Role of Substrate in Imparting Calcium and Phospholipid Requirements to Protein Kinase C Activation†

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ABSTRACT: The role of substrate in influencing the cofactor requirements of the phospholipid- and Ca^{2+}-dependent protein kinase C (PKC) was investigated by using several substrates. All of the substrates tested, including histone, troponin I, myosin light chain, protamine, poly(arginine, serine) (PAS), poly(lysine, serine) (PLS), and myelin basic protein (MBP), were found to interact with and aggregate phospholipid vesicles as well as phosphatidylserine (PS)–Triton mixed micelles. Phosphorylation of these different substrates by PKC indicated the presence of three distinct substrate categories: (A) substrates such as protamine requiring no cofactors; (B) substrates such as PLS, PAS, and MBP requiring only the presence of phospholipid; and (C) substrates such as histone, myosin light chain, and troponin I requiring the presence of Ca^{2+} and phospholipid. Diacylglycerol was a major cofactor only with category C substrates. These different requirements correlated with the interaction of the substrate with phospholipid and/or enzyme. The substrates in category A interacted strongly with and aggregated PKC in a binary mixture. In the absence of Ca^{2+}, PKC bound to substrates of category B directly but not to substrates in category C. Thus, substrate–enzyme binding eliminated the Ca^{2+} requirement of phosphorylation, and aggregation of substrate–enzyme complex eliminated the phospholipid requirements as well. Substrate–phospholipid interaction and substrate phosphorylation were inhibited by increasing salt concentrations, but the amount needed depended upon the substrate. Loss of PKC activity appeared to coincide with loss of substrate–PS aggregation while dissociation of PKC from the membranes required much higher salt concentrations. Poly(L-lysine) and poly(L-arginine), two potent inhibitors of PKC, also showed substrate-dependent inhibition characteristics. Both of these polymers interacted strongly with phospholipid vesicles and appeared to exert their inhibition by interfering with substrate–phospholipid binding. These studies indicated that the substrate plays an important role in imparting Ca^{2+}, diacylglycerol, and phospholipid requirements to the activation of PKC, and titrations of PKC activity with phospholipid were actually dominated by substrate–phospholipid interactions. Delivery of substrate to the active site appears to be a key event in PKC phosphorylation, and this process may exhibit a requirement for phospholipid and/or Ca^{2+} depending upon the choice of substrate.

The Ca^{2+}- and phospholipid-dependent protein kinase C (PKC)[1] is an important enzyme because of its possible role in many cell regulation events [for reviews, see Nishizuka (1984a,b, 1986a,b) and Kuo et al. (1985)]. At approximately cellular concentrations of Ca^{2+}, the activity of PKC is greatly stimulated by DAG or phorbol esters. Biologically active phorbol esters are potent tumor promoting agents, and several studies suggest that their primary target is PKC (Ashendel, 1985; Nishizuka, 1984b). Diacylglycerol, produced in membranes by signal-induced phosphatidylinositol turnover (Berridge, 1984; Nishizuka, 1984b), activates PKC in a manner that is presumed to be similar to that of phorbol esters. Consequently, the general picture of PKC is of a protein which binds Ca^{2+} and membranes, binds phorbol esters or DAG, and carries out phosphorylation of protein substrates, thereby altering critical cellular events.

PKC has a broad substrate specificity, and a number of in vitro substrates have been identified (Nishizuka, 1986a; Takia et al., 1985; Kuo et al., 1985). Small peptide substrates have also been produced, and the structural requirements appear to consist of several basic amino acids closely associated with serine or threonine residues (Ferrari et al., 1985; Kishimoto et al., 1985; Turner et al., 1985). PKC is considered to be a Ca^{2+}- and phospholipid-dependent kinase. However, PKC does not display these requirements for certain synthetic substrates (Ferrari et al., 1985; Turner et al., 1985). Furthermore, it is well-known that PKC phosphorylates protamine in a DAG-, Ca^{2+}-, and phospholipid-independent manner (Takia et al., 1977). The explanation for this substrate-dependent variation in cofactor requirements is poorly understood.

Many studies have determined the amounts of cofactors needed for appearance of PKC activity. The titration curves often appear as normal hyperbolic saturation curves so that apparent K_a values can be obtained. However, activation of PKC involves many interactions, and an unambiguous assignment of the interaction responsible for an apparent K_a can be complex. An interesting observation was that the quantity of micelles (Hannun et al., 1985) or phospholipid vesicles (Bazzi & Nelsestuen, 1987) required to activate PKC was always in great excess over the PKC enzyme concentration. In contrast, binding of PKC to membranes was of high affinity

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[1] Abbreviations: PKC, Ca^{2+}- and phospholipid-dependent protein kinase C; PLS, random copolymer of lysine, serine (3:1); PAS, random copolymer of arginine, serine (3:1); MBP, myelin basic protein; DAG, diacylglycerol or diolein; PS, phosphatidylserine; PI, phosphatidylinositol; PG, phosphatidylglycerol; PC, phosphatidylcholine; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; Rho-PE, *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine; dansyl-PE, dipalmitoyl-*N*-dansyl-L-α-phosphatidylethanolamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; BSA, bovine serum albumin.

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and occurred at equal weight concentrations of enzyme and phospholipid (Bazzi & Nelsestuen, 1987). Thus, the nature of the interaction(s) responsible for titration of activity by adding phospholipid is not known; it does not appear to consist of the enzyme–membrane binding step. Other potential interactions occurring in the activation mixture include substrate–enzyme and substrate–phospholipid. It is possible that the substrate plays a major role in these titration curves.

In this study, several substrates of PKC were used to assess their role in imparting the cofactor requirements of PKC activation. All of the PKC substrates tested were found to interact strongly with the phospholipid and cause aggregation of phospholipid vesicles or PS–Triton mixed micelles. All attempts to prevent aggregation of substrate and phospholipid resulted in loss of activity. These interactions appeared to be critical to phosphorylation. Protein kinase C appeared to be a nonspecific enzyme that required the delivery of substrate to its active site. Substrate–membrane interaction and subsequent aggregation appeared to be an effective in vitro means of providing substrate to the active site of the enzyme.

Experimental Procedures

Materials. Phosphatidylserine (egg yolk), phosphatidylinositol (soybean), phosphatidylglycerol (egg yolk), phosphatidylcholine (egg yolk), and diolein were purchased from Sigma Chemical Co. and were of the highest purity available. The phospholipids were reported to be greater than 98% pure. *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (NBD-PE) and *N*-(lissamine Rhodamine B sulfonyl)phosphatidylethanolamine (Rho-PE) were from Avanti Polar Lipids, Inc. Histone (type III-S), protamine, poly(lysine, serine) (3:1), and poly(arginine, serine) (3:1) were purchased from Sigma Chemical Co.

Protein Purifications. PKC was purified to apparent homogeneity from bovine brain as described previously (Bazzi & Nelsestuen, 1987). Myelin basic protein was purified from bovine brain (Lampe & Nelsestuen, 1982). Troponin I, purified from rabbit skeletal muscle (Strasburg et al., 1985), was the generous gift of Dr. G. Strasburg. Myosin light chain (rabbit muscle) and troponin complex were purchased from Sigma Chemical Co. and used without further purification.

Phospholipid Preparations. Phospholipid vesicles were prepared by a procedure similar to that of Huang (1969). The phospholipids were mixed in the organic solvent at the desired composition, dried under a stream of argon, then suspended in 20 mM Tris buffer (pH 7.5), and subjected to brief direct probe sonication. For light-scattering intensity and fluorescence energy-transfer measurements, the suspension of phospholipid vesicles was applied to a Sepharose 4-B column (30 × 1.5 cm) equilibrated with the same buffer, and only small single-bilayer phospholipid vesicles obtained from the column elution were used. For PKC activity measurements, the phospholipids were used without gel filtration. Phospholipid concentrations were determined by an organic phosphate assay (Chen et al., 1956) using a phospholipid:phosphorus weight ratio of 25.

PS–Triton mixed micelles were prepared essentially as described by Hannun et al. (1985). In all experiments performed with micelles, a 0.3% (w/w) Triton X-100 solution was used, and the phospholipid concentration was expressed as mole percent of the Triton concentration.

Light-Scattering Intensity Measurements. Light-scattering intensity measurements were used to estimate membrane–protein and protein–protein binding quantitatively and qualitatively. The light-scattering intensity (I_s) is proportional to several factors: $I_s = HCMP(\phi)$ where H is a solution constant, C is the weight concentration of scattering particles, M is the molecular weight, and $P(\phi)$ is the scattering function of the particle (Doty & Edsall, 1951). When the dimensions of the scattering particle are small in comparison with the wavelength of the light $P(\phi)$ is approximately 1.0, and light-scattering intensity measurements can be used to quantitatively measure membrane–protein binding by using the relationship (Nelsestuen & Lim, 1977):

$$\frac{I_2}{I_1} = \left(\frac{M_2}{M_1}\right)^2 \left(\frac{\partial n/\partial c_1}{\partial n/\partial c_2}\right)^2$$

where I_2 is the light-scattering intensity of the protein–lipid complex, I_1 is light-scattering intensity of the phospholipid, and $\partial n/\partial c$ is the refractive index increment of each species. However, when the dimensions of the particle approach or exceed the wavelength of the light, the scattering function $P(\phi)$ changes dramatically, and the light-scattering intensity may increase or decrease depending upon $P(\phi)$, particle size, shape, and properties [for reviews, see Tanford (1961) and Doty & Edsall (1951)]. In this study, only qualitative information was extracted in such cases.

Light-scattering intensity was measured on a Perkin-Elmer spectrofluorometer (Model MPF 44 A). The excitation and the emission wavelengths were set at 320 nm. The temperature was maintained at 25 °C.

Particle Size Measurements. Quasi-elastic light-scattering intensity measurements were used to estimate the average size of phospholipid vesicles and histone–phospholipid aggregates. The measurements were performed as described by Pletcher et al. (1980) except that a Langley-Ford LSA 2 particle sizing instrument was used.

Fluorescence Energy Transfer. Fluorescence energy-transfer measurements were used to measure PKC–membrane binding as a function of salt concentration and to assess the distribution of fluorescent phospholipids in micelles. PKC–membrane binding was performed by using phospholipid vesicles composed of PS/DAG/PC/dansyl-PE (30:10:50:10). PKC (0.5 μg) and phospholipid vesicles (0.4 μg) were mixed in the presence of NaCl at the desired concentration. Protein–membrane binding was monitored by the intensity of fluorescence intensity due to energy transfer from tryptophan residues in the protein to dansyl-PE in the membrane using an SLM 4800 spectrofluorometer. Excitation was at 284 nm, and a 500-nm cutoff filter was used in the emission path. The temperature was maintained at 25 °C. The binding was expressed as the percentage change of the fluorescence intensity due to energy transfer ($100\Delta I_i/\Delta I_0$), where ΔI_i is the fluorescence intensity due to energy transfer observed at salt concentration i and ΔI_0 is that observed in the absence of added salt.

Phospholipid distributions in Triton X-100 micelles were measured by using two populations of fluorescently labeled micelles. Micelles containing 5 mol % PS and 0.25 mol % of either NBD-PE or Rho-PE were prepared as described by Hannun et al. (1985). Fluorescence energy transfer from NBD-PE-containing micelles to those containing Rho-PE was measured by mixing equal volumes of the two micelles. In all of these experiments, the final concentration of Triton X-100 was 0.3%. The wavelength of excitation of NBD-PE was 480 nm. These experiments were performed on the Perkin-Elmer spectrofluorometer at 25 °C.

Activity Measurements. PKC activity measurements were performed as described by Kikkawa et al. (1983). The assay mixture (0.25 mL) contained 20 mM HEPES (pH 7.5), 10 mM Mg^{2+}, 0.5 mM Ca^{2+}, 400 μg/mL histone, 20 μM ATP,

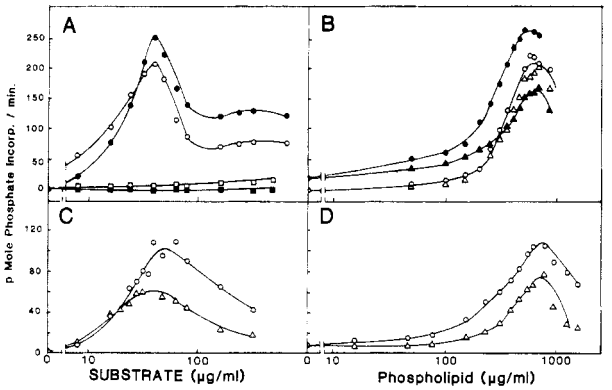

FIGURE 1: Phosphorylation of PLS (panels A and B) and PAS (panels C and D) by PKC. The assay measurements were performed at 480 μg/mL phospholipid vesicles (panels A and C) or at 54 μg/mL substrate (panels B and D). The phospholipid composition was 30% PS with (○, ●), or without (△, ▲) 10% DAG (the remaining phospholipid was PC). The activity measurements were performed in the presence of either 0.5 mM Ca^{2+} (open symbols) or 2.0 mM EGTA (closed symbols). Additional results shown in panel A include the experiments which omitted phospholipid (□) or PKC (■).

~25 ng of PKC, and 160 μg/mL phospholipid. Unless otherwise indicated, the phospholipid vesicle composition was 30% PS/10% DAG/60% PC.

Enzyme–substrate binding was measured by determining PKC activity in the supernatant after centrifugation of aggregates as described in the text. In these experiments, bovine serum albumin (1 mg/mL) was added to stabilize both substrate and PKC. This was an essential addition in the case of the substrate histone, since the latter was found to self-aggregate to a significant extent. In the presence of bovine serum albumin, the optimal concentration of PLS and PAS substrates was 80 μg/mL. In all of these experiments, additional substrate and phospholipid were added to the supernatant to replace that lost in the pellet. Several experiments with different additions were performed to ensure that maximum PKC activity in the supernatant was measured.

Unless indicated, the buffer used was 20 mM HEPES (pH 7.5), and the temperature of the experiments was 25 °C.

RESULTS

Synthetic Polymers as Substrates for PKC. Studies on the substrate specificity of PKC indicated a requirement for positively charged groups surrounding the phosphorylation sites (Ferrari et al., 1985; Turner et al., 1985). Random copolymers of PLS and PAS were found to be excellent substrates for PKC (Figure 1), and the maximum rates of phosphorylation were greater than those obtained with histone. However, activity titrations showed that the cofactor requirements for these substrates were substantially different from those reported for other substrates. In the absence of both phospholipid and Ca^{2+}, PLS was phosphorylated to a very small extent. Phosphorylation of PLS was greatly enhanced by addition of phospholipid alone (Figure 1A). Addition of 0.5 mM calcium actually inhibited phosphorylation. This result was in strong contrast with that obtained with histone, where both Ca^{2+} and phospholipid were essential for phosphorylation. Activity measured as a function of phospholipid concentration and under several experimental conditions showed an optimum phospholipid concentration of 480 μg/mL (Figure 1B). While calcium decreased the amount of phosphorylation, DAG caused a slight enhancement of PKC activity which was variable but was always less than 3-fold.

Similar results were obtained with PAS as the substrate (Figure 1C,D). PAS was phosphorylated by PKC in the absence of Ca^{2+}, and DAG caused only a slight enhancement of reaction. Both PLS and PAS exhibited maximum phosphorylation at a substrate concentration of about 54 μg/mL (phospholipid was 480 μg/mL), but PLS gave higher maximum rates of phosphorylation. However, both of these substrates functioned at lower concentrations than histone which required about 0.4 mg/mL for maximum activity.

Maximum activity of PKC toward PLS and PAS showed an optimum value for substrate (Figure 1A–D) rather than a saturation curve. Other substrates of PKC, including myelin basic protein and several types of histones, also showed optimum concentrations (Wise et al., 1982a). A possible explanation for this phenomenon is that PKC required a certain density of substrate on the membrane surface and alteration of either the phospholipid or the substrate concentration altered this optimum density.

Substrate–Phospholipid Interaction. The phospholipid required for maximum PKC activation always exceeded that required for PKC–membrane binding [see above and Bazzi & Nelsestuen (1987)]. Similar observations were reported by Hannun et al. (1985) using PS–Triton mixed micelles. The additional phospholipid may be required for substrate binding. Consequently, the interaction of PKC substrates with phospholipid vesicles was investigated.

Upon mixing histone with the membrane vesicles used for assay of PKC, large changes in light-scattering intensity occurred (Figure 2A). While an increase in the light-scattering intensity indicated protein–membrane binding, the changes observed in these experiments greatly exceeded the changes expected for simple association of the added protein with the membrane. In addition, the subsequent time-dependent decrease in light-scattering intensity indicated the formation of very large particles. Particle size measurements on this histone–phospholipid mixture indicated that the average size of the particles was greater than 15 μm. In addition, the materials could be sedimented by low-speed centrifugation (see below). While we are unaware of any previous report of this aggregation, the turbidity was not striking although it could be observed with the unaided eye.

Similar results were observed when the substrates protamine, myelin basic protein, PLS, and PAS (Figure 2A) were added to vesicles. Similar results (not shown) occurred for myosin light chain, troponin I, and troponin complex. Thus, it appeared that all in vitro assay systems that employ these substrates and phospholipid vesicles to measure PKC activity involve severely aggregated substrate–membrane complexes.

Since accurate assessment of substrate–phospholipid interactions is difficult in an aggregate, alternative approaches were evaluated. Hannun et al. (1985) developed a PS–Triton mixed micelle system which replaced the phospholipid vesicle requirements of PKC This system was proposed to provide major advantages over phospholipid vesicles since the micelles are of more well-defined composition. However, the results in Figure 2B showed that, under the conditions required for activity, extensive aggregation of these micelles took place with all of the substrates tested. Thus, the micelles were subject to the same problems encountered with phospholipid vesicles. These results indicated that all the activity measurements of PKC activation involved highly aggregated mixtures regardless of the use of well-defined initial phospholipid vesicles (Boni & Rando, 1985) or micelles (Hannun et al., 1985).

The micelles showed other properties inherent to their dynamic nature. The experiments shown in Figure 2C indicated that fluorescent phospholipids, originally dispersed in two different micelle populations, were rapidly randomized when

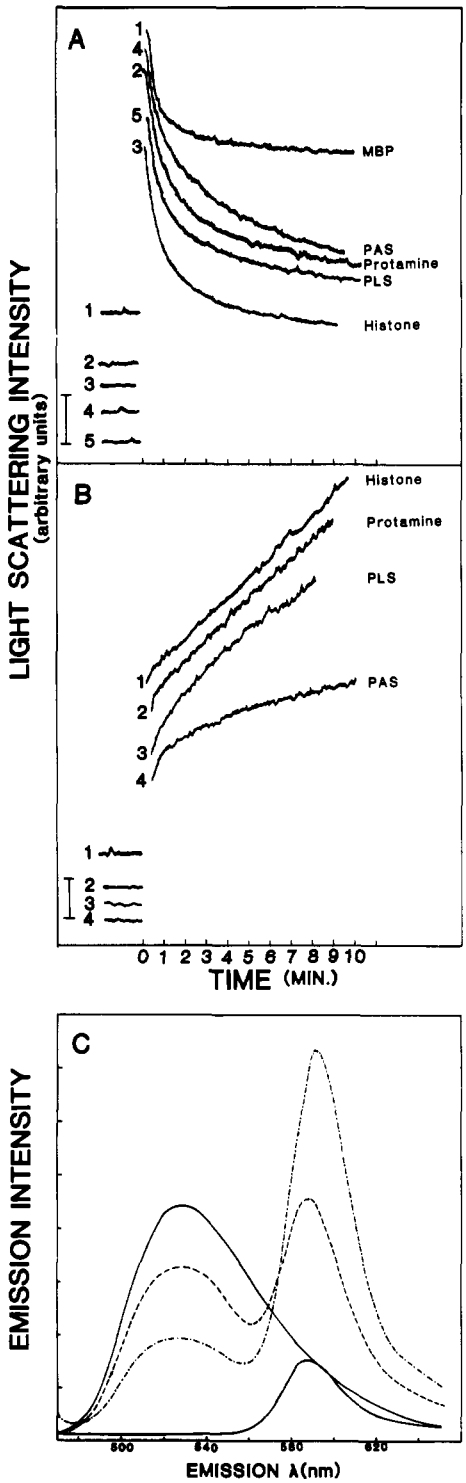

FIGURE 2: Aggregation of phospholipid components by PKC substrates. Changes in the light-scattering intensity of phospholipid vesicles (panel A) or PS–Triton mixed micelles (panel B) upon addition of the indicated substrates were monitored as a function of time. In both panels, the different tracings are offset for clarity; the numbers are provided to identify the tracings before and after addition of substrate. The light-scattering intensity of the phospholipid vesicles was the same in every experiment and was equal to the bar shown on the left of panel A. The scattering intensity of the micelles is given in panel B. 40 μg of phospholipid vesicles (30% PS, 10% DAG, and 60% PC, panel A) and PS–Triton mixed micelles (containing 8 mol % PS and 1.0 mol % DAG; panel B) were used in these studies. Panel C shows the fluorescence energy transfer between fluorescent lipids in PS–Triton micelles. In all cases, the reaction contained 0.3% (w/w) total Triton and 5 mol % PS plus fluorescent phospholipids in a total volume of 1.6 mL. The scans shown include the emission intensity of NBD-PE-containing micelles [0.25 mol % (—), upper line], Rho-PE-containing micelles [0.25 mol % (—), lower line], a mixture containing 0.25 mol % each of NBD-PE and Rho-PE (- - -) and the latter mixture after addition of 40 μg of histone (-·-).

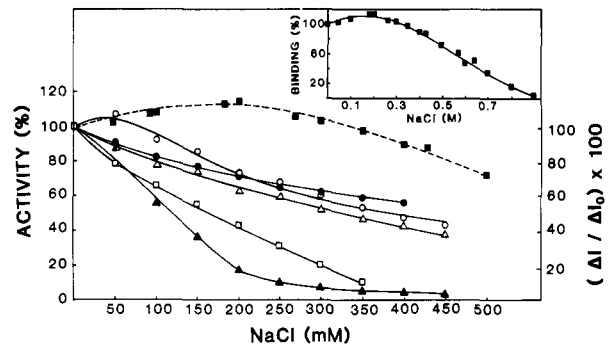

FIGURE 3: Effect of NaCl concentration on PKC activity and membrane binding. The activity of PKC was measured in the presence of 480 μg/mL phospholipid (containing 10% DAG) and 0.5 mM Ca^{2+} with 54 μg/mL PLS (▲), 400 μg/mL histone (□), 54 μg/mL PAS (△), or 400 μg/mL protamine (●). The activity of PKC was also measured with protamine as substrate in the absence of both phospholipid and Ca^{2+} (○). PKC–membrane binding (dashed line) was measured by fluorescence energy transfer as described under Experimental Procedures. The inset shows a titration of PKC–membrane binding as a function of NaCl concentration. The phospholipid consisted of 30% PS–10% DAG–10% dansyl-PE–60% PC. Each experimental determination used a different sample of PKC (0.31 μg/mL) and phospholipid (0.25 μg/mL). Loss of fluorescence energy transfer from PKC to dansyl-PE upon addition of salt indicated dissociation of PKC from the membrane.

the two populations were mixed. This was evinced by the increased fluorescence energy transfer from one type of phospholipid to the other (Figure 2C), a process that requires close physical proximity of the fluorescent phospholipids such as that obtained when they are in the same micelle. Addition of histones to this mixture resulted in further large increases in fluorescence energy transfer among the micelle components, suggesting greater proximity of the phospholipids. The fluorescence energy-transfer signal continued to gradually increase upon incubation of the mixture for 20 min (not shown). These results suggested that the phospholipid content underwent further alteration and the final composition of the lipid in the aggregate may differ from that of the original micelle mixture. For some purposes, vesicles may be a preferable source of defined lipid composition since they are relatively static structures which do not exchange molecules readily and can exist in a nonfused state even when aggregated by myelin basic protein (Lampe & Nelsestuen, 1982).

Effects of Salt on Aggregation and Activity. In an attempt to limit substrate–phospholipid aggregation events, the NaCl concentration of the medium was altered. Salt interfered with, and ultimately prevented, substrate–phospholipid aggregation as assessed by light-scattering changes. The approximate concentration of NaCl required to abolish substrate–phospholipid aggregation was dependent upon the substrate. For example, 300 mM NaCl was adequate to abolish vesicle aggregation by histone while 500 mM NaCl was needed to abolish aggregation with PLS. Protamine and PAS showed stronger interactions with phospholipid so that 1.0 and 1.6 M NaCl, respectively, were needed to prevent aggregation.

Unfortunately, NaCl also inhibited the activity of PKC in a substrate-dependent manner. The effect of NaCl concentration on the activity of PKC with various substrates is shown in Figure 3. The extent of PKC inhibition at each salt concentration was dependent on the substrate, and complete inhibition correlated approximately with loss of aggregation (compare NaCl concentrations given above with the activity results shown in Figure 3).

Binding of PKC to the membrane was examined by using fluorescence energy transfer (Figure 3, dashed line). PKC–membrane dissociation did not correlate with loss of activity

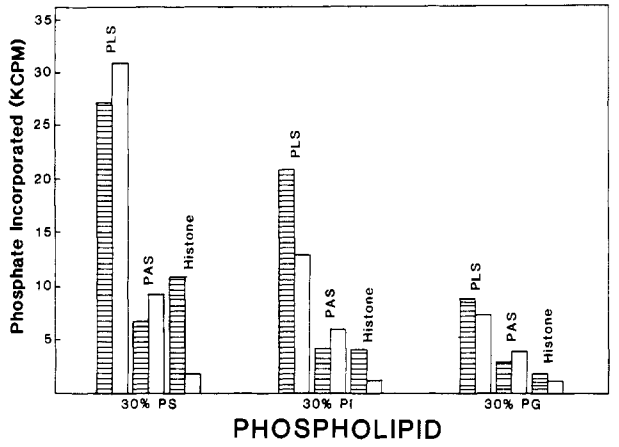

FIGURE 4: Phospholipid selectivity of PKC activation. PKC was assayed by using the indicated substrate in the presence of either 0.5 mM Ca^{2+} (cross-hatched bars) or 2.0 mM EGTA (open bars). The vesicles were composed of 30% of the indicated phospholipid along with 10% DAG and 60% PC. The phospholipid concentration was 480 μg/mL in every case.

in any case. PKC–membrane binding studies were conducted at approximately equal weight concentrations of protein and membrane while the activity measurements contained a large excess of phospholipid. Consequently, dissociation of enzyme from the membrane would require even more NaCl under the conditions of the activity measurement. Thus, inhibition of PKC activity by salt was clearly not the result of dissociation of PKC from the membrane. Loss of activity could be due to loss of aggregation, dissociation of substrate–phospholipid complex, or prevention of substrate–PKC interaction.

Phospholipid Selectivity of PKC Activation. It is well established that PS is the most effective phospholipid in supporting the activity of PKC, while other acidic phospholipids support the activity to a small extent (Kiabuchi et al., 1981; Wise et al., 1982b; Schatzman et al., 1983). Such selectivity has been attributed to a PKC–PS interaction. However, recent studies showed that PKC bound in a similar manner to PS, PI, or PG (Bazzi & Nelsestuen, 1987).

The phospholipid selectivity of PKC activation was examined by using histone, PLS, and PAS as substrates. The results (Figure 4) showed that all substrates displayed selectivity for vesicles containing PS regardless of whether or not Ca^{2+} was needed for activity (see PLS and PAS, Figure 4). Since Ca^{2+} is required for PKC–membrane binding (Wolf et al., 1985a; Bazzi & Nelsestuen, 1987), the preference for PS in the phosphorylations that occurred in the presence of EGTA did not appear to be attributable to a PKC–PS interaction.

In order to acertain whether the PKC (Figure 4) was attached to membranes through the positively charged polypeptides, fluorescence energy-transfer measurements were performed. In the absence of Ca^{2+}, there was no fluorescence energy transfer from PKC to dansyl-PE-containing membranes regardless of whether or not PLS or PAS was present. The limits of detection were less than 5% of the energy transfer observed in the presence of Ca^{2+}. Fluorescence energy transfer was observed only when Ca^{2+} was present in the medium. While it is possible that the phospholipid selectivity of PKC arose from a subtle interaction between PKC and membranes that was not detected by fluorescence energy-transfer measurements, it seemed more plausible that the phospholipid selectivity was the result of some property of substrate–phospholipid interaction. Further work is needed to identify the source of this phospholipid selectivity. However, it was clear that simple PKC–PS interactions were inadequate to explain this phospholipid selectivity.

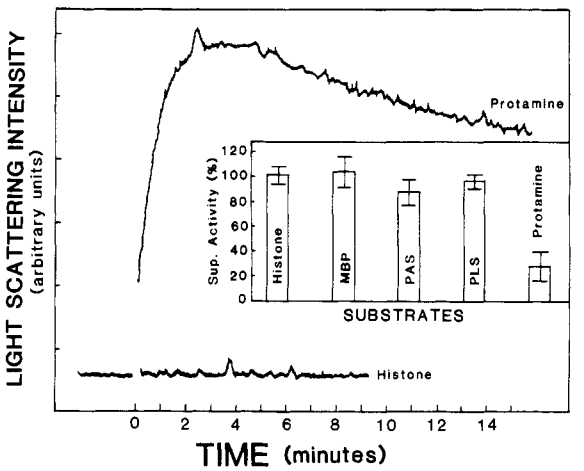

FIGURE 5: Interaction of PKC with substrates. The light-scattering intensity changes of solutions (1.5 mL) containing PKC (2.2 μg) were monitored after addition of 400 μg of protamine or histone. The changes induced by addition of PLS and PAS were very similar to those of histone (not shown). The inset shows activity measurements of the supernatant after centrifugation. PKC (25 ng) was incubated for 5 min (25 °C) with either PLS (54 μg/mL), MBP (96 μg/mL), histone (400 μg/mL), PAS (54 μg/mL), or protamine (400 μg/mL). The mixture was centrifuged for 5 min at 13000g, and the PKC activity remaining in the supernatant was measured as outlined under Experimental Procedures. The average and the standard deviation of four measurements are shown.

Substrate–PKC Interactions. A number of substrates are known to require Ca^{2+}, DAG, and phospholipid for activity including histone III-S (Takia et al., 1977), myosin light chain (Endo et al., 1982), troponin proteins (Katoh et al., 1983), and troponin I (Mazzei & Kuo, 1984). However, it is well-known that PKC phosphorylates protamine in a Ca^{2+}-, DAG-, and phospholipid-independent manner (Takia et al., 1977; Kikkawa et al., 1983). This behavior is even more unusual than that observed here for PLS and PAS and has been considered anomolous. Therefore, we studied the interaction of protamine and other substrates with PKC in order to understand the basis of such diverse selectivity for cofactors.

The data in Figure 5 showed that the addition of protamine to PKC resulted in large increases in the light-scattering intensity of the solution. This suggested extensive aggregation involving multiple copies of protamine associated with PKC. In fact, the light-scattering intensity of the free proteins was undetectable at the sensitivity settings of this experiment. In contrast, addition of histone, myelin basic protein, PLS, or PAS to PKC did not induce significant increases in the light-scattering intensity, indicating the absence of protein aggregation.

To establish that the PKC enzyme itself was present in the aggregates, the solutions were subjected to modest centrifugal forces, and the activity remaining in the supernatant was measured (Figure 5, inset). In the case of protamine, 76% of the activity was lost from the supernatant. All other substrates showed full recovery of activity in the supernatant. Consequently, PKC and protamine underwent mutual aggregation with at least 70% of the aggregates large enough to be sedimented by low-speed centrifugation.

The binding of PKC to other substrates was examined by using the ability of substrate to aggregate phospholipid vesicles. The results (Table I) showed that most of the PKC activity was recovered in the supernatant when histone, troponin complex, troponin I, or myosin light chain was added, indicating that PKC did not bind to any of these substrate–phospholipid aggregates. With PLS, PAS, or protamine, only a small fraction of the PKC activity remained in the super-

Table I: Substrate–PKC Binding and Cofactor Requirements

substrate	concn (μg/mL)	supernatant activity (%)[a]	cofactor requirements		
			Ca^{2+}	DAG	phospholipid
histone	400	81.0 ± 1.6	+	+	+
troponin I	100	95.3 ± 6.0	+	+	+
troponin complex	200	98.1 ± 13.0	+	+	+
myosin light chain	200	97.1 ± 6.5	+	+	+
PLS	56	7.3 ± 1.2	-	*b*	+
PAS	56	4.3 ± 0.5	-	*b*	+
protamine	400	11.4 ± 1.0	-	-	-
MBP	96	18.3 ± 1.0	-	ND[d]	+
MBP	96		27844 (0.5 mM Ca^{2+})[c]		
MBP	96		20311 (2 mM EGTA)[c]		
MBP	96		3234 (-PL)[c]		

[a] PKC was incubated with the indicated substrate at the given concentration in the presence of 480 μg/mL phospholipid vesicles, 10 mM Mg^{2+}, 1 mg/mL BSA, and 0.2 mM EGTA. The reaction mixture was centrifuged for 5 min at 13000*g*, and the activity remaining in the supernatant was compared to that originally present in the mixture. The results are the average and standard deviation of four measurements. [b] DAG caused a low level of activation (<3-fold). [c] Phosphate incorporated (cpm) into substrate under the standard conditions except in the presence and absence of Ca^{2+} and in the absence of phospholipid (-PL). Similar results were obtained with MBP available from a commercial source. [d] Not determined.

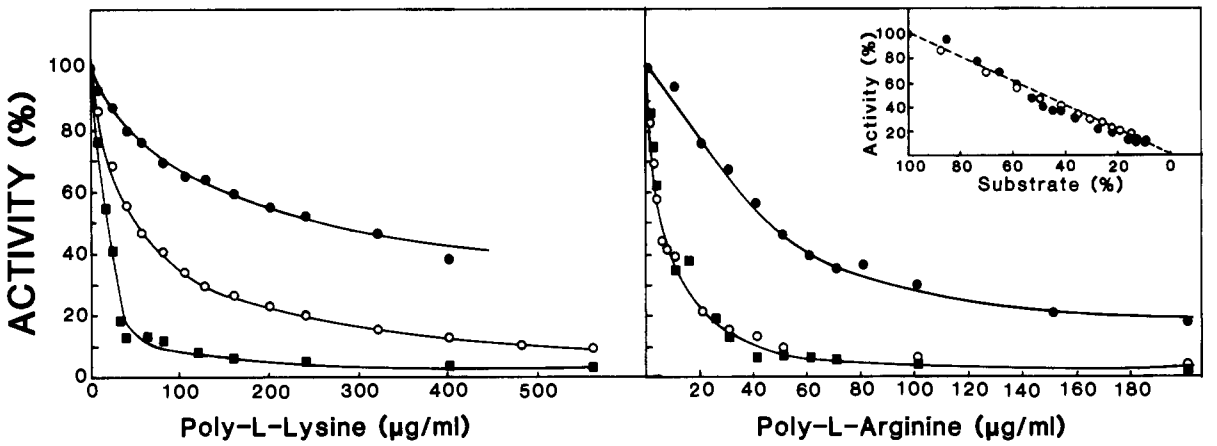

FIGURE 6: Inhibition of PKC by poly(L-lysine) and poly(L-arginine). Inhibition of PKC activity by poly(L-lysine) (left panel) or poly(L-arginine) (right panel) was measured by using PLS (54 μg/mL, ○), PAS (54 μg/mL, ●), or histone (400 μg/mL, ■). The inset in the right panel shows the inhibition of PLS by poly(L-lysine) (○) and PAS by poly(L-arginine) (●) as a percentage of the total peptide in solution: $100S/(S + I)$, where S is the substrate concentration and I is the inhibitor concentration.

natant, indicating that PKC bound to these substrates and sedimented with the substrate–phospholipid complex.

A potentially important correlation was evident in Table I. The substrates which bound PKC to the aggregate did not require Ca^{2+} for phosphorylation, while substrates which required Ca^{2+} for phosphorylation did not show significant enzyme–substrate binding. This correlation suggested a critical role of substrate–enzyme and substrate–phospholipid binding in determination of the cofactor requirements of PKC.

An unexpected result was that PKC bound to myelin basic protein aggregates. Subsequent measurements showed that greater than 70% of the phosphorylation activity was Ca^{2+} independent (Table I). This result contradicted a previous report that PKC phosphorylated myelin basic protein in a Ca^{2+}-dependent manner (Wise et al., 1982a). To test this property further, a partially purified myelin basic protein (available commercially from Sigma Chemical Co.) was tested and found to show a similar high degree of calcium-independent phosphorylation. The reason for the apparent conflict between these results and those reported by Wise et al. (1982a) is not known. Nevertheless, in this study, the properties of myelin basic protein maintained the correlation between substrate–enzyme binding and the lack of Ca^{2+} requirement for phosphorylation.

Inhibition of Phosphorylation by Poly(L-lysine) and Poly(L-arginine). Qi et al. (1983) reported that polyamines and poly(L-arginine) inhibited PKC. The inhibition appeared competitive or noncompetitive to either Ca^{2+} or phospholipid, depending on the polyamine used. Recently, it has been suggested that this inhibition may play a role in insulin secretion under physiological conditions (Thames et al., 1986). As shown above, straightforward interpretations of kinetic data are difficult in reactions involving a complex mixture. Polyamines may exert their influence on substrate interactions. The relationship between substrate and inhibition by poly(L-lysine) and poly(L-arginine) was studied further.

Phosphorylation of PLS, PAS, and histone was carried out by using a constant substrate concentration with varying concentrations of inhibitors. The results (Figure 6, left panel) showed that poly(L-lysine) displayed substrate-dependent inhibition properties. Histone phosphorylation was the most susceptible to inhibition while PAS was the least affected. Phosphorylation of either PLS or PAS was inhibited to a similar extent in the presence of 2.0 mM EGTA or 0.5 mM Ca^{2+}, a result indicating that Ca^{2+} was not directly involved in the mechanism of these latter inhibitions.

Poly(L-arginine) was a more potent inhibitor of PKC phosphorylation than was poly(L-lysine) (Figure 6, right panel) but still showed different inhibition curves for the different substrates. Again, PAS was the least susceptible to inhibition (Figure 6, right panel). However, both of these polypeptide inhibitors aggregated phospholipid vesicles extensively. While accurate quantitation was not possible, the nature of light-scattering intensity changes and the degree of visible turbidity

indicated that poly(L-arginine) was more potent than poly(L-lysine) in aggregating vesicles. Similarly, the NaCl concentration required to inhibit the interaction of PAS with phospholipid was greater than that required for either histone or PLS (see above). Consequently, it is possible that the relative strength of interaction of these inhibitors with the membranes was responsible for the different curves, and inhibition may result from displacement of substrate from the phospholipid or from the enzyme. It seems clear that inhibition was not due to displacement of enzyme from the membrane since that mechanism should show similar inhibition properties for all substrates.

The inhibition of PLS phosphorylation by poly(L-lysine) and PAS phosphorylation by poly(L-arginine) was replotted as a percentage of the total substrate plus inhibitor in the medium (Figure 6, inset of right panel). The plots showed that the activity of PKC toward substrate was nearly linear with respect to the percentage of the substrate present (dashed line in inset). This suggested that PKC had no preference for binding or interacting with either PLS vs. poly(L-lysine) or PAS vs. poly(L-arginine) and that the inhibition was "competitive" with respect to substrate. The complexity of the aggregation process disallowed analysis of the data by normal kinetic equations, and the term competitive is only used to indicate that substrate could overcome the inhibition.

Discussion

An understanding of the PKC activation mechanism requires knowledge of all interactions contributing to appearance of activity. PKC has been shown to bind to membrane vesicles or to Triton-solubilized phospholipids, and this interaction appears to consist of a simple association of protein with the membrane (Hannun et al., 1985; Wolf et al., 1985b; Bazzi & Nelsestuen, 1987). However, binding of PKC (±DAG) to membranes is not sufficient for the development of phosphorylation activity (Bazzi & Nelsestuen, 1987) so that other interactions contribute to the appearance of activity.

The present study indicated that the substrate interacted strongly with the phospholipid vesicles or micelles, and different substrates showed different Ca^{2+}, DAG, and phospholipid requirements for PKC activation. These results have two major implications regarding PKC mechanism and activation studies. First, any proposed mechanism for the activation of PKC must explain the role of substrate in conferring cofactor requirements on such activation. Second, caution must be exercised in mechanistic interpretation of activity studies. Due to aggregation that takes place, conclusions regarding reaction stoichiometries and other refined interpretations are greatly restricted.

The commonly perceived picture of PKC activation consists of the formation of a quaternary complex, PKC–DAG–phospholipid–Ca^{2+}, that exposes the active site of PKC (Ashendel, 1985; Ganong et al., 1985; Parker et al., 1986). However, it is clear that neither Ca^{2+} nor phospholipid is absolutely essential to development of the PKC active site. In fact, there are at least three categories of substrates characterized by different cofactor requirements: (A) those which require no cofactors, such as protamine; (B) those which require only phospholipid, such as PLS, PAS, and myelin basic protein; and (C) those which require all three cofactors such as histone and troponin.

A speculative model for in vitro PKC phosphorylation of substrates is presented in Figure 7. The major event that determines the cofactor requirements for protein phosphorylation is proposed to be presentation of substrate to the active site of the enzyme. Protamine represents category A substrates

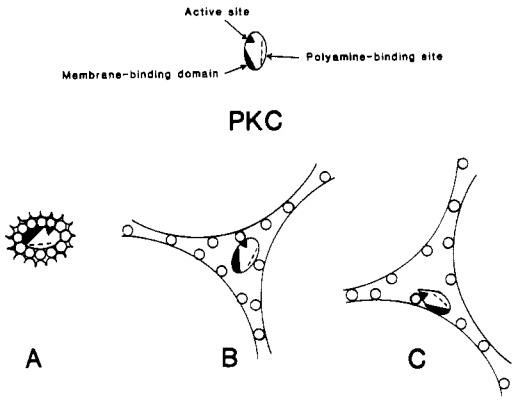

FIGURE 7: Schematic representation of possible in vitro PKC activation complexes. The enzyme and regions representing its different interactions are shown above. The substrate is depicted by the small circles and the membrane by the large curved surfaces. A key requirement is proposed to be presentation of the substrate to the active site of PKC. This may be accomplished by (A) enzyme–substrate aggregation, (B) enzyme binding to substrate–phospholipid aggregates, or (C) enzyme–membrane binding which introduces enzyme into substrate–membrane aggregates. DAG is only required by category C substrates and is proposed to cause a protein conformational change that makes the active site accessible to substrate.

(Figure 7) which bind to and aggregate PKC directly (Figure 5) so that no other cofactor is required. Category B substrates (PLS, PAS, and myelin basic protein) bind PKC but do not aggregate it (Figure 5 and Table I). Addition of phospholipid causes substrate–phospholipid aggregation, thereby delivering a second substrate molecule to the active site of PKC. Category C substrates do not bind PKC (e.g., histones; see Table I), and Ca^{2+} is needed to induce PKC–membrane binding. This binding incorporates PKC into the substrate–membrane complex where it can phosphorylate substrate molecules. Consistent with this model is the observation that PLS and PAS (category B substrates) and several types of histones (category C substrates) showed an optimum concentration for maximum activity rather than a saturation curve. In this model, the density of substrate on phospholipid vesicles, rather than absolute concentration, is an important factor in determining the availability of substrate.

From the available results, DAG is a major cofactor only when Ca^{2+} is needed for phosphorylation (category C substrates). Therefore, the model in Figure 7 was modified to suggest that membrane-bound PKC was unable to interact with substrate due to some factor such as steric hindrance and that DAG caused a conformational change that made the active site accessible. While further studies are clearly needed to understand the in vitro mechanism of PKC in greater detail, the model shown in Figure 7 has the attractive property of accommodating all three substrate categories in a common mechanism.

Aggregation was an unavoidable consequence of substrate addition to phospholipid vesicles or mixed micelles for all substrates tested. In addition to the proteins outlined in this study, less thoroughly studied substrates included bovine brain P2 protein and a synthetic peptide (YSRRRRRRG) which were also found to cause phospholipid vesicle and mixed micelle aggregation.[2] The latter peptide is the best short peptide substrate for PKC (Ferrari et al., 1985). In fact, the general characteristics of a good synthetic peptide substrate, which include several positive charges in close association with serine or threonine residues (Ferrari et al., 1985; Kishimoto et al.,

[2] M. Bazzi and G. L. Nelsestuen, unpublished data.

1985; Turner et al., 1985), also coincide with the predicted ability of the peptide to interact with membranes. Thus, the observed specificity for peptide sequence may represent substrate–phospholipid interaction as well as substrate–enzyme interaction.

The quantitative role of substrate–phospholipid aggregation is not known, but it appears that aggregation is not absolutely essential for phosphorylation.[2] PKC associates with membranes but does not cause aggregation (Bazzi & Nelsestuen, 1987), and it is well-known that PKC catalyzes autophosphorylation (Huang et al., 1986; Kikkawa et al., 1982; LePeuch et al., 1983). Aggregation may change the quantitative aspects of phosphorylation by enhancing the enzyme turnover or the rate of filling the active site of the enzyme. Nonaggregating substrates may simply give low rates of reaction and thereby be classified as poor substrates.

The biological relevance of these in vitro findings is not known. An extreme possibility is that PKC phosphorylates all of the in vitro substrates in an essentially artificial manner relative to the in vivo substrates. However, it is more probable that the in vitro results offer important clues for in vivo phosphorylations. Phosphorylation of substrates by PKC may proceed by two pathways. First, phosphorylation of the substrate could arise from a DAG-generating signal when both PKC and substrate are assembled on membranes (analogous to category C substrates). In this case, substrate–membrane binding and subsequent translocation or lateral diffusion across the membrane may be an effective means of providing substrates to the active site of the enzyme. Alternatively, other proteins may associate with PKC and introduce substrate molecules to the active site (analogous to category A and B substrates). The latter mechanism requires protein cofactors rather than membranes. In fact, preliminary results[2] show that phosphorylation of PLS and PAS, substrates in category B, was promoted by bovine serum albumin. The latter protein caused some aggregation of PLS and PAS. Consequently, category B substrates do not specifically require phospholipids for phosphorylation. The subcellular distribution of PKC in many tissues shows a majority of PKC activity in the cytosol (Anderson et al., 1985; Kuo et al., 1985; Kikkawa et al., 1983).

The aggregation phenomenon imposes substantial limitations on the types of studies and conclusions that can be applied to PKC function. For example, it was found that a minimum of 1 mol % DAG and 4 mol % PS in Triton micelles were required to form an active PKC complex (Hannun et al., 1985, 1986; Ganong et al., 1985). These properties have been interpreted as stoichiometric requirements for PKC–phospholipid binding. However, substrate–micelle aggregation appears to be the dominant feature of these activity measurements, and the observed phospholipid requirement may reflect the concentration needed for substrate–phospholipid interaction rather than PKC–phospholipid binding. Thus, the stoichiometry of the PS/PKC interaction is uncertain and needs to be reexamined if appropriate substrates or conditions can be identified for truely quantitative studies of this type. An additional problem is the observed slow dynamics of aggregation; the materials in the aggregates may not be in equilibrium with solution materials on the time scale of the assay (see Figure 2). Consequently, interpretation of activity titration curves by equilibrium equations such as the double-reciprocal plot, Hill plot, or Scatchard analysis, a common practice, may not be appropriate. Analysis of inhibition of PKC by polyamines, which has been reported to be competitive or noncompetitive when analyzed by standard steady-state kinetic equations (Qu et al., 1983), may suffer from the same difficulties. The results presented here (Figure 6) suggested that polyamines may simply function by displacing the substrate from the phospholipid.

The present studies addressed several factors concerning the nature of the in vitro PKC active complex, especially the role of substrates. The results may explain several features of the active complex. However, a number of in vitro characteristics of PKC activation are not explained by this model. For example, PKC displayed selectivity for PS-containing vesicles regardless of whether or not the enzyme was membrane bound (Figure 4). This selectivity did not appear to be related to PKC–membrane binding. In addition, the role of DAG in altering the cofactor requirements of PKC activation is poorly understood. DAG reduced the Ca^{2+} and phospholipid requirements of PKC activation when histone was used as substrate, and there was a strong "synergistic effect" among the various cofactors (Hannun et al., 1986; Bazzi & Nelsestuen, 1987; Wolf et al., 1985b). However, with random copolymers (PLS and PAS) as substrates, DAG had a small positive effect on activity but did not alter the phospholipid requirements (Figure 2). Further work is needed to fully understand the in vitro mechanism of protein kinase C activation.

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Adenosine Kinase from Human Erythrocytes: Kinetic Studies and Characterization of Adenosine Binding Sites†

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ABSTRACT: The reaction catalyzed by adenosine kinase purified from human erythrocytes proceeds via a classical ordered sequential mechanism in which adenosine is the first substrate to bind to and AMP is the last product to dissociate from the enzyme. However, the interpretation of the steady-state kinetic data is complicated by the finding that while AMP acts as a classical product inhibitor at concentrations greater than 5 mM, at lower concentrations AMP can act as an apparent activator of the enzyme under certain conditions. This apparent activation by AMP is proposed to be due to AMP allowing the enzyme mechanism to proceed via an alternative reaction pathway that avoids substrate inhibition by adenosine. Quantitative studies of the protection of the enzyme afforded by adenosine against both spontaneous and 5,5′-dithiobis(2-nitrobenzoic acid)-mediated oxidation of thiol groups yielded "protection" constants (equivalent to enzyme–adenosine dissociation constant) of 12.8 μM and 12.6 μM, respectively, values that are more than an order of magnitude greater than the dissociation constant (K_{ia} = 0.53 μM) for the "catalytic" enzyme–adenosine complex. These results suggest that adenosine kinase has at least two adenosine binding sites, one at the catalytic center and another quite distinct site at which binding of adenosine protects the reactive thiol group(s). This "protection" site appears to be separate from the nucleoside triphosphate binding site, and it also appears to be the site that is responsible for the substrate inhibition caused by adenosine.

Although adenosine kinase (EC 2.7.1.20) has been partially purified from several different sources (Caputto, 1951; Lindberg et al., 1967; Schnebli et al., 1967; Murray, 1968; Divekar & Hakala, 1971; Henderson et al., 1972; Shimizu et al., 1972; Namm & Leader, 1974; DeJong, 1977; Andres & Fox, 1979) and purified to apparent homogeneity from brewers' yeast (Leibach et al., 1971), rabbit liver (Miller et al., 1979), rat brain (Yamada et al., 1980), murine leukemia L1210 cells (Chang et al., 1980) and human liver (Yamada et al., 1981) and many of its properties have been studied, there remains some uncertainty regarding the mechanism of the reaction catalyzed by this enzyme. Kinetic studies of the enzyme from human placenta (Palella et al., 1980) and from Ehrlich ascites tumor cells (Henderson et al., 1972) originally indicated that the reaction sequence proceeded via an ordered sequential mechanism, but these two studies disagreed on the order of binding of adenosine and $MgATP^{2-}$ to the enzyme. In a more recent study, Chang et al. (1983) have suggested that adenosine kinase purified from murine leukemia L1210 cells catalyzes the phosphorylation of adenosine via a two-site ping-pong mechanism. While it is possible that the enzyme from different sources may catalyze the kinase reaction via different reaction mechanisms, it is much more likely that these differences result from difficulties in the interpretation of the kinetic data. These difficulties arise largely from the properties of the enzyme such as its lability in dilute solution, the substrate inhibition caused by adenosine, the inhibition caused by excess free magnesium ion, and the interrelationships between some of these properties and the pH of the assay.

The lability of adenosine kinase was first reported by Caputto (1951) during initial attempts to purify the enzyme from

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