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A Cluster of Basic Amino Acid Residues in Calcineurin B Participates in the Binding of Calcineurin to Phosphatidylserine Vesicles[1]

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Interactions between phospholipid membranes and the acyl chain and specific amino acid residues of myristoylated proteins are necessary for membrane association. In the present study we tested the effects of mutations of calcineurin B subunit amino acid residues $K^{20}K^{21}$, $K^{24}R^{25}$, $K^{27}K^{28}$ to Glu on the interactions between calcineurin and phosphatidylserine vesicles. Calcineurin–phosphatidylserine interactions were measured using binding assays and assays of phosphatidylserine-stimulated calcineurin phosphatase activity. The reverse-charge calcineurin B subunit mutant had a slower mobility in SDS-PAGE relative to wild-type calcineurin B. In addition, the myristoylated calcineurin B reverse-charge mutant had a slower mobility in SDS-PAGE compared to the non-myristoylated form, in contrast to the faster mobility of myristoylated wild-type calcineurin B relative to non-myristoylated calcineurin B. The reverse-charge mutations had no apparent effect on N-terminal myristoylation, Ca^{2+}-binding, or calcineurin heterodimer formation and stimulation of Ca^{2+}/calmodulin-dependent phosphatase activity. However, in contrast to the results obtained using native calcineurin, phosphatidylserine vesicles did not bind to or activate the phosphatase activity of calcineurin containing the calcineurin B reverse-charge mutant. These results indicate that calcineurin B contains an amino terminal basic residue cluster that is involved in the binding of calcineurin to acidic phospholipids.

Key words: calcineurin, calmodulin, membrane, myristoylation, phospholipids.

Calcineurin (CaN) is a widely expressed Ca^{2+}/CaM-dependent Ser/Thr protein phosphatase that plays a fundamental role in diverse physiological processes such as T cell activation, certain forms of cardiac hypertrophy, long term depression, and neuronal post-synaptic potential (*1–3*). CaN is evenly distributed between the cytosolic and particulate fractions of brain and dephosphorylates cytosolic, membrane-associated, and integral membrane proteins (*4–7*). CaN has been shown to associate with and be activated by acidic phospholipid vesicles *in vitro*, and has been purified from the plasma membrane fraction of cells lysed in the presence of Ca^{2+} (*8–10*). These findings suggest that the *in vivo* association of CaN with the particulate fraction may be due, in part, to CaN-membrane interactions. Several investigations of the mechanism of association between CaN and membrane phospholipids have focused on the role of the N-terminal myristate of the Ca^{2+}-binding regulatory CnB subunit (*11–13*). It has been reported that the association of CaN with membrane phospholipids is Ca^{2+}-dependent and specific for negatively charged phospholipids (*9*). It has also been reported that the association of CaN with membrane phospholipids is neither Ca^{2+}- nor myristoylation-dependent, but is still mediated by negatively charged phospholipids (*11*, *12*). The findings of these contrasting reports both indicate, however, that CaN associates with anionic phospholipids, suggesting that electrostatic interactions are involved in the binding of CaN to negatively charged phospholipids.

It has been shown that additional factors are required for the binding of myristoylated proteins to phospholipid membranes (*13*). Recoverin binds to phospholipid membranes by a Ca^{2+}- and myristoylation-dependent interaction of hydrophobic residues with neutral phospholipids (*14*). The proteins eNOS, MARCKS, ADP-ribosylation factor, and Src contain a cluster of basic residues that interact electrostatically with negatively charged phospholipids to anchor the proteins to the phospholipid membrane following the initial hydrophobic interactions between the N-terminal myristate and the non-polar phospholipid tails (*13*). The results from CaN-phospholipid cross-linking experiments showing the predominant labeling of CnB, suggest that a phospholipid

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Abbreviations: CaM, calmodulin; CaN, calcineurin; CnA, catalytic subunit of calcineurin; CnB, Ca^{2+}-binding regulatory subunit of calcineurin; CnB-BRC, calcineurin B subunit containing the basic residue cluster reverse-charge mutations; myr-CnB, non-myr-CnB, myristoylated and non-myristoylated calcineurin B subunit, respectively; myr-CnB-BRC, non-myr-CnB-BRC, myristoylated and non-myristoylated CnB-BRC, respectively; eNOS, endothelial nitric oxide synthase; HRP, horseradish peroxidase; PCOP, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylcholine PSOP, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylserine; IPTG, isopropyl-β-D-thiogalactopyranoside; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

binding site(s) is located on CnB (*10*). Since the N-terminal α-helix of EF-hand Ca^{2+}-binding loop I of CnB contains a cluster of basic residues, we hypothesized that electrostatic interactions between acidic phospholipids and this CnB N-terminal basic residue cluster are important for the Ca^{2+}- and myristoylation-dependent binding of calcineurin to the negatively charged phospholipid phosphatidylserine. A CnB reverse-charge mutant having the point mutations $KK^{20,21}$/EE, $KR^{24,25}$/EE, $KK^{27,28}$/EE (CnB-BRC) was constructed and co-expressed with CnA using baculovirus expression. CaN containing CnB-BRC was tested for its ability to bind to and be activated by phosphatidylserine vesicles. The $KK^{20,21}$/EE, $KR^{24,25}$/EE, $KK^{27,28}$/EE mutations did not affect N-terminal myristoylation, CnA-CnB subunit interactions or Ca^{2+}/CaM-stimulated phosphatase activity. However, CaN containing the CnB-BRC subunit did not bind to and was not activated by phosphatidylserine vesicles. These results indicate that a basic residue cluster in the N-terminal region of CnB is involved in the interaction between CaN and acidic phospholipids.

MATERIALS AND METHODS

Materials—The rabbit anti-CnB antibody was purchased from Affinity Bioreagents. The anti-CnA antibody was a gift of Kohji Fukunaga (Kumamoto University, Kumamoto). Bovine brain CaN, and horseradish peroxidase–conjugated goat anti–rabbit IgG were purchased from Chemicon. The palmitoyl, oleoyl form of phosphatidylserine (PSOP) was obtained from Avanti Polar Lipids. Histone H1, Grace's insect cell medium supplemented and non-supplemented, Pluronic F-68, antibiotic/antimycotic solution, and bacterial culture media, were obtained from GIBCO/BRL. Fetal bovine serum was purchased from Atlanta Biologicals. DEAE-Sephacel, Sephadex G-25, and CaM-Sepharose were purchased from Pharmacia. Oligonucleotides were obtained from Oligo's Etc. Antibiotics were from Sigma. EDTA-free protease inhibitor tablets and IPTG were obtained from Boehringer Mannheim. The catalytic subunit of PKA was obtained from Promega. [γ-^{32}P]ATP was from ICN. RII peptide was from American Peptide Company. All other materials and reagents were of the highest quality available commercially.

Recombinant Protein Expression and Purification—The expression and purification of baculovirus-expressed CaN containing the rat brain CnA αδ subunit and rat brain CnB was carried out as described with the following modifications (*15*). The 20,000 ×*g* supernatant was chromatographed over a DEAE Sephacel column (2 ml) that had been equilibrated in lysis buffer minus NaCl, and CaN was eluted in EGTA buffer (*15*). The peak A_{280} fractions (0.5 ml) were pooled and brought to 45% ammonium sulfate. The ammonium sulfate pellets were resuspended into EGTA buffer, brought to 1 mM free Ca^{2+}, and CaN purified by CaM-Sepharose chromatography as described (*15*). The CnB $KK^{20,21}$/EE, $KR^{24,25}$/EE, $KK^{27,28}$/EE mutations were generated by oligonucleotide-directed *in vitro* mutagenesis (Sculptor System, Amersham). Mutants were identified by DNA sequencing using the Sequenase DNA sequencing Kit (Amersham). Recombinant baculoviruses expressing CnB-BRC were generated using the Bac-N-Blu Transfection Kit (InVitrogen) and screened for expression by Western blotting of insect cell homogenates with anti CnB antibody. Recombinant baculovirus was titered by plaque assay as described (*16*). Bacterially expressed myristoylated or non-myristoylated CnB (myr-CnB, non-myr-CnB, respectively), and CnB-BRC (myr-CnB-BRC, non-myr-CnB-BRC, respectively) were partially purified by Ca^{2+}-dependent hydrophobic interaction chromatography as described (*17*).

Preparation of Sized, Sucrose-Loaded Phospholipid Vesicles—Phospholipid stocks in chloroform were dried in clean conical glass tubes under a stream of N_2. The dried phospholipids were resuspended into the appropriate volume of sucrose buffer (20 mM Tris-HCl pH 7.5, 170 mM sucrose) by vigorous vortexing, transferred to a microfuge tube, and then put through 5 freeze-thaw cycles to load the vesicles with sucrose. The sucrose-loaded vesicles were divided into aliquots, and stored at −20°C. On the day of their use, sucrose-loaded vesicles were sized using a LiposoFast Apparatus (Avestin) fitted with a 1 μm polycarbonate membrane. The sized, sucrose-loaded vesicles were diluted five-fold with wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl) and collected by centrifuging in a microfuge at maximum speed for 30 min at 4°C. The same volume of buffer that was added prior to centrifuging was removed and the sized, sucrose-loaded vesicles were resuspended by vigorous vortexing, and placed on ice until use.

CaN-Phospholipid Binding Assays—As described in the figure legend, bovine brain CaN or baculovirus-expressed CaN-BRC was incubated with the indicated amounts of sized, sucrose-loaded PSOP vesicles. The binding assays were carried out in a final volume of 45 μl of binding buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM $MgSO_4$, 0.0065% TritonX-100, 15 mM β-mercaptoethanol, and the indicated concentrations of Ca^{2+} or EGTA). The samples were vortexed for 30 min at room temperature and then microfuged for 30 min at 4°C to collect the phospholipid vesicles. The supernatants were transferred to a microfuge tube containing SDS-sample buffer plus 1 mM EGTA. The pellets were resuspended into 45 μl of binding assay buffer, and re-centrifuged. The supernatant was removed, and the pellet resuspended into SDS-sample buffer containing 1 mM EGTA. The pellet and supernatant fractions were boiled, and analyzed by SDS-PAGE (15%) or Western blotting and immunostaining as described (*18*). The SDS-sample buffer and SDS-PAGE gels contained 1 mM EGTA to prevent any Ca^{2+}-induced mobility shift of CnB (*5*).

Phosphorylation of Histone H1—Histone H1 was phosphorylated by the catalytic subunit of PKA as described, except 20 mM Hepes pH 7.2 was used instead of 20 mM Mops pH 7.0 (*19*). A stoichiometry of 0.8–1.0 mol phosphate per mol Histone H1 was attained by incubation at 30°C for 20 min, as determined by subtracting the cpm in the TCA supernatant (unincorporated activity) from the total cpm, and comparing this value with the expected value for 100% incorporation determined using the specific activity of the [γ-^{32}P]ATP and the molar concentration of Histone H1. The reaction mixture was then passed over a 5 ml Sephadex G-25 column equilibrated in TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl) to remove unincorporated nucleotide from the protein. The ^{32}P-Histone H1 sample was concentrated using a microcon 10 filter (Amicon), and its concentration calculated by determining the cpm/μl of the ^{32}P-Histone H1 sample and dividing this sample by the specific activity of the [γ-^{32}P]ATP (3,000–5,000 cpm/pmol). The non-protein-bound radioactivity was determined by TCA precipitation

of an aliquot of the ^{32}P-Histone H1 sample, followed by microfuging for 5 min and liquid scintillation counting of the supernatant. The radioactivity in the supernatant was typically less than 1% of the total radioactivity in the sample. Phosphorylation of RII peptide, removal of excess [γ-^{32}P]-ATP, and determination of its concentration were carried out as described (*18*).

Phosphatase Assays—Dephosphorylation of ^{32}P-Histone H1 by baculovirus-expressed CaN was determined using 15 μl reactions in triplicate. The concentrations of baculovirus-expressed CaN, CaM, PSOP, ^{32}P-Histone H1, and ^{32}P-RII peptide are indicated in the figure legends. The reactions were carried out in CaN assay buffer [40 mM Tris-HCl pH 7.5, 6 mM $Mg(C_2H_3O_2)_2$, 150 mM NaCl, 15 mM β-mercaptoethanol, 0.1 mM $CaCl_2$, and 0.5 mM $MnCl_2$]. The reactions were initiated by the addition of phosphorylated substrate, placed at 30°C for 30 min and then terminated by the addition of 75 μl of 20% trichloroacetic acid. Bovine serum albumin was added (10 μl of a 20 mg/ml stock) and each tube briefly vortexed and placed on ice. The amount of ^{32}P released was determined by microfuging each sample for 5 min, collecting 50 μl of the supernatant, and counting in a liquid scintillation counter. The concentration of ^{32}P-Histone H1 in the assay was calculated by determining the cpm/μl and dividing this value by the specific activity of the [γ-^{32}P]ATP. ^{32}P-RII peptide dephosphorylation was carried out as described (*18*).

RESULTS

The previous findings that CaN binds to the negatively charged membrane phospholipid phosphatidylserine, but not to the neutral phospholipid phosphatidylcholine (*9*) suggest that electrostatic interactions between negatively charged phospholipids and positively charged amino acid residues may be involved in the CaN-phospholipid interaction. Comparison of the amino acid sequence of CnB with other myristoylated proteins containing basic residue clusters indicates the presence of a cluster of N-terminal basic residues between CnB residues 19 and 30 (Fig. 1). The positively charged residues $KK^{20,21}$, $KR^{24,25}$, and $KK^{27,28}$ were changed to Glu to generate the CnB-BRC-reverse-charge mutant. In addition, the same reverse-charge mutations were made to the CnB-G^2/A mutant (N-terminal Gly mutated to Ala to prevent N-terminal myristoylation) to obtain non-myristoylated CnB-BRC. Sf21 insect cells were co-infected with recombinant baculovirus expressing CnA and CnB-BRC or non-myr-CnB-BRC, and heterodimeric CaN containing the reverse-charge CnB mutants purified as described in Experimental Procedures. SDS-PAGE and Western blot analysis of the purified CaN-BRC mutants indicate that CnB-BRC and non-myr-CnB-BRC have slower mobilities in SDS-PAGE than native or non-myr-CnB (Fig. 2, A and B). The slower mobilities of the CnB-BRC mutants are most likely due to the replacement of positively charged amino acid residues with negatively charged Glu residues. A CnB mutant in which $Val^{156/157/168/169}$ were all changed to Glu also exhibits a slower mobility in SDS-PAGE (*20*). The faint bands in lanes 3, 4, and 5 may be due to the presence of minor amounts of endogenous insect cell CnB. Interestingly, non-myr-CnB-BRC has a faster mobility than CnB-BRC (Fig. 2, A and B). For native CnB the faster and slower migrating protein bands represent myristoylated- and non-myristoylated-CnB, respectively (*18*). To confirm that the faster and slower migrating CnB-BRC protein bands represent non-myristoylated and myristoylated forms of the protein, respectively, CnB-BRC was bacterially-expressed with or without cultures expressing yeast *N*-myristoyltransferase, partially purified by phenyl-Sepharose chromatography, and analyzed by SDS-PAGE and Western blotting. As shown in Fig. 3, CnB-BRC co-expressed with yeast-*N*-myristoyltransferase migrates more slowly than CnB-BRC expressed without yeast-*N*-myristoyltransferase, providing direct evidence that myristoylated CnB-BRC, unlike native myristoylated CnB, has a slower mobility in SDS-PAGE than non-myristoylated CnB-BRC. These findings also strongly suggest that CnB myristoylation is unaffected by the reverse-charge muta-

CnB		
N. crassa	(24-33)	DRLRKRFMKL
yeast	(24-33)	ERLRKRFMKL
D. melanogaster	(20-30)	RRLGKRFRKL
mouse testis	(21-30)	RRLGKSFRKL
human brain	(20-29)	KRLGKRFKKL
Src	(5-17)	KSKPKDASQRRR
MARCKS	(151-175)	KKKKKRFSFKKSFFKLSGFSFKKNKK

Fig. 1. **Basic residue cluster sequences.** The amino acid sequences of CnB from several species (*33–37*) are compared to the basic residue clusters involved in membrane association from MARCKS and Src (*38, 39*). The numbers in parentheses refer to the location within the amino acid sequence. Basic residues are in bold.

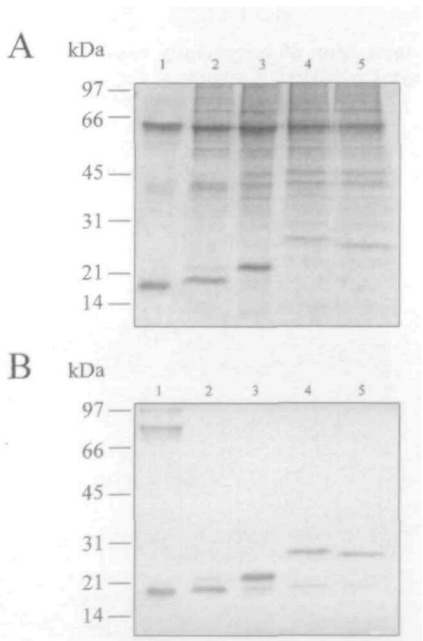

Fig. 2. **SDS-PAGE and Western blot of baculovirus-expressed CaN, non-myr-CaN, CaN-BRC, and non-myr-CaN-BRC.** A: Proteins were separated by SDS-PAGE (15%) and stained with Coomassie Brilliant Blue. B: Proteins were separated by SDS-PAGE (15%) and transferred to nitrocellulose. Immunostaining with anti CnB antibody was carried out as described in "MATERIALS AND METHODS." Lane 1, (4 μg panel A; 2 μg panel B) bovine brain CaN; lane 2, wild-type CaN; lane 3, non-myr-CaN; lane 4, CaN-BRC; lane 5, non-myr-CaN-BRC. 7.5 μg protein in lanes 2–5 of panel A; 4 μg protein in lanes 2–5 of panel B.

tions. If myristoylation of CnB-BRC was impaired by the KK^{20,21}/EE, KR^{24,25}/EE, KK^{27,28}/EE mutations, a faster migrating non-myr-CnB-BRC protein band would be present below the myr-CnB-BRC protein band. However, only a single band of myr-CnB-BRC is present in bacterially-expressed myr-CnB-BRC and in baculovirus-expressed CaN containing CnB-BRC (Figs. 2 and 3).

CnB-BRC Ca^{2+}-Binding—The KK^{20,21}/EE, KR^{24,25}/EE, KK^{27,28}/EE mutations are within the N-terminal α-helix of EF-hand Ca^{2+}-binding loop I. Since the binding of CaN to PSOP vesicles is Ca^{2+}-dependent, we tested whether Ca^{2+}-binding to CnB was affected by the reverse charge mutations. Ca^{2+} binding to CnB causes a conformational change that results in a small increase in mobility in SDS-PAGE (*5*). As seen in Fig. 4, wild-type CnB and non-myristoylated wild-type CnB both demonstrated similar Ca^{2+}-induced mobility shifts. In addition, both myristoylated- and non-myristoylated CnB-BRC demonstrate Ca^{2+}-induced increases in mobility that are similar to the Ca^{2+}-induced mobility shifts of wild-type CnB and non-myristoylated wild-type CnB. These results strongly suggest that similar to native CnB, the CnB-BRC mutant undergoes a Ca^{2+}-dependent conformational change caused by Ca^{2+} binding that results in its increased mobility in SDS-PAGE.

CaN Heterodimer Formation and Activation of Phosphatase Activity by the CnB-BRC Mutant—The above results indicate that the reverse charge mutations of the CnB-BRC mutant do not affect Ca^{2+}-binding or N-terminal myristoylation. The presence of a CnB protein band in the Coomassie-stained SDS-PAGE gel, and a CnB immunopositive band in the Western blot of the CaM-Sepharose–purified CaN containing CnB-BRC in Fig. 2 provide strong evidence that CnB-BRC binds to CnA. CaN is purified by Ca^{2+}-dependent CaM-Sepharose chromatography because CnA subunit contains a CaM-binding domain. The presence of a CnB protein band in Coomassie stained SDS-PAGE gels or immunostained Western blots of CaM-Sepharose–purified CaN is due to the binding of CnB to the B subunit-binding helix in CnA (*21*, *22*). Densitometry of the Coomassie stained CnA and CnB protein bands indicates A:B ratios of 2.85:1 and 2.8:1 in wild type CaN and CaN containing CnB-BRC, respectively. Based on the molecular masses of the A and B subunits, these findings suggest that the CaN heterodimers consist of a 1:1 ratio of A:B. These findings strongly suggest that the CnB reverse charge mutations have no effect on heterodimer formation. However, as observed for previous CnB mutants, these methods are not sufficiently quantitative to detect small differences in the affinity of the interactions (*22*). In addition, CnB mutants with similar affinities for CnA as wild-type CnB have been described, but which do not support Ca^{2+}/CaM-dependent stimulation of the phosphatase activity of CnA (*23*). Thus, to determine whether CnB-BRC binds to CnA with an affinity similar to that of wild-type CnB and supports Ca^{2+}/CaM-dependent stimulation of CnA phosphatase activity, we compared the Ca^{2+}/CaM-dependent stimulation of CaN containing CnB-BRC with that of wild-type CaN. It has previously been shown that full Ca^{2+}/CaM-dependent stimulation of CnA phosphatase activity requires the presence of an eqimolar amount (with respect

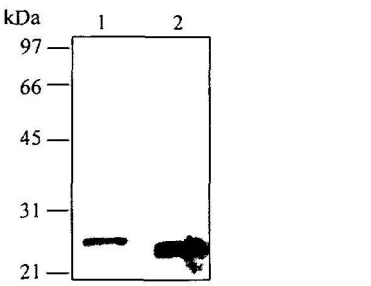

Fig. 3. **Western blot of bacterially-expressed myr-CnB-BRC and non-myr-CnB-BRC.** The mutant CnB proteins were partially purified by phenyl-Sepharose chromatography, separated by SDS-PAGE (15%) (3 μg/lane), and transferred to a nitrocellulose membrane. Immunostaining with anti CnB antibody was carried out as described in "MATERIALS AND METHODS." Lane 1, myr-CnB-BRC; lane 2, non-myr-CnB-BRC.

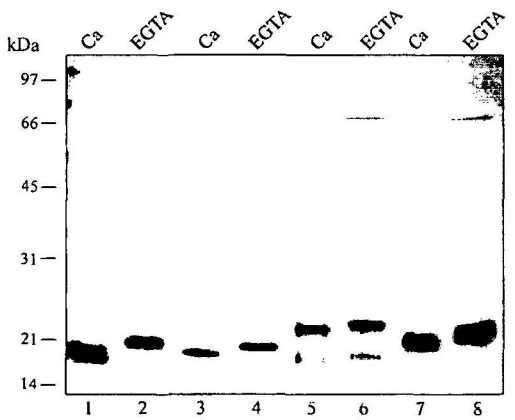

Fig. 4. **Ca^{2+}-induced shift in mobility of wild-type and mutant CnB proteins in SDS-PAGE.** Proteins (2 μg per lane) were separated by SDS-PAGE (15%) in the presence of Ca^{2+} (1 mM) or EGTA (1 mM) and transferred to a nitrocellulose membrane. Immunostaining with anti CnB antibody was carried out as described in "MATERIALS AND METHODS." Lanes 1 and 2, bovine brain CaN; lanes 3 and 4, CaN containing non-myr-CnB; lanes 5 and 6, myr-CnB-BRC; lanes 7 and 8, non-myr-CnB-BRC.

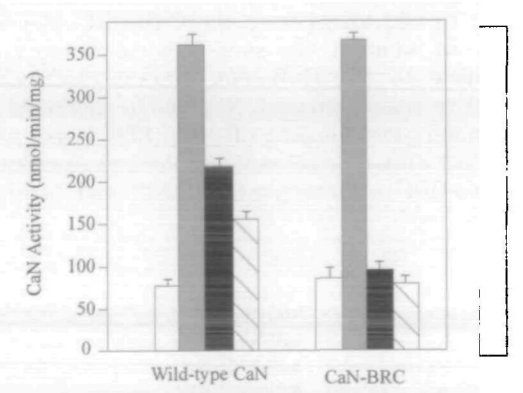

Fig. 5. **Activation of CaN or CaN-BRC phosphatase activity by Ca^{2+}/CaM or Ca^{2+}/PSOP.** The dephosphorylation of ^{32}P-Histone H1 (5 μM) or ^{32}P-RII peptide (10 μM) by baculovirus-expressed wild-type CaN or CaN-BRC (100 nM) was measured as described in "MATERIALS AND METHODS." As indicated, CaM or PSOP vesicles were present at concentrations of 300 nM and 0.5 μg/μl, respectively. Ca^{2+}/Mn^{2+}, □; Ca^{2+}/Mn^{2+}/CaM, ■; Ca^{2+}/Mn^{2+}/PSOP, ■; Ca^{2+}/Mn^{2+}/PSOP, ^{32}P-RII peptide, ⧅. Each bar represents the mean ± SD of data from three experiments.

to CnA) of CnB (*18*). Thus, CaN containing a sub-stoichiometric amount of CnB displays reduced Ca^{2+}/CaM-dependent stimulation relative to CaN composed of the native 1:1 CnA/CnB molar ratio because of the presence of un-complexed CnA subunit (*18*). As seen in Fig. 5, the Ca^{2+}/CaM-stimulated activity of CaN containing CnB-BRC is similar to that of baculovirus-expressed native CaN. These results strongly suggest that CnB-BRC binds to CnA with an affinity similar to that of wild-type CnB and supports Ca^{2+}/CaM-dependent stimulation of CnA phosphatase activity.

CnB $KK^{20,21}$/EE, $KR^{24,25}$/EE, $KK^{27,28}$/EE Mutations Prevent CaN Binding to Phosphatidylserine Vesicles—Having established that Ca^{2+} binding, N-terminal myristoylation, and binding to and activation of CnA by CnB-BRC are unaffected by the reverse charge mutations, we then compared the binding of CaN-BRC and bovine brain CaN to phosphatidylserine vesicles to test the hypothesis that these residues participate in the binding of CaN to phosphatidylserine. A tenfold excess of sucrose-loaded phosphatidylserine vesicles was incubated with bovine brain CaN or baculovirus-expressed CaN containing CnB-BRC in the presence of 0.5 mM Ca^{2+} as described in "MATERIALS AND METHODS." As shown by the anti-CnB immunostaining in Fig. 6, approximately 50% of bovine brain CaN associated with phosphatidylserine. However, CaN containing the CnB-BRC mutant did not associate with phosphatidylserine vesicles, as indicated by the absence of CnB immunostaining in the phosphatidylserine vesicle pellet (P) fractions. Neither bovine brain CaN nor CaN-BRC bound to phosphatidylcholine vesicles, confirming the specificity of native CaN for negatively-charged phospholipids. Additional evidence that residues $KK^{20,21}$, $KR^{24,25}$, and $KK^{27,28}$ are required for phosphatidylserine binding to CaN is provided by the results from our experiments examining the phosphatidylserine-dependent stimulation of CaN phosphatase activity. It has previously been reported that CaN phosphatase activity is stimulated by phosphatidylserine in a Ca^{2+}-dependent manner, using ^{32}P-Histone H1 as substrate, although the mechanism of activation is not known (*9*). As shown in Fig. 5, the Ca^{2+}/CaM-stimulated activities of native baculovirus-expressed CaN or CaN containing CnB-BRC are similar. These results indicate that the reverse charge mutations in CnB have no effect on the stimulation of CaN phosphatase activity by Ca^{2+}/CaM. As also shown in Fig. 5, the phosphatase activity of native CaN toward ^{32}P-Histone H1 was increased by phosphatidylserine. The levels of phosphatidylserine-stimulated phosphatase activity of native CaN were 60% of the levels of the Ca^{2+}/CaM-stimulated activity. In contrast, little or no stimulation of the phosphatase activity of CaN containing CnB-BRC by phosphatidylserine was observed. Phosphatidylserine also stimulated the dephosphorylation of ^{32}P-RII peptide by native CaN, although to a lesser extent than observed with ^{32}P-Histone H1. In addition, PSOP vesicles did not stimulate the phosphatase activity of CaN-BRC towards ^{32}P-RII peptide. The findings that CnB-BRC has no effect on the Ca^{2+}/CaM-stimulated activity of CaN strongly suggest that lack of activation by phosphatidylserine is not due to a deleterious effect of these mutations on CaN phosphatase activity. The findings that CaN containing the CnB-BRC mutant does not bind to and is not activated by phosphatidylserine vesicles provide strong evidence that residues $KK^{20,21}$, $KR^{24,25}$, and $KK^{27,28}$ of CnB are required for the Ca^{2+}-dependent binding of CaN to phosphatidylserine.

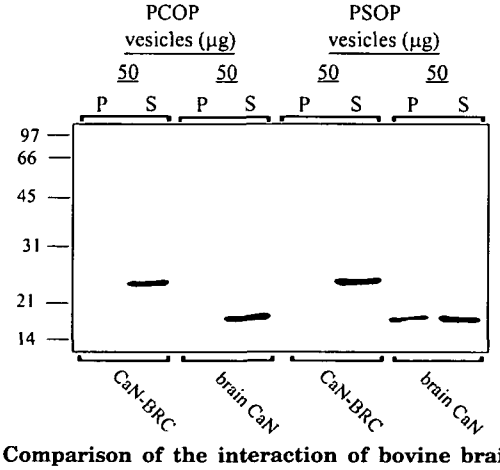

Fig. 6. **Comparison of the interaction of bovine brain CaN and CaN-BRC with PSOP.** Bovine brain CaN (5 μg) or CaN-BRC (5 μg) were each incubated with PSOP or PCOP vesicles in the presence of 1 mM $CaCl_2$ as described in "MATERIALS AND METHODS." The supernatant (S) and pellet (P) fractions from the binding assays were analyzed by SDS-PAGE and Western blotting. The anti-CnB, and HRP-conjugated goat-anti rabbit antibodies were each diluted 2,000-fold.

DISCUSSION

These studies examined the effect of reverse-charge mutations of a cluster of N-terminal basic residues in the CnB subunit on the Ca^{2+}- and myristoylation-dependent association of CaN with phosphatidylserine vesicles. The previous findings that CaN binds to negatively charged, but not neutral phospholipids suggests that, similar to MARCKS, positively charged amino acid residues participate in the myristoylation-dependent binding of CaN to phosphatidylserine vesicles (*13*). This hypothesis is supported by the present results indicating that additional factors besides myristoylation are involved in the binding of CaN to phosphatidylserine. Although the CnB-BRC mutant is myristoylated, the $KK^{20,21}$/EE, $KR^{24,25}$/EE, $KK^{27,28}$/EE mutations in CnB abolished the binding of CaN to phosphatidylserine vesicles. Similar to the findings for the MARCKS-membrane association, our findings support a three-component model for CaN association with negatively charged phospholipids in which hydrophobic interactions from the N-terminal myristate and electrostatic interactions from CnB basic residues $KK^{20,21}$, $KR^{24,25}$, and $KK^{27,28}$ are both required for the Ca^{2+}-dependent association of CaN with phosphatidylserine vesicles. However, unlike MARCKS, there is not a phosphorylatable Ser/Thr within the basic residue cluster of neuronal CnB to provide a mechanism for the introduction of negative charge and electrostatic repulsion necessary for reversible membrane association. Interestingly, the testis-specific isoform contains a Ser instead of an Arg at position 26. Whether this Ser in the testis-specific CnB modulates CnB binding to phosphatidylserine has not been examined. The crystal structure of bovine brain CaN shows that the N-terminal myristate of CnB is attached to a 14 residue loop that allows the myristate to lay against the hydrophobic side of the amphipathic N-terminal helix of EF-hand Ca^{2+}-binding domain I (*21*) (Fig. 7). The charged

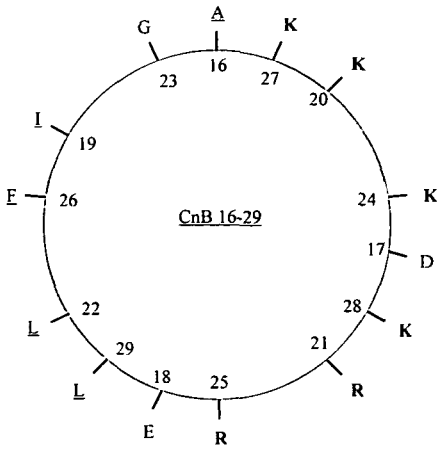

Fig. 7. **Helical wheel analysis of CnB amino acid residues 16–29.** The positions of amino acid residues 16–29 along the N-terminal α-helix of EF-hand Ca^{2+}-binding loop I of CnB are shown by helical wheel analysis. Basic residues are in bold. Hydrophobic residues are underlined.

side of the N-terminal helix contains the cluster of basic residues. CnB undergoes conformational changes upon Ca^{2+} binding that are transmitted to CnA and are involved in activation of phosphatase activity (*5*). These findings suggest a model for the association of CaN with phosphatidylserine vesicles based upon the mechanism of the Ca^{2+}- and myristoylation-dependent binding of recoverin to rod outer segment membranes. Recoverin is an N-terminally myristoylated EF-hand Ca^{2+}-binding protein expressed in photoreceptor cells that undergoes a Ca^{2+}- and myristoylation-dependent binding to the neutral membrane phospholipid phosphatidylcholine (*24*). In the absence of Ca^{2+} the myristate lies within a hydrophobic groove (*24*). Ca^{2+} binding to recoverin disrupts the hydrophobic interactions between the myristate and hydrophobic residues forming the groove, leading to myristate extrusion and exposure of the hydrophobic residues for binding to phosphatidylcholine (*24*). Similar to recoverin, Ca^{2+} binding to CnB could also cause conformational changes in the CnB N-terminal helix that disrupt the interaction with the myristate and expose the basic residue cluster. Together these changes would allow for the association of CaN with negatively charged membrane phospholipids.

CaN phosphatase activity is stimulated by negatively-charged phospholipids *in vitro* (*9*, *25*). Based upon these findings we compared the PSOP-dependent stimulation of wild-type CaN and CaN-BRC as an additional experimental approach to show that CaN-BRC does not associate with PSOP. The present study shows that PSOP vesicles stimulate the phosphatase activity of baculovirus-expressed mammalian brain CaN toward ^{32}P-Histone H1 (Fig. 6). In contrast the phosphatase activity of CaN-BRC was not increased by PSOP. These results provide additional evidence that the $KK^{20,21}$/EE, $KR^{24,25}$/EE, $KK^{27,28}$/EE mutations in CnB disrupt the association of CaN with phosphatidylserine. Using ^{32}P-Histone H1 or ^{32}P-RII peptide as substrates, the PSOP-stimulated phosphatase activities were 60, and 40%, respectively, of the CaM-stimulated phosphatase activity. Similar results were observed for the stimulation of CaN activity by phosphatidylinositol in the presence of Ca^{2+} (*25*). Phosphatidylinositol-stimulated CaN phosphatase activity was 30% of the CaM-stimulated activity (*25*). These differences in the levels of phospholipid-stimulated CaN phosphatase activity may be due differences in enzyme preparation, the lipid used, the activating divalent cation, and the substrate. Presently, ^{32}P-casein, ^{32}P-Histone H1, and ^{32}P-RII peptide are the only substrates that have been shown to be dephosphorylated by phospholipid-activated CaN *in vitro* (*9*, *25*). The physiological significance of these findings is unknown. However, several membrane proteins and membrane-associated proteins are CaN substrates (*2*, *26*), suggesting that phospholipid modulation of CaN activity may be involved in their regulation by CaN. Additional experiments investigating the phospholipid-stimulated dephosphorylation of known physiological substrates of CaN are necessary.

Similar to Type 1 protein phosphatase, targeting of CaN to specific subcellular locations or substrates appears to be a physiological mechanism to regulate CaN phosphatase activity (*27*). AKAP-CaN binding localizes CaN near the plasma membrane (*27*). CaN is dynamically associated with and regulates β2-adrenergic receptors, and is also associated with the 1,4,5-trisphosphate receptor via an interaction with FKBP12 (*28*, *29*). Physical association between CaN and NFAT1, or NFAT4 is necessary to initiate and maintain the nuclear localization of these transcription factors (*30*, *31*). Activation-induced T cell death is inhibited by the binding of CaN to Bcl-2 (*32*). As CaN-protein interactions target CaN to dephosphorylate specific substrates, CaN-membrane interactions may localize CaN to substrates in or associated with the plasma membrane. The Ca^{2+}- and myristoylation-dependent association of CaN with PSOP vesicles *in vitro* suggests a mechanism for the reversible association of CaN with phosphatidylserine in the plasma membrane.

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