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Lipid-dependent membrane enzymes. Kinetic modelling of the activation of protein kinase C by phosphatidylserine

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Key words: Protein kinase C; Lipid regulation; Phosphatidylserine; Activation; Kinetic model; Hill coefficient; Ligand trapping

A previously developed kinetic theory for lipid-dependent membrane enzymes (Sandermann, H. (1982) Eur. J. Biochem. 127, 123-128) is used to examine the activation of protein kinase C by phosphatidylserine. Hill-coefficients ranging up to 11 have been reported for activation in mixed micelles with Triton X-100. On the basis of this uniquely high degree of cooperativity, protein kinase C has been postulated to represent a new class of lipid-dependent membrane enzymes (Newton, A. and Koshland, D.E., Jr. (1989) J. Biol. Chem. 264, 14909-14915). In contrast, activation in the absence of Triton X-100 has led to Hill-coefficients of only <; 2.6. In order to resolve the apparent discrepancy, activation is now considered to involve binding of PS monomers to interacting sites on the enzyme, a non-activating PS trapping process also occurring in the presence of Triton X-100. Estimates for trapping are made for several sets of published data for micellar activation. The kinetic model developed here successfully fits each data set using a Hill-coefficient of only 3.0. An influence of Ca²⁺/ions or of a two-step mechanism of lipid-protein interaction are considered as possible molecular explanations. It is concluded (i) that lipid activation of protein kinase C may proceed without unique cooperativity and (ii) that ligand trapping could provide another means for 'threshold-type' kinetic regulation of membrane enzyme and receptor systems.

Introduction

The activity of membrane-bound enzymes is in most cases dependent on, or modulated by, the membrane lipid phase [1]. Lipid activation curves are as a rule sigmoidal and thus possess positive kinetic cooperativity [2,3]. In the special case of diacylglycerol kinase from Escherichia coli, a lipid-dependent enzyme has even been shown to be capable of autocatalytic self-activation [4].

The molecular basis for lipid regulation is as yet ill understood. Lipid/protein binding equilibria are at present accessible by two major approaches that have not yet merged. ESR-spectroscopy considers a 1:1 exchange of bound versus free lipid and leads to dimensional binding exchange constants [5,6] which are, however, not useful for biochemical kinetics [3].

The second approach is based on multiple binding site kinetics [2,3]. Kinetic cooperativity is attributed to either allosteric interactions between lipid binding sites or to a non-allosteric mechanism resulting from the lipid solvation of the membrane protein. A kinetic theory for both allosteric and non-allosteric cooperativity of lipid activation has been developed [7]. However, there is still a paucity of experimental systems where the theoretical models can be tested, and support by biophysical data is lacking. Na⁺/K⁺-ATPase [8,9] and β-hydroxybutyrate dehydrogenase [10,11] probably represent the best-defined systems.

Kinetic studies on membrane enzymes are generally difficult because the usual kinetic formalism refers to non-aggregated homogeneous solutions. The recognition and elimination of kinetic artifacts caused by micellar or liposomal aggregation processes is therefore of great importance in the study of membrane enzymes [3]. This type of kinetic analysis is presented here for

Abbreviations: v, actual enzyme velocity; V, maximal enzyme velocity; n_H, Hill coefficient; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PS, phosphatidylserine; [PS], concentration of total PS; [PS], concentration of complexed PS; [PS]₁₀, [PS]₅₀, [PS]₉₀, total phosphatidylserine concentrations required to reach 10%, 50% and 90%, of V, respectively.

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the activation of protein kinase C by phosphatidyl-
sine (PS). The lipid-dependence of this membrane
enzyme has been reviewed [12,13]. Protein kinase C is
of particular interest since it acts as a receptor for
phorbol diesters, and plays a central role in tumor
promotion and numerous other important physiological
events [14,15].

Bell and co-workers [16,17] have developed a mixed
micellar system (using Triton X-100) to allow system-
atic studies of the interactions of protein kinase C with
its most effective phospholipid activator, phosphatidyl-
sine (PS), as well as with other activators and inhibi-
tors. The detergent was reported to provide a ki-
netically inert matrix and to solubilize protein kinase C
in monomeric form [16,17]. Curves for the PS-depen-
dence of histone phosphorylation in micelles were
highly sigmoidal, with Hill-coefficients ($n_H$) of up to 11
[16–23]. In contrast, previous activation curves for PS
determined in liposomes had failed to display a pro-
nounced sigmoidal shape [24,25]. Activation in Triton
X-100 mixed micelles also gave high $n_H$ values for the
PS-dependent binding of phorbol-diesters and for the
autophosphorylation of the enzyme.

Hill-coefficients approaching 11 are unique and have
not been previously reported for any other enzyme.
Newton and Koshland [23] have therefore concluded
that protein kinase C represents a new group of lipid-
dependent enzymes, that interacts specifically and co-
operatively with ≥12 phospholipid activator molecules.
This far-reaching conclusion may, however, be based
on a kinetic artifact, as indicated by the present kinetic
analysis. When the phenomenon of ligand trapping is
taken into account, the published data sets reduce to a
fairly common Hill-coefficient, $n_H = 3$. At the same
time, ligand trapping is identified as a possible mecha-
nism for 'threshold-type' kinetic regulation.

Results

Hill coefficients for PS activation

Many data sets for activation of protein kinase C by
phosphatidylserine have been published. As shown in
Table I, activation in mixed micelles with Triton X-100
has led to Hill-coefficients in the range of 4.7–11,
whereas activation in the absence of Triton X-100 has
led to Hill coefficients in the range of 0.7–2.6. Table I
contains data from three separate laboratories where
reconstitution in the presence or absence of Triton
X-100 was studied under similar experimental condi-
tions.

Basic kinetic models

PS is proposed to bind cooperatively to protein
kinase C, with a stoichiometry of 4 [30] or of ≥12 [23]
lipid monomers per enzyme monomer, with the addi-
tional participation of $Ca^{2+}$ and a diacylglycerol or

<table>
<thead>
<tr>
<th>Amount of Triton X-100 used (w/v)</th>
<th>Ca$^{2+}$ concn. (mM)</th>
<th>$n_H$</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>0.1</td>
<td>4.8</td>
<td>16</td>
</tr>
<tr>
<td>0.3</td>
<td>0.1</td>
<td>≤8.8</td>
<td>17–22</td>
</tr>
<tr>
<td>0.3</td>
<td>0.1</td>
<td>4.67</td>
<td>26</td>
</tr>
<tr>
<td>0.3</td>
<td>0.1</td>
<td>5.4*</td>
<td>27 (Fig. 1B)</td>
</tr>
<tr>
<td>0.1</td>
<td>1.3</td>
<td>≤11</td>
<td>23</td>
</tr>
<tr>
<td>0</td>
<td>0.01</td>
<td>0.8*</td>
<td>26 (Fig. 2A)</td>
</tr>
<tr>
<td>0</td>
<td>1.3</td>
<td>≤2.6*</td>
<td>28 (Fig. 1)</td>
</tr>
<tr>
<td>0</td>
<td>0.6</td>
<td>≤1.02</td>
<td>29</td>
</tr>
<tr>
<td>0</td>
<td>0.2</td>
<td>0.7*</td>
<td>27 (Fig. 7)</td>
</tr>
</tbody>
</table>

* $n_H$ estimated by linear regression (Hill plot) from the published
data points between $[PS]_0$ and $[PS]_p$. The figures used are indi-
cated together with the reference numbers. The correlation coeffi-
cient was > 0.98 in all cases.

phorbol-diester activator molecule. As previously dis-

cussed, a number of mechanisms can lead to kinetic

cooperaivity in the absence of true cooperative inter-

actions [3]. In the present communication, a specific
alternative kinetic model is considered. This model

depends on binding of PS monomers at dis-


tinct interacting sites on protein kinase C. In addition,
a PS complexation process induced by Triton X-100 is

considered to occur as a non-activating trapping event.

This trapping phenomenon is similar to substrate de-
pletion by complexation which is known to convert

hyperbolic velocity curves to sigmoidal curves with $n_H$

> 1 [31].

Rate equation for ligand trapping

In the alternative kinetic model, PS in mixed mi-
celles with Triton X-100 may undergo two types of
interaction. Firstly, PS molecules are withdrawn by
some as yet undefined binding reaction that does not
occur in liposomal systems. The second possible inter-
action involves binding of PS monomers to activator
sites on the enzyme. A simplified kinetic model is
based on the following assumptions:

(1) Trapping proceeds with such high affinity that
all initial PS is consumed until the total possible con-
centration of complexed PS, $[PS]_p$, is reached.

(2) The concentration of excess free PS, available
for enzyme activation, is given by the difference be-
tween total and complexed PS concentrations, ($[PS]_t$

$-[PS]_p$).

The degree of kinetic cooperativity can be described
by a modified form of the nonlinear Hill-type equation (cf. Ref. 7):

\[ \frac{v}{(\text{[PS]}_0 - \text{[PS]}^c)^n} = \frac{(\text{[PS]}_0 - \text{[PS]}^c)^n + (\text{[PS]}_0 - \text{[PS]}_e)^n}{(\text{[PS]}_0 - \text{[PS]})^n} \]  

(1)

Here, \( v \) is actual velocity, \( V \) is maximal velocity, \( \text{[PS]}_0 \) is the total PS concentration required for half-maximal activation, and \( n_H \) is the Hill coefficient. Remember that the value of \( n_H \) is less than \( n \), the number of PS binding sites, but will approach \( n \) if the strength of cooperativity between the sites is high.

**Specific features of the trapping model**

According to Eqn. 1, activator trapping should significantly influence overall apparent kinetic cooperativity and Hill coefficients. Two types of calculated data are used to illustrate this point.

First, in Fig. 1 activation curves calculated with Eqn. 1 become significantly steeper with increased trapping of PS. Eqn. 1 predicts that at various amounts of trapping, 50% relative activity will always occur at the same value of \( \text{[PS]}_0 \). To achieve 50% activity at the same value of \( \text{[PS]} \), means that, in the presence of trapping, the overall cooperative affinity of the sites for PS must be correspondingly higher than if no trapping occurred.

Each curve of Fig. 1 was analyzed with the standard linearized Hill equation between \( \text{[PS]}_0 \) and \( \text{[PS]}_0^c \), not correcting for trapping. The apparent Hill-coefficients obtained are 2.8 (for \( \text{[PS]}_0^c = 0 \)), 3.2 (for \( \text{[PS]}_0^c = 1 \)), 4.4 (for \( \text{[PS]}_0^c = 3 \)), and 7.0 (for \( \text{[PS]}_0^c = 5 \)). Another index of cooperativity, the ratio \( \text{[PS]}_0^c/\text{[PS]}_0 \), progressively decreases from 4.7 (for \( \text{[PS]}_0^c = 0 \)) to 3.9 (for \( \text{[PS]}_0^c = 1 \)), 2.6 (for \( \text{[PS]}_0^c = 3 \)), and finally to 1.8 (for \( \text{[PS]}_0^c = 5 \)). Non-cooperative, hyperbolic kinetics would have a value of 8.1. This example shows that, if trapping did occur in an enzyme-activator system but was not accounted for in the analysis of the behavior, kinetic cooperativity versus the activator (and thus \( n_H \)) would appear larger.

Second, a complementary treatment was developed as follows. Three sets of activation data, \( (v/v^c) \) versus \( [PS]_0 \), were calculated from Eqn. 1 assuming no trapping (\( [PS]_0^c = 0 \)) and fixed values of \( [PS]_0^c = 8 \) mol%, and \( n_H = 2.8, 4 \) or 8. Then the data set for each fixed \( n_H \) value was fitted with Eqn. 1, now invoking increasing PS trapping. As summarized in Table II, the apparent value for \( n_H \) decreases when an increased trapping term (larger \( [PS]_0^c \)) is inserted into the fitting equation. Thus, in the presence of activator trapping, a smaller number of cooperative binding sites on the enzyme would be sufficient to explain an apparently high degree of kinetic cooperativity.

**Application of trapping model to published data**

In order to apply the present kinetic model to protein kinase C, three published data sets have been analyzed. In the original publications, the data were fit to the standard Hill equation (Fig. 2, dashed curves), and yielded the published \( n_H \) values of 4.8 (Fig. 2A) and 8 ± 2 (Fig. 2B) for histone phosphorylation and of 8.7 ± 0.9 (Fig. 2C) for autophosphorylation.

With values of PS trapping estimated from the data (see legend of Fig. 2), Eqn. 1 leads to calculated activation curves with a uniform value of \( n_H = 3.0 \) (Fig. 2; solid lines). Thus the various published high values for \( n_H \) are considerably lowered. The present and the previous treatments result in the solid and the dashed

<table>
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<tr>
<th>[PS]_0^c</th>
<th>Fitted Hill-coefficients</th>
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<td>set 1</td>
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<tr>
<td>0</td>
<td>2.0</td>
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<tr>
<td>1</td>
<td>2.5</td>
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<td>5</td>
<td>1.1</td>
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Fig. 2. Analysis of published PS-activation data with the trapping model. Data points and the values for \([\text{PS}]_0\) and for \(V\) were taken from the following sources: Fig. 3 of Ref. 16; Fig. 2A of Ref. 23; Fig. 1A of Ref. 23, data obtained with 7.5 mol% dioleoylglycerol. The dashed curves correspond to the previously published Hill-coefficients and were calculated with Eqn. 1 using \([\text{PS}]_0 = 0\). The theoretical curves for the trapping model (solid lines) were obtained using Eqn. 1 of the text with a value of \(n_H = 3.0\) and the following values for \([\text{PS}]_0\): (A) 2.48 mol%; (B) 4.2 mol%; (C), 4.5 mol%; each value for \([\text{PS}]_0\) was determined graphically as the \(x\)-intercept of a line drawn tangent to the mid-range of the published curve.

Discussion

Triton X-100 has been reported to act as an inert matrix in the activation of protein kinase C [16,17]. The data summarized in Table I show, however, that Triton X-100 has a drastic effect on apparent cooperativity. The reports on the micellar reconstitution method [16–22] ignored the lack of an apparent threshold in PS activation curves for liposomal systems (see, for example, Refs. 24 and 25). It should be noted that most bilayer titrations were carried out by increasing the ratio of total lipid to enzyme, at constant PS mole fraction. For a ligand such as PS in a bilayer, it is more appropriate to vary the mole fraction for the activator, PS, at constant total phospholipid; it has been shown for \(\beta\)-hydroxybutyrate dehydrogenase, for its activator PC in a bilayer 'background', that varying the PC mole fraction yields a higher Hill coefficient \((n_H = 2.4)\) than titration at constant PC mole fraction \((n_H = 1.8)\) [11,32]. However, it is clear that the unique degree of cooperativity observed for activation of protein kinase C by PS in the Triton X-100 detergent system has not been observed in bilayer systems.

There has been a tendency to accept the uniquely high Hill coefficients in Triton X-100 as a fact. The earlier model postulating a geometrically defined complex involving four PS molecules [30] has recently been replaced by a geometrically undefined complex with six PS molecules [22]. Protein kinase C is thus thought to interact in a highly cooperative fashion with 6 [22] or \(\geq 12\) PS molecules [23,28]. In contrast to these reports, all other previously investigated lipid-dependent enzymes had much lower degrees of kinetic cooperativity [3,7]. Three of the most highly cooperative lipid-activated enzymes known are \(\text{Na}^+\)/\(\text{K}^+\)-ATPase \((n_H \uparrow \text{up to } 2.7\); [8]), pyruvate oxidase \((n_H \uparrow \text{up to } 3.6\); [33]) and \(\beta\)-hydroxybutyrate dehydrogenase \((n_H \uparrow \text{up to } 2.4\);[11]). A Hill-coefficient in the same range (3.0) was obtained here for protein kinase C after taking ligand trapping into account. However, it became clear from Fig. 2 that the published data points were too imprecise to distinguish between the high cooperativity models and the present ligand trapping model. There is thus a need for better kinetic data and for direct PS binding data in order to decide between models. In view of the great physiological importance of protein kinase C, a more detailed discussion seems of interest.
Possible influence of Ca\(^{2+}\) ions

A complex phase equilibrium between PS, diacylglycerol, detergent, substrate and enzyme proteins, Ca\(^{2+}\) and Mg\(^{2+}\), ATP and buffer components exists in the kinase assay mixture used [16-23]. The complex and non-physiological mixtures employed make reliable conclusions difficult. Interactions between histones and PS are well known [22,27,29], but are perhaps not critical for the present kinetic analysis. Autophosphorylation and phorbol-diester binding had the same high Hill coefficients as histone phosphorylation, although each of these processes had a different PS dependence.

A possible role of Ca\(^{2+}\)-ions in the mixed micellar assay with Triton X-100 may result from the known strong interaction of Ca\(^{2+}\) with phosphatidylserine and the phosphatidylserine polar group alone [35]. The concentration of Ca\(^{2+}\) used in the micellar assays (\(\geq 100 \mu M\)) was much above the physiological range (\(\leq 10 \mu M\)) which was used in some of the liposomal activation studies [12,13,24,25]. When 0.3% w/v Triton X-100 was used in the complete absence of Ca\(^{2+}\), a Hill coefficient of \(\approx 2.5\) was obtained for PS activation of the Ca\(^{2+}\)-independent isoenzyme and of protein kinase C [36]. This Hill coefficient (estimated from Fig. 2A of Ref. 36) approximates the Hill coefficient of 3.0 found here for the Ca\(^{2+}\)-dependent isoenzymes of protein kinase C after correcting for PS trapping. Isoenzyme \(\epsilon\) lacks one of the sequence domains that are involved in the binding of Ca\(^{2+}\), but also in binding of diacylglycerol and PS [15]. On the other hand, when Ca\(^{2+}\) concentration was lowered in the standard micellar assay, no obvious decrease in the initial threshold became apparent [17]. The available data therefore allow no firm conclusion as to a role of Ca\(^{2+}\) or Ca\(^{2+}\)/Triton X-100 in PS trapping.

Two-step mechanism of lipid–protein interaction

Protein kinase C exists as a monomer in Triton X-100, and the monomer has been proposed to be the active species [16,17]. However, the enzyme may need oligomerization [37] or a bulk binding step to the micelle prior to the specific activation step. Oligomerization or bulk binding of protein may occur much more easily in liposomal than in micellar systems, and may initially consume PS in micelles. Other acidic phospholipids could conceivably replace PS in oligomerization or bulk binding, but not in the specific activation step. It has indeed been observed that equimolar phosphatidic acid significantly reduced the threshold for PS and also reduced the Hill coefficient for PS from 9.6 to 4.8 [23]. Other phospholipids (PC, PE, PG) also caused a significant though less pronounced decrease of the Hill coefficient for activation by PS [23]. Kinetic anomalies were also indicated by the described strong variation of Hill coefficients for PS activation when phorbol diester [18], diacylglycerol [17] or sphingosine [19] were included in the assay mixture. The idea of \(\geq 12\) PS molecules being the activating species [23] may furthermore be difficult to reconcile with the stereospecificity for an t-serine moiety in PS [38]. However, stereospecificity was not absolute, and the published data [38] did not clarify whether all or only part of the added PS had to contain t-serine.

Conclusion

In summary, it now appears that the published interpretations for the PS activation of protein kinase C may have been inconclusive because experimental artifacts may have occurred in the mixed micellar assay using the detergent Triton X-100. Although other alternative mechanisms may apply, the present analysis indicates that protein kinase C may be regulated not through the lipid activator concentration per se, but through the effective lipid activator concentration remaining after physical and chemical sequestering processes. Such processes may occur as artifacts, but they could also occur physiologically and provide another general mechanism for 'threshold-type' regulation of membrane enzyme and receptor systems.

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