Ca^{2+}/Calmodulin-Dependent Protein Kinase II Is Phosphorylated by Protein Kinase C in Vitro†

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ABSTRACT: Protein kinase C (PKC) phosphorylated a synthetic peptide (CBP) that included the Thr-286 phosphorylation sequence and calmodulin binding domain of Ca^{2+}/calmodulin-dependent protein kinase type II (CaM-kinase). Studies with a variety of truncated peptides suggested that the amino acid phosphorylated by PKC was Thr-286, the same amino acid that when autophosphorylated by Ca^{2+}/calmodulin activation of CaM-kinase results in Ca^{2+}/calmodulin-independent activity. These peptide studies also suggested that the C-terminal region of CBP is required to obtain maximal phosphorylation of Thr-286 by PKC. PKC also phosphorylated purified CaM-kinase from rat forebrain. Phosphopeptide analysis by one- and two-dimensional proteolytic maps of autophosphorylated CaM-kinase and CaM-kinase phosphorylated with PKC identified that there are both similar and unique sites phosphorylated. Phosphoamino acid analysis of CaM-kinase phosphorylated by PKC indicated that both Ser and Thr residues were phosphorylated. Even though Thr-286 of CaM-kinase appeared to be phosphorylated by PKC, no Ca^{2+}/calmodulin-independent activity was detected, and, additionally, no significant change in Ca^{2+}/CaM-dependent activation was detected. These results provide the first indication that these two important protein kinases may communicate directly through interenzyme phosphorylation.

Protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent protein kinase II (CaM-kinase) are responsible for a large proportion of the Ca^{2+}-dependent protein phosphorylation in brain and other tissues. Each kinase is dependent on specific second messengers for activation; PKC is activated by Ca^{2+}, phosphatidylserine, and diacylglycerol, while CaM-kinase is activated by Ca^{2+} and calmodulin (CaM). Each of these kinases, when activated, phosphorylates its own family of protein substrates on Ser and Thr residues [for review, see Schulman (1991)]. Studies with synthetic peptide substrates suggest that the minimum recognition sequence for phosphorylation between these two kinases are similar but have some distinctions. PKC prefers the amino acid recognition sequence -Arg-X-X-Ser/Thr-X-Arg- while CaM-kinase prefers the sequence -Arg-X-X-Ser/Thr- (Kemp & Pearson, 1990), although exceptions to this generality are known [for example, see Miller et al. (1988)]. Therefore, the potential seems to exist for PKC and CaM-kinase to phosphorylate similar amino acid sequences, although few instances of this case have been reported [however, see Hashimoto and Soderling (1987) and Haycock (1990)]. Both PKC and CaM-kinase autophosphorylate after activation, and some studies suggest that autophosphorylation must occur in both enzymes prior to other substrates being phosphorylated (Borner et al., 1989; Kwiatkowski et al., 1988). One of the growing number of unique properties of CaM-kinase autophosphorylation (on amino acid Thr-286 of the 50-kDa subunit) is the production of a Ca^{2+}/CaM-independent form of the kinase. Detailed studies on the autophosphorylation of Thr-286 along with the switch to a Ca^{2+}/CaM-independent form have been the focus of numerous studies over the last several years (Lickteig et al., 1988; Lou & Schulman, 1989; Miller & Kennedy, 1986; Miller et al., 1988; Theil et al., 1988; Waxham et al., 1990).

The analysis of second messenger regulation of cellular processes has in the past focused on the specificity of changes induced by activation of selective pathways. However, several recent papers have appeared suggesting that cross-talk between different intracellular signaling pathways occurs, adding a new layer of complexity to the regulation of intracellular processes. For example the production of IP_3, by activation of phospholipase C, causes liberation of Ca^{2+} from intracellular stores, and Ca^{2+} acting through CaM activates Ca^{2+}/CaM-dependent protein kinases as well as one subtype of adenylate cyclase, leading to increased cAMP levels and activation of cAMP-dependent protein kinase [PKA; see Yoshimasa et al. (1987), MacNicol et al. (1990), and MacNicol and Schulman (1992)]. PKC has also been shown to phosphorylate the catalytic subunit of adenylate cyclase both in vitro and in vivo, providing another example for cross-talk between cellular signaling pathways (Yoshimasa et al., 1987). Another established route of communication between second messenger activated protein kinase systems is when one kinase directly phosphorylates and regulates the second (Bading & Greenberg, 1991; Conti & Adelstein, 1981; Ikebe et al., 1985; Gomez & Cohen, 1991; Kelly & Schenolikar, 1988; Ramachandran et al., 1987; Roach, 1991). In this report, we provide results that PKC can directly phosphorylate CaM-kinase, thus providing initial evidence that this type of direct cross-talk between these two important kinases can be observed. This finding raises an interesting possibility that studies addressing presumed PKC-mediated phosphorylation (e.g., by addition of phorbol esters) may, in fact, be measuring an indirect response via some phosphorylation mediated change in CaM-kinase activity.

EXPERIMENTAL PROCEDURES

Kinase Purification. CaM-kinase was purified from rat forebrain as previously described (Kelly & Shenolikar, 1987) with the exception that gel filtration was omitted. No activity was detected (either autophosphorylation or peptide substrate

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Table I

synthetic peptide		PKC phosphorylation (K_m ± SEM)
-MHRQE$\overset{286}{\underset{*}{T}}$VDCLKKFNARRKLKGAILTTMLATRNFSGGK-	(50-kDa sequence)	
MHRQETVDCLKKFNARRKLKGAILTTMLA	(CBP)	~4 μM ± 2 μM
.....QETVDCLKKFNARRKLKGAILTTMLA	(CBP_{-3})	ND[a]
MHRQETVDCLKKFNARRKLKGA.....	(MB-I)	~40 μM ± 8 μM
MHRQETVDCLKKFNA.....	(CK–II LONG)	~60 μM ± 14 μM
MHRQETVDG.....	(NTP)	ND[a]
MHRQEAVDCLKKFNARRKLKGA.....	(MB-II)	ND[a]

[a] ND, not detectable; these peptides were not phosphorylated to detectable levels in these assays.

phosphorylation) when Ca^{2+}/phosphatidylserine/diacylglycerol (Ca^{2+}/PS/DAG) was added to the purified CaM-kinase preparation. Three separate preparations of purified CaM-kinase were used in these studies, and experiments with each produced similar results (two of the preparations were from our laboratory and the third was kindly provided by Dr. Howard Schulman). PKC was purified from rat brains essentially as described by Wooten et al. (1987). Briefly, forebrains from adult rats were homogenized for 2 min at 4 °C in buffer with protease inhibitors using a polytron. The soluble fraction, obtained by ultracentrifugation at 100000*g* for 1 h at 4 °C, was applied to a DEAE-Sephacel column, and the PKC was eluted with a 0.0–0.3 M linear gradient of NaCl in buffer. The fractions containing PKC activity that eluted between 0.1 and 0.2 M NaCl were combined, made to a final concentration of 1.5 M NaCl, and then applied to a Phenyl-Sepharose column. The column was washed and then eluted with a 0.6–0.0 M NaCl linear gradient, followed by an additional 10 mL of buffer without NaCl. The PKC peak, eluting near the end of the gradient, was pooled and stored at 4 °C and was used for up to 3 weeks. This fraction contained no detectable Ca^{2+}/CaM-stimulated or cAMP-stimulated protein kinase activity and was used in all assays. Seven individual preparations of PKC were used to analyze the phosphorylation of peptides and CaM-kinase. While the activity was variable between preparations of PKC [0.2–4.0 μmol/(min·mg) using histone as a substrate], the phosphorylation of CaM-kinase and synthetic peptides was seen in every preparation. Purified PKC from rat brain was also obtained from Lipidex [specific activity 1.4 μmol/(min·mg) also using histone as a substrate]. No differences were detected in reactions done with either preparation of PKC except that the Lipidex kinase exhibited minor (<5%) Ca^{2+}/PS/DAG-independent activity.

Phosphorylation Reactions. CaM-kinase was activated by Ca^{2+}/CaM and assayed for activity as previously described (Waxham et al., 1989). PKC activity was determined using either histone type IIIS, histone type H1, or the synthetic peptide substrate syntide (PLRRTLSVAA-amide). Final reaction conditions to measure PKC activity were 5 mM Pipes, pH 6.5, 1.6 mM $CaCl_2$, 16 μM DAG, 200 μM PS, 5 mM $MgCl_2$, 15 μM ATP, and 5–20 μCi of [^{32}P]ATP at 30 °C for 2.5–5 min, unless otherwise noted. Reactions were always initiated by the addition of enzyme. In some experiments, PKCi (a synthetic peptide inhibitor of PKC; RFARKGAL-RQKNV-amide; House & Kemp, 1987) was added to the reactions to verify that phosphorylation was specific to the activity of PKC. Synthetic peptides were added to the reactions as 10× stocks in water. Protein kinase activity was determined by spotting an aliquot of each reaction on P–81 filters and immersing the filters in 75 mM phosphoric acid. After being washed twice more in acid, the filters were rinsed with ethanol and air dried. ^{32}P incorporation was quantitated by counting Cerenkov radiation in a liquid scintillation counter. Kinetic constants were calculated using double-reciprocal plots fitted by a simple linear regression of the data. Phosphorylation of native CaM-kinase was quantitated by excising the 50- or 60-kDa protein bands from one-dimensional SDS gels and counting in a liquid scintillation counter.

Functional Assays. CaM-kinase was phosphorylated by PKC as described above but in the absence of [^{32}P]ATP and in the presence of 1 mg/mL bovine serum albumin to stabilize the kinase. Reactions were done for 5 min at 30 °C, and then each was cooled on ice for 2 min (stage 1). Aliquots of these reactions were then distributed to tubes with [^{32}P]ATP and NTP (MHRQETVD-amide), and reactions were continued at 30 °C for 1–2 min in increasing concentrations of CaM to assay CaM-kinase activity (stage 2). In experiments designed to assay for the production of Ca^{2+}/CaM-independent CaM-kinase activity after PKC phosphorylation, reactions were done with PKC and CaM-kinase in the presence of NTP. Since PKC does not phosphorylate NTP (see below), any Ca^{2+}/CaM-independent activity produced by PKC phosphorylation of CaM-kinase could be measured directly in the same reaction by CaM-kinase phosphorylation of NTP.

Synthetic Peptides and Antiserum. The synthetic peptides used in these studies are listed in Table I along with their amino acid sequences. All peptides were purified by reversed-phase high-performance liquid chromatography as described (Kelly et al., 1988). Immunoprecipitation experiments were accomplished using a rabbit polyclonal antibody produced against the synthetic peptide CBP (Weinberger et al., 1988). Immune complexes were formed using protein A–Sepharose and anti-CBP, and then autophosphorylated or PKC phosphorylated CaM-kinase was added and the complexes were allowed to form at room temperature for 2 h. Unbound radioactivity was removed by washing three times, and the precipitated proteins were analyzed by SDS–PAGE. All incubations and washes were done in NET buffer (0.01 M Tris-HCl, pH 7.4, 0.001 M EDTA, and 0.15 M NaCl) containing 0.1 mM phenylmethanesulfonyl fluoride and 5 mg/L leupeptin. Preimmune rabbit serum was also used in these experiments to control for nonspecific binding of the phosphorylated kinase. PKC-specific antibodies crossreactive with the α, β, and γ isotypes were obtained from Gibco/BRL and were used in western blot analysis exactly as described (Waxham et al., 1989).

Peptide Mapping and Phosphoamino Acid Analysis. One-dimensional phosphopeptide maps were accomplished by limited proteolysis using 3 μg per lane of *Staphylococcus aureus* V8 protease essentially as described (Waxham et al., 1989). Two-dimensional phosphopeptide maps were also

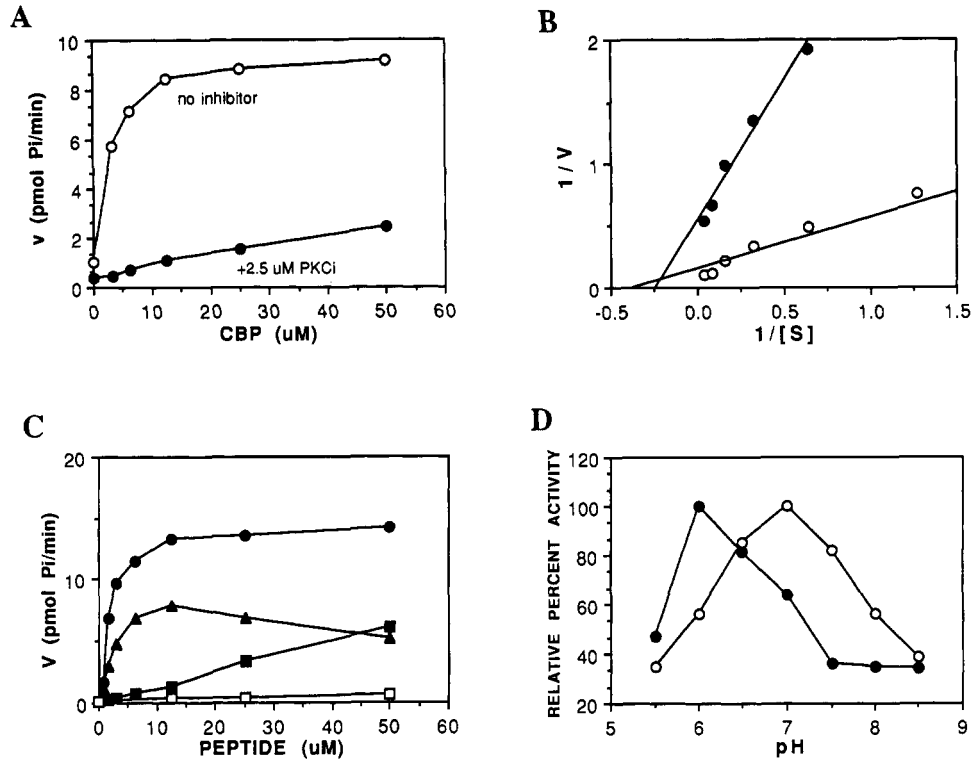

FIGURE 1: (A) CBP phosphorylated by PKC. Increasing concentrations of CBP were phosphorylated by PKC in the presence (●) or absence (○) of 2.5 μM PKC_i. The reactions were accomplished at 30 °C for 2.5 min. Reactions included 5 mM Pipes, pH 6.5, 1.6 mM $CaCl_2$, 16 μM DAG, 200 μM PS, 5 mM $MgCl_2$, 15 μM ATP, and 5 μCi of [^{32}P]ATP. (B) Kinetic analysis of PKC phosphorylation of syntide and CBP. Increasing concentrations of syntide (○) or CBP (●) were phosphorylated by PKC for 2.5 min exactly as described for panel A. The data are presented as a double-reciprocal plot fitted with a simple linear regression. (C) Relative phosphorylation of various peptides by PKC. Phosphorylation reactions were accomplished with increasing concentrations of syntide (●), CBP (▲), CK-II LONG (■), or CBP_{-3} (□). Reactions were for 2.5 min and were accomplished as described for panel A. (D) pH optimum for PKC phosphorylation of synthetic peptides. Phosphorylation reactions were done in HEPES, buffered to the indicated pH. Otherwise reactions were identical to those described for panel A. Relative percent activity for phosphorylation of syntide (○) or CBP (●) by PKC is shown.

accomplished using extensive digestion (16 h) with an excess of thermolysin (100 μg of enzyme per 0.5 μg of protein). Phosphopeptides were separated by electrophoresis in the first dimension and ascending chromatography in the second dimension. Phosphoamino acid analysis was accomplished by first eluting protein from the gel slices with tryptic hydrolysis, and then the peptides were collected (approximately 80% of the radioactivity was recovered) and lyophilized. The peptides were then hydrolyzed with 6 N HCl at 110 °C in vacuo for 6 h. Phosphoamino acids were separated by thin layer chromatography along with unlabeled phospho-Thr and phospho-Ser standards. Phosphoamino acids were visualized with ninhydrin, and [^{32}P]phosphoamino acids were visualized by autoradiography. For quantitation, the spots containing the individual [^{32}P]phosphoamino acids were scraped from the thin layer chromatography plate, and the radioactivity was measured by liquid scintillation spectroscopy.

RESULTS

CBP is a bifunctional 29 amino acid peptide that contains two inhibitory domains. It is both a CaM antagonist and an active-site-directed inhibitor of CaM-kinase and is phosphorylated very poorly, although to detectable levels, by the purified enzyme (Colbran et al., 1988; Kelly et al., 1988). CBP contains the autophosphorylation sequence Arg-Gln-Glu-Thr-286 that, when phosphorylated in native CaM-kinase, produces the switch from Ca^{2+}/CaM-dependent to Ca^{2+}/CaM-independent activity (Lou & Schulman, 1989; Miller et al., 1988; Theil et al., 1988). In an earlier study designed to determine the specificity of CBP inhibition, we found that CBP did not inhibit PKC (Malenka et al., 1989). In fact, PKC phosphorylated CBP in a Ca^{2+}/PS/DAG-dependent manner, and the phosphorylation could be inhibited by including the peptide inhibitor of PKC, PKCi (Figure 1A). No phosphorylation of synthetic peptides was ever detected when these PKC preparations were assayed in the absence of Ca^{2+}/PS/DAG or in the presence of Ca^{2+}/CaM (data not shown). Kinetic analyses showed that CBP was phosphorylated by PKC with a K_m of approximately 4 μM, which was similar to the K_m for the synthetic peptide substrate syntide, 2.6 μM (Figure 1B). However, CBP was phosphorylated with a lower V_{max} than syntide, approximately 0.6 and 1.2 μmol/(min·mg of total protein), respectively. PKC phosphorylation of increasing amounts of CBP often (>75% of the time) exhibited a biphasic curve (Figure 1C) suggesting that at higher concentrations CBP actually inhibits PKC activity. Smith et al. (1990) reported similar findings that CBP could inhibit PKC activity (IC_{50} of 38 μM) in experiments designed to test the specificity of these peptides as inhibitors of different protein kinases. Combined, these experiments suggest that CBP's interactions with PKC are complex and applying simple enzymic kinetic analyses must be interpreted with caution.

In attempts to determine the amino acid phosphorylated in CBP by PKC, a series of truncated peptides were used as substrates in phosphorylation experiments. CBP_{-3}, a peptide lacking three N-terminal amino acids from CBP, was not phosphorylated at all by PKC (see Figure 1C and Table I). CBP_{-3} is missing an Arg upstream from the Thr that together form the consensus sequence for CaM-kinase phosphorylation (Kelly et al., 1988). Since PKC does not phosphorylate CBP_{-3},

the Met-His-Arg sequence also seems necessary to PKC-dependent phosphorylation of CBP and implies that Thr-286 is the amino acid phosphorylated. Additional synthetic peptides were analyzed to further support the notion that Thr-286 in CBP was the amino acid being phosphorylated. In decreasing order of phosphorylation by PKC were CBP, MB-I, and CK-II LONG, although ^{32}P incorporation into MB-I and CK-II LONG was significantly slower than for CBP. Relative K_ms for MB-1 and CK-II long were 40 and 60 μM, respectively, suggesting these peptides had only moderate affinity for PKC. These results could also be explained if another amino acid (Thr-305 or Th-306) was also being phosphorylated by PKC, although the absence of phosphorylation of CBP_{-3}, which includes Thr-305 and Thr-306, argues against this possibility. CBP_{-3} and NTP (up to 50 μM) were not phosphorylated by PKC (Figures 1C and Table I). Also, the substitution of an Ala residue in place of Thr-286 in MB-I, called MB-II, completely eliminated the ability of the peptide to be phosphorylated by PKC (Table I). Together, these peptide phosphorylation experiments suggested that Thr-286 in CBP is the likely amino acid phosphorylated by PKC and that amino acids Thr-305 or Thr-306, other sites autophosphorylated in the 50-kDa subunit of CaM-kinase (Patton et al., 1990), were not phosphorylated. An additional set of experiments was done to determine the pH optimum of CBP phosphorylation by PKC. In contrast to syntide, which has a pH optimum for phosphorylation of approximately 7.2 (Figure 1D), CBP was phosphorylated best under slightly more acidic conditions. The pH optimum for CBP phosphorylation was near 6.0 (Figure 1D). These results suggest that CBP is an optimal substrate for PKC only under mildly acidic conditions and may reflect some change in the structure of the peptide. One possibility is that the His group five amino acids N-terminal to Thr-286 might be responsible for this transition since the side chain of His has a pK of around 6.5. The hypothesis would be that the His group would have to be in the protonated state for maximal PKC phosphorylation. In theory, this would provide additional positive charge near the already positively charged Arg residue that forms part of the consensus phosphorylation sequence. As described below, a similar pH optimum was obtained for the phosphorylation of purified CaM-kinase by PKC.

These peptide studies suggested that CaM-kinase itself might be a substrate for PKC. Figure 2 shows that CaM-kinase 50- and 60-kDa subunits were phosphorylated by PKC in the presence of Ca^{2+}/PS/DAG. No phosphorylation was detected if PKC was left out of the reaction (see below), and no phosphorylation of proteins in the 50-kDa region were phosphorylated in the PKC preparations alone stimulated by Ca^{2+}/PS/DAG (Figure 2, lane b). In addition, no phosphorylation was detected when Ca^{2+}/CaM was added to the PKC preparations (Figure 2, lane c), and no PKC activity was detected in the presence of EGTA (Figure 2, lane a). In addition, no activity was detected when Ca^{2+}/PS/DAG was not added to reactions containing PKC and CaM-kinase (data not shown). Increasing concentrations of CaM-kinase were also used to determine if there was a linear dose–response relationship in the incorporation of ^{32}P into CaM-kinase by PKC. When compared in the same experiment with the same concentrations of CaM-kinase activated by Ca^{2+}/CaM (Figure 2, lanes d, e, and f), PKC phosphorylation of CaM-kinase showed a definite dose–response relationship (Figure 2, lanes g, h, and i). The autophosphorylated PKC provides a convenient internal indication that a constant amount of PKC was added to each reaction. Although these reactions were

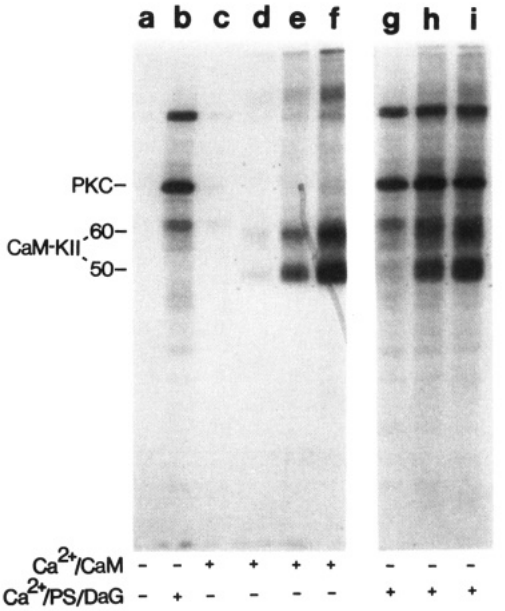

FIGURE 2: Autoradiograph of SDS–PAGE of phosphorylation reactions containing PKC and CaM-kinase. Lanes a–c contained only PKC. Lanes d–f contained only CaM-kinase, and lanes g–i contained both PKC and CaM-kinase. A constant amount (10 μL) of PKC was used in each reaction. The final concentration of CaM-kinase in these reactions (50 μL final reaction volume) was 20, 40, and 80 nM in lanes d and g, lanes e and h, and lanes f and i, respectively. Reactions were accomplished at 30 °C for 5 min. The locations of PKC and the 60- and 50-kDa subunits are indicated on the left of the autoradiograph. PKC was identified in these preparations by western blot analysis. Autoradiographic exposure was for 2 h.

difficult to saturate for CaM-kinase substrate, an estimated K_m for CaM-kinase phosphorylation by PKC was approximately 5–10 μM. Very similar results were obtained using CaM-kinase provided by Dr. Howard Schulman (data not shown). As noted above, we also found that a pH optimum exists for PKC phosphorylation of CaM-kinase. The optimum was pH 6.5, which decreased 4-fold when reactions were done at pH 7.5 (data not shown). Converse reactions were also accomplished activating CaM-kinase with Ca^{2+}/CaM in the presence of PKC. Results from these experiments indicated that PKC was not phosphorylated to detectable levels by CaM-kinase (data not shown).

With fresh preparations of PKC, CaM-kinase could be phosphorylated to the same level as that attained by autophosphorylation at 4 °C (compare Figure 3, lanes c and d), a protocol that reportedly incorporates about 1 mol of PO_4 per mol of 50-kDa subunit, predominantly on Thr-286 (Theil et al., 1988). In this experiment, the stoichiometry of PKC phosphorylation of CaM-kinase and autophosphorylation at 4 and 30 °C was 0.9, 0.9, and 1.7 mol of PO_4/mol of 50-kDa subunit, respectively. Also, as noted earlier, no phosphorylation is detected when Ca^{2+}/PS/DAG is added to purified CaM-kinase (Figure 3, lane b). The time course of PKC mediated phosphorylation showed a linear incorporation of ^{32}P into the 50- and 60-kDa subunits of CaM-kinase for up to 5 min which plateaued after 10 min (Figure 4B). The rate of PKC-dependent ^{32}P incorporation into CaM-kinase was approximately 0.8 μmol(min·mg) in this experiment. Note also that the autophosphorylation of PKC followed a similar time course to CaM-kinase phosphorylation, further suggesting that CaM-kinase is a relatively good substrate for PKC. Ca^{2+}/CaM-dependent autophosphorylation of CaM-kinase showed a more rapid time course, significant after 0.5 min, which plateaued within 5 min (Figure 4A). Heat denaturation of CaM-kinase (65 °C for 20 min) eliminated the ability of PKC

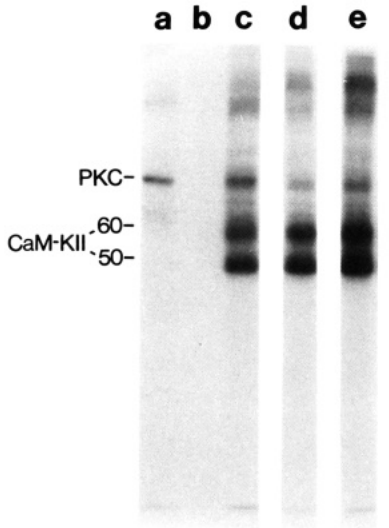

FIGURE 3: Autoradiograph of SDS–PAGE of CaM-kinase autophosphorylated at 4 and 30 °C and phosphorylated by PKC. Lanes a and c contained PKC (10 μL) and lanes b–e contained CaM-kinase (80 nM). Phosphorylation reactions in lanes a–c were activated by Ca^{2+}/PS/DAG. Phosphorylation reactions in lanes d and e were activated by Ca^{2+}/CaM. Phosphorylation was accomplished at 30 °C (lanes a–c and e) or 4 °C (lane d). Autoradiographic exposure was for 30 min.

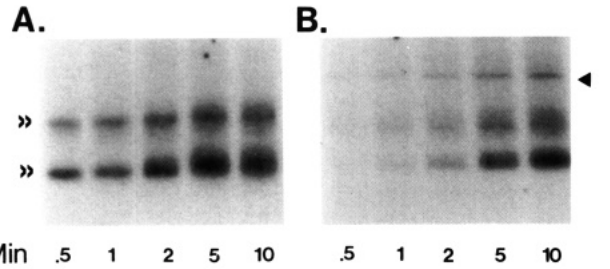

FIGURE 4: Autoradiograph of SDS–PAGE of a time course for phosphorylation reactions. (A) CaM-kinase (80 nM) was activated by Ca^{2+}/CaM and placed at 30 °C for the indicated times. (B) PKC (10 μL/reaction) and CaM-kinase (80 nM) were phosphorylated in the presence of Ca^{2+}/PS/DAG for the indicated times at 30 °C. Reactions were terminated by addition of SDS sample buffer. Double arrowheads on the left side of the figure indicate the location of the 50- and 60-kDa subunits of CaM-kinase. The location of PKC is indicated on the right side of the figure with an arrowhead. The autoradiographic exposure for both panels A and B was for 2 h.

to phosphorylate the enzyme (data not shown), suggesting that PKC was not simply phosphorylating denatured CaM-kinase that might have been present in these preparations.

One-dimensional peptide maps with *S. aureus* V8 protease indicated that there were both similarities and differences in the phosphopeptide patterns of autophosphorylated CaM-kinase and CaM-kinase 60- and 50-kDa subunits phosphorylated by PKC (Figure 5, lanes a–c and d–f, respectively). Ca^{2+}/CaM-dependent autophosphorylation was accomplished at 4 °C in attempts to limit phosphorylation to Thr-286, although there was little difference in the phosphopeptide maps of CaM-kinase autophosphorylated at 30 or 4 °C using this procedure (compare Figure 5, lanes a and b). Slight migration differences were seen in some of the phosphopeptides that appeared to be related to their degree of phosphorylation; however, the family of phosphopeptides identified with this technique were overlapping (compare Figure 5, lanes d–f). The 60-kDa subunit of CaM-kinase mapped after both autophosphorylation and after phosphorylation by PKC (Figure 5 planes a–c) showed that, like the 50-kDa phosphopeptides, there were both similar and unique peptides. In

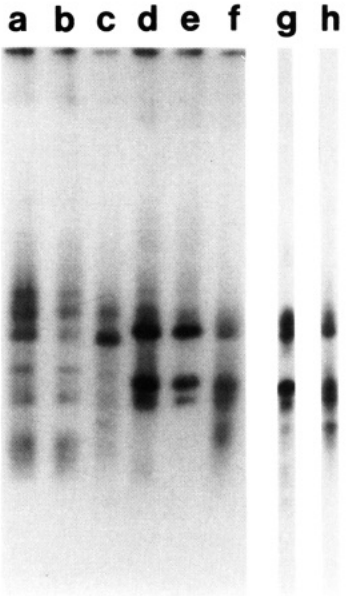

FIGURE 5: Autoradiograph of SDS–PAGE of *S. aureus* V8 protease digests of CaM-kinase phosphorylated by PKC and autophosphorylated CaM-kinase. Phosphorylation reactions were accomplished for 5 min at either 30 °C, lanes a, c, d, f, g, and h, or 4 °C, lanes b and e. The 60-kDa subunit, lanes a–c, or 50-kDa subunit of CaM-kinase, lanes d–h, were digested with *S. aureus* V8 protease (3 μg/lane). Lanes a, b, d, e, and g were from reactions containing only CaM-kinase activated by Ca^{2+}/CaM. Lanes c, f, and h are from reactions containing PKC and CaM-kinase stimulated by Ca^{2+}/PS/DAG. Lanes g and h represent CaM-kinase phosphorylated and then immunoprecipitated before mapping. Autoradiographic exposure was for 30 min (lanes a–f) or 4 or 2 h lanes g and h, respectively.

some of the PKC preparations, variable amounts of another minor phosphoprotein were evident that overlapped slightly with the 60-kDa subunit. The differences noted in the phosphopeptides for the 60-kDa subunit must therefore be interpreted with caution. Similar phosphopeptide maps were reproduced three separate times using two different CaM-kinase preparations and two different PKC preparations. To verify that the 50- and 60-kDa phosphoproteins phosphorylated by PKC were the two subunits of CaM-kinase, we immunoprecipitated PKC phosphorylated CaM-kinase and autophosphorylated CaM-kinase using an anti-synthetic peptide (CBP) antibody. Both subunits were precipitated under these conditions, and the 50-kDa subunits were again mapped by phosphopeptide analysis (Figure 5, lanes g and h). Again overlapping peptide maps were produced similar to those accomplished without immunoprecipitation (compare Figure 5, lanes g and h with lanes d–f).

Two-dimensional phosphopeptide maps of the autophosphorylated (at 4 °C) 50-kDa subunit of CaM-kinase and that phosphorylated by PKC were also compared (Figure 6, panels A and B, respectively). Extensive (16 h) thermolytic digestion was used in an attempt to attain complete proteolysis. Similar to the one-dimensional V8 maps, the two-dimensional thermolytic maps showed both similarities and differences. The diffuse radioactivity near the center of the autoradiograph is found when CaM-kinase is autophosphorylated at 4 °C and presumably represents the phosphopeptide(s) containing the Thr-286 phosphorylation sequence, although we have not verified this by sequence analysis. This diffuse phosphopeptide is also found when PKC is used to phosphorylate CaM-kinase, suggesting again that they have at least one site in common. There are also three distinct phosphopeptides found in the PKC phosphorylated CaM-kinase map marked by black arrowheads in Figure 6. Whether each of these phospho-

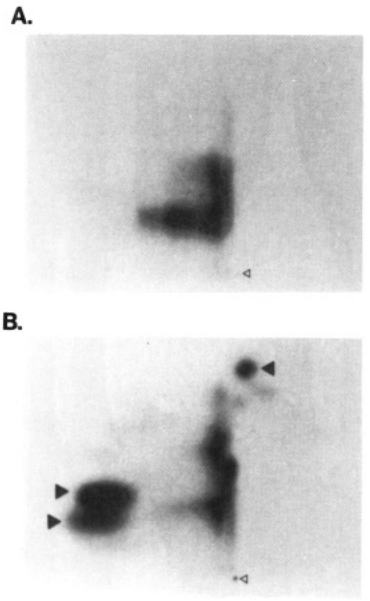

FIGURE 6: Autoradiographs of two-dimensional thermolytic peptide maps. Panel A represents the map of the 50-kDa subunit of CaM-kinase (0.5 μg) autophosphorylated for 5 min at 4 °C in the presence of Ca^{2+}/CaM. The protein isolated from the first dimension SDS gel was excised and digested with 100 μg of thermolysin. The lyophylized peptides were applied to a thin layer chromatography plate, electrophoresed in the first dimension, and separated by ascending chromatography in the second dimension. Panel B represents the map of the 50-kDa subunit of CaM-kinase phosphorylated by PKC for 5 min at 30 °C in the presence of Ca^{2+}/PS/DAG. The small arrowheads indicate the position of sample application in both panels. The large arrowheads in panel B represent the three unique peptides discussed in the text. Autoradiographic exposures were for 8 h.

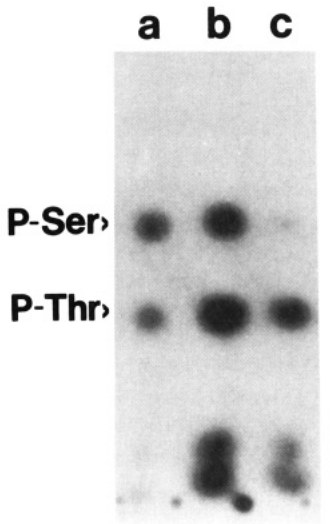

FIGURE 7: Autoradiograph of phosphoamino acids analyzed by thin layer chromatography. Lane a represents the phosphopeptides from the 50-kDa subunit of CaM-kinase phosphorylated by PKC for 5 min at 30 °C in the presence of Ca^{2+}/PS/DAG. Lanes b and c represent the phosphoamino acids from CaM-kinase 50-kDa subunit autophosphorylated for 5 min at either 30 or 4 °C, respectively, in the presence of Ca^{2+}/CaM. The positions of phospho-Ser and phospho-Thr standards, added to each sample, were determined by visualization after ninhydrin staining and are indicated on the left side of the figure.

peptides is unique is unknown. We are currently attempting to investigate the amino acid sequences of the common and unique phosphopeptides produced by PKC phosphorylation of CaM-kinase.

Phosphoamino acid analyses showed that unlike CaM-kinase autophosphorylated at 4 °C, which produced predominantly (90%) phospho-Thr (consistent with the idea that 4 °C phosphorylation is at Thr-286; Figure 7 lane c), CaM-kinase autophosphorylated at 30 °C produced both phospho-Ser and phospho-Thr, approximately 40% and 60%, respectively (Figure 7, lane b). The phospho-Ser to phospho-Thr ratios for PKC phosphorylated CaM-kinase were approximately 50:50 (Figure 7, lane a). These data indicated that both Ser and Thr residues in the CaM-kinase 50-kDa subunit were phosphorylated by PKC and further supports the phosphopeptide mapping data that at least two distinct sites in CaM-kinase were phosphorylated by PKC.

Functionally, autophosphorylation of CaM-kinase on Thr-286 produces an altered form of the enzyme that is independent of Ca^{2+}/CaM for sustained activity. The peptide phosphorylation data showed that Thr-286 might be phosphorylated by PKC, and the peptide mapping data also suggested that Thr-286 might be one of the phosphorylation sites in the 50-kDa subunit. To determine whether CaM-kinase phosphorylated by PKC exhibited Ca^{2+}/CaM-*in*dependent enzyme activity, we took advantage of the *in*ability of PKC to phosphorylate the CaM-kinase substrate, NTP. If reactions were composed of PKC, CaM-kinase, NTP, and the agents to activate PKC to permit CaM-kinase phosphorylation, NTP should be phosphorylated if CaM-kinase activity was produced. No activity was found when assays were accomplished in this manner (data not shown), even though parallel fractions showed that extensive CaM-kinase phosphorylation by PKC had occurred. These results suggested that even though Thr-286 appeared to be phosphorylated by PKC, no detectable Ca^{2+}/CaM-independent activity was produced. It is possible that some other factor, possibly binding of Ca^{2+}/CaM, is required for CaM-kinase to express activity even if Thr-286 is phosphorylated. Alternatively, these results could be explained if Thr-286 was not phosphorylated in native CaM-kinase. Although not conclusive, the phosphopeptide mapping data suggested that this was not the case. Additionally, we tested the sensitivity of CaM-kinase, with and without phosphorylation by PKC, to increasing amounts of Ca^{2+}/CaM to determine if phosphorylation by PKC might alter the sensitivity of CaM-kinase to Ca^{2+}/CaM activation. No significant increase or decrease in CaM-kinase's sensitivity could be detected in these assays (data not shown). Another conclusion from these experiments is that PKC phosphorylation of CaM-kinase does not significantly effect NTP phosphorylation, at least under the conditions of this assay and that CaM-kinase was not inactivated by PKC-dependent phosphorylation. At 500 nM CaM, NTP was phosphorylated to the same extent with our without phosphorylation of CaM-kinase by PKC (data not shown).

DISCUSSION

We demonstrated in this report that a synthetic peptide that mimics an autophosphorylation site that includes Thr-286 and the CaM-binding domain of the 50-kDa subunit of CaM-kinase is an excellent substrate for PKC. An interesting pattern emerged from the studies in that peptide substrates phosphorylated the best by CaM-kinase were phosphorylated the least by PKC and vice versa. For example, CBP is phosphorylated very poorly by CaM-kinase (Kelly et al., 1988) but quite well by PKC. In contrast, NTP is phosphorylated very well by CaM-kinase (Malenka et al., 1989) but not at all by PKC. By examining the order of phosphorylation of the peptides in Table I, it appears that the sequence of amino acids extending from the C-terminus of NTP are required for

maximal phosphorylation by PKC. At the same time, extending the C-terminus of NTP makes these peptides inhibitory for CaM-kinase so that while CK-II LONG is phosphorylated by CaM-KII, although not as well as NTP (unpublished observation), MB-I is an inhibitor of CaM-KII and is phosphorylated poorly. Apparently there is some conformational constraint imposed on the structure surrounding Thr-286 by amino acids extending in the C-terminal direction. What these constraints are is unknown, but the inverse relationships in which these peptides are used as substrates for the two different kinases is also consistent with this idea. CBP was also found to be a much less potent inhibitor of CaM-kinase when it was complexed with Ca^{2+}/CaM (Colbran et al., 1988; Kelly et al., 1988), lending further support that the peptide consists of two distinct functional regions that communicate through conformational changes. These conclusions are based on the assumption that Thr-286 is the only amino acid phosphorylated in CBP, an assumption based on the absence of phosphorylation of CBP_{-3}; however, we did not directly identify that Thr-286 was the only amino acid in CBP phosphorylated.

Both the 50- and 60-kDa subunits of purified CaM-kinase from rat forebrain were phosphorylated by PKC. PKC phosphorylated CaM-kinase to a similar stoichiometry as autophosphorylated CaM-kinase, suggesting that the purified enzyme, like the synthetic peptides, is a good substrate for PKC [approximate K_m between 5 and 10 μM; rate of phosphorylation, 0.8 μmol/(min·mg)]. Phosphopeptide mapping of the 50- and 60-kDa subunits and phosphoamino acid analysis of the 50-kDa subunit indicate some of these phosphorylation sites are in common with autophosphorylated CaM-kinase subunits, while at least one is unique. This unique phosphopeptide will provide a convenient marker for future in vivo investigations into this process. The phosphopeptide mapping studies suggested that Thr-286 was phosphorylated by PKC; however, this conclusion awaits isolation and sequencing of these phosphopeptides.

When autophosphorylated in the native enzyme, phosphorylatation of Thr-286 triggers the switch from a Ca^{2+}/CaM-dependent to a Ca^{2+}/CaM-independent enzyme (Lou & Schulman, 1989; Miller et al., 1988; Theil et al., 1988). We could not show a direct activation of CaM-kinase by PKC phosphorylation, although the situation might be different in more complicated settings. For example, PKC phosphorylation of Thr-286 might help to maintain CaM-kinase in a Ca^{2+}/CaM-independent state after initial Ca^{2+}/CaM-dependent autophosphorylation. This might be particularly important when considering the biochemical events underlying a specific physiologic response through combined activation of the PKC and CaM-kinase pathways (MacNicol et al., 1990; MacNicol & Schulman, 1992). One interesting example is a study done by MacNicol et al. (1990), who reported that activation of the phosphatidylinositol pathway in PC-12 cells produced autophosphorylation of CaM-kinase and generation of Ca^{2+}/CaM-independent activity. These authors suggested that CaM-kinase was activated by events coupled to Ca^{2+} elevation through the phosphatidylinositol signaling system. Our results suggest that a portion of the CaM-kinase phosphorylation seen in those studies might have been a direct effect by PKC phosphorylation. In fact, their two-dimensional maps examining the phosphopeptides of CaM-kinase produced by in vivo labeling after stimulation showed at least four unique peptides that were not present when purified CaM-kinase isolated from the PC-12 cells was autophosphorylated by Ca^{2+}/CaM stimulation. It is possible that one or more of these phosphopeptides is homologous to the unique phosphopeptide we have detected when PKC phosphorylates CaM-kinase in vitro (see Figure 7). These authors suggested that PKC did not phosphorylate CaM-kinase in vitro; however, we have shown that PKC can phosphorylate both CaM-kinase purified in our laboratory and CaM-kinase provided by Dr. Schulman. Methodological differences (e.g., pH of reactions, ATP concentration, PKC preparations) might explain this finding. Recent studies have also shown that Ca^{2+}/CaM-dependent autophosphorylation of Thr-286 produces an increase in the affinity of CaM-kinase for CaM, referred to as CaM-trapping (Meyer et al., 1992). We detected a negligible shift in the Ca^{2+}/CaM activation curve of CaM-kinase after phosphorylation by PKC, which suggests that this phosphorylation does not significantly affect CaM activation. This is in striking contrast to the orders of magnitude change detected in CaM-kinase's affinity for Ca^{2+}/CaM after Ca^{2+}/CaM-dependent autophosphorylation (Meyer et al., 1992).

There are numerous examples for protein kinase regulation occurring directly through phosphorylation, and this may, in fact, be a common mechanism for integration of intracellular signaling and control of cellular metabolism. One of the best studied examples is in the glycolytic pathway where phosphorylase b is phosphorylated and activated by phosphorylase kinase which is in turn phosphorylated and activated by PKA (Kelly & Shenolikar, 1987; Ramachandran et al., 1987). Another example is the PKA- and PKC-dependent phosphorylation of myosin light chain kinase that alters its activation by Ca^{2+}/CaM, leading to altered regulation of smooth muscle contraction (Conti & Adelstein, 1981; Ikebe et al., 1985; Kelly & Shenolikar, 1987). Bading and Greenberg (1991) and Gomez and Cohen (1991) have also recently shown that MAP-2 kinase (a Ser/Thr-specific kinase) is phosphorylated by a tyrosine-specific kinase, widening the scope of this type of cross-talk to the tyrosine kinase family as well. In fact, Gomez and Cohen (1991) have detailed an elaborate cascade of protein kinases involving no less than three different protein kinases. We have shown here that CaM-kinase is a substrate for PKC in vitro and suggest that this could be another example of protein kinase systems communicating by one kinase phosphorylating the other.

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