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MINI-REVIEW

Activation and Regulation of Protein Kinase C Enzymes

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Abstract

Protein Kinase C (PKC) has been a principal regulatory enzyme whose function has been intensely investigated in the past decade. The primary features of this family of enzymes includes phosphorylation of serine and threonine residues located on basic proteins and peptides in a manner that is stimulated by calcium, phospholipid, and either diacylglycerol or phorbol esters. An additional intriguing feature of the enzyme is its ability to form two membrane-associated states, one of which is calcium dependent and reversible and the second is an irreversible complex which has the characteristics of an intrinsic membrane protein. Formation of the irreversible membrane-bound form is greatly facilitated by calcium and the tumor-promoting phorbol esters but does not appear to include covalent changes in the PKC structure. The intrinsic membrane-bound form is a very different enzyme in that its activity is no longer dependent on the other cofactors. It is proposed that formation of the irreversible membrane-bound form may be a mechanism for generating long-term cell regulation events where transient cell signals and second messengers induce long-term changes in the distribution of an enzyme in the cell. This property may be common to a number of regulatory proteins that are known to be distributed between the cytosol and membrane-fractions in the cell. Unfortunately, many problems have confronted study of PKC mechanism using the *in vitro* assay. This assay involves aggregation of the substrate, phospholipid, and enzyme to form a discontinuous mixture. Such a complex system prevents straightforward interpretation of enzyme kinetic data. Although many compounds affect the *in vitro* activity of PKC, most appear to accomplish this by relatively uninteresting mechanisms such as interference with the aggregation process. While some highly potent inhibitors undoubtedly interact directly with PKC, they also inhibit other enzymes and there are no entirely specific inhibitors of PKC known. Speculation on the possible roles of PKC in cell regulation are abundant and exciting. However, delineation of the regulatory roles of PKC may require another decade of intense effort.

Key Words: Protein Kinase C; membranes; calcium; phorbol esters; regulation; long-term regulation; phospholipids; diacylclycerol.

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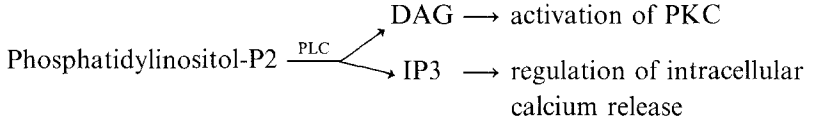
Introduction

Protein kinase C (PKC) has been one of the exciting research topics of the 1980's. This enzyme was discovered in 1977 as a protein kinase that required proteolytic cleavage for activation (Takai *et al.*, 1977; Inoue *et al.*, 1977). Subsequent studies have shown that the enzyme can be activated reversibly by calcium and phospholipids (Takai *et al.*, 1979a). Two seminal observations that have governed thinking with regard to this enzyme are that, at physiological concentrations of calcium, PKC is significantly activated by diacylglycerol (DAG) (Takai *et al.*, 1979b; Kishimoto *et al.*, 1980) or phorbol esters (PhE) (Castagna *et al.*, 1982). The activation of protein kinase C, therefore, involves multiple interaction between enzyme, calcium, phospholipid, substrate, and phorbol esters or diacylglycerol.

The actual function of PKC in cell regulation is not known. However, the influence of diacylglycerols on protein kinase C makes this enzyme a central player in what is known as the phosphatidylinositol cycle; a greatly simplified version is given below.



Other key proteins of this cycle are phospholipase C (PLC) and the receptors for inositol phosphates (IP_3). The inositol phosphates are subject to many metabolic changes that produce either active or inactive second messengers (for reviews, see Berridge and Irvine, 1984; Nishizuka, 1984; Majerus *et al.*, 1986). Several of the enzymes of the phosphoinositol cycle are known to consist of a complex family of proteins rather than of a single species. The phospholipase C family acts on the various forms of phospholipids. It is possible that the PKC functions in what should be known as the phospholipase C cycles.

Several aspects of PKC regulation have recently been reviewed including molecular heterogeneity (Nishizuka, 1989), enzymatic function (Nishizuka, 1986a,b; Kikkawa and Nishizuka, 1986), regulation by phospholipids (Rando, 1988) or phorbol esters (Blumberg, 1988; Ashendel, 1985). This review will concentrate on evaluation of the biochemical properties of PKC and their possible implication to observations *in vivo*.

Section 1 of this review presents a brief description of the enzyme itself. Section 2 provides a detailed description of the interaction of PKC with its cofactors, where many of the unique properties of PKC emerge. Section 3 deals with *in vitro* activation of PKC and describe the basic, but often overlooked, complications associated with interpretation of *in vitro* studies.

Finally, Section 4 presents some recent ideas regarding PKC function. We will also discuss the possibility that several of the most striking properties of PKC may be more general phenomena that apply to other regulatory enzymes as well.

1. Structure of Protein Kinase C

Since the early reports describing PKC, many efforts have been directed toward elucidation of its structure. The enzyme has been purified from different sources, and many of its structural characteristics have been described. With few exceptions (see below), the enzyme appears to be composed of a single polypeptide chain with a molecular weight of about 80,000 which binds acidic phospholipids or biological membranes in a Ca^{2+}-dependent manner, binds stoichiometeric amounts of phorbol esters with high affinity, and catalyzes the incorporation of phosphate into a wide variety of basic peptide substrates. The kinase belongs to a family of kinases that phosphorylate serine or threonine residues (Edelmann *et al.*, 1987). Like many protein kinases, PKC can undergo autophosphorylation (Newton and Koshland, 1987).

Sequence studies show a family of seven isozymes thus far (termed α, $\beta 1$, $\beta 2$, γ, δ, ε, ζ; see Nishizuka, 1989 for a review). Additional members of this family might include *n*PKC, a phorbol ester receptor that is related to the PKC family (Ohno *et al.*, 1988a), and protein kinase PAK II, a protease-activated kinase II (Gonzatti-Haces and Traugh, 1986). It may be possible to divide the family into two major subfamilies. The first includes the isozymes α, $\beta 1$, $\beta 2$, and γ, since these are the sequences identifiable under normal conditions used for screening *c*DNA libraries (Ohno *et al.*, 1987, 1988b; Parker *et al.*, 1986; Knopf *et al.*, 1986; Kikkawa *et al.*, 1987). The second subfamily has been identified under less stringent conditions and may include the remaining isozymes (δ, ε, ζ, and possibly *n*PKC or PAK II). The first subfamily typifies the classical definition of PKC; they catalyze the incorporation of phosphate into histone H1 (a commonly used substrate) in a Ca^{2+}-, phospholipid-, and DAG-dependent manner, and they all bind phorbol esters with high affinity. Members of the second subfamily, however, do not exhibit all of these properties. For example, ε-PKC phosphorylates histone H1 poorly (Schaap *et al.*, 1989), ζ-PKC does not bind phorbol esters (Ono *et al.*, 1989), and both *n*PKC and ζ-PKC require phospholipid but not calcium for activity (Ono *et al.*, 1989; Ohno *et al.*, 1988a).

While molecular approaches suggested the presence of at least seven isozymes of PKC, biochemical separations have not been as generous. Currently, only three peaks of protein kinase activity are obtained by fractionation on

hydroxyapatite (Huang *et al.*, 1986; Jaken and Kiley, 1987; Azhar *et al.*, 1987). These three forms were designated types I, II, and III, and currently are thought to correspond to γ, $\beta 1$ plus $\beta 2$, and α, respectively (Huang *et al.*, 1987). Studies from several laboratories have suggested that complete separation of even these three forms of PKC is not always possible. Adequate fractionation may require special considerations and materials (Huang *et al.*, 1986; Marais and Parker, 1989). The chromatographic behavior of other PKC isozymes remains to be established.

While PKC isozymes differ in their primary structure and genomic origin, they share many interesting features. PKC is often considered to have a catalytic domain and a regulatory domain that can be separated by proteolytic degradation by trypsin (Huang and Huang, 1986) or Ca^{2+}-dependent protease (Kishimoto *et al.*, 1983). The catalytic fragment of PKC, originally referred to as protein kinase M, is a 45–55,000 Dalton polypeptide that displays activity toward histone in the absence of phospholipid and Ca^{2+}. Nevertheless, phospholipid could still activate protein kinase M further (Nakadate *et al.*, 1987). The regulatory fragment of PKC is a 32–36,000 Dalton fragment that contains the phospholipid and phorbol ester binding domains. Separation of the catalytic fragment of PKC apparently did not alter the binding affinity for phorbol esters, even though it may have altered the calcium requirement of this binding (Huang and Huang, 1986; Lee and Bell, 1986). It may be important to note that separation of PKC into catalytic and regulatory fragments has been limited to PKC preparations from rat brain.

Recently, differential degradation of PKC isozymes by trypsin (Huang *et al.*, 1989) or Ca^{2+}-dependent neutral proteases (Kishimoto *et al.*, 1989) has been reported. All three types of PKC are susceptible to proteases but differ in the rate of their degradation. PKC type III is more resistant to trypsin and/or calpains than types I and II. However, the rate of PKC degradation is more pronounced in the presence of phospholipids and DAG or phorbol esters. Observation of increased susceptibility of the activated form of PKC may account for the phenomenon known as "down regulation" of PKC. Sustained activation of PKC by phorbol esters often results in depletion or down regulation of PKC in cells (Nishizuka, 1986a).

Several *in vitro* studies have contrasted the activation properties of PKC isozymes in terms of their requirements for phorbol esters or DAG, calcium, and phospholipids (Huang *et al.*, 1988; Sekiguchi *et al.*, 1988; Marais and Parker, 1989). There appear to be relatively minor differences in these parameters which were limited to the required quantity of a cofactor. Since the activation of PKC is measured in a complex and aggregated system (see below), standard steady-state kinetics analysis may not always give an accurate indication of the requirements. For example, one of the more striking differences among PKC isozymes was the calcium requirement for

activation. However, there are conflicting reports of whether type I PKC (Huang *et al.*, 1988) or type II PKC (Sekiguchi *et al.*, 1988) is less dependent on Ca^{2+}. Marais and Parker (1989) reported this discrepancy was due, at least in part, to the physical state of the phospholipid component (phospholipid–detergent micelles vs. phospholipid vesicles).

It seems probable that the different isozymes will have different properties. Speculation about the function of different isozymes is intriguing due to the tissue distribution and developmental expression of the different isozymes (Nishizuka, 1989). For example, γ-PKC is exclusively localized in brain and the spinal cord and its expression is related to development. The α and β forms are more widely distributed and are not as closely linked to development. However, since the biological function of PKC is not known, ascribing differences in physical properties to biological functions must await more detailed description of the roles of the individual kinases themselves.

2. Interactions of Protein Kinase C with Its Cofactors in the Purified State

2.1. Interaction of Protein Kinase C with Calcium

In a rapidly evolving field, the boundary lines between an established observation and a good assumption sometimes become blurred. Generating PKC activity usually requires the presence of calcium, and many Ca^{2+} chelators inhibit PKC. These properties led to the natural assumption that PKC is a Ca^{2+}-binding protein. However, there have been no direct measurements of the Ca^{2+}–PKC interaction. In addition, the three major functions of PKC (activity, phorbol ester binding, and phospholipid binding) each seem to display distinct Ca^{2+} requirements (Bazzi and Nelsestuen, 1987a). Since the primary sequence of PKC did not show evidence of known calcium-binding sites such as the EF-hand or calelectrin-like structures, calcium binding to free PKC would have to involve a new type of structure (Parker *et al.*, 1986; Ohno *et al.*, 1987). All of these properties may suggest something unusual.

Direct measurements with phospholipid vesicles (Bazzi and Nelsestuen, 1987a), erythrocytes (Wolf *et al.*, 1985a,b), or Triton–phosphatidylserine micelles (Hannun *et al.*, 1985) show that PKC is a member of a family of proteins that bind to phospholipid in a Ca^{2+}-dependent manner. The role of calcium in this process is usually described by one of two models, the calcium-bridge model or the protein conformational change model. In the first model, Ca^{2+} functions as a bridge between the protein and the membrane and one proposal suggests that this involves one Ca^{2+}, four phosphatidylserine (PS), one diacylglycerol, and one PKC molecule (Ganong *et al.*, 1986;

Hannun *et al.*, 1986a). To the extent that some of these stoichiometries are based on steady-state kinetic properties, they may need reexamination (see below). In the second model, binding of Ca^{2+} to protein induces a conformational change that exposes hydrophobic domains that allow membrane-protein attachment. Some data do suggest that PKC undergoes a conformational change in the presence of Ca^{2+} and becomes more hydrophobic. This property has been utilized to purify PKC (Walsh *et al.*, 1984), or to generate an artificial phorbol ester receptor (Bazzi and Nelsestuen, 1989a).

Preliminary results (Bazzi and Nelsestuen, 1990) suggest that the binding of PKC to Ca^{2+} is a complex process that is best represented, at the current state of knowledge, by a single equilibrium:

$$PKC + PL + 6\text{–}10\,Ca^{2+} \leftrightarrow PKC_{Ca}\text{–}PL$$

Direct measurements by equilibrium dialysis revealed that free PKC bound low levels of calcium with low affinity ($\leqslant 2$ calcium per PKC at 100 μM calcium, unpublished results). Addition of phospholipid vesicles greatly increased calcium binding to $\geqslant 6$ sites. While other mechanisms of calcium interaction are possible, it seems likely that calcium binds at the interface between the protein and phospholipid. The protein therefore may contain only a part of each calcium binding site. Depending on the affinity of these partial sites, binding of calcium to the protein alone would occur, but with such low affinity that it may go undetected in the physiological range of calcium concentrations. Such a property would raise the question of whether PKC should be considered a calcium binding protein. Since the large number of calcium sites per protein ($\geqslant 6$) will probably not occur in a contiguous manner on the peptide, they will not easily be identified from primary sequence.

Qualititatively, this calcium-binding property of PKC is shared by at least two other proteins isolated from bovine brain (Bazzi and Nelsestuen, unpublished observations). These proteins bound 10–12 calcium ions in the presence of phospholipid (100 μM calcium) but less than 2 calcium ions in the absence of calcium (100 μM calcium). In fact, "phospholipid-dependent calcium binding" may be characteristic of a relatively large class of proteins. Glenney (1986) reported that calpactin I (also known as lipocortin II) bound 2 moles calcium in the presence of phospholipids, but had very low affinity for calcium in the absence of phospholipids. Similarly, lipocortin I (or calpactin II) has been reported to bind about 4 moles of calcium in the presence of phospholipids, but had virtually undetectable calcium binding in the absence of phospholipids (Schlaepfer and Haigler, 1987). Indirect calcium binding measurements suggest that virtually all members of the lipocortin/calpactin group of proteins may exhibit these calcium-binding properties (for a review, see Klee, 1988). Additional candidates for such calcium-binding

properties may include phospholipase C and the large number of unidentified calcium-dependent membrane binding proteins described by Creutz *et al.* (1987).

The vitamin K-dependent plasma proteins may offer some similarities in terms of membrane binding characteristics (reviewed in Nelsestuen, 1988). Although these are true calcium binding proteins, they bind a larger number of metal ions when bound to phospholipid (Sommerville *et al.*, 1986). The X-ray crystal structure of the membrane-binding segment of prothrombin shows the alignment of 12 carboxyl groups on one face of the protein (Soriano-Garcia *et al.*, 1989). These carboxyls may be involved in calcium bridging to acidic phospholipids in the membrane. It is possible that clustering of carboxyl groups on a protein surface may be common to the entire class of calcium-dependent membrane binding proteins. Protein kinase C possesses such a clustering of carboxyl groups (Parker *et al.*, 1986; Ohno *et al.*, 1987).

2.2. Interactions of PKC with Phospholipids

The interaction of PKC with phospholipids provides some intriguing properties. Figure 1 shows the minimum number of interactions between PKC and phospholipids that are needed to explain current observations. For the sake of simplicity, these interactions are divided into reversible and irreversible categories.

2.2.1. Reversible PKC Interactions. At low Ca^{2+} concentrations, PKC binds to phospholipids in a reversible manner. This reaction requires acidic phospholipid with no strong preference for a particular head group (Bazzi and Nelsestuen, 1987a). While accurate equilibrium binding constants are

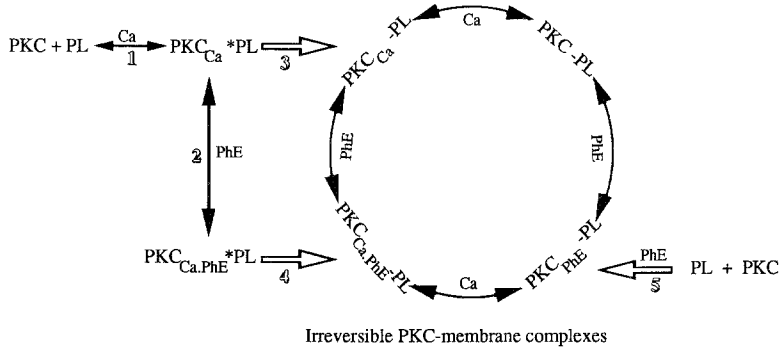


Fig 1. Equilibria describing the interactions of PKC with phospholipid (PL). Reversible protein–membrane interactions are described by an asterisk (*) while irreversible protein membrane complexes are described by dash (—). Also depicted is the probable influence of phorbol ester (PhE) and calcium (Ca) on these reversible and irreversible interactions.

currently not available, the binding is of high affinity ($K_D \leqslant 5$ nM; see Bazzi and Nelsestuen, 1987a). PKC–membrane interaction is presented in Fig. 1 as a single equilibrium step rather than a multiple step process beginning with calcium–PKC interaction. As stated above, the interaction of PKC with calcium is dependent on phospholipid.

The second reversible interaction shows binding of phorbol esters to PKC (reaction 2 in Fig. 1). A surprising property was that phorbol esters and diacylglycerol did not appear to influence the stability of the reversible PKC* membrane complex. In contrast, removal of calcium caused dissociation of PKC from membranes and release of the phorbol ester (Bazzi and Nelsestuen, 1989a). The widely reported stabilizing effect of phorbol esters on the PKC–membrane complex is probably due to the formation of the irreversible PKC–membrane complex (see reaction 3 or 5 in Fig. 1).

Apparently, the bilayer structure of the membrane is not required for the reversible associations. Hannun *et al.* (1985) reported the binding of PKC to Triton–PS mixed micelles and the subsequent binding of phorbol esters or diacylglycerol (Hannun and Bell, 1986). However, the use of nonionic detergents may alter the specificity of PKC–phospholipid interaction. For example, with phospholipid–Triton mixed micelles, interaction of PKC showed strong preference for PS over PG or PI (Hannun *et al.*, 1986a). In contrast, the binding of PKC to phospholipid vesicles did not display such a high preference (Bazzi and Nelsestuen, 1987a). This selective behavior was not unique to PKC. Histone IIIS, a substrate for PKC that interacts electrostatically with ionic phospholipids, also displayed preference for PS over PI or PG in Triton–phospholipid mixed micelles, but showed little specificity when these phospholipids were in vesicles (Bazzi and Nelsestuen, 1987b).

2.2.2. Irreversible PKC–Membrane Interactions Early reports of subcellular fractionation of PKC from brain tissue revealed that the enzyme existed in at least three states (Kikkawa *et al.*, 1982). One state was a cytosolic protein, another was a complex with the membrane that could be dissociated by calcium chelation, and a third had the properties of an integral membrane protein in that it could only be removed by solubilization of the membrane itself (Kikkawa *et al.*, 1982; see also Shearman *et al.*, 1987). Similar distribution of PKC has been observed in several cell lines (Anderson *et al.*, 1985). PKC that had been released from the membrane appeared similar to PKC isolated from the cytosol (Kikkawa *et al.*, 1983a; see also Bazzi and Nelsestuen, 1988a).

Phorbol esters have been shown to significantly alter the distribution of PKC. The effect of phorbol esters was initially described as "translocation" of PKC to the membrane (Kraft and Anderson, 1983; Kraft *et al.*, 1982) since phorbol esters caused a shift in the PKC population from the cytosolic to the membrane-bound form. Translocated PKC was EGTA-stable but detergent

extractable (Gopalakrishna *et al.*, 1986). It was initially suggested that phorbol esters functioned by enhancing affinity for calcium so that it could no longer be removed by EGTA (Bell, 1986). Such high-affinity calcium binding would be exceptional. Studies with purified materials showed that calcium was removed from the irreversible PKC–membrane complex without is dissociation (Bazzi and Nelsestuen, 1989b).

In vitro studies also showed two forms of membrane-bound PKC, reversibly and irreversibly bound forms (Bazzi and Nelsestuen, 1987a, 1988a). The forms which could not be dissociated by calcium chelators were observed with vesicles containing 30% PS in the presence or the absence of phorbol esters (reaction 3 in Fig. 1). Phorbol esters greatly enhanced formation of the tightly membrane-bound form (reactions 4 in Fig. 1) in a manner that appeared to mimic the events observed in cells and tissues. Both calcium and phorbol esters dissociated from the irreversible protein–membrane complex (Bazzi and Nelsestuen, 1989a,b), so that the stability of the complex was not due to residual cofactors.

A puzzling observation was that high phorbol ester concentrations (about 0.1 μM) promoted formation of membrane-inserted PKC (reaction 5 in Fig. 1). In contrast, there was no detectable interaction of phorbol esters with PKC in the absence of phospholipid (Bazzi and Nelsestuen, 1989b). In this respect, phorbol ester binding appears somewhat like calcium binding in that there is much stronger interaction with the protein–membrane complex. It is possible that low-affinity interactions between PKC and calcium or between PKC and phorbol esters are responsible for initiating the interactions in steps 4 and 5 of Fig. 1.

The biochemical basis for the irreversible interaction between PKC and the membranes is not known. Studies with phospholipid monolayers suggested that PKC–phospholipid interactions produced surface pressure changes consistent with protein penetration into the hydrocarbon region of the membrane (Bazzi and Nelsestuen, 1988b). Therefore, a likely explanation is a protein conformational change that allows insertion of a portion of PKC into the membrane to generate an integral membrane protein. For this reason, the irreversible form of membrane-bound PKC is referred to as "membrane-inserted" PKC.

A common method of generating integral membrane proteins is attachment of fatty acids or diglycerides which then attach irreversibly to the membrane bilayer (for a review, see Sefton and Buss, 1987). While such a mechanism has been proposed for PKC (Alkon and Rasmussen, 1988), it seems unlikely since irreversible binding to membrane could be achieved in pure systems that should be devoid of other enzymes. The irreversible membrane-bound form could be released by dissolving the membrane with detergent to generate PKC that seemed indistinguishable from the original

soluble enzyme (Bazzi and Nelsestuen, 1988a). Also, photoaffinity labeling suggested that PKC–vesicle interaction was consistent with PKC penetrating the phospholipid bilayer (Snoek *et al.*, 1986). Furthermore, high-pressure release of PKC from membranes required pressures (> 1500 atm) consistent with an integral membrane protein (Lester, 1989). Thus, if non-protein prosthetic groups are important in forming the integral membrane protein, they are probably present in the cytosolic form of the enzyme as well.

There are several striking differences between the reversibly membrane-associated PKC and membrane-inserted PKC. The most interesting property may be that membrane-inserted PKC is active without cofactors so that its activity is unaffected by calcium, DAG, or phorbol esters (Bazzi and Nelsestuen, 1988b,c). While caution must be exercised to avoid over-interpretation of kinetic results obtained in the *in vitro* assay (see below), this striking difference indicated that membrane-inserted PKC was a substantially different enzyme. This property even suggests that the traditional definition of PKC as the phorbol ester or DAG-requiring kinase may need reexamination since this description may exclude a substantial portion of the PKC in cells.

This great change in enzymatic behavior is generated in an irreversible manner and may illustrate one mechanism for generating long-term events in cell regulation from transient stimulation of second messengers (see below). Irreversible protein–membrane binding events may also result in ultrastructural changes in membranes, proteins, or membrane–protein arrangements so that, in effect, the cell remembers events that occurred in the past. Maintenance of memory by protein phosphorylation was proposed by Crick (1984) and this concept adjusts easily to the observed properties of PKC (Nelsestuen and Bazzi, 1989; Burgoyne, 1989).

While the biological relevance of the speculations in the preceding paragraphs are not known, the phenomenon which they represent may be fairly general; other regulatory proteins are known to be distributed between the cytosol and irreversible membrane-associated states. For example, a phosphotyrosine phosphatase (Tonks *et al.*, 1988a) can be isolated from the cytosol or from the membrane fraction of placenta. The two forms seem to have little or no structural difference (Tonks *et al.*, 1988b). Once again, the general purposes of the two populations may be similar to those of PKC. The calcium and calmodulin-stimulated protein phosphatase, calcineurin, is also found in cytosolic and membrane-associated forms (Tallant *et al.*, 1983). In fact, it is possible that many regulatory proteins from a wide range of places are found in similar two-state distributions. Thus, the distribution and properties of PKC may be representative of a common regulatory mechanism.

2.3. Protein Kinase C–Phorbol Ester Interactions

Since the report of activation of PKC by phorbol esters (Castagna *et al.*, 1982), and the demonstration that PKC is indeed a phorbol ester receptor (Kikkawa *et al.*, 1983b), several key studies have addressed properties of PKC–phorbol ester binding. With minor variations, all studies suggest interaction between PKC and phorbol esters that is specific and of high affinity (Kikkawa *et al.*, 1983b; Sando and Young, 1983; Niedel *et al.*, 1983; Leach *et al.*, 1983). Variations in the reported equilibrium constants (generally a K_d between < 1 to 15 nM is reported) may arise from methodology and the possibility that equilibrium is not achieved in some assay systems (Bazzi and Nelsestuen, 1989a).

Most studies suggested that high-affinity interaction between PKC and phorbol esters required that PKC be membrane-bound. Subsequently, in many cases, demonstration of phorbol ester binding requires the presence of acidic phospholipids and calcium (Konig *et al.*, 1985). However, recent studies have shown that materials other than calcium and membranes can support phorbol ester binding to PKC. These include polycations (Thompson *et al.*, 1988) and gel column matrix materials (Bazzi and Nelsestuen, 1988a). In addition, the calcium requirement of phorbol ester binding is dependent on whether the reversible or irreversible PKC-membrane complex is used. In contrast to reversibly bound PKC, phorbol ester–PKC binding to the irreversible complex occurred in a calcium-independent manner (see Fig. 1).

It is possible that the phorbol ester binding site is formed in a manner analogous to the many calcium-binding sites (see above); free protein may contain only a partial site that could be completed via attachment of the protein to membranes. A study with photoactivatable derivative of phorbol esters indicated that only phospholipids were labeled, though protein was an essential component for labeling (Blumberg *et al.*, 1984). Recently, new classes of PKC activators have been described (Nishizuka, 1986b; Blumberg, 1988). These reagents are structurally unrelated to phorbol esters, but presumably function by binding to the phorbol ester-binding site on PKC. A key structural feature common to all of these compounds, in addition to the spatial similarity (Wender *et al.*, 1986; Brasseur *et al.*, 1985), is an essential lipophilic arm. This may suggest that the binding of these reagents to PKC involves a membrane-related event. DAG also contains lipophilic hydrocarbons and is known to competitively inhibit phorbol ester binding to PKC (Sharkey *et al.*, 1984).

3. *In vitro* Activation of PKC

A reliable *in vitro* assay is imperative to elucidation of an enzyme's function. Enzyme mechanism as well as screening of materials for enzyme

inhibition or stimulation can be studied in a defined system where components can be varied in a systematic manner. The activity of PKC is usually measured in a complex mixture, and cofactors needed for activity are interpreted as enzyme requirements. With histone H1 as a substrate, PKC activity required the presence of phospholipids, Ca^{2+}, and phorbol esters or diacylglycerol. However, observations of these requirements necessitates careful selection of conditions and substrates; activity can often be generated with some or none of these cofactors. The value of an *in vitro* assay for the goals outlined above requires a thorough understanding of its properties and their ramifications. The properties of the assay determined to date indicate that some very challenging problems confront interpretation of mechanism and identification of specific regulators of PKC via the *in vitro* assay.

The commonly used substrates for *in vitro* PKC assay are polycationic peptides or proteins. Since the phospholipid is a polyanionic material, the substrate–phospholipid combination produces nonspecific ionic interactions and aggregates, often large enough to be observed with the unaided eye and pelleted by centrifugation. Attempts to prevent aggregation also prevented avid phosphorylation by PKC (Bazzi and Nelsestuen, 1987c). Assembly of PKC with its substrate on planar nonaggregated membranes in the presence of Ca^{2+} and phorbol esters was not sufficient to generate kinase activity (Bazzi and Nelsestuen, 1988b). It was concluded that aggregation of substrate and enzyme was an integral part of the *in vitro* assay. This property may help to explain the poor correlation between PKC–membrane binding and appearance of activity (Bazzi and Nelsestuen, 1987a).

A model for PKC activation was proposed in which delivery of substrate to the active site was a key step (Bazzi and Nelsestuen, 1987c). Three categories of *in vitro* substrates could be identified: category A substrates, typified by protamine sulfate, did not require any cofactor for phosphorylation. These substrates interacted directly with PKC and formed aggregates, thereby delivering substrate to the active site of the enzyme. Category B substrates, typified by myelin basic protein, required the presence of acidic phospholipids but not calcium. These substrates bound PKC directly, but required an aggregating agent (phospholipids) to deliver the substrate to the active sites. Category C substrates, typified by histone, required all three of cofactors and had weak interaction with PKC. The latter class of substrates, while still confronted by the problems of aggregation, are clearly the most interesting for use in mechanism studies.

The complexity of the *in vitro* assay and the aggregation process allows many compounds to influence PKC activity by relatively uninteresting mechanisms. These are discussed in the following sections. Earlier, we speculated that the interesting properties of PKC may be common to other enzymes (above). Unfortunately, it is also possible that the problems of the

PKC assay may be common to other enzymes as well. Specifically, enzymes that are stimulated by phospholipids need to be examined for possible aggregation during the assay. The complexity of another phospholipid-dependent system, which may produce a relatively uninteresting mechanism of inhibition, has been documented (Davidson *et al.*, 1987).

3.1. Problems for Determining Substrate Specificity

PKC phosphorylates many substrates *in vitro*, but there have been only a few *in vivo* substrates identified (Nishizuka *et al.*, 1986a). Several studies have attempted to determine the specificity of PKC for peptide substrates (Ferrari *et al.*, 1985, 1987; Turner *et al.*, 1985; Woodgett *et al.*, 1986; House *et al.*, 1987; House and Kemp, 1987). These studies suggested that good peptide substrates are characterized by a cluster of positively charged residues surrounding a serine or a threonine. Relatively low specificity is also illustrated by the fact that random polymers of lysine plus serine or arginine plus serine were among the best *in vitro* substrates for PKC (Bazzi and Nelsestuen, 1987c). While the structural specificity may result to some degree from enzyme–substrate interactions, several positive charges also allow the substrate to aggregate the anionic phospholipids. In fact, it may be very difficult to discriminate between substrate specificity arising from substrate interactions with the enzyme versus substrate interactions with the phospholipid.

3.2. Limitation on Kinetic Studies

An aggregate is a discontinuous mixture in which enzyme and substrate may be heterogeneous due to physical properties or accessibility. This is incompatible with the requirements and assumption of the standard methods for analysis of enzyme kinetics. The latter include steady-state kinetics (Michaelis–Menten or Hill equations), association and dissociation rate constants, and equilibrium binding constants. If kinetic data, obtained in a discontinuous mixture, are analyzed by steady-state equations, variations in K_m or V_{max}, the appearance of linear or curved Lineweaver–Burk plots, and apparent inhibition of a certain type (competitive or noncompetitive with respect to another component), Hill coefficients that are larger or smaller than 1.0, etc., may not have their standard mechanistic meaning. In fact, reagents that are likely to interfere with substrate–phospholipid aggregation seem to exhibit complex kinetic behavior. For example, derivatives of amino-acridines have been reported to inhibit PKC in a manner that differs with the derivative used (Hannun and Bell, 1988). Polyamines have also been reported to inhibit PKC by mechanisms that differ with the derivative used (Qi *et al.*, 1983). Since these inhibition mechanisms were based on steady-state enzyme kinetic properties, the conclusions may need to be reexamined. The effect of

polyamines may simply consist of removal of substrate from the enzyme or from the aggregate (Bazzi and Nelsestuen, 1987c).

Nevertheless, it is still possible to measure reaction velocity in a discontinuous mixture. Factors that affect velocity clearly do alter the assay system in some manner and it is necessary that other approaches, often of a less formal nature, be used to determine the basis of an observed kinetic effect.

3.3. Problems with Identification of Activators or Inhibitors of PKC

Many materials which influence PKC activity have been identified. However, to date, there are no compounds, other than phorbol esters, of which we are aware that are clearly specific for PKC. The most common types of stimulators or inhibitors are amphipathic molecules which are both lipid and ionic materials. An example is cationic hydrocarbons such as sphingosine. While some properties initially suggested that sphingosine was a specific inhibitor which bound to the regulatory domain of protein kinase C (Hannun *et al.*, 1986b), other studies showed that a direct correlation existed between the amount of acidic phospholipid used in the assay and the amount of sphingosine needed to inhibit the reaction (Bazzi and Nelsestuen, 1987d; Hannun *et al.*, 1986b). In fact, the properties of sphingosine action in the *in vitro* assay suggested that its major impact was on the aggregation process, a relatively nonspecific effect. It is always possible that sphingosine exerts an entirely different, specific effect on PKC in the unique conditions found in cells. Reports have differed on this point and have suggested either that sphingosine effects on cells are specific (reviewed by Merrill and Stevens, 1989; Hannun and Bell, 1989) or nonspecific (Pittet *et al.*, 1987; Krishnamurthi *et al.*, 1989; Winicov and Gerhengorn, 1988). In any event, the important point is that many materials can affect the *in vitro* assay in an uninteresting manner so that this assay alone is a poor predictor of enzyme effectors that will be important *in vivo*.

The nonspecificity of most known PKC inhibitors has been proposed by others (Hidaka and Hagiwara, 1987). These include anticalmodulin compounds such as W-7 and several positively charged reagents such as neurotoxins, polyamines, and several phospholipid interaction molecules. Whether or not the inhibition by the isoquinoline derivatives, H-7 (Garland *et al.*, 1987), staurosporine, K252, and UCN-01 (Rüegg and Burgess, 1989) is specific has also been questioned.

Due to their potential value, the search for specific inhibitors and stimulators of regulatory enzymes such as PKC must continue. In the future, care must be exercised to identify materials, like the phorbol esters, which modify PKC by direct interaction with the protein rather than by modification of other components of the cell or the *in vitro* assay. An initial assessment of

specificity might consist of the amounts of material needed to generate an effect. For example, biologically active phorbol esters exert their effect at nanomolar concentrations. In contrast, most nonspecific reagents require 10^3–10^4 times as much material and the concentrations often rival the concentration of phospholipids in the assay. This property may constitute a first indication of nonspecificity.

3.4. Selection and Preparation of Phospholipids Used in the Assay

A major complicating component of the PKC assay is the phospholipid. Knowledge of phospholipid role is increasingly important since a number of regulatory enzymes are stimulated by phospholipid. In the case of PKC, phospholipids provided in the form of vesicles or detergent-mixed micelles have been used. These two systems are not equivalent. Furthermore, the relevance of an observation may depend on the care and methods used in phospholipid preparation.

When dispersed in aqueous buffers, phospholipids form multiple bilayer structures with an undefined percentage of the total phospholipid exposed to the solution. Formation of small unilamellar vesicles usually requires sonication of these mixtures. Unfortunately, sonication is a rather destructive process and can produce significant levels of hydrolysis products. During storage, small vesciles will undergo fusion to form larger vesicles. Thus, mechanism studies involving phospholipid vesicles requires knowledge of their state and determination of whether or not subtle changes, occurring during lipid storage, are affecting enzyme activity.

A second concern regarding the phospholipid component is that bilayers composed exclusively of acidic phospholipids have some highly unusual properties. The intense charge of these membranes may give unusual interactions due to such things as charge repulsion between head groups, lack of positive interaction between head groups, and an increased exposure of the hydrocarbon region of the membrane. Well-known examples of behavior limited to membranes of high charge density include calcium-induced aggregation and fusion (Portis *et al.*, 1979). If the acidic phospholipid content is at or below 30%, as are most biological membranes, these events are virtually undetected during a normal experiment, even at very high calcium concentrations (Nelsestuen and Lim, 1977). Boni and Rando (1985) first showed that membranes of 25% phosphatidylserine were adequate for study of PKC.

In an attempt to avoid the problems of phospholipid bilayers, an assay based on phospholipids dispersed in Triton X-100 was developed (Hannun *et al.*, 1985). Such a system might have several advantages, the greatest of which may be homogeneity. Since the micelles are dynamic, they may be able

to reach a true equilibrium state and give the same structures regardless of the procedure used to generate them. Unfortunately, some preliminary evidence suggested that mixed micelles may fail to deliver this theoretical advantage. For example, sphingosine is a cationic detergent and an inhibitor of PKC. Like phospholipids, it should distribute itself randomly among the micelles. However, its inhibition properties differed greatly depending on whether sphingosine was mixed with the phospholipid in organic solvent before drying or dispersed independently in Triton micelles (Bazzi and Nelsestuen, unpublished observations). In addition, mixed micelles did not avoid the problems of aggregation (Brazzi and Nelsestuen, 1987b).

The membrane and mixed micelle systems are not equivalent. Acidic phospholipids provided in the form of vesicles can support the activity of PKC in the absence of DAG if calcium concentrations were high. In contrast, Triton–PS mixed micelles required the presence of both phosphatidylserine and DAG or phorbol esters. A second difference was that phospholipid vesicles supported both reversible and irreversible binding of PKC to membrane. Triton–PS mixed micelles only supported reversible interactions (Bazzi and Nelsestuen, 1989b). Consequently, phospholipid bilayers appear to provide the best-defined phospholipid component for mechanistic study of phospholipid-dependent enzymes. In addition, procedures for producing large unilamellar vesicles with minimal chemical modification are available (Hope *et al.*, 1985) and these membranes may provide the most well-defined phospholipid component.

4. Future Directions

The potential role of PKC in long-term cell regulatory events has been proposed by workers in the neurobiology field. Considerable effort has been put forward in this area with conflicting opinions of the importance of PKC in cell memory. Speculation on its possible role in long-term cell potentiation has been considerable (see, e.g., Alkon and Rasmussen, 1988; Alkon, 1989; Rasmussen, 1989). Furthermore, the observed ability of PKC to form reversible and irreversible membrane-bound complexes that have different catalytic properties, as outlined in this review, provides tantalizing biochemical support for such a mechanism for long-term cell regulation or cell memory. Indeed, the convergence of genetic approaches to detection of memory flaws, the intense study of nerve cells in advanced and primitive organisms, and the biochemical study of PKC and other translocatable enzymes may be at the point of producing important new findings on this subject.

In addition, it still appears necessary to continue to question the biological relevance of PKC stimulation by DAG. Although DAG is a known biological

molecule which is generated by the action of highly regulated enzyme(s) (the phospholipase C's), DAG must be added in relatively large amounts in order to stimulate PKC in the *in vitro* assay. Consequently, whether or not DAG is the true biological stimulator of PKC does not appear established. It is necessary to continue study of DAG and its actual role as a second messenger.

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