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The binding of [^{3}H]R-PIA to A_1 adenosine receptors produces a conversion of the high- to the low-affinity state

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Kinetic evidence for negative cooperativity on the binding of [^{3}H]R-PIA to A_1 adenosine receptors was obtained from dissociation experiments at different ligand concentrations and from the equilibrium isotherm. The dissociation curves indicate that there is an apparent ligand-induced transformation of high- to low-affinity states of the receptor. At concentrations of 18.2 nM R-PIA or higher there was only found the low-affinity state of the receptor. In view of these results equilibrium binding data were analyzed by the usual two-state model (assuming that there is an interconversion between them) and by the negative cooperativity model employing the Hill equation.

Adenosine receptor; Affinity state; Negative cooperativity; Pig brain

1. INTRODUCTION

From results obtained in different laboratories in recent years (see [1–4]), it appears evident that the A_1 adenosine receptor has two affinity states. The nomenclature has been established according to the relative affinity for adenosine agonists. The high-affinity species displays a K_d for [^{3}H]R-PIA around 0.2 nM whereas the K_d ([^{3}H]R-PIA) for the low-affinity center is one order of magnitude greater. It is generally agreed that the high-affinity receptor molecule is coupled to a regulatory G protein whereas the low-affinity species is uncoupled. In a previous report [4] we have demonstrated that the detection of both affinity forms requires the integrity of the membrane structures. Thus, for instance, treatment with detergents leads to one single type or soluble or membrane-bound species which displays high-affinity [^{3}H]R-PIA.

In this paper we demonstrate that the binding of the agonist [^{3}H]R-PIA provokes the apparent conversion from the high- to the low-affinity state. In view of these data the equilibrium isotherm was studied and simulation studies show that the results can be explained by assuming an equilibrium between forms which is modified by the agonist. Kinetically the overall results indicate negative cooperativity and, obviously, the equilibrium binding isotherm can be fitted to the Hill equation.

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2. EXPERIMENTAL

2.1. *Materials*

[adenine-2,8-^{3}H, ethyl-2-^{3}H]-N^6-Phenylisopropyladenosine ([^{3}H]R-PIA; 42.5 Ci/mmol) was purchased from New England Nuclear Research Products (Boston, MA, USA). N^6-(*R*) Phenylisopropyladenosine (R-PIA) and adenosine deaminase (EC 3.5.4.4) were obtained from Boehringer Mannheim (Germany). All other commercial compounds were purchased from Merck (Darmstadt, Germany) or Sigma Chemical Co. (St. Louis, MO, USA).

Pig brain cortical membranes were obtained as described elsewhere [4,5].

2.2. *Methods*

Protein was measured by the method of Lowry et al. [6] using bovine serum albumin as standard. [^{3}H]R-PIA binding to membranes (saturation isotherm) was performed as previously described [5]. Association-dissociation experiments were carried out as described by Casadó et al. [4], employing various ligand concentrations for association and a 300-fold excess of non-labelled R-PIA for dissociation.

2.3. *Analysis of binding data*

The individual saturation isotherms were analyzed by non-linear regression using the ENZFITTER program (Elsevier Biosoft) or other available programs [7–9] as described elsewhere [4,5]. Association-dissociation curves were fitted to data using the same nonlinear regression programs using the equations described by Casadó et al. [4]. Other published programs may be also used for this kind of analysis [10]. Five replicates of each point were performed and no further assumptions about errors were made.

Goodness of fit was tested according to the reduced χ^2 or SD values given by the programs. Modified *F*-test was used to analyze whether the fit to the two-state model significantly improved on the fit to the one-state model. The equation applied in this test is the following:

$$F = df_2\,(SS_1 - SS_2)/SS_2\,(df_1 - df_2)$$

where SS_1 and SS_2 are residual sums of squares with corresponding degrees of freedom df_1 and df_2, associated with the simpler and more complex model, respectively. The *F* values were calculated using df_1–df_2 degrees of freedom in the numerator and df_2 degrees of

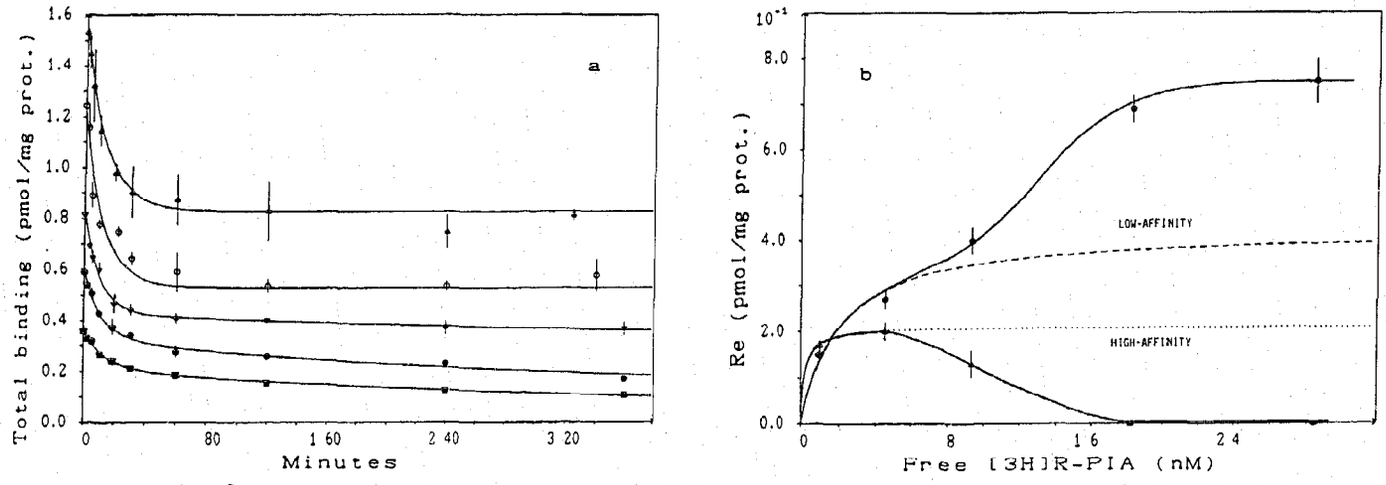

Fig. 1. Dissociation of [^{3}H]R-PIA to pig brain cortical membranes at different ligand concentrations used for association. (a) Dissociation curves corresponding to the different R-PIA concentrations. Membranes (0.7 mg protein/ml) were incubated with adenosine deaminase (0.2 IU/ml) for 30 min at 25°C in 50 mM Tris-HCl buffer, pH 7.4. Addition of 0.9 (■), 4.5 (●), 9.3 (▼), 18.2 (○) and 28.4 (▲) nM [^{3}H]R-PIA prepared in the same buffer were then made. After standing at 25°C for 2 h, dissociation was induced by addition of cold R-PIA to a final concentration of 300-fold greater than the radioligand concentration. At different time intervals, 500 μl aliquots were taken for filtration and subsequent analysis as indicated previously [4]. Nonspecific binding was determined in a separate sample with the simultaneous addition of cold R-PIA at zero time of radioligand association. All points represent mean ± SE of four replicates. The computer lines drawn correspond to the best fit obtained using the dissociation equations (one-site or two-sites) previously reported [4]. The goodness of the fit was tested as indicated in Materials and Methods. (b) Evolution of the binding at equilibrium (R_e) of the high- (▲) and low-affinity (●) components. This figure is derived from data shown in (a). Discontinuous curves are the theoretical curves assuming no interconversion among affinity states: (•••) high-affinity; (---) low-affinity. Upon increasing the R-PIA concentration used for association, 'high-affinity binding' disappears while 'low-affinity binding' appears.

freedom in the denominator [11]. In all cases it was considered that the two-site model led to a significant improvement over the one-site model when $P<0.001$. When no significant improvement over the one-site model was detected, the P values were greater than 0.30.

3. RESULTS AND DISCUSSION

Association of [^{3}H]R-PIA to cortical membranes was performed using different ligand concentrations; dissociation was induced by adding an excess of non-labelled R-PIA (Fig. 1). The dissociation curves of 18.2 and 28.4 nM [^{3}H]R-PIA are qualitatively different from the other since the horizontal asymptote is reached very soon. Data in Table I confirm this impression. First, the results obtained with 0.9 and 4.5 nM [^{3}H]R-PIA are very similar except for the obvious increase of R_e (binding at equilibrium) of the low-affinity state since at 0.9 nM [^{3}H]R-PIA this state was far from saturation (at 0.9 nM the high-affinity center is likely to be saturated). On the other hand, the results obtained by the dissociation of 18.2 and 28.4 nM [^{3}H]R-PIA are very revealing. Briefly, the high-affinity species was not detected. The R_e of the low-affinity increased as a con-

Table I

Kinetic parameters of [^{3}H]R-PIA dissociation from pig brain cortical membranes

Association conditions		Dissociation kinetic parameters					
		High affinity state			Low affinity state		
[[^{3}H]R-PIA] (nM)	Time (min)	k_{-1} (min^{-1})	R_e (pmol/mg prot.)	%[a]	k_{-1} (min^{-1})	R_e (pmol/mg prot.)	%[a]
0.9	120	0.0024 ± 0.0003	0.17 ± 0.01	53	0.07 ± 0.01	0.15 ± 0.01	47
4.5	30	0.006 ± 0.001	0.17 ± 0.02	44	0.14 ± 0.03	0.21 ± 0.02	56
	120	0.0033 ± 0.0008	0.20 ± 0.02	43	0.08 ± 0.01	0.27 ± 0.02	57
	240	0.0030 ± 0.0006	0.23 ± 0.03	48	0.06 ± 0.01	0.25 ± 0.02	52
9.3	120	0.002 ± 0.001	0.13 ± 0.03	25	0.09 ± 0.02	0.40 ± 0.03	75
18.2	120	–	–	0	0.08 ± 0.01	0.69 ± 0.03	100
28.4	10	–	–	0	0.07 ± 0.02	0.77 ± 0.07	100
	120	–	–	0	0.07 ± 0.01	0.75 ± 0.05	100

The values were obtained from dissociation experiments according to the equations previously described [5]. The experimental conditions are those described in Fig. 1. Values are means ± SD of five replicates.

[a] Percentages of low- and high-affinity centers are calculated with respect to the total binding for each R-PIA concentration, 100% corresponding to the sum of binding to high- and low-affinity sites, i.e. R_e for the low plus R_e for the high.

sequence of the conversion of high-affinity species to low-affinity sites (see Fig. 1b). By means of simulation, the possibility of a masking of the high-affinity site when using 18.2 or 28.4 nM [^{3}H]R-PIA and by the usual ways of fitting dissociation data, can be discarded (data not shown). It should be emphasized that, for a given ligand concentration, the distribution of high- and low-affinity species is time-independent. Thus the presence of two affinity states when 4.5 nM [^{3}H]R-PIA is used, is found irrespective of whether the association time is 30, 120 or 240 min. In the case of 28.4 nM [^{3}H]R-PIA the dissappearance of the high-affinity state is already evident at 10 min of association and the kinetic parameter (k_{-1}) for the single low-affinity state found was similar at 10 and 120 min (Table I).

Fig. 2 corresponds to an equilibrium binding isotherm (Fig. 2a) with its corresponding Scatchard plot (Fig. 2b) obtained after fitting data to two affinity states (see Fig. 2b) and parameter values therein). In the case of an enzyme, or even in the case of the insulin receptor, the appearance of Scatchard plots like that displayed in Fig. 2b may suggest the convenience of the fitting of data to the Hill equation in order to obtain the Hill coefficient. The results of the fit to the Hill equa-

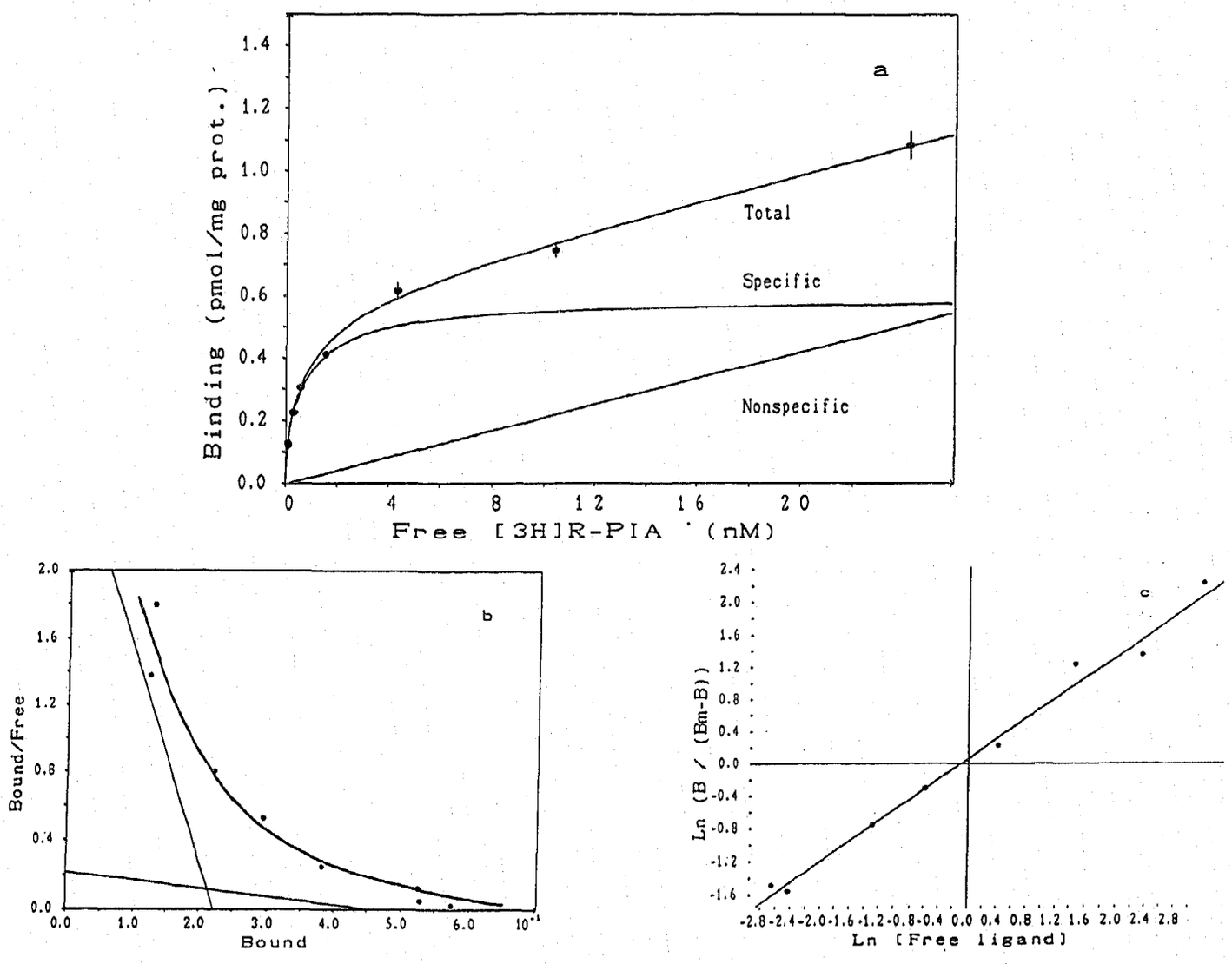

Fig. 2. Equilibrium binding isotherm curve of [^{3}H]R-PIA binding to pig brain cortical membranes. Membranes (0.7 mg protein/ml) were incubated with adenosine deaminase (0.2 IU/ml) for 30 min at 25°C in 50 mM Tris-HCl buffer, pH 7.4. Addition of [^{3}H]R-PIA prepared in the same buffer was then made. After standing at 25°C for enough time to achieve equilibrium for each ligand concentration used, free and bound ligand were separated for filtration and subsequent analysis as indicated previously [4,5]. All points represent the mean ± SE of five replicates. The computer lines drawn correspond to the best fit obtained using the equations previously described [4] or the Hill equation. The goodness of the fit was tested as indicated in Materials and Methods. (a) The equilibrium binding isotherm. (b) Scatchard plot of the computer derived specific binding data. (c) Hill plot. Estimates of the parameters were; $R_H = 0.16$ pmol/mg prot. and $K_{dH} = 0.05$ nM for the high-affinity state and $R_L = 0.42$ pmol/mg prot. and $K_{dL} = 1.1$ nM for the low affinity state when data were fitted to two-site model or $R = 0.69$ pmol/mg prot., $K_d = 0.94$ nM and $n = 0.63$ when data were fitted to the Hill equation.

TABLE II

Fitting results from two different simulated data sets

	R range	Calculated R	True K_d (nM)	Calculated K_d (nM)
High-affinity center	0.5-0	0.42	0.1	0.066
Low affinity center	0.5-1	0.57	2	2.6
High-affinity center	1-0	0.68	0.1	0.053
Low affinity center	0-1	0.32	2	6.6

Data were simulated assuming the existence of two pre-existing affinity states of K_d values: 0.1 and 2 nM, and 1 pmol/mg protein of total maximum binding ($R_L + R_H$). Simulated points corresponding to saturation isotherms were obtained at 9 different ligand concentrations (0.01, 0.1, 0.2, 0.5, 1, 2, 5, 15 and 30 nM) but assuming that there was a different proportion of high-/low-affinity states for each ligand concentration (0.5/0.5, 0.5/0.5, 0.5/0.5, 0.4/0.6, 0.4/0.6, 0.35/0.65, 0.35/0.65, 0.2/0.8 and 0/1, respectively). Another simulation was performed for the same ligand concentrations but at the following high-/low-affinity state proportions: 1/0, 0.9/0.1, 0.8/0.2, 0.7/0.3, 0.6/0.4, 0.5/0.5, 0.4/0.4, 0.2/0.8 and 0/1, respectively. Simulated data were then fitted by means of a non-linear regression program (ENZFITTER) to both a one-site model and a two-site model (see [4]). The results correspond to the fitting of a simulated isotherm to two centers (the fitting to one center is statistically very poor).

tion appear in Fig. 2c (legend). As expected by the shape of Fig. 2b, the Hill coefficient is less than one; in one system displaying cooperativity this would correspond to negative cooperativity.

Experimental results corresponding to the R-PIA binding to A_1 adenosine receptors are usually fitted to the two-affinity state model. If the agonist modifies the distribution between low- and high-affinity states it is obvious that the results of the fit performed, assuming a two-site model, are incorrect. By means of simulation we have demonstrated that the fit to the two-state model, in the case of interconversion between states, would lead to reasonably good results which are comparable to those described in the literature. In this simulation, a concentration-dependent interconversion between high- ($K_d = 0.1$ nM) and low-affinity sites ($K_d = 2$ nM) was assumed, and simulated data were fitted to either one or two sites. The fit to one center was very poor. Fitting to two sites was very good and the statistical parameters of the fit were similar to those described in the literature and to those obtained when fitting the data of Fig. 2a to two affinity states. From the simulation results, summarized in Table II, it is evident that the actual equilibrium parameters for the two affinity states are different from the apparent ones; K_{dH} (apparent) is less than the true value whereas K_{dL} (apparent) is higher than the true K_{dL}.

An important consequence of these results is that all data based on the pre-existence of two affinity states must be re-evaluated. Apart from the negative cooperativity possibility, the ligand-induced interconversion between affinity states makes K_d and R_{max} values for agonists described in the literature for A_1 adenosine receptors incorrect. Discrimination between the two pre-existing affinity state model or the negative cooperativity is difficult. A similar controversy surrounds the putative negative cooperativity of binding of insulin to its receptor [12] even after the demonstration of the heterotetrameric structure of the receptor [13], which would lead to a possible explanation of the cooperative effect. In the case of adenosine the final answer must await the structural characterization of the receptor and the receptor-G protein complex.

Recently, computer analyses of agonist competition, using the antagonist [^{3}H]CPX as ligand, in membranes from control and R-PIA-treated myocytes, revealed a conversion of the high-affinity A_1 adenosine receptor to a low-affinity form; the conversion was total after 24 h of 1 μM R-PIA exposure [14]. Since in this paper we present evidence that this conversion induced by R-PIA is yet possible in isolated membranes, we agree with these authors in that the conversion may be part of the ligand-induced desensitization mechanism of A_1 adenosine receptor.

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REFERENCES

[1] Frame, L.T., Yeung, S.M.H., Venter, J.C. and Cooper, D.M.F. (1986) Biochem. J. 235, 621-624.
[2] Morgan, P.F. and Marangos, P.J. (1987) Dev. Brain Res. 35, 269-274.
[3] Ramkumar, V. and Stiles, G.L. (1988) J. Pharmacol. Exp. Ther. 246, 1194-1200.
[4] Casadó, V., Cantí, C., Mallol, J., Canela, E.I., Lluis, C. and Franco, R. (1990) J. Neurosci. Res. 26, 461-473.
[5] Casadó, V., Martí, T., Franco, R., Lluis, C., Mallol, J. and Canela, E.I. (1990) Anal. Biochem. 184, 117-123.
[6] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
[7] Canela, E.I. (1984) Int. J. Biomed. Comput. 15, 121-130.
[8] López-Cabrera, A., Cabré, F., Franco, R. and Canela, E.I. (1988) Int. J. Biomed. Comput. 23, 9-20.
[9] Canela, E.I., Canela, M.A. and López-Cabrera, A. (1990) Int. J. Biomed. Comput. 25, 7-20.
[10] Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107, 220-239.
[11] Hoyer, D., Reynolds, E.E. and Molinoff, P.B. (1984) Mol. Pharmacol. 25, 209-218.
[12] Levitzki, A. (1981) Nature 289, 442-443.
[13] Kahn, C.R. and White, M.F. (1988) J. Clin. Invest. 82, 1151-1156.
[14] Liang, B.T. and Donovan, L.A. (1990) Circ. Res. 67, 406-414.