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Kinetic Analysis of Protein Kinase C: Product and Dead-End Inhibition Studies Using ADP, Poly(L-lysine), Nonhydrolyzable ATP Analogues, and Diadenosine Oligophosphates†

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ABSTRACT: The kinetic mechanism of protein kinase C (PKC) was analyzed via inhibition studies using the product MgADP, the nonhydrolyzable ATP analogue adenosine 5′-(β,γ-imidotriphosphate) (MgAMPPNP), the peptide antagonist poly(L-lysine), and several naturally occurring ATP analogues that are produced in rapidly growing cells, i.e., the diadenosine oligophosphates (general structure: Ap_nA; n = 2-5). By use of histone as the phosphate acceptor, the inhibition of PKC by MgAMPPNP and MgADP was found to be competitive vs MgATP (suggesting that these compounds bind to the same enzyme form), whereas their inhibition vs histone was observed to be noncompetitive. In contrast, the inhibition by poly(L-lysine) appeared competitive vs histone but uncompetitive vs MgATP, which is consistent with a model wherein MgATP binding promotes the binding of poly(L-lysine) or histone. With the diadenosine oligophosphates, the degree of PKC inhibition was found to increase according to the number of intervening phosphates. The diadenosine oligophosphates Ap_4A and Ap_5A were the most effective antagonists of PKC, with Ap_5A being approximately as potent as MgADP and MgAMPPNP. However, as opposed to MgADP and MgAMPPNP, Ap_4A and Ap_5A appear to act as noncompetitive inhibitors vs both MgATP and histone, suggesting that they can interact at several points in the reaction pathway. These studies support the concept of a steady-state mechanism where MgATP binding preferentially precedes that of histone, followed by the release of phosphorylated substrate and MgADP. Furthermore, these results indicate a differential interaction of the diadenosine oligophosphates with PKC, when compared to other adenosine nucleotides.

Protein kinase C (PKC) consists of a family of homologous calcium- and phospholipid-dependent enzymes that have been extensively characterized with respect to their role in cellular regulation (Nishizuka, 1986, 1988). PKC has been implicated in mediating hormone signal transduction, in modulating gene expression, and in controlling cellular growth and differentiation (Nishizuka, 1986, 1988). Because of the importance of PKC in these processes, many investigations have focused on its structure (Coussens et al., 1986; Knopf et al., 1986; Makowske et al., 1986; Housey et al., 1987; Ohno et al., 1987; Ono et al., 1988), as well as its regulation and physical properties (Nishizuka, 1986, 1988; Bazzi & Nelsestuen, 1987; Huang et al., 1988; Leach et al., 1988; Kaibuchi et al., 1989; Lowndes et al., 1990; Hannun & Bell, 1990).

In general, PKC phosphorylates substrates on serine/threonine residues located near basic amino acids, a process that usually requires calcium and phospholipid (Turner et al.,

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1985; Nishizuka, 1986, 1988; Bazzi & Nelsestuen, 1987; House et al., 1987). PKC can also be activated by certain diacylglycerols that are produced as the result of hormone-stimulated phosphoinositide (Nishizuka, 1986, 1988; Bell, 1986) or phosphatidylcholine hydrolysis (Exton, 1990). This activation by diacylglycerols can be mimicked by phorbol esters (Castagna et al., 1982), agents which have been useful for studies requiring PKC activation in intact cells. Conversely, several inhibitors of PKC have been identified that may act as physiological, pharmacological, or toxicological affectors, e.g., sphingosine (Hannun & Bell, 1989), staurosporine (Tamaoki et al., 1986), nucleoside analogues (Loomis & Bell, 1988), DNA intercalators (Hannun et al., 1989), aminoacridines (Hannun & Bell, 1988), and certain heavy metals (Spelzer et al., 1989).

Previous studies on the kinetics of PKC have indicated that in the absence of allosteric activators the enzyme is inhibited by an intrinsic pseudosubstrate domain that acts as a potent competitive inhibitor vs exogenous substrates (House & Kemp, 1987). In the presence of sufficient levels of calcium and phospholipid, the pseudosubstrate domain is displaced, and the enzyme is activated because its catalytic site appears to now be available to exogenous substrates. However, the specific kinetic mechanism of PKC following its activation by calcium and phospholipid is presently unclear. In this regard, recent initial velocity investigations using a PKC catalytic fragment (to avoid substrate inhibition at high substrate levels) have indicated a sequential rather than a ping-pong mechanism (Hannun & Bell, 1990), although the precise order of substrate binding and product release was not determined. In these studies, the double-reciprocal plots of the initial velocity data, obtained at nonsaturating levels of both substrates, intersected above the $1/[S]$ axis and off the $1/v$ axis. These results exclude a rapid equilibrium-ordered model but still allow for either a random mechanism or a steady-state mechanism wherein substrate addition occurs with some degree of preferred order.

To distinguish between these two possibilities, we have performed product and dead-end inhibition analyses using the product MgADP, the nonhydrolyzable ATP analogue adenosine-5′-(β,γ-imidotriphosphate) (MgAMPPNP), the peptide antagonist poly(L-lysine), and several naturally occurring ATP analogues that are produced in rapidly growing cells, i.e., the diadenosine oligophosphates (general structure: Ap_nA; n = 2–5) (Rapaport & Zamecnik, 1976; Zamecnik, 1983; Varshavsky, 1983). Ap_4A and Ap_5A have been reported to inhibit the protein-tyrosine kinase activity of $pp60^{src}$ (Maness et al., 1983), which is the transforming protein of the Rous sarcoma virus, but they do not appear to be antagonists of the cAMP- and cGMP-dependent protein kinases (Maness et al., 1983).

In this paper, we present evidence supporting a steady-state model (at 10 mM Mg^{2+}, 30 °C, pH 7.4) wherein MgATP binding preferentially precedes that of histone, followed by the apparent ordered release of products. In addition, we show that Ap_4A and Ap_5A, but not Ap_2A and Ap_3A, are effective inhibitors of PKC. The mode of PKC inhibition by Ap_4A and Ap_5A appears different from that observed with other adenosine nucleotides (i.e., MgADP and MgAMPPNP), suggesting that they are not simply acting as product or ATP-like dead-end inhibitors.

Materials and Methods

Materials. [γ-^{32}P]ATP was purchased from Amersham Corp. (Arlington Heights, IL). Na_2ADP, $Li_4AMPPNP$, $NaAp_2A$, NH_4Ap_3A, NH_4Ap_4A, $LiAp_5A$, NaATP, histone (type III-S), phosphatidylserine, poly(L-lysine) hydrobromide (M_r ~3800), and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO).

Protein Kinase C Purification. Protein kinase C was purified from rat brain as previously detailed (Walton et al., 1987). Briefly, the purification entailed three chromatographic steps: (i) ion exchange by DEAE-cellulose, (ii) hydrophobic interaction with phenyl-Sepharose, and (iii) affinity purification with protamine–agarose. Primarily α and β forms were purified as evidenced by sequence analysis and antibody reactivity (Walton et al., 1987; Woodgett & Hunter, 1987).

Protein Kinase C Assay. Unless otherwise stated, the enzyme activity was assayed in a 60-μL reaction buffer consisting of 20 mM HEPES buffer, pH 7.4, 100 μg/mL phosphatidylserine, 1 mM $CaCl_2$, 1 mM dithiothreitol, 10 mM $MgCl_2$, 10–100 ng of protein, and the indicated concentrations of inhibitors, histone, and [γ-^{32}P]ATP [sp act. ca. (0.3–2) × 10^4 cpm/pmol]. The samples were preincubated at 30 °C for 2 min, and the assays were initiated by the addition of $MgCl_2$/ATP as previously described (Walton et al., 1987). After 2 min, the reactions were terminated by the addition of 77 mM phosphoric acid (final concentration) and cooling to 0 °C. Phosphate incorporation into histone was measured by spoting an aliquot of the reaction mixture on phosphocellulose paper, rinsing several times with dilute phosphoric acid, drying, and counting for adsorbed radioactivity (Walton et al., 1987).

Kinetic Analysis. Enzyme kinetic data conforming to linear inhibitions, as determined by secondary replots of the slopes and/or intercepts from initial double-reciprocal plots ($1/v$ vs $1/A$) vs inhibitor concentration, were fitted to eqs 1–3 corresponding to competitive, uncompetitive, and noncompetitive inhibition models (Cleland, 1979; Greco et al., 1982):

$$v = VA/[K(1 + I/K_{islope}) + A] \quad (1)$$

$$v = VA/[K + A(1 + I/K_{iintercept})] \quad (2)$$

$$v = VA/[K(1 + I/K_{islope}) + A(1 + I/K_{iintercept})] \quad (3)$$

In these equations, V is the maximum velocity, K is the Michaelis constant for the varied substrate, A is the concentration of the varied substrate, I is the concentration of the inhibitor, and K_{islope} and $K_{iintercept}$ are the slope and intercept inhibition constants, respectively. Initial parameter estimates were obtained by a weighted multiple linear regression analysis using the reciprocal form of the appropriate equation (Cleland, 1979; Greco et al., 1982). A nonlinear regression routine was used to calculate the final parameter estimates (Greco et al., 1982). The best fit of the data to a given model was assumed on the basis of the least residual mean square value and on estimates of the kinetic parameters not encompassing zero.

Results

Product and Dead-End Inhibition Studies Using MgADP, MgAMPPNP, and Poly(L-lysine). Previous investigations on the kinetics of histone phosphorylation by PKC have indicated a sequential mechanism (Hannun & Bell, 1990), although it is unclear whether substrate addition and product release are random or exhibit some degree of order. To assess which of these mechanisms is involved, we utilized MgADP as a product inhibitor and MgAMPPNP and poly(L-lysine) as dead-end inhibitors and evaluated their mode of inhibition vs MgATP and histone. As shown in Table I, both MgADP and MgAMPPNP were competitive inhibitors vs MgATP and noncompetitive inhibitors vs histone. These patterns are consistent with either a steady-state mechanism that exhibits a preferential order of substrate addition [i.e., MgATP binds to the enzyme first and MgADP is released last (since they

Table I: Estimated Kinetic Constants for the PKC Inhibition by MgADP, MgAMPPNP, and Poly(L-lysine) with MgATP and Histone as the Varied Substrates[a]

varied substrate	inhibitor[b]	inhibition pattern	K_{islope}	$K_{iintercept}$
MgATP[c]	MgADP	competitive	73 ± 6 μM	
histone[d]	MgADP	noncompetitive	235 ± 36 μM	59 ± 13 μM
MgATP[c]	MgAMPPNP	competitive	77 ± 14 μM	
histone[d]	MgAMPPNP	noncompetitive	475 ± 64 μM	514 ± 81 μM
MgATP[e]	poly(L-lysine)	uncompetitive		359 ± 29 μg/mL
histone[f]	poly(L-lysine)	competitive	269 ± 27 μg/mL	

[a] All assays were carried out for 2 min at 30 °C with 10–100 ng of purified rat brain PKC as described under Materials and Methods. All samples were assayed in duplicate, and analogous results were obtained in two to four different experiments. [b] [MgADP] = 0, 100, 200, and 500 μM when either MgATP or histone was the varied substrate. [MgAMPPNP] = 0, 150, 450, and 1500 μM when histone was the varied substrate and 0, 100, 300, and 1000 μM when MgATP was the varied substrate. [poly(L-lysine)] = 0, 200, and 300 μg/mL when histone was the varied substrate and 0, 150, and 250 μg/mL when MgATP was the varied substrate. [c] The reactions contained 50 μg/mL histone and 2, 3, 5, and 20 μM [γ-^{32}P]ATP. [d] The assays consisted of 5 μM [γ-^{32}P]ATP and 20, 30, 50, and 200 μg/mL histone. [e] The reactions contained 20 μg/mL histone and 2, 3, 5, 10, and 20 μM [γ-^{32}P]ATP. [f] The assays consisted of 5 μM [γ-^{32}P]ATP and 20, 30, 50, and 100 μg/mL histone.

both compete for the same enzyme for)] or a random mechanism wherein the binding of the adenosine nucleotides is largely overlapping. A pathway that has histone preferentially binding prior to MgATP is excluded because in such a model MgAMPPNP would be expected to be an uncompetitive inhibitor vs histone and a competitive inhibitor vs MgATP, whereas MgADP would be predicted to be a noncompetitive inhibitor vs MgATP.

To distinguish between the steady-state mechanism wherein MgATP binding tends to precede that of histone vs a totally random mechanism where there is a relatively equal probability of either substrate binding first, it would be useful to have a competitive dead-end inhibitor vs histone. Such an inhibitor would be expected to be uncompetitive vs MgATP in the first model but noncompetitive vs MgATP in the second model. Earlier studies have indicated that both poly(L-lysine) and poly(L-arginine) are potent inhibitors of PKC phosphorylation of histone (Bazzi & Nelsestuen, 1987). However, arginine-rich compounds such as protamine can interact with PKC in the absence of calcium and phospholipid (Walton et al., 1987; Bazzi & Nelsestuen, 1987), suggesting that they may bind to multiple sites on the enzyme. Conversely, the phosphorylation of lysine-rich substrates such as histone is very calcium and phospholipid dependent (Takai et al., 1979), suggesting that they may interact primarily at the enzyme active site. Furthermore, poly(L-arginine) is more potent at aggregating phospholipid vesicles than poly(L-lysine) (Bazzi & Nelsestuen, 1987), and thus it is more likely to lead to multiple forms of inhibition. Because of these considerations, we chose to explore the mechanism of PKC inhibition using a small oligomer of poly(L-lysine) (HBr salt; M_r <4000), since this compound appears to be more specific for interaction at the enzyme active site.

The data in Table I reveal that the PKC inhibition by poly(L-lysine) is competitive vs histone and uncompetitive vs MgATP. However, at relatively high doses (>300 μg/mL), the inhibition by poly(L-lysine) appears mixed (data not shown), probably becaue of nonspecific effects such as binding to secondary sites on the enzyme and/or the induction of phospholipid aggregation (Bazzi & Nelsestuen, 1987). It is impotant to note that the uncompetitive inhibition of poly(L-lysine) vs MgATP is analogous to that obtained by inhibition studies using a synthetic peptide corresponding to the PKC pseudosubstrate domain, i.e., the pseudosubstrate peptide is also an uncompetitive inhibitor vs MgATP and a competitive inhibitor vs peptide substrates (House & Kemp, 1987). Because uncompetitive inhibition indicates that the varied substrate induces the enzyme form to which the inhibitor combines, these data are consistent with a steady-state model wherein MgATP binding generates the enzyme form to which

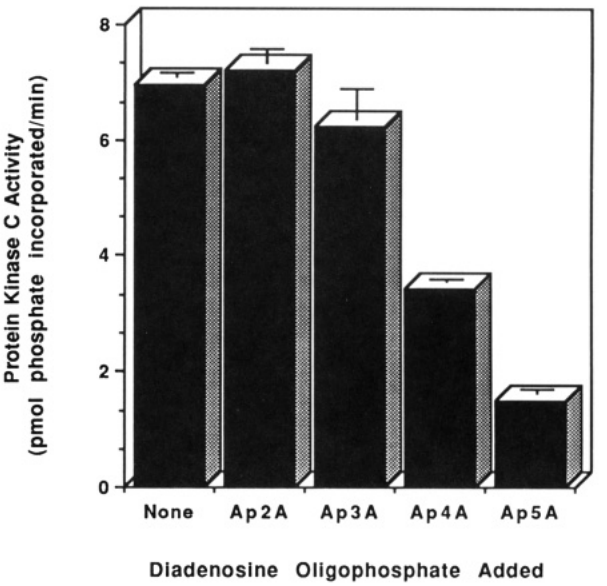

FIGURE 1: Influence of the diadenosine oligophosphates on PKC activity. Purified rat brain PKC (0.1 μg) was assayed for protein kinase activity as detailed under Materials and Methods. Each sample contained 1 μM [γ-^{32}P]ATP, 50 μg/mL histone, and 1 mM concentration of the indicated diadenosine oligophosphates. The data are shown as the average of duplicate determinations (± ranges), and similar results were obtained in two different experiments.

poly(L-lysine), histone, and the pseudosubstrate domain associate. That is, there appears to be a preferential order of substrate addition, rather than a totally random mechanism where either substrate can bind first with comparable affinity/probability.

Kinetic Analyses of PKC Inhibition by Diadenosine Oligophosphates. Because the diadenosine oligophosphates have been shown to be associated with cellular proliferation (Rapaport & Zamecnik, 1976; Zamecnik, 1983) and may act to inhibit pp60^{src} but not the cAMP- and cGMP-dependent protein kinases (Maness et al., 1983), it was of interest to assess the influence of these compounds on PKC activity. As shown in Figure 1, Ap_4A and Ap_5A were the most potent inhibitors of PKC, whereas Ap_2A and Ap_3A had little effect. In general, the relative potency of the diadenosine oligophosphates increased with the number of intervening phosphates.

Given that Ap_4A and Ap_5A were the most effective of the diadenosine oligophosphate antagonists of PKC, they were further analyzed with respect to their mechanism of inhibition vs MgATP (the substrate they most resemble) and histone. As illustrated in Table II, the inhibition by Ap_4A and Ap_5A is noncompetitive vs both MgATP and histone. A comparison of the kinetic constants estimated from these studies indicates

Table II: Estimated Kinetic Constants for the PKC Inhibition by Ap_4A and Ap_5A with MgATP and Histone as the Varied Substrates[a]

varied substrate	inhibitor	inhibition pattern	K_{islope} (μM)	$K_{iintercept}$ (μM)
MgATP[b]	Ap_4A	noncompetitive	356 ± 54	409 ± 85
histone[c]	Ap_4A	noncompetitive	772 ± 222	62 ± 13
MgATP[b]	Ap_5A	noncompetitive	73 ± 12	448 ± 214
histone[c]	Ap_5A	noncompetitive	229 ± 44	41 ± 16

[a] All assays were carried out for 2 min at 30 °C with 10–20 ng of purified rat brain PKC as described under Materials and Methods. When Ap_4A was used as the inhibitor, its concentration was set at 0, 30, 100, and 300 μM, whereas when Ap_5A was used as the inhibitor its concentration was set at 0, 30, 100, and 250 μM. All samples were assayed in duplicate, and analogous results were obtained in two different experiments. [b] The reactions contained 50 μg/mL histone and 2, 3, 5, and 20 μM [γ-^{32}P]ATP. [c] The assays were performed with 5 μM [γ-^{32}P]ATP and 20, 30, 50, and 200 μg/mL histone.

that Ap_5A is a higher affinity PKC inhibitor than Ap_4A (compare K_{islope} values in Table II) and that its potency is somewhat comparable to that determined for MgADP and MgAMPPNP (Table I).

The fact that Ap_4A and Ap_5A are structurally similar to ATP, and yet are not strictly competitive inhibitors vs ATP, suggested that they may interact at several points in the reaction pathway. Thus, the nature of the inhibition by Ap_4A and Ap_5A vs phosphatidylserine was evaluated because the binding of the negatively charged phospholipid may be affected by the negatively charged polyphosphate domain on the diadenosine nucleotides. As shown in Figure 2, Ap_4A and Ap_5A appear as noncompetitive inhibitors vs phosphatidylserine, since an analysis of the PKC inhibition data by these compounds yields intersecting lines on both Dixon and Cornish–Bowden plots (Dixon & Webb, 1979).

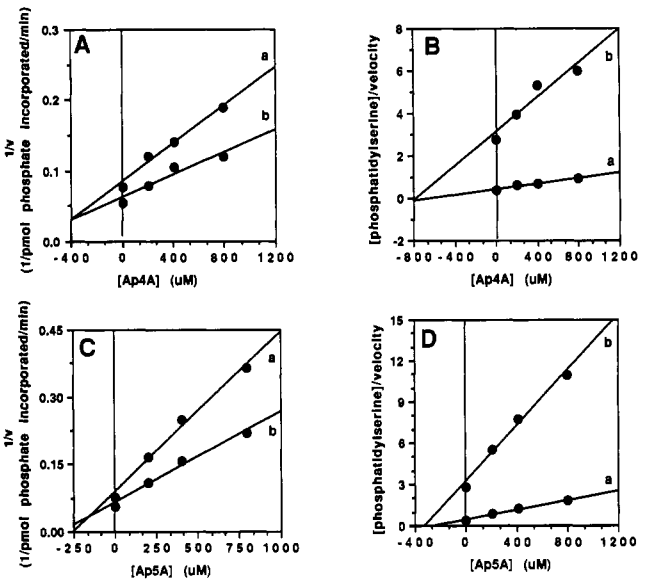

FIGURE 2: Influence of phosphatidylserine on the PKC inhibition by Ap_4A and Ap_5A. The assays were performed as described under Materials and Methods except that the incubations contained 5 μM [γ-^{32}P]ATP, 50 μg/mL histone, ~15 ng of purified rat brain PKC, and the following concentrations of phosphatidylserine: (a) 5 μg/mL; (b) 50 μg/mL. The results are the average of duplicate determinations, and similar results were obtained in two different experiments. (A) Dixon plot of the PKC inhibition by Ap_4A. (B) Cornish–Bowden replot of the Ap_4A inhibition data shown in (A). (C) Dixon plot of the PKC inhibition by Ap_5A. (D) Cornish–Bowden replot of the Ap_5A inhibition data presented in (C).

Discussion

Previous investigations of the kinetic properties of protein kinase C have suggested a sequential mechanism of substrate addition but have excluded a rapid equilibrium-ordered model (Hannun & Bell, 1990). However, the data from these initial velocity studies were still compatible with either a random mechanism, where there is no specific order of substrate addition, or a steady-state mechanism wherein there exists some degree of preferential order of substrate binding and product release. The results presented here indicate a kinetic mechanism for PKC where MgATP binding preferentially precedes that of histone, followed by catalysis and the apparent ordered release of products such that phosphorylated protein appears released first followed by MgADP.

This order of substrate binding is supported by the observations that the dead-end inhibitors poly(L-lysine) (this study) and the pseudosubstrate peptide (House & Kemp, 1987) are competitive inhibitors vs protein substrates but appear as uncompetitive inhibitors vs MgATP. Because uncompetitive inhibition indicates that the varied substrate promotes the binding of the inhibitor, these inhibition patterns are consistent with the model that MgATP helps generate the form of the enzyme to which histone, poly(L-lysine), or the pseudosubstrate peptide preferentially combines. Furthermore, the reverse order of addition, i.e., histone first followed by MgATP, is excluded on the basis of the fact that MgAMPPNP was observed to be a competitive inhibitor vs MgATP but a noncompetitive inhibitor vs histone. If histone bound first, followed by MgATP binding, then a competitive inhibitor (i.e., MgAMPPNP) vs MgATP would be expected to be an uncompetitive inhibitor vs histone. Finally, possible ordered product release is suggested by the observation that MgADP is a competitive inhibitor vs MgATP, supporting the concept that MgADP and MgATP bind to the same enzyme form. If MgADP was released before the phosphorylated substrate, then its inhibition would be predicted to be noncompetitive vs both MgATP and histone.

It should be noted that the present data do not rule out the possibility of some degree of randomness in the PKC kinetic mechanism. Rather, they suggest that under the present assay conditions (10 mM Mg^{2+}, 30 °C, pH 7.4) there is a preferred order of substrate addition. Interestingly, a similar mechanism has been proposed for the cAMP-dependent protein kinase (Kong & Cook, 1988). In the case of the cAMP-dependent protein kinase, several studies have provided evidence for an apparent ordered addition of substrates and release of products (Whitehouse & Walsh, 1983; Whitehouse et al., 1983). Further analyses using isotope partitioning have shown that although the reaction can proceed in a steady-state random fashion, there is a strong preference for MgATP binding to the enzyme first in the presence of high or physiological Mg^{2+} levels (Kong & Cook, 1988).

In addition to evaluating the kinetics of PKC using the adenosine nucleotides MgADP and MgAMPPNP, we have also explored the influence of several naturally occurring ATP analogues that appear to accumulate to micromolar levels during cellular proliferation (Rapaport & Zamecnik, 1976; Zamecnik, 1983) or following physiological stress (Varshavsky, 1983; Baker et al., 1987), i.e., the diadenosine oligophosphates. Diadenosine 5′,5‴-P^1,P^4-tetraphosphate (Ap_4A) is the major diadenosine oligophosphate identified in mammalian cells (Rapaport & Zamecnik, 1976; Zamecnik, 1983), and it has also been shown to occur at concentrations 50–1000-fold higher in rapidly growing cells vs arrested cells (Rapaport & Zamecnik, 1976). Although the mechanism of Ap_4A action is unclear, it is possible that it is involved in the modulation of cellular protein kinases. In this regard, Ap_4A and Ap_5A have been reported to inhibit the protein-tyrosine kinase activity

of pp60^{src} (Maness et al., 1983), which is the transforming protein of the Rous sarcoma virus, but they do not appear to be antagonists of the cAMP- and cGMP-dependent protein kinases (Maness et al., 1983).

In this paper, we have shown that two of these compounds, Ap_4A and Ap_5A, can serve as effective inhibitors of PKC. The inhibition is noncompetitive vs MgATP, histone, and phosphatidylserine, indicating that these compounds probably do not work by simply competing with MgATP or phosphatidylserine binding but rather they may be acting at multiple points in the reaction pathway. One possibility is that because the diadenosine oligophosphates are quite bulky when compared to MgATP or MgADP, their association with the enzyme not only blocks MgATP binding but may alter histone binding as well. In addition, it is conceivable that, given their large negative charge, they may also interact at other sites on the enzyme or directly with the positively charged substrate (histone) or calcium. These types of enzyme antagonism would be expected to create a mixed inhibition and thereby contribute to the generation of a noncompetitive inhibition pattern.

The present studies also indicate that the degree of PKC inhibition by these diadenosine nucleotides increases with the number of intervening phosphates. That is, with MgATP as the varied substrate, Ap_5A exhibits a lower K_{islope} [which is indicative of the dissociation constant of the inhibitor (Cleland, 1977)] relative to that measured for Ap_4A, whereas both inhibitors yield comparable $K_{iintercept}$ values. These observations are consistent with the concept that the greater the distance between the two adenosine moieties, the more efficiently the diadenosine nucleotide can associate with PKC and thus compete with MgATP binding, perhaps due to the attenuation of certain steric constraints. Although the MgATP binding domain in the PKC family shares significant homology with the cAMP- and cGMP-dependent protein kinases (Coussens et al., 1986; Knopf et al., 1986; Makowske et al., 1986; Housey et al., 1987; Ohno et al., 1987), the apparent differential sensitivity of these various kinases to inhibition by Ap_4A and Ap_5A (Maness et al., 1983; this paper) would suggest some divergent features within this region.

In terms of possible biological action, if sufficient elevations in diadenosine oligophosphates are achieved during rapid cell growth or following stress, then they may function, at least in part, by attenuating the negative feedback effects of PKC and other kinases on certain growth-promoting pathways, e.g., the epidermal growth factor receptor system (Cochet et al., 1984; Bertics et al., 1985). However, the level of Ap_4A necessary for substantial inhibition of PKC appears higher than the levels reported for mammalian cells in vivo ($\sim$5 μM) (Rapaport & Zamecnik, 1976). Still, it has been noted that Ap_4A is highly labile, and it exact site of synthesis is unclear (Rapaport & Zamecnik, 1976; Zamecnik, 1983). Thus, higher local concentrations of these compounds might be attained within the cell, when compared to that estimated in whole extracts, thereby allowing for their possible action as transient regulators of protein kinases such as PKC.

In sum, we have characterized the inhibition of PKC by the product inhibitor MgADP, as well as by the dead-end inhibitors MgAMPPNP, poly(L-lysine), and the diadenosine oligophosphates. These data, together with previous work, suggest a steady-state kinetic mechanism for PKC wherein there is a preferential order of substrate addition and product release.

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Supplementary Material Available

Analysis of the inhibition data of Tables I and II presented as PKC inhibition plots, slope replots, and intercept replots of MgADP vs histone, MgAMPPNP vs histone, Ap_4A vs MgATP, Ap_4A vs histone, Ap_5A vs MgATP, and Ap_5A vs histone, PKC inhibition plots and slope replots of MgADP vs MgATP, MgAMPPNP vs MgATP, and poly(L-lysine) vs histone, and PKC inhibition plot and intercept replot of poly(L-lysine) vs MgATP (11 pages). Ordering information is given on any current masthead page.

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Steady-State and Pre-Steady-State Kinetics of Propionaldehyde Oxidation by Sheep Liver Cytosolic Aldehyde Dehydrogenase at pH 5.2. Evidence That the Release of NADH Remains Rate-Limiting in the Enzyme Mechanism at Acid pH Values

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ABSTRACT: The k_{cat} value for the oxidation of propionaldehyde by sheep liver cytosolic aldehyde dehydrogenase increased 3-fold, from 0.16 s^{-1} at pH 7.6 to 0.49 s^{-1} at pH 5.2, in parallel with the increase in the rate of displacement of NADH from binary enzyme·NADH complexes. A burst in nucleotide fluorescence was observed at all pH values consistent with the rate of isomerization of binary enzyme·NADH complexes constituting the rate-limiting step in the steady state. No substrate activation by propionaldehyde was observed at pH 5.2, but the enzyme exhibited dissociation/association behavior. The inactive dissociated form of the enzyme was favored by low enzyme concentration, low pH, and low ionic strength. Propionaldehyde protected the enzyme against dissociation.

MacGibbon et al. (1977a,b,c) established that sheep liver cytosolic aldehyde dehydrogenase follows a compulsory order mechanism with NAD^+ as the leading substrate (Scheme I) at pH 7.6, and this was also found to be the case at pH 7.0 (Hart & Dickinson, 1982; Dickinson, 1985). In Scheme I, *E·NADH represents a conformationally rearranged form of the enzyme which must isomerize before NADH can be released from its binding site. Blackwell et al. (1987) have shown that the two-step release of NADH from the enzyme (Scheme I, eq 5) is rate-limiting in the mechanism for the oxidation of propionaldehyde at pH 7.6.

Scheme I

$$E + NAD^+ \rightleftharpoons E\cdot NAD^+ \quad (1)$$

$$E\cdot NAD^+ + \text{aldehyde} \rightleftharpoons E\cdot NAD^+\cdot\text{aldehyde} \quad (2)$$

$$E\cdot NAD^+\cdot\text{aldehyde} \rightleftharpoons E\cdot NADH\cdot\text{acyl} \quad (3)$$

$$E\cdot NADH\cdot\text{acyl} \rightarrow {}^*E\cdot NADH + \text{acid} \quad (4)$$

$$^*E\cdot NADH \rightleftharpoons E\cdot NADH \rightleftharpoons E + NADH \quad (5)$$

At high concentrations of propionaldehyde, a 3-fold activation of the steady-state rate occurs, and this has been explained on the basis of an increase in the rate of NADH release caused by binding of the aldehyde to the enzyme·NADH complexes (Dickinson, 1985). Studies with *trans*-4-(*N*,*N*-dimethylamino)cinnamaldehyde (DACA) (Buckley & Dunn, 1982; Dunn & Buckley, 1985) and with *trans*-cinnamaldehyde (Dickinson & Haywood, 1986) suggest that at acidic pH values acyl-enzyme hydrolysis is slow and thus becomes the rate-limiting step in the mechanism. The limited solubility of both *trans*-cinnamaldehyde and DACA restricts the concentration range over which kinetic measurements can be made with these substrates. No such problems are encountered when propionaldehyde is used as a substrate for the enzyme. The purpose of this study was therefore to investigate the mechanism of sheep liver cytosolic aldehyde dehydrogenase at low pH values using propionaldehyde as a substrate, to determine whether acyl-enzyme hydrolysis is the rate-limiting step in the oxidation for aliphatic aldehydes.

Experimental Procedures

Materials. NADH (grade III) and NAD^+ (grade III) were purchased from Sigma Chemical Co. (St. Louis, MO). Pro-

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