Optimal association–saturation procedure for estimating association and dissociation rate parameters in receptor studies

Application to solubilized A₁ adenosine receptors

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A method of obtaining estimates of the maximum binding and association and dissociation rate constants for a receptor–ligand interaction is described. This new procedure, the association–saturation method, is based on an exact mathematical equation which defines the model without simplifications. The method proposed is readily applicable to any system consisting of one ligand and one receptor. With only four determinations, each involving one ligand concentration and an association time, it is possible to determine the number of specific binding sites, the dissociation and association rate constants and the equilibrium dissociation constant with accuracy. No dissociation curve is required, and only one point of the association curve for each ligand concentration is necessary. In addition to the higher confidence in the estimates of the parameter values obtained, this method leads to important savings in radiolabelled compounds and experimental time. The results were compared with those obtained with standard association–dissociation curves and saturation isotherms. The optimum number of replicates of each experimental point to obtain reliable estimates of different parameters is discussed on the basis of simulation studies. The performance of the procedure is analysed by means of association–saturation experiments with the agonist [*adenine-2,8-³H,ethyl-2-³H]*N*⁶-phenylisopropyladenosine and solubilized A₁ adenosine receptors from pig brain cortex.*

INTRODUCTION

A complete study of physicochemical and pharmacological behaviour of receptor–ligand interaction requires the knowledge of the association and dissociation rate constants, as well as the binding parameters, i.e. equilibrium dissociation constant (K_D) and maximum binding (R_0) with good accuracy and precision (Rescigno *et al.*, 1982). To achieve this, a suitable model of the binding reaction is required, and the sequence of chemical reactions involved must be established. A system of differential equations representing the dynamics of the time-course variation of free ligand, free receptor and receptor–ligand complexes can be derived from these elemental reactions. Then, when analytical integration is possible, an equation expressing the concentration of bound ligand as a function of the initial concentration of receptor and ligand, the association and dissociation rate constants, and time, can be obtained.

Usually, instead of the exact equation representing the association–dissociation process, a simplified version has been used (Linden, 1984; Lohse *et al.*, 1984; Murray & Siebenaller, 1987; Zielke & Zielke, 1987; Leid *et al.*, 1988, 1989; Casadó *et al.*, 1990b). Only in one case has the exact equation been used, but non-specific binding was not included (Donner *et al.*, 1980). With an association curve it is theoretically possible to obtain k_1 and k_{-1} (Limbird, 1986). However, the values obtained for these kinetic constants from a single association curve are not precise. This methodology has been applied by Leung *et al.* (1990), who obtained discrepant values of K_D from equilibrium binding and association data. This problem is often overcome by performing the dissociation experiment from which a more precise estimate of k_{-1} can be obtained. The association constant, k_1 , can be calculated from the k_{-1} value and the data from the association curve. Obviously this indirect procedure leads to

accumulated errors that produce a very large spread of values for the kinetic parameters obtained in different experiments or in different laboratories.

Here we present a better way to obtain the various kinetic-parameter values by generating the association curves at different ligand concentrations. We name this kind of assay as the ‘association–saturation’ experiment. The novelty of the method is the use of various ligand concentrations, and the performance of the dissociation curve is unnecessary. The advantage is the opportunity to work with an association equation without simplifications but which includes the non-specific binding, thus increasing the confidence level of the parameter values calculated. This method is based on the D-optimality or Box–Lucas criterion (Box & Lucas, 1959; Fedorov, 1972), from which suitable determinations for carrying out the association–saturation experiment were calculated in the case of one-receptor–one-ligand interaction, with a computer program (Canela, 1985; Canela *et al.*, 1989).

The performance of the method was analysed by working with solubilized A₁ receptor from pig brain cortex. This receptor was chosen for many reasons, one of which is the inherent interest in describing the kinetic behaviour of the agonist binding to the receptor by which the adenosine exerts potent action on a wide range of physiological systems (Gerlach & Becker, 1986; Daly *et al.*, 1983). We have previously demonstrated the presence in crude membranes of an A₁ subtype receptor (Casadó *et al.*, 1990a) with high- and low-affinity states; however, in soluble extracts, only the high-affinity state is detected (Casadó *et al.*, 1990b). Thus A₁ subtype receptor solubilized from pig brain cortex constitutes a simple example of a one-receptor–one-ligand interaction. There is also a spread in the K_D values reported for A₁ adenosine receptor (Nakata & Fujisawa, 1983; Bruns *et al.*, 1986; Patel *et al.*, 1988), and this is more apparent

Abbreviations used: [³H]R-PIA, [*adenine-2,8-³H,ethyl-2-³H]*N*⁶-phenylisopropyladenosine; R-PIA, *N*⁶(R)-phenylisopropyladenosine.*

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for the solubilized A_1 receptor, for which K_D values varying between 0.3 nM and 3.3 nM have been reported using phenylisopropyladenosine (PIA) as agonist (Nakata & Fujisawa, 1983, 1984; Klotz *et al.*, 1986; Yeung *et al.*, 1987; Stiles, 1988). The determination of kinetic parameters (k_1 and k_{-1}) for ligand binding to solubilized adenosine receptor is difficult, owing to the experimental complexity of solubilized-receptor–ligand binding determination. In some cases the binding determination technique employing filtration through glass fibres presoaked with polyethylenimine has been successfully used (Leid *et al.*, 1989; Nakata, 1990), but in others more complex methods, as such as gel filtration, poly(ethylene glycol) treatment and subsequent filtration of the precipitated ligand–receptor complex, were required (Nakata & Fujisawa, 1983; Pérez-Reyes *et al.*, 1987; Casadó *et al.*, 1990b). The soluble A_1 receptor kinetic parameters have been determined by the association–dissociation method only in a case (Leid *et al.*, 1989) where it was possible to substitute the precipitation technique by a single filtration. This is usually impossible, and thus the experimental simplifications introduced by the method described here makes possible an easy determination of the kinetic parameters of soluble receptors, irrespective of whether or not the precipitation technique is used.

MATERIALS AND METHODS

Materials

[adenine-2,8- 3H , ethyl-2- 3H]N⁶-Phenylisopropyladenosine ([3H]R-PIA) (42.5 Ci/mmol) was purchased from New England Nuclear Research Products (Boston, MA, U.S.A.). N⁶(R)-Phenylisopropyladenosine (R-PIA), CHAPS and adenosine deaminase (EC 3.5.4.4) were obtained from Boehringer Mannheim. Polyethylenimine (50%, w/v), crystallized, poly(ethylene glycol) 8000 and bovine γ -globulins were purchased from Sigma Chemical Co. All other products were the best grade available and purchased from Merck. Deionized water further purified with a Millipore Milli-Q system was used throughout.

Membrane preparations

Porcine brains were obtained from the local slaughterhouse. Cortices (two to six brains) were dissected on ice and washed with 0.25 M-sucrose containing 5 mM-Tris/HCl buffer, pH 7.4. Tissue homogenization was performed in 10 vol. of the ice-cold buffer with a Polytron (Kinematica, PTA 20TS rotor, setting 4) for two periods of 5 s separated by an interval of 15 s. The homogenate was centrifuged at 105 000 g for 30 min at 4 °C, and the pellet was resuspended in 10 vol. of 50 mM-Tris/HCl buffer, pH 7.4, and centrifuged under the same conditions. The pellet was stored at –20 °C and was washed immediately before use as described above.

Receptor solubilization

Receptor solubilization was performed as previously described (Casadó *et al.*, 1990b). Briefly, washed cortical membranes were

suspended in a 50 mM-Tris/HCl buffer, pH 7.4, containing 0.5% (w/v) CHAPS and 0.5% (w/v) digitonin. The total detergent/protein ratio was 2:1. After incubation for 10 min at 22 °C, the suspension was centrifuged at 80 000 g for 90 min at 4 °C. The supernatant containing the soluble receptors was filtered through a 0.22 μ m-pore-size Millipore filter.

Protein determination

Protein was determined according to the method of Lowry *et al.* (1951), with BSA as the standard.

Association–saturation experiments

Portions of the solubilized receptors (0.5 mg of protein/ml) were incubated with adenosine deaminase (0.2 unit/ml) for 20 min at 25 °C in 50 mM-Tris/HCl buffer, pH 7.4. [3H]R-PIA (0.128, 0.555, 1.70 and 7.67 nM final concns.) was then added. To the aliquots of 500 μ l which were taken at 300 (0.128 nM), 7.25 (0.555 nM), 300 (1.70 nM) and 0 (7.67 nM) min, poly(ethylene glycol) 8000 and bovine γ -globulins were added to a final concentration of 10% (w/v) and 1.3 mg/ml respectively. After further incubation for 10 min at 0 °C, the suspension was removed and filtered through Whatman GF/C filters presoaked in 0.3% polyethylenimine (5 h, pH 10) to improve the performance of filtration. The filters were washed with 10 ml of the same ice-cold buffer and left overnight at room temperature with 10 ml of Aquasol 2 scintillation cocktail (New England Nuclear); the radioactivity in the vials was counted by using a Packard 1500 Tri-Carb liquid-scintillation counter with an efficiency of 50%.

Association–dissociation experiments

Association–dissociation experiments by the usual method described in the literature were carried out as follows. Association of [3H]R-PIA to solubilized receptors was performed after the incubation of the detergent extracts (0.5 mg of protein/ml) with adenosine deaminase (0.2 unit/ml) for 30 min at 25 °C in 50 mM-Tris/HCl buffer, pH 7.4, followed by the addition of 1 nM-[3H]R-PIA. Higher concentrations of adenosine deaminase yield identical results. For association measurements, aliquots of 500 μ l were taken at the indicated times and processed as described above. From an equal parallel incubation, after 120 min of association, dissociation was induced by addition of unlabelled R-PIA to a final concentration of 500 nM. At different time intervals aliquots of 500 μ l were taken for poly(ethylene glycol) precipitation, followed by filtration and radioactivity determination as described above.

Equilibrium binding isotherms

[3H]R-PIA binding to solubilized receptors was performed by incubation of detergent extracts (0.8 mg of protein/ml pretreated with 0.2 unit/ml of adenosine deaminase for 30 min at 25 °C) with [3H]R-PIA (0.2–25 nM) in 50 mM-Tris/HCl buffer, pH 7.4. After the time necessary (0.5–6 h) to achieve the equilibrium for

Table 1. Parameter values* of solubilized A_1 receptor

Method	k_{-1} (min ⁻¹)	k_{-1} (min ⁻¹ ·nM ⁻¹)	R_0 (pmol/mg)	w (pmol ⁻¹ ·nM·mg)	K_D (nM)
Association–saturation	$(3.05 \pm 0.9)10^{-3}$	$(1.6 \pm 0.2)10^{-2}$	0.91 ± 0.03	51 ± 3	0.22 ± 0.06 †
Association–dissociation	$(1.25 \pm 0.1)10^{-3}$	$(4.0 \pm 0.2)10^{-2}$	0.69 ± 0.02	50 ± 9	0.03 ± 0.003 †
Equilibrium-binding isotherm	–	–	0.90 ± 0.03	56 ± 8	0.25 ± 0.03

* Parameters are those deduced by eqns. (1) (association–saturation), (6) and (7) (association–dissociation) and (5) (equilibrium-binding isotherm).

† Calculated as k_{-1}/k_1 .

each ligand concentration at 25 °C, poly(ethylene glycol) precipitation, filtration and radioactivity determination were performed as described above.

Analysis of binding data

Data from association-saturation experiments were fitted by non-linear regression (Canela, 1984) to the following equation:

$$\text{Total binding} = \frac{\frac{a-b}{2k_1c}(1-e^{-bt})}{1 - \frac{a-b}{a+b}e^{-bt}} + (1-c)L_0 \quad (1)$$

developed as indicated in the Appendix, where:

$$w = \frac{k_{-2}}{k_2N_0} \quad (2)$$

$$c = \frac{w}{1+w} \quad (3)$$

$$a = k_1(R_0 + L_0)c + k_{-1} \quad (4)$$

$$b = \sqrt{(a^2 - 4k_1^2c^2R_0L_0)} \quad (5)$$

R_0 , L_0 and N_0 being the initial concentrations of receptor, ligand and non-specific-binding sites respectively. Eqn. (1) has no simplifications and includes the non-specific binding.

Data from association-dissociation experiments were fitted by the above stated non-linear regression program (Canela, 1984) to the following models:

$$\text{Dissociation: total binding} = R_e \times e^{-tk_{-1}} + \frac{L}{w} \quad (6)$$

$$\text{Association: total binding} = R_e \times (1 - e^{-t(k_{-1} + Lk_1)}) + \frac{L}{w} \quad (7)$$

where t is time, L is the free ligand concentration, R_e is the ligand specifically bound at equilibrium, k_{-1} denotes the dissociation rate constant and k_1 is the association rate constant. L/w is the non-specific binding and w is defined in eqn. (2).

The relationship between kinetic and equilibrium constants is:

$$\frac{k_{-1}}{k_1} = K_D \quad (8)$$

Equilibrium binding isotherms were analysed according to our previous methods (Casadó *et al.*, 1990a). Total binding was fitted to an equation corresponding to the sum of specific binding to the A_1 population plus non-specific binding (assuming that it was linear with respect to the ligand concentration):

$$\text{Total binding} = \frac{R_0 \times L}{K_D + L} + \frac{L}{w} \quad (9)$$

where R_0 stands for the maximum number of specific binding sites and K_D is the equilibrium dissociation constant. The individual saturation isotherms were analysed by non-linear regression (Canela, 1984).

In all cases, the fitting was done as stated (Canela, 1984) with a FORTRAN77 version of the non-linear program in a VAX 6310 or in a TANDON desk-top microcomputer. Fitting can be performed with other non-linear regression programs and computers.

Discrete D-optimal design to choose the experimental conditions for the association-saturation experiments

Discrete D-optimal design was carried out using eqn. (1), and the program, devised by Canela *et al.* (1989), run on a TANDON desk-top microcomputer. This program, written in FORTRAN77, runs on microcomputers or mainframes and can be obtained from the authors on request. Alternatively, other programs for D-optimal design could be used.

First of all, in order to perform a well-designed experiment, irrespective of the criterion selected, the experimental error should be known. Analysing a set of 2750 different experimental determinations obtained by our group during the last 2 years under the experimental conditions described here, the following empirical equation explaining the error pattern was deduced.

$$\text{Residual}^2 = 0.005356 \times z_{av.}^2 + 1.0 \times 10^{-6} \quad (10)$$

where $z_{av.}$ is the average of the replicates of each determination, z , and the residual is the difference between one replicate and the corresponding average. This error structure was entered, according to the specifications of the program, in the determination of the D-optimal design of the association-saturation method.

In general the user supplies to the computer the number of parameters of the model and their approximate values. For a defined model the approximate values can be obtained from the literature, by a less precise association-dissociation experiment or by any other procedure. In this case we use as guess estimates of the parameter values those we had obtained for the high-affinity membrane-bound A_1 receptor subtype (Casadó *et al.*, 1990b): $k_1 = 0.047 \text{ min}^{-1} \cdot \text{nm}^{-1}$, $k_{-1} = 0.003 \text{ min}^{-1}$, $R_0 = 0.8 \text{ pmol/mg}$ of protein and $w = 50 \text{ nm} \cdot \text{pmol}^{-1} \cdot \text{mg}$ of protein. The maximum and minimum concentrations of ligand and protein to be used, as well as the maximum and minimum time in which the determination can be performed, were also entered, considering the experimental limitations. In this case they were 0.1–30 nM of ligand, 0–300 min and 0.5–1 mg/ml respectively. The output of the program, for each determination, was the concentration of ligand and protein and the time in which the determination should be carried out.

Simulation of experiments

Each simulated experiment was D-optimally designed by considering the empirical error structure described above (see eqn. 10) and the parameters' values determined in the association-saturation experiment (see Table 1). Before analysis of the results it was verified that the generation of errors followed a normal distribution identical with that stated by us according to eqn. (1), as demonstrated by analysis of the mean, variance, skewness and kurtosis. Each simulated experiment was fitted to the model (eqn. 1), and the set of the 1000 estimates of each parameter was statistically analysed.

RESULTS

The experimental conditions of the association-saturation assay were chosen as indicated in the Materials and methods section and were 0.5 mg of protein/ml for each determination, [^3H]R-PIA concentrations of 0.128, 0.555, 1.70 and 7.67 nM with incubation times (min) of 300 (0.128 nM), 7.25 (0.555 nM), 300 (1.70 nM) and 0 (7.67 nM). With the selected experimental conditions, the association-saturation experiment was performed (see the Materials and methods section), and 12 replicates of each determination were made. The values obtained were fitted to eqn. (1) and the parameter values calculated are those indicated in the first row of the Table 1.

The four measurements of total binding fitted to eqn. (1) as a

Fig. 1. Three-dimensional association-saturation surface obtained by fitting the four determinations to eqn. (1)

The experiment was performed as indicated in the Materials and methods section. Each of the four points represent the means for 12 replicates. S.E.M. bars are less than the radius of the closed symbols. The data were fitted by a non-linear regression program (Canela, 1984) to eqn. (1). The plane of the bottom represents the non-specific binding.

Fig. 2. Saturation curves derived from Fig. 1

Projection of Fig. 1 on the total-binding-ligand-concentration plane. Bottom to top time increases from 10 min to 300 min every 10 min.

function of the ligand concentration and the time, for a protein concentration of 0.5 mg/ml, define the surface given in Fig. 1. As shown in this Figure, the association-saturation method combines some aspects of the equilibrium-binding-isotherm experiments and some of the association experiments. This becomes more obvious when Fig. 1 is projected on to the total-binding/ligand-concentration plane (Fig. 2) and on to the total-binding/time plane (Fig. 3). Fig. 2 consists of a set of saturation curves at different times of incubation of ligand and receptor showing that the equilibrium binding is only reached at higher times (upper curves). The straight line at $t = 0$ (Fig. 2, lower line) corresponds to the non-specific binding. Fig. 3 consists of a series of

Fig. 3. Association curves derived from Fig. 1

Projection of Fig. 1 on the total-binding-time plane. Bottom to top the ligand concentration increases from 1 nM to 30 nM every 1 nM.

Fig. 4. Association-dissociation of $[^3\text{H}]\text{R-PIA}$ to soluble receptor

The experiment was performed as indicated in the Materials and methods section. At various incubation times binding was measured. Dissociation was induced after 120 min of association. All points represent means $\pm$ S.E.M. for three replicates. The lines drawn correspond to the best fit to the eqns. (6) and (7) (see the Materials and methods section).

association curves which, after a given time for each ligand concentration, are parallel with the x -axis, thus indicating that the binding equilibrium is reached. The distance between two adjacent parallel lines divided by the increment of ligand concentration is the constant $(1/w)$ for non-specific binding.

Association-dissociation and equilibrium-binding experiments

We have compared the association-saturation method with the classical experiments of: (i) association-dissociation and (ii) equilibrium-binding isotherms.

The association-dissociation experiment, consisting of a set of 16 determinations with three replicates each, was carried out as indicated in the Materials and methods section. Eight association times and eight dissociation times were geometrically distributed between 0 and 120 min, and between 0 and 360 min respectively. The results were fitted to eqns. (6) and (7) as indicated in the Materials and methods section, and plotted as in Fig. 4. The estimates of different parameter values are stated in the second

Fig. 5. Equilibrium binding isotherm

Incubation of [3 H]R-PIA with soluble receptor was done as indicated in the Materials and methods section. All points represent means $\pm$ S.E.M. of five replicates. The data were fitted to the eqn. (9) (see Methods) with a non-linear regression program (Canela, 1984). The insert shows a Scatchard plot of the computer-derived specific binding data.

Fig. 6. Dependence of the S.D./mean ratio of the parameters on the number of replicates for the association-saturation method

The results correspond to the mean of 1000 simulations of the association-saturation experiment (eqn. 1) with different replicates of the four determinations. Simulation was performed assuming the error pattern described by eqn. (10). The parameters were: $\square$, k_{-1} ; $\blacksquare$, k_1 ; $\bullet$, R_0 ; and $\circ$, w .

row of Table 1. As can be seen, the two methods (association-saturation and association-dissociation) differ in the estimates of different parameter values, the K_D value having the greatest discrepancy.

Equilibrium-binding-isotherm analysis was carried out as described in the Materials and methods section. The results obtained were fitted to eqn. (9) and plotted as in Fig. 5. The estimates of different parameter values are shown in the third row of Table 1. This method provides a good estimation of maximum binding and K_D without information about the values

of the kinetic parameters (k_1 and k_{-1}). The values obtained agree well with those obtained with the association-saturation experiment.

Optimization of the number of replicates used for the association-saturation experiment

For the association-saturation experiment the minimum number of experimental points required in the case of one-ligand-one-receptor interaction is four, which is the number of parameters in the model. However, a design consisting of four experimental points is insufficient to obtain good estimates of the parameter values and therefore replicates of each of the four experimental measurements must be carried out.

The minimum number of replicates was determined as follows. We will consider a set of experiments consisting of determinations which are multiples of the number of parameters (8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60 and 400 determinations) and formed by four different points with the same number of replicates each. Since the model is non-linear, we simulated (see the Materials and methods section) 1000 experiments of each case (8, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48, 52, 56, 60 and 400 determinations). The results appear in Fig. 6. It should be noted that the standard deviations of the parameter estimates decrease with the number of determinations, but these decrements are asymptotic, as Fig. 6 shows. Therefore the response would be subjective, but we think that 11 or 12 replicates of each determination are sufficient to obtain accurate and precise estimates of the parameter values. The enhanced refinement achieved with more replicates may not justify for the additional experimental effort. Moreover, we have observed that performing 11 replicates of the experimental point with the highest importance in the k_{-1} estimation, i.e. that for which k_{-1} has the highest relative sensitivity (lowest ligand concentration and maximum time), and only five replicates of the other points, leads to an S.D./mean ratio for each parameter similar to that obtained when performing 11 replicates for all points. It is obvious that this is a subjective decision, but to improve the precision significantly, the experimental effort must be very high.

DISCUSSION

In the present paper, a simple and accurate method to determine the kinetic and equilibrium parameters of a ligand-receptor interaction is proposed. The method consists of a set of association-saturation curves (Figs. 1, 2 and 3) and combines the advantages of the two classical methods of dealing with this issue. The advantages of the association-dissociation curves are the low ligand concentrations used and the determination of estimates of the kinetic constants. The benefits of the saturation isotherms in which high ligand concentrations are used as the accurate determinations of the maximum binding sites (R_0) and K_D . From the results shown in Table 1, the values estimated for R_0 and K_D with the association-saturation method agree with those determined from the equilibrium-binding isotherm. In addition, the association-saturation results permit the calculation of the kinetic constants.

The kinetic parameter values obtained by the association-dissociation method disagree with those obtained by the association-saturation method, and the K_D values obtained by association-dissociation or obtained by saturation experiments are also different. Theoretically, one of the reasons for this last discrepancy is the empirical model used. If the simple bimolecular model used is incorrect because the actual system is more complex (i.e. $R + L \rightleftharpoons RL \rightleftharpoons RL^*$, followed by other ligand-receptor and ligand-enzyme reactions), the association-dissociation method would not yield the same K_D values as those

obtained by saturation isotherms. In this case the same discrepancy will be observed between the association-saturation method and the saturation isotherm, as is easy to prove by simulation analysis. Our results show that K_D values obtained by both methods, association-saturation and saturation isotherms, are the same; thus the simplest empirical model proposed can be used. The disagreement between our method and the classical association-dissociation one is probably due to two factors. The first is that the equation used to fit data in the classical method is only an approximation. The second is that the experimental determination of k_1 in the association-dissociation experiments needs the previous determination of k_{-1} from the dissociation data. This value is subsequently substituted in the association curve equation to obtain k_1 . As a result the error in k_1 is double, since it also includes the error of the k_{-1} determination. These facts together render the whole determination of kinetic constants more sensitive to experimental errors.

The most interesting aspect of the method described here is the accuracy and precision of the estimates of the parameter values obtained. This is due to the fact that the surface of the binding is completely defined by only four points, optimally selected by D-optimal design, and determined with many replicates and, consequently, with high precision. Moreover, the experimental protocol is very simple.

Obviously, the method requires previous knowledge of the model as well as an approximate estimation of the kinetic constants. There are many circumstances in which the model followed is known, but in others is not. There are two basic experimental details that should be borne in mind in order to determine whether the empirical behaviour of ligand-receptor binding fits to the simplest bimolecular interaction described here. One of them is the linearity of the Scatchard plot and the other is the linearity in the $\ln(\text{binding}/\text{binding}_{\text{equilibrium}})$ -versus-time plot, obtained from a simple dissociation experiment (Leung *et al.*, 1990). When these conditions are accomplished, the equation deduced in the Appendix of the present paper can be applied (eqn. 1), and these preliminary experiments can also give guess estimates of the parameter values.

In accordance with our previous results (Casadó *et al.*, 1990b) the solubilized A_1 adenosine receptor has high affinity for R-PIA in the absence of Mg^{2+} and, in contrast with two affinity states found in membranes (Casadó *et al.*, 1990b), there is only one in this case. The K_D value and those of the kinetic constants determined by association-saturation experiment agree with those for the high-affinity state found for the membrane-bound A_1 adenosine receptor subtype from the same source in the same conditions (Casadó *et al.*, 1990b), although a little decrease of the affinity is observed for the solubilized receptor (the K_D value is approximately twice that of the membrane). The decrease in the affinity induced by solubilization is due to a halving of the value of the association rate constant of the solubilized receptor compared with the association rate constant value of the high-affinity membrane-bound receptor, whereas the dissociation rate constant is the same for both forms of the receptor.

APPENDIX

Association-saturation equation

Let us consider the second-order kinetic reaction:

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where R, L and x represent, respectively, the free receptor, free ligand and receptor-ligand complex, and N and y denote the free non-specific binding site and non-specific-binding-site-ligand complex respectively.

Changes in x and y (the concentrations of x and y) are defined by the following system of differential equations

$$\frac{dx}{dt} = k_1 RL - k_{-1}x = k_1(R_0 - x)(L_0 - x - y) - k_{-1}x \quad (\text{A1})$$

$$\frac{dy}{dt} = k_2 NL - k_{-2}y = k_2(N_0 - y)(L_0 - x - y) - k_{-2}y \quad (\text{A2})$$

where R_0 , L_0 and N_0 denote the initial concentrations of receptor, ligand and non-specific-binding sites respectively.

Considering that non-specific binding is a rapid-equilibrium step, its effect is not significant in the receptor-ligand interaction at the usual times at which the measurements are taken. Therefore:

$$\frac{dy}{dt} = 0 \quad (\text{A3})$$

Also $N_0 \gg y$ at the concentrations of ligand used in the experiment. In consequence, eqn. (A2) becomes:

$$k_2 N_0 (L_0 - x - y) - k_{-2}y = 0 \quad (\text{A4})$$

and

$$y = \frac{(L_0 - x)}{(1 + w)} \quad (\text{A5})$$

where w stands for:

$$\frac{k_{-2}}{k_2 N_0} \quad (\text{A6})$$

Substituting y in eqn. (A1) we have:

$$\frac{dx}{dt} = k_1(R_0 - x)\left(L_0 - x - \frac{L_0 - x}{1 + w}\right) - k_{-1}x = k_1(R_0 - x)(L_0 - x)c - k_{-1}x \quad (\text{A7})$$

where

$$c = \frac{w}{1 + w} \quad (\text{A8})$$

In consequence

$$\frac{dx}{dt} = \frac{dx}{k_1 R_0 L_0 c - (k_{-1}(R_0 + L_0)c - k_{-1})x + k_1 c x^2} = \frac{\frac{ck_1}{b}dx}{x - \frac{a+b}{2k_1c}} - \frac{\frac{ck_1}{b}dx}{x - \frac{a-b}{2k_1c}} \quad (\text{A9})$$

where

$$a = k_1(R_0 + L_0)c + k_{-1} \quad (\text{A10})$$

$$b = \sqrt{(a^2 - 4k_1^2 c^2 R_0 L_0)} \quad (\text{A11})$$

Solving by integration

$$x = \frac{\frac{a-b}{2k_1c}(1 - e^{-bt})}{1 - \frac{a-b}{a+b}e^{-bt}} \quad (\text{A12})$$

It should be noted that the measured total bound ligand is $z = x + y$; therefore using eqn. (A5) we will have:

$$z = x + \frac{L_0 - x}{1 + w} = cx + (1 + c)L_0 \quad (\text{A13})$$

and substituting x by the expression given in eqn. (A12):

$$\text{Total binding} = z = \frac{\frac{a-b}{2k_1c}(1 - e^{-bt})}{1 - \frac{a-b}{a+b}e^{-bt}} + (1 + c)L_0 \quad (\text{A14})$$

This equation, eqn. (A14) (eqn. 1 of the main paper) is similar to that obtained by Rodbard & Weiss (1973) for immunoradiometric assays and used by Donner *et al.* (1980). However, the authors failed to take the non-specific binding into consideration.

In eqn. (A14) R_0 , N_0 and z are expressed as concentration units. In order to express these variables as mol/g of protein, R_0 , N_0 and z have to be substituted by R'_0 , N'_0 and $z'_0 = R_0/P$, $N'_0 = N_0/P$ and $z' = z/P$, P being the concentration of protein (g/l) used in the experiment.

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