myces, suggesting a broader distribution of this regulatory mechanism among bacterial species. In addition, activation has now been extended to slow as well as resistant sites.

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Stimulation of the ATPase Activity of Rat Brain Protein Kinase C by Phospho Acceptor Substrates of the Enzyme†

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ABSTRACT: We recently reported that autophosphorylated rat brain protein kinase C (PKC) catalyzes a Ca^{2+}- and phosphatidylserine- (PS-) dependent ATPase reaction. The Ca^{2+}- and PS-dependent ATPase and histone kinase reactions of PKC each had a $K_{m\,app}$(ATP) of 6 μM. Remarkably, the catalytic fragment of PKC lacked detectable ATPase activity. In this paper, we show that subsaturating concentrations of protein substrates accelerate the ATPase reaction catalyzed by PKC and that protein and peptide substrates of PKC induce ATPase catalysis by the catalytic fragment. At subsaturating concentrations, histone III-S and protamine sulfate each accelerated the ATPase activity of PKC in the presence of Ca^{2+} and PS by as much as 1.5-fold. At saturating concentrations, the protein substrates were inhibitory. Poly(L-lysine) failed to accelerate the ATPase activity, indicating that the acceleration observed with histone III-S and protamine sulfate was not simply a result of their gross physical properties. Furthermore, histone III-S induced the ATPase activity of the catalytic fragment of PKC, at both subsaturating and saturating histone concentrations. The induction of ATPase activity was also elicited by the peptide substrate Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val, when the peptide was present at concentrations near its $K_{m\,app}$. The induction of the ATPase activity by the nonapeptide provides strong evidence that the binding of phospho acceptor substrates to the active site of PKC can stimulate ATP hydrolysis. Taken together, our results indicate that PKC-catalyzed protein phosphorylation is inefficient, since it is accompanied by P_i production. Our results also provide support for a model of PKC catalysis in which the binding of the phospho acceptor substrate to the active site of PKC enhances the rate of phospho donor substrate hydrolysis.

Protein kinase C (PKC)[1] consists of a family of closely related Ca^{2+}- and phosphatidylserine- (PS-) dependent protein kinase isozymes that are activated in vivo by the second messenger diacylglycerol (Kikkawa et al., 1989; O'Brian & Ward, 1989a). We recently reported that purified, autophosphorylated rat brain PKC has an intrinsic Ca^{2+}- and PS-dependent ATPase activity. The Ca^{2+}- and PS-dependent histone kinase and ATPase activities of PKC each had a

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[1] Abbreviations: BSA, bovine serum albumin; cAMP, adenosine cyclic 3′,5′-phosphate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; PS, phosphatidylserine; RRKASGPPV, Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val; TCA, trichloroacetic acid; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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$K_{m\,app}$(ATP) value of 6 μM and similar metal ion cofactor requirements, providing evidence that the ATPase reaction may represent the bond-breaking step of the protein kinase reaction (O'Brian & Ward, 1990). In addition, agents that inhibit PKC-catalyzed protein phosphorylation, including polymyxin B (Mazzei et al., 1982) and melittin (Katoh et al., 1982; O'Brian & Ward, 1989b), also inhibited the ATPase reaction (O'Brian & Ward, 1990). Compared with the histone III-S kinase activity of PKC, the amount of PKC-catalyzed ATPase activity observed in the absence of protein substrates was substantial (O'Brian & Ward, 1990). Remarkably, the catalytic fragment of PKC, which is a fully active Ca^{2+}- and PS-independent protein kinase generated by limited proteolysis (Inoue et al., 1977), lacked detectable ATPase activity (O'Brian & Ward, 1990).

The cAMP-dependent protein kinase (PKA) also has an intrinsic ATPase activity (Moll & Kaiser, 1976; Sugden et al., 1976; Armstrong et al., 1979). As in the case of PKC, the metal ion requirements and $K_{m\,app}$(ATP) of PKA are similar for the ATPase and protein kinase activities of that enzyme (Armstrong et al., 1979). Furthermore, the ATPase reaction is stimulated by cAMP (Armstrong et al., 1979) and inhibited by the inhibitor protein of PKA (Whitehouse & Walsh, 1983) and by active site directed oligopeptide inhibitors of PKA (Salerno et al., 1990).

Since the substrate histone II-S (200 μg/mL) accelerates the ATPase reaction catalyzed by the type I bovine brain PKA holoenzyme (Moll & Kaiser, 1976), it has been hypothesized that, in general, protein substrates bound at the active site of PKA might facilitate the formation of the active site geometry necessary for phosphoryl transfer to protein substrates or to water (Armstrong et al., 1979). On the basis of our recent observations that PKC catalyzes an ATPase reaction and that its catalytic fragment does not, we have hypothesized that the ATPase activity of PKC may be accelerated by PKC phospho acceptor substrates and by the pseudosubstrate region of the regulatory domain of PKC (which contains a sequence that is closely related to PKC substrates) (O'Brian & Ward, 1990). In this paper we demonstrate that, at subsaturating concentrations, protein substrates accelerate the ATPase reaction catalyzed by PKC and that both subsaturating and saturating concentrations of protein and peptide substrates induce ATPase catalysis by the catalytic fragment of PKC. Our results indicate that PKC-catalyzed protein phosphorylation is inefficient, since it is accompanied by P_i production. Our results also provide support for a model of PKC catalysis in which the binding of the phospho acceptor substrate to the active site of PKC can enhance the rate of phospho donor substrate hydrolysis.

Experimental Procedures

Materials. Histone III-S, grade X protamine sulfate (salmine), poly(L-lysine) hydrobromide (15 000–30 000 Da), fatty acid free bovine serum albumin, phenylmethanesulfonyl fluoride, TPCK-treated trypsin from bovine pancreas (10 000–13 000 BAEE units/mg of protein), DEAE Sephacel, PS, ATP, 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 (0.5–3 Ci/mmol) was obtained from Amersham Corp. (Arlington Heights, IL), and frozen rat brains were from Pelfreez (Rogers, AR). The synthetic peptides Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val and [Ser 25]PKC(19–31) were purchased from Peninsula Labs (Belmont, CA).

Purification and Phosphotransferase Assay of Rat Brain PKC. Rat brain PKC was purified to near homogeneity according to silver-stained polyacrylamide gels as previously described in a procedure that entailed elution of PKC from melittin agarose with MgATP (O'Brian & Ward, 1989b, 1990). The histone kinase activity of the PKC preparation was stimulated approximately 10-fold by 1 mM Ca^{2+} and 30 μg/mL PS but was not stimulated by either Ca^{2+} or PS alone. The preparation did not incorporate ^{32}P upon exposure to Mg[γ-^{32}P]ATP under standard phosphotransferase conditions in the absence of histone III-S, indicating that PKC was fully autophosphorylated (O'Brian & Ward, 1990).

The histone kinase activity of PKC was assayed as previously described (O'Brian et al., 1984) in reaction mixtures containing 20 mM Tris-HCl, pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$ (or 1 mM EGTA), 60 μg/mL PS (or none), 6 μM [γ-^{32}P]ATP (3500–6000 cpm/pmol), 0.67 mg/mL histone III-S, and purified rat brain PKC. In indicated cases, the substrate histone III-S was replaced by protamine sulfate. PKC was added to the reaction mixtures to initiate the reactions, which proceeded from 5 to 10 min at 30 °C with linear kinetics. Reactions were terminated by pipetting an aliquot of the reaction mixture onto phosphocellulose paper. The radioactivity incorporated into the protein or peptide substrate was then measured as previously described (O'Brian et al., 1984).

Generation of a Catalytic Fragment of PKC. In indicated experiments, a fully active, catalytic fragment of PKC was generated by previously described methods (O'Brian & Ward, 1989b,c). Briefly, equal volumes of 1300 units/mL trypsin in 20 mM Tris-HCl, pH 7.5, and purified PKC (10–20 nmol of ^{32}P min^{-1} mL^{-1}) were incubated together for 30 min at 4 °C, and proteolysis was terminated by the addition of PMSF (final concentration = 1 mM). The histone kinase activity of the proteolyzed PKC preparation was stimulated less than 2-fold by Ca^{2+} and PS, and the overall yield of activity was reproducibly >50% (O'Brian & Ward, 1989b,c).

ATPase Assay of PKC. The ATPase activity of rat brain PKC was assayed according to a previously described protocol (Pollard & Korn, 1973; O'Brian & Ward, 1990). ATPase assay 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), 60 μg/mL PS (or none), purified rat brain PKC, and 10 μM [γ-^{32}P]ATP (4000–6500 cpm/pmol). In indicated experiments, one of either histone III-S, protamine sulfate, Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val, poly(lysine), or BSA was included in the ATPase reaction mixtures. Reactions were initiated by adding [γ-^{32}P]ATP to the assay mixtures and were allowed to proceed for 20 min at 30 °C, which is a time interval that yields linear kinetics (O'Brian & Ward, 1990). Reactions were terminated on ice by the addition of 10 μL of 140 mM EDTA. [^{32}P]P_i was extracted from the reaction mixtures by vortexing 2 mL of 1:1 2-butanol–benzene and 0.5 mL of 3 N H_2SO_4 containing 4% silicotungstic acid together briefly, adding the resultant mixture to each reaction mixture, followed by brief vortexing, and then adding 0.2 mL of 10% ammonium molybdate to the resultant mixtures, followed by vortexing for 15 s. After the phases separated, 0.6 mL of the organic phase was transferred to a scintillation vial, 5 mL of Aquasol was added to each vial, and the samples were counted.

Results

The intrinsic ATPase activity of PKC (O'Brian & Ward, 1990) provides a means of analyzing the influence of protein substrates on the rate of PKC-catalyzed consumption of its

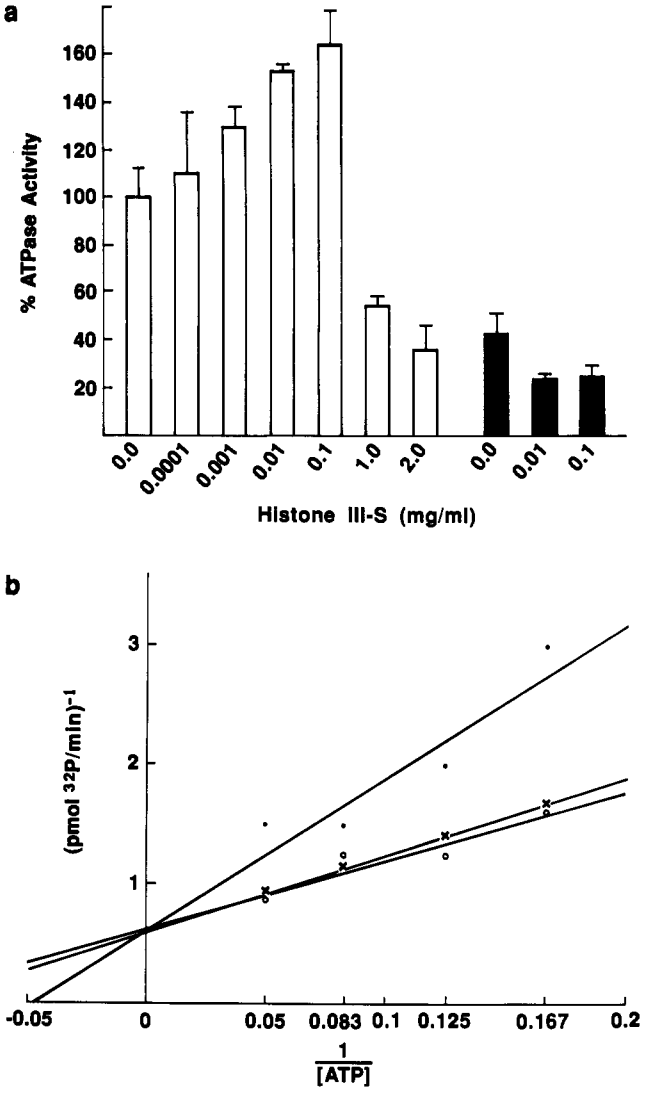

FIGURE 1: Regulation of the ATPase activity of PKC by the phospho acceptor substrate histone III-S. (a) The ATPase activity of PKC was assayed as described in Experimental Procedures in the presence of indicated concentrations of histone III-S . At 100%, the ordinate (% ATPase activity) represents the level of activity observed in the presence of Ca^{2+} and PS and in the absence of histone III-S (100% ATPase activity = 1.03 ± 0.12 pmol of $[^{32}P]P_i$/min). Open bars = assays done in the presence of 1 mM Ca^{2+} and 60 μg/mL PS. Solid bars = assays done in the presence of Ca^{2+} alone. Each bar represents an average of triplicate determinations. (b) The rates of the total ATPase activity (Ca^{2+}- and PS-dependent and basal components) of PKC in the presence of varying histone III-S concentrations were determined in assay mixtures containing 1 mM Ca^{2+} and 60 μg/mL PS. pmol of ^{32}P/min represents the rate of formation of $[^{32}P]P_i$, and [ATP] represents the micromolar concentration of $[\gamma\text{-}^{32}P]$ATP present in the assays. Each point represents an average of triplicate determinations: (●) no histone III-S; (○) 0.01 mg/mL histone III-S; (×) 0.05 mg/mL histone III-S. Linear correlation coefficients were 0.912 (no histone), 0.939 (0.01 mg/mL histone), and 0.999 (0.05 mg/mL histone). For experimental details, see Experimental Procedures.

phospho donor substrate. Figure 1a (open bars) shows that the PKC substrate histone III-S accelerates the ATPase activity of PKC in the presence of Ca^{2+} and PS at histone concentrations of 1–100 μg/mL but inhibits the ATPase activity at concentrations of 1–2 mg/mL. A comparison of Figure 1a with the histone kinase activity of PKC indicates that subsaturating concentrations of histone III-S (1–100 μg/mL histone III-S) stimulate the ATPase activity in the presence of Ca^{2+} and PS and that saturating histone concentrations (1–2 mg/mL histone III-S) inhibit the activity (data not shown). In the reaction mixtures containing 1–100 μg/mL histone III-S, <15% of the substrate $[\gamma\text{-}^{32}P]$ATP was consumed in the presence of Ca^{2+} and PS by the PKC-catalyzed histone kinase and ATPase reactions. However, 27 ± 3% and 26 ± 3% of the $[\gamma\text{-}^{32}P]$ATP were consumed under these conditions when histone III-S was present at concentrations of 1 and 2 mg/mL, respectively. To determine whether the inhibition of the ATPase activity by saturating histone could be accounted for by the depletion of $[\gamma\text{-}^{32}P]$ATP from the reaction mixtures, we measured the effects of 1 and 2 mg/mL histone III-S on the rate of the ATPase reaction in the presence of 33 μM $[\gamma\text{-}^{32}P]$ATP, in order to ensure that the ATP concentration in the reaction mixture would still be much larger than the $K_{m\,app}$(ATP) value of the ATPase reaction (6 μM) at the end of the reaction time course. The ATPase activity was inhibited 28 ± 7% and 52 ± 10% by 1 and 2 mg/mL histone III-S, respectively, despite the fact that approximately 20 μM $[\gamma\text{-}^{32}P]$ATP was present in each reaction mixture after the termination of the reaction. Thus, inhibition of the ATPase reaction by saturating histone appears to entail more than simply consumption of the substrate ATP. To test whether subsaturating concentrations of histone III-S stimulated the ATPase activity of PKC simply by stabilizing the enzyme in solution, we examined the effects of bovine serum albumin (BSA) on the ATPase activity. BSA had no effect on the ATPase activity at BSA concentrations of 0.1 μg/mL–1.0 mg/mL (data not shown).

In contrast with the effects of histone III-S on the ATPase activity of PKC in the presence of Ca^{2+} and PS, subsaturating histone III-S concentrations inhibited the basal ATPase activity that is observed in the presence of Ca^{2+} alone (solid bars) (Figure 1a). Similar inhibitory effects were achieved by histone III-S when the basal ATPase activity was measured in reaction mixtures that contained 1 mM EGTA instead of $CaCl_2$ (data not shown). This inhibitory activity of histone III-S could not be accounted for by depletion of $[\gamma\text{-}^{32}P]$ATP in the reaction mixtures, since less than 2% of the $[\gamma\text{-}^{32}P]$ATP was consumed by the ATPase and histone kinase reactions under these conditions (data not shown). We previously reported a marked difference between the degrees of activation of the ATPase and histone kinase activities of PKC by Ca^{2+} and PS (O'Brian & Ward, 1990). In that report, the ATPase activity was monitored in the absence of histone III-S, and the histone kinase activity was measured with 0.67 mg/mL histone III-S. However, a comparison of the Ca^{2+}- and PS-mediated stimulation of the ATPase activity of PKC in the presence of 0.1 mg/mL histone III-S (Figure 1a) and the stimulation of the histone kinase activity of PKC by these cofactors (data not shown) indicates that both reactions are activated by Ca^{2+} and PS approximately 6.5-fold under these conditions.

We next analyzed the kinetics of the acceleration of the ATPase activity by subsaturating levels of histone III-S in the presence of Ca^{2+} and PS. Figure 1b shows that histone III-S reduced the $K_{m\,app}$(ATP) for the ATPase reaction but did not detectably affect the $V_{max\,app}$ value. Previously, we reported that the $K_{m\,app}$(ATP) for the Ca^{2+}- and PS-dependent component of the PKC-catalyzed ATPase reaction is 6 μM (O'Brian & Ward, 1990). In this report, we determined from the kinetic analyses shown in Figures 1b and 2b that the $K_{m\,app}$(ATP) for the total ATPase activity of PKC observed in the presence of Ca^{2+} and PS (Ca^{2+}- and PS-dependent ATPase activity plus basal ATPase activity) was 28 ± 6 μM in the absence of protein substrates. Thus, the inclusion of the basal component in the kinetic analysis of the ATPase activity of Ca^{2+}- and PS-stimulated PKC resulted in an ap-

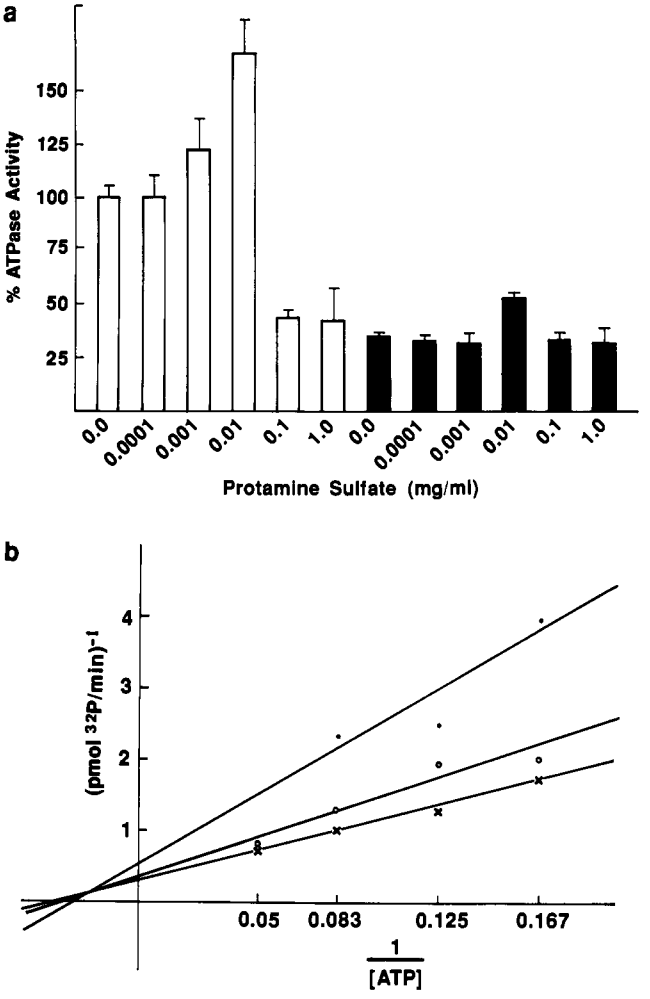

FIGURE 2: Regulation of the ATPase activity of PKC by the phospho acceptor substrate protamine sulfate. (a) The ATPase activity of PKC was assayed as described in Experimental Procedures in the presence of indicated concentrations of protamine sulfate (salmine). At 100%, the ordinate (100% ATPase activity, 0.95 ± 0.06 pmol of $[^{32}P]P_i$/min) represents the level of activity observed in the presence of Ca^{2+} and PS and in the absence of protamine sulfate. Open bars = assays done in the presence of 1 mM Ca^{2+} and 60 μg/mL PS. Solid bars = assays done in the presence of 1 mM EGTA. Each bar represents an average of triplicate determinations. (b) The rates of the total ATPase activity of PKC were determined in the presence of varying protamine sulfate concentrations in assay mixtures that contained 1 mM Ca^{2+} and 60 μg/mL PS. pmol of ^{32}P/min represents the rate of formation of $[^{32}P]P_i$, and [ATP] represents the micromolar concentration of [γ-^{32}P]ATP. Each point is an average of triplicate determinations: (●) 0 μg/mL protamine sulfate; (○) 1 μg/mL protamine sulfate; (×) 10 μg/mL protamine sulfate. Linear correlation coefficients were 0.901 (no protamine sulfate), 0.949 (1 μg/mL protamine sulfate), and 0.991 (10 μg/mL protamine sulfate).

parent reduction in the potency of the substrate ATP. Figure 1b indicates that histone III-S reduced the $K_{m\,app}$(ATP) value of the total ATPase activity to 9 and 11 μM, at histone III-S concentrations of 10 and 50 μg/mL, respectively.

Protamine sulfate is a unique PKC substrate insofar as it is phosphorylated in a Ca^{2+}- and PS-independent manner (Takai et al., 1979). Figure 2a shows that 10 μg/mL protamine sulfate accelerated both the Ca^{2+}- and PS-stimulated ATPase activity of PKC and the basal ATPase activity observed in the absence of Ca^{2+} and PS. In each case, the ATPase activity was accelerated by 10 μg/mL protamine sulfate approximately 1.6-fold, and 10 μg/mL protamine sulfate was approximately one-fourth as effective as Ca^{2+} and PS in stimulating the basal ATPase activity (Figure 2a). The acceleration of the ATPase activity in the presence of Ca^{2+} and PS was not simply a consequence of the effects of protamine sulfate on the basal activity of the enzyme, since the magnitude of acceleration was about 4 times greater in the presence of Ca^{2+} and PS than in the absence of those cofactors (Figure 2a). As in the case of histone III-S, subsaturating concentrations of the substrate protamine sulfate (0.01 mg/mL protamine sulfate) stimulated the ATPase activity, and saturating concentrations of protamine sulfate (0.1–1.0 mg/mL protamine sulfate) inhibited the ATPase reaction in the presence of Ca^{2+} and PS (data not shown). [γ-^{32}P]ATP was consumed to the extents of 38 ± 3% and 49 ± 4% in reaction mixtures containing Ca^{2+} and PS, in the presence of 0.1 and 1.0 mg/mL protamine sulfate, respectively, whereas <15% of the [γ-^{32}P]ATP was consumed in the presence of ≤10 μg/mL protamine sulfate (data not shown). To determine whether the inhibition of the ATPase reaction exerted by protamine sulfate in the presence of Ca^{2+} and PS could be accounted for by depletion of [γ-^{32}P]ATP, we measured the effect of 1.0 mg/mL protamine sulfate on the ATPase reaction in assay mixtures containing 33 μM [γ-^{32}P]ATP. Under these conditions, 1.0 mg/mL protamine sulfate inhibited the ATPase reaction 57 ± 2%, and 8 μM [γ-^{32}P]ATP remained in the reaction mixtures at the end of the assay. Thus, the degrees of inhibition in assays containing 10 μM [γ-^{32}P]ATP (Figure 2a) and 33 μM [γ-^{32}P]ATP were similar, suggesting that inhibition was not simply a consequence of the consumption of ATP. Kinetic analysis of the acceleration of the total ATPase activity of PKC by protamine sulfate in the presence of Ca^{2+} and PS indicated that, at concentrations of 1 and 10 μg/mL, protamine sulfate stimulated the ATPase activity primarily by enhancing the apparent V_{max} value of the ATPase reaction, although it did reduce the apparent K_m value to a minor extent (Figure 2b).

Protamine sulfate and histone III-S are both highly basic polypeptides with molecular weights in the range 10000–20000. Therefore, their abilities to accelerate the ATPase activity of PKC could be a result of their overall physical properties rather than their capacities to serve as PKC substrates. To address this possibility, we examined the effects of poly(L-lysine) (15000–30000) on the ATPase activity, since this synthetic polypeptide resembles histone III-S and protamine sulfate in its overall physical properties but is distinguished from them by its inability to serve as a PKC substrate.

We found that poly(L-lysine) inhibited the ATPase activity of PKC in the presence of Ca^{2+} and PS, at concentrations where protamine sulfate and histone III-S accelerated the reaction (micrograms per milliliter equivalents). Poly(L-lysine) exerted inhibitory effects against both the Ca^{2+}- and PS-stimulated histone kinase and ATPase activities of PKC at poly(L-lysine) concentrations of 0.01–1.0 mg/mL, although the polypeptide was markedly more potent against the histone kinase activity. At concentrations of 0.01, 0.1, and 1.0 mg/mL, poly(L-lysine) inhibited respectively 22 ± 5%, 30 ± 8%, and 45 ± 4% of the ATPase activity of PKC observed in the presence of Ca^{2+} and PS (100% activity = 0.78 ± 0.11 pmol of $[^{32}P]P_i$/min), whereas at each of these concentrations poly(L-lysine) inhibited greater than 95% of the histone kinase activity observed under identical conditions. Furthermore, poly(L-lysine) failed to accelerate the ATPase reaction in the presence of Ca^{2+} and PS across a concentration range of poly(L-lysine) that spanned four logs (0.1 μg/mL–1 mg/mL). These data provide strong evidence that the acceleration of the ATPase activity by histone III-S and protamine sulfate observed in the presence of Ca^{2+} and PS is not simply a consequence of their overall physical properties.

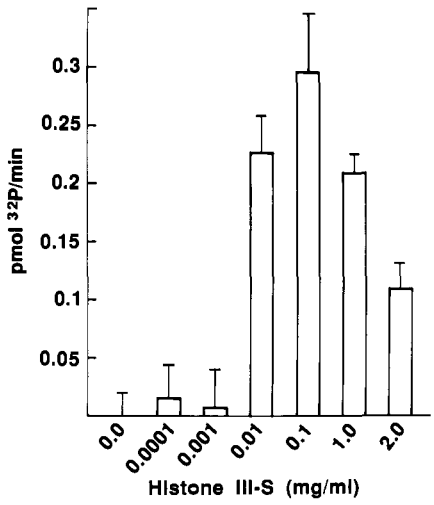

FIGURE 3: Activation of the ATPase activity of the catalytic fragment of PKC by histone III-S. The ordinate (pmol of ^{32}P/min) represents the production of [^{32}P]P_i catalyzed by the catalytic fragment of PKC in the presence of indicated concentrations of histone III-S. Reaction mixtures lacked Ca^{2+} and PS, and they contained 1 mM EGTA. Each bar represents the average of triplicate determinations. For experimental details, see Experimental Procedures.

We next measured the ATPase activity of the catalytic fragment of PKC in the presence of histone III-S in reaction mixtures that lacked Ca^{2+} and PS and contained 1 mM EGTA. We recently reported that the catalytic fragment lacks a detectable ATPase activity when assayed in the absence of protein substrates (O'Brian & Ward, 1990). Figure 3 shows that, at both subsaturating and saturating concentrations, histone III-S induced the catalysis of detectable levels of ATPase activity by the catalytic fragment of PKC. The induced ATPase activity could not be attributed to catalysis by residual intact PKC, since histone III-S inhibited the ATPase activity of intact PKC under these reaction conditions. Ca^{2+}- and PS-activated PKC and the catalytic fragment of PKC phosphorylate histone III-S optimally under mutually exclusive conditions (O'Brian & Ward, 1989a). Therefore, our observation that similar concentrations of histone III-S enhance ATPase catalysis by Ca^{2+}- and PS-stimulated PKC and by the catalytic fragment (in the presence of EGTA) provides further evidence that the observed acceleration of ATP hydrolysis by histone III-S results from the productive binding of histone III-S at the protein substrate-binding site of PKC.

The synthetic peptide Arg-Arg-Lys-Ala-Ser-Gly-Pro-Pro-Val (RRKASGPPV) contains the sequence of the major phosphoacceptor site of the PKC substrate histone III-S and is itself phosphorylated by PKC with a $K_{m\,app}$ of 130 μM and a $V_{max\,app}$ value that is approximately one-sixth of the $V_{max\,app}$ observed with histone III-S (O'Brian et al., 1984). We tested the capacity of RRKASGPPV to mimic histone III-S in the acceleration of the ATPase reaction, under the conditions described for Figure 1a. RRKASGPPV (10–400 μM peptide) failed to alter the rate of the ATPase reaction catalyzed by PKC under these conditions (data not shown). However, RRKASGPPV clearly induced detectable levels of the ATPase activity of the catalytic fragment of PKC, and the induction was optimal at peptide concentrations near its $K_{m\,app}$ value (Table I). While the lack of stimulation of the ATPase activity of intact PKC by the peptide may be due to interactions between the peptide and PS or the relative weakness of the peptide as a PKC substrate compared to histone III-S, the stimulation of the ATPase activity of the catalytic fragment by RRKASGPPV provides strong evidence that the binding of phospho acceptor substrates to the active site of PKC can accelerate the rate of PKC-catalyzed phospho donor substrate hydrolysis. Furthermore, we found that the catalytic fragment phosphorylated <1% of RRKASGPPV during the ATPase assay, when the peptide concentration was 200 μM (data not shown), providing strong evidence that the observed acceleration was due to the substrate RRKASGPPV rather than the product RRKA(P)SGPPV.

Table I: Stimulation of the ATPase Activity of the Catalytic Fragment of PKC by RRKASGPPV

peptide concn (μM)	pmol of [^{32}P]P_i/min[a]	peptide concn (μM)	pmol of [^{32}P]P_i/min[a]
0	0.000 ± 0.004	100	0.072 ± 0.005
10	0.030 ± 0.023	200	0.095 ± 0.020
50	0.041 ± 0.009	400	0.058 ± 0.013

[a] Rate of production of [^{32}P]P_i catalyzed by the catalytic fragment of PKC. Peptide concentration is the concentration of RRKASGPPV. Reaction mixtures contained 1 mM EGTA and lacked Ca^{2+} and PS. For experimental details, see Experimental Procedures.

Previously, we determined that the peptide PKC inhibitor [Ala 25]PKC(19–36) (0.5 μM) inhibited approximately 50% of the basal ATPase activity of PKC but had no effect on the Ca^{2+}- and PS-dependent component of the activity (O'Brian & Ward, 1990). For comparison, we tested the effects of a Ser-containing analogue of the inhibitory peptide (House & Kemp, 1987) on the ATPase activity. The PKC substrate [Ser 25]PKC(19–31) ($K_{m\,app}$ = 0.2 μM), at concentrations of 0.05–5.0 μM, failed to accelerate the ATPase reaction under the conditions of Figure 1a, and it induced the ATPase activity of the catalytic fragment of PKC only to about 15% of the extent achieved by histone III-S (data not shown). Thus, [Ser 25]PKC(19–31) may be a particularly efficient PKC substrate.

Discussion

Previously we reported that, in the absence of protein substrates, PKC catalyzes a Ca^{2+}- and PS-dependent ATPase reaction at a rate that is about 5 times slower than the rate of PKC-catalyzed Ca^{2+}- and PS-dependent histone III-S phosphorylation (O'Brian & Ward, 1990). In this report, we demonstrate that PKC-catalyzed peptide and protein phosphorylation reactions are inefficient, since they are accompanied by significant levels of P_i production. We show that, at subsaturating concentrations, the PKC substrates histone III-S and protamine sulfate exacerbate the inefficiency of the protein kinase activity of PKC by accelerating the PKC-catalyzed ATPase reaction (Figures 1 and 2). We found that, under each set of conditions where PKC catalyzed a peptide or protein kinase reaction, the enzyme also catalyzed an ATPase reaction. In the absence of exogenous protein substrates, the catalytic fragment of PKC represents a unique form of the enzyme insofar as it is devoid of protein kinase activity, since it is incapable of autophosphorylation (Newton & Koshland, 1987). We recently reported that, under these conditions, the catalytic fragment also lacked a detectable ATPase activity (O'Brian & Ward, 1990). In this report, we show that the addition of protein and peptide substrates to reaction mixtures containing the catalytic fragment of PKC not only turns on its protein kinase activity but also induces ATPase activity. Taken together, our results show that an ATPase reaction is associated with the protein kinase activity of PKC under diverse reaction conditions, providing evidence that the ATPase activity of PKC is an essential feature of its protein kinase activity.

This is the first report to show that a synthetic peptide substrate of a protein kinase can accelerate the rate of protein kinase catalyzed production of ADP + P_i from ATP. While

Moll and Kaiser (1976) previously reported that histone II-S accelerates the ATPase reaction catalyzed by a type I PKA holoenzyme, our study of PKC demonstrates that the ability to stimulate the ATPase reaction of PKC and/or its catalytic fragment is not a property unique to the substrate histone but is instead a property shared by diverse phosphoacceptor substrates of PKC, since it is observed with protamine sulfate, histone III-S, and the synthetic peptides [Ser 25]PKC(19–31) and RRKASGPPV. However, acceleration of the ATPase activity of PKC by histone III-S and protamine sulfate was observed only at subsaturating concentrations of the protein substrates, and the substrates were actually inhibitory at saturating concentrations. Depletion of the phospho donor substrate and product inhibition may contribute to the inhibition of the ATPase activity by saturating protein substrates, but they cannot completely account for the inhibition. Thus, it is possible that the proteins are intrinsically more efficient substrates at saturating concentrations. Our results with subsaturating protein substrates are not complicated by substrate depletion and product inhibition and are therefore amenable to a straightforward interpretation.

The acceleration of the ATPase activity of PKC by subsaturating protein substrates is consistent with a model of protein kinase catalysis proposed by Whitehouse et al. (1983) in a study of the kinetic mechanism of PKA catalysis. In that model, the binding of a peptide substrate to the active site of PKA induces a conformational change in the terminal anhydride of ATP that facilitates the transfer of the terminal phosphate of ATP to the peptide (peptide kinase reaction) and also to water (ATPase reaction). Our observation that both subsaturating and saturating concentrations of histone III-S induce the ATPase activity of the catalytic fragment of PKC is also consistent with this model. Further studies are needed, however, to test the relevance of this model to PKC catalysis. Kinetic studies of PKC-catalyzed phosphorylation of a defined peptide substrate are needed to determine whether the kinetic mechanisms of PKA (Whitehouse et al., 1983) and PKC catalysis are comparable. It should be noted that a meaningful comparison of the kinetic mechanisms of PKC-catalyzed histone phosphorylation (Hannun & Bell, 1990) and PKA-catalyzed histone phosphorylation cannot be made, because studies of PKA-catalyzed histone phosphorylation have yielded inconsistent results [for review, see Whitehouse et al. (1983)]. Since the sequences of PKC-encoding cDNAs indicate that PKC may have two functional ATP-binding sites (Coussens et al., 1986; Kikkawa et al., 1987), it is also necessary to ascertain whether the ATPase activity of PKC is catalyzed exclusively at the active site of the enzyme. Therefore, the determination of whether the recently described mutated form of PKCα, which contains an Arg in place of the active site residue Lys 368 (Ohno et al., 1990), has an ATPase activity could be particularly informative.

While diverse phospho acceptor substrates of PKC accelerate its ATPase reaction under certain conditions, the PKC inhibitors [Ala 25]PKC(19–36) (House & Kemp, 1987; O'Brian & Ward, 1990) and poly(L-lysine) (Bazzi & Nelsestuen, 1987), which structurally resemble and compete with phospho acceptor substrates of PKC, do not. Our observations that [Ala 25]PKC(19–36) and poly(L-lysine) inhibit the ATPase activity of PKC parallel observations that the ATPase activity of PKA is inhibited by the inhibitor protein of PKA (Whitehouse & Walsh, 1983) and by active site directed oligopeptide inhibitors of PKA (Salerno et al., 1990). Taken together with the striking homology observed between the catalytic domains of PKA and PKC (Hanks et al., 1988), these observations strongly suggest that the mechanisms of PKC and PKA catalysis are closely related.

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