Biochemical Characterization of Copine: A Ubiquitous Ca^{2+}-Dependent, Phospholipid-Binding Protein†

Jose Luis Tomsig and Carl E. Creutz*

Department of Pharmacology, University of Virginia, Charlottesville, Virginia 22908

Received August 22, 2000; Revised Manuscript Received October 28, 2000

ABSTRACT: The copines are a novel group of Ca^{2+}-dependent, phospholipid-binding proteins first isolated from *Paramecium tetraurelia* [Creutz, C. E., et al. (1998) *J. Biol. Chem. 273*, 1393−1402] and found in a wide range of organisms, from plants to humans. They have a Ca^{2+} and phospholipid-binding domain consisting of two C2 domains and a core domain in the C-terminal portion that is homologous to the A domain found in certain integrins. We provide here the first description of the properties and distribution of a native mammalian copine, copine I. This protein is expressed in all major adult rat organs as demonstrated by probing Western blots of rat organ homogenates with anticopine antibodies. The highest levels of copine are found in the spleen. A protocol for purifying copine to homogeneity from bovine spleen is described. Purified native copine is a 58 kDa monomer that exhibits Ca^{2+} self-association to form higher-order multimers, and Ca^{2+}-dependent, phospholipid binding activity with preference for negatively charged phospholipids over neutral phospholipids and selectivity for Ca^{2+} over Mg^{2+}. Half-maximal association with vesicles enriched in phosphatidylserine occurs at Ca^{2+} concentrations between 1 and 10 μM. Copine I exhibits Mn^{2+} binding activity that is strongly competed by Mg^{2+} and partially competed by Ca^{2+}, suggesting that the copine I A domain may be a functional MIDAS metal binding site similar to that found in integrins [Lee, J. O., et al. (1995) *Cell 80*, 631−638]. Roles for copine in binding membranes and target proteins or small molecules are discussed.

Recent studies of Ca^{2+}-dependent, phospholipid-binding proteins in *Paramecium tetraurelia* led to the discovery of copine, a 55 kDa protein characterized by the presence of two C2 domains and a C-terminal core domain of unknown function (*1*). Comparison of the copine sequence with sequences in contemporary databases revealed that the copines constitute a family of proteins widely distributed among species of the animal and plant kingdoms.

Proteins containing C2 domains play a number of roles in signal transduction and in membrane trafficking. The entry of Ca^{2+} into the cytoplasm of a cell may recruit C2 domain-containing proteins to the membrane surface and lead to the activation of catalytic domains under the control of the C2 domain (*2*, *3*). The biological role of this new member of the family of C2 domain proteins is unknown, but the fact that it is a highly conserved protein suggests that it may play an important physiological role in eukaryotic cells. The primary structure of the core domain of copine is not highly similar to that of any other protein, suggesting that its function may be unique. It has been shown however that the copine core domain bears a distant relationship (*1*) to a module known as the "A domain" found in the extracellular portion of some integrins and which exhibits Mg^{2+} and Mn^{2+} binding activity and is required for the interaction of integrins with target ligands in the extracellular matrix (*4*). Moreover, critical residues thought to be involved in the chelation of Mg^{2+} by the A domain are conserved in copine. On the basis of these analogies, the core domain of copine is also termed the copine A domain.

We have previously expressed recombinant human copines in *Escherichia coli* as GST[1] fusion proteins (*1*) under the control of the lac promoter. Large amounts of fusion proteins were produced upon induction, but unfortunately, they were mostly insoluble and thus inadequate for biochemical studies. We however were able to produce highly specific polyclonal anticopine antibodies using as an antigen the recombinant copine I A domain obtained by solubilization of bacteria with sarkosyl, an alkyl-anionic detergent (*5*). Here we show the distribution of copine in adult mammalian organs as revealed by Western blots probed with these antibodies.

Our tissue distribution studies show that copine is expressed in all major mammalian organs, although as a minor component of the pool of Ca^{2+}-dependent, phospholipid-binding proteins. Accordingly, purification of copine from mammalian tissue is technically more complex than in the

† This work was supported by NIH Research Grant GM53266. The W. M. Keck Biomedical Mass Spectrometry Laboratory at the Virginia Biomedical Research Facility is supported in part by grants from the W. M. Keck Foundation and the University of Virginia Pratt Committee.

* To whom correspondence should be addressed: Department of Pharmacology, University of Virginia Health System, P.O. Box 800735, 1300 Jefferson Park Ave., Charlottesville, VA 22908-0735. Phone: (804) 924-5029. Fax: (804) 982-3878. E-mail: cec3n@virginia.edu.

[1] Abbreviations: BSA, bovine serum albumin; DTT, DL-dithiothreitol; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; EDTA, ethylenediaminetetraacetic acid; GST, glutathione *S*-transferase; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Tris, tris(hydroxymethyl)aminomethane; PA, phosphatidic acid; PC, phosphatidylcholine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

10.1021/bi0019949 CCC: $19.00
Published on Web 11/30/2000

case of copine from *Paramecium* whose major Ca^{2+}-dependent, phospholipid-binding protein is copine (*1*). We here present a protocol for obtaining mammalian copine purified to homogeneity based on binding to phospholipid vesicles, ammonium sulfate precipitation, and anion exchange chromatography. Using this protocol, we have obtained sufficient material to conduct the first detailed biochemical study of a native copine. We focused our analysis on properties of copine that relate to its hypothetical role in membrane trafficking, the metal binding properties of its putative, integrin-like A domain, and its possible role as a nucleotide-requiring enzyme.

MATERIALS AND METHODS

Expression of the GST−Copine I A Domain Fusion Protein and Production of Anticopine Antibodies. The recombinant copine I A domain was expressed as a GST fusion protein in *E. coli* strain XL1 Blue (Stratagene) harboring the pGEX-KG vector (*6*) with the human copine I A domain as previously described (*1*). Polyclonal anticopine antibodies were prepared by immunization of rabbits as previously described (*1*) but using a more purified immunogen prepared as described below. Bacteria were induced with 0.1 mM IPTG (Boehringer) for 2 h at 37 °C, collected by centrifugation, resuspended in ice-cold PBS containing 5 mM EGTA, 5 mM DTT, 10 mg/mL PMSF, 0.025 mg/mL leupeptin, and 0.01% aprotinin solution, and sonicated for 2 min with a probe-type sonicator. The GST−copine I fusion protein was solubilized by supplementing the bacterial lysate with 0.5% sarkosyl as described previously (*5*). The mixture was then centrifuged at 9500*g* for 15 min. The supernatant was supplemented with 1% Triton X-100 and incubated with glutathione−agarose beads (Sigma, catalog number G-4510) for 1 h. Beads were then washed five times with 10 volumes of the same buffer supplemented with 1% Triton X-100 and five times with 10 volumes of cleavage buffer [150 mM NaCl, 50 mM Tris (pH 8.0), and 2.5 mM $CaCl_2$] and resuspended at a concentration of 40% in cleavage buffer. The copine portion was cleaved by incubating the beads for 40 min at room temperature with 19 units of thrombin (Sigma, catalog number T-0553) per milliliter of beads. Digested proteins were then eluted from the beads with 2× sample buffer and separated on SDS−PAGE gels. The 33 kDa band corresponding to the copine I A domain was excised and used as the immunogen as described previously (*1*). Purification of anticopine antibodies was carried out by affinity purification of the antiserum using the immunogen immobilized on nitrocellulose (*7*). The protein concentration in the purified antibody stock solution was 0.18 mg/mL, calculated on the basis of A_{280} using BSA as the standard. The results presented here were obtained using the most specific antibodies that were obtained (lot 8680). We have previously reported the use of anticopine antibodies obtained using a less purified immunogen (lot 11837) to detect purified copine and to identify chromobindin 17 as a copine (*1*). These antibodies, and other anticopine antibodies we have tested, including anticopine IV antibodies, exhibited a high affinity for copine but poor specificity even after affinity purification. The high specificity of lot 8680 as shown in Figure 1 may be the result of the higher purity of the immunogen used in this case.

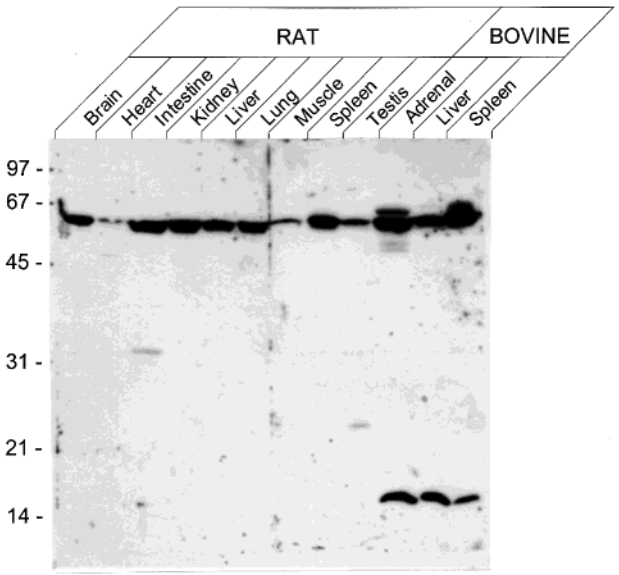

FIGURE 1: Western blot of rat and bovine organs probed with anticopine antibodies. Samples of homogenates from rat and bovine organs containing 100 μg of protein were run in SDS−PAGE, electroeluted onto a nitrocellulose membrane, and probed with polyclonal, affinity-purified anticopine antibodies. Detection was carried out using goat anti-rabbit secondary antibodies linked to a chemiluminescence detection system. Primary antibodies were produced using the A domain of recombinant human copine I as the immunogen. A high titer of primary antibody (1:500) was used to make sure that all bands recognized by the antibody are detected. A titer of 1:10000 of this antibody (lot 8680) was usually enough to demonstrate the 58 and 18 kDa bands in bovine organs. The lane identified as Adrenal in the figure corresponds to adrenal medullary tissue. The migration positions of molecular mass standards are marked on the left with the corresponding masses in kilodaltons.

Purification of Mammalian Copine from Bovine Spleen. Bovine spleens were obtained from a local slaughterhouse and kept on ice during transportation back to the laboratory (~90 min). Spleens were freed of surrounding fat tissue, but the capsules were left intact. They were cut in small cubes and ground in a meat grinder. Tissue obtained from individual spleens (700−800 g) was frozen at −80 °C no more than 4 h after the sacrifice of the animals and stored for up to 6 months. Preparation was started by thawing ~750 g of tissue. Once thawed, it was homogeneized in a Waring blender at high speed for 5 min in 2 L of 150 mM NaCl, 50 mM HEPES/NaOH (pH 7.3), 5 mM EGTA, and 0.5 mM PMSF (added as a 10 mL solution in ethanol) and 10 mL of aprotinin solution. The process was completed by further homogeneization of 300 mL aliquots of the initial homogenate for 5 min with a Polytron PT 3000 apparatus at 30 000 rpm. The homogenate was centrifuged at 10000*g* for 1 h. The resulting supernatant was then filtered through 10 layers of cheesecloth and centrifuged at 100000*g* for 1 h. The final 100000*g* supernatant (soluble fraction), usually 1.8−1.9 L, was kept on ice overnight (~16 h) and then supplemented with multilamellar phospholipid vesicles and $CaCl_2$ to extract Ca^{2+}-dependent, phospholipid-binding proteins. Vesicles were prepared using a commercial Folch Fraction I preparation from bovine brain (Sigma, catalog number B-1502) that contains approximately 50% PS, 10% PI, and minor amounts of several other brain lipids. Two grams of brain phospholipids was suspended in 100 mL of 150 mM NaCl and 50 mM HEPES/NaOH (pH 7.4) by vigorously stirring for 1 h

under argon and sonication of 33 mL aliquots in a probe-type sonicator for 5 min at 40% maximum power. Vesicles were then centrifuged at 25000*g* for 15 min, resuspended in the same buffer, and centrifuged one more time to obtain a final pellet. This pellet was resuspended in the soluble fraction using a Dounce homogenizer with a tight-fitting glass pestle. The soluble fraction was then stirred and supplemented drop by drop with 0.5 M $CaCl_2$ solution in H_2O to obtain 8 mM total $CaCl_2$; i.e., 3 mM free Ca^{2+}. The mixture was then stirred for an additional 15 min and centrifuged at 25000*g* for 1 h. The resulting dark red supernatant was discarded, and the walls of the tubes and the top of the pellets were carefully washed twice with buffer to remove the remaining supernatant. Pellets were then resuspended and centrifuged two times in 2 L of 150 mM NaCl, 50 mM HEPES/NaOH (pH 7.3), 2 mM $CaCl_2$, and 0.2 mM PMSF (first wash) and 2 L of 50 mM HEPES/NaOH (pH 7.3) and 2 mM $CaCl_2$ (second wash). In both cases, supernatants were discarded, the walls of the tubes and the top of the pellets washed, and pellets resuspended using a Dounce homogenizer. The last supernatant was completely clear; the presence of visible traces of the first, dark red supernatant was avoided as it indicates contamination with proteins that are poorly removed by the subsequent purification steps. If contamination was observed, the last wash was repeated. To recover the bound proteins, pellets were resuspended using a Dounce homogenizer in 100 mL of 20 mM HEPES/NaOH (pH 7.3) and 10 mM EGTA and centrifuged at 100000*g* for 1 h (the pH of this buffer was critical; deviations from 7.3 resulted in a less effective purification by ammonium sulfate precipitation). The supernatant was carefully decanted and centrifuged one more time at 100000*g* for 1 h to remove all traces of particulate material. This supernatant has a yellowish appearance and usually contained some 30 mg of protein, 5% of which is copine (see Figure 3, lane 1). The supernatant was then slowly supplemented while being stirred with a saturated solution of ammonium sulfate to a final concentration of 35% saturation. A saturated ammonium sulfate solution was prepared by adding 761 g of ammonium sulfate to 1 L of 14.4 mM EDTA (pH 7.3) in H_2O to obtain the following final concentrations: 0.53 g/L and 10 mM, respectively. After 24 h, the mixture was centrifuged at 50000*g* for 30 min. The supernatant was recovered, supplemented with saturated ammonium sulfate to obtain 52% saturation, allowed to precipitate for 24 h, and then centrifuged at 50000*g* for 30 min. The resulting pellet (usually invisible or slightly white) was resuspended in 15 mL of 20 mM HEPES/NaOH (pH 7.3) and 10 mM EGTA and dyalized using a 6−8 kDa molecular mass cutoff membrane for 24 h against 4 L of the same buffer with one buffer change after 12 h. The dialysate was then centrifugated for 15 min at 1000*g* to remove small amounts of particulate material that often can be found in this fraction and will clog the anion exchange column if not removed. The clarified dialysate usually containing 1−2 mg of protein, 80% of which is copine (see Figure 3, lane 2), was loaded into a 5 cm × 0.5 cm diameter column packed with the anion exchange resin Poros Q (Perseptive Biosystems, Foster City, CA) connected to a Pharmacia FPLC system and equilibrated with 20 mM histidine (pH 6.4). After the column had been washed with 10 column volumes, proteins were eluted by means of a 10 mM/min linear gradient of KCl at a flow rate of 1 mL/min.

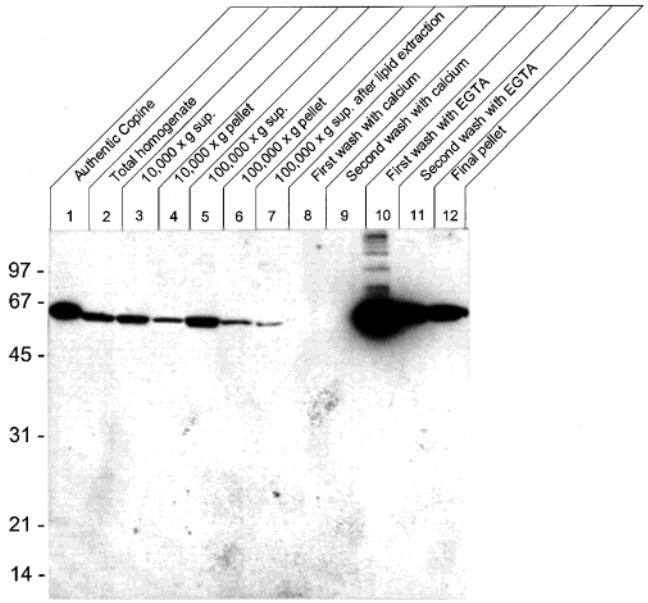

FIGURE 2: Characterization of copine as a Ca^{2+}-dependent, phospholipid-binding protein. The soluble fraction from a rat spleen homogenate was obtained by successive low-speed (10000*g*) and high-speed (100000*g*) centrifugation. Ca^{2+}-dependent, phospholipid-binding proteins were extracted from the soluble fraction by binding to brain phospholipid vesicles in the presence of Ca^{2+}. Vesicles were washed twice in Ca^{2+}-containing buffer (fractions W1 and W2) and extracted twice with EGTA-containing buffer to recover the proteins bound to the vesicles (fractions E1 and E2). Samples of all fractions obtained during this procedure were analyzed by SDS−PAGE, electroeluted onto a nitrocellulose membrane, probed with 1:10000 anticopine antibodies, and detected by chemiluminescence. The lanes were loaded as follows: lane 1, authentic copine from bovine liver, i.e., a sample whose copine content was confirmed by amino acid sequencing (see Table 1); lane 2, total homogenate; lane 3, 10000*g* supernatant; lane 4, 10000*g* pellet; lane 5, 100000*g* supernatant; lane 6, 100000*g* pellet; lane 7, 100000*g* supernatant after extraction with phospholipids in the presence of Ca^{2+}; lane 8, first wash (W1); lane 9, second wash (W2); lane 10, first extraction with EGTA (E1); lane 11, second extraction with EGTA (E2); and lane 12, final lipid pellet. Lanes 2−9 were loaded with 100 μg of protein. Lanes 10−12 were loaded with 10 μg of protein. The migration positions of molecular mass standards are marked on the left with the corresponding masses in kilodaltons.

Purified copine was obtained in fractions containing 110−120 mM KCl. The purity of the fractions was routinely checked by SDS−PAGE. All the procedures were carried out at 4 °C except for the anion exchange chromatography step that was performed room temperature.

Ca^{2+}-Dependent, Phospholipid-Binding Proteins from Rat and Bovine Organs. Bovine organs were obtained from a local slaughterhouse as described above. Rat organs were obtained from adult male Wistar rats (∼300 g body weight) sacrificed by decapitation. Rat uterus was obtained from female rats 2 h after delivery. Ca^{2+}-dependent, phospholipid-binding proteins, i.e., E1 fractions, were obtained using a scaled-down version of the preparative protocol described above. In this case, however, no supernatants or pellets were discarded so they could be analyzed by immunoblotting along with the rest of the fractions. In addition, a second extraction of the vesicles with EGTA was performed and the resulting supernatant and pellet (fractions E2 and final pellet) were analyzed as well. Three to five grams of tissue was homogenized in 12 mL of buffer using a motor-driven Teflon

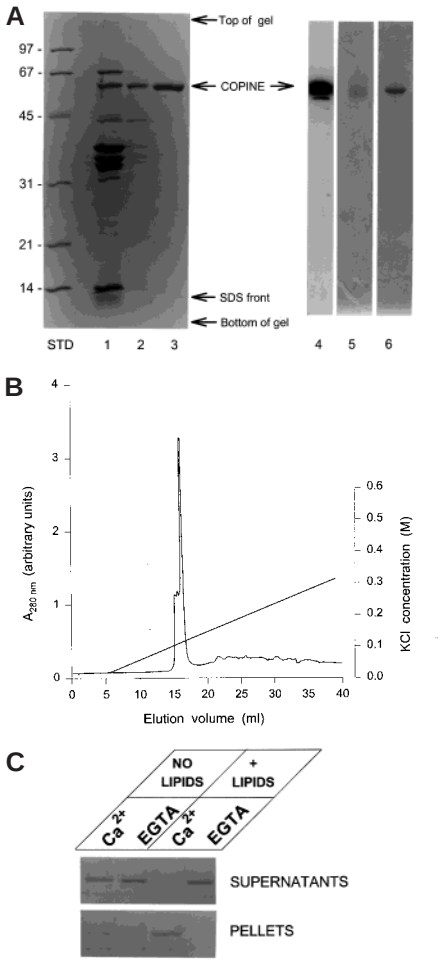

FIGURE 3: Purification of copine from bovine spleen. The fraction containing Ca^{2+}-dependent, phospholipid-binding proteins (E1 fraction) from a bovine spleen was obtained as described in Figure 2. Copine was then purified from that fraction by ammonium sulfate precipitation and anion exchange chromatography. (A) Purification steps and initial characterization. Lanes 1−3 are SDS−PAGE lanes stained with Coomassie Blue. Lane 1 contained fraction E1, lane 2 the portion of E1 precipitated between 35 and 52% ammonium sulfate, and lane 3 a FPLC fraction obtained at approximately 120 mM KCl ("purified copine"). Lanes 1−3 were loaded with 43, 2.5, and 10 μg of protein, respectively. The average molecular mass of purified copine was 58 ± 0.2 kDa ($n = 5$ independent preparations). Lanes 1 and 2 were loaded with an equivalent amount of material on a per volume basis to demonstrate the recovery after salting out. Lane 4 was loaded with 0.5 μg of purified copine, run in a SDS−PAGE gel, electroeluted into a nitrocellulose membrane, and probed with anticopine antibodies. Lanes 5 and 6 are adjacent lanes loaded with 5 μg of purified copine, run in a SDS−PAGE gel under nonreducing conditions, i.e., absence of DTT (lane 5), or standard reducing conditions (lane 6), and stained with Coomassie Blue. The migration positions of molecular mass standards (shown in lane STD) are marked on the left with the corresponding masses in kilodaltons. (B) Chromatogram of the anion exchange chromatography step. The absorbance at 280 nm of the material eluted from the Poros Q column is shown. Each fraction represents 1 mL. The flow rate was 1 mL/min. The straight line indicates the concentration of KCl in each fraction. The main peak (fractions 15−17) corresponds to the elution of copine. (C) Ca^{2+}-dependent binding of purified copine to brain phospholipids. Samples of purified copine (5 μg) and brain phospholipid vesicles (~350 μg) were resuspended in 100 μL of 120 mM KCl, 20 mM histidine (pH 6.4), and 2.5 mM EGTA or 2 mM $CaCl_2$. After incubation for 10 min at room temperature, mixtures were spun for 10 min at 10000*g* and lipid pellets and supernatants were analyzed by SDS−PAGE. A control was carried out in the absence of lipids. The gel was stained with Coomassie Blue.

pestle homogenizer. After the low- and high-speed centrifugations, the tissue soluble fractions were extracted with ~20 mg of brain phospholipids vesicles. After the washes, vesicles were extracted twice with 1 mL of EGTA-containing solution (fractions E1 and E2). To study the organ distribution of copine, samples of the homogenates were centrifuged at 500*g* for 30 min and the supernatants analyzed by immunoblotting with anticopine antibodies. The presence of copine in E1 fractions was analyzed by running samples containing 10 μg of protein in SDS−PAGE gels.

Binding of Copine to Lipid Vesicles of Defined Composition. To prepare vesicles from pure phospholipids, 10 mg/mL stock solutions of phospholipids in chloroform were used. Mixtures containing 40% of PC solution and 60% of the phospholipid under study were dried by evaporation of the solvent under a stream of argon and by exposure to high vacuum for 1 h. Dried phospholipids were resuspended in 1 mL of 150 mM NaCl and 50 mM HEPES/NaOH (pH 7.3) and sonicated for 30 s at 5% maximum power. Vesicles were centrifuged at 10000*g* for 10 min and washed two times with the same buffer. Aliquots of the vesicle suspension were then centrifuged at 10000*g* for 10 min and the pellets resuspended in 50 μL of 120 mM KCl and 20 mM histidine (pH 6.4), except in the cases of vesicles containing PC, PE, or sphingomyelin. In these three cases, a lower-density buffer consisting of 40 mM KCl and 6.7 mM histidine (pH 6.4) was used to prevent incomplete sedimentation. Mixtures were then supplemented with appropriate volumes of divalent cation stock solutions or EGTA/Ca^{2+} buffer to obtain the desired final divalent cation concentrations. Finally, an appropriate volume of purified copine solution was added and the final volume of the mixture was adjusted to 120 μL with the same buffer. After 10 min at room temperature, tubes were centrifuged at 10000*g* for 10 min. The pellets were resuspended in 120 mM KCl and 20 mM histidine (pH 6.4). Supernatants and resuspended pellets were then mixed with 24 μL of 6× SDS−PAGE sample buffer and analyzed by SDS−PAGE. In the case of vesicles containing PC, PE, or sphingomyelin, the buffer concentrations in the supernatant were corrected to 120 mM KCl and 20 mM histidine (pH 6.4) with a concentrated stock before electrophoresis to obtain gel bands of uniform size and thus facilitate comparison between different phospholipids. All experiments were carried out in low protein binding tubes (catalog number 145530, Research Products International Corp., Mount Prospect, IL). Pure phospholipid solutions were obtained from Avanti Polar Lipids (Alabaster, AL) as 10 mg/mL solutions in chloroform. They were brain L-α-PC, L-α-PE, L-α-PS, and sphingomyelin (catalog numbers 840053, 840022, 840032, and 860061, respectively), liver L-α-PI (catalog number 840042), and egg L-α-PA and L-α-PG (catalog numbers 840101 and 841138, respectively).

Native Polyacrylamide Gels. Composite gels having lanes with different concentrations of acrylamide were used to avoid differences in migration due to factors other than acrylamide-induced retardation. Thus, the migration of copine and standards under exactly the same electric field and for exactly the same time was measured at six different concentrations of acrylamide (7, 8, 9, 10, 11, and 12%). Gels were prepared by appropriately diluting the acrylamide stock solution (see General Methods and Materials) and a Tris/EGTA stock buffer (pH 8.3) to obtain final concentrations

of 50 mM Tris and 5 mM EGTA. Gels were polymerized with a TEMED/ammonium persulfate mixture and run at room temperature using 50 mM Tris (pH 8.3) and 5 mM EGTA as the electrophoresis buffer. The same buffer supplemented with 10% glycerol and bromophenol blue was used as the sample buffer. No stacking gel was used. Electrophoresis in the presence of Ca^{2+} was carried out by substituting EGTA in all buffers with 2 mM $CaCl_2$. Egg albumin (Sigma, catalog number A-5253, molecular mass of 43 kDa), BSA monomer (Sigma, catalog number A-4503, molecular mass of 67 KDa), and BSA dimer (Sigma, catalog number A-4503, molecular mass of 134 kDa) were used as molecular mass standards.

Dynamic Light Scattering. Time-dependent fluctuations of the light scattered at 90° by purified copine in the presence and absence of Ca^{2+} were assessed using a DynaPro-801 DLS instrument equipped with a 25 mW, 750 nm wavelength solid-state laser (Protein Solutions, Charlottesville, VA). The exponential decay of the scattered light due to optical phase shift caused by molecular Brownian motion was analyzed by photon correlation spectroscopy using software provided by the manufacturer. The translational diffusion coefficient was then calculated from the rate of optical decay. The hydrodynamic radii of the species present in the samples were calculated using the translational diffusion coefficient according to the Stokes−Einstein formula. Molecular masses were calculated from the hydrodynamic radius using a calibration curve for globular proteins. For measurements, a purified copine solution was concentrated to ~1 mg/mL using a Centricon 10 concentrator and filtered through a 0.02 μm filter (Whatman Anodisc 13, catalog number 68097003) and 22 μL samples were placed in a microcuvette maintained at 20 °C by a thermostatic cuvette holder. For studies in the presence of Ca^{2+}, a $CaCl_2$ stock solution was added to the samples after filtration.

Binding of $^{54}Mn^{2+}$ to Purified Copine. Experiments were carried out as described previously (*4*) with minor modifications. Pieces of nitrocellulose paper were spotted with 20 μL of 200 μg/mL purified copine and after 10 min placed in 15 mL conical tubes filled with overlay buffer [60 mM KCl and 30 mM imidazole (pH 6.8)]. Tubes were attached to a rotator and rotated for 30 min, changing the buffer every 10 min. Washed papers were then incubated for 15 min in overlay buffer containing $^{54}MnCl_2$ (4 μCi/mL) (NEN, NEZ 040, 34.7 Ci/mmol). Papers were then placed in Eppendorf tubes containing 1.5 mL of overlay buffer and rotated for 10 min, changing the buffer at 5 min. Finally, papers were dipped in 50% ethanol, dried, and radioautographed using photographic paper. After radioautography, radioactivity was eluted from papers by incubation for 2 h in 200 μL of 10 mM $MnCl_2$ and counted in a liquid scintillation counter using Scintiverse (Fisher) scintillation fluid (efficiency of counting ~ 0.4). Competition experiments were carried out by supplementing the $^{54}MnCl_2$ solution with 2 mM $MnCl_2$, $MgCl_2$, or $CaCl_2$. Controls were carried out using 200 μg/mL purified BSA, 80 μg/mL recombinant rat C2A synaptotagmin domain (*8*), or 200 μg/mL boiled copine.

ATP Binding. Partially purified copine fractions, i.e., anion exchange fractions containing some 30% copine and 1 μg of total protein, were incubated for 30 min at room temperature in 120 μL of 20 mM Tris (pH 7.4), 1 mM $MgCl_2$, 12.5 nM azido-[α-^{32}P]ATP ([α-^{32}P]-8-azidoadenosine 5′-triphosphate, 19.8 Ci/mmol; ICN), irradiated with UV light for 2 min, run in SDS−PAGE gels, and radioautographed using a Phosphoimager. Competition with ATP was carried out by including unlabeled ATP (Sigma, catalog number A-5394) in the initial mixtures at concentrations ranging from 10^{-9} to 10^{-2} M. Experiments were also carried out in the presence of 2.5 mM $CaCl_2$, 5 mM EGTA, or 2.5 mM $CaCl_2$ and 50 μg of brain phospholipids.

Liquid Chromatography−Tandem Mass Spectrometry. Spectrometry was performed by the V. M. Keck Biomedical Mass Spectrometry Laboratory at the University of Virginia as described previously (*9*).

General Methods and Materials. Twelve percent SDS−PAGE gels were prepared as described previously (*10*) using a commercial 40% acrylamide, 29:1 acrylamide:bisacrylamide solution (Fluka, catalog number 01708). The same solution was used for native gels. Western blotting, performed on nitrocellulose membranes (0.2 μm Trans-Blot, Bio-Rad), was carried out as described previously (*11*). Detection of copine in immunoblots was carried out using 1:10000 polyclonal goat anti-rabbit, peroxidase-labeled antibodies (American Qualex) and a chemiluminiscence kit (Pierce Supersignal). EGTA/Ca^{2+} buffers were prepared as described previously (*12*, *13*), and the pCa values were checked with a Ca^{2+}-selective electrode (Orion Research). Protein was quantified using a commercial kit (Bio-Rad protein assay) based on the Bradford dye-binding method (*14*) and BSA as a standard (Bio-Rad protein assay standard II). HEPES; Tris; EGTA, free acid; EDTA, disodium salt; L-histidine, free base; $CaCl_2 \cdot 2H_2O$; PMSF; leupeptin; aprotinin solution (catalog number A-6279); DTT; and ammonium sulfate were from Sigma. Sarkosyl (*N*-laurylsarcosine, sodium salt) was from International Biotechnologies. TEMED, ammonium persulfate, and SDS were from Bio-Rad.

RESULTS

Identification of Copine in Rat and Bovine Organs. The presence of copine in rat and bovine organs was investigated by probing Western blots of organ homogenates with anticopine antibodies generated using as an antigen a recombinant human copine I A domain produced in *E. coli*. Figure 1 shows that anticopine antibodies stained a 58 kDa band in all organs that were studied, in close agreement with the calculated molecular mass of copine I (58 928 Da). The amount of copine appears to be variable from organ to organ. Organs such as spleen, kidney, or the large intestine seem richer in copine as compared with, for instance, organs containing mainly striated muscle, i.e., heart and skeletal muscle. We examined Coomassie Blue-stained lanes of organ homogenates paired with corresponding immunoblots and found that no Coomassie Blue bands can be matched with the bands detected by our antibody, indicating that copine is not a major component of the protein pool of any tissue.

Figure 1 also demonstrates that our antibody has an excellent specificity in rat tissues; i.e., only one major band is detected in blots of total organ homogenates. In bovine tissues, an additional, strongly reactive 18 kDa band is also demonstrated. This protein does not seem to be a copine fragment since the same rat organs prepared under identical conditions showed no traces of this 18 kDa band. Although

the identity of this protein is unknown at this time, we have established that it is not a Ca^{2+}-dependent, phospholipid-binding protein. An analysis similar to that shown in Figure 2 (see below) carried out with bovine adrenal medullary tissue shows that this 18 kDa band is a soluble protein that does not bind brain phospholipid vesicles in the presence of Ca^{2+}. We have also observed in bovine tissues, although inconsistently and at high antibody titer, a ~61 kDa band that can be seen in the adrenal and spleen lanes of Figure 1. Finally, a few, weakly stained bands are demonstrated in some organs such as intestine, testis, and the adrenal gland. Except for the 58 kDa band and the 18 kDa band in bovine tissues, the rest of the bands seem to be cases of unspecific staining probably because some 20 times the minimum titer of antibody necessary to demonstrate the 58 kDa band was used to probe the blot shown in Figure 1. At lower antibody titer, all the additional bands (except the 18 kDa band in bovine tissues) are no longer stained.

To demonstrate the phospholipid binding properties of the 58 kDa band, we obtained Ca^{2+}-dependent, phospholipid-binding proteins from all the organs shown in Figure 1. Soluble fractions were extracted with brain phospholipid vesicles, and after two washes with Ca^{2+}-containing buffer (fractions W1 and W2), bound proteins were recovered by washing the vesicles twice with EGTA-containing buffer (fractions E1 and E2). These fractions and all the intermediate fractions obtained in the course of the preparation were analyzed by immunoblotting. A typical result is shown in Figure 2, in this case, the preparation of phospholipid-binding proteins from rat spleen. The 58 kDa band comigrates with authentic copine (lane 1) and exhibits a pattern that matches the expected behavior for a soluble, Ca^{2+}-dependent phospholipid-binding protein. (A) The bulk of the protein remains in the supernatants of the low- and high-speed centrifugations (lanes 3 and 5). The small amounts observed in the corresponding pellets (lanes 4 and 6) probably correspond to contamination with supernatant. In fact, some enrichment on a per microgram of protein basis is already apparent when lanes 2 (total homogenate) and 5 (soluble fraction) are compared. These observations show that the 58 kDa protein is soluble in EGTA-containing medium and that most of it can be recovered in the soluble fraction. (B) The 58 kDa protein is almost completely extracted from the soluble fraction by binding to phospholipid vesicles in the presence of Ca^{2+} as revealed by the comparison of lane 5 (soluble fraction before extraction) with lane 7 (soluble fraction after extraction). The absence of the protein in lanes 8 and 9 (washes in the presence of Ca^{2+}) shows that the protein remains bound to phospholipid vesicles when Ca^{2+} is present in the medium. Finally, the protein is released when the phospholipid vesicles are washed with EGTA as shown in lanes 10 and 11.

Fraction E2 (lane 11) and the final lipid pellet (lane 12) seem to contain an amount of 58 kDa protein comparable to that in fraction E1 (lane 10), suggesting that the 58 kDa protein is evenly distributed among these fractions. However, Coomassie Blue-stained gels of these three fractions (not shown) demonstrate that the E1 fraction contains most of the 58 kDa protein and that the remainder is usually found in the lipid pellet. The inability of the immunoblot to reveal the correct distribution of the 58 kDa protein is due to the nonlinear response of the chemiluminescent detection system. The presence of some copine in the final pellet may be due to the trapping of some copine inside the lipid vesicles as a result of vesicle breakage and resealing during resuspension of the pellets.

Purification of Copine from Bovine Spleen. Figure 2 shows that a copine-rich fraction (E1 fraction) can be obtained by EGTA wash of proteins bound to phospholipid vesicles, suggesting that this may be a good starting material with which to attempt the purification of mammalian copine. We analyzed by SDS−PAGE E1 fractions from all the organs shown in Figure 1 and found that in some cases a 58 kDa band that reacts with our antibody can be demonstrated by Coomassie Blue staining in lanes loaded with 10 μg of protein. Unfortunately, in most cases, copine is a minor component of the mixture due to the abundance of annexins in the fraction. We found, however, that the spleen is an exception to this observation. As shown in Figure 3A (lane 1), copine is easily identified as a sizable component of the proteins present in the spleen E1 fraction. Densitometric analysis of Coomassie Blue-stained gels indicates that it constitutes ~5% of the protein of this fraction. Accordingly, we decided to attempt the purification of mammalian copine from spleen tissue.

Initial attempts to purify mammalian copine from bovine spleen E1 fractions by anion exchange chromatography using a protocol similar to that used to purify *Paramecium* copine (*1*) were unsuccessful because of the presence of large amounts of contaminant proteins, in particular the 32−36 kDa annexins, and the unexpected finding that mammalian copine, in contrast to *Paramecium* copine, does bind the Poros Q anion exchange resin. Although some degree of purification was achieved because the 32−36 kDa annexins elute at higher salt concentrations in comparison to copine, we found it impossible to separate copine from these proteins in a single chromatographic step.

Having ruled out anion exchange chromatography as the first step, we explored precipitation with ammonium sulfate as a means of obtaining enriched material that could be further purified by anion exchange. We found that copine completely precipitates at 45% ammonium sulfate and that the rest of the proteins precipitate at either lower or higher concentrations. Accordingly, we carried out two successive precipitations of the E1 fraction at 35 and 52% ammonium sulfate. As Figure 3A shows, the bulk of the contaminants is removed by this procedure. The fact that similar amounts of copine are observed before and after precipitation shows that no copine loss occurs at this step since both lanes were loaded with equivalent amounts of protein solution. Densitometric analysis of lane 2 shows that copine constitutes 80% of the protein, indicating a factor for enrichment by ammonium sulfate precipitation of ~16. This fraction constitutes a fairly purified copine preparation that can be used in experiments where purity is not a critical factor. Moreover, purity can be slightly improved at the expense of the recovery by choosing closer ammonium sulfate cuts. In particular, choosing a lower concentration in the second precipitation will reduce the presence of the most abundant contaminant, i.e., the group of annexins at 32−36 kDa, since all these proteins precipitate at higher ammonium sulfate concentrations. In our case, we decided to maximize recovery over purity because of the further purification that can be obtained by anion exchange chromatography. As shown in Figure 3A,

Table 1: Alignment of Peptides Obtained by Trypsin Digestion of Copine from Liver and Spleen with the Sequence of Human Copine I[a]

	sequence	"start end"	"domain"	Li	Sp
1	VRNCSSPEFSK	50−60	C2I	•	
2	TLQLEYRFETVQK	61−73	C2I	•	
3	FGIYDIDNK	76−84	C2I	•	•
4	GTITVSAQELK	125−135	C2I	•	•
5	VVTMEVEAR	139−147	C2II	•	•
6	DFLGK	153−157	C2II		•
7	SDPFLEFFR	158−166	C2II	•	•
8	WHLVYR	172−177	C2II	•	
9	NNLNPTWK	183−190	C2II	•	•
10	QALPQVR	378−384	core	•	•
11	LFGPTNFAPIINHVAR	385−400	core	•	•
12	DIVQFVPYR	479−487	core	•	•
13	FQNAPR	489−494	core		•
14	EALAQTVLAEVPTQLVSYFR	495−514	core		•
15	AQGWAPLKPLPPSAK	515−529	core	•	

[a] Samples of purified copine from bovine liver and spleen were run in SDS−PAGE as shown in Figure 3 (lane 3). After Coomassie Blue staining, copine bands were cut, destained, and digested with trypsin. Digests were then analyzed by liquid chromatography−mass spectrometry. Peptides found in each digestion (Li, liver; Sp, spleen) were matched with the sequence of human copine I. All the peptides, except peptide 11, which shows a Y → F substitution in the second residue, are exact matches to the sequences of human copine I residue numbers shown in the start end column. The domain localization of the match is indicated in the domain column. The amino acids L and I cannot be differentiated by mass spectrometry. Therefore, the correct residues (according to the database sequence) are shown above in the places where an L or I amino acid was detected. The combined lengths of the liver peptides (123 amino acids) and spleen peptides (109 amino acids) represent 23 and 20%, respectively, of the 537 amino acids of human copine I. Both sets of peptides represent a total of 154 amino acids (29% of the sequence of copine I).

purification by anion exchange chromatography further removes contaminants and yields a much more purified copine preparation. The stacking gel (not shown) was also stained and analyzed. No stainable material was observed on top of lane 3, indicating that no other protein or copine aggregates were present in the fraction. Since lane 3 was loaded with 10 μg of protein, it can be concluded that no single contaminant exceeds 5% of the total protein (taking 0.5 μg as the limit of detection). SDS−PAGE gels loaded with smaller amounts of purified copine (1−5 μg) did not reveal the presence of more than one copine band (not shown), and all peptides obtained by digestion of purified copine as shown in Table 1 match the sequence of copine I. We found that elution from the anion exchange column at acidic pH greatly improves the separation because copine, in contrast to contaminant proteins, is very sensitive to the pH of the elution buffer and can be eluted at lower salt concentrations at more acidic pHs. Elution, as Figure 3B shows, occurs at 110−120 mM KCl. The optimal pH for copine purification under our experimental conditions was between 6.2 and 6.4. We chose pH 6.4 because binding to the anion exchanger is incomplete at lower pH.

The molecular mass of purified copine as revealed by SDS−PAGE is indistinguishable from that obtained by immunoblotting of organ homogenates (Figure 1), indicating that no degradation occurs during the purification protocol. The purified copine also demonstrates Ca^{2+}-dependent, phospholipid binding activity as seen in Figure 3C. Analysis of purified copine by immunoblotting reveals the presence of minute amounts of an additional, lower-molecular mass species. Figure 3A shows that our antibody detects a 58 kDa band that corresponds to the band stained with Coomassie Blue and a small, 53 kDa band. It is possible that this band represents another copine type or minute amounts of degraded copine. This lower-molecular mass species was not observed in immunoblots of organ homogenates, cruder copine fractions, or samples of purified copine stained with Coomassie Blue, indicating that it is a very small component that can only be detected in purified fractions by immunostaining.

Using this purification procedure, we usually obtain in a single preparation starting from a whole bovine spleen (700−800 g) approximately 1−2 mg of protein after ammonium sulfate precipitation which corresponds to approximately 0.8−1.6 mg of copine. Therefore, the copine I content of the bovine spleen can be roughly estimated as 1−2 μg/g of tissue, assuming 100% recovery in all steps. This is certainly the case in the ammonium sulfate precipitation step (see Figure 3) and perhaps in the lipid extraction step (see Figure 2). Some copine is however lost in the pellets during the low- and high-speed centrifugations (see Figure 2), indicating that the actual copine content may be somewhat higher. The final yield is however much lower, typically some 200 μg of purified copine in a single preparation, because a large amount of copine is lost as FPLC fractions contaminated with annexins.

Amino Acid Sequence of Bovine Spleen and Liver Copine. To further establish the identity of the putative copine, bands were eluted from gels of copine purified from bovine liver and spleen, digested with trypsin, and analyzed by liquid chromatography−tandem mass spectrometry. Table 1 shows a list of the peptides identified in the liver and spleen digests. All the peptides, except peptide 11, are exact matches to the sequences from human copine I. The peptide 11 mismatch consists of substitution of Y with F in the second residue. Comparison of the peptide sequences with protein sequences in databases shows that peptides 2, 14, and 15 match no proteins other than copine I. Partial matches, all corresponding to other members of the copine family, can be found for these peptides as well as for peptide 11.

Studies on Native Copine. An initial evaluation of copine in its native form is shown in Figure 3A. Analysis of purified copine by SDS−PAGE under nonreducing conditions revealed the presence of no other bands in addition to monomer copine in both the resolving (Figure 3, lane 5) and stacking gel (not shown). The copine band is more diffuse under these conditions which may reflect the presence of intramolecular disulfide bonds possibly formed by oxidation during purification.

To extend these observations and obtain information about copine in its native, nondenatured state, we carried out a series of analytical studies on purified bovine spleen copine. We found that copine in a Ca^{2+}-free solution behaves electrophoretically as a negatively charged monomer with a molecular mass similar to that measured by SDS−PAGE. Figure 4A shows that copine appears as a single, negatively charged band in nonreducing, native (i.e., SDS-free) polyacrylamide gels run in the presence of EGTA. No copine bands were observed when electrophoresis was carried out after reversing the electrodes, indicating the absence of positively charged molecules in our copine preparation. The molecular mass of native copine was calculated by studying

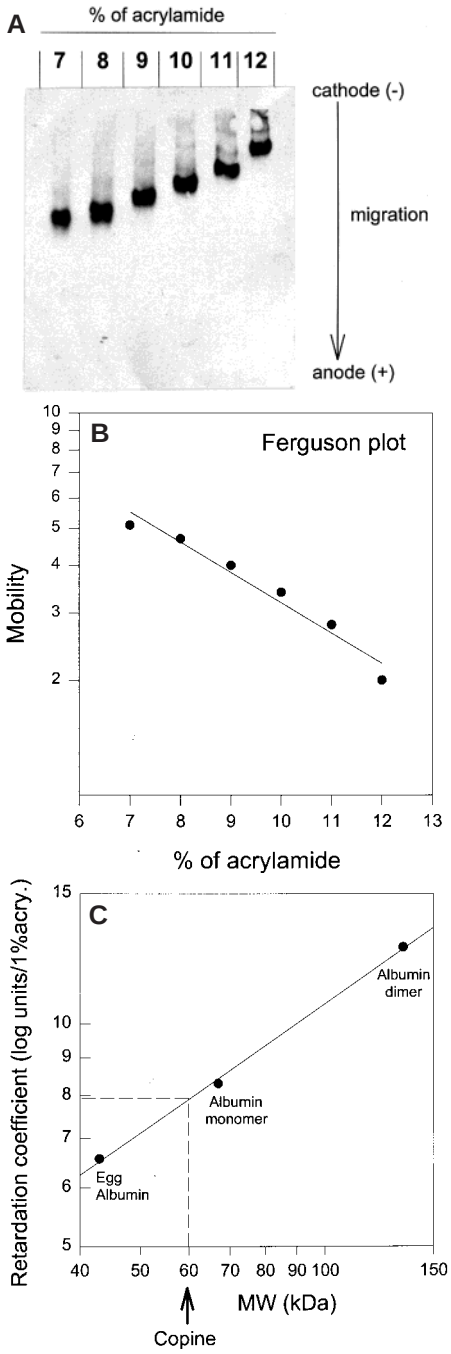

FIGURE 4: Analysis of purified copine by nondenaturing polyacrylamide gel electrophoresis. Samples of purified copine (5 μg) were run in polyacrylamide gels in the absence of SDS or DTT. The concentration of acrylamide was different for each lane and varied from 7 to 12%. Gels were prepared and run in a buffer consisting of 50 mM Tris (pH 8.3) and 5 mM EGTA. The anode was connected to the end of the gel opposite to where the samples were loaded. After the run, gels were electroeluted into a nitrocellulose membrane. (A) Migration of copine as revealed by detection with anticopine antibodies and goat anti-rabbit secondary antibodies linked to a chemiluminescence detection system. (B) Calculation of the coefficient of retardation for copine from mobility data shown in panel A by means of a Ferguson plot. A straight line was fitted to the data points by linear regression. The slope or coefficient of retardation was 7.94 log units/1% acrylamide concentration. (C) Calculation of the molecular mass of native copine by interpolation of the coefficient of retardation of copine in a log−log plot of the coefficients of retardation of molecular mass standards vs their molecular masses. The coefficients of retardation for the standards were obtained as described for copine (not shown). A straight line was fitted to the data points by linear regression.

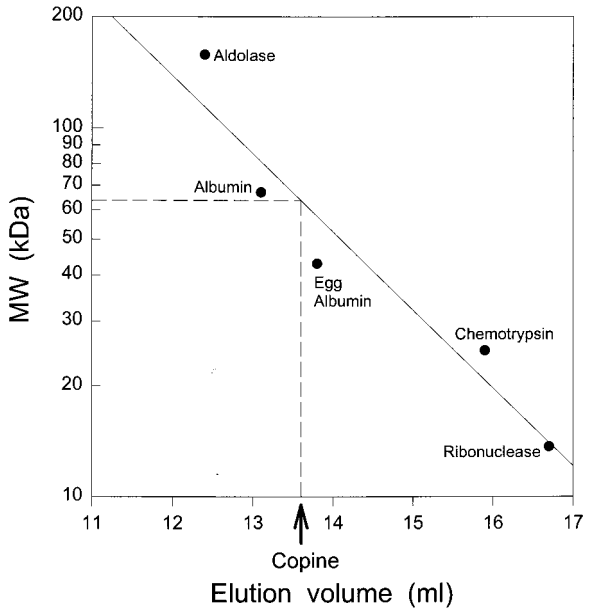

FIGURE 5: Analysis of purified copine by molecular exclusion chromatography. A 100 μg sample of purified copine was chromatographed in a Superose 12 HR 10/30 column in nominally Ca^{2+}-free buffer consisting of 120 mM KCl and 20 mM histidine (pH 6.4) at a flow rate of 0.3 mL/min. The elution volume for copine and standards was calculated according to the absorbance of the eluate at 280 nm. The presence of copine in the eluted material (collected as 0.3 mL fractions) was also analyzed by probing immunoblots of eluate samples with anticopine antibodies. In the experiment whose results are shown here, copine eluted at 13.6 mL which corresponds to 64 kDa, according to the calibration curve prepared using the proteins shown in the figure as standards. Fractions between 8 (excluded volume) and 21 mL (1.7 kDa) were analyzed by immunoblotting, and no copine peaks or traces were detected other than the 13.6 mL peak. The excluded volume of the column, measured using blue dextran, was 9 mL.

the reduction of its electrophoretic mobility as a function of the concentration of acrylamide according to the method of Ferguson (*15*). Because larger proteins are more effectively slowed by acrylamide, the molecular mass of an unknown protein can be calculated as a function of the mobility retardation induced by acrylamide. The coefficient of retardation was calculated as the slope of the plot of log mobility versus the percentage of acrylamide (Ferguson plot). Figure 4B shows a Ferguson plot for mobility data obtained with purified copine. Interpolation of this value in a log−log plot of the retardation coefficients of standards with known molecular masses versus their molecular masses resulted in a molecular mass for native copine of 60 kDa (Figure 4C).

Molecular exclusion chromatography studies similar to that shown in Figure 5 yielded an average molecular mass for native copine in absence of Ca^{2+} of 67 ± 3 kDa (mean ± the standard deviation obtained in three independent copine preparations, including the results shown in Figure 5). Similar results were obtained in the presence of EGTA. By analyzing the eluate by immunoblotting with anticopine antibodies, we ruled out the presence of copine in fractions other than the peak corresponding to monomeric copine.

In the presence of Ca^{2+}, we found that copine no longer exists as a single species. The most dramatic change occurred at concentrations of Ca^{2+} in the millimolar range. Under these conditions, we found it impossible to define the aggregation state of copine by either nondenaturing PAGE or molecular

exclusion chromatography; no defined bands or peaks could be observed under these conditions. We noticed, however, that in the presence of 2 mM Ca^{2+} copine no longer migrates toward the anode, indicating that it becomes either uncharged or positively charged. Attempts to define the migration of copine by reversing the polarity of the electrodes proved to be inconclusive. We observed that at least some copine migrates toward the cathode in the presence of Ca^{2+} in the form of smears along the lanes, possibly indicating a dynamic equilibrium between Ca^{2+}-bound and Ca^{2+}-free copine and/or monomeric and multimeric forms of copine. By molecular exclusion chromatography, we found that, as the Ca^{2+} concentration of the elution buffer increases, less copine is recovered as a monomer and at approximately 1 mM Ca^{2+} copine can no longer be recovered as a monomer. In fact, hardly any copine is recovered in the presence of Ca^{2+}, presumably because of precipitation inside the column or binding to the gel matrix. No changes in the elution pattern of copine were observed in the presence of 2 mM Mg^{2+}.

To further study the influence of Ca^{2+} on the aggregation state of copine, we analyzed samples of purified spleen copine by dynamic light scattering. We observed that, within the time resolution of this technique (~2 min with hand mixing), addition of Ca^{2+} to copine at 20 °C results in the immediate formation of multimolecular aggregates. Figure 6 shows that the average hydrodynamic radius of the molecular species present in a copine sample substantially increases upon addition of Ca^{2+}. Measurements obtained with three independent copine preparations (including the results shown in Figure 6) yielded average hydrodynamic radii of 3.7 ± 0.3 and 11.9 ± 0.4 nm in the absence and presence Ca^{2+}, respectively. This corresponds to molecular masses of 72 ± 12 and 1200 ± 100 kDa or an average of 17 copine molecules per aggregate. In both cases, samples exhibited low polydispersity indices, suggesting monodisperse molecular populations. The aggregation process exhibited an end point at 1−2 mM Ca^{2+}, and no further aggregation was observed by addition of more Ca^{2+}. At intermediate Ca^{2+} levels, our data indicate the formation of aggregates of intermediate size, although we were not able to single out complexes of a specific size as consistently as in the end point cases shown in Figure 6. The changes induced by Ca^{2+} were reversible; aggregated copine reverted to its monomeric form upon chelation of Ca^{2+} with EGTA.

To obtain information about the effect of Ca^{2+} on copine aggregation over a range of Ca^{2+} concentrations, we carried out ultracentrifugation studies of copine samples. As Figure 7 shows, substantial copine sedimentation was observed at 2 mM Ca^{2+}. Less but significant sedimentation was also observed down to 10^{-5} M Ca^{2+}, indicating the interaction between Ca^{2+} and copine at micromolar levels of Ca^{2+}.

Ca^{2+} Concentration Dependence and Metal Selectivity of Copine Binding to Brain Phospholipids. We explored the binding of copine to brain phospholipids at physiologically relevant concentrations of Ca^{2+} and the ability of other divalent cations to promote phospholipid binding. Figure 8 shows a SDS−PAGE analysis of supernatants and lipid pellets obtained from mixtures of brain phospholipids and purified spleen copine. In Figure 8A, we used mixtures containing free Ca^{2+} at concentrations ranging between 0 and 2 mM to demonstrate that half-maximal precipitation

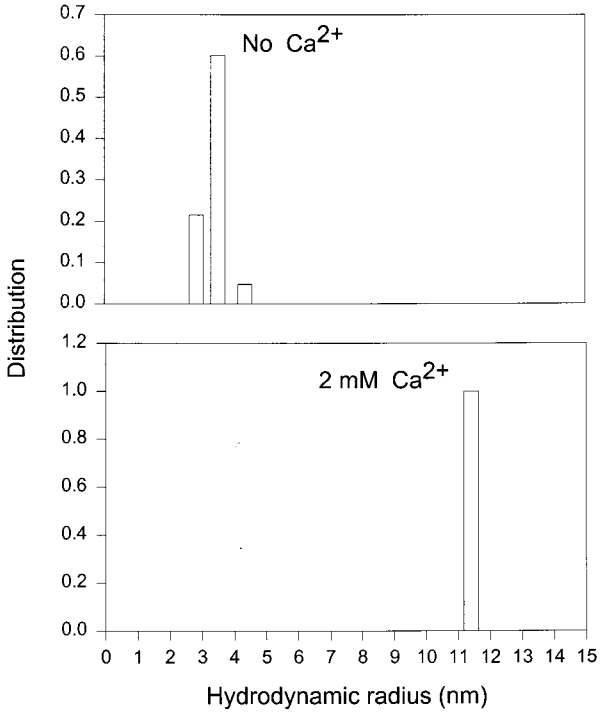

FIGURE 6: Analysis of purified copine by dynamic light scattering. Samples of purified copine were studied by analyzing the scattering of light at 90° in the presence and absence of Ca^{2+}. A dynamic light scattering instrument was used to calculate the hydrodynamic radius of the species present in the samples based on measurements of time-dependent fluctuations of the scattered light. Measurements were carried out at 20 °C in samples containing ~1 mg/mL purified copine in nominally Ca^{2+}-free medium or ~0.3 mg/mL purified copine in Ca^{2+}-containing medium. In both cases, a buffer consisting of 120 mM KCl and 20 mM histidine (pH 6.4) was used. Ca^{2+} was added as $CaCl_2$ to a final concentration of 2 mM. The figure shows the distribution of radii observed in the absence (top) and presence (bottom) of Ca^{2+}. In both cases, monomodal forms of light decay were observed, indicating the monodispersity of the molecules present in the samples. The average hydrodynamic radii were 3.95 and 11.4 nm, respectively. The molecular masses corresponding to these magnitudes calculated according to a standard curve prepared using globular proteins were 81 and 1060 kDa, respectively. Control measurements with BSA showed a hydrodynamic radius for this molecule of 3.8 ± 0.09 nm (mean ± the standard deviation, $n = 3$) which corresponds to 72 ± 4 kDa. No changes in the hydrodynamic radius of BSA were observed by addition of 2 mM $CaCl_2$.

occurs between pCa values of 5.5 and 5.0, i.e., 3.16 μM Ca^{2+} < K_{50} < 10 μM Ca^{2+}.

To study the divalent cation selectivity of copine, lipid/copine mixtures were supplemented with 2 mM unbuffered divalent cations. Figure 8B shows that copine has a very high selectivity for Ca^{2+} over Mg^{2+}. Sr^{2+} was slightly less effective than Ca^{2+} in promoting phospholipid binding, whereas Ba^{2+} was marginally effective. Controls show that metals in the absence of lipids cannot account for the observed effect, although some precipitation was apparent in tubes containing Ca^{2+} and Sr^{2+}, presumably due to divalent cation-induced precipitation similar to that shown in Figure 7.

Lipid Selectivity of Copine. The lipid selectivity of copine was assayed by analyzing the ability of copine to bind liposomes made out of mixtures of pure phospholipids containing 40% phosphatidylcholine and 60% of the phospholipid being studied. Copine/lipid mixtures were resus-

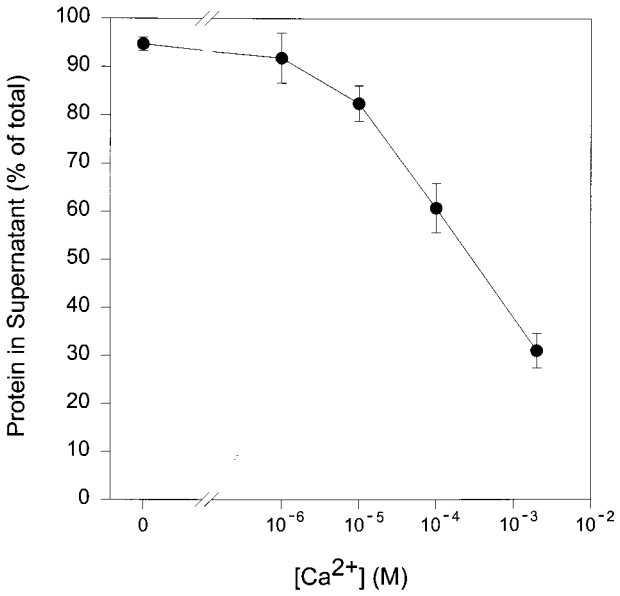

FIGURE 7: Analysis of purified copine by centrifugation at high speed in the presence of Ca^{2+}. The sedimentation of purified copine was studied in the presence of different concentrations of free Ca^{2+}. Aliquots (150 μL) of buffer consisting of 120 mM KCl and 20 mM histidine (pH 6.4) containing 10 μg of purified copine were supplemented with either $CaCl_2$ or a 2.5 mM EGTA/Ca^{2+} buffer to obtain concentrations of free Ca^{2+} of $<10^{-4}$ M. Samples were loaded in 7 mm $\times$ 20 mm polyallomer tubes (Beckman) and then centrifuged at 180000g_{max} for 5 h using the Beckman 42.2 TI rotor. The amount of protein in the supernatant was then measured according to the method of Bradford as described in Materials and Methods. Results are the amounts of protein in the supernatants expressed as a percentage of the total amount of protein present in the tubes. Values represent means $\pm$ the standard deviation of three determinations carried out with protein obtained in two independent preparations. A control carried out in the presence of 2 mM $MgCl_2$ showed no significant sedimentation. The protein concentration in the supernatant was in this case 94 $\pm$ 2% (mean $\pm$ the standard deviation, $n = 3$).

pended in buffer containing 60 μM free Ca^{2+} and centrifuged, and the supernatant and pellets were analyzed by SDS−PAGE. As expected, copine bound liposomes containing PS, i.e., the major component of the phospholipid mixture used for its purification (not shown). Binding was also observed in the cases of the other negatively charged phospholipids studied here: PG, PI, and PA (Figure 8C). In the case of PI, binding was only partial. We observed, however, that complete precipitation can be obtained at 2 mM free Ca^{2+} (not shown). At this high concentration of Ca^{2+} and at the applied centrifugal force, some copine precipitates due to self-aggregation (see Figure 3C). The rest is presumably bound to PI vesicles. We interpret this result as suggesting that copine has a weaker affinity for PI in comparison to other negatively charged phospholipids. PA appears to be a special case: binding of copine to PA liposomes is Ca^{2+}-independent. As Figure 8C shows, copine appears in the pellet of PA-containing liposomes even in the presence of EGTA. Finally, copine did not bind neutral phospholipids (PC, PE, and sphingomyelin).

Integrin Properties of Copine. The similarity between the core domain of copine and the A domain of certain integrins (*1*) prompted us to investigate the possibility that copine may exhibit some properties that are characteristic of integrins. It has been shown by $^{54}Mn^{2+}$ overlay that the integrin A domain is an autonomous divalent cation binding site that

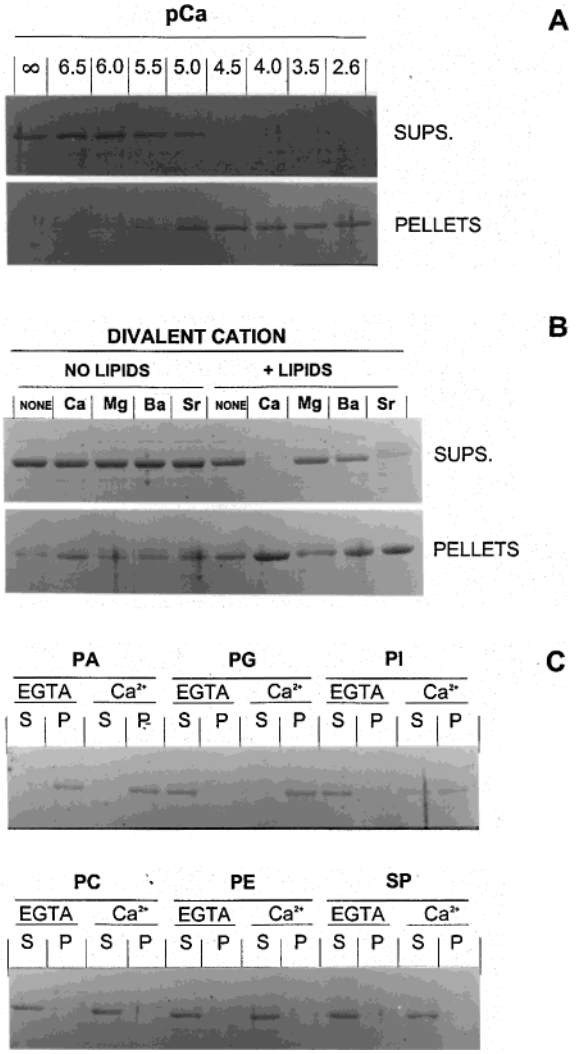

FIGURE 8: Binding of purified copine to phospholipid vesicles: Ca^{2+} concentration dependence, divalent cation, and phospholipid selectivity. The phospholipid binding activity of purified copine was assayed by SDS−PAGE analysis of supernatants and pellets obtained by centrifugation of copine/phospholipid vesicle mixtures. Mixtures were spun at 10000*g* for 10 min, and resuspended pellets and supernatants were supplemented with SDS−PAGE sample buffer and run in 12% acrylamide gels. Gels were stained with Coomassie Blue. Similar results were obtained in another set of experiments carried out using an independent copine preparation. (A) Ca^{2+} concentration dependence. Purified copine (5 μg) and phospholipid vesicles (~300 μg) were resuspended in 100 μL of a buffer containing 120 mM KCl, 20 mM histidine (pH 6.4), and different concentrations of free Ca^{2+} set by a 2.5 mM EGTA/Ca^{2+} buffer. No Ca^{2+} buffer was used to obtain free Ca^{2+} concentrations of $\geq$100 μM. The figure shows the copine bands in the supernatants and pellets at the indicated pCa values, between zero (pCa = ∞) and 2 mM free Ca^{2+} (pCa = 2.6). (B) Divalent cation selectivity. Purified copine (7 μg) and phospholipid vesicles (~300 μg) were resuspended in 100 μL of a buffer containing 120 mM KCl, 20 mM histidine (pH 6.4), and the chloride salts of different divalent cations (2 mM). The figure shows the copine bands in supernatants and pellets obtained with the indicated divalent cations. (C) Phospholipid selectivity. Purified copine (5 μg) and phospholipid vesicles (~200 μg) were resuspended in 100 μL of a buffer containing a 2.5 mM EGTA/Ca^{2+} buffer prepared to obtain a free Ca^{2+} concentration of 60 μM. Phospholipid vesicles made out of pure phospholipids consisted of 40% PC and 60% of the phospholipid being studied. Assays were carried out in 120 mM KCl and 20 mM histidine (pH 6.4), except when PC, PE, and sphingomyelin were studied. In this case, the buffer was 40 mM KCl and 6.7 mM histidine (pH 6.4). The figure shows the copine bands in supernatants and pellets obtained with the indicated phospholipids.

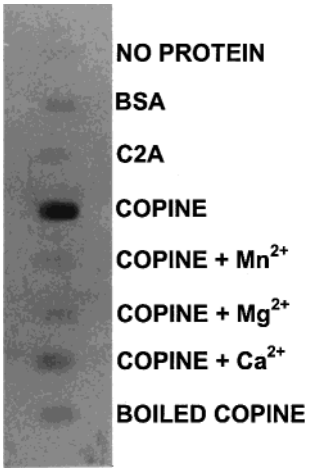

FIGURE 9: Binding of $^{54}Mn^{2+}$ to purified copine. The ability of purified copine to bind Mn^{2+} was assayed by overlaying dot blots of purified copine with $^{54}Mn^{2+}$ in the absence and presence of competitor divalent cations. Pieces of nitrocellulose paper were spotted with 20 μL of 0.2 mg/mL purified copine, washed, incubated with $^{54}Mn^{2+}$ in the presence and absence of 2 mM cold Mn^{2+}, Mg^{2+}, or Ca^{2+}, and washed in divalent cation-free buffer. Controls were carried out using nitrocellulose paper spotted with buffer or 0.2 mg/mL solutions of BSA or boiled copine. An additional control was included using 80 μg/mL recombinant C2A domain from rat synaptotagmin. The figure shows radioautographs obtained by exposure of photographic film for 24 h to experimental and control blots. Radioactivity eluted from papers after exposure was as follows (copine = 100%): no protein, 7.5%; BSA, 15%; C2A, 11%; copine, 100%; copine with Mn^{2+}, 8.3%; copine with Mg^{2+}, 9.5%; copine with Ca^{2+}, 39%; and boiled copine, 23%. Similar results were obtained in experiments carried out using an independent copine preparation.

exhibits specificity for Mg^{2+} and Mn^{2+} over Ca^{2+} (*4*). We carried out similar $^{54}Mn^{2+}$ overlay assays and found that purified spleen copine exhibits $^{54}Mn^{2+}$ binding activity that is sensitive to competition by Mn^{2+} and Mg^{2+} and, to a lesser extent, to competition by Ca^{2+}. As shown in Figure 9, copine shows intense $^{54}Mn^{2+}$ labeling in the absence of competitors and only background levels in the presence of an excess of Mg^{2+} or Mn^{2+}. The extent of labeling in the presence of Ca^{2+} was reduced but not eliminated, suggesting that binding occurs at a site that is not a Ca^{2+} selective site. A control carried out with a recombinant C2A domain from rat synaptotagmin shows no $^{54}Mn^{2+}$ binding, suggesting the C2 domain of copine may not be responsible for the observed binding of Mn^{2+}. Finally, thermal denaturation of copine substantially reduced the level of binding of $^{54}Mn^{2+}$, indicating that only native copine exhibits $^{54}Mn^{2+}$ binding activity.

ATP Binding. In a previous report (*1*), we discussed the possibility that copine may be an ATP binding protein on the basis of the similarities of the A domain of copine and the integrin A domain which has a Rossman fold structural motif. We here explored this possibility by investigating the binding of 8-azido-[α-^{32}P]ATP to partially purified copine, i.e., a FPLC fraction containing about 30% copine. We did not observe specific ATP binding; copine was labeled by the reagent, but no competitive inhibition occurred in the presence of additional unlabeled ATP. The same result was obtained irrespective of the presence of Ca^{2+} or brain phospholipids. We chose a partially purified preparation of copine to carry out these experiments because some of our FPLC fractions contain a 45 kDa protein that specifically binds ATP. We have not characterized this protein, but it served as a useful positive control.

DISCUSSION

In this paper, we have presented information about the organ distribution, purification, and biochemical properties of mammalian copine. We have shown that copine is expressed in all major adult mammalian organs. In contrast to *Paramecium* copine which is the major Ca^{2+}-dependent, phospholipid-binding protein in this ciliate (*1*), mammalian copine is a minor component of the pool of Ca^{2+}-dependent, phospholipid-binding proteins present in mammalian organs. Even in the spleen, the richest source of copine studied here, copine represents only 5% of the extractable Ca^{2+}-dependent, phospholipid-binding proteins. We have described a protocol for purifying mammalian copine I to homogeneity from bovine spleen and characterized the biochemical properties of this protein in relation to its Ca^{2+}-dependent binding to phospholipids and to the possibility of a functional relationship with the integrin family of proteins.

Purified mammalian copine I exhibited a molecular mass measured by SDS−PAGE (58 kDa) similar to that predicted from amino acid sequences derived from human ESTs (58 928 Da), showing that the SDS binding capacity or the intrinsic charge of copine does not deviate from average values and that no major carbohydrate moieties are attached to copine. Further analysis showed that purified copine exists as a monomer in Ca^{2+}-free solutions. Native electrophoresis yielded a molecular mass (60 kDa) that closely agrees with the SDS−PAGE value and showed that copine is a negatively charged species at pH 8.3 as expected for a protein whose isoelectric point, calculated from its amino acid sequence, is 5.52. A PI value of 5.8 was measured by isoelectric focusing for a protein, originally named "chromobindin 17" (*16*), that was recently identified as copine (*1*). Somewhat higher molecular mass values were obtained by molecular exclusion chromatography (67 kDa) and dynamic light scattering (72 kDa), possibly suggesting that copine may be a slightly asymmetric molecule as compared with the standards.

The most important observations provided by the dynamic light scattering experiments are the substantial increase in the hydrodynamic radius upon addition of Ca^{2+}, the presence of an end point, i.e., a concentration of Ca^{2+} beyond which no further increases of the hydrodynamic radius were observed, and the reversibility of the effect of Ca^{2+}. The increase in the hydrodynamic radius indicates the formation of larger particles, and the end point means that there is a maximum size for those particles. To interpret these data in terms of the number of molecules of copine per particle, an assumption must be made about the shape of the aggregates. If a globular model is assumed, our data indicate that 17 molecules of copine aggregate to form a particle at saturating Ca^{2+} concentrations. At intermediate Ca^{2+} levels, the data indicate the formation of aggregates of intermediate size, although the interpretation of this information in terms of actual aggregates of a particular size is less certain. First, we were unable to identify any intermediate species as consistently as the two forms mentioned above, and second, high polydispersity indices were measured under this condition, meaning that data cannot be as reliably interpreted in terms of the properties of specific molecular species.

Our results show that the interaction between copine and membranes occurs at concentrations of Ca^{2+} that are likely to occur in the cytosol of stimulated cells but not in resting cells. This may indicate a possible role of copine in signal transduction mechanisms modulated by Ca^{2+}. As expected for a molecule involved in such a process, mammalian copine, like *Paramecium* copine (*1*), does not exhibit Mg^{2+}-dependent binding to membranes. The ability of Sr^{2+} to replace Ca^{2+}, although probably not physiologically relevant, may eventually prove useful in biochemical studies or in a protocol for purification of copine from phospholipid-binding proteins with lower affinity for Sr^{2+}.

The mechanism by which C2 domain-containing proteins bind phospholipids is not fully understood. Some proteins such as PKC and synaptotagmin bind mainly acidic phospholipids, whereas others, such as $cPLA_2$, prefer phosphatidylcholine, a zwitterionic molecule (*3*), possibly reflecting the more hydrophobic nature of the $cPLA_2$ C2 domain (*17*). In agreement with observations on *Paramecium* copine (*1*), we have demonstrated here that mammalian copine binds phosphatidylserine and other negatively charged phospholipids but not phosphatidylcholine or other neutral phospholipids, suggesting that copine binds phospholipids by a mechanism similar to that of PKC or synaptotagmin. Binding to phosphatidic acid, a negatively charged phospholipid, constitutes a special and interesting case; under our experimental conditions, the binding was Ca^{2+}-independent. A similar phenomenon was reported for the binding of the recombinant PKCα C2 domain to PA/PC membranes (*18*). In this case, however, it was shown that Ca^{2+}-independent binding occurs only at a high PA:PC ratio. Binding to PA is an interesting property since evidence has accumulated during the past few years supporting a role for PA as an intracellular messenger (*19*), including direct activation of PKC and other kinases (*20*). In the cell, the affinity of copine for PA may cause copine to be translocated to the plasma membrane in a Ca^{2+}-independent manner. Although PA is usually a minor component of the membrane, it is rapidly formed by the breakdown of phosphatidylcholine into PA and choline as a result of the activation of phospholipase D. This enzyme is activated by a wide variety of extracellular and intracellular mediators and is linked to other signaling pathways and membrane trafficking events (*21*).

From the point of view of the mechanisms of interaction of C2 domain proteins with membranes, the notion of Ca^{2+}-independent binding seems to be an intriguing one. All models proposed for the interaction of C2 domain proteins with membranes require Ca^{2+} (*2*) and are mostly based on observations carried out on membranes containing PS. In general, Ca^{2+} is thought to bridge protein and phospholipid negative charges and thus promote binding. Recent crystallographic evidence however has pointed out the importance of hydrogen bonds and hydrophobic interactions for the binding of the C2 domain of PKCα to membranes (*22*). It is possible that in the case of PA, a phospholipid with a smaller headgroup, the requirement for Ca^{2+} bridging is relaxed and other interactions may take place even in the absence of Ca^{2+} when a large number of PA molecules are available for binding. In the presence of fewer molecules of PA, Ca^{2+} may be necessary to obtain the correct orientation of the sparse interacting molecules.

We have explored the possibility that copine may exhibit some properties related to the similarity between the primary structure of its core domain and the A domain of certain integrins that includes critical residues involved in the chelation of Mg^{2+}. Integrins are known to bind extracellular ligands in a divalent cation-dependent fashion, although in many cases the exact location of these binding sites as well as their role is still unclear. Michishita et al. (*4*) have demonstrated that the A domain may function as an autonomous, divalent cation and ligand binding locus by showing that a recombinant form of the A domain of the α-subunit of the CR3 integrin, the main integrin expressed in neutrophils, presents a metal binding site that is essential for ligand recognition. Specifically, $^{54}Mn^{2+}$ binding experiments show that, in comparison to Ca^{2+}, this site exhibits high specificity for Mn^{2+} and Mg^{2+}, two cations usually associated with the facilitation of the interaction of integrins with their ligands. We carried out similar experiments with spleen copine and observed a similar pattern. Interpretation of our results is however complicated by the fact that copine possesses two divalent cation-binding domains, the A domain and the C2 motifs. We observed binding of $^{54}Mn^{2+}$ that can be competed by Mn^{2+} or Mg^{2+} and only partially by Ca^{2+}, suggesting the presence of a site with Mn^{2+}/Mg^{2+} selectivity. This is not the expected selectivity of a C2 domain, and thus, it can be hypothesized that binding occurs at a site other than the lipid and Ca^{2+}-binding domain. Controls carried out with a recombinant C2 domain from synaptotagmin showed no $^{54}Mn^{2+}$ binding activity, supporting the idea of a distinct site.

In the case of the integrin A domain, binding of Mg^{2+} is required for the integrin to bind its target ligands in the extracellular matrix. It is interesting to speculate that the copine A domain may also be involved in the interaction of copine with certain target proteins. A potential copine ligand was identified (*23*) in a yeast two-hybrid screen (see below). However, this ligand, protein OS-9, was demonstrated to bind the C2 domain portion of copine.

No biological role has yet been defined for the copine family of proteins. The presence of C2 domains suggests the involvement in processes of signal transduction and/or membrane trafficking. We have shown in this study that copine is present in all major mammalian organs, suggesting that copine may be an essential protein. We found however that the amount of copine seems to vary from organ to organ, although there was no obvious correlation with the function of the organs studied here. The spleen is an interesting case. Our results show that the amount of copine in this organ seems to be greater than in other organs, suggesting that copine-rich cells may be involved in physiological processes occurring in the spleen such as phagocytosis or immune phenomena.

Recently, a new member of the copine family has been described, a 62 kDa protein named N-copine, for neural copine, on the basis that it appears to be brain-specific (*24*). Several putative roles with different degrees of experimental support have been advanced for this protein. It has been postulated that N-copine may be involved in synaptic long-term potentiation and synaptic plasticity based on the increased level of expression of N-copine mRNA observed in the hippocampal area of mice injected with kainate and in hippocampal slices after electrical stimulation. It has also

been reported that N-copine is localized in neurons of the hippocampus and the olfactory bulb of the mouse brain and hypothesized that N-copine may play a role in synaptic plasticity and memory formation (*25*). Using a yeast two-hybrid system, a protein BOS-9B that binds the C2B domain of N-copine through its carboxyl-terminal region in a Ca^{2+}-dependent manner has been identified (*23*). On the basis of the interaction between N-copine and OS-9 in cultured cells transfected with vectors for both proteins and on the basis of the fact that mRNAs for N-copine and OS-9 are expressed in the human brain, it has been postulated that this interaction may be physiologically relevant and, in particular, related to synaptic plasticity phenomena. We have not observed 62 kDa immunoreactive bands in homogenates or Ca^{2+}-dependent, phospholipid-binding fractions from rat brain, suggesting that either our anticopine antibody does not recognize N-copine or the 58 kDa protein recognized by our antibody was not resolved from N-copine.

Further support for the idea of a role for copine in nerve cells is provided by the observation that a *Caenorhabditis elegans* gene (ORF B0495.10, Genbank entry AAA62529) homologous to the human copine I gene shows a neuronal expression pattern. Studies using β-galactosidase as a reporter gene (Sanger Centre Wormbase, expression pattern report Expr130) show that the promoter for this copine is active in only eight neurons, four in the *C. elegans* head and four in its tail. This suggests that this member of the copine family may play a specific role in nerve cell function.

Future identification of the functions of neural copines as well as of the more widely expressed copines such as copine I will likely require identification of the role of the A domain as an enzyme or as a specific protein−protein interaction domain.

ACKNOWLEDGMENT

We thank Sandra Snyder and Zhiding Qian for their excellent technical assistance. We are indebted to the staff of Protein Solutions Inc. (Charlottesville, VA), to Dr. Robert Kretsinger, and to Charles Gawthrop for their excellent assistance with the dynamic light scattering experiments.

REFERENCES

1. Creutz, C. E., Tomsig, J. L., Snyder, S. L., Gautier, M. C., Skouri, F., Beisson, J., and Cohen, J. (1998) *J. Biol. Chem. 273*, 1393−1402.
2. Nalefski, E. A., and Falke, J. J. (1996) *Protein Sci. 5*, 2375−2390.
3. Rizo, J., and Südhof, T. C. (1998) *J. Biol. Chem. 273*, 15879−15882.
4. Michishita, M., Videm, V., and Arnout, M. A. (1993) *Cell 72*, 857−867.
5. Frangioni, J. V., and Neel, B. G. (1993) *Anal. Biochem. 210*, 179−187.
6. Guan, K., and Dixon, J. E. (1991) *Anal. Biochem. 192*, 262−267.
7. Olmsted, J. B. (1981) *J. Biol. Chem. 256*, 11955−11957.
8. Damer, C. K., and Creutz, C. E. (1994) *J. Biol. Chem. 269*, 31115−31123.
9. Mandal, A., Naaby-Hansen, S., Wolkowicz, M. J., Klotz, K., Shetty, J., Retief, J. D., Coonrod, S. A., Kinter, M., Sherman, N., Cesar, F., Flickinger, C. J., and Herr, J. C. (1999) *Biol. Reprod. 61*, 1184−1197.
10. Laemmli, U. K. (1970) *Nature 227*, 680−685.
11. Burnette, W. N. (1981) *Anal. Biochem. 112*, 195−203.
12. Zacks, W. J., and Creutz, C. E. (1990) *Biochim. Biophys. Acta 1029*, 149−160.
13. Durham, A. C. H. (1983) *Cell Calcium 4*, 33−46.
14. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
15. Ferguson, K. A. (1964) *Metabolism 13*, 985−1002.
16. Creutz, C. E., Dowling, L. G., Sando, J. J., Villar-Palasi, C., Whipple, J. H., and Zacks, W. J. (1983) *J. Biol. Chem. 258*, 14664−14670.
17. Davletov, B., Perisic, O., and Williams, R. L. (1998) *J. Biol. Chem. 273*, 19093−19096.
18. García-García, J., Corbalán-García, S., and Gómez-Fernández, J. C. (1999) *Biochemistry 38*, 9667−9675.
19. Waggoner, D. W., Xu, J., Singh, I., Jasinska, R., Zhang, Q., and Brindley, D. N. (1999) *Biochim. Biophys. Acta 1439*, 299−316.
20. English, D., Cui, Y., and Siddiqui, R. A. (1996) *Chem. Phys. Lipids 80*, 117−132.
21. Gomez-Cambronero, J., and Keire, P. (1998) *Cell. Signalling 10*, 387−397.
22. Verdaguer, N., Corbalán-García, S., Ochoa, W. F., Fita, I., and Gómez-Fernández, J. C. (1999) *EMBO J. 18*, 6329−6338.
23. Nakayama, T., Yaoi, T., Kuwajima, G., Yoshie, O., and Sakata, T. (1999) *FEBS Lett. 453*, 77−80.
24. Nakayama, T., Yaoi, T., Yasui, M., and Kuwajima, G. (1998) *FEBS Lett. 428*, 80−84.
25. Nakayama, T., Yaoi, T., and Kuwajima, G. (1999) *J. Neurochem. 72*, 373−379.

BI0019949