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Conodipine-M, a Novel Phospholipase A_2 Isolated from the Venom of the Marine Snail *Conus magus**

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We describe the purification and first biochemical characterization of an enzymatic activity in venom from the marine snail *Conus magus*. This enzyme, named conodipine-M, is a novel phospholipase A_2 with a molecular mass of 13.6 kDa and is comprised of two polypeptide chains linked by one or more disulfide bonds. The amino acid sequence of conodipine-M shows little if any homology to other previously sequenced phospholipase A_2 enzymes (PLA_2s). Conodipine-M thus represents a new group of PLA_2s. This is remarkable, since conodipine-M displays a number of properties that are similar to those of previously characterized 14-kDa PLA_2s. The enzyme shows little, if any, phospholipase A_1, diacylglycerol lipase, triacylglycerol lipase, or lysophospholipase activities. Conodipine-M hydrolyzes the *sn*-2 ester of various preparations of phospholipid only in the presence of calcium and with specific activities that are comparable to those of well known 14-kDa snake venom and pancreatic PLA_2s. The *Conus* enzyme binds tightly to vesicles of the negatively charged phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol and catalyzes the hydrolysis of this substrate in a processive fashion. Conodipine-M does not significantly discriminate against phospholipids with unsaturated *versus* saturated fatty acids at the *sn*-2 position or with different polar head groups. Linoleoyl amide and a phospholipid analog containing an alkylphosphono group at the *sn*-2 position are potent inhibitors of conodipine-M. We suggest that the functional resemblance of conodipine-M to other PLA_2s might be explained by the utilization of similar catalytic residues.

Conus are a group of predatory marine snails that prey on fish, molluscs, and polychaete worms (1). Venom from cone snails are extraordinarily complex and often contain more than 100 distinct components. Most venom characterizations have focused on small disulfide-rich peptides that target selectively to receptors and ion channels on nerve and muscle cells. Known targets include calcium channels (ω-conotoxins) (2–4), sodium channels (μ- and δ-conotoxins) (5–8), *N*-methyl-D-aspartate receptors (conantokins) (9–11), nicotinic acetylcholine receptors (α-conotoxins) (12–17), and vasopressin receptors (conopressins) (18, 19). Small neuroactive molecules, *e.g.* serotonin, have also been found (20).

Although larger protein components are present in cone venoms, these are generally less well characterized biochemically. Reports have included a convulsion-inducing peptide from *Conus geographus*, with an apparent molecular weight of 13,000 (21); a cardiotonic glycoprotein (approximate molecular weight of 25,000) from *Conus striatus*, which appears to act on voltage-sensitive sodium channels (22, 23); a protein from *Conus magus* (molecular weight estimated to be between 45,000 and 65,000), which induces powerful rhythmic contractions of guinea pig vas deferens (24); and a 25,000 molecular weight protein from *Conus distans*, which inhibits neurotransmitter release in rat hippocampus (25).

In this study, we have characterized a 13.6-kDa component from the venom of the fish-eating cone snail, *C. magus*. Novel ω- and α-conotoxins have been isolated from this species previously (13, 26). We detail the isolation and characterization of a polypeptidic venom component that we show to be a novel phospholipase A_2 (PLA_2).[1] This is the first biochemical characterization of an enzymatic activity from *Conus* venoms.

Low molecular mass secreted PLA_2s (14–18 kDa) have been reported from a wide variety of other venoms (snakes, insects, and lizards) as well as mammalian pancreatic and inflammation fluids (27–30). These enzymes hydrolyze the ester at the *sn*-2 position of phosphoglycerides. Snake venom and pancreatic PLA_2s are particularly well characterized (31, 32). All require millimolar concentration of calcium as a catalytic cofactor. In addition to PLA_2 activity, some of these venom proteins show potent neurotoxicity (33–35). For instance β-bungarotoxin, from the Formosan banded krait (*Bungarus multicinctus*) (36) and notexin isolated from the Australian tiger snake *Notechis scutatus scutatus* (37) exert potent neurotoxic action by inhibiting the release of acetylcholine from nerve terminals in neuromuscular junctions (38). β-Bungarotoxin consists of a PLA_2 chain covalently bonded by a disulfide bridge to a polypeptide (molecular weight ~ 20,000) (29). The PLA_2 chain of β-bungarotoxin is homologous to notexin. En-

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[1] The abbreviations used are: PLA_2, phospholipase A_2; DMPM, 1,2-dimyristoyl-*sn*-glycero-3-phosphomethanol; [^{3}H]DPG, 1-palmitoyl-2-[9,10-[^{3}H]]palmitoyl-*sn*-glycerol; [^{3}H]DPPA, 1-palmitoyl-2-[9,10-[^{3}H]] palmitoyl-*sn*-glycero-3-phosphate; [^{3}H]DPPC, 1-palmitoyl-2-[9,10-[^{3}H]] palmitoyl-*sn*-glycero-3-phosphocholine; [^{3}H]DPPE, 1-palmitoyl-2-[9,10-[^{3}H]]palmitoyl-*sn*-glycero-3-phosphoethanolamine; [^{14}C]PAPC, 1-palmitoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphocholine; [^{14}C]SAPI, 1-stearoyl-2-[1-^{14}C]arachidonyl-*sn*-glycero-3-phosphoinositol; [^{3}H]SAPS, 1-stearoyl-2-[5,6,8,9,11,12,14,15-[^{3}H]]arachidonyl-*sn*-glycero-3-phosphoserine; HPLC, high performance liquid chromatography; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

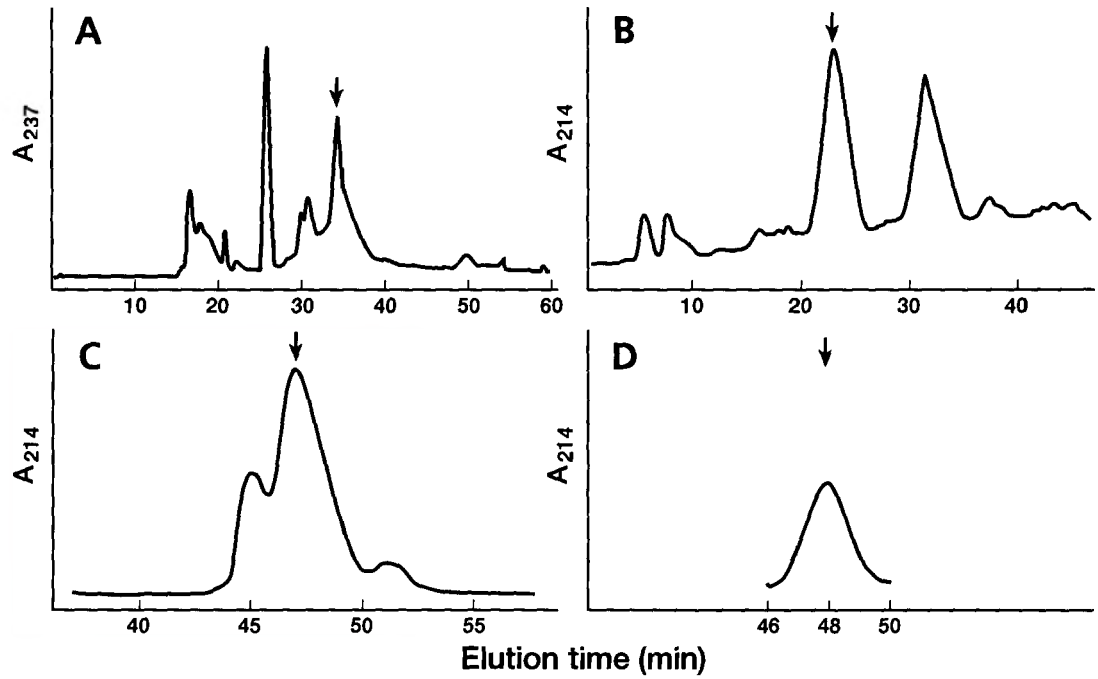

FIG. 1. **HPLC purification of conodipine-M.** *Panel A*, extracted crude venom (see "Experimental Procedures") was applied to a Vydac reversed phase C18 column (10-μm particle size; 22-mm inner diameter × 25-cm length) and eluted with a gradient that began at 95% A, 5% B for 5 min and increased to 100% B over 28 min. B was held at 100% for 5 min and then decreased to 5% over 4 min. The flow rate was 4 ml/min. Fractions were collected every 3 min beginning at 12 min. *Panel B*, material eluting at 34.3 min (*arrow* in *panel A*) was run on a Vydac reversed phase C18 column (10-μm particle size, 10-mm inner diameter × 25-cm length) and eluted by beginning at 70% A, 30% B for 4 min; increasing to 36% B over 6 min; and then increasing to 56% B over 60 min. The flow rate was 3 ml/min. Fractions were collected every 1.0–1.25 min, beginning at 3 min. *Panel C*, material eluting at 23.3 min (*arrow* in *panel B*) was run on a Microsorb MV reversed phase C18 column (5-μm particle size, 4.6-mm inner diameter × 25-cm length). The gradient began at 40% C, 60% buffer D for 1 min; increased to 70% D over 30 min; and increased to 80% D over 60 min. The flow rate was 1 ml/min. Fractions were collected according to visual inspection of the protein absorbance. *Panel D*, material eluting near the peak at 47.4 min (*arrow* in *panel C*) was run again using the same conditions as in *panel C*. Buffer A was 0.1% trifluoroacetic acid; buffer B was 0.1% trifluoroacetic acid, 90% acetonitrile in *panels A* and *B*. Buffer C was 0.1% trifluoroacetic acid, 2% MeOH, 98% H_2O; buffer D was 0.09% trifluoroacetic acid, 90% MeOH, 10% H_2O in *panels C* and *D*. It should be noted that prolonged use of trifluoroacetic acid/methanol in the Microsorb MV column is not recommended by the manufacturer because of potential hydrolysis. The absorbance was monitored at 237 nm in *panel A* and at 214 nm in *panels B–D*.

zymes isolated from the venoms of *Naja naja atra* and *Naja niggricollis*, however, show more potent PLA_2 activity, have less presynaptic effects, and are much less lethal (39). More than 50 proteins with PLA_2 activity have been isolated from snake venoms and show substantial sequence homology with each other and with PLA_2 enzymes isolated from mammalian pancreas and inflammatory exudates (40, 41). Sequence information reveals that the *C. magus* enzyme reported here differs substantially from any other PLA_2 characterized to date and defines a new group of these enzymes.

EXPERIMENTAL PROCEDURES

Materials

C. magus specimens were collected in the Philippines. Venom was removed from dissected ducts, lyophilized, and stored at −70 °C until use (42). β-Mercaptoethanol was from Pierce, dithiothreitol was from Boehringer Mannheim, trifluoroacetic acid (sequencing grade) was from Aldrich, and acetonitrile (UV grade) was from Baxter.

The preparation of 1-*O*-hexadecyl-2-deoxy-2-*S*-thiohexadecanoyl-*sn*-glycero-3-phosphocholine and DMPM has been described previously (43, 44). [^{3}H]DPPC (50 Ci/mmol), [^{14}C]PAPC (50 mCi/mmol), and [^{14}C]SAPI (50 mCi/mmol) were from DuPont NEN. [^{3}H]DPPE (10 mCi/mmol), [^{3}H]DPPA (5 mCi/mmol), and [^{3}H]SAPS (400 mCi/mmol) were prepared as described (45, 46). [^{3}H]DPG was synthesized by treating 0.15 mg of [^{3}H]DPPC (50 mCi/mmol) in 0.2 ml of 200 mM sodium phosphate, 400 μM $ZnCl_2$, 1 mM 2-mercaptoethanol, pH 7.0, with 30 μg of phospholipase C (*Bacillus cereus*, Sigma P-7147) for 2 h at 37 °C with vigorous shaking. The reaction mixture was extracted three times with ether, the extract was dried over anhydrous Na_2SO_4, and the residue was dissolved in ether and applied to a silica gel TLC plate. The plate was developed with chloroform:ether (9:1), the product band (visualized with iodine) was scraped from the plate, and the product was eluted with ether. The PLA_2 inhibitors linoleoyl amide and MG14 were prepared as described (47–49). Porcine pancreatic PLA_2 was obtained as a generous gift from Professor M. K. Jain (University of Delaware) (50), bee venom PLA_2 was from Boehringer Mannheim, and pancreatic lipase was from Sigma (L-0382).

Protein Purification and Structural Analysis

Venom Extraction—Crude lyophilized venom (1.99 mg) was dissolved in 8 ml of ice-cold buffer containing 50 mM *Tris*·HCl, pH 7.4 at room temperature, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$ and sonicated five times for 10 s each at setting 8 using a Sonifier (model LS-75, Branson Instruments). During sonication the vessel was cooled with an ice bath. The mixture was centrifuged at 17,200 × *g* for 10 min at 4 °C. The supernatant was retained, and an additional 2 ml of buffer was added to the pellet, and the mixture was sonicated three times as above. The mixture was centrifuged as above, and the supernatants were combined and centrifuged again. Supernatants were placed in an empty polypropylene spin column with a cut-to-size GF/C filter (Whatman) placed on the column support. The column was then centrifuged at 200 × *g* at 4 °C, and 25% of the filtrate was used for further purification by HPLC. The remaining filtrate was stored at −70 °C.

HPLC Purification—HPLC was carried out with HPXL pumps and a Dynamax model UV1 detector (Rainin Instruments, Woburn, MA). Vydac and Microsorb MV columns were from Rainin. See Fig. 1 for column specifications and buffer conditions. The concentrations of purified conodipine-M were determined from the OD_{280} using an extinction coefficient of 10/cm/percent (OD = 1 is 1 mg/ml). This number was derived from yields of protein obtained during amino acid sequencing of conodipine-M.

Protein Reduction and Alkylation—In Experiment 1, disulfide bonds were reduced by adding 50 μl of conodipine-M (200 pmol dissolved in HPLC elution buffer, Fig. 1*D*) to 200 μl of reducing buffer 1. Reducing buffer 1 is 50 mM Na_2HPO_4, 10 mM β-mercaptoethanol, 1 mM dithio-

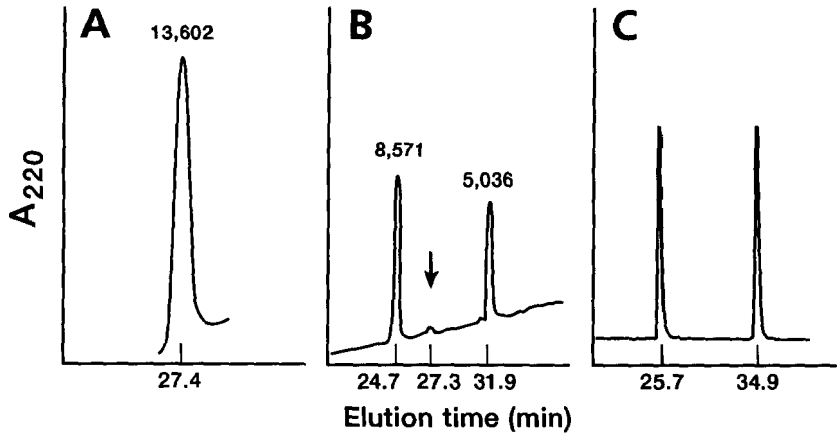

FIG. 2. **Conodipine-M has two subunits.** Native and modified conodipine-M were analyzed by HPLC. HPLC conditions are the same for each *panel* and are described in Experiment 1 under "Experimental Procedures." *Panel A*, unmodified conodipine-M, when chromatographed as described below, elutes at 27.4 min. Electrospray mass spectrometry of this material shows a molecular mass of 13,602. *Panel B*, reduction of conodipine-M (described under "Experimental Procedures") produces two primary protein absorbances with migration times (24.7 and 31.9 min) which differ from unmodified material. Mass spectroscopy of these proteins shows molecular masses of 8,571 and 5,036. The *arrow* indicates the expected migration time of unmodified conodipine-M. The sum of the molecular masses of these two proteins approximates that of the intact protein. *Panel C*, reduction and alkylation of conodipine-M (described in under "Experimental Procedures") also results in two protein absorbances with migration times of 25.7 and 34.9 min.

threitol, and 1 mM EDTA adjusted to pH 7.3 with H_3PO_4 and bubbled with nitrogen. The reaction vessel was flushed with nitrogen and incubated at 65 °C for 15 min. The reduced protein was analyzed by HPLC. A portion of the reduced protein subunits (see Fig. 2*B*) was submitted for mass spectrometry analysis. The remainder was alkylated by adding Tris base directly to the HPLC fractions to raise the pH to between 7 and 8 (measured by pH paper) and adding 4 μl of 4-vinylpyridine. The solution was reacted at room temperature in the dark for 25 min, and alkylated peptides were purified by HPLC. In Experiment 2, conodipine-M was reduced, alkylated, and then purified by HPLC (Fig. 2*C*). For this experiment, conodipine-M (3.2 nmol in 800 μl of HPLC elution buffer; Fig. 1*D*) was added to 80 μl of reducing buffer 2. Reducing buffer 2 was 0.25 M Na_2HPO_4, 100 mM β-mercaptoethanol, 10 mM dithiothreitol, and 10 mM EDTA (pH not adjusted; solution was bubbled with nitrogen). The pH of the combined conodipine-M and reducing buffer 2 solution was between 7 and 8 as measured by pH paper. The reaction vessel was flushed with nitrogen and incubated at 65 °C for 15 min and then allowed to cool to room temperature. Six μl of 4-vinylpyridine was then added and the solution reacted at room temperature for 25 min in the dark. The reaction mix was then diluted to 2.4 ml using 0.1% trifluoroacetic acid (to lower the pH) and analyzed by HPLC. For all HPLC protein reduction and alkylation reactions, a Vydac (218TP54) C18 reversed phase column was used (5-μm particle size, 4.6-mm inner diameter × 25-cm length). The solvent gradient used was 20% B until the base-line absorbance decreased to within 10% of its original value and then to 100% B over 40 min. Buffer A was 0.1% trifluoroacetic acid; buffer B was 0.1% trifluoroacetic acid, 60% acetonitrile. The flow rate was 1 ml/min, and detection was at 220 nm.

Enzymatic Digests

Pyroglutamate Aminopeptidase—The pyroglutamate at the NH_2 terminus was removed by adding purified α-chain of conodipine-M (66 μl in HPLC elution buffer, CH_3CN blown off with N_2; see Fig. 2) to 284 μl of buffer (50 mM Na_2HPO_4, 10 mM β-mercaptoethanol, 1 mM dithiothreitol, and 1 mM EDTA adjusted to pH 7.3 with H_3PO_4) containing 0.4 mg (16 μg of protein) of freshly dissolved pyroglutamate aminopeptidase preparation (Boehringer Mannheim). The reaction mix was incubated for 1 h at 37 °C. The cleaved protein was purified using a reversed phase C18 Microsorb MV column (Rainin Instruments, 86-203-C5). Elution buffer A was 0.1% trifluoroacetic acid; buffer B was 0.1% trifluoroacetic acid, 60% acetonitrile. A linear gradient of 20–100% B over 80 min was used to elute the peptide. The flow rate was 1 ml/min.

Endoproteinase Lys-C—α-Chain of conodipine-M was initially dissolved in 5 μl of CH_3CN followed by 40 μl of 0.1 M $NaHCO_3$, pH 8.5. To this was added 1.0 μl containing 0.1 μg of endoproteinase Lys-C (Boehringer Mannheim, 1047825) in 50 mM Tricine, pH 8.0, 10 mM EDTA. The reaction mix was incubated overnight at 37 °C. Digestion fragments were purified using a reversed phase C18 column (Vydac 218TP52). Elution buffer A was 0.1% trifluoroacetic acid; buffer B was 0.1% trifluoroacetic acid, 80% acetonitrile. A linear gradient of 5–95% B over 90 min was used to elute the peptides. The flow rate was 0.15 ml/min.

Endoproteinase Arg-C—α-Chain of conodipine-M was initially dissolved in 5 μl of CH_3CN. Endoproteinase Arg-C (Boehringer Mannheim 1370529), 0.5 μg in 95 μl of buffer (90 mM Tris-HCl, 8.5 mM $CaCl_2$, 5 mM dithiothreitol, 0.5 mM EDTA, pH 7.6), was added, and the reaction mixture was incubated for 18 h at 37 °C. Digestion fragments were purified as described above for endoproteinase Lys-C.

Sequence Analysis—Amino acid sequencing was performed with Edman chemistry on an Applied Biosystems 477A Protein Sequencer at the Protein/DNA Core Facility at the University of Utah Cancer Center.

Mass Spectrometry—Molecular masses of conodipine-M and its subunits were determined using a triple quadrupole mass spectrometer TSQ 700 (Finigan-Mat, San Jose, CA) equipped with an electrospray source.

Membrane Binding

Membrane Preparation—Neocortices were bluntly dissected from eight Sprague-Dawley rats (200–220 g, male) and placed in 150 ml of ice-cold 50 mM HEPES buffer, pH 7.4 using NaOH. The tissue was homogenized with a Brinkmann Polytron for 30 s using a PTA 20S probe. The homogenate was further diluted to 200 ml and centrifuged at 1,000 × *g* for 10 min at 4 °C. The supernatant was removed and centrifuged at 48,000 × *g* for 10 min. The supernatant was discarded, and the pellet was resuspended in a total of 200 ml of buffer and centrifuged at 48,000 × *g* for 10 min. The supernatant was discarded, the pellet was weighed, and 1 ml of buffer/125 mg of pellet was added. The tissue was suspended using a Potter glass-Teflon homogenizer using eight strokes at 800 rpm. Membranes were frozen in liquid nitrogen at −70 °C until use.

[^{3}H]Isradipine Binding—Neocortical membranes (50 μg as measured using the Bio-Rad microprotein assay) were incubated at 37 °C for 60 min in 2 ml of 50 mM Tris-HCl, pH 7.4, 1 mM $CaCl_2$, 1 mg/ml lysozyme (Sigma L-6876), with 100 pM [^{3}H]isradipine ((+)-[*methyl*-^{3}H]PN 200-110, 70–85 Ci/mmol, Amersham) in the presence or absence of venom fractions (typically 1/10,000 of the fraction during preparative phases of the purification, see Fig. 1). Venom fractions were routinely dried using a Speed Vac (Savant) in the presence of 50 μg of lysozyme to decrease nonspecific adsorption to the tubes. Nonspecific binding of [^{3}H]isradipine was determined using 100 nM isradipine. The reaction was stopped by filtering the samples through GF/C filters, followed by two 4-ml washes with ice-cold buffer. Samples were then analyzed using a scintillation counter. Fractions that showed 95–100% inhibition were subsequently tested at lower concentrations to determine the relative amount of activity present in all of the fractions. For later experiments with purified conodipine-M, the reaction volume was decreased to 500 μl, [^{3}H]isradipine was increased to 200 pM, membrane protein was increased to 500 μg, lysozyme was reduced to 0.1 mg/ml, and nonspecific binding was defined with 10 μM nifedipine (Sigma) (prepared initially as a 20 mM stock in ethanol). Samples were filtered and washed

for 24 s using a Classic cell harvester from Skatron Instruments (Sterling, VA) using Skatron 11734 filtermats. The latter protocol reduced the amount of venom material needed and allowed 12 samples to be filtered simultaneously.

Lipolytic Assays

For all lipolytic assays, stock solutions of conodipine-M were in water containing 1 mg/ml lysozyme (added during lyophilization). Spectrophotometric assays to detect PLA_2 activity were carried out with 1-*O*-hexadecyl-2-deoxy-2-*S*-thiohexadecanoyl-*sn*-glycero-3-phosphocholine as described previously (44). The reaction mixture contains 0.5 mM thiolester, 4 mM Triton X-100, 0.1 M KCl, 10 mM $CaCl_2$, 0.8 mM 5,5′-dithiobis(2-nitrobenzoic acid) (Aldrich), and 25 mM Tris, pH 8.0. The reaction mixture (0.35 ml) was placed in a thermostatted cuvette (25 °C), 0.1 μg of conodipine-M was added, and the absorbance at 412 nm was monitored.

Hydrolysis of small unilamellar DMPM vesicles was carried out using a pH-stat as described previously (43), and 4-ml reaction mixtures contained 0.6 mM $CaCl_2$, 1 mM NaCl, 0.6 mg of DMPM, and typically 0.3 μg of conodipine-M. For the analysis of inhibitors, assays were carried out under conditions that measure the initial velocity (51), and 4-ml reaction mixtures contained 2.5 mM $CaCl_2$, 1 mM NaCl, 20 μg of polymyxin B sulfate (Sigma), 0.6 mg of DMPM, and typically 0.1 μg of conodipine-M.

In some cases, radiometric assays of PLA_2 activity were carried out. Covesicles of nonlabeled DMPM vesicles containing radiolabeled phospholipids were prepared as described previously (45). The reaction mixtures were extracted with organic solvent, and the amounts of liberated radioactive fatty acids were analyzed as described previously (45). In this way the calcium dependence of PLA_2 activity of conodipine-M was measured using reaction mixtures with 0.3 mg of DMPM vesicles containing 120,000 cpm of [^{3}H]DPPC in 2 ml of 10 mM HEPES, 100 mM NaCl, 100 mM EGTA, pH 7.4. The mixtures also contained different amounts of 1 M K_2CaEGTA solution to give the desired concentrations of free calcium (52). Reactions were initiated by the addition of 0.04 μg of conodipine-M and were quenched with organic solvent after a 10-min incubation at 20 °C. Reactions for determining the pH dependence of conodipine-M-catalyzed vesicle hydrolysis were carried out as above except that the buffer was 10 mM HEPES, 100 mM NaCl, 1 mM $CaCl_2$, pH 6–9.

Competitive substrate specificity studies using the double radioisotope method were carried out as described (45). The reaction mixtures contained 0.6 mg of sonicated DMPM vesicles with 1×10^5–8×10^5 cpm of ^{3}H- and ^{14}C-labeled phospholipids in 4 ml of 0.6 mM $CaCl_2$, 1 mM NaCl, 20 μg of polymyxin B at 21 °C. Reactions were carried out in the pH-stat with 0.07 μg of conodipine-M (added last) and were quenched with organic solvent after 120–130 nmol of titrant was consumed. Reactions with DMPM vesicles containing [^{14}C]PAPC and [^{3}H]DPG were quenched and extracted with organic solvent as described above. The residue from the extract was dissolved in low boiling petroleum ether:ether:acetic acid (70:30:1), and nonlabeled arachidonic acid and 1,2-dioleoyl-*sn*-glycerol (Sigma) were added as markers. The mixture was applied to a silica gel TLC plate (4 × 12 cm), and the plate was developed with the same solvent. Regions corresponding to fatty acid and diacylglycerol were scraped from the plate, and the silica was soaked in scintillation fluid for 1 h followed by scintillation counting. Relative k_{cat}/K_m values for the radioactive substrates were calculated using Equation 4 of (45).

Studies to measure the positional specificity of the phospholipase activity of conodipine-M were carried out with reaction mixtures of 200 μg of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphomethanol in 4 ml of 2.5 mM $CaCl_2$, 1 mM NaCl, pH 8.0, containing 20 μg of polymyxin B at 21 °C in the pH-stat. After about 30% of the substrate was hydrolyzed (30 μl of 3 mM NaOH titrant), the reaction was quenched by the addition of 4 ml of 0.1 M EDTA, 2 M NaCl, pH 7.0, followed by 0.56 ml of 88% formic acid and 4 ml of ethyl acetate. After mixing on a vortex mixer, the layers were separated by centrifugation, and the organic layer was transferred to a new tube. The aqueous phase was extracted with a second portion of ethyl acetate, the extracts were combined, and the solvent was removed with a stream of nitrogen. To the residue were added 0.5 ml of ether and 50 μl of methanol, and sufficient diazomethane in ether was added until the yellow color persisted. After 30 min at room temperature, the solvent was removed with a stream of nitrogen. The residue was taken up in ether, and a portion was analyzed by gas chromatography on a DB-5 column (J & W Scientific). Peaks corresponding to methyl oleate and methyl palmitate were integrated, and the peak areas obtained from a control reaction (omission of conodipine-M) were subtracted from those obtained in the presence of enzyme.

RESULTS

Purification of Conodipine-M—Conodipine-M was originally identified in a general screen of *Conus* venoms for new calcium channel blockers. A number of crude cone venoms were examined for their ability to inhibit the binding of [^{3}H]isradipine (a ligand specific for L-type calcium channels) (53, 54) to rat neocortical membranes. Several *Conus* venoms were found to inhibit [^{3}H]isradipine binding. The inhibition of this binding by *C. magus* venom was found to be exceptionally potent (55) and was therefore targeted for purification.

Crude venom from *C. magus* was extracted and initially chromatographed using a preparative HPLC column (see Fig. 1*A*). Fifteen fractions were collected and tested, and fraction 8 eluting at 34.3 min was found to inhibit [^{3}H]isradipine binding to rat brain membranes. This fraction was rechromatographed using a semipreparative column and a shallower gradient. The eluant resolved into two major broad peaks, as well as a number of minor components (see Fig. 1*B*). Activity (as measured by the isradipine binding assay) was associated with the earlier eluting major peak in Fig. 1*B*.

Further purification was carried out using an analytical HPLC column (4.6-mm inner diameter × 25-cm length). Resolution was difficult because of the presence of at least three closely migrating components (Fig. 1*C*). The trifluoroacetic acid/acetonitrile buffer system used in the initial steps of purification did not fully resolve these components (data not shown). The best resolution was obtained with a very slow gradient of trifluoroacetic acid/methanol buffer (see Fig. 1*C* for buffer conditions) using a reversed phase C18 Microsorb MV column. Using these conditions, a center cut of the middle peak reliably gave a homogeneous appearing peak on rechromatography (see Fig. 1*D*). Although the material in Fig. 1*C* did not appear chromatographically homogeneous, the amount of [^{3}H]isradipine binding activity was proportional to the protein absorbance across all peaks, suggesting that the active material might be present as multiple isoforms. The major component was purified (see Fig. 1*D*) and was the material that was further characterized below, which we have called conodipine-M.

Biochemical Characterization of Conodipine-M—Preliminary analysis of conodipine-M suggested that the purified material was not a typical *Conus* peptide of 10–30 amino acids in length. Chromatography on reversed phase HPLC columns gave a late eluting, broad peak, much more characteristic of larger polypeptides than of small peptidic conotoxins characterized previously. When characterized by mass spectrometry, conodipine-M was found to have a molecular mass of 13,602, indicating a large polypeptide. Conodipine-M was reduced and rechromatographed on HPLC. Two protein peaks were detected and when analyzed by mass spectrometry, gave values of M_r = 8,571 and 5,036 (see Fig. 2). These results suggest two polypeptide chains, joined together by interchain disulfide bonding. As detailed below, amino acid sequence analysis of reduced and alkylated conodipine-M verified the presence of two polypeptide chains.

Initial attempts at amino acid sequencing revealed that the NH_2 terminus of the α-chain is blocked. The α-chain was reduced, alkylated (pyridylethylated), and purified by reversed phase HPLC. The alkylated α-chain was unblocked by digestion with pyroglutamate aminopeptidase, repurified, and sequenced successfully through the first 34 residues. To analyze the remainder of the chain, digests with endoproteinase Lys-C (cleaves peptide bonds at the COOH-terminal side of Lys residues) and endoproteinase Arg-C (cleaves peptide bonds COOH-terminally at Arg residues) were performed. Fragments were isolated by reversed phase HPLC and sequenced. The β-chain of conodipine-M was reduced, pyridylethylated, and sequenced.

TABLE I
Sequence analysis of conodipine-M

The sequences of the α- and β-chains of conodipine-M are shown. Enzymatic cleavage products are indicated. The β-chain of conodipine-M is compared with the β-chain of β-bungarotoxin (29). The first 34 amino acids of the α-chain of conodipine-M were analyzed after digestion with pyroglutamate aminopeptidase to unblock the NH_2 terminus. Endoproteinase Lys-C and Arg-C digestion fragments were analyzed to determine the remainder of the α-chain sequence. *X* = uncertain amino acid. Underlined residue = enzymatic cleavage site.

TABLE II
PLA_2 sequence comparisons

Sequences for porcine pancreas PLA_2 (28, 29), the α-chain of the two-subunit β-bungarotoxin (29), the viper *Bitis gabonica* PLA_2 (27), the cloned sequence for bee venom PLA_2 (30), and the α-chain of conodipine-M are compared (see also Table IV). Note that conodipine-M lacks the Tyr/Trp-Cys-Gly-Xaa-Gly (underlined) found in all other secreted PLA_2s. This sequence is postulated to be a calcium binding loop critical to catalytic activity. Also note the significantly smaller size of conodipine-M. The table was modified from Kondo *et al.* (29). Standard one-letter amino acid abbreviations are used. *X* = uncertain amino acid. Z = pyroglutamate.

	1234567890123456789012345678901234567890
Porcine (I)	ALWQFRSMIKCAIPGSHPLMDFNNYGCYCGLGGSGTPVDE
β-BTX (I)	NLINFMEMIRYTIPCEKTWGEYADYGCYCGAGGSGRPIDA
B. gabonica (II)	DLTQFGNMIN——KMGESVFDYIYYGCYCGWGGKGKPIDA
Bee (III)	IIYPGTLWCGHGNKSSGPNELGRFKHTDACCRTHDMCPDV
Conodipine-M (V)	ZXPSTAELCKINSNACSVPFSXIPCQKXFLAACDRHDTCY
Porcine	LDRCCETHDNCYRDAKNLDSCKFLVDNPYTESYSYSCSNT
β-BTX	LDRCCYVHDNCYGDAEKKHKCNPKTSQ—YS——YKL—TKR
B. gabonica	TDRCCFVHDCCYGKMGTYDT—KWTSYN———YEI—QNG
Bee	MSAGESKHGLTNTASHTRLSCDCDDKFYDCLKNSADTISS
Conodipine-M	HCGKHFGFKQDDCDDAFFRDMTALCAHGTDDEGXCPX
Porcine	EITCNSKNNA—CEAFICNCDRNAAICFSKAPYNKEHKNLD
β-BTX	TIICYGAAGGTC—RIVCDCDRTAALCFGQSDYIEEHKNID
B. gabonica	GIDCDEDPQK——KEL—CECDRVAAICFANNRNTYNSNYFG
Bee	YFVGKMYFNLIDTKCYKLEHPVTGCGERTEGRCLHYTVDK
Porcine	TKKY—C
β-BTX	TARF—CQ
B. gabonica	HSSSKCTGTEQC
Bee	SKPKVYQWFDLRKY

Results of the sequence analysis are summarized in Table I. It is noteworthy that the sequence of the α-chain of conodipine-M is considerably shorter than the α-chain of other two-subunit PLA_2s (*e.g.* compare β-bungarotoxin in Table II). Mass spectrometry measurements of conodipine-M, however, confirmed the size of the indicated sequences of the α- and β-chains (see below).

In the conodipine-M sequences, a few residues could not positively be assigned (indicated by *X* in the tables). In several cases, these residues appear as blanks in the sequencing run. These blanks appeared in multiple sequencer runs, always in the same position. Peptides from *Conus* contain a number of unusual amino acids and post-translational modifications which appear as blanks during sequencing (9). It is likely that the unassigned residues in the middle portions of the sequence represent such modifications (1 or 2 residues at the COOH terminus could not be assigned with certainty because of a diminishing signal). The size of any post-translational modification is small, however. By comparing the observed mass (measured by mass spectrometry) with the calculated mass of the sequence shown in Table I, the average mass for the unassigned residues in the α-chain is 132.2 ((8,571 − 7,910)/5) and in the β-chain 137.0 ((5,036 − 4,625)/3).

Although conodipine-M potently inhibits the binding of isradipine to L-type calcium channels, several lines of evidence suggest that this might not be the result of direct competition. As shown in Fig. 3 the inhibition of isradipine binding depends on the time of preincubation of conodipine-M with cortical membranes. These results raise the possibility that the inhibition may be indirect.

The larger size of conodipine-M and the time course of isradipine binding led us to investigate whether conodipine-M had any enzymatic activity. Since many venoms have been reported to contain PLA_2s, we tested conodipine-M for its ability to hydrolyze phospholipids. As shown in the following section, conodipine-M is indeed a PLA_2.

The sequence of conodipine-M can be compared to other known PLA_2s. The sequence of the α-chain of conodipine-M and group I–III PLA_2s is shown in Table II. Although conodipine-M shows little homology to any known PLA_2, certain catalytic residues may be similar (see Tables II, IV and "Discussion").

Conodipine-M Has PLA_2 Activity—Initial studies of the PLA_2 activity of conodipine-M were carried out with a spectrophotometric assay using the *sn*-2 thiolester phospholipid analog 1-*O*-hexadecyl-2-deoxy-2-*S*-thiohexadecanoyl-*sn*-glycero-3-phosphocholine dispersed in Triton X-100 micelles. Hydrolysis

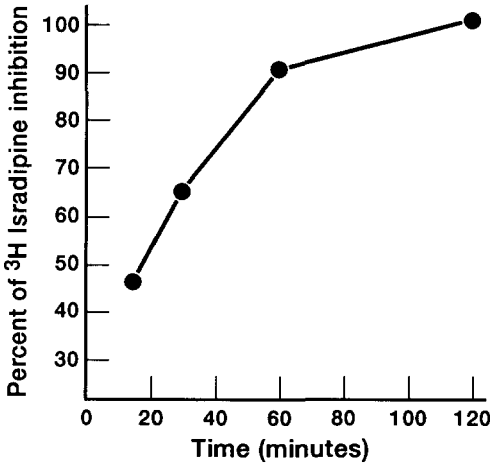

FIG. 3. **Time dependence of conodipine-M inhibition of [^{3}H]isradipine binding.** Cortical rat brain membrane was incubated in the presence or absence of 2 nM conodipine-M at 37 °C for the indicated lengths of time. Specific binding was defined as the difference between [^{3}H]isradipine counts bound in the absence (total binding) and presence (nonspecific binding) of 10 μM nifedipine (see "Experimental Procedures"). Data were acquired in duplicate and the results averaged.

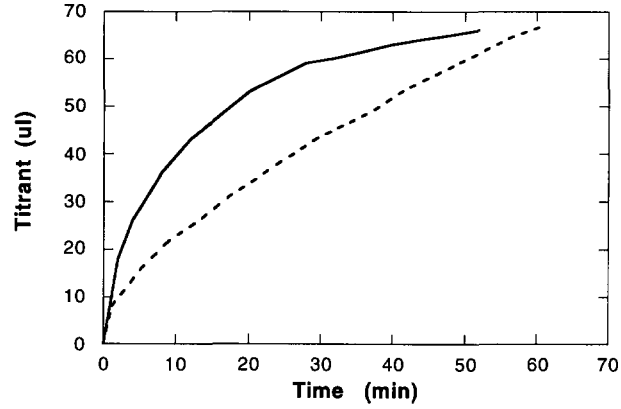

FIG. 4. **Reaction progress curves for the hydrolysis of small unilamellar vesicles by 0.3 μg of porcine pancreatic PLA_2 (*solid line*) and 0.3 μg of conodipine-M (*dashed line*).** Other conditions are given under "Experimental Procedures."

of the *sn*-2 thiolester generates an SH group that is detected with Ellman's reagent. Using the conditions given under "Experimental Procedures," conodipine-M displays significant PLA_2 activity; the specific activity was 22 mmol/min/mg for conodipine-M *versus* 4 mmol/min/mg for bee venom PLA_2 measured under identical conditions.

Vesicles of the anionic phospholipid DMPM have been used extensively to detect and characterize the kinetics of hydrolysis of the *sn*-2 ester of phospholipids by PLA_2s (43, 56, 57), and a brief overview of this system is given here as it relates to the action of conodipine-M on DMPM vesicles. Secreted PLA_2s (molecular masses of 14 kDa) bind irreversibly to DMPM vesicles and hydrolyze all of the substrate in the outer layer in a processive fashion termed scooting mode hydrolysis (enzyme does not leave the surface). The reaction progress stops when all of the outer layer substrate in enzyme-containing vesicles has been hydrolyzed. At this point the reaction products remain in enzyme-containing vesicles, and the vesicles remain structurally intact. As shown in Fig. 4, the reaction ceases after a limited amount of total DMPM is hydrolyzed by porcine pancreatic PLA_2. This is because the inner layer DMPM is not hydrolyzed, and there are more vesicles than enzyme, and the enzyme does not hop from one vesicle to another. Thus, the total mol of product formed is equal to the mol of catalytically active enzyme times the mol of substrate in the outer layer of a DMPM vesicle (58).

As shown in Fig. 4, the addition of conodipine-M to DMPM vesicles leads to the immediate onset of lipolysis. With conodipine-M, the hydrolysis of DMPM vesicles does not stop abruptly, but a steady-state velocity is seen later in the reaction progress (Fig. 4). This continued reaction could be caused either by slow dissociation of conodipine-M from vesicles followed by rebinding to different vesicles (slow hopping) and/or by conodipine-M-promoted fusion of vesicles; such fusion would bring substrate into substrate-depleted, enzyme-containing vesicles. In any case, the fact that both conodipine-M- and porcine pancreatic-catalyzed reactions produce similar amounts of products before the reaction ceases (or slows) indicates that a large fraction, perhaps all, of the conodipine-M mass has lipolytic activity, and thus this activity is not due to a minor contaminant.

The addition of the cationic peptide polymyxin B leads to rapid intervesicle exchange of DMPM, and this replenishes the substrate in enzyme-containing vesicles. As a result, the initial reaction velocity (when the mol fraction of substrate in the vesicle is close to 1) is prolonged, and the progress curves are linear for several min (51). Under such conditions conodipine-M displays a specific activity of 200 s^{-1}, which is similar to that of porcine pancreatic PLA_2 (320 s^{-1}) (59). Further support that conodipine-M has lipolytic activity comes from an additional experiment. Conodipine-M was chromatographed on an HPLC column. The lipolytic activity of each fraction was measured using the DMPM/polymyxin B assay, and as shown in Fig. 5, the profile of lipolytic activity exactly matches the peak of protein eluting from the column. Further indication of the purity of conodipine-M is evident from HPLC analysis of reduced and alkylated material. As shown in Fig. 2*C*, when alkylated conodipine-M (43.5 μg) is chromatographed, two sharp protein absorbances are seen (A and B chains). Contaminant peaks (if any) represent no more than 0.1% of the total protein absorbance at 220 nM.

Vesicles of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphomethanol were used to test the positional specificity of the esterase activity of conodipine-M. Such vesicles were readily hydrolyzed, and gas chromatographic analysis of the extracted fatty acid fraction shows that the major product is oleic acid (<5% of the fatty acids formed is palmitic acid). Thus, conodipine-M has little, if any, PLA_1 activity.

Conodipine-M Is a Calcium-dependent PLA_2—When $CaCl_2$ in the reaction buffer was replaced by 1 mM EDTA, no hydrolysis of DMPM vesicles by conodipine-M was detected. The activity measured in the presence of 0.1 mM or 2.5 mM $CaCl_2$ is the same within experimental error. To measure the dependence of the reaction velocity on concentrations of calcium below 0.1 mM, a radiometric PLA_2 assay was utilized with buffered calcium and pH to establish the precise concentration of free calcium (see "Experimental Procedures"). In these studies the hydrolysis of [^{3}H]DPPC present in DMPM vesicles is measured. The results in Fig. 6 show that the reaction velocity increases over the range 0–80 μM calcium and gradually decreases as the calcium concentration approaches 300 μM. The pH-rate profile of the PLA_2 activity of conodipine-M was measured under the same conditions, and the rate increased as the pH was increased from 6.0 to 7.5 and then remained constant up to pH 9.0 (not shown).

Substrate Specificity of Conodipine-M—Substrate preferences of conodipine-M for different phospholipids were analyzed using the double radioisotope method (45). In this analysis DMPM vesicles containing small amounts of ^{3}H- and ^{14}C-labeled substrates are used, and the substrates compete with

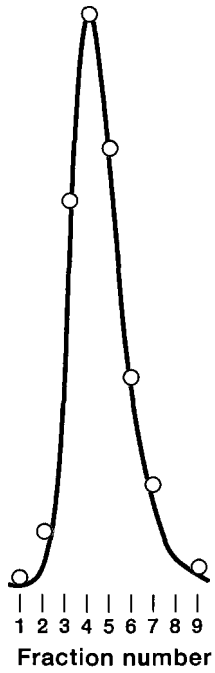

FIG. 5. **Conodipine-M (23 μg) was run on a HPLC column using conditions described in *panel C* of Fig. 1.** Fractions were collected every 30 s as indicated. Absorbance was monitored at 220 nM (*solid line*). One percent of each fraction was tested for PLA_2 activity using the DMPM/polymyxin B assay described under "Experimental Procedures." Activity (nmol of NaOH titrant used per min in the pH-stat) is normalized such that the maximum activity (10.6 nmol/min) is plotted at the same ordinate value as the UV absorbance. Note that the PLA_2 activity profile (*open circles*) also corresponds to the protein absorbance profile.

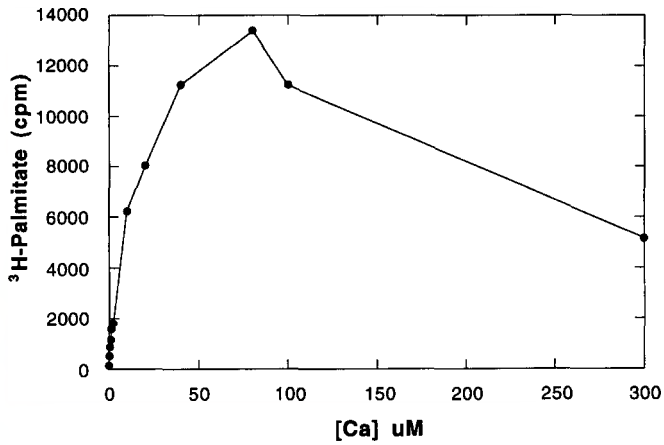

FIG. 6. **Calcium dependence of the conodipine-M-catalyzed hydrolysis of [^{3}H]DPPC in DMPM vesicles.** Conditions are given under "Experimental Procedures."

each other for hydrolysis by the enzyme. Substrate specificity studies using vesicles containing single species of phospholipids are less meaningful because the observed preferences will be due, in part, to differential amounts of enzyme bound to the different vesicles (60). The competitive double radiolabel analysis gives the intrinsic substrate specificities of the enzyme which are defined by the relative k_{cat}/K_m values for the various substrates. Thus, if the vesicle contains an equal concentration of two competing substrates, the ratio of velocities for hydrolysis of the different phospholipids is equal to the ratio of k_{cat}/K_m values for the same phospholipids. Relative k_{cat}/K_m values for the action of conodipine-M on vesicles are given in Table III. The general feature is that conodipine-M has broad specificity toward different phospholipids. The enzyme does not

TABLE III
Substrate specificity of conodipine-M

Radiolabeled substrates are present in DMPM vesicles. See "Experimental Procedures" for additional details.

Radiolabeled substrates	$(k_{cat}/K_m)^3/(k_{cat}/K_m)^{14}$
[^{3}H]DPPC *vs.* [^{14}C]SAPC	1.04
[^{3}H]DPPC *vs.* [^{14}C]SAPI	0.71
[^{3}H]DPPE *vs.* [^{14}C]SAPC	0.20
[^{3}H]DPPA *vs.* [^{14}C]SAPC	0.13
[^{3}H]SAPS *vs.* [^{14}C]SAPC	0.33

discriminate between *sn*-2 palmitoyl *versus sn*-2 arachidonyl chains, and only modest discriminations (up to 8-fold) are seen for phospholipids with various polar head groups. When conodipine-M is added to DMPM vesicles containing [^{3}H]DPG and [^{14}C]SAPC, only the phospholipid is hydrolyzed. Thus, conodipine-M has no diacylglycerol lipase activity.

Additional phospholipids were tested as possible substrates of conodipine-M. The reactions were carried out in 4 ml of 0.6 mM $CaCl_2$, 1 mM NaCl, pH 8.0, containing 0.6 mg of phospholipid, 20 μg of polymyxin B, and 0.07 μg of conodipine-M at 21 °C in the pH-stat. The enzyme readily hydrolyzes vesicles of 1,2-dioleoyl-*sn*-glycero-3-phosphomethanol, but vesicles of the D-stereoisomer were not detectably hydrolyzed. Thus, conodipine-M, like other PLA_2s, is stereospecific for phospholipids with the natural stereochemistry at the glycerol backbone. Under the same conditions the lysophospholipid 1-myristoyl-*sn*-glycero-3-phosphocholine is not a substrate. Conodipine-M hydrolyzes vesicles of the zwitterionic phospholipid 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, although the turnover number of 10 s^{-1} is considerably less than 200 s^{-1} measured with DMPM vesicles. Since fully vesicle-bound conodipine-M does not significantly discriminate between phospholipids with different polar head groups, the low turnover rate on zwitterionic vesicles may be due to poor binding of enzyme to the interface as is the case for many 14-kDa PLA_2s (45, 56, 61), but this was not investigated further.

Conodipine-M has no triacylglycerol lipase activity. Adding 0.15 μg of conodipine-M to 7 mM tributyrin in 4 ml of 0.6 mM $CaCl_2$, 1 mM NaCl, 20 μg of polymyxin B, pH 8.0, at 21 °C in the pH-stat did not result in detectable hydrolysis, whereas adding 0.25 μg of pancreatic lipase gave rise to an immediate reaction. Conodipine-M also failed to hydrolyze 0.3 mM triolein in a reaction mixture containing 20 mM deoxycholate, 0.6 mM $CaCl_2$, 1 mM NaCl, pH 8.0, at 21 °C in the pH-stat. Under such conditions, hydrolysis was detected after adding pancreatic lipase.

Conodipine-M did not detectably hydrolyze [^{3}H]DPG present in DMPM vesicles, although [^{14}C]PAPC present in the same vesicles was hydrolyzed. Thus, conodipine-M has no diglyceride lipase activity.

Inhibitors of Conodipine-M—As shown in Fig. 7 conodipine-M is inhibited by two compounds, linoleoyl amide and MG14, which have been previously shown to inhibit 14-kDa PLA_2s (47–49). Inhibition studies were carried out by adding inhibitors to DMPM vesicles and measuring the initial velocity for the hydrolytic reaction. Since the enzyme is tightly bound to DMPM vesicles, the inhibition must be due to a direct enzyme/inhibitor interaction rather than to a decrease in the fraction of enzyme bound to the membrane (62, 63). The mol fractions of linoleoyl amide and MG14 in DMPM vesicles which cause a 50% decrease in the initial velocity are 0.001 and 0.003, respectively, and thus these compounds are potent conodipine-M inhibitors.

DISCUSSION

The results demonstrate that conodipine-M is a novel PLA_2; this is the first enzymatic activity extensively characterized

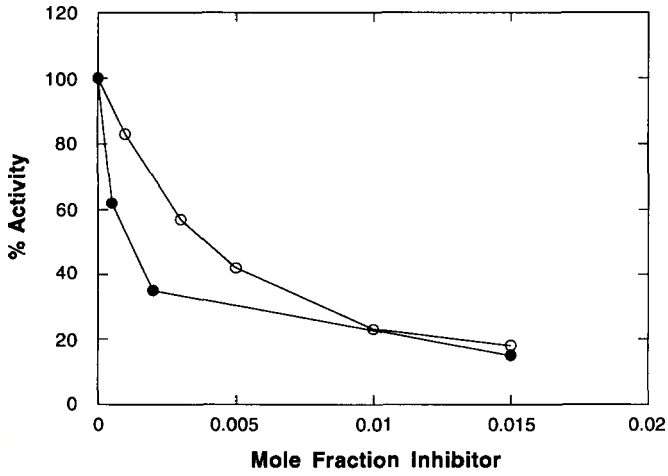

FIG. 7. **Inhibition of conodipine-M-catalyzed hydrolysis of DMPM vesicles by linoleoyl amide (*filled circles*) and MG14 (*open circles*) present in DMPM vesicles at the indicated mol fraction.** Other conditions are given under "Experimental Procedures."

from cone snail venom. We have named this enzyme conodipine-M and anticipate that this is only the first of a family of PLA_2 enzymes present in these venoms. In another *Conus* venom, *Conus caracteristicus*, a related enzyme has been partially characterized.[2] Thus, we propose that other PLA_2 enzymes belonging to this family should also be referred to as conodipines (the major enzyme from *C. caracteristicus* would be referred to as conodipine-C; sequence variants of conodipine-M from *C. magus* would be referred to as conodipine-M1, conodipine-M2, etc.).

Conodipine-M was identified by an unusual route; the assay actually used to purify this PLA_2 activity was not enzymatic activity but rather the inhibition of binding of a dihydropyridine drug to L-type calcium channels in rat brain membranes. We have demonstrated that purified conodipine-M potently inhibits [^{3}H]isradipine binding under standard assay conditions. There are two general possibilities. (i) The inhibition may be relatively nonspecific, *i.e.* PLA_2 may be solubilizing the L-type calcium channel; if binding sites can no longer be filtered, this would appear as inhibition. (ii) An alternative explanation is that the PLA_2 produces a metabolite (perhaps derived from arachidonic acid) which inhibits binding of isradipine to L-type calcium channels. These possibilities are presently being investigated. It is notable, however, that a 10-fold greater concentration of conodipine-M than is required to inhibit completely the binding of [^{3}H]isradipine to L-type calcium channels exhibits no detectable effect on the binding of ^{125}I-labeled ω-conotoxin GVIA to N-type calcium channels, even though both assays employ filtration of membranes (55).

Conodipine-M has two distinct polypeptide chains that are apparently linked to each other through one or more disulfide bonds; upon reduction of disulfides, conodipine-M is dissociated in two unequally sized subunits. A similar situation is found with several snake venom PLA_2s that have multiple subunits. In some cases, at least one of the subunits appears to be used for targeting the PLA_2 to a particular membrane component (*e.g.* the β-chain of bungarotoxin interacts with potassium channels) (64). It is possible that one of the two subunits of conodipine-M is responsible for the catalytic activity, with the other subunit involved in targeting the enzyme to specific sites. The physiological function of PLA_2 within *C. magus* venom remains a matter for conjecture, but it is of interest that this activity is apparently present in other *Conus* venoms, which specialize on different prey.

[2] S. R. Naisbitt and J. M. McIntosh, unpublished results.

TABLE IV
Sequence comparison of catalytic residues

Sequences containing catalytic residues in group I–III PLA_2s are compared with potentially similar sequences in conodipine-M. Proposed group numbers are shown in parentheses. BTX, bungarotoxin.

Enzyme	Sequence	Residue no.
Porcine pancreas (I)	R C C E T **H D** N C Y	43–52
β-BTX (I)	R C C Y V **H D** N C Y	39–48
B. gabonica (II)	R C C F V **H D** C C Y	37–46
Bee venom (III)	A C C R T **H D** M C P	29–38
Consensus	X C C X X **H D** X C X	
Conodipine-M (V)	A A C D R **H D** T C Y	31–40

Enzyme	Sequence	Residue no.
Porcine pancreas (I)	C N C **D** R N A A I C F	96–106
β-BTX (I)	C D C **D** R T A A L C F	84–94
B. gabonica (II)	C E C **D** R V A A I C F	78–88
Bee venom (III)	C D C **D** D K F Y D C L	61–71
Consensus	C X C **D** X X X X C X	
Conodipine-M (V)	D D C **D** D A F F R D M	51–61

More than 50 PLA_2s from venoms have been sequenced to date, and three sequence groups are apparent (32). Group I is composed of PLA_2s from elapid and Old World snakes. Members of group II include crotalids and viperids of the New World. Group I and group II sequences are highly homologous to each other. The major differences lie in the positioning of the one of the seven disulfides, and group II enzymes have a short COOH-terminal extension. Group III PLA_2s, which include the enzymes from bee and lizard venoms, are only about 20% homologous to group I and II enzyme (41). A novel PLA_2, group IV, has recently been purified from the cytosol of a number of proinflammatory mammalian cells. It has a molecular weight of 85,000, and its amino acid sequence shows no homology to group I–III enzymes (21, 65, 66).

The amino acid sequence of conodipine-M does not reveal any obvious homology with group I–IV PLA_2s. However, some similarity may exist in sequences including and near amino acid residues that are known to function in the active site. In group I–III PLA_2s a histidine residue, which functions as a general base in assisting the attack of a water molecule on the substrate ester, is followed by an aspartate that is one of the ligands for the active-site calcium cofactor. This pair of residues is flanked by 3 cysteines (Tables II and IV). The α-chain of conodipine-M has a histidine-aspartate pair flanked by 2 cysteines instead of three, but it is not known whether these residues function in a way analogous to those in group I–III PLA_2s. Group I–III PLA_2s have an aspartate that is hydrogen-bonded to the catalytic histidine residue and is flanked by 3 cysteines (Tables II and IV). The α-chain of conodipine-M has an aspartate residue with only 1 nearby cysteine (Tables II and IV). The sequence Tyr/Trp-Cys-Gly-Xaa-Gly is found in all group I–III PLA_2s. The carbonyl oxygens of the 2 glycines of this sequence are ligands to the calcium cofactor. Conodipine-M lacks such a sequence.

Like the other enzymes, conodipine-M requires calcium for its PLA_2 activity, although the concentration of calcium that gives 50% of maximal activity (about 20 μM) is considerably lower than typical values for group I–III enzymes (a few hundred μM). It is thus significant that conodipine-M lacks the otherwise conserved calcium binding residues. All sequence factors considered, conodipine-M clearly represents a very structurally diverse enzyme, and we therefore propose to classify it as a group V PLA_2.

Given the low homology to other PLA_2s, it is remarkable that conodipine-M displays many other features of venom PLA_2s as well. The phospholipid analog MG14 is a transition state analog inhibitor of group I–III enzymes, and the fact that it is also a potent inhibitor of conodipine-M suggests that the catalytic mechanism of the *Conus* enzyme may be similar to the other venom enzymes. Also, the pH-rate profile for conodipine-M is similar to those for group I–III enzymes. For these latter enzymes the pH dependence reflects, in part, that the active-site histidine is in the nonprotonated form to function as a general base in the catalytic reaction (67). Conodipine-M, like other venom PLA_2s, does not significantly discriminate between phospholipids with different polar head groups or unsaturated *versus* saturated acyl chains. Further structural studies of conodipine-M will be required to understand fully its catalytic mechanism and to compare it with other known PLA_2s. It is not known which conodipine-M subunit contains the phospholipase activity, but as discussed above, the α-chain may have similar catalytic sequence motifs. The amino acids that we have postulated as key catalytic residues need to be confirmed experimentally. Our speculation clearly predicts, however, that catalytic activity will be carried out primarily by the α-chain.

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