

THE “TWO-STATE DIMER RECEPTOR MODEL”. A GENERAL MODEL FOR RECEPTOR DIMERS

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R-PIA: (-)-N⁶-(2-Phenylisopropyl)adenosine

[³H]R-PIA: [³H(G)] R-PIA

SCH 23390: (R)-(+)-7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine

[³H]SCH 23390: [N-methyl-³H] SCH 23390

SKF 38393: (+/-)-1-Phenyl-2,3,4,5-tetrahydro-(1H)-3-benzazepine-7, 8-diol

ABSTRACT

Non-linear Scatchard plots are often found for agonist binding to G-protein-coupled receptors. Since there are clear evidences of receptor dimerization these non-linear Scatchard plots can reflect cooperativity on agonist binding to the two binding sites in the dimer. According to this, the “two-state dimer receptor model” has been recently derived. In this paper the performance of the model has been analyzed in fitting data from A₁ adenosine receptors agonist binding, which is an example of receptor displaying concave downward Scatchard plots. Analysis of agonist/antagonist competition data for dopamine D₁ receptors using the “two-state dimer receptor model” has also been performed. Although fitting to the “two-state dimer receptor model” is similarly good than the fitting to the “two-independent-site receptor model”, the former is simpler and a discrimination test selects the “two-state dimer receptor model” as the best. This model was also very robust in fitting data of estrogen binding to the estrogen receptor, which display concave upward Scatchard plots. On the one hand, the model would predict the already demonstrated existence of estrogen receptor dimers. On the other hand, the model would predict that concave upward Scatchard plots reflect positive cooperativity, which cannot be neither predicted nor explained by assuming the existence of two different affinity states. In summary the “two-state dimer receptor model” is good for fitting data of binding to dimeric receptors displaying either linear, concave upward or concave downward Scatchard plots

INTRODUCTION

In 1983 Fuxe et al. (Fuxe et al., 1983) formulated the hypothesis about the existence of homodimers for different types of heptaspanning membrane receptors and in the same year Avissar et al. (Avissar et al., 1983) published the first demonstration of G protein-coupled receptor homodimers and homotetramers of muscarinic receptors. The authors suggested that the muscarinic receptor exists in oligomeric forms and that a dimer and tetramer may exist as interconvertible species. This was not seriously considered until ten year later when the demonstration of further receptor homodimers in cells expressing recombinant receptors and in membranes from mammalian brain came (Ciruela et al., 1995; Ng and George, 1994). Among others, D₁ and D₂ dopamine, A₁ and A_{2A} adenosine, muscarinic, peptide P, GABA, metabotropic glutamate, opioid, adrenergic, histamine, serotonin and chemokine receptors can be found as homodimers in living cells (Bouvier, 2001; Milligan and White, 2001; Agnati et al., 2003; Bai, 2004; Urizar et al., 2005 and references therein). For A_{2A} adenosine receptors and probably for other receptors, dimers are the physiological species that are activated by the physiological ligand, thus whereas monomers and dimers of A_{2A} adenosine receptors appear in living cells, the dimers are found predominantly on the cell surface (Canals et al., 2004). These data strongly suggest a key role of dimers in the nonlinear Scatchard plots found for ligand binding and in the mechanism of operation of heptaspanning receptors.

As occasionally speculated (Avissar et al., 1983; Wreggett and Wells, 1995; Lazareno et al., 1998; Trankle et al., 2003; Urizar et al., 2005), it is readily obvious that the available experimental evidence points out the impossibility of explaining the operation of heptaspanning receptors without considering dimers as the minimum structure for many heptaspanning receptors. This evidence has led us to revisiting the existing models to take into account dimers and to devise a novel model that includes dimers as basic units (Franco et al., 2005). This model, “the two-state dimer receptor model”, considers a ligand-induced conformational change from one component of the dimer is communicated to the other (Figure 1). The “two-state dimer

receptor model” is based on the communication between the two subunits of the receptor dimer. The model is an extension of the “two state model of receptor activation” but considering dimeric structures able to bind one molecule to the orthosteric centre in each monomer. Assuming receptor isomerization between inactive (R_2) and active (R_2^*) species, the model is able to explain the behaviour of heptaspanning membrane receptors. Negative or positive cooperativity is naturally explained by assuming that binding of the first ligand modifies the equilibrium parameters defining the binding of the second ligand molecule. Among other features, the two-state-dimer model predicts that cooperativity in the binding of all type of molecules to the orthosteric centre would depend on the degree of constitutive activity (Franco et al. 2005). In this paper we test the performance of the model using saturation binding and competition binding data for G-protein-coupled dimeric receptors and for dimeric receptor not coupled to G-proteins.

MATERIALS AND METHODS

Membrane preparation and protein determination

Membrane suspensions from lamb brain cortex or striatum were obtained as described previously (Casadó et al., 1990). Tissue was disrupted with a Polytron homogenizer (Kinematica, PTA 20 TS rotor, setting 3; Brinkmann Instruments, Westbury, NY) for three 5-sec periods in 10 volumes of 50 mM Tris-HCl buffer, pH 7.4. Tissue debris were separated by centrifugation ($900 \times g$, 10 min, 4°C). Membranes were then obtained by centrifugation at $105,000 \times g$ (40 min, 4°C) and the pellet was resuspended and recentrifuged under the same conditions. The pellet was stored at -80°C and was washed once more as described above and resuspended in 50 mM Tris-HCl buffer for immediate use.

Protein was quantified by the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL, U.S.A.) using bovine serum albumin as standard.

Radioligand binding experiments

Membrane suspensions from lamb brain cortex or striatum (0.2-0.3 mg of protein/ml) were incubated with increasing radioligand concentrations: triplicates of 8 different concentrations from 0.01 nM to 24 nM of the A₁ adenosine receptor agonist [³H]R-PIA (30.5 Ci/mmol, Moravék Biochemicals Inc, Brea California) or from 0.09 nM to 10.5 nM of the D₁ dopamine receptor antagonist [³H]SCH 23390 (85 Ci/mmol, Perkin Elmer Life Sciences, Boston, MA) at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ and for [³H]R-PIA binding, 0.2 U/ml adenosine deaminase (ADA, EC 3.5.4.4, Roche Diagnostics, S.L., Sant Cugat del Vallès, Spain). After enough time of incubation to achieve the equilibrium for the lowest radioligand concentration (5 h), free and membrane-bound radioligand were separated as described later. Non-specific binding was defined as the binding remaining in the presence of 50 μM R-PIA (SigmaAldrich Chemical Co.) (Casadó et al., 1990), or 50 μM SCH23399 (Tocris Cookson Ltd., Avonmouth, UK).

Competition experiments were performed by incubating (90 min) membranes from lamb brain striatum (0.3 mg of protein/ml) at 25°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM MgCl₂ and 0.2 U/ml adenosine deaminase with 1.2 nM of [³H]SCH23399 in the absence or presence of increasing concentrations (triplicates of 12 different competitor concentrations from 0.1 nM to 50 μM) of non-labelled D₁ dopamine receptor agonist SKF 38393 (Tocris Cookson Ltd., Avonmouth, UK). Non-specific binding was determined in the presence of 50 μM SCH23399.

In all cases, free and membrane-bound ligand were separated by rapid filtration of 500 μl aliquots in a Brandel (Gaithersburg, MD, U.S.A.) cell harvester through Whatman GF/C filters embedded in 0.3% polyethylenimine, which were subsequently washed in 5 seconds with 5 ml of ice-cold Tris-HCl buffer. The filters were incubated with 10 ml of Ecoscint H scintillation cocktail (National Diagnostics, Atlanta, GA, U.S.A.) overnight at room temperature and radioactivity counts were determined using a Packard 1600 TRI-CARB scintillation counter with an efficiency of 62% (Sarrió et al., 2000).

Data analysis

The specific binding data from the saturation or competition experiments were all analyzed by nonlinear regression, using the commercial Grafit curve-fitting software (Erithacus Software, Staines, Middlesex, UK). This software consists of an iterative procedure based on the Marquardt algorithm. This procedure allows the use of two or more independent variables (e.g., time and concentration), which was necessary for the analyses reported in this paper.

Results are given as parameter values \pm SEM and differences respect to controls have been tested for significance ($P < 0.05$) using Student's t-test for unpaired samples. Goodness of fit was tested according to reduced χ^2 value given by the non-linear regression program. The test of significance for two different model population variances was based upon the F-distribution (see Casadó et al., 1990 for details). Using this F test, a probability greater than 95% ($P < 0.05$) was considered the criterion to select a more complex model over the simplest one. In all cases a probability of less than 70% ($P > 0.30$) resulted when one model was not significantly better than the other.

RESULTS

Saturation binding isotherms for receptors coupled to G-proteins

Agonist binding to A₁ Adenosine receptors

The A₁ adenosine receptor (A₁R) is a prototypic example of heptaspanning membrane receptor with apparently complex kinetics of agonist binding. The binding of different full agonists displays nonlinear biphasic Scatchard plots, indicating apparent negative cooperativity (Casadó et al., 1991, Franco et al., 1996). This corresponds to an equation of second degree numerator and second degree denominator (or 2:2 functions) and not to simple michaelian functions (or 1:1 functions). To investigate the behaviour of agonist binding to A₁R, the binding of increasing concentrations of the specific A₁R agonist, R-PIA, to brain cortical membranes was performed. The experimental data (Figure 2) were numerically analyzed using different 2:2 functions and,

for comparison, using a 1:1 function. Data were also fitted to a classical “two-independent-state model” using the following equation:

$$[A]_{Bound} = \frac{R_1[A]}{K_1 + [A]} + \frac{R_2[A]}{K_2 + [A]} \quad (1)$$

where K_1 and K_2 stand for the equilibrium dissociation constant for the high- and low-affinity states and being R_1 and R_2 the number of high- and low-affinity states. The parameter values are shown in Table 1.

Considering that A_1R forms homodimers (Ciruela et al., 1995) a more realistic model for this receptor is the “two-state dimer receptor model” shown in the Figure 1. The functional A_1R unit is the homodimer having each monomer a binding site for the agonist. Binding of agonists to these dimers is represented by the function:

$$[A]_{Bound} = \frac{K[A] \cdot (1 + \alpha L) + 2 \cdot K^2 \mu [A]^2 \cdot (1 + \alpha \beta L)}{1 + L + K[A] \cdot (1 + \alpha L) + K^2 \mu [A]^2 \cdot (1 + \alpha \beta L)} [R_T] \quad (2)$$

As shown in Figure 1, the homodimer can be in inactive form (R_2) or in active form (R_2^*). L is the equilibrium constant for the isomerization of the receptor from R_2 to R_2^* . R_2 and R_2^* can bind reversibly two molecules of ligand (A), one to each receptor in the dimer. As defined previously (Franco et al., 2005), the affinity constants for the first and second binding of A to the inactive form are K and μK , respectively, where μ is the intrinsic association constant for the binding of A to the second receptor in R_2 , αK and $\mu \beta K$ are the affinity constants for the first and second binding of A to the active form R_2^* , and α and β reflect the intrinsic efficacy of the first and second molecule of A entering the dimer.

As there are only three independent parameters, the ligand binding equation can be rewritten as follows:

$$[A]_{Bound} = \frac{c_1[A] + 2 \cdot [A]^2}{c_2 + c_1[A] + [A]^2} R_T \quad (3)$$

where c_1 and c_2 stand for:

$$\begin{aligned}c_1 &= \frac{(1 + \alpha L)}{K \cdot \mu \cdot (1 + \alpha \beta L)} \\c_2 &= \frac{(1 + L)}{K^2 \mu \cdot (1 + \alpha \beta L)}\end{aligned}\tag{4}$$

Data from Figure 2 were fitted to this equation and R_T , c_1 , and c_2 were determined. Values appear in Table 1. It should be noted that $c_2 = ([A]_{50})^2$, i.e., the square of the semisaturation concentration. Thus, $\sqrt{c_2}$ gives information about the receptor affinity for the ligand.

Interestingly, c_1 is related to cooperativity. $c_1 = 2 \cdot [A]_{50}$ denotes noncooperativity and in this case $c_2 = c_1^2/4$ and, therefore, the 2:2 binding isotherm simplifies to a 1:1 function. Values of $c_1 < 2 \cdot [A]_{50}$ indicate positive cooperativity and $c_1 > 2 \cdot [A]_{50}$ indicates negative cooperativity.

As shown in Figure 2 and Table 1, an F-test demonstrates that the equations of “two-independent-site receptor model” and “two-state dimer receptor model” fit the data significantly better than the 1:1 equation. Since no improvement ($P > 0.30$; probability of less than 70%) is obtained with one extra parameter (“two-independent-site receptor model”), the simplest model explaining the experimental data for the binding of R-PIA to A_1 adenosine receptors is the “two-state dimer receptor model”. Also, the fact that these receptors are dimers is a strong confirmation for the “two-state dimer receptor model”. In the “two-state dimer receptor model”, the value of $[A]_{50}$ yields information about the receptor affinity for R-PIA (0.313 nM). This parameter can be interpreted as the K_D in models assuming receptor monomers. An interesting aspect of this model is that fitting experimental data to the equation 3 gives direct information about cooperativity. As it can be observed in Table 1, the value of c_1 is 1.42 nM and therefore significantly ($P < 0.01$) greater than $2 \cdot [A]_{50}$ (2×0.313), which is an indication of negative cooperativity. This would indicate that R-PIA binds to a dimeric receptor (R_2) giving the complex (R_2)-R-PIA, and that a second molecule of R-PIA binds to this complex with less affinity then yielding the complex R-PIA-(R_2)-R-PIA. Both complexes would isomerize giving the corresponding active forms.

Competition experiments for receptors coupled to G-proteins

Antagonist binding to D₁ dopamine receptor

For some receptors a saturation curve is not the best choice to analyze ligand binding behaviour, i.e., in cases where ligands have low affinity or ligands are not available in radiolabeled form, competition experiments are useful to evaluate binding characteristics. The pharmacology of the D₁ dopamine receptor, a heptaspanning G-protein-coupled membrane receptor, is usually analyzed by competition experiments. Here the affinity of the specific D₁ receptor agonist, SKF 38393 was determined from competition assays using the specific antagonist [³H]SCH 23390 as radioligand bound to brain striatal membranes.

Irrespective of the model considered, to deduce the binding parameters of a ligand acting as competitor it is necessary to know in advance the binding parameters of the radioligand. Thus, as described above for A₁ adenosine receptors, the saturation curve corresponding to the antagonist, [³H]SCH 23390, binding to D₁ receptors was first analyzed using the “two-state dimer receptor model” according to the equation 3. As c_2 was not significantly different from $2 \cdot [A]_{50}$ ($P > 0.30$), the antagonist binding to D₁ dopamine receptor is non-cooperative.

According to the data obtained by the “two-state dimer receptor model”, the Scatchard plot was linear indicating noncooperativity or neutral cooperativity (Figure 3). The values of R_T , $[A]_{50}$ and c_1 were deduced and appear in Table 2. As it is easily deduced for noncooperativity, the ligand affinity ($[A]_{50}$) is $c_1/2$ and, therefore, the affinity of [³H]SCH 23390 was estimated to be 0.78 nM.

An extension of the “two-state dimer receptor model” was used to fit the data of competition of antagonist, [³H]SCH 23390, binding (A) with increasing concentrations of agonist SKF 38393 (B). The simultaneous antagonist and agonist binding to a dimeric receptor is now taken into account as indicated in Figure 4. According to this scheme, the suitable equation to fit competition data deduced as indicated in Supplementary Material is

$$[A]_{Bound} = \frac{c_1[A] + 2 \cdot [A]^2 + c_5[A] \cdot [B]}{[c_2 + c_1[A] + [A]^2] + [c_3 + c_5[A]] \cdot [B] + c_4[B]^2} R_T \quad (5)$$

being c_1 to c_5 a combination of constants (see Supplementary Material).

Fitting the experimental data (Figure 5) to equation (5) and taking into account that $c_2 = c_1^2/4$ and $c_1 = 1.6$ nM (see Table 2) and that the concentration of the radioligand is 1.2 nM, the parameters deduced are indicated in Table 3. Values of c_3 and c_4 give information about affinity and cooperativity of agonist (B) binding to the receptor. Thus, the quotient c_2/c_4 corresponds to $([B]_{50})^2$ giving information about the affinity for the agonist ($[B]_{50}$). The value of 78 nM indicates a relatively low affinity of the agonist SKF 38393. Moreover the quotient c_3/c_4 is related to cooperativity: values of $c_3/c_4 = 2 \cdot [B]_{50}$ indicate noncooperativity; values of $c_3/c_4 < 2 \cdot [B]_{50}$ point out positive cooperativity and values of $c_3/c_4 > 2 \cdot [B]_{50}$ indicate negative cooperativity. With the data provided in Table 3, $c_3/c_4 > 2 \cdot [B]_{50}$ (3,300 nM versus 156 nM), which indicates that a strong negative cooperativity exists in the binding of SKF 38393 to D_1 receptors. The value of c_5 indicates the relative tendency to form the complex $A(R_2)B$ respect to that of forming $A(R_2)A$ or $B(R_2)B$.

Saturation curve for receptors not coupled to G-proteins

Agonist binding to estrogen receptor

The dimer model was tested for radioligand binding to receptors forming dimers but that are not coupled to G proteins. The estrogen receptor is a convenient choice since binding of agonists show positive cooperativity (Figure 6), which is rarely found for heptaspanning G-protein-coupled membrane receptors. Data of specific [3 H]estradiol binding to estrogen receptor from calf uteri, which was reported already in 1981 by Notides et al. is shown in Figure 6. The “two-independent-site receptor model” is not suitable for this system since it cannot explain positive cooperativity. Here experimental data were examined using the two-state dimer receptor model. Data were fitted to equation (3) and the parameters deduced are shown in Table 4. As c_2 is the

square of the semisaturation concentration, a value of $[A]_{50}$ of 0.642 nM is deduced which corresponds to the affinity of [^3H]estradiol for the estrogen receptor. More interestingly, c_1 denotes a strong positive cooperativity. In fact the product $2 \cdot [\text{Estrogen}]_{50}$ is higher than c_1 ($1.284 > 0.178$) indicating positive cooperativity.

DISCUSSION

After the pioneering studies of receptor models devised by Colquhoun (1973) and Thron (1973), different models have been developed to describe the behaviour of heptaspanning membrane receptors or G-protein coupled receptors (De Lean et al., 1980; Costa et al., 1992; Samama et al., 1993; Lefkowitz et al., 1993; Leff, 1995; Weiss et al., 1996a-c). Practically all published models are based on the “noncooperative mechanism” devised by del Castillo and Katz (1957) to explain the behaviour of nicotinic acetylcholine receptors.

One of the most useful models to date is the “two-state model of receptor activation”, which assumes the occurrence of two different conformational states of the receptor molecule, R and R* (Figure 7a). In terms of signal transduction, the R form would be inactive whereas the R* form would be the active or productive form. These two conformational states, which are in equilibrium, have an orthosteric or “competitive” centre for agonist binding. Filling this centre shifts the equilibrium towards the productive form. On the other hand, there are synthetic compounds, which could be of pharmacological interests, which compete with the physiological agonist, i.e. they bind to the orthosteric centre, leading to a variety of outputs, from full agonism/antagonism to partial/inverse agonism. The occurrence of an equilibrium between the unproductive and the productive or active form may explain why some degree of signalling may happen in the absence of agonists, the so-called constitutive activity. Synthetic compounds that upon binding to the orthosteric centre displace the equilibrium towards the inactive form act as inverse agonists or negative antagonists by reducing the constitutive activity.

There are more complex models derived from the “ternary complex model” proposed by De Lean et al. (1980) (Figure 7b) which apart from the orthosteric centre includes an allosteric or

regulatory site where compounds not structurally related to agonists, for example G proteins, can bind. The “ternary complex model of allosteric modulation” (Figure 7c) is a modification of the “ternary complex model” (Figure 7b) in which the allosteric modulation is not restricted to G protein and includes different compounds, which may have pharmacological activity, acting on allosteric sites (see Lazareno et al. (1998) and references therein). Samama et al. (1993) expanded this model and developed the “extended ternary complex model” (Figure 7d), which included different affinity states (R and R*) for the receptor uncoupled to G protein. As in the two-state model, R is the unproductive form and R* the active form. In this model it is assumed that the G protein binds to a specific and allosteric site in R*. The G protein, acting as an allosteric modulator, modifies the agonist binding, and/or affects the equilibrium between R and R*. Since the allosteric modulator, the G protein in this case, does not compete with orthosteric compounds, maximum binding is not affected but K_D is. The “cubic ternary complex model” (Weiss et al., 1996a-c) (Figure 7e) expands the “extended ternary complex model”, allowing the binding of G to R and R*.

More recently the “allosteric two-state model” developed by Hall (2000) combines the “ternary complex model of allosteric modulation” (Figure 7c) and the “two-state model of receptor activation” (Figure 7a). This model (Figure 7f), which is similar but more complex than the “cubic ternary complex model”, predicts the behaviour of receptors when both, orthosteric and allosteric compounds are present. More complex models, including the “quaternary complex model” of allosteric interactions have been proposed assuming similar principles as those described above (Christopoulos and Kenakin, 2002).

Experimental data giving linear Scatchard plots were easily fitted to a single affinity state. However, the binding of agonists to heptaspanning receptors quite often give concave upward Scatchard plots. The above models do not explain suitably this behaviour since they predict linear Scatchard plots for agonist binding to the orthosteric site. Concave upward Scatchard plots would be explained considering the existence of two separated (non interconvertible forms) of the receptor: a high affinity form (R* or G-protein coupled) and a low affinity form (R

or G-protein uncoupled). The above described models are based on an equilibrium between R and R* and therefore they would only explain nonlinear Scatchard plots if the concentration of G protein is lower or similar than that of the receptor, something not occurring in physiological conditions (Neubig, 1994). Moreover, it has been observed that the agonist induces changes in the proportions of the so called “high” and “low” affinity states, which strongly suggests that these two states cannot exist separately but they are interconnected (Wong et al., 1986) and this apparent interconversion between states is independent of the G protein (Casadó et al., 1991). Working with adenosine A₁ receptors we showed that a full agonist led to an apparent change in the proportion of receptor in high and low affinity (Casadó et al., 1991). If the agonist is able to vary the proportion of high and low affinity states, these two forms would be in equilibrium and consequently the “two-independent-site receptor model” cannot accurately represent the behaviour of the receptors if the Scatchard plot is nonlinear.

Taken this into account we claim that this type of nonlinear Scatchard plots is a consequence of negative cooperativity in the binding. Furthermore, concave downward Scatchard plots have been reported for agonist binding to muscarinic receptors (Lazareno et al., 1998), for agonist binding to mu and delta opioid receptors (Tomassini et al., 2003) and for agonist binding to H₂ and H₃ histamine receptors (Sinkins and Wells, 1993) and also in these cases the reported models cannot explain these Scatchard plots that likely reflect positive cooperativity.

We have postulated that to fully explain receptor behaviour in terms of ligand binding but also of receptor activation, both intramolecular and intermolecular interactions should be relevant (Franco et al., 2003). Ten years ago it was supposed that heptaspanning receptors were constituted by monomeric molecules. Then we proposed a model of heptaspanning receptor operation based on intermolecular receptor-receptor, receptor-protein and receptor-lipid interactions. The “cluster-arranged cooperative model” (Franco et al., 1996) was the first formulated model able to explain nonlinear Scatchard plots for agonist binding to heptaspanning receptors in basis of negative cooperativity. We were aware of the requirement for receptor-protein and receptor-lipid interactions to achieve the conformational changes to be transmitted

to the different receptor molecules and that would account for both negative and positive cooperativity. To explain the behaviour of A₁ adenosine receptor two extreme conformational states were assumed, which correspond to high and low-affinity binding states. Also it was assumed the existence of a number of infinite conformational states with intermediate affinities. All of these forms would be in an equilibrium which would be affected by the agonist. According to the apparent negative cooperativity in the ligand binding and to the fact that agonist is able to cluster the receptors, it was assumed that receptors within the cluster (or microdomain) display a decreased affinity. A relevant feature in this model is the assumption that each agonist molecule that binds to the receptor is able to infinitesimally modify the affinity of subsequent agonist molecules interacting with the “empty” receptors in the cluster. This agonist-induced global change in the affinity is able to explain the negative cooperativity in the binding of adenosine to A₁ receptors (Franco et al., 1996).

A model consisting of a receptor molecule with more than one orthosteric site would also be able to explain cooperativity (positive, neutral and negative). A receptor with more than one orthosteric site could be a monomer able to bind more than one agonist molecule or an oligomeric molecule having each monomer one binding site (Franco et al., 1996; Franco et al., 2003). Here, the binding of an agonist, R-PIA, to A₁ adenosine receptor has been analyzed using the “two-state dimer receptor model” and the results have demonstrated that this is a suitable method for analyzing binding of radioligands to G protein coupled receptors showing or not cooperativity. Fitting the experimental data to a relatively simple equation (equation (3)) the parameters of ligand affinity and receptor cooperativity are easily deduced, thus characterizing the receptor behaviour. The deduced parameter estimates agree with those obtained using the classical “two-independent-site receptor model”. Data from competition assays can also be fitted using the equations derived from the “two-state dimer receptor model” (equation (5)). It is demonstrated that the competition by an agonist, whose binding to the receptor shows cooperativity, of an antagonist binding to dopamine D₁ receptors has been well solved by the model and can be generalized to other dimeric receptors. Although the estrogen receptor is not

coupled to G proteins, it is a dimer of identical, interacting subunits (Bond et al., 1992) and estradiol binding to the receptor present positive cooperativity. In cases like this, the classical models are not suitable to deduce the binding parameters since they cannot explain positive cooperativity. Data of estrogen binding is well fitted using the “two-state dimer receptor model” allowing calculating ligand affinity and the degree of positive cooperativity using equation (3). Thus the “two-state dimer receptor model” is reliable not only for studying heptaspanning G-protein-coupled membrane receptors forming dimers but to study other receptors susceptible of forming dimeric structures.

In conclusion, equation (3) and equation (5), derived from the “two-state dimer receptor model” are simple equations to fit saturation experiments or competition experiments to obtain information about ligand affinity and cooperativity for dimeric receptors.

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FOOTNOTES

Footnote to the title:

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FIGURE LEGENDS

Figure 1. “Two-state dimer receptor model”. K is the equilibrium association constant of A to R_2 ; L is the equilibrium receptor isomerization constant; α is the intrinsic efficacy of A binding to unoccupied receptors; β is the intrinsic efficacy of A binding to single-occupied receptor; and μ is the intrinsic association constant of A.

Figure 2. Saturation curves (bottom) and Scatchard plot (top) for the agonist [3 H]R-PIA binding to A_1 adenosine receptors from isolated lamb brain cortical membranes. The experiment was performed as described in Materials and Methods. Data were fitted according to the “two-independent states receptor model” (red), to the “two-state dimer receptor model” (black), and “1:1 functions” (green). The parameter values are indicated in Table 1.

Figure 3. Scatchard plot for the antagonist [3 H]SCH 23390 binding to D_1 dopamine receptors from isolated lamb brain striatal membranes. The experiment was performed as described in Materials and Methods. Data were fitted according to the “two-state dimer receptor model”. The parameter values are indicated in Table 2.

Figure 4. Scheme of the “two-state dimer receptor model” when two ligands are used in competition assays. K , L , α , β and μ are defined in the legend to Figure 2. M is the equilibrium association constant of B to R_2 ; θ is the intrinsic efficacy of B binding to unoccupied receptors; ω is the intrinsic efficacy of B binding to single-occupied receptor; ϕ is the binding cooperativity between first and second B molecule: ratio of affinity of B for A(RR) and (RR); γ is the binding cooperativity between A and B: ratio of affinity of A for B(RR) and R or of B for A(RR) and RR, and δ is the activation cooperativity between A and B: ratio of affinity of A for B(RR)* and B(RR) or of B for A(RR)* and A(RR).

Figure 5. Competition of 1.2 nM [3 H]SCH 23390 binding to lamb brain striatal D_1 dopamine receptors with SKF 38393. The experiment was performed as described in Materials and

Methods. Data were fitted according the “two-stated dimer receptor model”. The parameter values are indicated in Table 3.

Figure 6. Scatchard plot for the agonist estradiol binding to the estrogen receptor from calf uteri. Data were obtained from Notides et al. (1981). Data were fitted according to the “two-state dimer receptor model”. The parameter values are indicated in Table 4.

Figure 7. Models for heptaspanning membrane receptor function. a) two-state model of receptor activation, b) the ternary complex model, c) the ternary complex model of allosteric modulation, d) the extended ternary complex model, e) the cubic ternary complex model, and f) the allosteric two-state model.

Table 1 Parameters values obtained fitting the data of the agonist [³H]R-PIA binding to A₁ adenosine receptors, shown in Figure 2, to different models*

Two-state dimer receptor model ^{&}		Models in Figure 7 (1:1 equation)		Two-independent-site receptor model [§]	
Parameter	Value	Parameter	Value	Parameter	Value
R_T^{**}	0.509 ± 0.008 pmols/mg protein	R_1	0.925 ± 0.019 pmols/mg protein	R_1	0.63 ± 0.05 pmols/mg protein
C_1	1.42 ± 0.18 nM	K_1	0.228 ± 0.023 nM	K_1	0.100 ± 0.014 nM
C_2	0.098 ± 0.014 nM ²			R_2	0.42 ± 0.04 pmols/mg protein
[A] ₅₀	0.313 nM			K_2	2.6 ± 0.9 nM

* Nonspecific binding was determined in the presence of an excess of R-PIA as described in Saura et al. (1996). The determined value was 0.029 pmols/(mg protein · nM). Data are mean ± SEM values given by the regression program (GRAFIT[®])

[&] P < 0.05 comparing models represented by a 1:1 equation and the “two-state dimer receptor model”

[§] P > 0.30 comparing the classical “two-independent-site” and the “two-state dimer receptor model”

**Maximum binding = 2 · R_T = 1.018 pmols/mg protein

Table 2

Parameter values obtained fitting the data of the antagonist [³H]SCH 23390 binding to D₁ dopamine receptors shown in Figure 3 to the “two-state dimer receptor model”*

Parameter	Value
R_T^{**}	0.178 ± 0.003 pmols/mg protein
C_1	1.6 ± 0.1 nM
[A] ₅₀	0.78 nM

* Nonspecific binding was determined in the presence of an excess of SCH 23390 as described in Materials and Methods. The determined value was 0.039 pmols/(mg protein · nM). Data are mean ± SEM values given by the regression program (GRAFIT[®])

**Maximum binding = 2 · R_T = 0.356 pmols/mg protein

Table 3

Parameter values obtained fitting the data of competition of the antagonist [³H]SCH 23390 binding to D₁ dopamine receptors with SKF 38393, shown in Figure 5, to the “two-state dimer receptor model” *

Parameter	Value
R_T^{**}	0.19 ± 0.01 pmols/mg protein
C_3	0.33 ± 0.03 nM
C_4	$(1.00 \pm 0.01) \times 10^{-4}$
C_5	0.0806 ± 0.015
[B] ₅₀	78 nM
C_3/C_4	3,300 nM

* Nonspecific binding was determined in the presence of an excess of SCH 23390 as described in Materials and Methods. The determined value was 0.039 pmols/(mg protein · nM). Data are mean ± SEM values given by the regression program (GRAFIT[®])

** Maximum binding = $2 \cdot R_T = 0.30$ pmols/mg protein

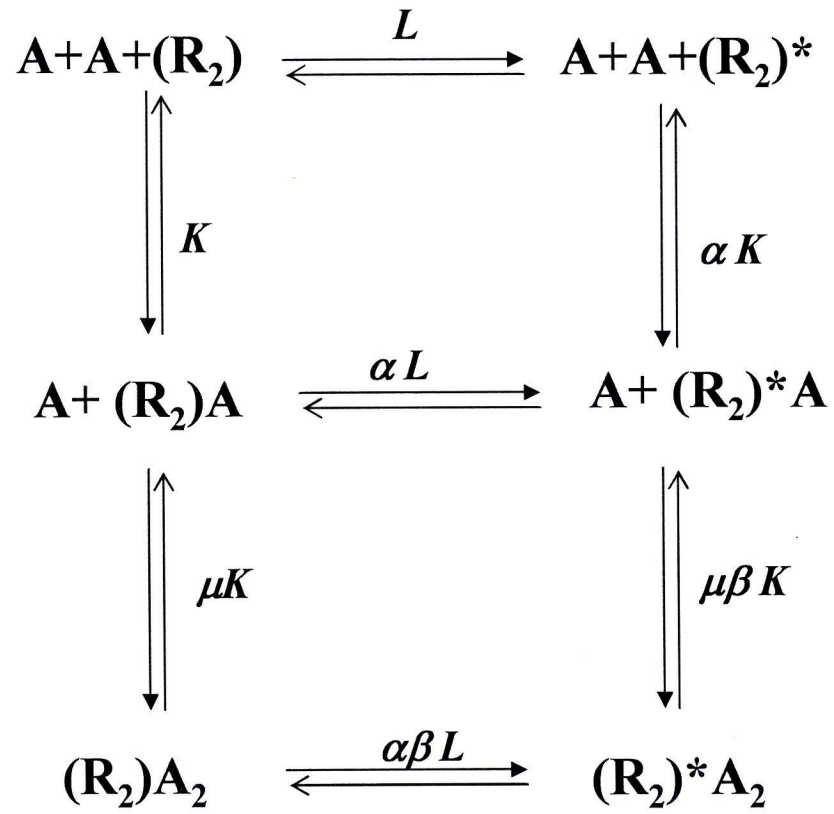
Table 4

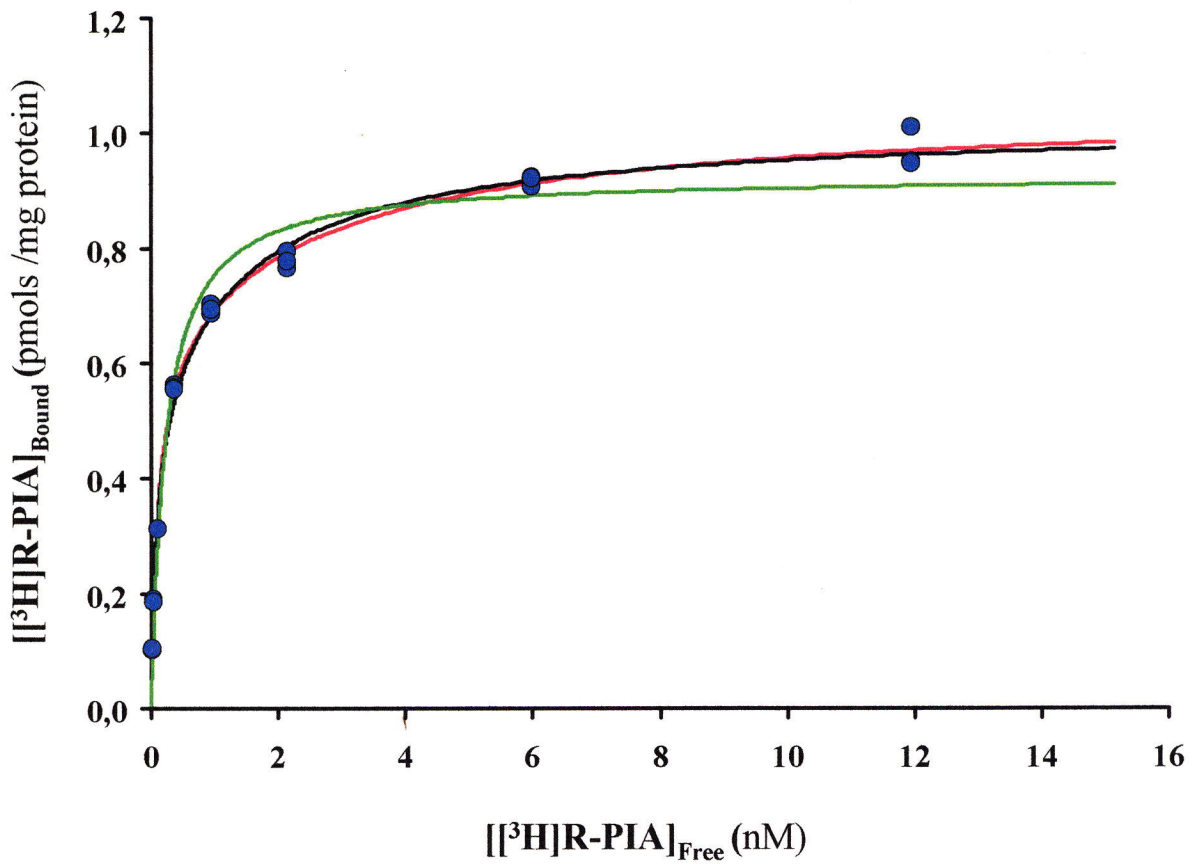
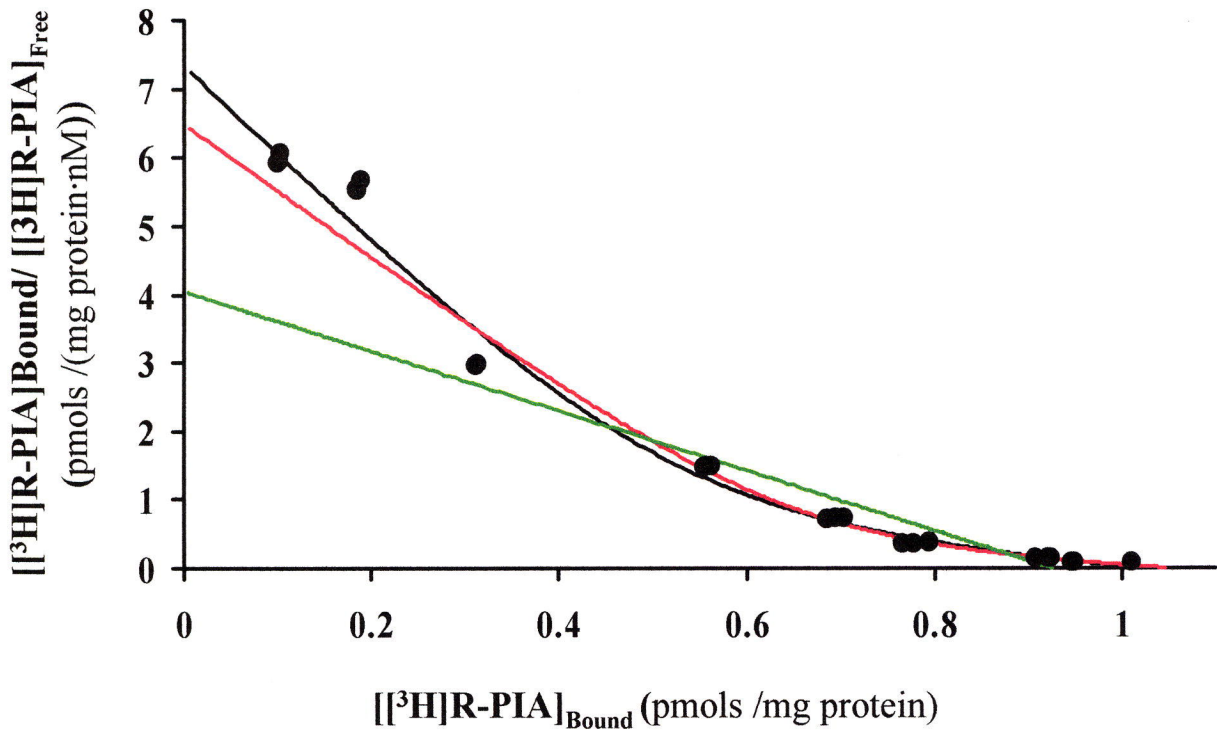
Parameter values obtained fitting the data of the agonist estradiol binding to the estrogen receptors, shown in Figure 6, to the “two-state dimer receptor model”*

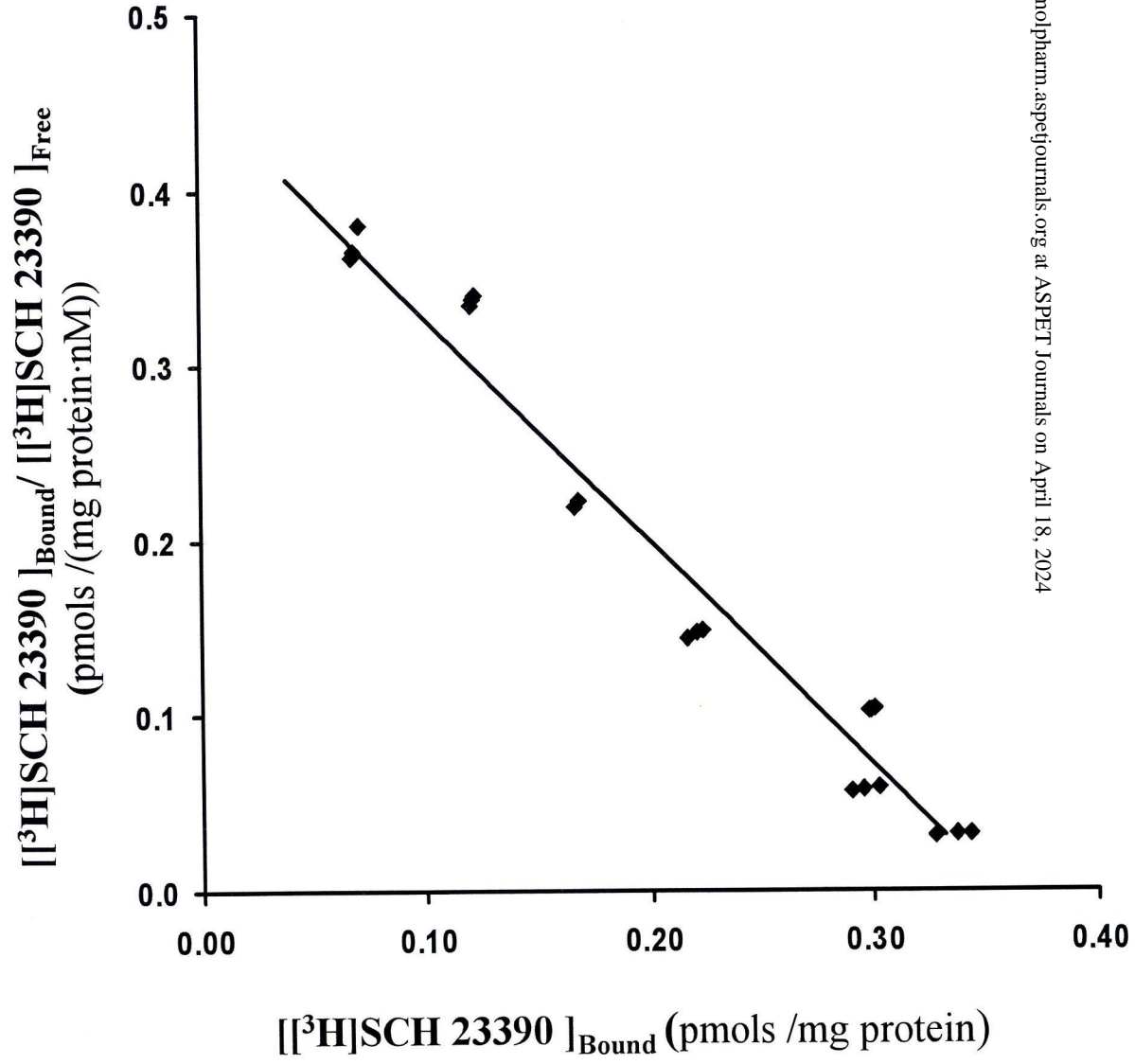
Parameter	Value
R_T^{**}	2.44 ± 0.05 pmols/mg protein
C_1	0.178 ± 0.010 nM
C_2	0.42 ± 0.04 nM ²
$[A]_{50}$	0.642 nM

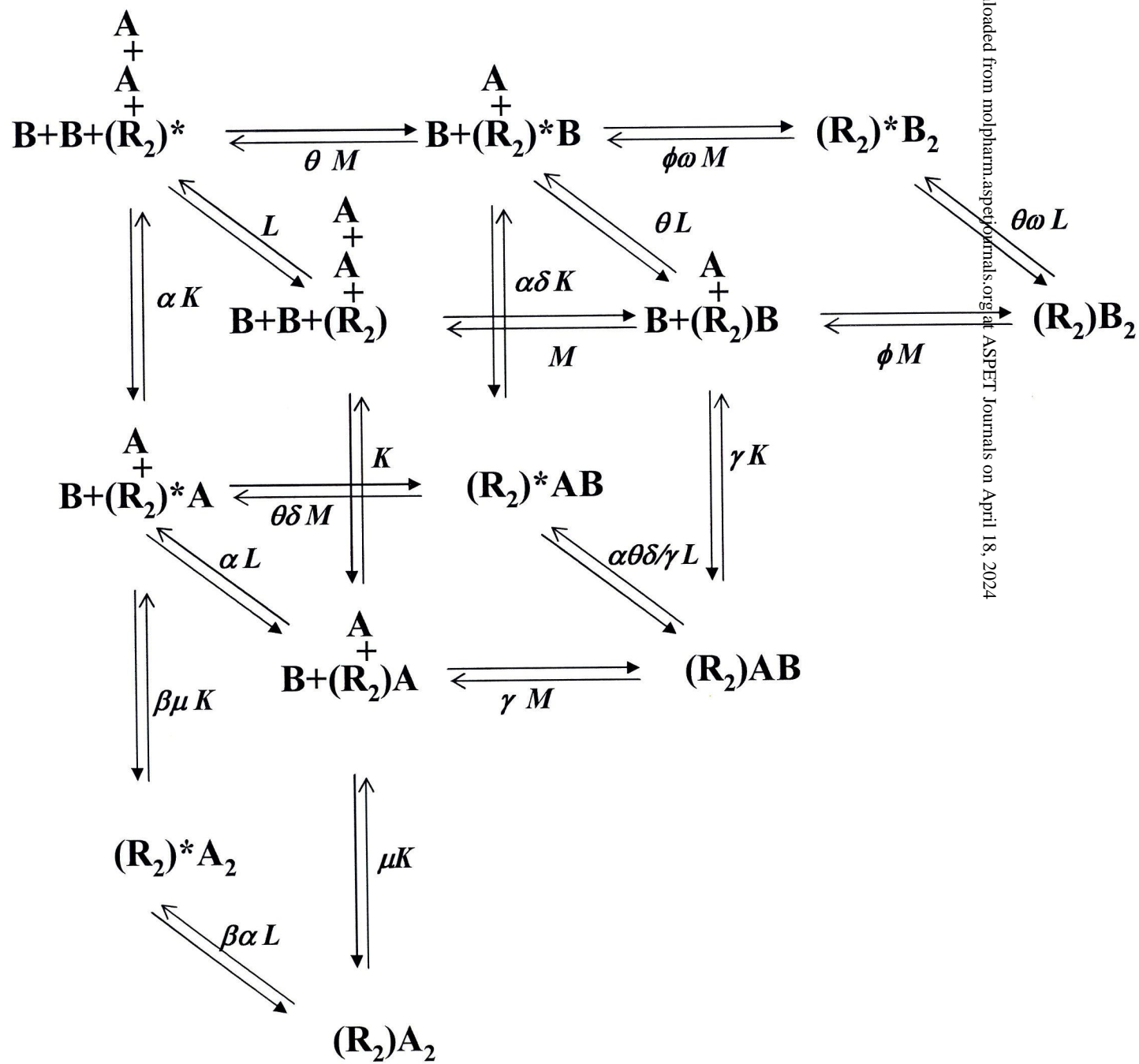
* Data were obtained from Figure 2 in Notides et al., 1981. Data are mean \pm SEM values given by the regression program (GRAFIT[®])

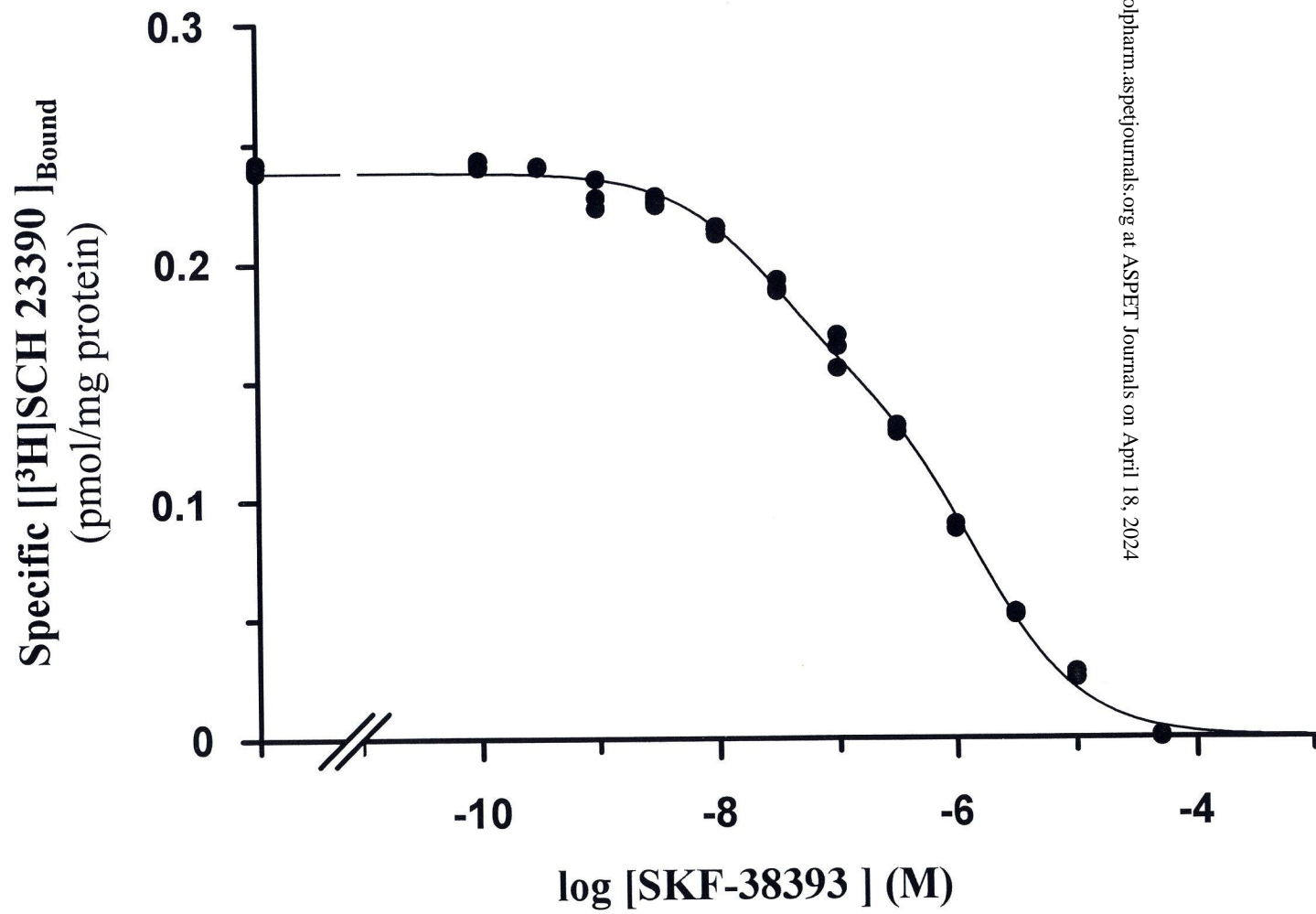
**Maximum binding = $2 \cdot R_T = 0.489$ pmols/mg protein

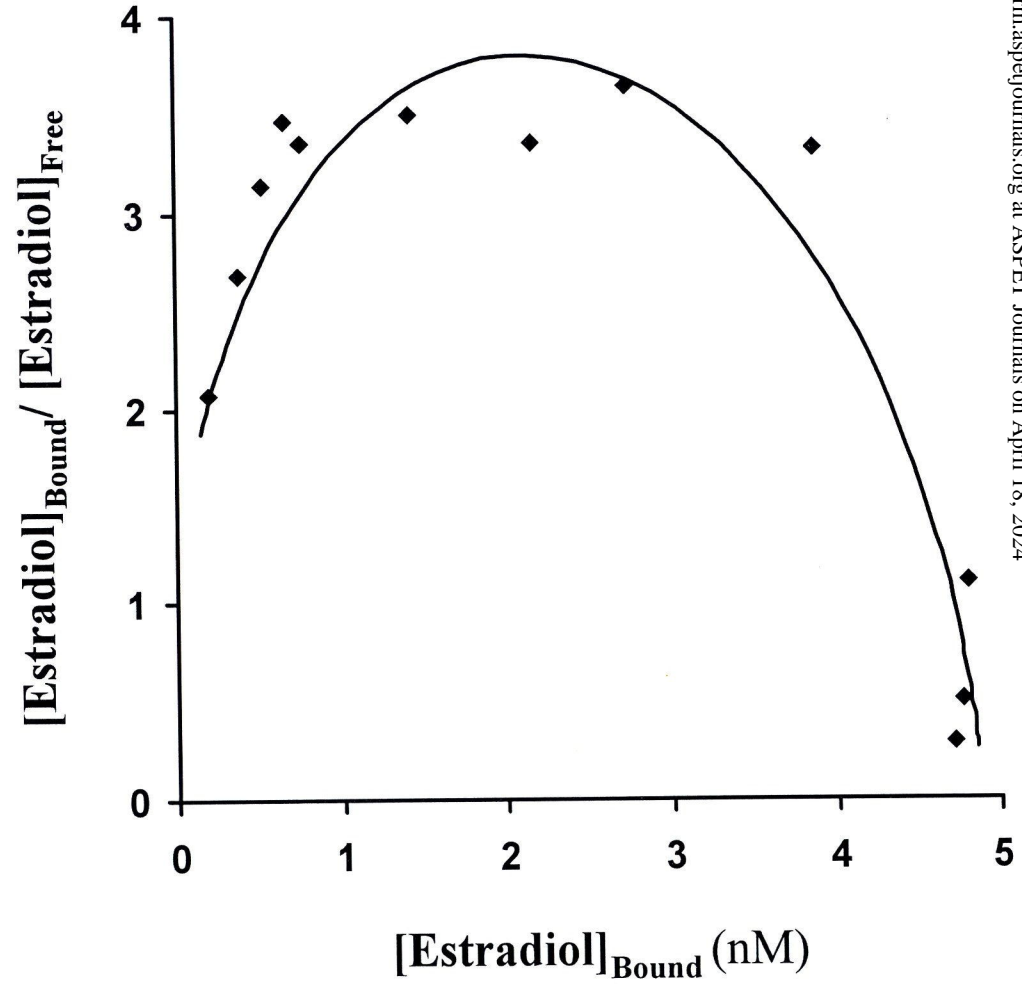












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