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PROTEIN KINASE AND CYCLIC 3′,5′-AMP: SIGNIFICANCE OF BINDING AND ACTIVATION CONSTANTS

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SUMMARY

There have been many recent studies of the stimulation of protein kinases by cyclic AMP which activates the most common kinases by binding to an inactive regulatory subunit. In this paper, we show that conventional graphical representation of data (as Lineweaver–Burk and Scatchard plots) does not allow the evaluation of constants for the action of cyclic AMP on such protein kinase.

Moreover, it is demonstrated that, for such enzymes, the so-called dissociation constant (K_d) and activation constant (K_a) have no meaning. The apparent discrepancy between the in vitro results on cyclic AMP concentrations which activate protein kinases and intracellular concentration of cyclic AMP is thus explained at least in part.

Graphical methods of data representation to discriminate between models of protein kinase activation are demonstrated.

INTRODUCTION

In a wide variety of hormone-sensitive cells, cyclic AMP mediates its actions through the activation of protein kinases. A mechanism of activation has been demonstrated for the most prevalent enzyme: a reversible dissociation of the inactive protein kinase (R–C) by binding of cyclic AMP to an inactive regulatory subunit (R) and release of a free fully active catalytic subunit (C) [1–3]:

$$\text{cyclic AMP} + \text{R–C} \rightleftharpoons \text{R–cyclic AMP} + \text{C}$$

Many recent quantitative studies of this binding mechanism and of the resulting increase in kinase activity include the determination of two constants, the equi-

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librium constant of the reaction (K_{eq}) and the so-called "activation constant", defined as the concentration of cyclic AMP needed for half-maximal activation of the kinases (K_a). As experimental determinations of such constants were made before the reaction mechanism was known they were often performed using the conventional linear graphical representations of the enzymatic data, namely Lineweaver–Burk and Scatchard plots [2, 4–6]. The values found for K_a ($1–5 \times 10^{-8}$ M) were much lower than the cyclic AMP concentrations (about 10^{-6} M) measured in unstimulated cells, which implied that in such cells, protein kinases should be fully activated and no regulation through cyclic AMP could occur unless it was postulated that a large part of this cyclic AMP was not available to the kinase, i.e. was sequestrated [7–13]. Until now the search for a sequestrating structure has been unfruitful.

The purpose of this article is to show that the conventional Lineweaver–Burk and Scatchard representations are not compatible with the only known mechanism of protein kinase activation and that when this mechanism applies the constants estimated from such an analysis have no meaning. Adequate methods for discriminating between mechanism of activation and for estimating the constants are proposed. It is shown that the so-called K_a is not a constant.

METHODS AND RESULTS

Cyclic AMP binding mechanism

The first historical, and simplest, hypothesis about the binding of cyclic AMP to protein kinase was implicit: it involved one ligand and one site on a receptor protein [5]. If R–C is the protein kinase the reaction is

$$\text{R–C} + \text{cyclic AMP} \rightleftharpoons \text{cyclic AMP–R–C} \qquad \text{(Model 1)}$$

For a compound X, (X) refers to equilibrium concentration and $(X)_t$ to total concentration. At equilibrium:

$$K_{eq}^{(1)} = \frac{(\text{cyclic AMP–R–C})}{(\text{R–C})(\text{cyclic AMP})} \qquad (1)$$

This $K_{eq}^{(1)}$ has the dimensions of the reciprocal of a concentration. In order to express the concentration at which half of the kinase is bound, biochemists often prefer to use $K_d = 1/K_{eq}^{(1)}$ which has the dimensions of a concentration [4, 6, 14–26].

In the case of the kinase dissociated by cyclic AMP the reaction is

$$\text{cyclic AMP} + \text{R–C} \rightleftharpoons \text{R–cyclic AMP} + \text{C} \qquad \text{(Model 2)}$$

and at equilibrium:

$$K_{eq}^{(2)} = \frac{(\text{R–cyclic AMP})(\text{C})}{(\text{R–C})(\text{cyclic AMP})} \qquad (2)$$

$K_{eq}^{(2)}$ is a dimensionless constant. Although the most studied kinases correspond to Model 2 [1, 2, 4], they have been investigated according to Model 1 and their equilibrium constant has thus been expressed in terms of the reciprocal of a concentration, which has no sense and may lead to great confusion in interpretations.

To determine the characteristics of cyclic AMP binding to the protein kinase,

the concentrations of cyclic [^{3}H]AMP bound to an unknown but fixed constant amount of kinase are measured at equilibrium for varying concentrations of free cyclic [^{3}H]AMP. In Model 1, Eqn 1 can be rewritten under the classical forms of Lineweaver–Burk:

$$\frac{1}{(\text{cyclic AMP–R–C})} = \frac{1}{(\text{cyclic AMP})} \cdot \frac{1}{K_{eq}^{(1)}\,(\text{R–C})_t} + \frac{1}{(\text{R–C})_t} \tag{3}$$

and Scatchard

$$\frac{(\text{cyclic AMP–R–C})}{(\text{cyclic AMP})} = K_{eq}^{(1)}\,[(\text{R–C})_t - (\text{cyclic AMP–R–C})] \tag{4}$$

where

$$(\text{R–C})_t = (\text{R–C}) + (\text{cyclic AMP–R–C}) \tag{5}$$

Although Scatchard plots as originally described by Scatchard would involve the plotting of r/(cyclic AMP) against r where $r = (\text{cyclic AMP–R–C})/(\text{R–C})_t$, we use in our study the most common plot encountered in the literature about the cyclic AMP binding [16, 19], i.e. the ratio of bound to free cyclic AMP is plotted against the concentration of bound cyclic AMP. This slight modification is a simple normalization and does not affect the results. When data corresponding to Model 1 are plotted according to Lineweaver–Burk and Scatchard representations, they fall in straight lines which can be fitted easily and used to estimate $K_{eq}^{(1)}$ (Fig. 1 A, C).

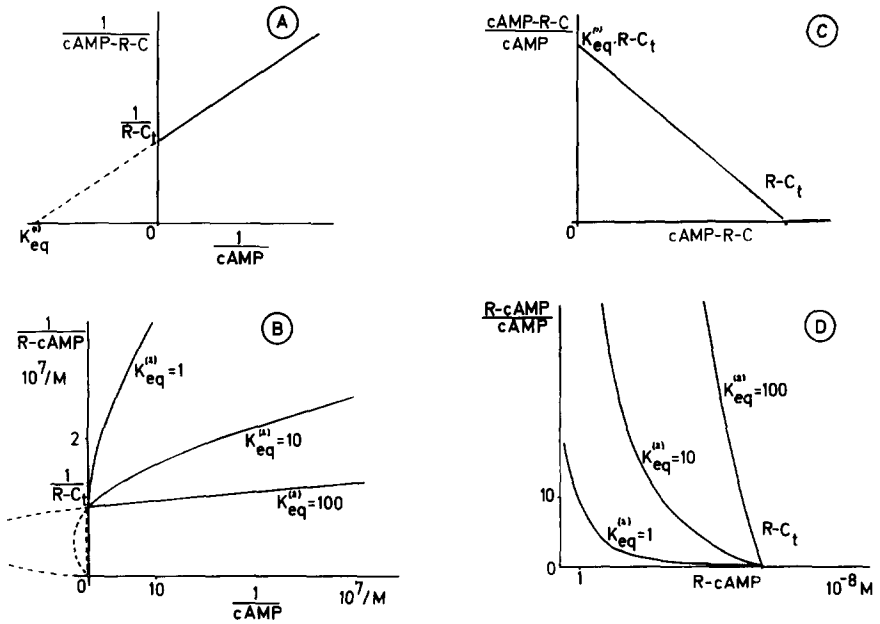

Fig. 1. Theoretical Lineweaver–Burk plots for Model 1 (A) and Model 2 (B) and theoretical Scatchard plots for Model 1 (C) and Model 2 (D). For Model 2, three curves are plotted for the following values of $K_{eq}^{(2)}$: 1; 10; 100. If these curves were fitted by straight lines as if the reaction occured according to Model 1, one could estimate the $K_{eq}^{(1)}$: 10^8/M; $2.5 \cdot 10^8$/M, $12 \cdot 10^3$/M. cAMP: cyclic AMP.

When Eqn 2 corresponding to Model 2, i.e. to the dissociable kinase, is treated in the same way, very different results are obtained. The Lineweaver–Burk rearrangement gives:

$$\frac{K_{eq}^{(2)}\,(\text{R–C})_t}{(\text{R–cyclic AMP})^2} - \frac{K_{eq}^{(2)}}{(\text{R–cyclic AMP})} - \frac{1}{(\text{cyclic AMP})} = 0 \tag{6}$$

where

$$(\text{R–C})_t = (\text{R–C}) + (\text{R–cyclic AMP}) \tag{7}$$

and under the assumption $(R)_t = (C)_t$, thus

$$(\text{C}) = (\text{R–cyclic AMP}) \tag{8}$$

Eqn 6 describes a parabola (Fig. 1 B).

Using Eqns 7 and 8, the Scatchard rearrangement gives Eqn 9:

$$\frac{(\text{R–cyclic AMP})}{(\text{cyclic AMP})} = K_{eq}^{(2)}\,\frac{(\text{R–C})_t - (\text{R–cyclic AMP})}{(\text{R–cyclic AMP})} \tag{9}$$

This equation describes a hyperbola (Fig. 1 D).

Until now, the relative physiological concentrations of catalytic and regulatory subunits have not been measured. The assumption $(R)_t = (C)_t$ has been chosen on account of its simplicity. Moreover, in most of the reported binding experiments, protein kinase had been purified by a method which would exclude separate R and C subunits. Anyway, unequal concentrations of subunits would affect only the parameters of the conic curves but not the nonlinearity of the two representations nor the aspect of the curves.

In both representations data corresponding to Model 2 do not therefore fall in a straight line, although they may at first sight seem to do so. In any case, the estimation from such lines of a $K_{eq}^{(1)}$ corresponding to Model 1 would have no meaning. It is of interest that several cases have been found for which the Scatchard plot of binding data did not fall in a straight line [21, 22]. Experimental data corresponding to Model 2 could be fitted by Eqns 6 and 9 and thus allow the calculation of $K_{eq}^{(2)}$ and $(\text{R–C})_t$. However, it would be more convenient to find a linear representation from which graphical estimates could be derived. Eqn 9 can be rearranged as:

$$\frac{(\text{R–cyclic AMP})^2}{(\text{cyclic AMP})} = K_{eq}^{(2)}\,[(\text{R–C})_t - (\text{R–cyclic AMP})] \tag{10}$$

which will give a representation very similar to the Scatchard plot (Fig. 2). This method is as general as the Scatchard representation and could be applied to determine the equilibrium constant of all reactions of the type $W + X \rightleftharpoons Y + Z$ where $(Y) = (Z)$.

In practice, the Lineweaver–Burk method, the Scatchard method and the proposed representation should be used to plot binding data. The nonlinearity in the first two representations will exclude Model 1. However, the linearity of such plots will not prove the validity of this model. In this case $K_{eq}^{(1)}$ should be estimated for

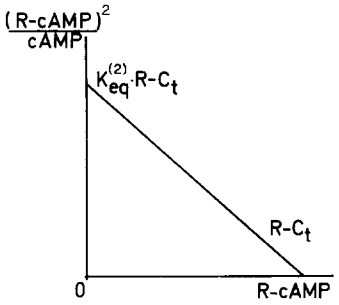

Fig. 2. Theoretical linear representation defined for Model 2 by Eqn 10. cAMP: cyclic AMP.

various concentrations of kinase and, if different $K_{eq}^{(1)}$ values are found, Model 1 should be rejected. A similar argument can be made for Model 2 and the representation we propose. Of course, a nice fitting of the data as a straight line in a given representation is never a proof of the validity of the model implied by this representation.

In all equations discussed above, (cyclic AMP) represents the concentration of free cyclic AMP at equilibrium. If the kinase is dialysed against cyclic AMP, (cyclic AMP) is given by:

$$\text{(cyclic AMP)} = \text{(cyclic AMP)}_t - h\,\text{(cyclic AMP–R–C)} \qquad (11) \text{ for Model 1}$$

$$\text{(cyclic AMP)} = \text{(cyclic AMP)}_t - h\,\text{(R–cyclic AMP)} \qquad (12) \text{ for Model 2}$$

where $h = v/V$, v is the volume of the dialysis bag and V is the total volume (including v). If the binding reaction is held in a closed system, i.e. all the components of the binding reaction are distributed in the same volume, h is equal to 1. The assumption (cyclic AMP) = (cyclic AMP)$_t$ is valid only if the product of h by equilibrium concentration of bound cyclic AMP is much lower than the total concentration. Otherwise, one could verify that, in the case of the first model for instance, the replacement of (cyclic AMP) by (cyclic AMP)$_t$, affects the linearity of the representations (Fig. 3). For $h = 0$, we find again the linear representation. Thus, in order to avoid some additional approximations in the graphical determination of $K_{eq}^{(1)}$, the values of (cyclic AMP) should be computed with the aid of Eqn 11 or measured, which is rarely done [15, 17]. Of course, whatever the model which is to be applied to the binding of cyclic AMP to protein kinase, the equilibrium constants should be determined under conditions similar to those existing in the cell in vivo (with regard to temperature, pH, ionic composition of the medium, etc...) if they have to have any physiological significance. It should be pointed out in this regard that most binding studies are carried out at 4 °C and acid pH.

Cyclic AMP activation of protein kinase

In the literature on cyclic AMP activation of protein kinase, a confusion between Model 1 and Model 2 also exists. Under conditions where substrate is saturating and substrate by itself does not modify the equilibrium of the binding reaction, the rate of the enzymatic reaction should be proportional to the concentration of the

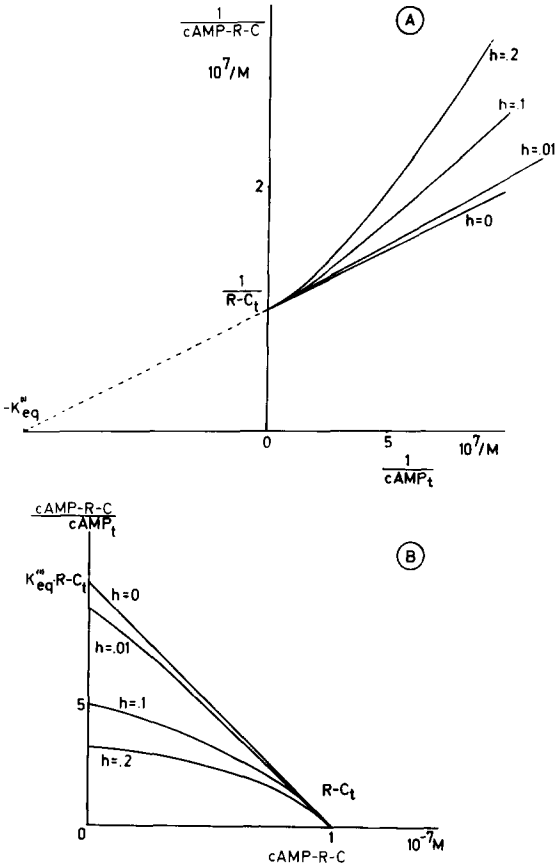

Fig. 3. Hyperbolic representations defined for Model 1 and deduced from Lineweaver–Burk plot (A) and Scatchard plot (B) when (cyclic AMP) is replaced by (cyclic AMP)$_t$. By letting $K_{eq}^{(1)} = 10^8$/M and (R–C)$_t = 10^{-7}$ M, four curves are plotted for varying values of h (0.2; 0.1; 0.01; 0). The graphical evaluate of $K_{eq}^{(1)}$ gives, respectively, the following values: $4.5 \cdot 10^7$/M; $6.5 \cdot 10^7$/M; $9.5 \cdot 10^7$/M; 10^8/M. cAMP: cyclic AMP.

active enzyme, i.e. to the concentration of kinase to which cyclic AMP is bound. Thus, curves of cyclic AMP activation of protein kinase should parallel curves of cyclic AMP binding when activity is plotted versus free cyclic AMP concentration. Depending on the type of binding (Model 1 or Model 2), the curves of activation will therefore be very different in both Lineweaver–Burk and Scatchard representations. In the literature, K_a has always been estimated assuming Model 1 and the validity of Scatchard and Lineweaver–Burk representations [4, 5, 14, 17–20, 24, 28–40]. However, generally, in such experiments, only the total cyclic AMP concentration (cyclic AMP)$_t$, i.e. the concentration added to the medium, is known. Half-maximal activation is obtained at the cyclic AMP concentration (K_a) at which half of the total kinase is activated, i.e. is bound to cyclic AMP. In the case of Model 1, setting

$$\text{(cyclic AMP–R–C)} = \frac{1}{2}\,\text{(R–C)}_t \text{ and } K_a^{(1)} = \text{(cyclic AMP)}_t$$

in Eqn 1, we can arrive at the simple form:

$$K_a^{(1)} = \frac{1}{2}(\text{R–C})_t + \frac{1}{K_{eq}^{(1)}} \tag{13}$$

In a similar way, we can evaluate $K_a^{(2)}$ for the Model 2. Setting (R–cyclic AMP) = 1/2 (R–C)$_t$ and $K_a^{(2)}$ = (cyclic AMP)$_t$ in Eqn 2 we obtain

$$K_a^{(2)} = \frac{1}{2}(\text{R–C})_t \left(1 + \frac{1}{K_{eq}^{(2)}}\right) \tag{14}$$

A plot of K_a against (R–C)$_t$ will be linear in the two cases, but the slopes are different, 1/2 and 1/2 (1 + $1/K_{eq}^{(2)}$) respectively, and the intersection of the curve with the y-axis is equal to $1/K_{eq}^{(1)}$ for the first model, whereas the curve passes through the point of origin for the second model. Thus, whatever the mechanism, when expressed as the total cyclic AMP concentration in the reaction used, K_a is not a constant and is not equivalent to K_d. However, for small concentrations of kinase (e.g. 10 times less than K_d) one can, as has been done, equate K_d and K_a in Model 1. These equations also show that whatever model is used it is necessary to know the concentration of kinase in the cell in order to estimate the level of activation of intracellular protein kinase by a given cyclic AMP concentration.

If free cyclic AMP, i.e. (cyclic AMP) were measured in kinase activation experiments, and used in Eqns 1 and 2, K_a would be equivalent to K_d for Model 1.

In model 1, $K_a^{(1)} = \frac{1}{K_{eq}^{(1)}} \equiv K_d$

In model 2, $K_a^{(2)} = \frac{(\text{R–C})_t}{2\,K_{eq}^{(2)}}$

However, in the literature, we have not found activation curves related to free cyclic AMP concentrations. In practice, when only total cyclic AMP concentration is known, activation curves of protein kinase by cyclic AMP should be shown by the direct representation, i.e. rate of reaction versus cyclic AMP concentration. The Scatchard and Lineweaver–Burk representations are only valid in the case of Model 1 and if protein kinase concentration is markedly lower than K_d.

Activation curves should be obtained for various concentrations of protein kinase and K_a derived from each curve. The plot of K_a versus kinase concentration should give a straight line (Fig. 4). If this line passes through the origin, Model 1 is excluded and the validity of Model 2 suggested; if the line crosses the ordinate above the origin, Model 2 is excluded, Model 1 is suggested and the ordinate of the intersection gives an estimate of K_d in this model. Whatever the model, if K_{eq} is known, the total concentration of protein kinase can be evaluated from the slope of the line (Fig. 4).

The two models considered in this article do not account for the existence of kinase activity in the absence of cyclic AMP. If the basal and cyclic AMP-activated activities belong to the same enzyme, this basal activity could be explained by the presence of free catalytic subunit resulting from (C)$_t$ > (R)$_t$ or from a spontaneous

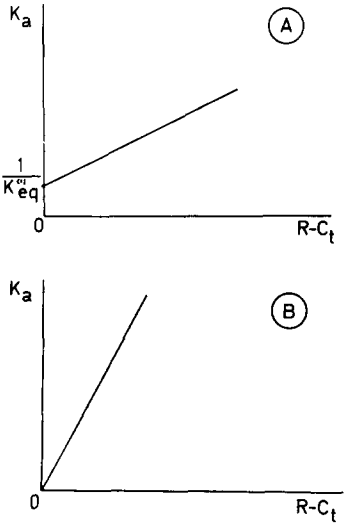

Fig. 4. Theoretical plots of K_a versus the total concentration of kinase, for Model 1 (A) and Model 2 (B). The slopes of the curves are respectively $^1/_2$ and $^1/_2$ $(1 + 1/K^{(2)}_{eq})$. cAMP: cyclic AMP.

reversible dissociation of the complex R–C. It is unlikely that such assumptions would account for the basal activity (10–20% of the maximal activity) as addition of regulatory subunit or of a catalytic subunit inhibitor does not affect this activity [3].

The influence of protein substrate and Mg-ATP concentrations have not been considered as it is assumed that these substrates only take part in the phosphorylation reaction after dissociation, and as they are then always used at saturating concentrations.

DISCUSSION

There is an apparent discrepancy in the literature between the concentration of cyclic AMP in the cell ($\sim 10^{-6}$ M) and the concentration of cyclic AMP needed to bind or to activate half the protein kinase ($\sim 2 \cdot 10^{-8}$ M) in such cells. If these two sets of data were valid, all the cyclic AMP-activated protein kinases should be fully activated in the unstimulated cells and cyclic AMP could not act as a regulatory agent on this kinase. In fact, in cases where both have been measured there is a close parallelism between intracellular cyclic AMP concentration and protein kinase activity [8, 41, 42]. To explain these facts, it has been postulated that a large part of the intracellular cyclic AMP is sequestred from the kinase [5, 7–12, 41]. However, the search for a sequestrating structure has until now been unfruitful. In this article it is shown that the discrepancy may arise in part because (1) the K_d, i.e. the concentration of free cyclic AMP at which half of the kinase is bound to cyclic AMP, has no meaning for an enzyme which dissociates when activated; (2) the K_a, i.e. the total concentration of cyclic AMP at which half of the kinase is activated has no meaning for a dissociable enzyme; even for an enzyme does not dissociate when activated K_a is not a constant. Kinetic methods to distinguish, even in relatively crude preparations, the reaction by which cyclic AMP binds to and activates protein kinase, and to determine the

equilibrium constant of such reactions are presented. A similar reasoning may be applied to other reactions of the same type for example the cyclic 3′,5′-GMP protein kinases. It is stressed that methods and representations used to analyse kinetic data and models to which these data apply should correspond.

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