Characterization of a Ca^{2+}- and Phospholipid-Dependent ATPase Reaction Catalyzed by Rat Brain Protein Kinase C†

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Received October 18, 1989; Revised Manuscript Received January 12, 1990

ABSTRACT: Protein kinase C (PKC) consists of a family of Ca^{2+}- and phospholipid-dependent protein kinases that catalyze the transfer of the γ-phosphate of ATP to phosphoacceptor serine or threonine residues of protein and peptide substrates. In this report, we demonstrate that purified, autophosphorylated rat brain PKC catalyzes a Ca^{2+}- and phospholipid-dependent ATPase reaction, that appears to represent the bond-breaking step of its phosphotransferase reaction. The histone kinase and ATPase activities of PKC each had a $K_{m_{app}}$ of 6 μM for ATP, and their metal ion cofactor requirements were similar. The rate of the Ca^{2+}- and phospholipid-dependent PKC-catalyzed ATPase reaction was approximately 5 times slower than the rate of histone phosphorylation, but the basal rates of the PKC-catalyzed ATPase and histone kinase activities differed by less than a factor of 2. The mechanism of the ATPase reaction could entail either direct hydrolysis of ATP by water or formation of a stable phosphoenzyme (PKC–P) followed by its hydrolysis (PKC + P_i). The latter mechanism appears unlikely since [γ-^{32}P]ATP failed to label autophosphorylated PKC. Furthermore, the PKC preparation did not contain contaminating protein phosphatases, excluding the possibility that the ATPase activity represented dephosphorylation of contaminating PKC substrates. Therefore, our results suggest that water may effectively compete with protein substrates of PKC for the γ-phosphate of ATP. Using PKC inhibitors and activators, we found that the ATPase and protein kinase activities of PKC were regulated analogously, providing evidence that allosteric activation of PKC involves facilitation of the bond-breaking step of the phosphotransferase reaction. Use of the ATPase reaction in the analysis of PKC catalysis may shed further light on the active-site chemistry of PKC and on the allosteric regulation of its protein kinase activity.

Protein kinase C (PKC)[1] consists of a family of Ca^{2+}- and phospholipid-dependent protein kinases that catalyze the transfer of the γ-phosphate of ATP to phosphoacceptor serine and threonine residues of protein and peptide substrates (Kikkawa et al., 1989; O'Brian & Ward, 1989a). Although numerous PKC activators, such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (Castagna et al., 1982), phosphatidylserine (PS) (Takai et al., 1979a), diglyceride (Takai et al., 1979b), and phosphatidylinositol 4,5-diphosphate (O'Brian et al., 1987a; Chauhan & Brockerhoff, 1988), and a large number of PKC inhibitors, including tamoxifen (O'Brian et al., 1985, 1988; Su et al., 1985), chlorpromazine (Mori et al., 1980; O'Brian et al., 1987b), and melittin (Katoh et al., 1982; O'Brian & Ward, 1989b), have been identified and characterized, very little is known concerning the active-site chemistry of the enzyme. The active-site chemistry of PKC has not been subjected to detailed analysis by either chemical modifications or site-specific mutagenesis. It does appear to be closely related to that of the cAMP-dependent protein kinase (PKA), since sequence analysis of PKC isozymes inferred from PKC-encoding cDNAs indicates a high degree of homology between the catalytic domains of PKC and PKA (Hanks et al., 1988).

Previous studies have shown that PKA catalyzes a cAMP-dependent ATPase reaction (Moll & Kaiser, 1976; Armstrong et al., 1979). In this report, we demonstrate that purified, autophosphorylated rat brain PKC catalyzes a Ca^{2+}- and phospholipid-dependent ATPase reaction. Using PKC inhibitors and activators, we show that ATPase and phosphotransferase activities of PKC are regulated analogously. The rate of the Ca^{2+}- and PS-dependent PKC-catalyzed ATPase reaction is approximately 5 times slower than that of histone phosphorylation, although the basal rates of the PKC-catalyzed ATPase and histone phosphorylation reactions differ by less than a factor of 2. This indicates that substantial ATP hydrolysis can be catalyzed by autophosphorylated PKC when the peptide substrate binding region of its active site is not occupied by a phosphoacceptor substrate.

Experimental Procedures

Materials. Histone III-S, PS, ATP, TPA, TCA, Tris-HCl, and ammonium molybdate tetrahydrate were purchased from Sigma Chemical Co. (St. Louis, MO). Silicotungstic acid, 2-butanol, benzene, and phosphocellulose paper, grade p81, were purchased from Fisher Scientific (Houston, TX). [γ-^{32}P]ATP and [α-^{32}P]ATP were purchased from Amersham Corp. (Arlington Hts., IL), and frozen rat brains were from Charles River Breeding Laboratories (Wilmington, MA). Poly(ethylenimine) sheets were from Curtin Matheson Scientific (Houston, TX); the peptide PKC-(19–36) was from Peninsula Labs (Belmont, CA).

Purification of Rat Brain PKC. Rat brain PKC was purified to near homogeneity according to silver-stained polyacrylamide gels by a procedure involving elution of PKC from a melittin–agarose affinity column with MgATP (O'Brian & Ward, 1989b). The resultant PKC preparation was fully autophosphorylated and did not incorporate detectable

† This work was supported by Grant G1141-02 from the Robert A. Welch Foundation. This paper is dedicated to the memory of Professor E. Thomas Kaiser.

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[1] Abbreviations: H-7, 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine; H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide; PKA, cAMP-dependent protein kinase; HA-1004, *N*-(2-guanidinoethyl)-5-isoquinolinesulfonamide; PKC, protein kinase C; PS, phosphatidylserine; TCA, trichloroacetic acid; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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amounts of ^{32}P upon exposure to Mg[γ-^{32}P]ATP under standard phosphotransferase assay conditions in the absence of histone III-S. The PKC preparation was stimulated 10–30-fold by 1 mM Ca^{2+} and 30 μg of PS/mL but was not stimulated by either Ca^{2+} or PS alone.

Phosphotransferase Assay of Rat Brain PKC. The phosphotransferase activity of PKC was assayed as previously described (O'Brian et al., 1984). Reaction mixtures contained 20 mM Tris-HCl at pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 30 μg of PS/mL (or none), 10 μM [γ-^{32}P]ATP (300–600 cpm/pmol), 0.67 mg of histone III-S/mL, and purified rat brain PKC. Reactions were initiated by the addition of PKC and proceeded for 5–10 min at 30 °C, which is within the linear phase of the time course. Reactions were terminated by pipetting a 40-μL aliquot of the reaction mixture onto phosphocellulose paper, and the radioactivity incorporated into histone was measured as previously described (O'Brian et al., 1984). In indicated experiments, phosphotransferase reactions were terminated by TCA precipitation using 20% TCA–1% PP_i, as previously described (O'Brian et al., 1984).

ATPase Assay of Rat Brain PKC. The ATPase activity associated with rat brain PKC was measured by applying the procedure of Pollard and Korn (1973). Reaction mixtures (60 μL) contained 20 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 30 μg of PS/mL (or none), purified rat brain PKC, and 10 μM [γ-^{32}P]ATP. Reactions were initiated by the addition of [γ-^{32}P]ATP (3000–5000 cpm/pmol), proceeded at 30 °C for the indicated interval of time, and were terminated on ice by the addition of 10 μL of 140 mM EDTA. To precipitate the protein and extract the product [^{32}P]P_i from the reaction mixtures, we vortexed 2 mL of 1:1 2-butanol–benzene and 0.5 mL of 3 N H_2SO_4 containing 4% silicotungstic acid together briefly and then added this to each reaction mixture, followed by brief vortexing. To the resultant mixtures was added 0.2 mL of 10% ammonium molybdate, and the samples were vortexed for 15 s. After the phases separated, 0.6 mL of the organic phase (upper phase) was pipetted into a scintillation vial. Five milliliters of Aquasol was added to each vial, and the samples were counted.

In experiments indicated under Results, an alternative ATPase assay was also employed. In these assays, reaction mixtures were identical with those described above, except that [α-^{32}P]ATP (4000–6000 cpm/pmol) was present in lieu of [γ-^{32}P]ATP, and reaction volumes were 30 μL. Reactions proceeded at 30 °C and were terminated on ice with 5 μL of 140 mM EDTA. The product [α-^{32}P]ADP was recovered by thin-layer chromatography of a 5-μL aliquot of the reaction mixture on poly(ethylenimine) sheets (developed with 1 M KH_2PO_4 at pH 3.4) and identification under UV light, as previously described (Moll & Kaiser, 1976; Armstrong et al., 1979). Spots were cut from sheets and counted in 6 mL of Aquasol. To quantitate residual protein phosphorylation activity in these assays, parallel experiments were done using [γ-^{32}P]ATP and measuring TCA-precipitable counts, as previously described (O'Brian et al., 1984). ATPase activity was calculated as the difference between the formation of [α-^{32}P]ADP from [α-^{32}P]ATP and the production of TCA-precipitable ^{32}P from [γ-^{32}P]ATP. TCA-precipitable ^{32}P amounted to <10% of the [α-^{32}P]ADP produced in parallel assays.

Results

In order to address the question of whether PKC could catalyze the hydrolysis of ATP, we first measured the production of [^{32}P]P_i from [γ-^{32}P]ATP in the presence of >95% pure, autophosphorylated rat brain PKC. We observed an ATPase reaction that was linear over a 20-min time course (Figure 1). The reaction rate was enhanced 1.5–2-fold by Ca^{2+} plus PS. A comparison of the Ca^{2+}-plus PS-dependent rates of the ATPase reaction and of PKC-catalyzed histone phosphorylation indicated that the ATPase reaction rate was 23 ± 3% as fast as the rate of histone phosphorylation. However, the basal rate of the ATPase reaction was 42% ± 4% faster than the basal rate of histone phosphorylation catalyzed by PKC. The $K_{m_{app}}$ of ATP in Ca^{2+}- and PS-dependent PKC-catalyzed histone phosphorylation was 6 μM (Kikkawa et al., 1982), and the $K_{m_{app}}$ value we obtained by Lineweaver–Burk analysis of the Ca^{2+}- and PS-dependent ATPase activity associated with PKC was also 6 μM (linear correlation coefficient = 0.996). In parallel experiments, we measured the rate of formation of [α-^{32}P]ADP from [α-^{32}P]ATP catalyzed by the autophosphorylated PKC preparation (see Experimental Procedures). [α-^{32}P]ADP formation was linear over a 20-min time course and was stimulated by Ca^{2+} and PS. The Ca^{2+}- and PS-dependent production of [α-^{32}P]ADP amounted to 2.2 ± 0.8, 8.4 ± 1.4, and 21.0 ± 1.8 pmol of [α-^{32}P]ADP at 1, 5, and 20 min, respectively. A comparison of these data with the results illustrated in Figure 1 indicates that similar rates of ATP hydrolysis were observed whether the production of [α-^{32}P]ADP from [α-^{32}P]ATP or the production of [^{32}P]P_i from [γ-^{32}P]ATP was measured. Thus, our nearly homogeneous PKC preparation was associated with a substantial amount of ATPase activity according to two independent ATPase assay systems.

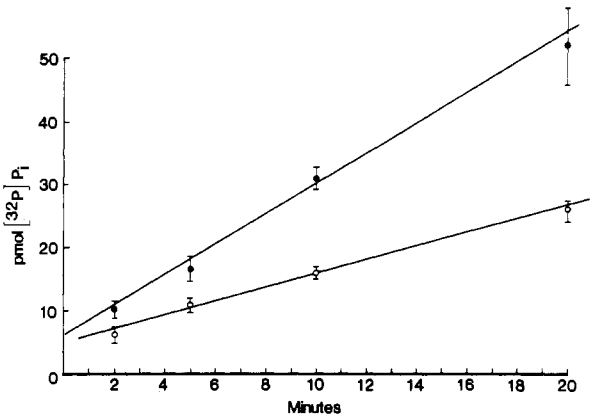

FIGURE 1: Time course of the ATPase reaction associated with purified rat brain PKC. The rate of formation of [^{32}P]P_i from [γ-^{32}P]ATP (picomoles of [^{32}P]P_i) catalyzed by a purified and autophosphorylated rat brain PKC preparation was measured in the presence of Ca^{2+} and PS (●) and in the absence of these cofactors (○). For experimental details, see Experimental Procedures. Each point represents the average of triplicate determinations (±SD), and this experiment was reproducible in its entirety.

The phosphotransferase activity of PKC can be stimulated by Ca^{2+} plus PS, but not by Ca^{2+} or PS alone (Kikkawa et al., 1989; O'Brian & Ward, 1989a). Similarly, we found that Ca^{2+} plus PS enhanced the ATPase activity associated with PKC and that neither Ca^{2+} nor PS alone could stimulate the activity, according to assays containing [γ-^{32}P]ATP that monitored [^{32}P]P_i formation (Figure 2) as well as assays containing [α-^{32}P]ATP that monitored [α-^{32}P]ADP formation (data not shown). Furthermore, we found that TPA, a highly selective PKC activator (Castagna et al., 1982), also stimulated the ATPase activity of our PKC preparation in the presence of PS (Figure 2), albeit to a lesser extent than Ca^{2+} plus PS. Previously, we reported that TPA does not significantly enhance the phosphotransferase activity of PKC in the presence

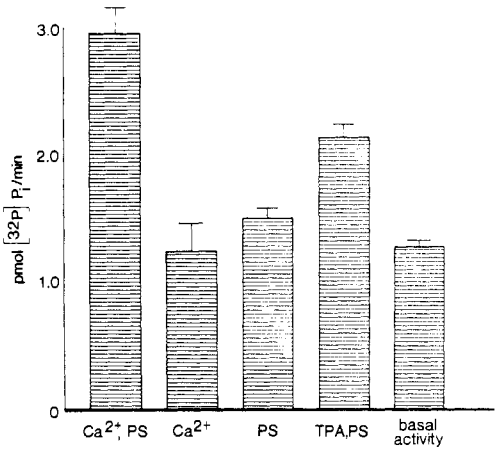

FIGURE 2: Allosteric regulation of the ATPase reaction associated with PKC. The rate of formation of $[^{32}P]P_i$ from $[\gamma\text{-}^{32}P]ATP$ catalyzed by a purified and autophosphorylated preparation of rat brain PKC (picomoles of $[^{32}P]P_i$ per minute) was measured as described under Experimental Procedures. The reactions proceeded for 20 min at 30 °C, in the presence of the indicated cofactors. Cofactor concentrations were 1 mM Ca^{2+}, 30 μg of PS/mL, and 100 nM TPA. Reaction mixtures lacking Ca^{2+} contained 1 mM EGTA. Each bar represents the average of triplicate determinations (±SD).

of Ca^{2+} and phospholipid (O'Brian et al., 1984). Consistent with these results, we found that neither TPA nor its endogenous analogue diacylglycerol could enhance the ATPase activity of our PKC preparation in the presence of 1 mM Ca^{2+} and 30 μg/mL PS. Relative to the ATPase activity that we observed with Ca^{2+} and PS (100%) (Figure 2), we observed 102 ± 15% and 104 ± 13% of the ATPase activity with 100 nM TPA and 10 μg/mL diolein, respectively, in the presence of Ca^{2+} and PS. Thus, the allosteric regulation of the phosphotransferase activity of PKC by Ca^{2+}, PS, and TPA is qualitatively analogous to the regulation of the ATPase activity associated with our purified PKC preparation. However, the ATPase and phosphotransferase activities differed markedly in their degrees of dependence on PS plus either Ca^{2+} or TPA. While Ca^{2+} and PS stimulated PKC-catalyzed histone phosphorylation 10-fold, they stimulated the ATPase activity associated with PKC approximately 2-fold. In addition, TPA and PS stimulated PKC-catalyzed histone phosphorylation 10-fold under conditions where they stimulated the ATPase reaction associated with PKC about 1.5-fold (Figure 2).

The phosphotransferase activity of PKC has a strict requirement for the metal cofactor Mg^{2+}. We found that substitution of Mn^{2+} or Ca^{2+} for Mg^{2+} in standard PKC phosphotransferase assays changed the extent of histone phosphorylation from 100% ± 7% to 0% ± 2% and 0% ± 1%, respectively. Similarly, the substitution of either Mn^{2+} or Ca^{2+} for Mg^{2+} in ATPase assays abolished the Ca^{2+}- and PS-dependent ATPase activity and most of the basal ATPase activity associated with PKC (Figure 3). These results indicate that the metal ion requirements for the phosphotransferase activity of PKC and the ATPase activity associated with the enzyme are similar.

We further compared the Ca^{2+}- and PS-dependent histone kinase activity of PKC with the Ca^{2+}- and PS-dependent ATPase activity associated with our PKC preparation by analyzing the effects of cationic–amphiphilic PKC inhibitors on the ATPase activity. Melittin, polymyxin B, and *N*-desmethyltamoxifen are cationic–amphiphilic PKC inhibitors that inhibit 50% of the Ca^{2+}- and phospholipid-dependent histone kinase activity of the enzyme at concentrations of 3 μM (O'Brian & Ward, 1989b), 6 μM (Mazzei et al., 1982), and 8 μM (O'Brian et al., 1986), respectively. We found that

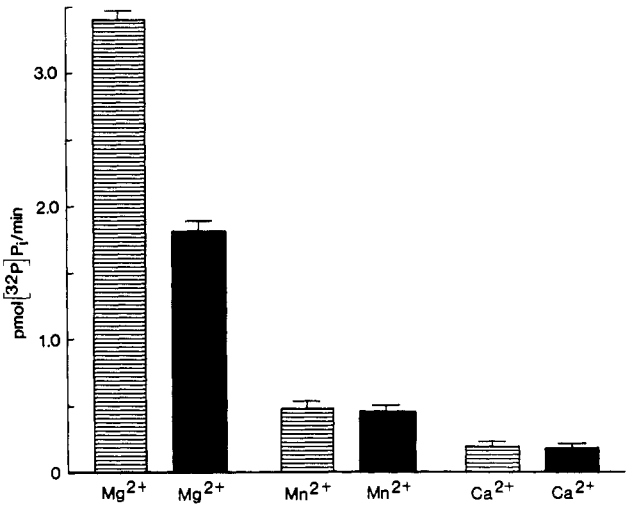

FIGURE 3: Metal cofactor requirements for the ATPase reaction associated with purified rat brain PKC. The ATPase reaction associated with rat brain PKC was measured either in the presence of 1 mM Ca^{2+} and 30 μg of PS/mL (striped bars) or in the absence of these cofactors (solid bars) as described in the legend to Figure 2, and the metal cofactor Mg^{2+} was replaced with Mn^{2+} or Ca^{2+} as indicated. Metal cofactor concentrations were 10 mM Mg^{2+}, 10 mM Mn^{2+}, and 1 mM Ca^{2+}. (Higher concentrations of Ca^{2+} were precluded by its inhibitory effects on PKC at elevated concentrations.) Each bar represents the average of triplicate determinations (±SD). This experiment was reproducible in its entirety.

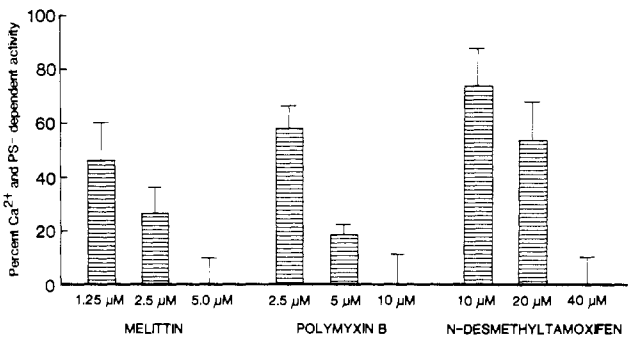

FIGURE 4: Inhibition of the ATPase activity associated with purified rat brain PKC by cationic–amphiphilic PKC inhibitors. The Ca^{2+}- and PS-dependent component of the ATPase activity associated with purified rat brain PKC (picomoles of $[^{32}P]P_i$ per minute) was calculated as the difference between the ATPase activity observed in the presence of 1 mM Ca^{2+} and 30 μg of PS/mL and the activity observed in the absence of these cofactors, in a 20-min reaction at 30 °C. Bars represent the percentage of the Ca^{2+}- and PS-dependent ATPase activity remaining in the presence of the indicated PKC inhibitors (melittin, polymyxin B, *N*-desmethyltamoxifen) at the specified concentrations. Each bar represents triplicate assays (±SD).

these agents inhibited the ATPase activity associated with PKC with potencies that differed from their inhibitory activities against PKC-catalyzed histone phosphorylation by less than a factor of 3 (Figure 4), providing further evidence that the allosteric regulation of PKC-catalyzed histone phosphorylation is related to that of the ATPase reaction. A fully active catalytic fragment of PKC can be generated by limited proteolysis (O'Brian & Ward, 1989a). We generated an active catalytic fragment of PKC by limited tryptic digestion as previously described (O'Brian et al., 1988). We found that the proteolytic fragment had no detectable ATPase activity. According to our limits of detection, the ATPase activity of the catalytic fragment was <10% of the ATPase activity observed with an equivalent number of phosphotransferase units of PKC (data not shown).

Isoquinolinesulfonamides, such as H-7, form a class of selective protein kinase inhibitors that compete with the substrate

ATP (Hidaka et al., 1984). Although H-7 inhibits the activities of several protein kinases with K_i's <150 μM, it is a very weak inhibitor of ATPases. H-7 and the related compound H-8 inhibit actinomyosin ATPase and (Ca^{2+}–Mg^{2+})-ATPase with K_i's >700 μM (Hidaka et al., 1984). Thus, H-7 can be informative in studies aimed at distinguishing the active sites of ATPases and protein kinases. H-7 inhibits histone phosphorylation catalyzed by PKC approximately 60% at a concentration of 30 μM (Nakadate et al., 1988). We found that H-7 inhibited the ATPase activity associated with PKC with a similar potency. At concentrations of 20 and 40 μM, H-7 inhibited the Ca^{2+}- and PS-dependent ATPase activity 38% ± 10% and 72% ± 9%, respectively. In addition, 40 μM H-7 inhibited 50% ± 4% of the basal ATPase activity. HA-1004 is a structural analogue of H-7 that is about 15% as potent as H-7 in the inhibition of PKC (Hidaka et al., 1984). We found that 40 μM HA-1004 inhibited the Ca^{2+}- and PS-dependent ATPase activity 57% ± 9% and the basal ATPase activity 30% ± 5%. Our observation that isoquinolinesulfonamide ATP antagonists inhibit PKC-catalyzed histone phosphorylation and the ATPase activity associated with purified PKC with IC_{50}'s <100 μM indicates either that PKC itself catalyzes the ATPase reaction or that the reaction is catalyzed by a contaminating ATPase that is activated by PKC. However, the latter possibility is highly unlikely in view of the prolonged exposure of the PKC preparation to MgATP during its purification on melittin–agarose and our observation that the potencies of HA-1004 against the ATPase and protein kinase activities of the PKC preparation differed markedly.

The synthetic octadecapeptide PKC-(19–36) is a specific PKC inhibitor that contains a sequence located in the regulatory domain of PKC. PKC-(19–36) inhibits PKC-catalyzed Ca^{2+}- and PS-dependent glycogen synthase peptide phosphorylation with an IC_{50} of 0.18 ± 0.02 μM and myosin light chain kinase phosphorylation with an IC_{50} of 0.21 ± 0.05 μM (House & Kemp, 1987). We found that 0.5 μM PKC-(19–36) inhibited the basal ATPase activity associated with purified PKC 55% ± 8% but did not inhibit the Ca^{2+}- and PS-dependent ATPase activity (percent inhibition = 0% ± 10%). This observation provides strong evidence that the ATPase activity is catalyzed by PKC itself rather than by a PKC substrate that copurifies with PKC to near-homogeneity and is activated by the enzyme, since PKC-(19–36) inhibits Ca^{2+}- and PS-dependent protein phosphorylation reactions catalyzed by PKC (House & Kemp, 1987).

To address the possibility that the mechanism of the ATPase reaction observed with autophosphorylated PKC involved dephosphorylation of autophosphorylated PKC by PKC itself or by a contaminating phosphatase, we measured the labeling of autophosphorylated PKC in the presence of [γ-^{32}P]ATP under the conditions of the ATPase assay by TCA precipitation (see Experimental Procedures). Under these conditions, Ca^{2+}- and PS-dependent labeling of PKC was not detectable. According to the limits of detection of the experiment, the enzyme was labeled to an extent of less than 0.03 pmol of ^{32}P/pmol of PKC during the ATPase assay. Thus, it appears unlikely that the mechanism of the ATPase reaction entails dephosphorylation of autophosphorylated PKC.

To directly examine the possibility that a protein phosphatase contaminated our PKC preparation and contributed to the observed ATPase reaction by dephosphorylating either PKC or a contaminating PKC substrate, we labeled histone IIIS with [γ-^{32}P]ATP by PKC catalysis under standard conditions and purified ^{32}P-histone from [γ-^{32}P]ATP and PKC by passing it through a DEAE-Sephacel column equilibrated in 20 mM Tris-HCl, pH 7.5. We then measured the dephosphorylation of ^{32}P-histone by our PKC preparation in the presence of 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. The PKC preparation failed to detectably dephosphorylate ^{32}P-histone over a 1-h time course at 30 °C. Our limits of detection indicated a rate of ^{32}P-histone dephosphorylation that was less than 0.04 pmol of ^{32}P/min. These results, taken together with our observation that autophosphorylated PKC cannot be labeled with [γ-^{32}P]ATP, indicate that the ATPase reaction associated with PKC cannot be accounted for by a contaminating protein phosphatase activity.

Discussion

We conclude that the ATPase activity associated with PKC is catalyzed by PKC itself on the basis of the following evidence: (1) The ATPase activity was associated with a PKC preparation that was nearly homogeneous, according to silver-stained gels (O'Brian & Ward, 1989), and the specific activities of the basal ATPase and histone kinase activities were similar. (2) The histone kinase activity of PKC and the associated ATPase activity appeared to be catalyzed by the same active site since they each had a $K_{m_{app}}$ of 6 μM for ATP, and since their metal ion cofactor requirements were indistinguishable. (3) The allosteric regulation of the ATPase activity by Ca^{2+}, PS, TPA, and cationic–amphiphilic PKC inhibitors qualitatively resembled the regulation of PKC-catalyzed histone phosphorylation, indicating that the reaction was catalyzed by either PKC or a contaminating PKC substrate that could be activated by PKC. Since the PKC preparation present in the assays was fully autophosphorylated due to prolonged exposure to MgATP during chromatography on melittin–agarose (O'Brian & Ward, 1989), it is highly unlikely that any contaminating PKC substrates in the PKC preparation were incompletely phosphorylated. Therefore, the Ca^{2+} and PS dependence of the ATPase activity provides strong evidence that the ATPase activity associated with purified PKC was catalyzed by PKC itself. (4) If PKC were the catalyst, one would expect that ATP antagonists would inhibit both the ATPase and protein phosphorylation activities of the enzyme, and that peptide substrate antagonists of PKC-catalyzed protein phosphorylation might be relatively ineffective antagonists of the ATPase reaction. Consistent with this model, we found that isoquinolinesulfonamide ATP antagonists (Hikada et al., 1984) inhibited the ATPase and histone kinase activities (albeit with somewhat different potencies), whereas the peptide substrate antagonist PKC-(19–36) (House & Kemp, 1987) inhibited only the basal ATPase activity associated with purified PKC and had no effect on the Ca^{2+}- and PS-dependent ATPase activity. This was despite the fact that PKC-(19–36) is a potent inhibitor of PKC-catalyzed Ca^{2+}- and PS-dependent protein phosphorylation (House & Kemp, 1987). Furthermore, since PKC-(19–36) is a specific PKC inhibitor, its inhibition of the basal ATPase activity also provides evidence that PKC itself catalyzes the ATPase reaction. Taken together, our results provide strong evidence that the ATPase activity associated with purified PKC represents the bond-breaking step of its phosphotransferase reaction.

In this report, we demonstrate that rat brain PKC catalyzes a Ca^{2+}- and PS-dependent ATPase reaction that is about one-fifth as fast as Ca^{2+}- and PS-dependent PKC-catalyzed histone phosphorylation. The basal rate of the ATPase reaction was about 1.5 times that of histone phosphorylation. The mechanism of the ATPase reaction could involve either direct hydrolysis of ATP by water or formation of a stable

phosphoenzyme (PKC–P) followed by its hydrolysis (PKC + P_i). The latter mechanism appears unlikely since [γ-^{32}P]ATP failed to label autophosphorylated PKC. Furthermore, the PKC preparation did not contain contaminating protein phosphatases, excluding the possibility that the ATPase activity represented dephosphorylation of contaminating PKC substrates. Our results therefore suggest that water may effectively compete with protein substrates of PKC for the γ-phosphate of ATP. The modes of stimulation of the ATP hydrolysis and histone phosphorylation activities by Ca^{2+}, PS, and TPA were qualitatively similar, providing evidence that allosteric activation of PKC involves facilitation of the bond-breaking step of the phosphotransferase reaction. Furthermore, cationic–amphiphilic PKC inhibitors, such as melittin and polymyxin B, inhibit PKC activation (Katoh et al., 1982; Mazzei et al., 1982), and we found that these agents inhibited the Ca^{2+}- and PS-dependent histone kinase and ATPase activities of PKC with comparable potencies. These results suggest that PKC inhibition by cationic amphiphiles involves inhibition of ATP hydrolysis. The octadecapeptide PKC-(19–36) is believed to inhibit PKC-catalyzed protein phosphorylation by binding to the peptide substrate binding region of the active site of the enzyme (House & Kemp, 1987). PKC-(19–36) may inhibit the basal ATPase activity of PKC by stabilizing the occlusion of the active site of the enzyme by its regulatory domain. The inability of PKC-(19–36) to inhibit the ATPase activity of activated PKC suggests that the peptide is insufficient to block the region of the active site responsible for ATP hydrolysis on its own.

Studies with PKA provide evidence that the protein substrate histone can accelerate the ATPase reaction of that enzyme (Moll & Kaiser, 1976). In this paper, we report that although the catalytic fragment of PKC is an efficient phosphotransferase catalyst, it is relatively ineffective as an ATPase. Our results are consistent with a model in which PKC phosphoacceptor substrates and the pseudosubstrate region of the regulatory domain of PKC (which contains a sequence that is closely related to PKC substrates) (House & Kemp, 1987) accelerate the ATPase reaction of PKC. Thus, the rate of ATP hydrolysis would be slowed in the absence of the regulatory domain and PKC phosphoacceptor substrates, i.e., in standard ATPase assays of the catalytic fragment of PKC. Studies are now in progress to determine the effects of PKC phosphoacceptor substrates on the rate of the PKC-catalyzed ATPase reaction.

According to sequence analysis, the catalytic domains of PKC and PKA are closely related (Hanks et al., 1988). In addition to phosphotransferase reactions, PKA catalyzes a cAMP-dependent ATPase reaction (Moll & Kaiser, 1976; Armstrong et al., 1979). In this report, we show that PKC catalyzes an ATPase reaction with metal cofactor requirements similar to those observed with PKA. However, while the ATPase and phosphotransferase activities of PKA appear to be regulated by cAMP by the same mechanism (Armstrong et al., 1979), the mechanisms of regulation of these activities of PKC appear to be related but not identical. The discrepancies between the apparent modes of activation of the ATPase and phosphotransferase activities of PKC cannot be ascribed to the introduction of a protein substrate into the reaction mixtures, since the regulation of the ATPase reaction catalyzed by PKC that we report here differs markedly in its properties from the regulation of PKC autophosphorylation (Huang et al., 1986).

In conclusion, our results provide strong evidence that PKC catalyzes an ATPase reaction that represents the bond-breaking step of its phosphotransferase reaction. Use of this partial reaction in the analysis of PKC catalysis may shed further light on the active-site chemistry of PKC and on the allosteric regulation of its phosphotransferase activity.

Acknowledgments

We thank Patherine Greenwood for her secretarial assistance in preparing the manuscript.

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