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The Intrinsic ATPase Activity of Protein Kinase C Is Catalyzed at the Active Site of the Enzyme†

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ABSTRACT: We recently reported that autophosphorylated protein kinase C (PKC) has an intrinsic Ca^{2+}- and phospholipid-dependent ATPase activity and that the ATPase and histone kinase activities of PKC have similar metal-ion cofactor requirements and $K_{m,app}$(ATP) values. We hypothesized that the intrinsic ATPase activity of PKC may represent the bond-breaking step of its protein kinase activity. The rate of the ATPase reaction is several times slower than the histone kinase reaction rate. At subsaturating concentrations, various peptide and protein substrates stimulate the ATPase reaction by as much as 1.5-fold. In contrast, non-phosphorylatable substrate analogs are not stimulatory. These observations support a mechanism of PKC catalysis in which the productive binding of phosphoacceptor substrates enhances the rate of phosphodonor substrate (ATP) hydrolysis at the active site of PKC. However, this mechanism contains an assumption that the ATPase activity of PKC is catalyzed at the active site. In fact, sequence analysis indicates that PKC contains a potential second nucleotide binding site outside of its active site. In this report, we provide a detailed analysis of the relationship between the active site of PKC and the intrinsic ATPase activity of the enzyme. We show that the regulatory and catalytic properties of the ATPase reactions of three PKC isozymes are similar, despite critical differences among the isozymes in their consensus sequences for the potential non-active-site nucleotide binding site in their catalytic domains. We also show that the ATPase and histone kinase reactions of each isozyme have similar $K_{m,app}$(ATP) values. Furthermore, we demonstrate that an active-site-directed anti-PKC monoclonal antibody has parallel stimulatory effects on the ATPase and histone kinase activities of PKC and that monovalent salts have parallel inhibitory effects against these activities. Finally, we report that H7, which inhibits protein kinases but not other ATP-utilizing enzymes by competition with ATP, inhibits the ATPase activity of PKC with predominantly competitive kinetics. Taken together, the data presented in this report provide convincing evidence that the ATPase activity of PKC is catalyzed at the active site of the enzyme. Our results also indicate the usefulness of the ATPase reaction as a diagnostic tool in studies of the bond-breaking step of the protein kinase reaction of PKC.

Protein kinase C (PKC) is a family of closely related Ca^{2+}- and phospholipid-dependent protein kinases that are stimulated in vivo by the second-messenger *sn*-1,2-diacylglycerol (Bell & Burns, 1991; Bishop & Bell, 1988; O'Brian & Ward, 1992). While the regulation of PKC activity has been well characterized in recent years (Bell & Burns, 1991; Epand & Lester, 1990), much less is known regarding the active-site chemistry of the enzyme (O'Brian & Ward, 1990, 1992). The cAMP-

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dependent protein kinase (PKA) has an intrinsic ATPase activity that is thought to represent a partial reaction of its protein kinase activity (Moll & Kaiser, 1976; Sugden et al., 1976; Armstrong et al., 1979), and intrinsic ATPase activity is also a feature of phosphorylase kinase (Paudel & Carlson, 1991). We recently reported that purified, autophosphorylated PKC has an intrinsic Ca^{2+}- and phosphatidylserine (PS)-dependent ATPase activity and that the ATPase and histone kinase activities of PKC have similar metal-ion cofactor requirements and $K_{m,app}$(ATP) values (O'Brian & Ward, 1990). The rate of the ATPase reaction of PKC was approximately 5–10 times slower than the histone kinase reaction rate, but in the presence of subsaturating concentrations of various peptide and protein substrates, the rate of the ATPase reaction was stimulated by as much as 1.5-fold (O'Brian & Ward, 1990, 1991). Moreover, a catalytic fragment of PKC that fully retains its protein kinase activity can be generated by limited proteolysis (Kikkawa et al., 1989). The catalytic fragment is devoid of autophosphorylation activity (Newton & Koshland, 1987) and of ATPase activity in the absence of phosphoacceptor substrates (O'Brian & Ward, 1990). However, both protein and synthetic peptide substrates of PKC potently induce the intrinsic ATPase activity of the catalytic fragment (O'Brian & Ward, 1991). In contrast, synthetic peptide substrate analogs of PKC and PKA that inhibit their respective protein kinase reactions also inhibit their respective ATPase reactions (Salerno et al., 1990; O'Brian & Ward, 1990). The stimulation of the ATPase activity of PKC by phosphoacceptor substrates and the lack of stimulation by inhibitory substrate analogs suggest that productive occupation of the phosphoacceptor substrate binding site of PKC enhances the rate of phosphodonor substrate hydrolysis at the active site of PKC (O'Brian & Ward, 1991). However, it is not yet clear whether the ATPase reaction of PKC is catalyzed at the active site of the enzyme. PKC isozyme-encoding cDNA sequences indicate that the catalytic domain of PKC contains a second potential nucleotide binding site outside of the active-site region (Coussens et al., 1986; Kikkawa et al., 1987). It is not yet known whether the putative second nucleotide binding site inferred from consensus sequences is actually functional (Kikkawa et al., 1989; Ward & O'Brian, 1992). In this report, we provide a detailed analysis of the relationship between the active site of PKC and the intrinsic ATPase activity of the enzyme. We compare the intrinsic ATPase activities of PKC isozymes that have important differences in their consensus sequences for the second putative nucleotide binding site. We also compare the effects of active-site-directed anti-PKC monoclonal antibodies, substitution of the substrate ATP with GTP, and monovalent salts on the ATPase and protein kinase reactions of PKC. Finally, we determine the kinetics of inhibition of the ATPase reaction of PKC by an agent that inhibits the protein kinase reaction of the enzyme with competitive kinetics with respect to ATP. Taken together, the results of these complementary approaches provide convincing evidence that the intrinsic ATPase activity of PKC is catalyzed at the active site of the enzyme.

Experimental Procedures

Materials. Ammonium molybdate tetrahydrate, ATP, GTP, PS, DEAE-Sepharose, fatty acid-free bovine serum albumin, histone III-S, leupeptin, melittin, phenylmethanesulfonyl fluoride, and Tris-HCl were purchased from Sigma Chemical Co. (St. Louis, MO); DNA-grade hydroxylapatite resin, protein assay solution, and a silver stain kit were from Bio-Rad Labs (Richmond, CA); and silicotungstic acid, 2-butanol, benzene, and phosphocellulose paper (p81) were from Fisher Scientific (Houston, TX). The monoclonal antibody anti-type III protein kinase C (M6) was purchased from UBI (Lake Placid, NY). Frozen Sprague Dawley rat brains were purchased from Pelfreez (Rogers, AR), [γ-^{32}P]ATP and [γ-^{32}P]GTP were from Amersham Corp. (Arlington Heights, IL), and the monoclonal antibody anti-protein kinase C (1.9) was from GIBCO BRL (Gaithersburg, MD). Mellitin–agarose was prepared using cyanogen bromide-activated resin as previously described (O'Brian & Ward, 1989).

Purification of Rat Brain PKC and the Isozymes PKC-α, PKC-β, and PKC-γ. Rat brain PKC was purified to near-homogeneity according to silver-stained polyacrylamide gels by a procedure that involved elution of PKC from melittin–agarose with MgATP (O'Brian & Ward, 1989; 1990). The resultant preparation was fully autophosphorylated and was not radiolabeled upon exposure to Mg[γ-^{32}P]ATP under standard phosphotransferase conditions (O'Brian & Ward, 1991). The isozymes PKC-α and PKC-γ were purified from the nearly-homogeneous PKC preparation by subjecting the preparation to hydroxylapatite chromatography under previously described conditions (Huang et al., 1986), using a 50-mL 40–115 mM linear potassium phosphate gradient at pH 8.3 followed by step-elution of PKC-α with 20 mL of 180 mM potassium phosphate at pH 8.3. Collection tubes contained bovine serum albumin at a final concentration of 0.1 mg/mL. PKC-β was not recovered by hydroxylapatite chromatography of the nearly-homogeneous PKC preparation. To obtain PKC-β, PKC was partially purified from rat brains on DEAE-Sepharose using a linear gradient of 0.0–0.3 M NaCl as described previously (O'Brian et al., 1984), and the partially purified PKC preparation was chromatographed on a hydroxylapatite column (3 mL) by the procedure used for the purification of PKC-α and PKC-γ. Consistent with previous reports (Huang et al., 1986; Sekiguchi et al., 1988; Kosaka et al., 1988; Ward & O'Brian, 1992), rat brain PKC-α, PKC-β, and PKC-γ eluted at 130, 105, and 62 mM potassium phosphate, respectively. Following hydroxylapatite chromatography, each PKC isozyme was dialyzed against 20 mM Tris-HCl (pH 7.5)–2 mM EDTA–2 mM EGTA–15 mM 2-mercaptoethanol–10 μg/mL leupeptin, in order to remove inorganic phosphate from the isozyme preparations. The histone kinase activity of the nearly-homogeneous PKC preparation and of each PKC isozyme preparation was stimulated approximately 10-fold by Ca^{2+} plus PS but not by either Ca^{2+} or PS alone in standard assays that contained 0.67 mg/mL histone III-S. Purified PKC and the isozyme preparations were stored in 50% glycerol at −20 °C.

Histone Kinase Assay. The histone kinase activity of the purified PKC preparation and of each isozyme was assayed as previously described (O'Brian et al., 1984). Briefly, reaction mixtures (120 μL) contained 20 mM Tris-HCl (pH 7.5), 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 30 μg of PS/mL (or none), 6 μM [γ-^{32}P]ATP (3500–6000 cpm/pmol), 0.67 mg/mL histone III-S, and purified rat brain PKC. Where indicated, histone III-S was present at a concentration of 0.1 mg/mL. Reactions were initiated by the addition of PKC and proceeded with linear kinetics for 10 min at 30 °C. Reactions were terminated by pipetting an aliquot of the reaction mixture onto phosphocellulose paper, and the radioactivity incorporated into histone III-S was measured as previously described (O'Brian et al., 1984). All data points represent average values obtained from assays conducted in triplicate.

ATPase Assay. The ATPase activity of the nearly-homogeneous PKC preparation and of the PKC isozymes was as-

sayed by our standard procedure (O'Brian & Ward, 1990, 1991), in reaction mixtures (60 μL) containing 20 mM Tris-HCl, pH 7.5, 1 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 30 μg/mL PS (or none), 6 μM [γ-^{32}P]ATP (6000–15 000 cpm/pmol), and purified rat brain PKC. In indicated experiments, the ATPase reaction mixtures included 0.1 mg/mL histone III-S. ATPase reactions were initiated by the addition of [γ-^{32}P]ATP. After a 20-min incubation at 30 °C, reactions were terminated on ice by the addition of 20 mM EDTA (final concentration). To extract [^{32}P]P_i from reaction mixtures, 2 mL of 1:1 2-butanol–benzene and 0.5 mL of 3 N H_2SO_4 containing 4% silicotungstic acid were vortexed together briefly, and the resultant mixture was added to each reaction mixture, followed by brief vortexing. Next, 0.2 mL of 10% ammonium molybdate was added to each mixture, and each sample was vortexed for 15 s. Following phase separation, a 0.6-mL aliquot of the organic phase (upper phase) was counted in 5 mL of Aquasol (O'Brian & Ward 1990, 1991). Each ATPase activity value in this report represents an average of triplicate determinations.

Monoclonal Antibodies. The monoclonal antibody anti-PKC(1.9) (Mochly-Rosen & Koshland, 1987) was stored as a 2 mg/mL stock solution in phosphate-buffered saline at −70 °C. Immediately prior to use, anti-PKC(1.9) was diluted with 20 mM Tris-HCl, pH 7.5, to the desired concentrations. To determine the effects of anti-PKC(1.9) on the ATPase and histone kinase activities of PKC, PKC and the antibody were incubated together for 5 min at 30 °C prior to the execution of the assays. The monoclonal antibody anti-type III protein kinase C (M6) (Leach et al., 1988; Jaken et al., 1989) was stored as a powder at 4 °C. M6 was reconstituted with water to 1 mg/mL and diluted as required with 20 mM Tris-HCl (pH 7.5)–0.1% bovine serum albumin. The reconstituted antibody was used within 12 h.

Kinetic Analysis. The $K_{m,app}$(ATP) values in Table II were obtained by least-squares regression analysis of Lineweaver–Burk plots. In the plots, ATP concentrations varied across a range of 1–20 μM. The kinetics of inhibition of the ATPase reaction of PKC by H7 (Figure 3) were also analyzed by Lineweaver–Burk plots that were generated by least-squares regression analysis.

Results

Characterization of the ATPase Reactions Catalyzed by Rat Brain PKC-α, PKC-β, and PKC-γ. Previously we characterized the ATPase reaction of a purified preparation of rat brain PKC which consisted of a mixture of isozymes (O'Brian & Ward, 1990, 1991). In this report, as a first step toward defining the relationship between the active site of PKC and the ATPase reaction of the enzyme, we compared the capacities of purified PKC isozymes to catalyze an ATPase reaction. We purified PKC-α and PKC-γ from the nearly-homogeneous rat brain PKC preparation that was employed in studies which demonstrated that PKC catalyzes an ATPase reaction (O'Brian & Ward, 1990, 1991). Under standard assay conditions, purified PKC-α and PKC-γ typically catalyzed Ca^{2+}- and PS-dependent ATPase reactions that were respectively 4.7% and 2.6% as fast as their histone kinase reactions. For PKC-α, 100% Ca^{2+}- and PS-dependent ATPase activity was 0.085 ± 0.020 pmol of ^{32}P/min, when 100% Ca^{2+}- and PS-dependent histone kinase activity was 1.80 ± 0.08 pmol of ^{32}P/min. For PKC-γ, the 100% ATPase activity value was 0.061 ± 0.018 pmol of ^{32}P/min, when 100% histone kinase activity was 2.34 ± 0.27 pmol of ^{32}P/min. By comparison, 100% ATPase activity of the purified mixed PKC isozyme preparation was 0.46 ± 0.08 pmol of ^{32}P/min, when 100% of its histone kinase activity was 5.16 ± 0.35 pmol of ^{32}P/min; i.e., the Ca^{2+}- and PS-dependent ATPase reaction was 9% as fast as the histone kinase reaction.

Table I: Regulation of the ATPase Reactions of PKC Isozymes by Allosteric Cofactors and Histone III-S

reaction conditions[a]	ATPase act. (pmol of ^{32}P/min) PKC-α[b]	PKC-β[c]	PKC-γ[b]
Ca^{2+}, PS	0.064 ● 0.006	0.247 ● 0.025	0.098 ± 0.004
Ca^{2+}	0.031 ± 0.010	0.135 ± 0.026	0.045 ± 0.010
x-fold activn by PS in absence of histone	2.1	1.8	2.2
Ca^{2+}, PS, histone	0.128 ± 0.005	0.964 ± 0.039	0.216 ± 0.012
Ca^{2+}, histone	0.020 ± 0.012	0.160 ± 0.024	0.024 ± 0.004
x-fold activn by PS in presence of histone	6.4	6.0	9.0

[a] Where indicated, Ca^{2+} was present at 1 mM, PS at 30 μg/mL, and histone III-S at 0.1 mg/mL. For a complete description of reaction conditions, see Experimental Procedures. [b] PKC-α and PKC-γ were isolated from a nearly-homogeneous preparation of rat brain PKC, as described under Experimental Procedures. [c] Partially purified PKC-β was prepared from rat brains by successive DEAE and hydroxylapatite chromatographies (see Experimental Procedures).

Table II: $K_{m,app}$(ATP) Values for the Ca^{2+}- and PS-Dependent Components of the ATPase and Histone Kinase Reactions Catalyzed by PKC Isozymes

reaction	$K_{m,app}$(ATP) (μM)[a] PKC-α[b]	PKC-β[b]	PKC-γ[b]
ATPase act. in absence of histone	9.3 ± 2.4	2.6 ± 1.3	4.7 ± 1.6
ATPase act. in presence of histone[c]	4.5 ± 1.2	2.5 ± 0.3	2.5 ± 0.1
histone kinase act.[c]	1.8 ± 0.5	3.0 ± 1.4	4.8 ± 0.4

[a] Each $K_{m,app}$(ATP) value represents an average of two determinations. [b] PKC isozymes were obtained as described in the legend to Table I. [c] Histone III-S was present at a concentration of 0.1 mg/mL, and the ATPase and histone kinase reactions were assayed under identical conditions. For a complete description of reaction conditions, see Experimental Procedures. The Ca^{2+}- and PS-dependent components of the PKC-catalyzed reactions were calculated by subtracting the activity observed in the presence of Ca^{2+} alone from the activity observed in the presence of Ca^{2+} plus PS.

We previously reported that Ca^{2+} plus PS stimulates the ATPase activity of a purified mixture of PKC isozymes 1.5–2.0-fold (O'Brian & Ward, 1990) and that 0.1 mg/mL histone III-S stimulates the ATPase activity of a purified PKC isozyme mixture in the presence of Ca^{2+} and PS (O'Brian & Ward, 1991). In this study, we extended these observations to PKC-α and PKC-γ (Table I). Table I shows that Ca^{2+} plus PS stimulated the ATPase activities of PKC-α and PKC-γ 2.1-fold and 2.2-fold, respectively, in the absence of histone. Ca^{2+} plus PS stimulates the ATPase activity of the purified PKC isozyme mixture 6.5-fold in the presence of 0.1 mg/mL histone III-S (O'Brian & Ward, 1991), and under these conditions, the ATPase activities of PKC-α and PKC-γ were stimulated by Ca^{2+} plus PS by 6.4-fold and 9.0-fold, respectively (Table I). Thus, the ATPase reactions of the purified PKC isozyme mixture (O'Brian & Ward, 1990, 1991), PKC-α, and PKC-γ appear to be regulated similarly.

Next, we compared the $K_{m,app}$(ATP) values for the Ca^{2+}- and PS-dependent components of the ATPase reactions of PKC-α and PKC-γ in the presence and absence of 0.1 mg/mL histone III-S, and we also determined the $K_{m,app}$(ATP) values for the Ca^{2+}- and PS-dependent components of the histone kinase reactions of PKC-α and PKC-γ in reaction mixtures that contained 0.1 mg/mL histone III-S (Table II). In the case of PKC-γ, $K_{m,app}$(ATP) values that differed by less than a factor of 2 were observed for the Ca^{2+}- and PS-dependent components of the histone kinase reaction, the ATPase reaction in the absence of histone, and the ATPase reaction in the presence of histone (Table II). In addition, the $K_{m,app}$(ATP)

values for the Ca^{2+}- and PS-dependent components of the ATPase reaction of PKC-α in the presence of histone and the histone kinase reaction of PKC-α differed by less than a factor of 3. In the absence of histone, a somewhat higher $K_{m,app}$-(ATP) value was observed for the Ca^{2+}- and PS-dependent ATPase reaction of PKC-α (Table II). For both PKC-α and PKC-γ, the closely related $K_{m,app}$(ATP) values observed for the ATPase and histone kinase reactions when the reactions were assayed under identical conditions in the presence of 0.1 mg/mL histone III-S (Table II) provide evidence that the ATPase reaction of each isozyme may be catalyzed at the active site. If the ATPase reaction of PKC were catalyzed by a region of the enzyme other than the active site, the most likely site would be the putative second nucleotide binding site of the enzyme (O'Brian & Ward, 1991). Since a critical Lys residue (Hanks et al., 1988) present in the nucleotide binding motif of the putative second ATP binding site in rat brain PKC-α is replaced by Arg in rat brain PKC-γ (Kikkawa et al., 1987), one would expect striking differences between the properties of the ATPase reactions of PKC-α and PKC-γ if ATPase catalysis occurred at the putative second nucleotide binding site. For example, the replacement of the Lys residue with Arg at protein kinase active sites completely abolishes protein kinase activity (Hanks et al., 1988). Therefore, the similarity of the ATPase reactions catalyzed by PKC-α and PKC-γ reported here (Tables I and II) argues against ATPase catalysis at the putative second ATP binding site and thus provides a line of evidence that the ATPase reaction of each isozyme is catalyzed at the active site.

We also examined the ATPase reaction associated with a partially purified preparation of rat brain PKC-β that was obtained by successive DEAE and hydroxylapatite chromatographies (see Experimental Procedures). The ATPase activity associated with PKC-β was stimulated 1.8-fold by Ca^{2+} plus PS (Table I). As observed with the PKC isozyme mixture (O'Brian & Ward, 1991), PKC-α, and PKC-γ (Table I), the Ca^{2+}- and PS-dependent ATPase activity associated with PKC-β was further stimulated by 0.1 mg/mL histone III-S, and Ca^{2+} plus PS stimulated the ATPase activity associated with PKC-β 6.0-fold when 0.1 mg/mL histone III-S was present. The $K_{m,app}$(ATP) values for the Ca^{2+}- and PS-dependent components of the ATPase activities associated with PKC-β in the presence and absence of histone III-S and for the histone kinase reaction were nearly identical (Table II). Thus, the Ca^{2+}- and PS-dependent ATPase activity associated with partially purified PKC-β resembles the intrinsic ATPase activities of PKC-α and PKC-γ and therefore appears to be catalyzed by the β isozyme. However, because the PKC-β employed in this study is a partially purified preparation, we cannot rule out the possibility that contaminating activities, such as phosphatases, ATPases, or other protein kinases, contribute to the basal ATPase activity shown for PKC-β in Table I.

Comparison of the Effects of an Active-Site-Directed Anti-PKC Monoclonal Antibody on the ATPase and Histone Kinase Reactions of the Enzyme. The monoclonal antibody anti-PKC(1.9) has been reported to inhibit the histone kinase reaction of diverse rat brain PKC isozymes and their catalytic fragments when the histone substrate is present at saturating concentrations, and the inhibitory potency of anti-PKC(1.9) is reduced by an excess of ATP (Mochly-Rosen & Koshland, 1987, 1988). Thus, anti-PKC(1.9) appears to act near the active-site region of the catalytic domain of PKC (Mochly-Rosen & Koshland, 1987). As a second approach to investigating the relationship between the ATPase reaction of PKC

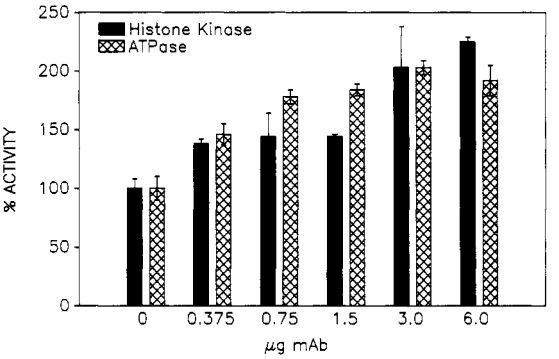

FIGURE 1: Stimulation of the ATPase and histone kinase activities of PKC in the presence of Ca^{2+} and PS by an active-site-directed monoclonal antibody. Purified rat brain PKC was preincubated with the indicated amount of the monoclonal antibody anti-PKC(1.9) for 5 min at 30 °C, and then the ATPase and histone kinase activities of the enzyme were assayed under identical conditions as described under Experimental Procedures in the presence of indicated amounts of anti-PKC(1.9), 1 mM $CaCl_2$, 30 μg/mL PS, and 0.1 mg/mL histone III-S. In each case, activity was calculated by subtracting the background cpm (the cpm obtained using reaction mixtures lacking PKC) corresponding to either [^{32}P]P_i (ATPase assay) or [^{32}P]histone-P (histone kinase assay) from the cpm obtained using PKC-containing reaction mixtures. Under these conditions and in the absence of the antibody, histone is phosphorylated suboptimally, and the ATPase reaction is accelerated by histone. 100% ATPase activity is 0.96 ± 0.09 pmol of ^{32}P/min, and 100% histone kinase activity is 1.99 ± 0.15 pmol of ^{32}P/min. This experiment was reproducible in its entirety. Solid bars = histone kinase activity; cross-hatched bars = ATPase activity.

and the active-site region of the enzyme, we used anti-PKC-(1.9) as a probe of the active site of PKC and determined the effects of the antibody on the ATPase and histone kinase reactions of our nearly-homogeneous PKC preparation. Although the ATPase reaction of PKC is stimulated by 0.1 mg/mL histone III-S in the presence of Ca^{2+} and PS, higher histone III-S concentrations inhibit this activity (O'Brian & Ward, 1991). Therefore, in order to compare the effects of anti-PKC(1.9) on the ATPase and histone kinase reactions of PKC under identical conditions, we employed 0.1 mg/mL histone III-S in the assays. Surprisingly, at this subsaturating concentration of histone III-S, anti-PKC(1.9) actually stimulated the histone kinase activity of PKC in the presence of 1 mM Ca^{2+} and 30 μg/mL PS (Figure 1). Furthermore, anti-PKC(1.9) stimulated the ATPase reaction of PKC to a comparable extent under identical conditions (Figure 1). The maximal activation achieved by anti-PKC(1.9) for each reaction was approximately 2-fold (Figure 1). As a control, we determined that murine IgG2a (1–6 μg) was without effect on the ATPase and histone kinase activities of PKC under these conditions (data not shown). In contrast with the findings of Mochly-Rosen and Koshland (1987), in other control experiments we found that anti-PKC(1.9) also stimulated the histone kinase activity of PKC and that of its catalytic fragment when histone III-S was present at a saturating concentration (data not shown). Although the effects of anti-PKC(1.9) on our PKC preparation differ from effects reported by Mochly-Rosen and Koshland (1987), our observation that anti-PKC(1.9) has similar effects on the catalytic activities of PKC and its catalytic fragment does support the conclusion of Mochly-Rosen and Koshland (1987, 1988) that anti-PKC(1.9) is an active-site-directed antibody. The parallel effects of anti-PKC(1.9) on the histone kinase and ATPase reactions of PKC (Figure 1) provide evidence that the reactions are catalyzed at the same site. Thus, Figure 1 provides a second line of evidence that the ATPase reaction of PKC is catalyzed at the active site. Furthermore, the anti-PKC-α

monoclonal antibody M6 inhibits the protein kinase activity of PKC-α through direct interactions with the catalytic domain of the isozyme (Leach et al., 1988; Jaken et al., 1989), and we found that, subsequent to a 30-min preincubation with purified PKC-α at 30 °C, M6 (2–6 μg/reaction mixture) inhibited the histone kinase and ATPase activities of PKC-α with similar potencies, in the presence of Ca^{2+}, PS, and 0.1 mg/mL histone III-S (data not shown).

Parallel Effects of Monovalent Salts on the Histone Kinase and ATPase Activities of PKC. Various monovalent salts, including NaCl, KCl, and potassium acetate, inhibit the Ca^{2+}- and phospholipid-dependent histone kinase reaction of PKC (Bazzi & Nelsesteun, 1987; Hannun & Bell, 1990), the Ca^{2+}- and phospholipid-independent histone kinase reaction of the catalytic fragment of PKC, and the autophosphorylation reaction of the enzyme with comparable potencies (Hannun & Bell, 1990). Thus, monovalent salts appear to exert direct inhibitory effects against productive interactions between the catalytic domain of PKC and substrates of the enzyme (Hannun & Bell, 1990). In our third approach to the question of whether the ATPase reaction of PKC is catalyzed by the active site of the enzyme, we compared the effects of monovalent salts on the histone kinase and ATPase reactions of PKC. We found that NaCl, KCl, and potassium acetate inhibited the Ca^{2+}- and PS-dependent histone kinase reaction of PKC with IC_{50}'s that ranged from approximately 75 to 180 mM, when histone III-S was present at the subsaturating concentration of 0.1 mg/mL (Figure 2A). Furthermore, under identical conditions, each salt also inhibited the Ca^{2+}- and PS-dependent ATPase reaction catalyzed by PKC (Figure 2A). The IC_{50}'s of the salts against the Ca^{2+}- and PS-dependent ATPase reaction of PKC ranged from about 90 to 200 mM (Figure 2A). Similar results were obtained with sodium acetate (data not shown). The range of IC_{50}'s observed with the salts in the inhibition of the Ca^{2+}- and PS-dependent histone kinase reaction of PKC provides evidence that the inhibitory potencies of the salts reflect not only their presumed contributions to the ionic strength of the assay mixture but also specific interactions between the activated enzyme complex and certain salts, under the conditions of Figure 2A. Likewise, specific effects of some of the salts on enzyme activity may account for the range of IC_{50}'s observed with the salts for the Ca^{2+}- and PS-dependent ATPase reaction in Figure 2A. However, although minor discrepancies are evident between the inhibitory potencies of each salt against the Ca^{2+}- and PS-dependent ATPase and histone kinase reactions of PKC, Figure 2A clearly shows that diverse monovalent salts effect parallel inhibition of the Ca^{2+}- and PS-dependent ATPase and histone kinase reactions of PKC. Moreover, in the absence of Ca^{2+} and PS, the monovalent salts inhibited the basal ATPase and histone kinase reactions of PKC in parallel (Figure 2B). Since monovalent salts appear to inhibit PKC by direct effects on its catalytic activity (Hannun & Bell, 1990), Figure 2 provides an independent line of evidence that the ATPase reaction of PKC is catalyzed at the active site of the enzyme.

Comparison of the Nucleotide Substrate Specificities of the ATPase and Histone Kinase Reactions of PKC. In our fourth line of investigation into whether the ATPase reaction of PKC is catalyzed at the active site of the enzyme, we compared the capacities of the NTPase and histone kinase reactions of PKC to utilize [γ-^{32}P]GTP as a substrate. Mg[γ-^{32}P]GTP served as a substrate of both the NTPase and the histone kinase reactions of PKC in the presence of 1 mM Ca^{2+} and 30 μg/mL PS. With Mg[γ-^{32}P]GTP as the phosphodonor

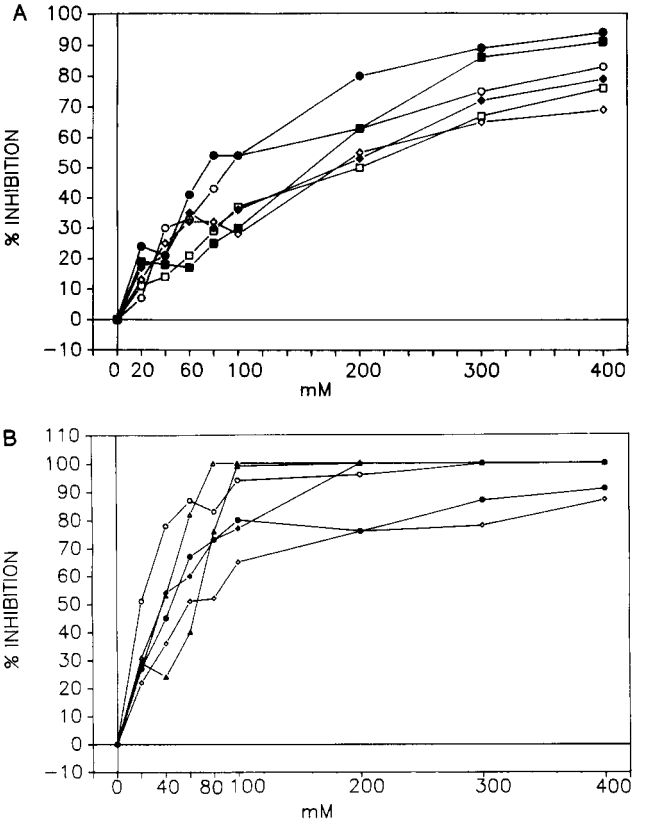

FIGURE 2: Inhibition of the ATPase and histone kinase reactions of PKC by monovalent salts. Inhibition of the Ca^{2+}- and PS-dependent ATPase and histone kinase reactions of PKC (A) and inhibition of the basal ATPase and histone kinase reactions of the enzyme (B) by monovalent salts are shown. The ATPase and histone kinase activities of PKC were assayed as described under Experimental Procedures under identical conditions. The reactions were assayed both in the presence and in the absence of 1 mM Ca^{2+} and 30 μg/mL PS, and all reaction mixtures contained 0.1 mg/mL histone III-S. Monovalent salts were included in the reaction mixtures as indicated. Ca^{2+}- and PS-dependent activities were calculated by subtracting the activity observed in the absence of Ca^{2+} and PS from the activity observed in their presence. Basal activities were calculated by subtracting background cpm from the cpm obtained when PKC was assayed in the absence of Ca^{2+} and PS. Each experiment was reproducible in its entirety. Closed symbols = histone kinase activity; open symbols = ATPase activity. Monovalent salts are NaCl (circles), KCl (squares), sodium acetate (triangles), and potassium acetate (diamonds).

substrate, each reaction was catalyzed at a rate that was 2–4% as fast as the rate observed with Mg[γ-^{32}P]ATP, when the NTP concentration was 50 or 100 μM and the Mg^{2+} concentration was 10 mM. When Mg^{2+} was replaced by Mn^{2+}, [γ-^{32}P]GTP no longer served as a substrate of either reaction. Thus, the relative rates of hydrolysis of Mg[γ-^{32}P]ATP and Mg[γ-^{32}P]GTP in the NTPase reaction of PKC were similar to the relative catalytic rates observed with these metal nucleotides in the histone kinase reaction of the enzyme. In addition, neither Ca^{2+} nor Mn^{2+} could serve as a metal-ion cofactor for [γ-^{32}P]GTP in either reaction. These results support the evidence that the ATPase reaction of PKC is catalyzed at the active site of the enzyme.

Kinetics of ATPase Inhibition by H7, an Agent That Competes with ATP in the Inhibition of PKC-Catalyzed Histone Phosphorylation. If the ATPase reaction of PKC is a partial reaction of the protein kinase activity of the enzyme, then inhibitors of the protein kinase activity that function simply by competition with ATP should also inhibit the ATPase reaction with competitive kinetics (see Discussion). The isoquinolinesulfonamide H7 is an inhibitor of at least several

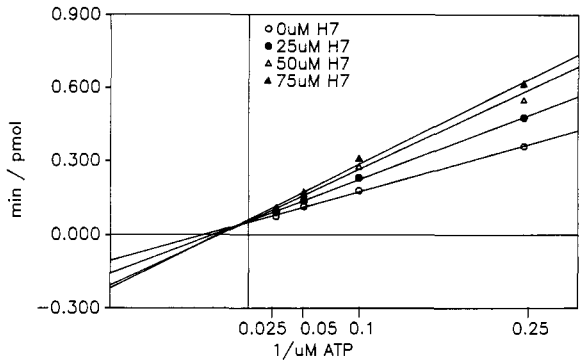

FIGURE 3: Kinetics of inhibition of the ATPase reaction of PKC by H7. Inhibition of the ATPase reaction of PKC by H7 in the presence of Ca^{2+} and PS was subjected to Lineweaver–Burk analysis. Reaction mixtures contained 1 mM Ca^{2+}, 30 μg/mL PS, and 0.1 mg/mL histone III-S. Assays were conducted as described under Experimental Procedures. ATPase activity was calculated by subtracting the cpm corresponding to $[^{32}P]P_i$ that were extracted from reaction mixtures that lacked PKC from the cpm extracted from PKC-containing reaction mixtures. Each point represents a mean of triplicate determinations. Lines were determined by least-squares regression analysis. Linear correlation coefficients are 0.999 (0 μM H7), 0.997 (25 μM H7), 0.999 (50 μM H7), and 0.997 (75 μM H7).

protein kinases including PKC that functions by competition with ATP (Hidaka et al., 1984). At micromolar concentrations, H7 appears to interact exclusively with protein kinase active sites, since it does not inhibit other ATP-utilizing enzymes, e.g., ATPases (Hidaka et al., 1984). Previously, we reported that H7 inhibits the ATPase and histone kinase reactions of PKC with similar potencies (O'Brian and Ward, 1990). Figure 3 shows that the kinetics of H7 inhibition of the ATPase reaction of the mixed PKC isozyme preparation in the presence of Ca^{2+} and PS are mixed competitive/noncompetitive (Palmer, 1985) with respect to ATP. The competitive component of the inhibitory kinetics is predominant. For example, at 50 μM H7 the $K_{m,app}$(ATP) value is increased by 52%, and the $V_{max,app}$ value is reduced by merely 10%. The pronounced competitive character of these inhibitory kinetics provides further evidence that the ATPase reaction of PKC is catalyzed at the active site of the enzyme.

Discussion

Previously we demonstrated that autophosphorylated PKC catalyzes an ATPase reaction. We proposed that the intrinsic ATPase activity of PKC could represent the bond-breaking step of the protein kinase reaction catalyzed at the active site of the enzyme. According to this model of PKC catalysis, protein substrates would compete with water for the γ-phosphate of ATP at the active site, and the products of catalysis would be phosphoproteins and inorganic phosphate (O'Brian & Ward, 1990).

In this report, we employed several independent experimental approaches to test our hypothesis that the intrinsic ATPase activity of PKC is a partial reaction of the protein kinase activity of the enzyme. With each approach, we obtained evidence that the ATPase reaction of PKC is catalyzed at its active site and is intimately linked with its protein kinase reaction. These results can be summarized as follows. (1) The regulatory and catalytic properties of the ATPase reactions of the isozymes PKC-α, PKC-β, and PKC-γ are similar (Tables I and II), despite important differences among the isozymes in their consensus sequences for a potential nonactive-site nucleotide binding site in their catalytic domains (Coussens et al., 1986; Kikkawa et al., 1987). Thus, it is likely that ATPase catalysis either does not occur at the putative second nucleotide binding site of PKC or occurs at that site only to a negligible extent. Because the putative second nucleotide binding site of PKC appears to be the only logical alternative to the active site for ATPase catalysis, these studies support our hypothesis that the ATPase reaction of PKC is catalyzed at the active site. In future studies, it will be important to characterize the catalytic activities of recombinant PKC isozymes that contain deletions or mutations at the second nucleotide binding site, in order to determine whether that site plays any role in the ATPase and protein kinase activities of PKC. In the present study, we also demonstrate that PKC-α, PKC-β, and PKC-γ each catalyze Ca^{2+}- and PS-dependent ATPase reactions with $K_{m,app}$(ATP) values that are closely related to the $K_{m,app}$(ATP) values of their histone kinase reactions, providing evidence that the ATPase and histone kinase reactions of PKC isozymes are intimately related. (2) The active-site-directed monoclonal antibody anti-PKC(1.9) (Mochly-Rosen & Koshland, 1987) has parallel stimulatory effects on the ATPase and histone kinase reactions of PKC in the presence of 1 mM Ca^{2+}, 30 μg/mL PS, and 0.1 mg/mL histone III-S (Figure 1). Because monoclonal antibodies are highly selective probes of specific regions of protein structure, the modulation of the ATPase activity of PKC by an active-site-directed monoclonal antibody provides evidence that ATPase catalysis occurs at the active site of the enzyme. Furthermore, the similarity of the effects of the antibody on the ATPase and histone kinase reactions of PKC provides evidence that these catalytic events are intimately related. (3) Monovalent salts inhibit the Ca^{2+}- and PS-dependent ATPase and histone kinase reactions of PKC and the corresponding basal reactions of the enzyme in parallel (Figure 2). Presumably, PKC undergoes subtle conformational changes as the monovalent salt concentraton is gradually increased. Thus, the parallel inhibition of the ATPase and histone kinase reactions of PKC across various salt gradients provides strong evidence that the ATP and histone kinase reactions not only occur to the same site but also follow the same pathway. (4) The ATPase and histone kinase reactions of PKC both utilize MgGTP as a substrate, and the relative rates observed for each reaction with ATP and GTP are similar. (5) The isoquinolinesulfonamide H7 inhibits the histone kinase reaction of PKC by competition with ATP (Hidaka et al., 1984). Studies of product and dead-end inhibition provide evidence that the mechanism of PKC-catalyzed histone phosphorylation is steady-state preferred ordered with MgATP binding to PKC prior to histone III-S (Leventhal & Bertics, 1991). Therefore, if the ATPase reaction of PKC is the bond-breaking step of its protein kinase reaction, inhibitors of the histone kinase reaction that competes with ATP should also inhibit the ATPase reaction of PKC by competition with ATP. In fact, H7 does inhibit the ATPase reaction of PKC with kinetics that are predominantly competitive (Figure 3). Taken together, the data presented in this report provide convincing evidence that the ATPase reaction of PKC is catalyzed at the active site of the enzyme. These data also indicate the usefulness of the ATPase reaction of PKC as a diagnostic tool in studies of the bond-breaking step of the protein kinase reaction of the enzyme.

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