The Calcium-dependent Association and Functional Coupling of Calmodulin with Myocardial Phospholipase A2

IMPLICATIONS FOR CARDIAC CYCLE-DEPENDENT ALTERATIONS IN PHOSPHOLIPOLYSIS*

(Received for publication, May 1, 1996, and in revised form, June 13, 1996)

Matthew J. Wolf and Richard W. Grosst
From the Division of Bioorganic Chemistry and Molecular Pharmacology, Departments of Medicine, Chemistry, Pharmacology, and Molecular Biology, Washington University School of Medicine, St. Louis, Missouri 63110

Herein we demonstrate the calcium-dependent regulation of myocardial phospholipase A2 activity, which is mediated by a cytosolic protein constituent that can be chromatographically resolved from, and subsequently reconstituted with, purified myocardial phospholipase A2. Purification of this protein by sequential chromatographies revealed an 18-kDa doublet, which was identified as calmodulin by Western blotting, calcium-dependent precipitation with W-7 agarose beads, and reconstitution of calcium-mediated phospholipase A2 inhibition with authentic homogeneous calmodulin. Calcium-induced calmodulin-mediated inhibition of myocardial phospholipase A2 was titrated by physiologic increments of calcium ion (Kd = 200 nm). Moreover, ternary complex affinity chromatography with calmodulin-Sepharose demonstrated that inhibition of myocardial phospholipase A2 activity by calmodulin resulted from the direct interaction of calmodulin with the myocardial phospholipase A2 catalytic complex. Exposure of cultured A-10 muscle cells to three structurally disparate calmodulin antagonists (W-7, trifluoperazine, and calmidazolium) resulted in the robust release of arachidonic acid, which was entirely ablated by pretreatment of cells with (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2-H-tetrahydropyran-2-one. Collectively, this study identifies a novel mechanism whereby latent phospholipase A2 activity can be released from tonic inhibition by alterations in the interactions between the phospholipase A2 catalytic complex, calcium ion, and the intracellular calcium transducer, calmodulin.

In myocardium the majority of phospholipase A2 activity is catalyzed by a calcium-independent phospholipase A2, which is selective for plasmanalogen substrate containing arachidonic acid (1–3). Since this enzyme neither requires calcium as an obligatory cofactor in catalysis nor employs calcium for membrane association, it has traditionally been assumed that calcium ion does not directly regulate the activity of this enzyme (1–6). However, alterations in calcium homeostasis play prominent roles in cardiac physiology, the predominant phospholipid constituents in the electrically active membrane of myocytes are plasmalogens containing arachidonic acid (7, 8), and both reaction products of phospholipase A2 catalysis are potent modulators of ion channel function (9–12). Since prior results demonstrated that calcium ion could inhibit the activity of crude (i.e. cytosolic) myocardial phospholipase A2 activity (1), we sought to identify a pathway that could integrate alterations in myocardial phospholipase A2 activity with changes in myocytic calcium homeostasis and electrophysiologic function. Herein we describe a novel mechanism through which calcium ion regulates nominally “calcium-independent” myocardial phospholipase A2 through its physical association with, and functional coupling to, the intracellular calcium transducer, calmodulin. We now report that myocardial phospholipase A2 specifically and tightly binds to calmodulin in a calcium-dependent fashion, that this interaction is titrated over physiologic increments of calcium ion, that the interaction between calmodulin and the phospholipase A2 catalytic complex regulates phospholipase A2 activity in vitro, and that pharmacologic ablation of this interaction by three structurally disparate calmodulin antagonists results in the release of arachidonic acid by nominally “calcium-independent” phospholipase A2 in intact muscle cells.

EXPERIMENTAL PROCEDURES

Materials—The preparation of rabbit myocardial cytosolic calcium-independent phospholipase A2 and the synthesis of 1-(O-(2-hexadecyl-1-ethyl-2-[9,10-3H]octadec-9-enyl)-2-[9,10-3H]octadec-9-enyl-2-[9,10-3H]octadec-9-enyl-sn-glycero-3-phosphocholine and (E)- (bromomethylene)-3-(1-naphthalenyl)-2-H-tetrahydropryan-2-one (BEL) were performed as described previously (13, 14). Recombinant 85-kDa calcium-dependent phospholipase A2 was purified from a baculovirus expression system as described previously (15). A-10 muscle cells (ATTC CRL 1476) were cultured and labeled according to established methods (16).

Purification of the Calcium-dependent Inhibitor of Myocardial Phospholipase A2—Ventricular myocardium from New Zealand White rabbits (300–400 g) were homogenized in ice-cold buffer (250 mM sucrose, 10 mM potassium phosphate, 1.0 mM KCl, 5 mM EDTA, pH 7.5) and homogenized using a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 20,000 g for 20 min, and the supernatant was saved (90°C for 3 min) and rapidly cooled, and precipitated protein was pelleted by centrifugation (20,000 g for 20 min). The resultant supernatant was heated treated (90°C for 3 min) and cooled, and precipitated protein was pelleted by centrifugation (20,000 g for 20 min). This supernatant was then adsorbed protein was eluted by a linear gradient of NaCl (100 mM–1 M NaCl). The calcium-dependent inhibitor was concentrated (Amicon Centripullus-10) and diluted 3-fold with buffer A, and 500 μg of protein was loaded onto a PC 1.65 Mono-Q column (Pharmacia Biotech Inc.) prior to elution with a linear 1 M NaCl gradient in buffer A.

Enzyme Assays—Calcium-dependent inhibition of myocardial phospholipase A2 was assessed by incubating partially purified phospholipase A2 (150 μg) with column fractions (25 μl) or bovine brain calmodulin in a final volume of 200 μl in 100 mM Tris-HCl, pH 7.0, containing either 10 mM CaCl2 or 4 mM EGTA for 2 min at 25°C. Phospholipase A2 activity was quantified by the release of [1H]oleic acid from 1-O-(Z)-hexadec-1-yl-2-[9,10-3H]octadec-9-enyl-sn-glycero-3-phosphocholine (plasmanolylcholine) (2 μM) as described previously (2).

The abbreviations used are: BEL, (E)-6-(bromomethylene)-3-(1-naphthalenyl)-2-H-tetrahydropryan-2-one; PAGE, polyacrylamide gel electrophoresis.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Division of Bioorganic Chemistry and Molecular Pharmacology, Washington University School of Medicine, 660 South Euclid, Box 8020, St. Louis, MO 63110. Tel.: 314-362-2690; Fax: 314-362-1402.

‡ To whom correspondence should be addressed: Division of Bioorganic Chemistry and Molecular Pharmacology, Departments of Medicine, Chemistry, Pharmacology, and Molecular Biology, Washington University School of Medicine, St. Louis, Missouri 63110.

© 1996 by The American Society for Biochemistry and Molecular Biology, Inc.
indicated free calcium ion concentrations employed in the assays were prepared using buffered CaCl2/EGTA solutions (17).

Calmodulin-Sepharose and W-7 Agarose Chromatography—Protein samples (500 μM CaCl2 final concentration) were loaded onto calmodulin-Sepharose columns pre-equilibrated with 50 mM Tris-HCl (pH 7.0) (buffer B) containing 500 μM CaCl2. After application of 10 column volumes of equilibration buffer, columns were washed with buffer B containing 4 mM EGTA. For experiments involving W-7 agarose, 75 μl of Mono-Q-purified calcium-dependent inhibitor was adjusted to either 1 mM CaCl2 or 4 mM EGTA prior the addition of W-7 agarose equilibrated in 20 mM imidazole, pH 7.5. After a 60-min incubation at 4°C, the W-7 agarose was pelleted by centrifugation, and the pellet was washed (3 times) with 20 mM imidazole, pH 7.5, containing either 1 mM CaCl2 or 4 mM EGTA prior to resuspension.

RESULTS AND DISCUSSION

Previously, we have demonstrated that calcium ion inhibits over 80% of crude myocardial cytosolic phospholipase A2 activity (1) and have repeatedly observed this effect in multiple independent preparations (x = −96 pmol/mg min in the presence of EGTA and −18 pmol/mg min in the presence of 10 mM calcium ion). During the course of investigating this phenomenon, we observed that highly purified preparations of myocardial phospholipase A2 did not manifest the calcium-mediated inhibition of enzymic activity, which was present in the crude cytosolic fraction (Fig. 1). The calcium-mediated inhibition of purified phospholipase A2 activity could be reconstituted by the addition of heat-treated cytosol (which did not contain measurable phospholipase A2 activity) to highly purified preparations of myocardial phospholipase A2 (Fig. 1A). Furthermore, incubation of heat-treated cytosol with trypsin completely ablated its ability to reconstitute calcium-mediated inhibition of purified myocardial phospholipase A2 activity. Collectively, these experiments demonstrate that a heat-stable protein constituent present in myocardial cytosol was responsible for the calcium-mediated inhibition of myocardial phospholipase A2 activity.

To identify the protein constituent mediating these effects, heat-treated cytosol was subjected to anion exchange chromatography. A single, well resolved peak of calcium-dependent inhibition was identified (Fig. 1B), pooled, and subjected to Mono-Q chromatography (Fig. 1C). The calcium-dependent inhibitor from Mono-Q chromatography was loaded onto a Mono-Q column prior to elution utilizing a linear gradient of 1 M NaCl as described under “Experimental Procedures.” O, 10 mM CaCl2; ●, 4 mM EGTA. D, proteins from Mono-Q chromatography were resolved by SDS-PAGE and subjected to either silver staining (top) or Western blotting utilizing mouse monoclonal anti-calmodulin-IgG1, (bottom) as described under “Experimental Procedures.”
against calmodulin. The 18-kDa protein doublet, which cochromatographed with calcium-dependent inhibition, was recognized by anti-calmodulin monoclonal antibody (Fig. 1). Moreover, authentic bovine brain calmodulin both cochromatographed with the homogeneous calcium-dependent inhibitor of myocardial phospholipase A₂ (utilizing identical conditions for Mono-Q chromatography) and inhibited myocardial phospholipase A₂ activity in a calcium-dependent fashion.

To further substantiate the identity of the calcium-dependent inhibitor of myocardial phospholipase A₂ as calmodulin, W-7 agarose affinity resin was employed (20). W-7 agarose beads bound the Mono-Q-purified calcium-dependent inhibitor in the presence of calcium ion, and the inhibitor was completely released by subsequent incubation with 4 mM EGTA (Fig. 2A). No binding was manifest in the presence of buffer containing 4 mM EGTA. The partitioning of the protein mediating calcium-dependent inhibition onto W-7 agarose in the presence of calcium ion and its subsequent release with EGTA correlated with the amount of calcium-dependent inhibition of phospholipase A₂ (Fig. 2, B and C). Since the 18-kDa polypeptide, which cochromatographed with calcium-dependent inhibition of myocardial phospholipase A₂, was: 1) recognized by antibodies directed against calmodulin; 2) cochromatographed with authentic calmodulin; 3) bound to W-7 agarose beads in a calcium-dependent fashion; 4) possessed identical electrophoretic characteristics as authentic calmodulin; and 5) calmodulin entirely reproduced the calcium-dependent inhibition of myocardial phospholipase A₂, we conclude that the cytosolic protein constituent mediating calcium-dependent inhibition of myocardial phospholipase A₂ was calmodulin.

To determine the calcium dependence of calmodulin-mediated inhibition of myocardial phospholipase A₂ activity, measurements of phospholipase A₂ activity were conducted with highly purified myocardial phospholipase A₂ (50,000-fold purified; 10 ng of protein) and authentic calmodulin. Half-maximal inhibition was manifest at 200 nM calcium ion (Fig. 2D), which closely parallels the Kᵣ of calcium ion for calmodulin (21–23). This calcium-induced calmodulin-mediated inhibition was reversible by subsequent chelation of calcium ion.

To distinguish between the potential mechanisms that were responsible for the calmodulin-induced alterations in myocardial phospholipase A₂ activity (i.e. phospholipid substrate sequestration versus a direct interaction between calcium-activated calmodulin and myocardial phospholipase A₂) calmodulin-Sepharose chromatography was employed (24, 25). The application of myocardial cytosol (in buffer containing 500 μM CaCl₂) to a calmodulin-Sepharose affinity column resulted in the adsorption of less than 5% of the applied protein while myocardial phospholipase A₂ activity was completely adsorbed. Phospholipase A₂ activity was quantitatively desorbed by application of buffer containing 4 mM EGTA (Fig. 3A). The load and the void contained an identical protein banding pattern and protein masses (39 versus 38.5 mg for load and void, respectively), while the EGTA eluent contained only 0.5 mg of protein, which possessed a completely different banding pattern (Fig. 3B). Myocardial cytosolic phospholipase A₂ activity applied to a calmodulin-Sepharose column in the presence of EGTA was not adsorbed, demonstrating that activated calmodulin was required for association with the myocardial phospholipase A₂ catalytic complex. Moreover, 100-fold increases in substrate concentration (from 2 to 200 μM) did not attenuate calmodulin-mediated phospholipase A₂ inhibition.

To determine whether the interaction between activated calmodulin and myocardial phospholipase A₂ present in myocardial cytosol was an inherent component of the myocardial phospholipase A₂ complex, highly purified myocardial phospholipase A₂ (>50,000-fold purified from the ATP affinity chromatography eluent from Ref. 2) was utilized. Similar to results with crude fractions, activity was quantitatively absorbed in the presence of calcium ion and desorbed by washing with 4 mM EGTA. For comparison, highly purified recombinant 85-kDa calcium-dependent phospholipase A₂ was not adsorbed onto calmodulin-Sepharose.

To determine the biologic significance of calmodulin-mediated regulation of phospholipase A₂ in intact cells, we examined the effects of three structurally disparate calmodulin antagonists on arachidonic acid release in cultures of A-10 muscle cells, a cell line that predominantly contains "calcium-independent" phospholipase A₂ (14). Exposure of prelabeled A-10 muscle cells to W-7 resulted in over an 8-fold increase in [³H]arachidonic acid release (Fig. 4). Furthermore, half-maximal effects were manifest at a concentration of W-7, which parallels the Kᵣ for binding of W-7 by calmodulin (26–28). Moreover, preincubation of A-10 muscle cells with BEL prior to the addition of W-7 ablated the release of [³H]arachidonic acid (Fig. 4). Finally, utilization of two structurally disparate calmodulin antagonists, calmidazolium and trifluoperazine, resulted in similar increases in [³H]arachidonic acid release from A-10 cells with effective concentrations that approximated the Kᵣ for the binding of calmidazolium and trifluoperazine to calmodulin (<1 μM and ~1 μM, respectively) (29–33). No significant differences in [³H]arachidonic acid incorporation into phospholipids were manifest in the presence of W-7.

The results of this study identify the physical association of...
Calmodulin Modulates Myocardial Phospholipase A2

Acknowledgment—We gratefully acknowledge the technical expertise of Jian Wang in the cell culture experiments.

Fig. 3. Calmodulin-Sepharose chromatography of phospholipase A2 from myocardial cytosol. A, dialyzed rabbit heart cytosol (39 mg of protein) was loaded onto a 1-ml column of calmodulin-Sepharose, myocardial phospholipase A2 activity was eluted by the application of buffer containing 4 mM EGTA, and phospholipase A2 activity was quantified as described under “Experimental Procedures.” The void volume contained 38.5 mg of total protein, and the EGTA 1 fraction contained 0.5 mg of total protein. B, calmodulin-Sepharose column fractions were individually diluted to a final concentration of 100 μg/ml protein with 10% SDS and 0.05% β-mercaptoethanol, and the proteins were resolved on a 10% polyacrylamide gel and subsequently stained with silver. Calmodulin-Sepharose column fractions were assayed for calcium-dependent phospholipase A2 activity as described under “Experimental Procedures.”

Calmodulin antagonists stimulate the calcium-independent phospholipase A2-catalyzed release of arachidonic acid from A-10 muscle cells. A-10 muscle cells (2.75 × 10^7) were prelabelled in media containing 50 μCi of [3H]arachidonic acid for 16 h as described under “Experimental Procedures.” Next, the cells were preincubated with 10 μM BEL or vehicle alone for 15 min prior to exposure to W-7 (0, 10, 25, or 50 μM) for 60 min. The results are expressed as the disintegrations/min/plate of released radiolabeled fatty acid. Forty thousand cpm represents the release of ~40% of [3H]arachidonate incorporated into cellular phospholipids during the 16-h prelabeling interval.

Fig. 4. Calmodulin with the myocardial phospholipase A2 catalytic complex, the role of calcium ion in facilitating this association, and the importance of this interaction in modulating phospholipase A2 activity in intact muscle cells. Since calcium ion is neither an obligatory cofactor in catalysis nor necessary for the membrane association of this enzyme (in contrast to secretory phospholipase A2 (34) and calcium-dependent phospholipase A2 (35–37)), it has traditionally been assumed that calcium ion does not play an important role in modulating the activity of this enzyme in intact cells (1–6). These results clearly demonstrate the importance of calcium ion and the intracellular calcium transducer, calmodulin, in regulating the activity of nominally “calcium-independent” myocardial phospholipase A2. The calmodulin antagonists resulted in release of [3H]arachidonic acid from cellular phospholipids even at ambient cytoso-
The Calcium-dependent Association and Functional Coupling of Calmodulin with Myocardial Phospholipase A2: IMPLICATIONS FOR CARDIAC CYCLE-DEPENDENT ALTERATIONS IN PHOSPHOLIPOLYSIS
Matthew J. Wolf and Richard W. Gross

J. Biol. Chem. 1996, 271:20989-20992.
doi: 10.1074/jbc.271.35.20989

Access the most updated version of this article at http://www.jbc.org/content/271/35/20989

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 40 references, 20 of which can be accessed free at http://www.jbc.org/content/271/35/20989.full.html#ref-list-1