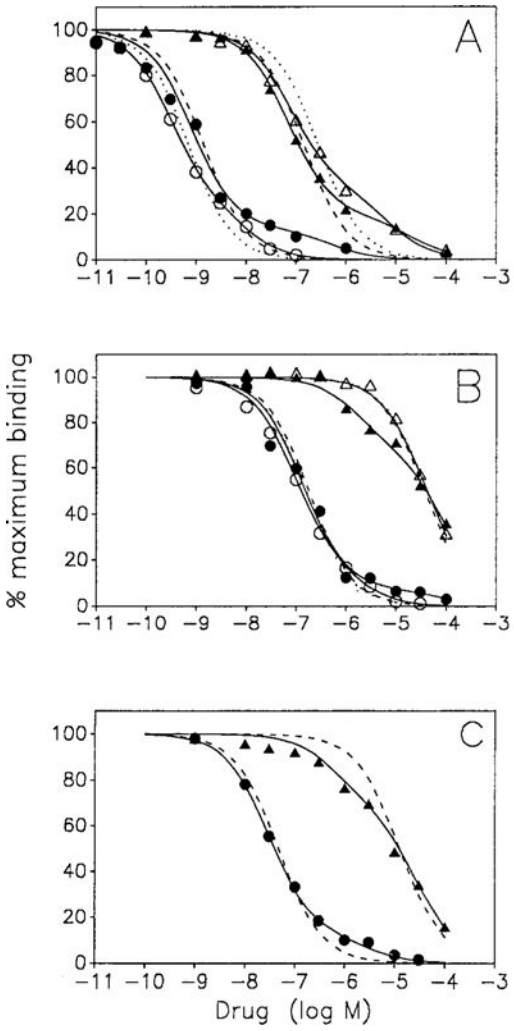

Fig. 1.

A–C. Inhibition curves for antagonist radioligand binding by R-RIA (*circles*) and NECA (*triangles*) to sites in cortical (*open symbols*) and cardiac (*closed symbols*) membranes. Panel A [^{3}H]XAC, control membranes. Panel B [^{3}H]XAC, NEM-pretreated membranes in presence of 0.1 mM Gpp(NH)p. Panel C [^{3}H]CPX: NEM-pretreated membranes in presence of 0.1 mM Gpp(NH)p. *Points* shown are averages of duplicate determinations. Nonspecific binding as defined by theophylline (5 mM) is substracted from all data. The two site fits are shown as *solid tines.* One site fits are shown as – – – (heart) or ⋯ (Brain)

Table 1

Inhibition of [^{3}H]XAC[a] binding by adenosine receptor agonists and antagonists in bovine cortical and cardiac membranes. K_H and K_L are the dissociation constants for the high and low affinity states determined by LIGAND. %H is the percentage of high affinity binding sites. Values are means ± SEM for 3 experiments

Compound	Cortex			Heart		
	K_H, nM	K_L, nM	%H	K_H, nM	K_L, nM	%H
R-PIA	0.07 ± 0.02	4.5 ± 2.1	81 ± 7	0.36 ± 0.16	4000 ± 1600	57 ± 7
S-PIA	1.30 ± 0.40	140.0 ± 40.0	83 ± 7	5.30 ± 2.20	5500 ± 2200	61 ± 6
CHA	0.14± 0.04	8.3 ± 3.1	76 ± 6	0.67 ± 0.44	5200 ±3200	66 ±11
IABA	0.21 ± 0.11	18.0 ± 12.9	78 ±10	0.40 ± 0.21	4700 ±2700	65 ± 9
NECA	13.0 ± 5.2	700 ± 400	74 ± 10	14.7 ± 16.0	900 ± 600	41 ± 14
NCCA	5.3 ± 5.5	1376 ± 886	51 ± 13	3.8 ± 7.6	1000 ± 800	41 ± 25
Theophylline	9600 ± 1400	–	–	32500 ± 7900	–	–
XAC	0.17 ± 0.01	–	–	0.20 ± 0.07	–	–

[a][^{3}H]XAC was present in concentrations of 0.06 – 0.07 nM for cortical membranes and 0.15–0.25 nM for cardiac membranes

Table 2

Dissociation constants of adenosine receptor agonists for binding to sites present in NEM-pretreated cortical and cardiac membranes estimated by inhibition of [^{3}H]XAC[a] binding

Compound	Cortex[b]	Heart	
	Kd, nM	K_1, nM (% total)	K_2, nM (% total)
R-PIA	26 ± 6	17 ± 9 (86%)	28 248 ± 30322 (14%)
IABA	55 ± 10	98 ± 44 (90%)	15480 ± 17421 (10%)
NECA	6329 ±347	124 ± 80 (36%)	16155 ± 3836 (64%)
NCCA	6329 ± 336	107 ± 68 (33%)	12121 ± 2159 (67%)

[a] [^{3}H]XAC was present in concentrations of 0.6 – 0.7 nM for both cortical and cardiac membranes. The Kd values of [^{3}H]XAC under these conditions was 0.20 ± 0.0.02 nM (B_{max} 101 ± 14 fmol/mg, *N* = 3) for cardiac membranes and 0.09 ± 0.01 nM (B_{max} 780 ± 75 fmol/mg, *N=3*) for cortical membranes

[b] Inhibition curves were monophasic giving a single Kd value

Table 3

Dissociation constants of adenosine receptor agonists for binding to sites present in NEM-preteated cardiac membranes estimated by inhibition of [^{3}H]CPX[a] binding

Compound	K_1, nM (% total)	K_2, nM (% total)
R-PIA	5 ± 3 (84%)	152 ± 96 (16%)
IABA	25 ± 4 (100%)	–
NECA	89 ± 44 (30%)	4016 ± 398 (70%)
NCCA	110 ± 66 (24%)	4049 ± 354 (76%)

[a]The K_d for [^{3}H]CPX was 0.12 ± 0.01 nM (B_{max} 100 ± 8 fmol/mg; N = 3). 0.6 nM [^{3}H]CPX was used in the inhibition experiments