Determinants of Calcineurin Binding to Model Membranes†

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ABSTRACT: The biochemical factors that lead to membrane targeting of the Ser/Thr protein phosphatase calcineurin were examined using model phospholipid membranes. The interaction of myristoyl- and non-myristoylcalcineurin with lipid surfaces was investigated as a function of negatively charged phospholipids, diacylglycerol, Ca^{2+}, and calmodulin. The data indicate that calcineurin binding to phospholipid monolayers both is myristoyl-independent and is mediated by anionic phospholipids and/or diacylglycerol. Although the effect of Ca^{2+} on calcineurin−lipid binding is minor, calmodulin altered the binding of calcineurin to the lipid membrane in a Ca^{2+}-dependent manner. Experiments with a constitutively active form of calcineurin that does not bind calmodulin indicated that the effect required the interaction of calcineurin with calmodulin. Our results suggest that phosphatidylserine, diaclyglycerol, and calmodulin may mediate the lipid binding properties of calcineurin *in vivo*.

Calcineurin is a Ser/Thr protein phosphatase comprised of two subunits. The 58 kDa calcineurin A subunit contains the active site and shares homology with the other members of the Ser/Thr protein phosphatase family (*1*, *2*). The calcineurin B subunit is a 19 kDa Ca^{2+}-binding subunit and is a member of the EF-hand family of Ca^{2+}-binding proteins, including members such as calmodulin and troponin C. The primary sequence of calcineurin B is well conserved in higher eukaryotes and contains the sequence requirements for N-terminal myristoylation (*3*), a modification that is conserved in calcineurin B from yeast to mammals (*4*−*6*). Non-myristoylcalcineurin has yet to be isolated from a biologic source, suggesting that myristoylation of calcineurin B is quantitative and irreversible.

For many myristoylated proteins, including calcineurin, the biological role of the myristoyl moiety is not clear. Until recently, the predominant view of myristoylation and fatty acylation in general was that attachment of a fatty acid caused the protein to associate with membranes. It is now known that myristoylation is not sufficient to anchor a parent protein to biomembranes. Electrostatic interactions and myristoylation each account for approximately half of the energetic contribution to membrane binding in model peptide studies (*7*−*9*). Thus, disruption of either of these forces may result in dissociation of the protein from the lipid surface. In fact, myristoyl-dependent membrane localization has recently been shown to be reversible by several means, including phosphorylation (*10*, *11*), Ca^{2+} binding (*12*−*14*), and pH (*15*, *16*). In addition, it has been demonstrated for the MARCKS1[1] protein and eNOS that protein−lipid binding may also be regulated by Ca^{2+}/calmodulin (*10*, *17*).

Although a significant amount of research indicates calcineurin is capable of binding lipid surfaces both *in vitro* (*18*−*20*) and *in vivo* (*21*, *22*), there is evidence that calcineurin is cytoplasmically localized (*23*) and may be associated with either cytoskeletal (*24*) or integral membrane proteins (*25*). In fact, membrane targeting of calcineurin may represent a regulatory mechanism since calcineurin translocates from the cytoplasm to the cytoplasmic membrane upon coexpression with Bcl-2 in BHK cells (*26*). Calcineurin bound to Bcl-2 is active but is no longer able to promote the nuclear localization of NF-AT, a transcription factor necessary for interleukin-2 transcription. Recent work has indicated that myristoylation of calcineurin was not required for association with lipid monolayers (*20*), in agreement with experiments examining the localization of myristoyl- and non-myristoylcalcineurin in yeast (*22*). In contrast to a membrane-localizing function, it was found that myristoylation of calcineurin significantly affected the thermostability of the enzyme (*20*) in a manner similar to that of cAMP-dependent protein kinase (*27*). Since calcineurin−lipid interactions do not appear to be myristoyl-dependent, other mechanisms such as electrostatics or insertion of hydrophobic residues into the bilayer must account for the energetically favorable lipid-binding properties of calcineurin.

In this study, recombinant myristoyl and non-myristoyl forms of calcineurin were used to further examine the requirements for lipid association. The effects of anionic phospholipid, diaclyglycerol, Ca^{2+}, and calmodulin on cal-

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[1] Abbreviations: CaN, nonmyristoylated calcineurin heterodimer; CaNB, nonmyristoylated calcineurin B subunit; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; eNOS, endothelial nitric oxide synthase; FKBP12, FK-506 binding protein; LUV, large unilamellar vesicle; MARCKS, myristoylated alanine-rich C kinase substrate; M-CaN, myristoylated calcineurin heterodimer; M-CaNB, myristoylated calcineurin B subunit; PODG, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylglycerol; POPC, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylcholine; POPS, 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylserine.

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cineurin−membrane interactions were studied using both phospholipid monolayers and vesicles. Our results indicate that calcineurin binding to model membranes is mediated in part by negatively charged phospholipid and/or diacylglycerol. Although Ca^{2+} alone did not significantly influence calcineurin−lipid interactions, Ca^{2+}/calmodulin caused an apparent reorientation of calcineurin at the buffer−lipid interface.

EXPERIMENTAL PROCEDURES

Materials. 1-Palmitoyl-2-oleoyl-*sn*-3-phosphatidylglycerol (PODG) was purchased from Sigma (St. Louis, MO). The phospholipids 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylcholine (POPC) and 1-palmitoyl-2-oleoyl-*sn*-3-phosphatidylserine (POPS) were from Avanti Lipids (Alabaster, AL).

Expression of Calcineurin A, CaNB, and Myristoyl-CaNB in E. coli. The cDNAs for the α isoform of rat calcineurin A and rat calcineurin B were kindly provided by Dr. Brian Perrino and Dr. Tom Soderling (*28*, *29*). Plasmid pBB131 encoding the yeast *N*-myristoyltransferase gene was a gift from Dr. Jeffrey Gordon (*30*).

Rat calcineurin A was purified as previously described (*31*). Cultures of *E. coli* expressing myristoylcalcineurin B (M-CaNB) (*20*) and non-myristoylcalcineurin B (CaNB) (*31*) were grown in a 10 L New Brunswick Bioflo 3000 fermentor. Cells were grown at 37 °C in 2× TY media supplemented with 100 μg/mL each of kanamycin and ampicillin. The pH was continuously monitored and maintained at 7.1 by addition of either NH_4OH or H_3PO_4. Dissolved oxygen was maintained at 20% saturation (relative to air) using a stir rate of 400 rpm and sparging with either compressed air (low cell density) or oxygen (high cell density). The culture was grown to a cell density corresponding to an absorbance at 595 nm of ≈4 after which the cells were induced by addition of IPTG to 1 mM. Growth was allowed to proceed 8 h post-induction, reaching a final absorbance at 595 nm of ≈8, after which the cells were harvested and lysed as previously described (*31*).

Reconstitution of Calcineurin A with CaNB or M-CaNB. Calcineurin A was reconstituted with an excess of either CaNB or M-CaNB, followed by purification using calmodulin−Sepharose affinity chromatography and Sephacryl S-300 gel filtration chromatography as described (*31*). The purity of M-CaN or CaN was >95%, as judged by 13% SDS−PAGE gels stained with Coomassie blue (*20*, *31*). All protein concentrations were determined using a Coomassie blue dye-binding assay (Pierce, Rockford, IL), with bovine serum albumin as a standard.

Preparation of Trypsinized Calcineurin. Limited proteolysis of calcineurin by trypsin produces a truncated, 43 kDa form of calcineurin A that binds calcineurin B but no longer binds calmodulin (*32*). Briefly, 10 μM M-CaN in 50 mM Tris-HCl, 0.1 mM $CaCl_2$, and 10 mM β-mercaptoethanol, pH 7.0, was incubated with 100 nM bovine pancreatic trypsin (Sigma) at 30 °C for 5 min after which the reaction was immediately placed on ice and then chromatographed over a Superose 6 gel filtration column (1.6 × 50 cm) equilibrated in 20 mM Tris-HCl, 0.15 M NaCl, 1.0 mM dithiothreitol, 0.1 mM magnesium acetate, and 0.1 mM EGTA. Fractions containing trypsinized calcineurin were identified via 13% SDS−PAGE, pooled, and concentrated using a Centricon-30 ultrafiltration membrane (Amicon, Beverly, MA).

Assays Investigating the Interaction of Calcineurin with Phospholipid Monolayers. The interaction of M-CaN and CaN with lipid monolayers was studied using a Wilhelmy-type film balance (*33*, *34*) to measure changes in surface tension as previously described (*20*). The surface pressure (π) is defined as the difference in surface tension (γ) between buffer alone (γ_0) and buffer with the lipid film (γ_{mono}). In all experiments, the concentration of either M-CaN or CaN was 0.1 μM. Where noted, 0.2 M $CaCl_2$ or EGTA was injected into the subphase to a final concentration of 0.1 mM. Initial surface pressures in the range of 10−37 mN/m were explored. The surface pressure−area diagrams for the lipid mixtures used were determined (data not shown) and exhibited no phase transitions over the conditions explored.

Measurement of [^{14}C]M-CaN or [^{14}C]CaN Binding to 50 mol % POPS/50 mol % POPC Monolayers. M-CaN or CaN was labeled with [^{14}C]formaldehyde (55 Ci/mol) by reductive methylation (*35*). The labeling reaction was dialyzed in a 12−14 kDa cutoff dialysis bag against two changes of buffer (1 L each of 20 mM Tris-HCl, 0.15 M NaCl, 1.0 mM magnesium acetate, 0.1 mM EGTA, and 1.0 mM dithiothreitol, pH 7.5) at 4 °C. The specific activity of the radiolabeled protein was 108 Ci/mol for M-CaN and 110 Ci/mol for CaN, corresponding to approximately two modifications per mole of protein. Mock labeling reactions indicated that modified calcineurin retained ≈80% activity compared to samples that did not include formaldehyde. Additionally, the surface pressure changes induced by addition of either [^{14}C]M-CaN or [^{14}C]CaN to 50 mol % POPS/50 mol % POPC monolayers were identical to the unmodified forms of calcineurin.

[^{14}C]M-CaN and [^{14}C]CaN monolayer binding measurements were carried out using the procedure for measuring calcineurin−lipid monolayer interactions with the following additions. During the period of monolayer equilibration but prior to protein addition, a 20 μL aliquot of 10 mM ^{32}P-labeled inorganic phosphate, 0.1 M NaCl (final concentration of orthophosphate = 10 μM) was injected into the subphase to allow for correction of unbound protein. After the protein−monolayer interface had reached equilibrium, the monolayer was harvested by adsorption to a 7-cm diameter piece of Whatman 1PS hydrophobic filter paper (*36*). The paper was cut into small squares for ^{32}P and ^{14}C dual-label counting on a Beckman LS 3801 scintillation counter. The amount of ^{32}P and ^{14}C present in a 1 mL sample of the subphase was also counted for determination of aqueous buffer adhering to the paper. The volume of subphase carried over onto the filter paper was determined by dividing the ^{32}P dpm present on the filter paper by the dpm/mL of ^{32}P present in the subphase, yielding the volume carried over. Since the dpm/mL of [^{14}C]calcineurin in the subphase is known, the amount of unbound protein can be determined to correct for carryover. The corrected amount of protein bound to the paper (defined as surface excess, Γ) was expressed in units of picomoles bound per square centimeter.

Preparation of Lipid Vesicles. Phospholipids in chloroform were mixed according to the desired mole fractions, and the chloroform was evaporated under high vacuum for ≈1 h. The lipids were suspended in 25 mM Tris-HCl, 0.15 M NaCl, and 1.0 mM dithiothreitol, pH 7.5. Large unilamellar vesicles (LUVs) were prepared by extrusion through 100 nm pore-size polycarbonate membranes according to the manufacturer's directions (Avanti, Alabaster, AL).

Equilibrium Binding Measurements of Calcineurin to Phospholipid Vesicles. Binding of M-CaN to LUVs was determined by quasi-elastic light scattering as described (*37*, *38*), using eq 1:

$$\frac{I_{s2}}{I_{s1}} = \left(\frac{\partial n_2/\partial c_2}{\partial n_1/\partial c_1}\right)^2 \left(\frac{M_2}{M_1}\right)^2$$

where I_{s2} and I_{s1} are the scattered light intensities of the protein−vesicle complexes and the lipid vesicles alone, respectively, $\partial n_2/\partial c_2$ and $\partial n_1/\partial c_1$ are the change in refractive indices for protein−vesicles and vesicles, respectively, and M is the molecular weight of the scattering species. All light scattering measurements were corrected for scattering due to unbound protein. The approximation $\partial n_2/\partial c_2 = \partial n_1/\partial c_1$ was used to simplify the equation. As a result, an error of up to 15% will be reflected in the final determination of M_2/M_1. For comparative purposes, however, the approximation is valid (*37*). Thus, the ratio of light scattering intensities is proportional to molecular mass.

Light scattering at 320 nm was measured using a FluoroMax fluorescence spectrometer (JY/Spex Instruments) at 90° relative to the incident light in buffer consisting of 25 mM Tris-HCl, 0.15 M NaCl, and 1.0 mM dithiothreitol, pH 7.5. The scattered light intensities of samples containing lipid alone were compared to samples containing both lipid and protein. The final lipid and calcineurin concentrations were 25 μg/mL and 0.32 μM, respectively. For both M-CaN and CaN, $\leq$0.1 μM was saturable for the observed increases in light scattering. Where indicated, calmodulin was added to the appropriate final concentration using a concentrated stock solution to minimize any dilution effects. A molecular mass of 1.15×10^8 Da for 50 mol % POPS/50 mol % POPC LUVs was used to quantify the amount of calcineurin bound to extruded vesicles (*39*).

RESULTS

Effect of Myristoylation, POPS, and Diacylglycerol on Calcineurin Interactions with Phospholipid Monolayers. All lipid films were in the liquid-expanded state and exhibited no phase transitions in the surface pressure range between 0.1 mN/m and monolayer collapse. For both CaN and M-CaN, a protein concentration of 0.1 μM was sufficient to saturate the observed surface pressure changes. Lipid compositions ranging from 100 mol % POPS/0 mol % POPC to 0 mol % POPS/100 mol % POPC, as well as 60 mol % PODG/20 mol % POPS/20 mol % POPC, were investigated. Protein−lipid binding data acquired using the Wilhelmy technique are most often plotted as the change in surface pressure ($\Delta\pi$) as a function of the initial surface pressure (π_i). The *x*-intercept of such plots ($\pi_i|_{\Delta\pi=0}$) represents the maximum surface pressure of the monolayer at which the interaction of protein and lipid is energetically favorable for protein insertion.

Figure 1 shows the $\Delta\pi-\pi_i$ plots for M-CaN and CaN for four different lipid compositions. The isotherms for both forms of calcineurin are indistinguishable, regardless of lipid composition, indicating myristoylation does not contribute to model membrane association. Interestingly, the mole fraction of anionic lipid (POPS) raised the maximum surface pressure at which both CaN and M-CaN will bind in a concentration-dependent fashion, indicating an electrostatic

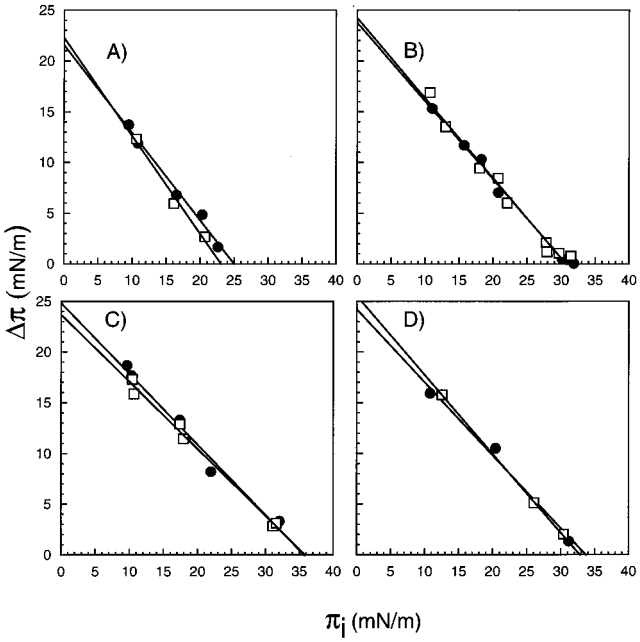

FIGURE 1: Effect of lipid composition on the interaction of CaN and M-CaN with phospholipid monolayers. The effect of POPS on the ability of M-CaN (filled circles) and CaN (open squares) to bind phospholipid monolayers composed of POPS and/or POPC overlying buffer (25 mM Tris-HCl, 0.15 M NaCl, 0.1 mM EGTA, and 1.0 mM dithiothreitol, pH 7.5) was examined as described under Experimental Procedures. The resultant change in surface pressure induced by protein binding was plotted as a function of initial monolayer surface pressure. (A) 100 mol % POPC; (B) 50 mol % POPS/50 mol % POPC; (C) 100 mol % POPS; (D) 20 mol % POPS/20 mol % POPC/60 mol % PODG. For all measurements, the final protein concentration was 0.1 μM. A least-squares fit was applied to the data for each monolayer composition.

Table 1: Effect of Phosphatidylserine and Diacylglycerol on CaN and M-CaN Lipid Binding[a]

monolayer composition (mole fraction)			$\pi_i\|_{\Delta\pi=0}$ (mN/m)	
POPS	POPC	PODG	M-CaN	CaN
	1.0		25	23
0.2	0.8		27	25
0.5	0.5		31	31
1.0			36	36
0.2	0.2	0.6	34	33

[a] The maximum surface pressure ($\pi_i|_{\Delta\pi=0}$) at which CaN and M-CaN will insert into a monolayer of the indicated composition was determined from the *x*-intercept of the respective $\Delta\pi-\pi$ plot.

contribution to membrane binding (Table 1). Similarly, the presence of diaclyglycerol in the monolayer raised the maximum insertion pressure in comparison to a monolayer without diacylglycerol, but the presence of the myristoyl group again had no effect (Figure 1, Table 1).

Since a Ca^{2+}-dependent mechanism of myristoyl protein−lipid association has recently been identified for recoverin (*12*−*14*), and calcineurin is a Ca^{2+}-binding protein, we also tested the effect of Ca^{2+} on the lipid-binding properties of M-CaN and CaN to 50 mol % POPS/50 mol % POPC monolayers. Comparing the data in Figure 2A,B, the $\pi_i|_{\Delta\pi=0}$ values for M-CaN and CaN in the presence of Ca^{2+} are virtually identical ($\approx$31 nN/m). A small effect of Ca^{2+} on the slope of both the myristoyl- and the non-myristoylcalcineurin $\Delta\pi-\pi_i$ plot is observed, perhaps suggesting that Ca^{2+} may affect the lipid-binding properties of calcineurin to membranes at reduced surface pressure. The surface

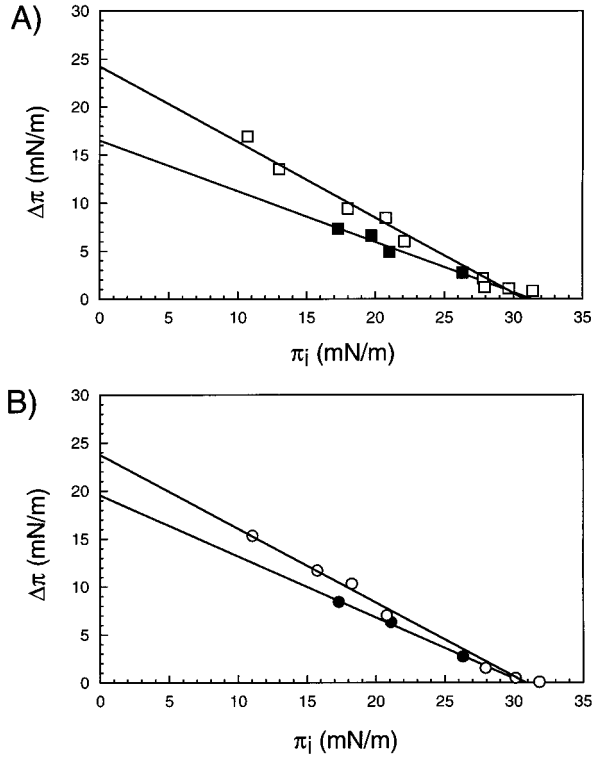

FIGURE 2: Effect of Ca^{2+} on the interaction of CaN and M-CaN with 50 mol % POPS/50 mol % POPC monolayers. The effect of Ca^{2+} on the ability of CaN (A) and M-CaN (B) to bind 50 mol % POPS/50 mol % POPC monolayers overlying buffer (25 mM Tris-HCl, 0.15 M NaCl, 0.1 mM EGTA or 0.1 mM $CaCl_2$ where indicated, and 1.0 mM dithiothreitol, pH 7.5) was examined as described under Experimental Procedures. The resultant change in surface pressure induced by protein binding was plotted as a function of initial monolayer surface pressure. For all measurements, the final CaN or M-CaN concentration was 0.1 μM. (A) CaN + EGTA (open squares) or CaN + Ca^{2+} (closed squares); (B) M-CaN + EGTA (open circles) or M-CaN + Ca^{2+} (closed circles). A least-squares fit was applied to the data for each monolayer composition.

pressure−area diagrams for 50 mol % POPS/50 mol % POPC monolayers in the presence of either 0.1 mM Ca^{2+} or 0.1 mM EDTA were determined and showed no phase transitions over the range of experimental surface pressures (data not shown).

A limitation of the Wilhelmy method is that surface pressure changes may not correlate with the amount of protein bound unless this relationship is defined through a direct binding measurement, usually with radiolabeled protein (*40*). As shown in Figure 3, a correlation between the amount of protein bound to the lipid surface (Γ, surface excess) and increases in surface pressure after protein addition was confirmed in experiments using [^{14}C]M-CaN or [^{14}C]CaN and 50 mol % POPS/50 mol % POPC monolayers. The amount of protein bound is lower (≈1 pmol/cm^2) than the amount of protein that could theoretically bind (3−7 pmol/cm^2), based on the available surface area of the monolayer (20.35 cm^2) and the molecular dimensions of calcineurin (87 Å × 61 Å × 37 Å) (*41*), assuming no free space between molecules. However, other proteins such as the hydrophilic lipid-binding protein apolipoprotein A-IV saturate a phosphatidylcholine monolayer at 2.2 pmol/cm^2 (*42*). In fact, the amount of apo A-IV bound at 25 mN/m was only 0.2 pmol/cm^2. Thus, these measurements are consistent with a mechanism in which calcineurin is capable of binding lipid membranes in a myristoyl-independent

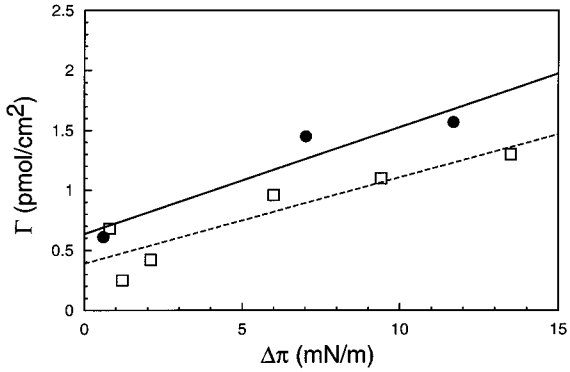

FIGURE 3: Relationship between surface excess (Γ) and $\Delta\pi$ for M-CaN and CaN. Amount of [^{14}C]CaN or [^{14}C]M-CaN bound (Γ, pmol/cm^2) to 50 mol % POPS/50 mol % POPC monolayers overlying buffer (25 mM Tris-HCl, 0.15 M NaCl, 0.1 mM EGTA, and 1.0 mM dithiothreitol, pH 7.5) as a function of the change in surface pressure ($\Delta\pi$). For all measurements, the final protein concentration was 0.1 μM. A least-squares fit was applied to the data for CaN (open squares) and M-CaN (closed circles).

manner. Moreover, the *y*-intercept of ≈0.5 pmol/cm^2 for both M-CaN and CaN provides further evidence for an electrostatic contribution to membrane binding in the absence of a surface pressure change.

Determination of protein bound along with the accompanying surface pressure changes allows the calculation of the average membrane surface area occupied by a calcineurin molecule. From the relationship $F_p = 1 - (A_f/A_i)$, one can calculate the fraction of the surface occupied by protein (F_p) where A_i is the initial area per phospholipid headgroup and A_f is the final. Dividing the amount of protein bound by F_p and converting units, one obtains an area of ≈1500 Å^2/molecule of calcineurin at an initial surface pressure of 18 mN/m. This value agrees qualitatively with the molecular dimensions of calcineurin and suggests that a substantial portion penetrates the monolayer.

Binding of M-CaN to Phospholipid Vesicles. To further investigate the correlation of calcineurin binding to monolayers and bilayers, light scattering was used to measure calcineurin binding to lipid vesicles. As shown in Figure 4, incubation of M-CaN with 50 mol % POPS/50 mol % POPC LUVs increased the mass of the vesicle population by 3.5%, corresponding to ≈50 calcineurin molecules bound per vesicle or 0.3 pmol/cm^2 (assuming a spherical vesicle with radius = 50 nm). This value is in good agreement with the data obtained by the Wilhelmy method at surface pressures π_i near 30 mN/m ($\Delta\pi = 0$, ≈0.5 pmol/cm^2, Figure 3).

Effect of Calmodulin on Calcineurin Interactions with Phospholipid Monolayers Measured Using the Wilhelmy Film Balance. Since calmodulin is a biologic effector of calcineurin function, we examined its effect on CaN and M-CaN lipid interactions. A mechanism by which calmodulin binds to its target proteins and decreases their affinity for membranes has been previously demonstrated for MARCKS and eNOS (*10, 17*). As shown in Figure 5, calmodulin decreased $\pi_i|_{\Delta\pi=0}$ for both CaN and M-CaN at 50 mol % POPS/50 mol % POPC with an *x*-intercept of ca. 27 mN/m. Additionally, the $\Delta\pi/\pi_i$ isotherms for CaN/calmodulin and M-CaN/calmodulin were virtually identical. Interestingly, addition of calmodulin to the subphase of a 50 mol % POPS/POPC monolayer previously equilibrated with M-CaN or CaN resulted in a decrease in surface pressure (Figure 6). Thus, following equilibration of the monolayer

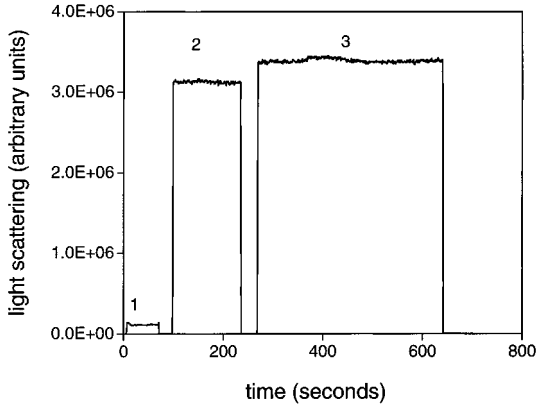

FIGURE 4: Binding of M-CaN to 50 mol % POPS/50 mol % POPC LUVs as determined by light scattering. The intensity of scattered light at 320 nm was measured as a function of time to assess M-CaN binding to phospholipid vesicles. The conditions for each are the same as the previous condition but with the indicated addition: (1) buffer alone (25 mM Tris-HCl, 0.15 M NaCl, and 1.0 mM dithiothreitol, pH 7.5); (2) +25 μg/mL 50 mol % POPS/50 mol % POPC LUVs; (3) +0.3 μM M-CaN. The results shown are representative of three independent trials, each yielding similar results.

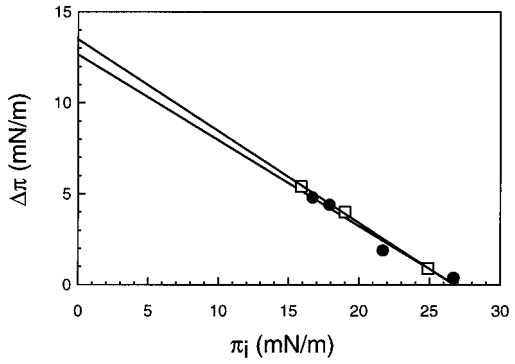

FIGURE 5: Effect of Ca^{2+}/calmodulin on the interaction of CaN and M-CaN with 50 mol % POPS/50 mol % POPC monolayers. The effect of Ca^{2+}/calmodulin on the ability of CaN and M-CaN to bind 50 mol % POPS/50 mol % POPC monolayers overlying buffer (25 mM Tris-HCl, 0.15 M NaCl, 0.1 mM $CaCl_2$, and 1.0 mM dithiothreitol, pH 7.5) was examined as described under Experimental Procedures. The resultant change in surface pressure induced by protein binding was plotted as a function of the initial monolayer surface pressure. For all measurements, the final CaN or M-CaN concentration was 0.1 μM; CaN (open squares), M-CaN (closed circles). A least-squares fit was applied to the data for both proteins.

at two different surface pressures, the addition of M-CaN (asterisks, Figure 6A) led to an increase in the surface pressure. Addition of calmodulin resulted in a subsequent decrease in surface pressure (Figure 6A, lower curve). This effect was Ca^{2+}-dependent (upper curve, Figure 6A), and the final surface pressure after calmodulin addition was similar to that observed in trials where calmodulin was added concomitantly with M-CaN (Figure 5). Calmodulin alone had only a very small, positive effect on the surface pressure change (data not shown). Thus, calmodulin clearly modifies the interaction of CaN with the monolayer.

To further probe the role of calmodulin in mediating calcineurin−lipid interactions, experiments using trypsinized M-CaN were performed. Limited proteolysis of calcineurin with trypsin results in the removal of the calmodulin binding and autoinhibitory domains (*32*). As shown in Figure 6B, injection of trypsinized M-CaN to a final concentration of

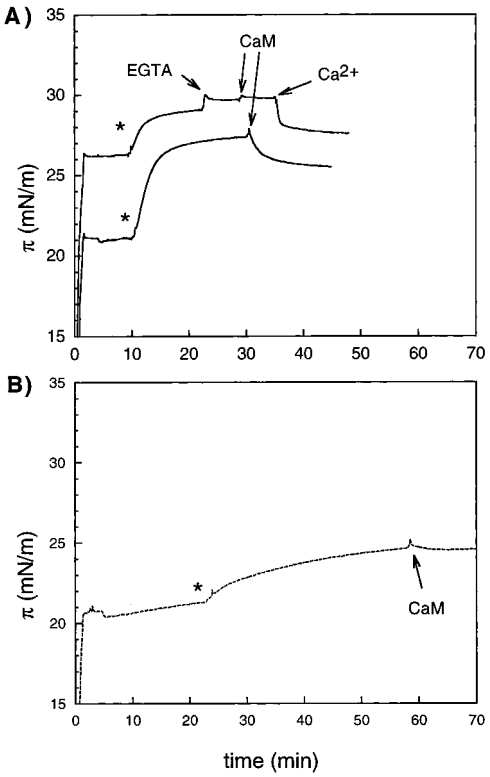

FIGURE 6: Effect of calmodulin on M-CaN and trypsinized M-CaN interactions with 50 mol % POPS/50 mol % POPC monolayers. Shown are plots of surface pressure as a function of time for M-CaN (A) and tryspinized M-CaN (B) interacting with a 50 mol % POPS/50 mol % POPC monolayer overlying buffer (25 mM Tris-HCl, 0.15 M NaCl, 0.1 mM $CaCl_2$, and 1.0 mM dithiothreitol, pH 7.5). The surface pressure changes prior to protein addition (indicated by an asterisk) represent lipid solvent evaporation and monolayer stabilization. Following addition of M-CaN and stabilization of any increases in surface pressure, aliquots of EGTA, calmodulin, or Ca^{2+} were added (arrows). (A) M-CaN: Phospholipid monolayers were spread at two different initial starting pressures (26 and 21 mN/m) over buffer containing 0.1 mM $CaCl_2$. At the times indicated by arrows, EGTA (0.15 mM), calmodulin (0.2 μM), or $CaCl_2$ (0.20 mM) was injected into the subphase to the final concentrations indicated. (B) Trypsinized M-CaN: At the time indicated by the arrow, calmodulin was injected into the Ca^{2+}-containing subphase to a final concentration of 0.2 μM. Abbreviations: CaM, calmodulin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

0.1 μM beneath a 50 mol % POPS/50 mol % POPC monolayer (asterisk) resulted in a positive surface pressure change, indicating protein binding. However, subsequent injection of calmodulin had no effect on the surface pressure, in contrast to the decrease in surface pressure it caused with full-length M-CaN (Figure 6A). These results indicate that calmodulin perturbs the binding of calcineurin to lipid monolayers either by (1) changing the orientation of calcineurin at the lipid surface such that a smaller fraction of calcineurin penetrates the surface or by (2) dissociating calcineurin from the interface.

DISCUSSION

Based on a report that calcineurin phosphatase activity is mediated by anionic phospholipid vesicles (*18*) and recent studies highlighting the necessity of both electrostatic and hydrophobic contributions to myristoyl-protein−lipid binding (*7*, *9*−*11*, *43*), we examined whether the lipid-binding properties of calcineurin may be charge-dependent. Interest-

ingly, we observe that calcineurin lipid binding is myristoyl-independent as evidenced by comparable binding properties of M-CaN and CaN to phospholipid monolayers over several different lipid compositions and in the presence versus absence of either Ca^{2+} or calmodulin. Both forms of calcineurin insert into the monolayer, resulting in a positive change in surface pressure. The maximum surface pressure at which the various forms of calcineurin bind is dependent on the composition of the monolayer. Increasing the mole fraction of POPS in the monolayer leads to an increase in $\pi_i|_{\Delta\pi=0}$ for both CaN and M-CaN (Table 1). More importantly, increasing the mole fraction of phosphatidylserine brings calcineurin monolayer binding into the accepted "physiologic range" (30−35 mN/M) (Table 1) (*44*). Similarly, inclusion of diacylglycerol in the monolayer also raises the maximum surface pressure into which calcineurin will insert (Table 1). Although the percentage of phosphatidylserine at which membrane binding is within the physiologic range exceeds the measured percentage of phosphatidylserine found in biomembranes (10−20%) (*45, 46*), the existence of locally high concentrations, or "patches", of phospholipids and/or diglyceride has been postulated in numerous studies (*47*−*50*). A stimulus-specific mechanism of achieving locally high concentrations of phospholipid/diglyceride might serve as a mechanism for regulating calcineurin localization in vivo.

The excellent agreement for the amount of calcineurin bound to either phospholipid monolayers at $\pi_i = 30$ mN/m or bilayers (∼0.5 pmol/cm^2 in each case) provides further evidence that the equivalence point of LUV bilayers and monolayers is in the surface pressure range of 30−35 mN/m. The debate over the biologic surface pressure of a bilayer has been the subject of a considerable amount of study and has yet to be conclusively resolved. Although estimates range from values as low as 12.5 mN/m up to 50 mN/m [reviewed in (*44*)], it is generally agreed that the biologic surface pressure is most likely in the range of 30−35 mN/m. This estimate is supported by results from several different techniques, including lipolytic enzyme action on monolayers for phospholipases C, D, and A_2 (*51, 52*), activation of protein kinase C in monolayers (*53*), and measurement of anesthetic binding to monolayers (*54*) as well as biophysical measurements of phase transitions (*55*). Since the surface pressure range over which calcineurin will bind model membranes spans the accepted physiological range of surface pressures (Table 1), a mechanism in which calcineurin lipid binding is regulated by the local concentration of anionic lipid or diacylglycerol seems plausible.

The ability of calmodulin to alter the binding of calcineurin to membranes may represent a mechanism for regulating calcineurin function. A Ca^{2+}/calmodulin-dependent mechanism of reversible myristoyl-protein−membrane association has recently been described for MARCKS (*10*) and eNOS (*17*). It is thought that calmodulin binding to these proteins covers a polybasic domain important for membrane association, lowering the affinity of the complex for the lipid surface. Calmodulin binds calcineurin at a polybasic site on the A subunit (residues 400−423, numbered according to the rat α isoform) that contains eight basic residues, suggesting a similar role for calmodulin binding. Our data indicate that calmodulin appears to alter the membrane-binding properties of calcineurin through an undefined mechanism (Figures 5 and 6). The decrease in surface pressure could reflect a reorientation of the calmodulin−calcineurin complex at the interface such that fewer residues of calcineurin penetrate the monolayer. A mechanism involving a calmodulin-dependent change in membrane association may explain the effect of acidic phospholipids on the calmodulin-dependent phosphatase activity of calcineurin observed previously by Politino and King (*18*).

Perturbation of the membrane-binding potential of calcineurin by calmodulin may also affect its localization. Calcineurin is a central component in the T-cell receptor signal transduction pathway which leads to the production of interleukin-2 (*56*). Interleukin-2 transcription requires nuclear import of the transcription factor NF-AT; the preexisting cytoplasmic subunit of NF-AT is a phosphoprotein substrate of calcineurin, and its dephosphorylation and subsequent nuclear import are blocked by the calcineurin inhibitors cyclosporin A and FK506 (*57, 58*). Bcl-2 disrupts NF-AT signaling and may involve the binding and sequestering of calcineurin to membrane surfaces (*26*). In addition, membrane localization of calcineurin may also be accomplished by other factors, including "adapter" proteins. An A-kinase anchoring protein (AKAP) was recently shown to colocalize calcineurin, cAMP-dependent protein kinase, and protein kinase C to the cytoskeleton (*59, 60*). Calcineurin has also been shown to interact with the inositol 1,4,5-trisphosphate receptor−FKBP12 complex, a membrane-bound entity, and regulate Ca^{2+} flux (*25*). It is not known whether myristoylation is required for these interactions, but it is possible that the observed localization of calcineurin to membrane fractions may represent a cell type-dependent combination of both calcineurin−membrane and calcineurin−protein interactions that may eventually bring the myristoyl moiety into play. Furthermore, it is likely that factors which govern calcineurin localization may also be regulated by Ca^{2+}/calmodulin.

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