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Ca^{2+}- and Myristoylation-Dependent Association of Calcineurin with Phosphatidylserine[1]

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It has been proposed that N-terminal myristoylation of calcineurin B is necessary for the membrane association of calcineurin. We tested the effects of Ca^{2+} and myristoylation on the binding of calcineurin B alone or heterodimeric calcineurin to phosphatidylserine or phosphatidylcholine vesicles. In the presence of excess phosphatidylserine, 50–60% of total calcineurin associated with phosphatidylserine in a Ca^{2+}-sensitive manner. Calcineurin did not associate with phosphatidylcholine. Calcineurin containing both the α and β catalytic subunit isoforms bound to phosphatidylserine. Calmodulin interfered with the association of calcineurin with phosphatidylserine. In the presence of Ca^{2+}, myristoylated calcineurin B alone did not bind to phosphatidylcholine but did bind to phosphatidylserine, although to a lesser extent than the calcineurin heterodimer. Non-myristoylated calcineurin B alone, or calcineurin containing non-myristoylated calcineurin B did not associate with phosphatidylserine in the presence of Ca^{2+}. These results indicate: (i) Both isoforms of calcineurin bind to phosphatidylserine. (ii) A phospholipid binding site is located on the calcineurin B subunit. (iii) Calcineurin B myristoylation is required for the Ca^{2+}-sensitive binding of calcineurin to phosphatidylserine vesicles *in vitro*.

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

The Ca^{2+}/CaM-dependent Ser/Thr protein phosphatase calcineurin (CaN) is a ubiquitously expressed multi-functional phosphatase involved in the regulation of diverse physiological processes such as neuronal post-synaptic potential, hippocampal long-term depression, T cell activation, and cardiac hypertrophy (*1–6*). In addition, several ion channels and the membrane-associated proteins GAP-43, dynamin-1, and eNOS are CaN substrates (*7–10*). CaN is a heterodimer of a 58–61 kDa catalytic A subunit (CnA) and a 19 kDa Ca^{2+}-binding regulatory B subunit (CnB) (*7*). CnB is post-translationally modified by *N*-terminal myristoylation (*8*). Protein *N*-myristoylation has been shown to be to be important for membrane localization, enzymatic activity, protein–protein interactions, Ca^{2+}-binding, and thermal stability (*9*, *10*). Myristoylation has been shown to increase the thermal stability of CaN, but is not required for heterodimer formation or Ca^{2+}-binding to CnB (*11–13*). Also, there is no difference in the kinetic parameters of CaN containing myristoylated or non-myristoylated CnB (*11*). However, the requirement of CnB myristoylation for the membrane association of CaN is still unclear. It has been reported that myristoylation is not required for CaN binding to phospholipid monolayers *in vitro*, and that equal amounts of CaN containing myristoylated or non-myristoylated CnB are found in the membrane fraction of yeast (*11*, *14*). Together these findings suggest that myristoylation is not required for CaN binding to membranes. However, it has also been reported that CaN binds to acidic phospholipids in a Ca^{2+}-dependent manner, and that CnB contains the phospholipid-binding site (*15*, *16*). Furthermore, phosphatidylserine stimulated CaN phosphatase activity toward Histone H1 (*15*). CaN has also been shown to associate with lymphocyte plasma membranes in a Ca^{2+}-dependent manner (*17*). In addition, the amount of membrane-associated CaN is increased in kindled rat brains (*18*).

In order to initiate studies aimed at clarifying the role of CnB myristoylation in the binding of CaN to membrane phospholipids we have used baculovirus expression to obtain heterodimeric CaN composed of both myristoylated and non-myristoylated CnB (myr-CnB and non-myr-CnB, respectively). We have also used bacterial expression to obtain myr-CnB or non-myr-CnB alone. In this report we present the results of our experiments in which we tested these proteins for their ability to bind to phospholipid vesicles composed of phosphatidylserine or phosphatidylcholine in the absence or presence of Ca^{2+}. Our results indicate that CaN associates with phosphatidylserine in a Ca^{2+}-sensitive manner and that CnB myristoylation is required for

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

CaN-phospholipid binding. In addition, we show that myr-CnB by itself binds to phosphatidylserine in a Ca^{2+}-sensitive manner, providing the first direct evidence that CaN contains a phospholipid binding site on CnB.

MATERIALS AND METHODS

Materials—Bovine brain CaN, rabbit anti-CnAα, rabbit anti-CnAβ, and horseradish peroxidase–conjugated goat anti–rabbit IgG were purchased from Chemicon. Rabbit anti-CnB antibody was purchased from Affinity Bioreagents. The palmitoyl, oleoyl forms of phosphatidylserine (PSOP) and phosphatidylcholine (PCOP) were obtained from Avanti Polar Lipids. Grace's supplemented insect cell medium, Pluronic F-68, antibiotic/antimycotic solution, and bacterial culture media, were obtained from GIBCO/BRL. Fetal bovine serum was purchased from Atlanta Biologicals. Sephacryl S-300, and CaM-Sepharose were purchased from Pharmacia. Antibiotics were from Sigma. BM-Fast Stain and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Boehringer Mannheim. 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, or the CnAαδ subunit alone were carried out as described (*12*, *20*). For bacterial expression of CnB, an *Nco*I restriction site was engineered into the initiator ATG of rat brain CnB cDNA by oligonucleotide-directed *in vitro* mutagenesis (Sculptor System, Amersham). Mutants were identified by DNA sequencing using the Sequenase DNA sequencing Kit (Amersham). The *Nco*I–*Pst*I fragment of the CnB cDNA was ligated into pSE420 (InVitrogen). Bacterially expressed myr-CnB or non-myr-CnB was obtained by Ca^{2+}-dependent hydrophobic interaction chromatography as described, except that following addition of IPTG (0.5 mM), the bacterial cultures were grown at 30°C for 3 h (*19*). The phenyl-Sepharose purified CnB samples were then further purified by gel filtration chromatography on a 1 × 100 cm Sephacryl S-300 column that had been calibrated with Blue Dextran and cytochrome c and equilibrated in EGTA buffer (2 mM EGTA pH 7.0, 0.5 mM dithiothreitol). Fractions (1.8 ml) containing CnB were identified by SDS-PAGE and Coomassie staining using BM Fast-Stain of aliquots (40 μl) of fractions from the expected elution volume of CnB. Fractions containing CnB were pooled, concentrated using Centriprep 10 filters, and stored at −70°C. Protein concentration was determined by the Bradford assay using bovine gamma globulin as standard. The purified CnB was greater than 95% pure as judged by SDS-PAGE (15%) and Coomassie staining using BM-Fast Stain.

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- or CnB-Phospholipid Binding Assays—As described in the figure legends, bovine brain CaN, baculovirus-expressed CaN or CnA alone, and bacterially expressed myr-CnB or non-myr-CnB, were incubated with the indicated amounts of sized, sucrose-loaded phospholipid vesicles composed of either PSOP or PCOP. 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

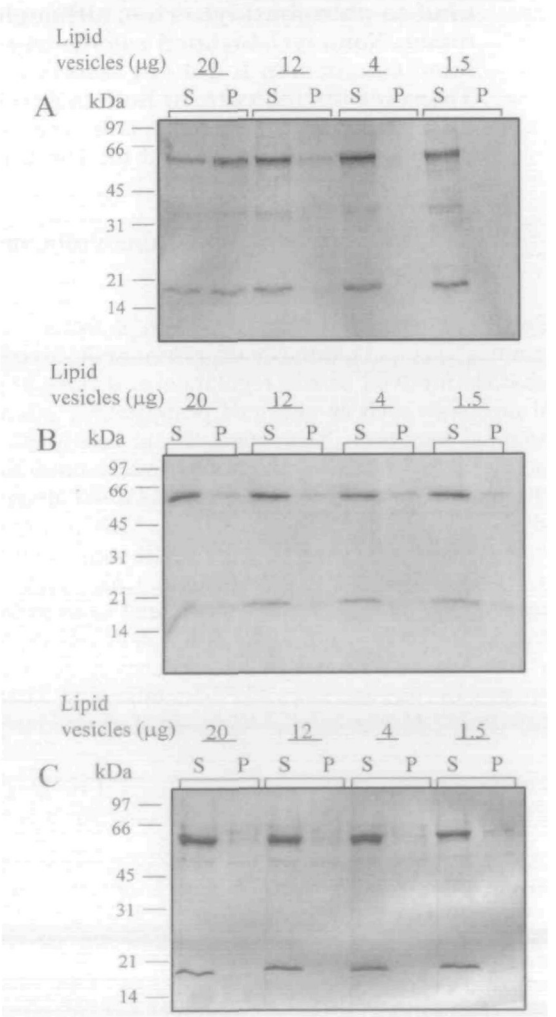

Fig. 1. **Comparison of the interaction of CaN with PSOP or PCOP.** Bovine brain CaN (4 μg) was incubated with the indicated amounts of PSOP or PCOP in the presence of 0.5 mM $CaCl_2$, or 1 mM EGTA, and supernatant (S) and pellet (P) fractions from CaN-phospholipid binding assays were obtained and analyzed by SDS-PAGE and Coomassie staining as described in "MATERIALS AND METHODS." (A) PSOP, 0.5 mM $CaCl_2$. (B) PSOP, 1mM EGTA. (C) PCOP, 0.5mM $CaCl_2$. These results are representative of three separate experiments.

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 (*20*). The SDS-sample buffer and SDS-PAGE gels contained 1 mM EGTA to prevent any Ca^{2+}-induced mobility shift of CnB (*21*).

RESULTS

Association of CaN with Phospholipids—Previous work has shown that bovine brain CaN associates with acidic, but not neutral, phospholipids in a Ca^{2+}-dependent manner *in vitro*, as assayed by gel filtration of CaN in the absence or presence of phospholipid vesicles (*15*). A shift of the protein (CaN) peak to the void volume (phospholipid vesicle peak) as determined by an increase in A_{595} absorbance, indicates an association of CaN with the phospholipid (*15*). We utilized a more rapid protocol in order to directly observe and more conveniently compare the binding of recombinant CaN composed of the CnA α isoform, brain CaN (composed of CnA α and β isoforms), and CnB alone to phospholipids, and to test directly the effect of CnB myristoylation on the association of CaN or CnB alone with membrane phospholipids.

We first incubated brain CaN with increasing amounts of phospholipid vesicles, microcentrifuged the mixtures to pellet the lipids, and collected the supernatant and pellet fractions. In the presence of Ca^{2+} (0.5 mM), the amount of brain CaN in the PSOP pellet fractions (P) increased as the amount of PSOP increased (Fig. 1A). Densitometry of the CnA protein bands in Fig. 1A indicate that with the five-fold excess of PSOP, 67% of the total CaN was present in the pellet fraction (Fig. 1A). Increasing the amount of PSOP to a tenfold excess did not increase the amount of PSOP pellet-associated CaN (data not shown). There was no increase in the amount of brain CaN associated with the PSOP pellet fractions in the presence of EGTA (Fig. 1B). In addition, in the presence of Ca^{2+} brain CaN was almost entirely absent from the PCOP pellet fractions (Fig. 1C). Using a different experimental protocol, these data are consistent with previous findings showing the Ca^{2+}-dependent association of brain CaN with the negatively charged phospholipid phosphatidylserine but not the neutral phospholipid phosphatidylcholine (*15*). The results showing that CaM decreases the amount of phosphatidylserine required to activate CaN phosphatase activity suggests that Ca^{2+}/CaM-binding to CnA increases the affinity of CaN for phosphatidylserine (*15*). However, the effect of CaM on the association of CaN with phosphatidylserine was not tested. To determine the effect of CaM on the Ca^{2+}-dependent association of CaN with PSOP, binding assays were carried out in which a threefold molar excess of CaM was present during the incubation of bovine brain CaN with phosphatidyl-

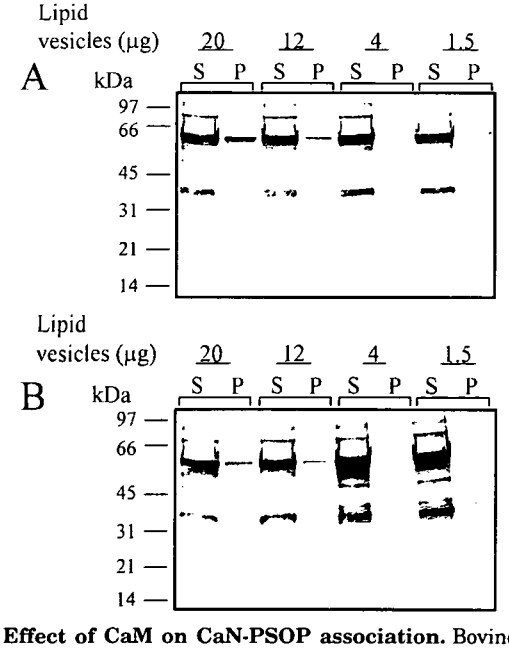

Fig. 2. **Effect of CaM on CaN-PSOP association.** Bovine brain CaN (4 μg) was incubated without (A) or with (B) CaM (1.8 μg) and the indicated amounts of PSOP in the presence of 0.5 mM $CaCl_2$. The supernatant (S) and pellet (P) fractions from CaN-phospholipid binding assays were analyzed by SDS-PAGE and Western blotting as described in "MATERIALS AND METHODS." The anti-CnA, and HRP-conjugated goat–anti rabbit IgG antibodies were each diluted 1000-fold. These results are representative of three experiments.

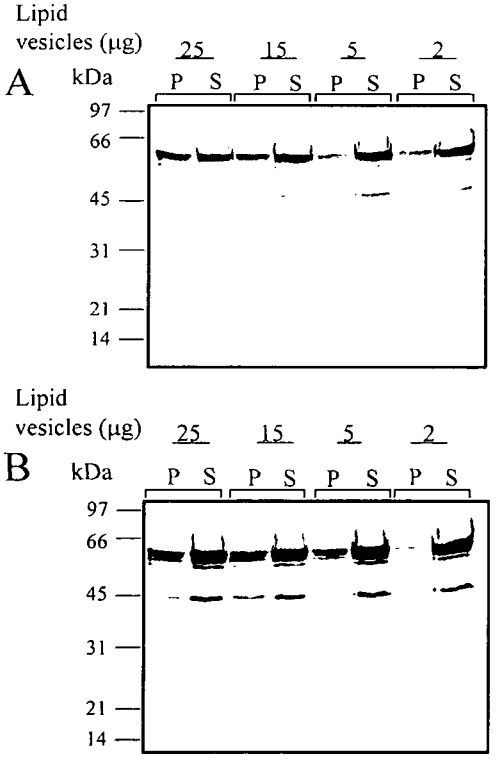

Fig. 3. **CaN composed of either the CnAα or CnAβ isoform associates with PSOP.** Bovine brain CaN (5 μg) was incubated with the indicated amounts of PSOP in the presence of 0.5 mM $CaCl_2$. The supernatant (S) and pellet (P) fractions from the CaN-PSOP binding assays were analyzed by SDS-PAGE and Western blotting as described in "MATERIALS AND METHODS." The anti-CnAα, (A) anti-CnAβ, (B) and HRP-conjugated goat–anti rabbit IgG antibodies were each diluted 1,000-fold. These results are representative of three experiments.

serine vesicles. As seen in Fig. 2 the amount of CaN present in the PSOP pellet fraction did not increase in the presence of a threefold molar excess of CaM (with respect to CaN). However, the amount of CaN present in the PSOP pellet fraction appears to be decreased in the presence of CaM. These findings indicate that CaM influences the association of CaN with phosphatidylserine.

Association of CaN Containing the α and β Isoforms of CnA with Phosphatidylserine—The α and β isoforms of CnA constitute approximately 80 and 20%, respectively, of bovine brain CaN (*22*). A possible reason for the findings shown in Figs. 1 and 2 that only a portion of the total brain CaN associates with the PSOP vesicles is that the binding of CaN to PSOP is isoform specific. To determine whether only one of the CnA isoforms associates with acidic phospholipids, the pellet and supernatant fractions from binding assays between brain CaN and PSOP vesicles were analyzed with CnA α or CnA β isoform-specific antibodies. The Western blot in Fig. 3 shows that the portion of brain CaN that associates with phosphatidylserine is composed of both the CnA α and CnA β isoforms, indicating that the association of CaN with phosphatidylserine is not isoform-specific.

Association of CnB with Phospholipids—The data from previous crosslinking experiments strongly suggest that the CnB subunit contains the phospholipid binding site(s) (*16*). To determine directly whether CnB is the subunit responsible for the interaction of CaN with acidic phospholipids, the association of myr-CnB or non-myr-CnB by themselves with membrane phospholipids was measured as described in the "EXPERIMENTAL PROCEDURES." Figure 4A shows that in the presence of EGTA, little or no myr-CnB was observed in the PSOP pellet fractions even when a 40-fold or greater excess of PSOP was present. Also, as seen in Fig. 4B, little or no myr-CnB associated with PCOP in the presence of Ca^{2+}. However, Fig. 4C shows that a portion of the myr-CnB binds to PSOP in the presence of Ca^{2+}. Densitometry of the CnB protein bands in the S and P fractions from the binding assay in which a 40-fold excess of PSOP was present indicates that 27% of the total myr-CnB associated with the PSOP pellet. These results indicate that similar to heterodimeric CaN, myr-CnB by itself associates with PSOP, but not PCOP, in a Ca^{2+}-sensitive manner. In contrast, non-myr-CnB does not bind to PSOP in the presence of Ca^{2+}, as indicated by the absence of a CnB protein band in the PSOP pellet fractions (Fig. 4D). These results directly demonstrate that CnB myristoylation is required for the Ca^{2+}-dependent association of CnB alone with phosphatidylserine. However, compared to total heterodimeric CaN, a lower percentage of total myr-CnB associated with PSOP. The association of CnB with PSOP vesicles occurred at PSOP:CnB ratios of 40:1, and 20:1, suggesting that the association of myr-CnB alone with PSOP requires a greater excess of phospholipid. CnB accounts for 25% of the total protein in the CaN heterodimer (*21*). Thus, using 4 μg of bovine brain CaN results in 1 μg of CnB present in the PSOP-brain CaN binding assays (Fig. 1). When the results in Fig. 1 are expressed as the ratio of PSOP:CnB, the heterodimeric CaN-PSOP association occurred at PSOP:CnB ratios (20:1, 12:1) that are lower than those observed with myr-CnB alone, and a higher percentage of heterodimeric CaN associated with the 20-fold excess of PSOP. These findings suggest that CnA may contribute to CaN-PSOP binding. Binding assays between PSOP and CnA alone were carried out to determine whether CnA by itself can interact with PSOP. As shown in Fig. 5, little or no binding was observed between CnA by itself and PSOP in the presence of Ca^{2+}. These results, and the findings that myr-CnB

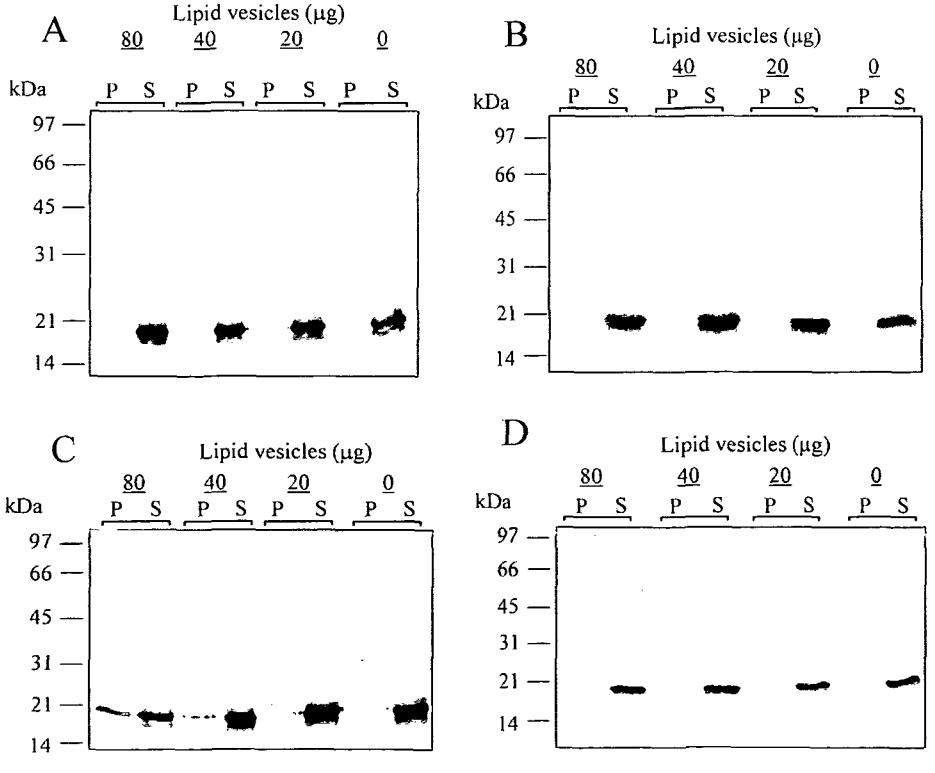

Fig. 4. **Comparison of the interaction of myr-CnB and non-myr-CnB with PSOP or PCOP.** Bacterially expressed myr-CnB (2 μg) or non-myr-CnB (2 μg) were each incubated with the indicated amounts of PSOP or PCOP in the presence of 0.5 mM $CaCl_2$, or 1 mM EGTA as described in "MATERIALS AND METHODS." The 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 IgG antibodies were each diluted 1,000-fold. (A) myr-CnB, PSOP, 1 mM EGTA. (B) myr-CnB, PCOP, 0.5 mM $CaCl_2$. (C) myr-CnB, PSOP, 0.5 mM $CaCl_2$. (D) non-myr-CnB, PSOP, 0.5 mM $CaCl_2$. Non-myr-CnB did not associate with PCOP in the presence of Ca^{2+} or with PSOP in the presence of EGTA (data not shown). These results are representative of at least three experiments.

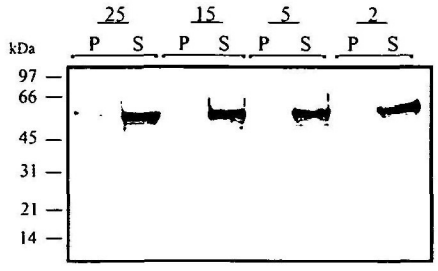

Fig. 5. **Interaction of CnA with PSOP.** Sf9 expressed CnA alone (8 μg) was incubated with the indicated amounts of PSOP in the presence of 0.5 mM $CaCl_2$. The supernatant (S) and pellet (P) fractions from CnA-PSOP binding assays were analyzed by SDS-PAGE and Western blotting as described in "MATERIALS AND METHODS." The anti-CnA, and HRP-conjugated goat–anti rabbit IgG antibodies were each diluted 1,000-fold. These results are representative of three experiments.

alone binds to PSOP support the earlier conclusion that the PSOP-binding site(s) of CaN is located on CnB (*19*).

Association of CaN Containing myr-CnB and Non-myr-CnB with Phosphatidylserine—The findings shown in Fig. 4 strongly suggest that CnB myristoylation plays a role in the Ca^{2+}-dependent association of heterodimeric CaN with phosphatidylserine. To determine directly the requirement of CnB myristoylation for the Ca^{2+}-dependent association of CaN with phosphatidylserine, we took advantage of the findings that baculovirus-expressed CaN is composed of both myr-CnB and non-myr-CnB (*12*). Non-myr-CnB is distinguished from myr-CnB by its slower mobility in SDS-PAGE (*12, 13*). The results in Fig. 6, A and B, show that, as expected, baculovirus-expressed brain CaN (composed of the CnA α isoform) associates with PSOP in the presence of Ca^{2+}. Baculovirus-expressed brain CaN does not bind to PSOP in the presence of EGTA or to PCOP in the presence of Ca^{2+} (data not shown). As also shown in Fig. 6, A and B, the baculovirus-expressed CaN used in these studies is composed of both myr-CnB and non-myr-CnB, as indicated by the presence of two CnB protein bands in the supernatant (S) fractions of the binding assays. If CnB myristoylation is not required for the Ca^{2+}-dependent association of CaN with PSOP, then both CnB protein bands should also be present in the pellet (lipid-binding) fractions of the binding assays. Interestingly, only the faster migrating protein band of myr-CnB is present in the PSOP pellet fractions (Fig. 6A). Eight micrograms of CaN was used in each binding condition to ensure the presence of a sufficient amount of CnB in the pellet and supernatant fractions for detection by Coomassie staining. However, in addition, the pellet and supernatant fractions from the CaN-PSOP binding assay were analyzed for CnB content by Western blotting and anti-CnB immunostaining to increase the sensitivity of detection of CnB. As shown in Fig. 6B, each supernatant fraction contains both myr-CnB and non-myr-CnB, but the pellet fractions from the binding assays containing the 5- or 3-fold excess, or equal amount of PSOP vesicles contain only the faster migrating myr-CnB. These results suggest that the absence of the non-myr-CnB protein band in the lipid-binding fraction is not a result of the lower sensitivity of Coomassie staining. Thus, these findings directly demonstrate the requirement of CnB myristoylation for the Ca^{2+}-sensitive association of CaN with phosphatidylserine.

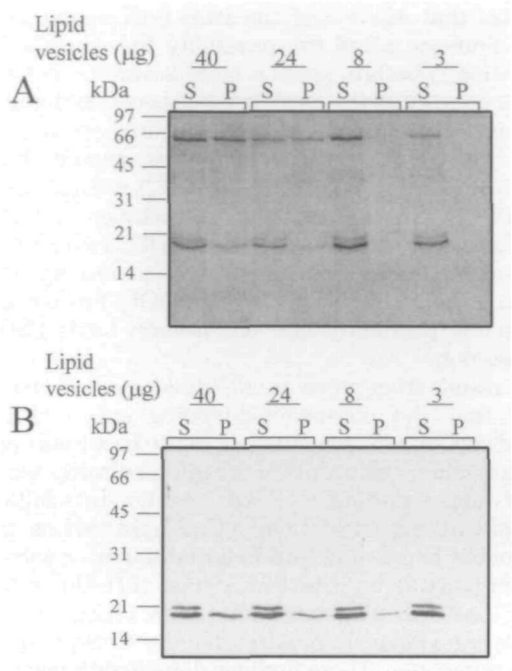

Fig. 6. **Interaction of CaN containing myr-CnB and non-myr-CnB with PSOP.** Sf9 expressed CaN (8 μg) was incubated with the indicated amounts of PSOP in the presence of 0.5 mM $CaCl_2$. The supernatant (S) and pellet (P) fractions from CaN-PSOP binding assay were analyzed by SDS-PAGE and Coomassie staining (A) or SDS-PAGE and Western blotting (B) as described in "MATERIALS AND METHODS." The anti-CnB and HRP-conjugated goat–anti rabbit IgG antibodies were each diluted 1,000-fold. These results are representative of three experiments.

DISCUSSION

Protein *N*-myristoylation has different functional consequences for different proteins (*9*). Protein-protein interactions, enzyme activity, Ca^{2+} binding, thermal stability, and membrane association have all been shown to be influenced by *N*-myristoylation (*9, 23*). For CaN, myristoylation does not influence subunit interactions, Ca^{2+} binding, or enzyme activity, but has been shown to provide thermal stability to the enzyme (*10–13*). In addition, there are reports describing the Ca^{2+}- and myristoylation-independent association of CaN with membranes and membrane phospholipids *in vitro* (*11, 14*), while other studies have described the Ca^{2+}-dependent association of CaN with membranes, and with negatively charged membrane phospholipids *in vitro* (*15, 17*). In the present study we utilized an additional approach to further investigate CaN-phospholipid interactions. Uniformly sized, sucrose-loaded phospholipid vesicles were incubated with bovine brain CaN, baculovirus-expressed rat brain CaN, myr-CnB, or non-myr-CnB with or without Ca^{2+}, and pelleted by microcentrifugation to obtain phospholipid vesicle–binding (pellet) and non-binding fractions (supernatant). The results of the present study show that mammalian brain CaN (native or recombinant) associates with PSOP vesicles in a Ca^{2+}- and myristoylation-dependent manner. CaN did not associate with vesicles composed of the neutral phospholipid PCOP. These findings support the hypothesis that CaN associates with acidic phospholipids in a Ca^{2+}- and myristoylation-sensitive manner. Densitometric analysis of the binding of CaN to PSOP

indicates that 40–70% of the total CaN bound to PSOP. These findings raised the possibility that the CaN-PSOP association is isoform specific, since bovine brain CaN contains a mixture of the two CnA isoforms (*22*). Immunoblot analysis of the composition of the lipid-associated CaN using isoform-specific CnA antibodies showed that CaN composed of either the CnA α or CnA β isoform associated with PSOP, indicating that the association of CaN with phosphatidylserine is not isoform-specific. In addition, 50% of recombinant heterodimeric CaN (containing only the CnA α isoform) associated with PSOP, further arguing against the possibility that one isoform binds PSOP and one does not.

The results from previous cross-linking experiments indicating that the phospholipid-binding site(s) of CaN is located on CnB (*16*) prompted us to use bacterially expressed mammalian brain CnB to determine directly the ability of CnB alone to bind to PSOP vesicles. In addition, the effect of CnB myristoylation on CnB-lipid binding could be analyzed by expressing CnB in bacteria with or without the gene for yeast *N*-myristoyltransferase (*11*). Our results indicate that CnB alone associates with phosphatidylserine and myristoylation is required for the CnB-phosphatidylserine interaction. These findings directly demonstrate that a phospholipid-binding site(s) of CaN is located on CnB. However, relative to heterodimeric CaN, a lower percentage of total myr-CnB bound to PSOP vesicles. Two possibilities raised by these findings are that the CnA subunit also contains binding site(s) for PSOP or that CnA-CnB subunit interactions influence the affinity of CnB for PSOP. Our finding that CnA by itself exhibits little or no association with the PSOP pellet supports the latter possibility.

The findings that a portion of the total CaN or myr-CnB associated with PSOP in the presence of Ca^{2+} are similar to findings that have been reported with recoverin. Recoverin is an EF-hand Ca^{2+}-binding protein that undergoes a Ca^{2+}-dependent association with membrane phospholipids (*23*). Dizhoor *et al.* reported that in the presence of excess membrane phospholipid, only 40% of total recoverin associated with the lipids (*24*). It was concluded that recoverin has a weak affinity for membrane phospholipids (*24*). Our findings that in the presence of up to a 40-fold excess of PSOP (with respect to CnB) only 27–67% of total CnB alone or CaN, respectively, binds to PSOP suggests that the interaction of PSOP with CnB in the CaN heterodimer or CnB alone may also be characterized by a weak affinity. These findings may explain, in part, why only half of neuronal calcineurin is associated with the particulate fraction. Furthermore, our results also indicate that both isoforms of calcineurin associate with phosphatidylserine, suggesting that the equal distribution of neuronal calcineurin between the soluble and particulate fractions is not due to isoform-specific binding of calcineurin to the plasma membrane.

The previous report that Ca^{2+}/CaM lowers the concentration of phosphatidylserine required for activation of CaN phosphatase activity suggests that Ca^{2+}/CaM increases the affinity of CaN for phosphatidylserine (*15*). This finding prompted us to determine whether Ca^{2+}/CaM would enhance the association of CaN with PSOP, as indicated by increased CaN binding to PSOP vesicles. However, our results indicate that Ca^{2+}/CaM interferes with the Ca^{2+}-sensitive association of CaN with PSOP. The different findings may be due to the fact that the effect of Ca^{2+}/CaM on phosphatidylserine-stimulated CaN phosphatase activity was obtained using Ni^{2+}-activated CaN (*15*), while we were studying the CaN-PSOP interaction in the presence of Ca^{2+}. Ni^{2+}-activated CaN exhibits unusual characteristics compared to Ca^{2+}-stimulated CaN, including prolonged enzymatic activity and antibody distinguishable conformational changes (*25*). We are currently investigating the regulation of CaN phosphatase activity by Ca^{2+}, CaM, and phosphatidylserine.

In contrast to our findings and the earlier report of Politino and King (*15*), Kennedy *et al.* reported a Ca^{2+}- and myristoylation-independent interaction of CaN with phosphatidylserine/–phosphatidylcholine monolayers (*11*). Although we used baculovirus-expressed mammalian brain CaN and Kennedy *et al.* used mammalian brain CaN reconstituted *in vitro* from bacterially-expressed subunits, purified bovine brain CaN was also used in all three studies. The different findings may be due to the different experimental protocols used to measure CaN-phospholipid interactions. Our results showing Ca^{2+} and myristoylation dependent association of mammalian brain CaN or CnB alone are not consistent with an earlier report of the myristoylation-independent association of yeast CaN with membrane and particulate fractions (*14*). It was reported that similar to the even distribution of mammalian brain CaN between soluble and particulate fractions, native yeast CaN or yeast CaN containing non-myr CnB is also distributed between the soluble and particulate fractions. In addition, Triton X-100 released an equal amount of CaN containing myr- or non-myr CnB from the particulate fraction, suggesting that CnB myristoylation is not necessary for membrane association of CaN in yeast (*14*). However, Cyert and Thorner reported that yeast CaN is found exclusively in the soluble fraction, suggesting that additional factors, such as strain differences, may play a role in yeast CaN membrane association (*26*). Furthermore, the amino acid sequences of mammalian brain and yeast CnB are 53% identical and 82% homologous (*27*). Mammalian brain CaN and yeast CaN may have different affinities for membrane phospholipids, due to differences in amino acid sequences involved in binding myristoylated proteins to membranes. We are currently carrying out studies to localize the phospholipid binding site(s) on mammalian brain CaN.

A wide variety of proteins ranging from the transcription factors Elk-1 and NFATc to the signaling proteins DARPP-32, Inhibitor-1, and the RII subunit of cAMP-dependent protein kinase are regulated by CaN (*28*, *29*). In addition, several ion channels and membrane-associated proteins such as adenylyl cyclase, GAP-43, and dynamin-1 are CaN substrates (*30–33*), suggesting CaN may be localized to the membrane to be positioned close to its substrates. The colocalization of CaN and its substrates at the membrane could allow for rapid dephosphorylation and regulation of activity. Additional studies are needed to determine the mechanism by which CaN associates with membrane phospholipids, and whether membrane-associated CaN dephosphorylates membrane-associated proteins.

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