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Analysis of effects of corticotropin, forskolin and fluoride on activity of adenylate cyclase of bovine adrenal cortex

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Abstract

A mathematical model relating the activity of adenylate cyclase (AC) with concentrations of stimulators, equilibrium dissociation constants, specific activity and efficacies of AC depending on the states of its binding sites has been developed and used for analysis of the data on activation of AC of bovine adrenal cortex plasma membranes presented in (De Foresta et al. (1987) FEBS Lett. 216, 107–112). Equilibrium dissociation constants, x_h and x_l, corresponding to high- and low-affinity forskolin-binding sites were estimated to be 0.37 and 17 μM; these constants characterize forskolin's potency more adequately than does ED_{50}, the concentration eliciting half-asymptotic activity of AC. Corticotropin does not affect the affinity of AC for forskolin whereas fluoride increases this affinity, thus augmenting forskolin's potency. Hormone receptor of adenylate cyclase of bovine adrenal cortex has been suggested to have two or more binding sites for corticotropin. Some unidentified factor(s) may be responsible for the differences found in adenylate cyclase activity in different experiments carried out under similar conditions. The model applied for the analysis may be thought to be the best means for the moment to relate dose-response dependencies with what is known or can be hypothesized about the mechanisms underlying activation of adenylate cyclase.

Keywords: Adenylate cyclase; Forskolin; Corticotropin; Fluoride; Mathematical model

1. Introduction

Adenylate cyclase, the enzyme converting ATP into cyclic AMP (cAMP) [1,2] is considered to be composed of three types of structural components [1]: receptors for a variety of hormones, neurotransmitters or other regulatory molecules [1–8], stimulatory (G_s) and inhibitory (G_i) guanine nucleotide-binding proteins (G-proteins) mediating the stimulation or inhibition [9] and catalytic component, catalytic (sub)unit or catalyst [1]. Alternately, in G-protein-centred reviews, adenylate cyclase (or more precisely, its catalyst) is considered as an effector in a transmembrane signal transduction system, G-protein playing the role of a transducer [9–11]. cAMP synthesis can be stimulated beyond the receptors by diterpene forskolin [3–5], by fluoride acting via G_s [12–15] or affected by other agents. Adenylate cyclase (i.e., the 3-component complex) has been shown to have two forskolin-binding sites, the low-affinity site being associated with the catalyst, and the high-affinity one being attributed to the activated complex of the catalyst with G-protein [16,17]. Although forskolin interacts directly with the catalyst [18,19], G-protein is necessary for the full expression of the effect of forskolin [20,21]. For understanding the processes of control of activity of the cyclase, of all available data on its stimulation or inhibition those providing more details are of major interest.

In de Foresta and co-authors' article [6] dose-response curves for both forskolin and hormone are presented as well as curves for combined effects of those agents and for forskolin and fluoride; divergence is demonstrated between combined effect of forskolin and hormone and that of forskolin and fluoride; difference in dose-response curves for forskolin and for hormone can be seen: the curve for forskolin extends over a wider range of concentrations than that for hormone.

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A simple mathematical model has been developed to relate the dose-response curves with known or putative properties of the cyclase and the agents affecting its activity (see Appendix). The subject of this article is to analyze and interpret the results of the quoted work [6] in terms of the model with the view to elucidate the mechanisms underlying control of activity of adenylate cyclase.

2. Methods and model

Materials and methods used are described in [6]. Briefly, bovine adrenal cortex plasma membranes were prepared by homogenizing the cortex essentially as in [22] as modified in [6]. The preparation included the preparation of a mitochondrial-lysosomal fraction, followed by a centrifugation at equilibrium in a discontinuous sucrose gradient. Adenylate cyclase activity was determined at pH 7.5 and 30° C [6]. The cAMP formed was determined as described in [23] using an Amersham assay kit.

The model is based on the following: (1) interactions between hormone and its receptor and between forskolin and adenylate cyclase are reversible [3,4]; (2) affinity of hormone receptor for hormone and those of forskolin-binding sites for forskolin either (i) remain unaltered irrespective of the states of other sites (free or occupied) or the receptor (inactive or active) or (ii) are altered as a result of interaction(s) of forskolin or hormone with other site(s) or the receptor; (3) adenylate cyclase complex is supposed to assume any of several fixed states (and to exhibit any of several fixed efficacies) depending on the states of its hormone receptor (either inactive or active) and its forskolin-binding sites (either free or occupied) (Fig. 1); (4) the total observable activity is comprised of the activities of all the complexes of the cyclase in all the states, the contribution of the complexes in each state being proportional to the efficacy of the complex and the number of complexes in this state depending on the concentrations of stimulators.

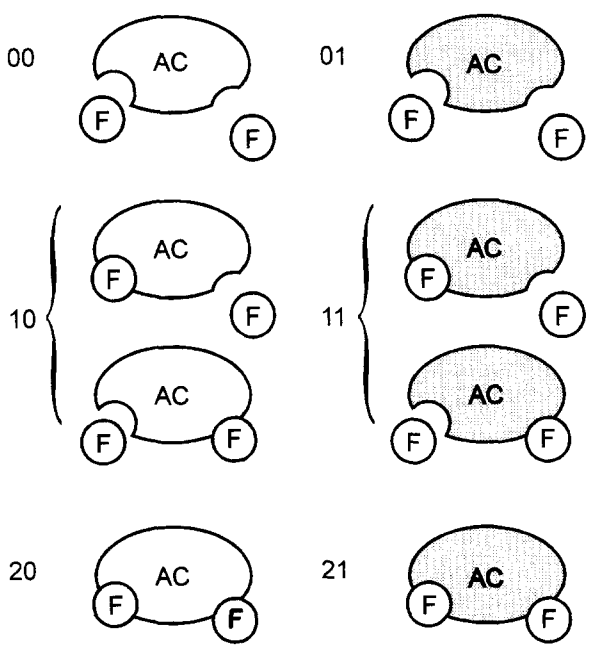

Fig. 1. Schematic representation of interactions between adenylate cyclase complex (AC) and forskolin (F). Possible states of the complex with regard to the states of its hormone receptor (unshaded figures corresponding to inactive states of hormone receptor, the shaded ones, to active states) and occupancy of its forskolin-binding sites are designated as 00,...,21. Forskolin-binding sites are characterized by equilibrium dissociation constants, the above states of the cyclase, by their efficacies (see text for details).

Under these assumptions, modelling of dose-response curves is a matter of technique, even if complicated. Relative concentrations of adenylate cyclase complexes in each state (Fig. 1) can be found in principle from the system of equilibrium equations based on the Law of Mass Action. This approach, however, is impracticable because of a number of possible states. In addition, the alternatives in statement (2) mean that several models are possible. The simplest case corresponding to the alternative (i) is developed in the Appendix.

According to the model (see Eq. (A-9) and Eqs. (A-3)–(A-8)), the activity of adenylate cyclase depends on the concentration of forskolin, x, and that of hormone or other agent, y, as follows:

$$A = \alpha\left[\frac{x_h x_l}{(x+x_h)(x+x_l)}e_{00} + \frac{(x_h+x_l)x}{(x+x_h)(x+x_l)}e_{10} + \frac{x^2}{(x+x_h)(x+x_l)}e_{20}\right](1-P_r) + \alpha\left[\frac{x_h x_l}{(x+x_h)(x+x_l)}e_{01} + \frac{(x_h+x_l)x}{(x+x_h)(x+x_l)}e_{11} + \frac{x^2}{(x+x_h)(x+x_l)}e_{21}\right]P_r \quad (1)$$

where α is specific activity of the cyclase; x_h and x_l are equilibrium dissociation constants corresponding to high- and low-affinity forskolin-binding sites; $e_{00} \geq 0$, ..., $e_{21} \geq 0$ are relative efficacies of adenylate cyclase complex corresponding to the states of forskolin-binding sites and that of the receptor, the numerical subscripts, 0, 1 and 2 referring to no one, any one and both forskolin-binding sites occupied (the first subscript), 0 and 1 referring to inactive and active states of the receptor (the second one); P_r is the probability for hormone receptor to be active (i.e., to be able to affect the state of the catalyst) which in case of single hormone-binding site is determined by

$$P_r = \frac{y}{y+y_r} \quad (2)$$

where y_r is equilibrium dissociation constant.

For $x = 0$ Eq. (1) is reduced to 1-agent model

$$A = \alpha e_{00}(1 - P_r) + \alpha e_{01} P_r \quad (3)$$

which can be applied to the activation of the cyclase by hormone alone; P_r in the simplest case is determined by (2). Similarly, $y = 0$ in Eq. (1) yields 1-agent 2-binding-sites model

$$A = \alpha e_{00} \frac{x_h x_l}{(x + x_h)(x + x_l)} + \alpha e_{10} \frac{(x_h + x_l)x}{(x + x_h)(x + x_l)} + \alpha e_{20} \frac{x^2}{(x + x_h)(x + x_l)} \quad (4)$$

which is applicable to the cyclase activation by forskolin alone.

Model fitting to the experimental data was carried out by the method of least squares using a special computer program (Juška, A., unpublished data). Analysis of variance (Fisher's F-criterion) [24] was used to test the goodness-of-fit of the model as well as to verify the homogeneity of two sets of experimental data.

3. Model fitting and data analysis

There is not much sense in fitting a model with 9 unknown parameters (such as model (1)) to a set of experimental data based on 8 or less independent measurements (corresponding to 8 or less fixed concentrations of an activator; see Figs. 1 and 2). Eq. (4), however, has only 5 independent parameters and can be applied to a set of data on activation of adenylate cyclase by forskolin alone (Fig. 2, left, lower solid curve). While applying the model, one of the relative efficacies, e_{00}, was taken for reference; it was assumed that $e_{00} = 1$; other parameters have been estimated as a result of fitting of this model to the data and presented in Table 1.

Application of analysis of variance to the model and the data results in F-ratio,

$$F = 2.4406 < 4.0662 = F(0.05;3,8)$$

where $F(0.05;3,8)$ is Fisher's critical value for 0.05 confidence level and 3 and 8 degrees of freedom, i.e., number of independent measurements (i.e., measurements at different fixed concentrations of stimulators) minus number of the parameters and total number of measurements minus number of independent measurements. On the basis of the above inequation the model has been assumed to be in agreement with the data. However, this model deviates from data points presented on Fig. 3, left (lower dashed curve and corresponding data points) for similar experiment. In these experiments two different preparations of the plasma membranes were used (Fig. 1, left, corresponding to preparation A, Fig. 3, to B), therefore, while analyzing these data two possibilities had to be considered: (1) the results obtained on A and B preparations are significantly different, and (2) the difference is statistically non-significant. By analysis of variance significant difference between the two sets of data has been found, and because of that fitting of the model to these data sets was carried out separately, assuming parameter α to be different for each set.

It can be seen (Fig. 3, left and right) that several combinations of concentrations of stimulators are represented twice, both on the curves for a range of concentrations of forskolin and on the ones for corticotropin (e.g., 0 concentrations of both stimulators). The activity of the cyclase of the same preparation was considered to be

Table 1
Parameters of models (1) and (7) estimated [a] by their fitting to experimental data obtained on three plasma membrane preparations (A, B and C)

Parameter	Symbol	Value			Dimension
		A	B	C	
Specific activity of the cyclase	α	14.9	12.9	14.9	pmol/min per mg protein
Equilibrium dissociation constant					
	x_h	0.368	0.368	1.41	μM
	x_l	16.7	16.7	16.7	μM
	x_r	– [b]	356	–	nM
Relative efficacy					
	e_{10}	4.26	4.26	4.26	dimensionless
	e_{20}	11.1	11.1	7.98	dimensionless
	e_{01}	–	7.92	–	dimensionless
	e_{11}	14.3	12.1	–	dimensionless
	e_{21}	19.2	14.6	–	dimensionless
Efficacy enhancement factor					
	ϵ_0	–	7.51	6.87	dimensionless
	ϵ_1	–	1.55	1.41	dimensionless
	ϵ_2	–	1.09	1.21	dimensionless
Potentiation factor	β	–	0.09	0.09	dimensionless

[a] e_{00} has been assumed to be equal to 1.
[b] Not determined.

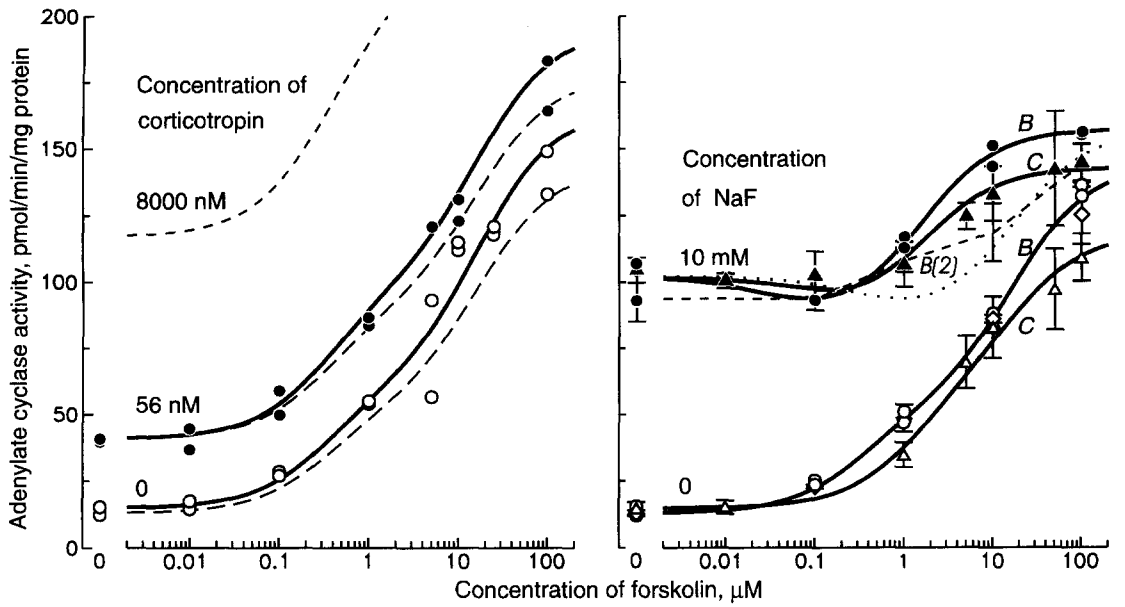

Fig. 2. Dependencies of activity of adenylate cyclase of bovine adrenal cortex plasma membranes obtained with preparations A (left), B and C (right) on concentration of forskolin applied alone (○, △ and ◇, lower solid curves) or in presence of 56 nM ACTH(1–24) (left, ●, upper solid curve) or 10 mM NaF (right, ● and ▲, upper solid curves). The curves correspond to Eq. (4) (the lower ones), Eq. (1) (left, solid and dashed ones) and Eq. (7) (right, upper solid ones) whose parameters are presented in Table 1. (Right) The dotted curve corresponds to model (7) with $\beta = 1$; the dashed broken line connects the means of data of experiment 2 carried out with preparation B; the bars correspond to S.E. of more than two (three or four) determinations.

independent of any factors other than stimulators of given concentrations. This allowed to consider all the measurements corresponding to the same combination as replicates of *one* independent measurement and to take data points corresponding to the same combination from one curve and plot them additionally on the other and vice versa and

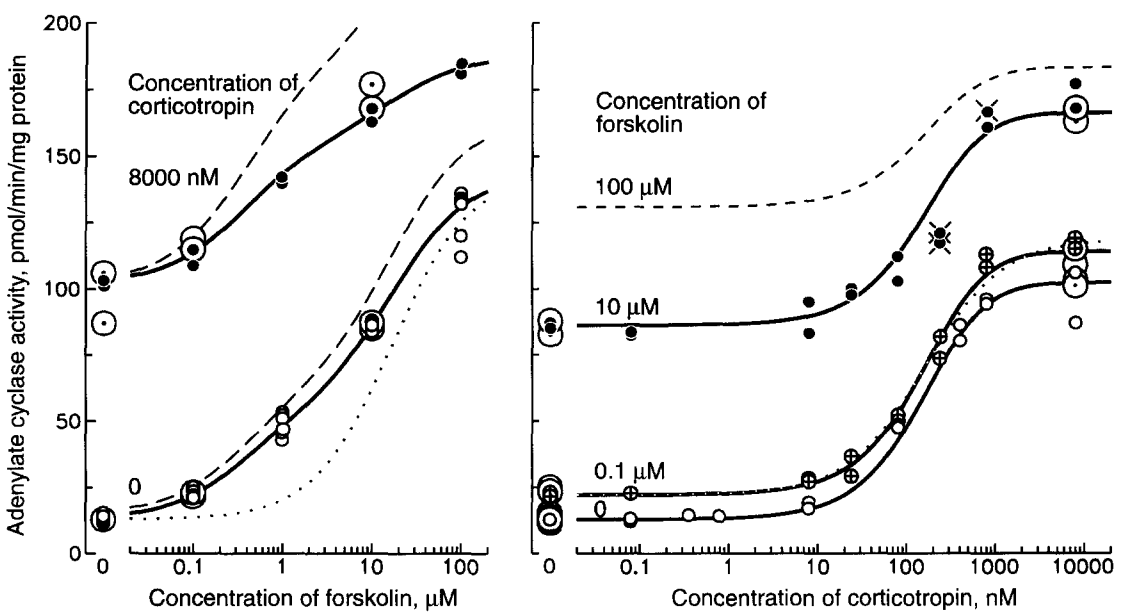

Fig. 3. Dependencies of activity of adenylate cyclase (preparation B) on concentration of forskolin (left) or ACTH (right) applied alone (○, lower solid curves) or in presence of (left) 8000 nM ACTH (●, upper solid curve); the lower dashed curve corresponds to α determined for preparation A (see Table 1), the upper dashed one, to e_{11} and e_{21} determined for preparation A; the dotted curve corresponds to Eq. (9). (Right) The lower solid curve corresponds to model (3) with P_r determined by Eq. (5), the middle and the upper solid curves as well as the upper dashed one correspond to model (1) with P_r determined by Eq. (5); the dotted one corresponds to Eq. (1) and P_r determined by Eq. (2). The encircled data points on one side of the Figure correspond to the data at the same concentrations of stimulators on the other side and vice versa. The data points marked by the symbols × were excluded from the analysis.

to use these 'additional' data (encircled data points on Fig. 3) for model fitting. Note that the encircled data points are close to the corresponding non-encircled ones.

All the parameters of the model were assumed to be the same for all the experimental data obtained on the same preparation. To estimate α for preparation B, Eq. (3) with P_r determined by Eq. (2) was applied to the data on activation of the cyclase by hormone alone. On the basis of Fisher's F-criterion the model fitted could be accepted.

With α estimated above, Eq. (4) fits better to the data obtained on the cyclase from preparation B (Fig. 2, right, and Fig. 3, left), the number of independent measurement not being sufficient to verify the goodness-of-fit. However, Eq. (4) taken together with Eq. (3) and considered as one model can be applied to the data for forskolin alone and hormone alone taken together (considering the data corresponding to 0 concentrations of the stimulators as data of *one* independent measurement) and analysis of variance can be applied to this model and these data. On the basis of Fisher's F-criterion the model could be accepted as well.

Eq. (1) with P_r determined by Eq. (2) applied to the data on combined effect of hormone and forskolin (Fig. 3, right, dotted curve) is rather close to the data points, the number of measurements being insufficient again to verify the goodness-of-fit. Taken into account the data for forskolin alone, the model *could not* be accepted. Experimental data points (see also the points for higher concentration of forskolin) seem to indicate a sharper bend of the curve at high concentrations of hormone than that at low concentrations whereas the model curve – a hyperbola – is symmetric (if plotted in semilogarithmic scale), its shape being determined, in the last analysis, by the Law of Mass Action. No more data are available to justify any modification of the model by introduction of additional parameters which would (formally, at least) solve the problem. With this in mind, Eq. (2) was substituted (formally) by

$$P_r = \frac{y(y + 2y_r)}{(y + y_r)^2} \tag{5}$$

which in case of stimulation by hormone alone results in

$$A = \alpha e_{00} \frac{y_r^2}{(y + y_r)^2} + \alpha e_{01} \frac{y(y + 2y_r)}{(y + y_r)^2} \tag{6}$$

This model fitted to the data is equally acceptable as that using Eq. (2):

$$F = 1.3974 < 2.8524 = F(0.05;5,16)$$

(see Fig. 3, right, lower solid curve). Eq. (5) will be substantiated and discussed in more detail in the next section. Further parameters estimated are presented in Table 1.

As a result of further fitting (Fig. 3, left, upper solid curve) the rest parameters of Eq. (1) with P_r determined by Eq. (5) for the combined effect of forskolin and hormone have been estimated (Table 1). The F-ratio for the upper curve, left, and the lower one, right (Fig. 3), taken together,

$$F = 2.7735 < 3.0491 = F(0.05;3,22),$$

and the model has been accepted.

Model curves (Eq. (1) with P_r determined by (5) and all the parameters estimated above) are rather close to experimental data points corresponding to combined effect of hormone and forskolin (Fig. 3, right, middle and upper solid curves) without any further fitting; this model fits better to the data than that with P_r determined by Eq. (2) (compare middle solid curve and dotted one, Fig. 2, right). Application of Fisher's F-criterion to the middle solid curve, right, and the upper one, left (Fig. 3) yields

$$F = 2.6360 < 3.0725 = F(0.05;3,21);$$

on this basis model (1) with P_r determined by Eq. (5) has been accepted.

It can be seen that a few data points deviate considerably from the upper solid curve (Fig. 3, right). The model could be considered consistent with these data only if some most deviating data points were excluded from the analysis. These deviating data points may indicate to a more complicated mechanism of the combined stimulation of the cyclase by forskolin and corticotropin than that the model is based on, or they may not. To solve this problem, more detailed data are necessary.

Again, as seen from Fig. 2 (left, middle dashed curve), model (1) with the values of e_{11} and e_{21} estimated above does not fit to the data on combined effect of forskolin and corticotropin obtained on preparation A. The model was fitted to this set of data separately, assuming e_{11} and e_{21} to be different from those for set B. As a result, the above efficacies for this set have been estimated (Table 1). In this case, again, the number of independent measurements is not sufficient to verify the goodness-of-fit of the model. No more data on this preparation are available.

There is good reason to apply Eq. (1) to the analysis of activation of the cyclase by forskolin and fluoride. Since in the work under analysis [6] data on activation of the cyclase by fluoride in a range of concentrations are not available, P_r in Eq. (1) cannot be determined. However, it is clear that for a fixed concentration of fluoride, $y = y_0$, the activity of the cyclase can be considered as a function of a single variable, x, concentration of forskolin. This function has been shown [25] to be similar to Eq. (4) in which the efficacies are changed by some factors independent of x and depending on y_0.

In the experiments of adenylate cyclase activation by forskolin and fluoride, in addition to preparation B, one more plasma membrane preparation, C, was used; the data of these experiments had to be analyzed separately again. In case of preparation C, in absence of activation by fluoride, Eq. (4) could be fitted only under assumption that one of the dissociation constants, x_h, and one of the

efficacies e_{20}, are different from those estimated above (Table 1: see also Fig. 2, right, lower solid curves labeled B and C).

The data for the combined effect of forskolin and fluoride obtained in 2 experiments on preparation B proved to be *non*-homogeneous, so these data had to be analyzed separately. Eq. (4) with the above modifications and the dissociation constants, x_h and x_l, remaining the same as estimated above could not be fitted to either set of data; its shape, however, could be made similar to that which fits well to the less scattered data, but remains shifted along the concentration axis (Fig. 2, right, dotted curve). It could not be fitted by changing either x_h or x_l alone: it was necessary to change both x_h and x_l. For this reason these constants were multiplied by a formal parameter, β, whose meaning will be discussed in the next section, to yield

$$A = \alpha e_{01}\epsilon_0 \frac{\beta^2 x_h x_l}{(x+\beta x_h)(x+\beta x_l)} + \alpha e_{11}\epsilon_1 \frac{\beta(x_h+x_l)x}{(x+\beta x_h)(x+\beta x_l)} + \alpha e_{21}\epsilon_2 \frac{x^2}{(x+\beta x_h)(x+\beta x_l)} \quad (7)$$

β determining the shift of the curve, ϵ_0, ϵ_1 and ϵ_2 being the factors altering the efficacies of the cyclase due to its association with fluoride, their subscripts referring to the number of forskolin molecules associated with the cyclase. As a result of fitting of model (7) to the less scattered data (let it be the data of experiment 1) for the combined effect of forskolin and fluoride (Fig. 1, right, upper solid curve labeled B) its parameters have been estimated (Table 1). To verify the goodness-of-fit of the model, the number of independent measurements in this case is not sufficient, either, still visually the model is quite acceptable.

Eq. (7) also has been fitted to the data obtained on preparation C; the parameters estimated are presented in Table 1. Taken separately from Eq. (1), this model can be considered to have only 5 independent parameters, so its goodness of fit to the data of 8 independent measurements could be verified. The F-ratio

$$F = 1.0081 < 3.0280 = F(0.05;3,23);$$

and the model has been accepted.

Model (7) could not be fitted to the set of more scattered data (of experiment 2) either without a shift ($\beta = 1$) or with a shift ($\beta < 1$) of the curve along the concentration axis. This discrepancy will be discussed in more detail in the next section. It should be noted that the data for forskolin alone in both experiments are *homogeneous* (Fig. 2, right); this allowed to pool these data together (Fig. 3, left, lower solid curve).

In summary, model (1) is in agreement with the data of 8 (of total 10) adenylate cyclase activation experiments (total 124 measurements), whereas it does not fit to the data of 1 experiment, model curve still being close to the data points and deviating considerably from 3 points of 16; goodness-of-fit of the model to the data of another experiment (16 measurements) could not be verified. Model (7) is in agreement with the data of 2 (of 3) experiments whereas no model has been fitted to the data of 1 experiment.

For non-linear models (which is the case), it is extremely difficult to determine confidence intervals for the parameters estimated. Moreover, in the case of multiparameter models those intervals are strongly interdependent. The subject of the present analysis, however, was to relate experimental dose-response curves with the properties of cAMP-generating system expressed via the parameters of the model rather than determine exact values and confidence intervals of those parameters.

4. Discussion

It can be seen that the first term of Eq. (1) corresponds to the contribution of adenylate cyclase which is not activated by hormone whereas the second one corresponds to that of the cyclase which is activated by hormone to its observable activity, the terms in the square brackets corresponding to the cyclase associated with none, 1, and 2 molecules of forskolin.

As follows from Eqs. (4) and (6), for $x = 0$ and $y = 0$ (in absence of any activation) $A = \alpha e_{00}$ or, keeping in mind that e_{00} has been assumed to be equal to 1, $A = \alpha$. Thus α, the parameter which has been defined as specific activity of the cyclase is equivalent to its background activity.

For $x \gg x_l$ in Eq. (4), $A = \alpha e_{20}$, i.e., αe_{20} is equivalent to the asymptotic activity of the cyclase when activated by forskolin alone.

Similarly, for $y \gg y_r$ in (6) $A = \alpha e_{01}$, i.e., αe_{01} is equivalent to the asymptotic activity of the cyclase activated by hormone in absence of forskolin.

And finally, for both $x \gg x_l$ and $y \gg y_r$ (which means $P_r \approx 1$ in Eq. (1)) $A \approx \alpha e_{21}$, i.e., αe_{21} is equivalent to the asymptotic activity of the cyclase activated by a combination of forskolin and hormone.

The relative efficacies e_{01}, e_{20} and e_{21}, relate, therefore, the asymptotic activities of the cyclase with its background activity, e_{01} (e_{20} or e_{21}) being equal to e_{01}-fold (e_{20}-fold or e_{21}-fold) stimulation of adenylate cyclase by hormone (forskolin or combination of forskolin and hormone) over its background activity.

Such an immediate interpretation as above is not possible for e_{10} or e_{11}, their role not being observable directly. It is clear, however, that these parameters reflect the contribution of those molecules of the cyclase which are associated with one molecule of forskolin to the observable activity of the cyclase.

It is worth noting the importance of absence of interdependence between the parameters. Let, e.g., e_{10} be expressed as a combination of other parameters,

$$e_{10} = e_{00}\frac{x_l}{x_h + x_l} + e_{20}\frac{x_h}{x_h + x_l} \quad (8)$$

Then substitution of e_{10} in Eq. (4) from (8) yields

$$A = \alpha e_{00}\frac{x_l}{x + x_l} + \alpha e_{20}\frac{x}{x + x_l} \quad (9)$$

which is hyperbolic and whose shape for given lower and upper levels (determined by e_{00} and e_{20}, as pointed out above) cannot be changed; the curve can only be shifted along the axis of concentrations by changing x_l. It could not be fitted to the data on activation of the cyclase by forskolin (see Fig. 3, left, dotted curve). It should be noted as well that x_h and x_l are different (see Table 1), their ratio determining the extent of the curve over a range of concentrations, provided that (8) is not true. These constants characterize forskolin's potency more adequately than, e.g., does ED_{50} which is conventionally used for this purpose.

The values of x_h and x_l estimated (0.37 and 17 μM) can be compared with those, e.g., for binding sites of rat brain membranes (15 nM and 1.1 μM, respectively) determined by centrifugation assay [16] or those for adenylate cyclase of rat ovaries (0.76 μM and 37 μM) estimated at the Institute of Biochemistry, Vilnius, by the same method as here [25].

As shown above (see Fig. 3, right, dotted curve), Eq. (1) with P_r defined by Eq. (2) does not fit to the data on the combined effect of corticotropin and forskolin on the activity of the cyclase. The model fitted (with P_r defined by Eq. (5)) was based on the assumption that the receptor has two corticotropin-binding sites, both sites being identical (to assume those sites being different would involve introducing more free (independent) parameters; that would make the model loser, less definite). Under this assumption, there are three possible states of the receptor concerning its binding sites: (1) both its sites free, (2) one site occupied and (3) both sites occupied by molecules of corticotropin. The situation can be illustrated by Fig. 1 if AC is assumed to symbolize hormone receptor, F, hormone molecule. To find the probabilities for the receptor to be in any of the above states the model developed in Appendix for forskolin-binding sites can be used. Substitution of y for x and y_r for x_h and x_l in Eq. (A-6)–Eq. (A-8) for $P_r = 1$ yields:

$$P_1 = \frac{y_r^2}{(y + y_r)^2}, \quad P_2 = \frac{2y_r y}{(y + y_r)^2}, \quad P_3 = \frac{y^2}{(y + y_r)^2}$$

In general, the activity of the receptor (i.e., its ability to stimulate adenylate cyclase) in the 2nd and 3rd state might be different. Such an assumption would, however, involve more parameters again, so it has been abandoned. The receptor in the 1st state has been assumed to be inactive. There remain three possibilities for its activity to be linked with its states defined above: it is active when being (i) in state 2, (ii) in state 3 and (iii) in state 2 or state 3. This is equivalent to three possibilities for P_r in Eq. (3):

(i) $P_r = P_2$, (ii) $P_r = P_3$ and (iii) $P_r = P_2 + P_3$

In case (i) Eq. (3) yields a bell-shaped curve (if plotted in semilogarithmic scale) with a pointed peak. No evidence is available on a peak or a decline of activity of the cyclase at high concentrations of corticotropin (see Fig. 3, right). Thus this possibility has been rejected. It can be seen that by assuming $P_r = P_2/2 + P_3$ the 2-binding-site model under analysis is reduced to Eq. (2), i.e., to 1-binding-site model. The model which has been accepted was based on the assumption (iii), i.e., that

$$P_r = P_2 + P_3$$

(see Eq. (5)) which means the receptor being active when at least one of its two hormone-binding sites is occupied. In other words, binding of another hormone molecule to the receptor does not affect its activity. Keeping in mind that stimulatory G protein transducing the activation from the receptor to the catalyst is a GTPase which is considered to act as a switch triggered by the receptor [26], the above conclusion seems to be quite reasonable. Indeed, having been triggered 'on', the switch is no longer affected by the receptor and, consequently, by another hormone molecule. As shown above, this two-binding-sites model is in agreement with experimental data whereas one-binding-site (hyperbolic) model is not. That does not mean that no other model can be fitted to the data. Alternative models (see, e.g., [27,28] could be tested if more detailed data were available.

In summary, in spite of the apparent complexity of model (1) having 9 parameters, there is good reason to believe that it is the simplest possible model relating dose-response dependencies with what is known or can be hypothesized about the mechanisms of adenylate cyclase activation. All the parameters of the model are indispensable; there is no interdependence between the parameters.

The parameters of the model were supposed to be useful as quantitative measures of intrinsic properties of adenylate cyclase and to be independent of any other factors. The difference in the values of α for two different preparations (see Table 1) does not seem surprising: α was estimated as the rate of cAMP production *per milligram* of *protein* rather than *per (pico)mol* of *adenylate cyclase*; possible difference in the purity of the cyclase in different plasma membrane preparations (see section Methods and model) could account for the difference found; α should be better regarded for this reason to characterize a preparation and only indirectly adenylate cyclase itself.

The differences in further parameters, e_{11} and e_{21}, cannot be explained this way; besides, $e_{21} > e_{20} + e_{01}$ for preparation A but $e_{21} < e_{20} + e_{01}$ for B (see Table 1). That

means the combined effect of forskolin and corticotropin to be lesser than additive in the second case (as pointed out in [6]) but greater than that in the first one. To demonstrate the difference, a curve with parameters e_{11} and e_{21}, estimated for preparation A is plotted on Fig. 3 corresponding to B (left, upper dashed curve). Model curves are plotted as well for high concentrations of corticotropin (Fig. 2, left, upper dashed curve) and forskolin (Fig. 3, right, upper dashed curve). Note the tendency of the curves to get away from each other for preparation A and to get closer to each other for preparation B with the increase of concentration of forskolin or corticotropin.

It should be noted that additivity or non-additivity of the effects of two stimulators cannot serve as a criterion for sorting out the possible mechanisms of adenylate cyclase activation. As seen from Eqs. (1), (4) and (6), the effect of two stimulators, in general, is not additive. That is because the most essential element of the system – the catalyst – is the same for all the stimulators whatever the pathway of activation – via receptors, G-protein or beyond these elements.

Likewise, the concept of synergism useful to characterize observable effects of two stimulators turns out to be misleading when judging about the mechanisms underlying those effects. As seen above, different effects (in terms of synergism) can be produced by the same mechanism which is described by the same model.

Concerning the differences discussed, the following problems arise: (i) do those differences reflect any difference in properties of the cyclase used in different experiments? (in other words, was the cyclase different in different preparations?); if no, (ii) what are the causes of the differences found? and (iii) how to relate those differences with the model?

A positive answer to the first question does not seem plausible, still ruling out such a possibility would be imprudent. Results obtained at the Institute of Biochemistry in Vilnius [25] show that some properties of adenylate cyclase from the cells of the same organs – from ovaries of intact rats, ovarian malignant cells and ovaries of tumour-bearing rats – are slightly different.

As for the possible causes of the differences found, they have to be bound up, presumably, with the conditions of the experiments: some unknown factor(s) had to be present in one case while being absent in the other or vice versa.

The model discussed (see Eq. (1)) implies the activity of adenylate cyclase to be independent of any factors other than concentrations of two stimulators. To take into account the unknown factor(s), the model has to be extended and include some additional parameter(s) related with the unknown factor(s); in such an extended model the parameters e_{11} and e_{21} would remain the same for both preparations.

As shown above, Eq. (1) had to be modified to take into account the effect of fluoride on x_h and x_l, the dissociation constants for forskolin (see Eq. (7)). It should be reminded that Eq. (1) was based on the assumption, among others, that the affinities of forskolin-binding sites for forskolin remain unaltered irrespective of interaction(s) of other agent(s) with other site(s). For combined effect of forskolin and fluoride this assumption proved to be false. Decrease of the dissociation constants means increase of the affinities of the sites for forskolin and, eventually, augmentation forskolin's potency; the formal parameter, β, determining the lowering of the constants, has been denoted for this reason as a potentiation factor (see Table 1). It should be noted that model (7) with $\beta \neq 1$ (which is supposed to be the case) is valid only if *all* the adenylate cyclase complexes can be considered to be affected by fluoride. For the concentration of NaF used (10 mM) this assumption seems to be quite justified (see dose-response curves for fluoride, e.g., in [5,12,14]). Indeed, model (7) based on this assumption is in agreement with the data of 2 (of total 3) experiments of combined stimulation of the cyclase by forskolin and fluoride. However, as mentioned above, this model could not be fitted to the data of experiment 2. Greater variance is characteristic to the data of experiment 2 which suggests possible effect of some unknown factor(s) in this experiment.

In absence of stimulation by forskolin, adenylate cyclase activity is affected by fluoride acting via G-protein, the observable effect depending on the concentration of Al, Be, or Mn ions [13,14]. Possible variation in the concentration of these ions could account for the variance of the observable activity of the cyclase and could leave a fraction of the cyclase with the affinity for forskolin *unaltered* making model (7) invalid in this case. No more data are available to support or reject this possibility. It should be noted that although model (7) is not supported by the data of experiment 2, it cannot be rejected on the basis of these data, either. The main assumption of the model – augmentation of forskolin's potency by fluoride – could be rejected only if model (7) with $\beta = 1$ (i.e., with *no* potentiation) was in agreement with the data.

It is of interest that activity of the cyclase as a function of concentration of forskolin for the concentration of fluoride used has a minimum (see Fig. 2, right); respectively, $e_{11} < e_{01}$ for this combination of stimulators whereas $e_{11} > e_{01}$ for forskolin and hormone (see Table 1). Similar curves with a minimum can be found for other stimulators, e.g., for GppNHp [4], for GTPγS [29].

5. Conclusions

(1) All the parameters of the models discussed are related with the intrinsic properties of adenylate cyclase; there is no interdependence between the parameters.

(2) Equilibrium dissociation constants, x_h and x_l (0.37 and 17 μM), corresponding to high- and low-affinity forskolin-binding sites characterize forskolin's potency more adequately than does ED_{50}, the concentration elicit-

ing half-maximal or, more accurately, half-asymptotic activity.

(3) Corticotropin does not affect the affinity of the catalyst for forskolin whereas fluoride increases this affinity, thus augmenting forskolin's potency.

(4) There is good reason to believe that hormone receptor of adenylate cyclase bovine adrenal cortex has two or more binding sites for corticotropin.

(5) Some unidentified factor(s) may be responsible for the differences found in adenylate cyclase activity in different experiments carried out under similar conditions.

(6) The models discussed may be thought to be the best means for the moment to relate dose-response dependencies with what is known or can be hypothesized about the mechanisms underlying activation of adenylate cyclase.

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Appendix 1

Assumption (2) with the alternative (i) allows to apply the Law of Mass Action to any class of binding sites or the receptor separately. E.g., the fraction of occupied high-affinity forskolin-binding sites (which is convenient to be considered as the probability for the site to be occupied), Q_h, is

$$Q_h = \frac{x}{x + x_h} \tag{A-1}$$

where x is concentration of forskolin, x_h is equilibrium dissociation constant. Obviously, the probability for the site to be free, R_h, is

$$R_h = \frac{x_h}{x + x_h} \tag{A-2}$$

The probability for adenylate cyclase complex to assume a concrete state is determined by the probabilities for the states of corresponding binding sites and that for hormone receptor. If the probability for hormone receptor to be active is P_r then the probability for the complex to assume, e.g., state 00 (see Fig. 1), S_{00}, is

$$S_{00} = R_h R_l (1 - P_r)$$

or

$$S_{00} = \frac{x_h x_l}{(x + x_h)(x + x_l)}(1 - P_r) \tag{A-3}$$

the subscript h referring to the low affinity site. Similarly,

$$S_{10} = (Q_h R_l + R_h Q_l)(1 - P_r)$$

or

$$S_{10} = \frac{(x_h + x_l)x}{(x + x_h)(x + x_l)}(1 - P_r) \tag{A-4}$$

and

$$S_{20} = \frac{x^2}{(x + x_h)(x + x_l)}(1 - P_r) \tag{A-5}$$

$$S_{01} = \frac{x_h x_l}{(x + x_h)(x + x_l)} P_r \tag{A-6}$$

$$S_{11} = \frac{(x_h + x_l)x}{(x + x_h)(x + x_l)} P_r \tag{A-7}$$

$$S_{21} = \frac{x^2}{(x + x_h)(x + x_l)} P_r \tag{A-8}$$

It can be seen that

$$S_{00} + S_{10} + S_{20} + S_{01} + S_{11} + S_{21} = 1$$

i.e., (back in terms of concentrations) all the partial concentrations of adenylate cyclase in all the possible states make the total concentration which is assumed here to be equal to 1. All the partial concentrations known, the observable activity, in accordance with assumption (4), can be expressed as

$$A = \alpha(S_{00}e_{00} + S_{10}e_{10} + S_{20}e_{20} + S_{01}e_{01} + S_{11}e_{11} + S_{21}e_{21}) \tag{A-9}$$

where α is specific activity of adenylate cyclase, $e_{00} \geq 0$, ..., $e_{21} \geq 0$ are efficacies of adenylate cyclase complexes in states 00, ..., 21, respectively (Fig. 1). Substitution of S_{00}, ..., S_{21} (Eqs. (A-3)–(A-8)) in Eq. (A-9) yields Eq. (1) in the main text.

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