The site in calcineurin, the Ca\(^{2+}\)/calmodulin (CaM)-dependent protein phosphatase, which is phosphorylated by Ca\(^{2+}\)/CaM-dependent protein kinase II (CaM-kinase II); has been identified. Analyses of \(^{32}\)P release from tryptic and cyanogen bromide peptides derived from \(^{32}\)P-calcineurin plus direct sequence determination established the site as -Arg-Val-Phe-Ser(PO\(_2\))-Val-Leu-Arg-, which conformed to the consensus phosphorylation sequence for CaM-kinase II (Arg-X-X-Ser/Thr-). This phosphorylation site is located at the C-terminal end of the putative CaM-binding domain in calcineurin (Kincade, R. L., Nightingale, M. S., and Martin, E. M. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5893–5897), thereby accounting for the observed inhibition of this phosphorylation when Ca\(^{2+}\)/CaM is bound to calcineurin. Since the phosphorylation site sequence also contains elements of the specificity determinants for Ca\(^{2+}\)/phospholipid-dependent protein kinase (protein kinase C) (basic residues both N-terminal and C-terminal to Ser/Thr), we tested calcineurin as a substrate for protein kinase C. Protein kinase C catalyzed rapid stoichiometric phosphorylation, and the characteristics of the reaction were the same as with CaM-kinase II: 1) the phosphorylation was blocked by binding of Ca\(^{2+}\)/CaM to calcineurin; 2) phosphorylation partially inactivated calcineurin by increasing the \(K_m\) (from 9.9 ± 1.1 to 17.5 ± 1.1 \(\mu\)M \(^{32}\)P-labeled myosin light chain); and 3) \(^{32}\)P-calcineurin exhibited very slow autodephosphorylation but was rapidly dephosphorylated by protein phosphatase IIA. Tryptic and thermolytic \(^{32}\)P-peptide mapping and subsequent phosphoamino acid sequence analysis confirmed that protein kinase C and CaM-kinase II phosphorylated the same site.

CaN\(^{1}\) is a Ca\(^{2+}\)/CaM-dependent protein phosphatase com-posed of two polypeptides, A and B, with molecular sizes of 58–61 and 19 kDa, respectively (1–3). The A subunit contains the CaM-binding (4, 5) and catalytic domains (6, 7), whereas the B subunit binds 4 eq of Ca\(^{2+}\) with high affinity (8, 9). Although physiological substrates for CaN have not been firmly established, the enzyme catalyzes dephosphorylation of not only phosphoserines/phosphothreonine but also phosphotyrosine (1–3, 10–12) under in vitro conditions, suggesting that CaN may have a rather wide physiological and regulatory role.

CaN has been purified from bovine brain (5, 13), bovine heart (14, 15), rabbit skeletal muscle (16), and human platelets (17). Based on immunological data (18), specific protein phosphatase assays (19), and enzyme purification data, CaN has been thought to be significantly enriched in brain and skeletal muscle relative to other tissues. Purified CaN contains up to 0.6 eq of endogenous phosphate/mol of A subunit (20), suggesting that CaN may be regulated by phosphorylation. Recently, we showed (21) that CaN is phosphorylated by the autophosphorylated form of CaM-kinase II. This phosphorylation is blocked when Ca\(^{2+}\)/CaM is bound to CaN, suggesting interaction between the phosphorylation site and the CaM-binding domain. Although this phosphorylation does not appear to affect the binding of Ca\(^{2+}\)/CaM to CaN, it does result in partial inactivation of CaN due to an increase in \(K_m\) for protein substrates. In this paper, we have identified the regulatory phosphorylation site and localized it relative to the CaM-binding domain. Furthermore, we have also shown that this same site can also be phosphorylated in vitro by protein kinase C, which, like CaM-kinase II, is very abundant in brain (22–24).

**EXPERIMENTAL PROCEDURES**

**Materials**—CaN (20), a gift from Dr. Marita M. King (Ohio State University), and CaM (25) were purified from bovine brain. Protein kinase C (26) and CaM-kinase II (27) were purified from rat brain by Dr. Roger J. Colbran (Vanderbilt University). The catalytic subunit of cAMP-dependent protein kinase (28) and the catalytic subunits of protein phosphatase I (29) and pig brain phosphatase II A (30) were kindly provided by Drs. Jackie D. Corbin (Vanderbilt University), Balwant S. Khatra (California State University at Long Beach), and Shiw-Der Yang (National Tsing Hua University, Republic of China), respectively. Rabbit skeletal muscle myosin light chain and myosin light chain kinase were generous gifts from Drs. Edwin G. Krebs and Peter J. Kennelly (University of Washington). \([gamma]^{32}\)P]ATP was prepared (31) using carrier-free \(^{32}\)P04 from ICN Pharmaceuticals. Synctide-2, a peptide substrate of CaM-kinase II, was synthesized (24). Other materials were obtained as follows: FS, diolein, trypsin, and thermolysin, Sigma; cytochrome c and X-OMat RP film, Eastman; phosphocellulose paper, Whatman; Sephadex G-25 (fine) and G-15, Pharmacia LKB Biotechnology, Inc.; and Millipore filters (0.45 \(\mu\)m), Nihon Millipore Kogyo K. K. (Japan).

**Phosphorylation, Dephosphorylation, and Activity Assay of CaN—**

FS (0.2 mg) and diolein (0.02 mg) in chloroform were dried under a
stream of \( \text{N}_2 \) suspended in 200 \( \mu \text{l} \) of \( \text{H}_2\text{O} \) and sonicated with a microtip sonicator (Brown Sonifier, cell disruptor 185) using two 8-s bursts (output control = 2) at 4 °C. Phosphorylation of CaN (4-15 \( \mu \text{M} \)) by protein kinase C (0.43 \( \mu \text{M} \)) was performed in a 10-\( \mu \text{l} \) reaction containing 50 nm HEPES (pH 7.5), 0.1 \( \mu \text{M} \) \( \gamma\text{-}[32\text{P}]\text{ATP} \), 10 mM magnesium acetate, 0.4 nm CaCl\(_2\), 1 mg/ml bovine serum albumin, and dispersed lipids (0.1 mg/ml PS and 0.01 mg/ml diolein). At the desired times, aliquots were spotted on phosphocellulose papers (32) or added to sample buffer (2% sodium dodecyl sulfate, 750 mM 2-mercaptoethanol) for SDS-PAGE according to Laemmli (33). Phosphorylation of CaN by the autophosphorylated form of CaM-kinase II, activity assay of CaN, and dephosphorylation of \( [32\text{P}] \)CaN were performed as described previously (21).

**Proteolytic Digestion and Analysis of Phosphopeptides**—The phosphorylation reaction containing \( [32\text{P}]\)CaN was subjected to proteolysis, diluted with 0.1% trifluoroacetic acid, and applied to a disposable C18 reverse-phase cartridge (Burdick & Jackson Laboratories Inc.). Free \( \gamma\text{-}[32\text{P}]\text{ATP} \) was removed by washing with 0.1% trifluoroacetic acid, and the \( 32\text{P} \)-peptides were eluted by a stepwise wash with 70% acetonitrile in 0.1% trifluoroacetic acid and concentrated using a Speed-Vac. In some experiments, the phosphorylation reaction mixture was first subjected to SDS-PAGE, and the 60-kDa \( 32\text{P} \)-subunit of CaN was extracted and subjected to proteolysis. Proteolysis was conducted at 30 °C for 16 h with 1 mg/ml trypsin in 0.1 M potassium phosphate (pH 8) or at room temperature for 16 h with 0.12 g/ml cyanogen bromide in 70% formic acid. The \( 32\text{P} \)-peptides were analyzed by reverse-phase HPLC on a Beckman C8 Ultrasphere ODS column equilibrated in 0.1% trifluoroacetic acid and eluted with a gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Fractions of 1 ml were collected, and their Cherenkov radiations were determined. Greater than 95% of applied radioactivity was recovered from the column during the gradient. For localization of the \( 32\text{P} \)-residue, the \( 32\text{P} \)-peptides were subjected to automated Edman degradation in a Beckman Model 890C Sequencer; and at each cycle, the release of \( ^{32}\text{PO}_{4} \) was determined. For determination of the peptide sequence, an Applied Biosystems Gas-Phase Sequencer was used.

**Other Methods**—Identification of \( 32\text{P} \)-amino-acids was determined by partial acid hydrolysis (6 N HCl for 2 h at 100 °C), high voltage paper electrophoresis at pH 1.9 and 2500 V for 1 h (34), autoradiography, and liquid scintillation counting. Concentrations of protein kinase C and CaM-kinase II were determined by the Bradford assay (35) using bovine serum albumin as standard. Concentrations of CaN and CaM were determined spectrophotometrically using absorbance indices for 1% protein solutions of 9.3 at 279 nm (36) and of 1.8 at 276 nm (37), respectively.

**RESULTS**

**Identification of Phosphorylation Site**—CaN, \( 32\text{P} \)-labeled to a molar stoichiometry of about 1 using the autophosphorylated form of CaM-kinase II (21), was cleaved with cyanogen bromide; and the digest was subjected to reverse-phase HPLC (Fig. 1A). The single \( 32\text{P} \)-peptide, when subjected to automated Edman sequence analysis, exhibited a burst of \( ^{32}\text{PO}_{4} \) release at cycle 5 of the sequence (Fig. 1A, inset). The cyanogen bromide \( 32\text{P} \)-peptide was subdigested with trypsin and chromatographed on Sephadex G-15 and then by reverse-phase HPLC. One sharp \( 32\text{P} \)-peptide and corresponding absorbance (210 nm) peak were detected (Fig. 1B). When the tryptic \( 32\text{P} \)-peptide was subjected to Edman analysis, \( ^{32}\text{PO}_{4} \) release occurred at cycle 3 (data not shown). Amino acid composition of the tryptic peptide indicated a rather hydrophobic composition (Table I), and gas-phase sequence analysis yielded the sequence Val-Phe-Ser-Val-Leu-Arg.

**Phosphorylation of CaN by Protein Kinase C**—When CaN (5 \( \mu \text{M} \)) was incubated with protein kinase (0.43 \( \mu \text{M} \)) in the

![Fig. 1. Purification of \( 32\text{P} \)-labeled peptide obtained by digestion of phosphorylated CaN. CaN (4 nmol) was phosphorylated by the autophosphorylated form of CaM-kinase II (33 pmol) in the presence of CaM (45 pmol) as described (21). At 5 min, the reaction was stopped by addition of excess EDTA, and \( [32\text{P}] \)CaN was digested with CNBr and loaded onto a reverse-phase HPLC column (A). Fractions at retention times of 43-47 min were collected. Part of the \( [32\text{P}] \)CaN was subjected to Edman sequence analysis for \( ^{32}\text{PO}_{4} \) release (A, inset). The remainder was redigested in 0.1 mM potassium phosphate (pH 8) with 1 mg/ml trypsin at 30 °C for 30 min, and the \( 32\text{P} \)-peptide was further purified on Sephadex G-15 (bed volume of 50 ml; data not shown) and then by reverse-phase HPLC (B).
Amino acid composition of phosphopeptide of CaN obtained by digestion with cyanogen bromide and then trypsin

The mixture of fractions 37 and 38 in Fig. 1B was analyzed using a Waters Pico-Tag analyzer. Amino acids not shown were less than 10 pmol. The numbers in parentheses are the relative numbers of residues assumed to be present in the phosphopeptide.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>pmol</th>
</tr>
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<tbody>
<tr>
<td>Ser</td>
<td>101.41 (1)</td>
</tr>
<tr>
<td>Gly</td>
<td>21.27</td>
</tr>
<tr>
<td>Arg</td>
<td>117.58 (1)</td>
</tr>
<tr>
<td>Ala</td>
<td>14.34</td>
</tr>
<tr>
<td>Tyr</td>
<td>26.47</td>
</tr>
<tr>
<td>Val</td>
<td>234.25 (2)</td>
</tr>
<tr>
<td>Cys</td>
<td>11.0</td>
</tr>
<tr>
<td>Leu</td>
<td>138.90 (1)</td>
</tr>
<tr>
<td>Phe</td>
<td>117.22 (1)</td>
</tr>
</tbody>
</table>

Fig. 2. Phosphorylation of CaN by protein kinase C. CaN (5 µM) (○; lanes 1–5) or CaN storage buffer (lane 6) was incubated in 50 mM HEPES (pH 7.5), 10 mM magnesium acetate, 0.4 mM CaCl₂, 1 mg/ml bovine serum albumin, 0.1 mM [γ-32P]ATP, dispersed lipids (0.1 mg/ml PS and 0.01 mg/ml diolein), and protein kinase C (0.43 µM) in the absence (○; lanes 1–4, and 6) or in the presence ⏯; lane 5) of 0.1 mM CaM. At the indicated times, aliquots were spotted on phosphocellulose papers to determine phosphorylation stoichiometries or added to Laemmli sample buffer (33) for SDS-PAGE and autoradiography. Molecular sizes are indicated (in kilodaltons) in the inset.

Effect of Phosphorylation on Phosphatase Activity—CaN phosphatase activity of CaN was then measured using varying concentrations of 32P-labeled myosin light chain. Reactions were initiated with addition of CaN. Aliquots were mixed with trichloroacetic acid (final concentration of 20%) at 1, 2, and 3 min and put on ice for at least 10 min after addition of bovine serum albumin to 2.5 mg/ml. Following centrifugation, radioactivity in the supernatant was measured. Values are the mean ± S.E. (n = 5).

<table>
<thead>
<tr>
<th>Nonphosphorylated</th>
<th>Phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>µM</td>
</tr>
<tr>
<td>9.1 ± 0.1</td>
<td>0.260 ± 0.035</td>
</tr>
</tbody>
</table>

32P<sub>O</sub> release at cycle 3 (Fig. 3, inset). Subdigestion of these two tryptic 32P-peptides with thermolysin resulted in identical shifts in the HPLC elution patterns (data not shown).

Effect of Phosphorylation on Phosphatase Activity—CaN was phosphorylated by protein kinase C to stoichiometries of 0.9–1.0 within 20 min (Fig. 2). This phosphorylation was not observed if PS and diolein (data not shown) or protein kinase C was omitted from the reaction. Phosphoamino acid analysis revealed that greater than 95% of the radioactivity was on serine (data not shown). The inclusion of CaM (6 µM) in excess of CaN effectively inhibited the phosphorylation reaction (Fig. 2). The inhibition by CaM appears to be substrate-directed, i.e., due to the interaction of Ca<sup>2+</sup> with CaM, since phosphorylation of a synthetic peptide, syntide-2, by protein kinase C was not affected by 6 µM Ca<sup>2+</sup>CaM (data not shown). There was no detectable phosphorylation of the 19-kDa subunit of CaN.

The 60-kDa 32P-subunit was extracted by SDS-PAGE and subjected to digestion for 16 h with 1 mg/ml trypsin. Peptide mapping by HPLC showed only a single 32P-peptide which eluted at about 30% acetonitrile (Fig. 3). When the tryptic 32P-peptides derived from CaN 32P-labeled with either protein kinase C or CaM-kinase II were mixed and subjected to HPLC, they coeluted (data not shown). Analysis of these tryptic 32P-peptides by sequential Edman sequencing gave
Phosphorylation reactions were stimulated by Ca²⁺ and strongly inhibited by Ca²⁺-independent autophosphorylation, probably occurring at the C terminus and removing the CaM-binding domain. Knowledge of the consensus sequence for sites phosphorylated by CaM-kinase II (Arg-X-X-Ser/Thr; Ref. 42) allowed us to identify the sequence Met-Ala-Arg-Val-Phe-Ser-Val-Leu-Arg-Glu-Glu-Ser-Glu-Ser-Val-Leu-Thr-Leu-Lys-Gly-Leu-Thr-Pro-Thr-Gly-Met-(residues 192-217 in Ref. 38) as the only consensus sites for phosphorylation. This tentative assignment was attractive since Ser-197 and Ser-203 would be positioned on the C-terminal boundary of the putative CaM-binding domain (assigned to residues 177-200). Thus, it would not be surprising that binding of Ca²⁺/CaM might block phosphorylation of either of these 2 serines, as was observed for phosphorylation of CaN by CaM-kinase II (21).

Determination of the site of phosphorylation was obtained by analysis of Ca²⁺PO₄ release at cycle 5 of the cyanogen bromide ⁴⁵³P-peptide (cleavage C-terminal to Met) and cycle 3 of the tryptic ⁴⁵³P-peptide. Since there was no release of Ca²⁺PO₄ at cycle 11 from the cyanogen bromide ⁴⁵³P-peptide, this indicated that only Ser-197, and not Ser-203, was phosphorylated. Although this analysis of Ca²⁺PO₄ release strongly suggested Ser-197 as the unique site of phosphorylation, we could not be certain of this conclusion since the entire sequence of CaN was not published. Quantitative subdigestion of the cyanogen bromide ⁴⁵³P-peptide required high concentrations of trypsin (1 mg/ml), probably due to 2 Glu residues C-terminal to Arg-200. Elution of the tryptic ⁴⁵³P-peptide at about 30% acetonitrile suggested a rather high degree of hydrophobicity for a peptide of only six amino acids, consistent with the predicted peptide from residues 195 to 200. Therefore, we decided to subject the tryptic ⁴⁵³P-peptide to gel filtration to remove contaminating larger tryptic fragments which would elute at this relatively high concentration of acetonitrile. This strategy was successful in that, during subsequent reverse-phase HPLC, the contaminating tryptic fragments coeluting from gel filtration eluted at a lower acetonitrile concentration, and a single absorbance peak was detected with the ⁴⁵³P-peptide which gave the correct amino acid composition. Subsequent amino acid analysis confirmed the sequence at Val-Phe-Ser-Val-Leu-Arg.

Although the phosphorylation of CaN is blocked by binding of Ca²⁺/CaM, ⁴⁵³P-CaN is still activated by Ca²⁺/CaM and binds to CaM-Sepharose (21). We were unable to detect an effect of this phosphorylation on the concentration required for half-maximal activation of CaN, but this question needs to be explored in a more quantitative manner. However, several other CaM-binding proteins are known to have phosphorylation sites, some of which affect binding of CaM. There are two cAMP-dependent protein kinase-catalyzed phosphorylation sites in smooth muscle myosin light chain kinase, one of which is within the CaM-binding domain; and phosphorylations at both sites cause decrease in affinity for binding of Ca²⁺/CaM (43). Likewise, CaM-kinase II has at least two regulatory autophosphorylation sites. Phosphorylation of Thr-286 (44), which is within the autoinhibitory domain just N-terminal to the CaM-binding domain (45-47), converts the kinase to the partially Ca²⁺-independent form (27, 48-51). Removal of CaM from this form of CaM-kinase II promotes additional Ca²⁺-independent autophosphorylation, probably at either Thr-305 or Thr-306 within the CaM-binding domain (52), which completely blocks binding of Ca²⁺/CaM (53). The 60- and 63-kDa isozymes of brain CaM-dependent cyclic nucleotide phosphodiesterases are specifically phosphorylated...
by cAMP-kinase (54) and CaM-kinase II (55), respectively, resulting in decreased affinity for Ca**2+**/CaM. Phosphorylation of neurenomodulin (also known as P-57, GAP-43, B-50, and F-1) by protein kinase C inhibits the binding of CaM, which occurs in a Ca**2+**-independent manner, to this membrane protein (56).

The presence of Arg residues in close proximity on not only the N-terminal but also the C-terminal sides of the phosphorylated serine in CaN suggested to us that this site may also be phosphorylated by protein kinase C (57). Indeed, CaN was rapidly and stoichiometrically phosphorylated by protein kinase C. This phosphorylation reaction exhibited all the same features as phosphorylation by CaM-kinase II: 1) inhibition by binding of Ca**2+**/CaM to CaN; 2) phosphorylation resulting in an increase in \( K_{\text{cat}} \), for myosin light chain with no change in \( V_{\text{max}} \); and 3) low rate of autodephosphorylation of [**32**P]CaN, but rapid dephosphorylation by protein phosphatase II A > phosphatase I. Proteolytic [**32**P]-peptide mapping studies confirmed that protein kinase C phosphorylated the same site on CaM-kinase II. Our results are at variance with those reported by Lim Tung (68), who reported phosphorylation of CaN (0.5–1.0 mol) by protein kinase C with no effect on phosphatase activity using [**32**P]casein as substrate. Details of the kinetic analysis were not given, but since [**32**P]casein is not a very good substrate for CaN, it could enhance phosphorylation of those proteins nor-

Acknowledgments—We wish to thank Dr. Marita M. King for helpful discussions and Dr. Randall L. Kincaid for making the partial sequence of CaN available to us prior to its publication.

REFERENCES


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1 Y. Hashimoto, unpublished observation.
Regulation of Calcineurin by Phosphorylation