Myofilament anchoring of protein kinase C-epsilon in cardiac myocytes

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Summary

Regulatory proteins on muscle filaments are substrates for protein kinase C (PKC) but mechanisms underlying activation and translocation of PKC to this non-membrane compartment are poorly understood. Here we demonstrate that the epsilon isoform of PKC (ε-PKC) activated by arachidonic acid (AA) binds reversibly to cardiac myofibrils with an EC_{50} of 86 nM. Binding occurred near the Z-lines giving rise to a striated staining pattern. The delta isoform of PKC (δ-PKC) did not bind to cardiac myofibrils regardless of the activator used, and the alpha isoform (α-PKC) bound only under strong activating conditions. Three established PKC anchoring proteins, filamentous actin (F-actin), the LIM domain protein Cypher-1, and the coatamer protein β′-COP were each tested for their involvement in cytoskeletal anchoring. F-actin bound ε-PKC selectively over δ-PKC and α-PKC, but this interaction was readily distinguishable from cardiac myofilament binding in two ways. First, the F-actin/ε-PKC interaction was independent of PKC activation, and second, the synthetic hexapeptide LKKQET derived from the C1 region of ε-PKC effectively blocked ε-PKC binding to F-actin, but was without effect on its binding to cardiac myofilaments. Involvement of Cypher-1 was ruled out on the basis of its absence from detergent-skinned myofibrils that bound ε-PKC, despite its presence in intact cardiac myocytes. The ε-PKC translocation inhibitor peptide EAVSLKPT reduced activated ε-PKC binding to cardiac myofibrils in a concentration dependent manner, suggesting that a RACK2 or a similar protein plays a role in ε-PKC anchoring in cardiac myofilaments.

Key words: Protein kinase C, Protein binding, Myofilament, Cardiac myocyte

Introduction

Protein kinase C (PKC) regulates cytoskeletal function in a variety of cell types (Dorn and Mochly-Rosen, 2002). PKC phosphorylation of non-membrane proteins such as desmin (Huang et al., 2002), myosin light chain (Venema et al., 1993) and troponin (Jideama et al., 1996; Huang et al., 1997) is well documented in cardiac cells, with functional consequences ranging from changes in mechanical integrity of the heart to regulation of actomyosin ATPase activity and force generation. PKC protein and PKC activity are elevated in various types of heart disease (Bowling et al., 1999) and phosphorylation of cytoskeletal or myofilament proteins may contribute to the progression of chronic heart disease to heart failure (Solaro, 2002). Mechanisms underlying translocation and activation of this lipid-dependent kinase family at non-membrane sites are poorly understood. The intracellular signals that regulate PKC translocation and phosphorylation of cytoskeletal proteins, and the isoform(s) of PKC responsible are also not clear.

PKC represents a family of at least 12 distinct isoforms each with a unique developmentally regulated and tissue-dependent pattern of expression (Nishizuka, 1992; Rybin and Steinberg, 1994). Prevailing models of PKC activation and translocation feature PKC binding to membranes where its lipid activators, phosphatidylserine and diacylglycerol (DAG), are located (Newton, 2001). Yet, there is accumulating evidence that proteins play a central role in translocation and that certain PKC isoforms interact directly with specific cytoskeletal proteins (Dorn and Mochly-Rosen, 2002). The β-isoform of PKC has been shown to bind in a saturable and high affinity manner to an anchoring protein termed RACK1 (receptor for activated C-kinase-1) initially identified in detergent-insoluble subcellular fractions (Ron et al., 1994). βII-PKC and ε-PKC have been reported to bind to F-actin (Blobe et al., 1996; Prekeris et al., 1996), and the α-PKC isoform is recruited to focal contacts (Hyatt et al., 1994; Baciu and Goetinck, 1995; Buensuceso et al., 2001).

How the lipid requirements for PKC activation are met upon translocation to the cytoskeleton remains uncertain. One hypothesis focuses on the ability of *cis*-unsaturated fatty acids to activate PKC in the absence of phosphatidylserine (Blobe et al., 1995). Another hypothesis focuses on the unique ability of F-actin to serve both as PKC activator and cytoskeletal anchoring site (Blobe et al., 1996; Prekeris et al., 1996). Other candidates include the Cypher family of adapter proteins, such as Cypher-1, which contains both a LIM domain PKC binding motif and a PDZ-like α-actinin binding domain in the same molecule (Zhou et al., 1999). Cypher proteins are highly expressed in ventricular myocytes and are capable of directing PKC to cardiac Z-lines (Zhou et al., 1999). Finally, tyrosine phosphorylation of PKC may lead to activation and a loss of normal lipid cofactor requirements for activation (Konishi et al., 1997).

PKC isoforms highly expressed in cardiac myocytes include α-, δ- and ε-PKC, whereas β-PKC is poorly expressed except in diseased heart muscle (Rybin and Steinberg, 1994; Steinberg et al., 1995; Huang et al., 1997). ε-PKC occurs in a cross-striation pattern in neonatal and adult cardiac myocytes after activation (Disatnik et al., 1994; Huang et al., 1997) and is thought to mediate at least some of the physiological effects of angiotensin II, endothelin-1 and α-adrenergic agonists on the heart (Clerk et al., 1994; Jiang et al., 1996; Clerk et al., 1998). A coatamer subunit β′-COP has recently been identified as a RACK for activated ε-PKC (Csukai et al., 1997), but it remains unclear whether β′-COP (also known as RACK2) is membrane- or myofilament-associated or is predominantly cytosolic. The V1 region in ε-PKC was identified as protein-protein interface for activated ε-PKC/myofilament binding (Csukai et al., 1997).

Since both the C1 and V1 regions of ε-PKC have been implicated in ε-PKC anchoring, we selected two peptides, one from the C1 region and one from the V1 region, to investigate the properties of ε-PKC binding to cardiac myofibrils devoid of membranes. The results of our studies show that the *cis*-unsaturated fatty acid arachidonic acid (AA) is a good activator for ε-PKC anchoring to cardiac myofibrils, but is ineffective in promoting α-PKC or δ-PKC anchoring. Immunoblotting and the peptide inhibition data indicate that RACK2 (or a related protein), but not F-actin or Cypher-1, contributes to high affinity anchoring of ε-PKC to cardiac myofilaments.

Materials and Methods

All animals used in the study were handled in accordance with the guidelines of the University of Wisconsin Research Animal Resource Committee and the Institutional Animal Care and Use Committee at Florida Atlantic University.

Preparation of PKC isoforms

Insect cells (Sf9) infected with recombinant baculovirus encoding α-PKC, ε-PKC or δ-PKC were kindly provided by T. Rogers, University of Maryland, Baltimore. The recombinant PKC isoforms were purified to apparent homogeneity by successive chromatographic runs on DEAE-cellulose, serine-Sepharose and Mono Q columns according to published methods (Schaap and Parker, 1990). The specific activity of the purified enzyme was 1-2 μmoles phosphate/minute/mg protein using myelin basic protein as substrate.

Preparation of peptides

Peptides were synthesized on a Synergy 432a automated synthesizer using FMOC chemistry. Because N-terminal glutamate residues can cyclize and cause problems and because we found a large contaminant during work up of EAVSLKPT whose amino acid composition was consistent with a cyclized form, we prepared this peptide in an *N*-acetylated and C-amide form and used this peptide for the experiments described. Peptides were purified by reverse-phase HPLC in acetonitrile/water/trifluoroacetic acid mixtures. Peptide compositions were confirmed by amino acid analysis and mass spectrometry; LKKQET, mass spec., expected: 746.7, found: 746.7; Ac-EAVSLKPT-NH_2, mass spec. expected: 886.0, found: 886.3. All data were expressed as mean±s.e.m. Numerical data were analyzed by Student's *t*-test with a value of <0.05 considered significant.

Standard curve for ε-PKC on western blots

In order to make the binding assay as quantitative as possible, a standard curve of ε-PKC versus enhanced chemiluminescent (ECL) signal was generated. Different concentrations of purified recombinant ε-PKC were electrophoresed on 12% SDS-polyacrylamide gels then transferred to nitrocellulose membranes. After blocking non-specific sites for 1 hour with blotto (5% powdered milk in TBS-T solution), the nitrocellulose was incubated with primary antibody (1:2500) for 1 hour at room temperature. An ε-PKC polyclonal antibody was used for western blots. The antibody was raised in rabbits at the University of Wisconsin Antibody Production Facility, then affinity purified as described previously (Huang et al., 1997). The nitrocellulose was washed in TBS-T solution three times, and then incubated for 1 hour at room temperature with horseradish peroxidase-linked secondary antibody (1:5000). The blots were again washed three times in TBS-T solution. Bound secondary antibody was visualized by the ECL method and analyzed by quantitative densitometry. The standard curve was used to identify a linear range for detection and quantification of ε-PKC on blots (Fig. 1). Also, three internal standards of ε-PKC (50 ng, 100 ng and 200 ng) were included on every blot to control for variation in transfer efficiency and exposure time.

ε-PKC binding assay

F-actin

Rabbit skeletal muscle F-actin was purified as described previously (Pardee and Spudich, 1982). The ε-PKC/F-actin binding assay was carried out according to the method of Terrian and co-workers (Prekeris et al., 1996) with the following modifications. One hundred μg of purified F-actin was pre-incubated for 30 minutes at room temperature in buffer solution containing 4 mM Tris-HCl pH 7.5, 10 μM $CaCl_2$, 30 mM KCl, 120 mM potassium propionate, 1 mM $MgCl_2$,

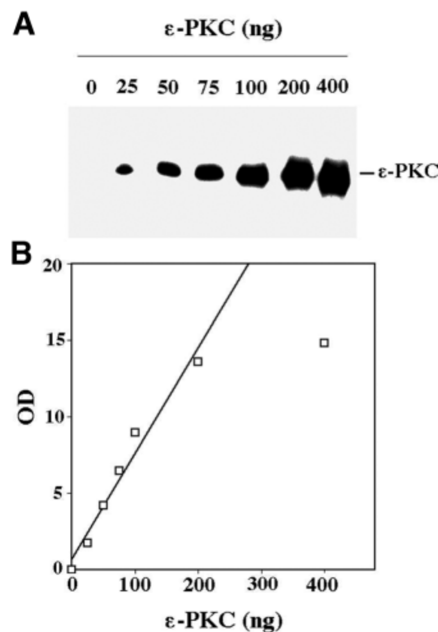

Fig. 1. ε-PKC western blot standard curve. Purified ε-PKC was subjected to SDS-PAGE and immunoblotted as described in Materials and Methods. (A) Bands were visualized by ECL and quantified by densitometry. (B) Integrated optical densities (OD) were plotted as a function of ε-PKC mass. To ensure linearity of ε-PKC binding in all subsequent experiments, only OD values below 13 were used for quantification.

150 μM ATP, 100 μM $ZnCl_2$ before addition of varying amounts of purified ε-PKC up to 1 μM. Arachidonic acid (AA), phorbol 12-myristate 13-acetate (PMA) or vehicle was then added and tubes were incubated for 1 hour at 30°C. The final volume of the incubation mixture was 220 μl. Tubes were then centrifuged at 78,000 ***g*** for 30 minutes at 4°C to pellet F-actin. Pellets were resuspended in 50 μl of sample buffer then 5 μl was subjected to SDS-PAGE and immunoblot analysis.

Myofilaments

Ventricular myocytes were isolated by retrograde perfusion of adult male rat hearts as described previously (Huang et al., 1996). Isolated myocytes were treated in skinning solution (0.5-1% Triton X-100 in relaxing solution containing 4 mM MgATP, 100 mM KCl, 10 mM imidazole, 2 mM EGTA, 1 mM $MgCl_2$, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM benzamidine, 0.01 leupeptin, pH 7.5) at room temperature for 6 minutes. Protein concentration was measured by the Bicinconinic acid assay (Sigma). 200 μg of skinned rat ventricular myocytes (containing about 60 μg F-actin, assuming 30% by weight) were incubated in relaxing solution with ε-PKC and PKC activator (AA, PMA or vehicle) for 1 hour at 30°C. The myofilaments were then sedimented at 1000 ***g*** for 2 minutes and washed three times with relaxing solution. Pellets were then resuspended in 50 μl of sample buffer and 5 μl was subjected to SDS-PAGE and immunoblot analysis.

Immunofluorescence

The skinned rat cardiac myocytes were collected after centrifugation (800 ***g*** for 5 minutes) and washed with relaxing solution three times. The myofibril suspension was incubated with recombinant ε-PKC (Sigma Co. St Louis, MO) in the presence of 50 μM AA for 1.5 hours at 30°C. At the end of the incubation, the myofibrils were collected by centrifugation and washed three times with relaxing solution. The myofibril suspension was then gently smeared over no. 1.5 coverslips and myofibrils were fixed in cold methanol for 1 minute. Slides were then incubated with a monoclonal antibody anti-ε-PKC (1:100) (BD Bioscience Transduction Laboratories, Lexington, KY, USA) in RB solution (74 mM KCl, 2 mM $MgCl_2$, 1 mM EGTA, 1 mM NaN_3, 5 mM KH_2PO_4, 1 mM dithiothreitol, adjusted to pH 7.0 with HCl) at 4°C for 2 hours and then incubated with secondary antibody, fluorescein-conjugated goat anti-mouse IgG (1:50) at 25°C for 1 hour. Slides were viewed with an Olympus AX 70 microscope equipped for phase contrast and epifluorescence illumination. Images were obtained using a ×100 objective and a CCD camera controlled with Image-Pro Plus image analysis software. Images were analyzed with Adobe Photoshop software.

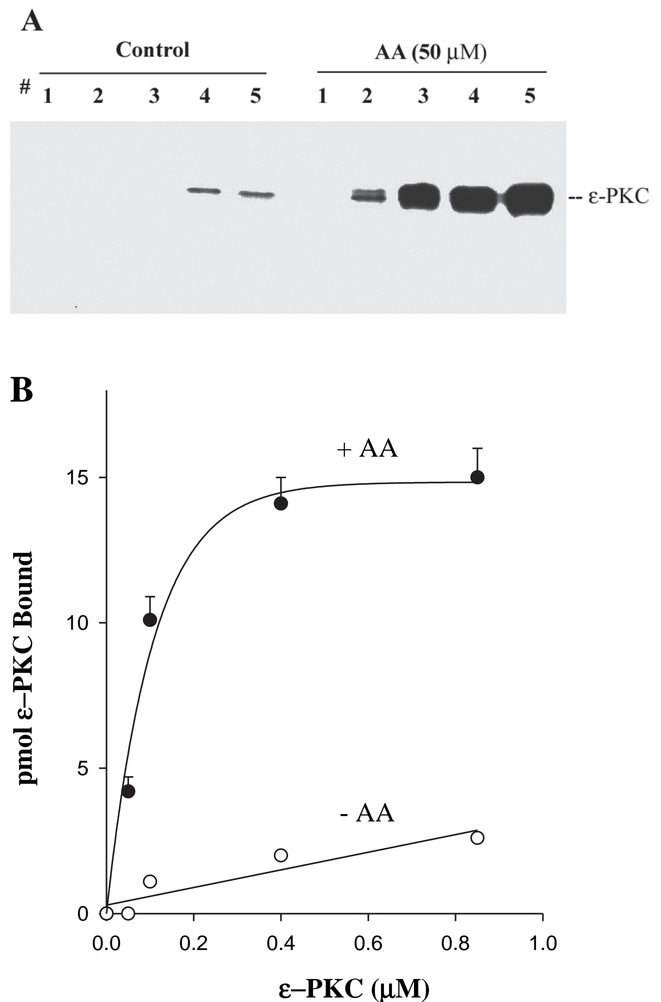

Fig. 2. Binding of ε-PKC to cardiac myofilaments. (A) Western blot of myofilament pellets showing binding of purified ε-PKC in absence or presence of 50 μM arachidonic acid (AA). ε-PKC concentrations (in μM): lane 1, 0; lane 2, 0.1; lane 3, 0.2; lane 4, 0.5; lane 5, 1. (B) Summary of ε-PKC binding to myofilaments. The solid circles (in the presence of AA) represents a fit to B/B_{max}=[ε-PKC]/([ε-PKC] + EC_{50}), with EC_{50}=86 nM and B_{max}=15 pmol (or 75 pmol/mg protein). The stoichiometry of binding was 15 pmols ε-PKC bound per 2 nmoles actin monomers or a 1/130 ratio. The open circles represent a linear regression of ε-PKC binding in the absence of AA.

Results

Properties of ε-PKC binding to cardiac myofilaments

In the absence of PKC activators, ε-PKC interacted only weakly and non-specifically with Triton X-100-skinned cardiac myocytes. Binding was greatly enhanced by 50 μM AA (Fig. 2) or 100 nM PMA (not shown). ε-PKC binding saturated above 0.5 μM free ε-PKC with a true saturation of sites because the total amount of ε-PKC binding was in the linear range for detection by ECL (see Fig. 1). The binding data were well fit by a hyperbolic binding isotherm with an EC_{50} of 86 nM and B_{max} of 75 pmol/mg protein (Fig. 2B), indicating a relatively high affinity interaction between ε-PKC and sites on cardiac myofilaments. Electron microscopic inspection revealed that the 0.5% Triton X-100 skinning protocol removed the vast majority of membranes including transverse tubules (T-tubules). The dihydropyridine (DHP) receptor protein, a T-tubule marker, and the ryanodine receptor protein, a sarcoplasmic reticulum marker, were both absent from the skinned myocytes, as determined by radiolabeled ligand binding and western blot analysis (not shown). Moreover, at the higher concentration of 1% Triton X-100, ε-PKC binding properties of skinned myocytes were unaltered. The preparation was thus highly enriched in cardiac myofilament proteins and largely devoid of membranes.

Immunofluorescence was used previously to show translocation of endogenous ε-PKC to the I-band near Z-lines, showing a cross-striation pattern in cardiac myocytes stimulated with AA or PMA (Huang et al., 1997). In the present experiments, addition of recombinant ε-PKC to skinned cardiac myocytes in the presence of a PKC activator

gave a similar cross-striation pattern (Fig. 3). While in the absence of PKC activator or incubation of slides only with the secondary antibody did not show any striated staining (not shown).

Isoform specificity of PKC binding

The three major PKC isoforms expressed in adult cardiac myocytes, ε, α and δ, were each tested for binding to cardiac myofilaments. ε-PKC binding was the most robust of the three isoforms, showing a complete activation of binding in the presence of 50 μM AA alone (Fig. 4). δ-PKC showed no binding with 50 μM AA or the more potent mixture of 50 μM AA and 25 μM DAG (Fig. 4), despite δ-PKC being fully catalytically active under these latter conditions (not shown). The behavior of α-PKC was intermediate between ε- and δ-PKC isoforms. 50 μM AA did not stimulate α-PKC binding to cardiac myofilaments but the more potent AA/DAG mixture did (Fig. 4). In addition, in subcellular translocation assays we observed that the majority of α-PKC was moved from cytosol to membranes in intact cardiac cells after activation. Thus, the behavior of α-PKC in intact cells is also different from the translocation pattern of the ε-PKC in intact cells in which most ε-PKC was moved to myofibrils after activation (Huang et al., 1997).

Inhibition of ε-PKC binding by synthetic peptides

It has been reported that the V1 region is required for anchoring of activated ε-PKC and that inhibition of anchoring by the V1 domain or by a near N-terminal peptide (residues 14-21; εV_{1-2}) are sufficient to inhibit translocation and function of the activated ε-PKC in intact cardiac cells (Johnson et al., 1996). Here, the nature of ε-PKC binding was probed with this eight-amino-acid peptide whose sequence was derived from the V1 region of the regulatory domain of ε-PKC itself. Under our assay conditions, the εV_{1-2} peptide with a sequence of EAVSLKPT reduced ε-PKC binding to adult cardiac myofilaments in a concentration-dependent manner (Fig. 5). 50 μM EAVSLKPT peptide reduced ε-PKC binding to myofilaments by up to 60%, whereas over the same concentration range the peptide, LKKQET, derived from the C1 region of ε-PKC, was without effect (Fig. 5).

PKC binding to F-actin

Of the three PKC isoforms examined only ε-PKC bound to F-actin under the experimental conditions employed. Recombinant ε-PKC bound to F-actin in a saturable manner with no obvious difference in the extent or affinity of binding in the absence or presence of the PKC activators AA (Fig. 6A) or PMA (not shown). Data with and without AA were included together in a fit that was well described by a hyperbolic binding isotherm with an EC_{50} of 110 nM and a B_{max} of 180 pmol/mg protein (Fig. 6A). The synthetic peptide LKKQET reduced ε-PKC binding to F-actin in a concentration-dependent manner (Fig. 6B), with up to 60% inhibition at a concentration of 50 μM peptide. Therefore, ε-PKC and LKKQET functioned as previously reported with regard to F-actin binding (Prekeris et al., 1996). However,

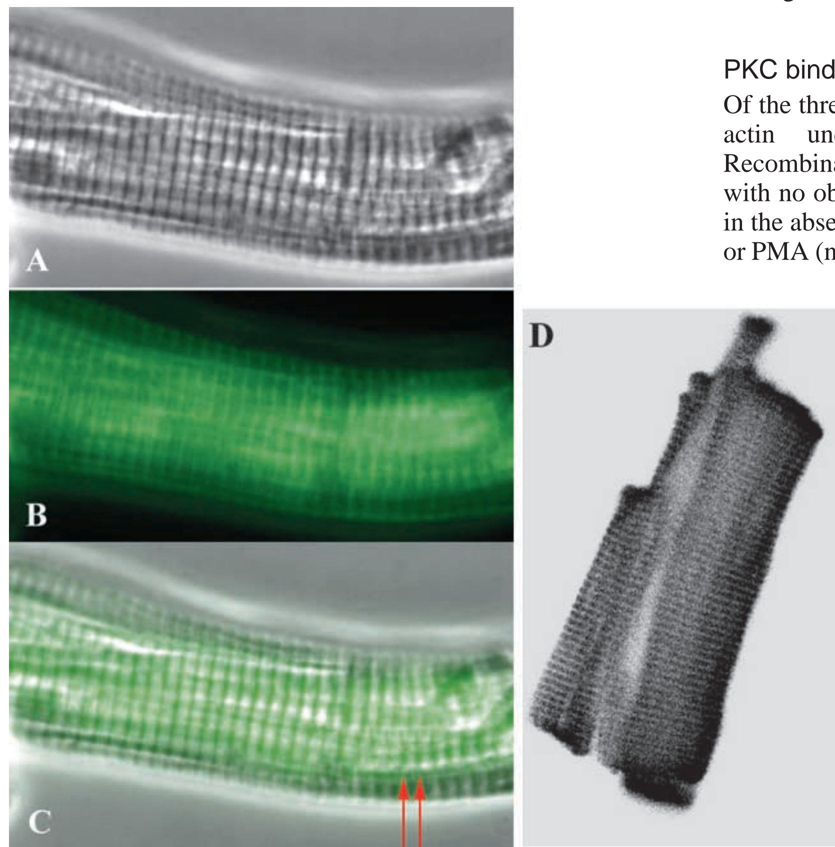

Fig. 3. Phase contrast (A) and fluorescent (B) images of myofilament associated ε-PKC. ε-PKC is activated with 50 μM arachidonic acid (AA) and the activated ε-PKC is associated with myofilaments near Z-lines as indicated by arrows in the overlay image (C). A confocal image of a skinned myocyte decorated with ε-PKC, an anti-ε-PKC primary antibody and Alexa 488 secondary antibody (D) Magnification ×1000.

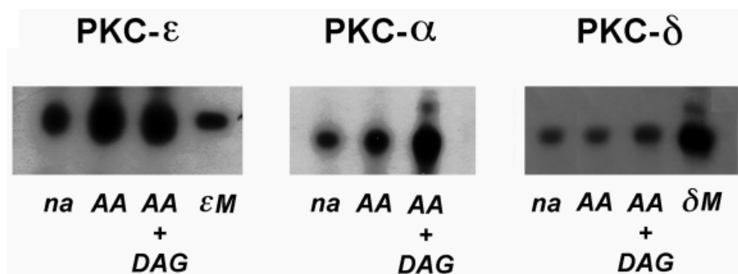

Fig. 4. Isoform specificity of PKC binding to cardiac myofibrils. (A) Western blots of myofibril pellets after incubation with α-PKC, δ-PKC or ε-PKC. Below each lane is indicated the PKC activators used to stimulate binding. na, No activators. AA, 50 μM arachidonic acid. AA + DAG, 50 μM AA plus 25 μM dioctanoylglycerol. εM is ε-PKC gel/blot marker, and δM is δ-PKC gel/blot marker.

LKKQET had no effect on the binding of ε-PKC to cardiac myofilaments in this same concentration range (Fig. 5). Likewise, EAVSLKPT had no effect on ε-PKC binding to F-actin (Fig. 6B). These reciprocal actions of peptide inhibitors on ε-PKC binding to F-actin versus cardiac myofilaments indicated that ε-PKC interacts with structures other than F-actin in cardiac myofilaments. Moreover, ε-PKC required a lipid activator to initiate myofilament binding, whereas F-actin alone appeared capable of serving the dual roles of ε-PKC activator *and* ε-PKC anchor under these experimental conditions.

Cypher-1 in cardiac myocytes

To explore a potential role for Cypher-1 in Z-line anchoring in cardiac myofilaments, a western blot analysis was performed on cardiac myofibrils after skinning. Virtually no Cypher-1 was detectable in skinned cardiac myofibrils, despite a robust Cypher-1 signal in intact myocytes (Fig. 7). The absence of Cypher-1 in cardiac myofibrils argues against it being responsible for Z-line anchoring in the experiments described here. It cannot be ruled out, however, that Cypher-1 plays a significant part in directing PKC translocation in intact ventricular myocytes.

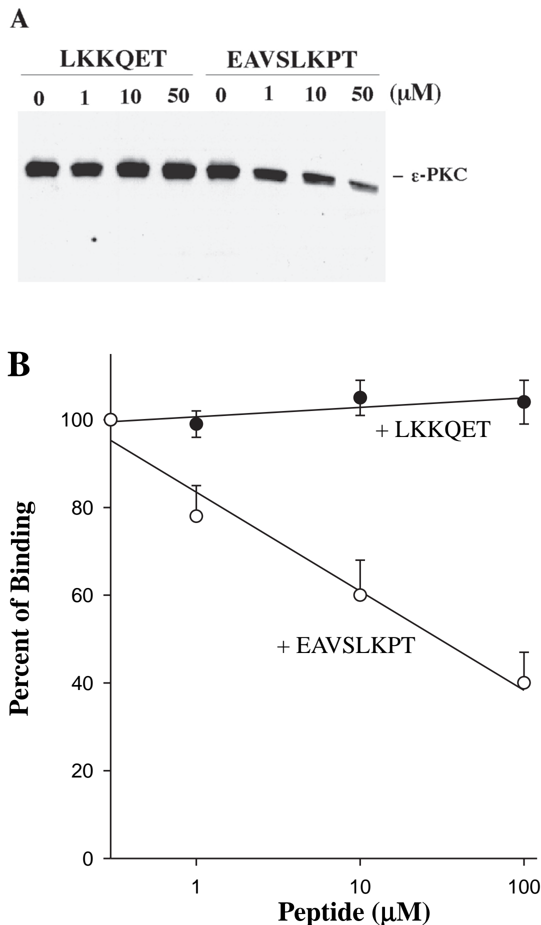

Fig. 5. Inhibition of ε-PKC binding to cardiac myofibrils by synthetic peptides. (A) A Western blot shows ε-PKC in the myofibril pellet is reduced in a concentration-dependent manner by the octapeptide, EAVSLKPT. The F-actin binding hexapeptide LKKQET had no effect of ε-PKC binding to myofibrils over the same concentration range. ε-PKC concentration was 100 nM and peptide concentrations in μM are given above each lane. (B) Summary of peptide inhibition of ε-PKC binding to cardiac myofibrils. Bars represent mean±s.e.m. of five experiments.

Discussion

The present results show that purified recombinant ε-PKC binds to cardiac myofilaments with a relatively high affinity.

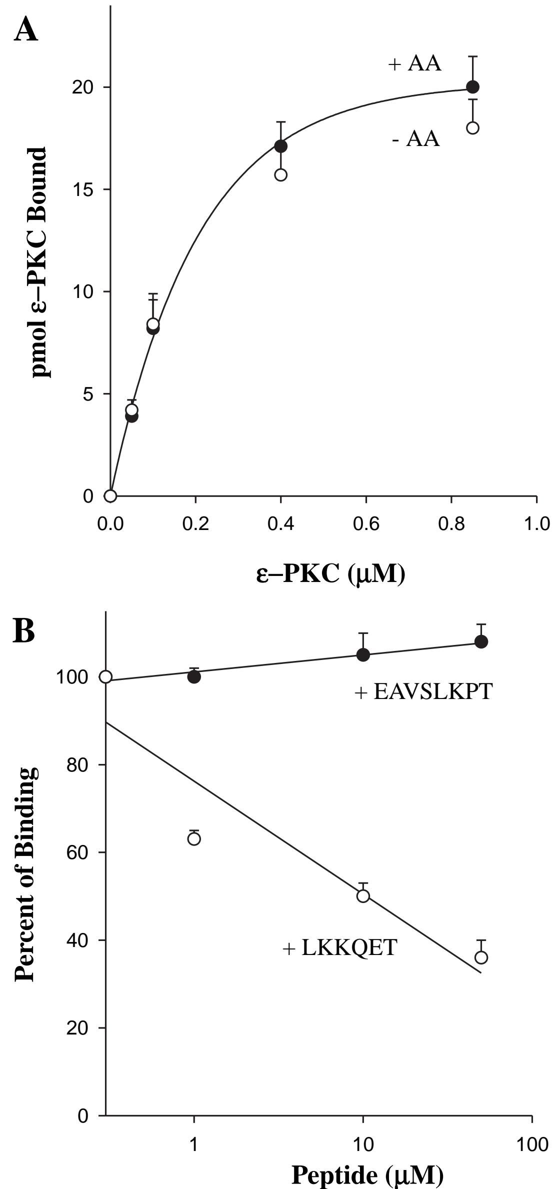

Fig. 6. Properties of ε-PKC binding to isolated F-actin. (A) Summary of ε-PKC binding to F-actin in the absence and presence of 50 μM arachidonic acid (AA). The line with solid circles (in the presence of AA) represents a fit to: B/B_{max}=[ε-PKC]/([ε-PKC] + EC_{50}), with EC_{50}=110 nM and B_{max}=18 pmol (or 180 pmol/mg protein). The stoichiometry of binding was 18 pmols ε-PKC bound per 2 nmoles actin monomers or a 1/110 ratio. Open circles represent ε-PKC with F-actin in the absence of AA. (B) Summary of inhibition of ε-PKC binding to F-actin by synthetic peptides. Data is represented as mean±s.e.m. from five experiments.

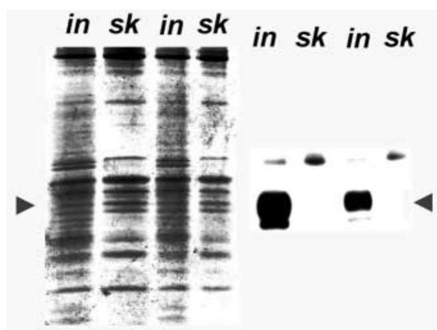

Fig. 7. Absence of Cypher-1 in skinned cardiac myofibrils. Left Panel: Coomassie Blue-stained SDS-PAGE gel of intact (*in*) and Triton X-100 skinned (*sk*) rat ventricular myocytes. Protein loads were 20 μg (left two lanes) and 10 μg (right two lanes). Right panel: Western blot of same samples as in left panel. Arrowheads mark the mobility of Cypher-1.

Binding required activation of ε-PKC for which inclusion of arachidonic acid (AA) or PMA sufficed. These observations are consistent with earlier observations in intact neonatal (Disatnik et al., 1994) and adult cardiac myocytes (Huang et al., 1997) where PKC activation caused a large-scale translocation of endogenous ε-PKC from cytosol to myofilaments, giving rise to a cross-striated staining pattern. Again, both AA and PMA strongly promoted ε-PKC binding. Arachidonic acid has been shown to selectively induce ε-PKC translocation in human breast carcinoma cells and in other cell types (Huang et al., 1997; Mackay and Mochly-Rosen, 2001; Palmantier et al., 2001). It is also apparent that PKC activation by arachidonic acid differs from that by diacylglycerol in terms of cofactor, Ca^{2+} and phospholipid requirements (Koide et al., 1992; Huang et al., 1993).

In the present study, ε-PKC anchoring to myofilaments in the presence of arachidonic acid was specific, since neither δ-PKC nor α-PKC bound under these conditions. This is probably a consequence of arachidonic acid being a more potent and effective activator of ε-PKC than of other PKC isoforms, and a reflection of ε-PKC having a higher affinity for anchoring sites on the myofilaments. The α- and δ-PKC isoforms have been shown to localize preferentially to other unique subcellular sites such as plasma membrane and perinuclear membranes (Disatnik et al., 1994; Huang et al., 1997). In only a few cases has the anchoring protein been identified, although there is no shortage of candidates including PIC-1, RACK1, β′-COP (RACK2), AKAPs, F-actin and Cypher-1.

Myofilament anchoring of ε-PKC has been shown to occur in the vicinity of the Z-lines in both neonatal and adult cardiac myocytes (Disatnik et al., 1994; Huang et al., 1997). The Z-line represents a key location in cardiac cells where sarcomeric actin filaments are tethered, where one end of the elastic protein titin is grounded, where transverse tubules integral to excitation-contraction coupling and its regulation interact with the cytoskeleton, and where a wide variety of signaling molecules congregate. Most of the targets of PKC phosphorylation at the Z-line remain to be identified. One protein, the Z-line-associated cytoskeletal protein desmin, has recently been shown to be phosphorylated by PKC in hamster cardiac myocytes (Huang et al., 2002). Desmin phosphorylation by PKC has been linked to myofibril disarray and cardiomyopathy in hamsters, but the isoform(s) of PKC involved and the role of anchoring proteins remain unknown. Very recently, Pyle et al. reported that phenylephrine-mediated myofilament/ε-PKC association was decreased in transgenic mice with reduced actin capping protein (CapZ), suggesting that the Z-line-associated CapZ might serve as a useful target in the management of myofilament function by PKC (Pyle et al., 2002).

In an effort to begin addressing this gap in understanding of PKC anchoring, we tested the involvement of several possible candidates for anchoring of ε-PKC activated by arachidonic acid. Filamentous actin has been suggested to be a principal anchoring protein for ε-PKC within intact nerve endings (Prekeris et al., 1996) and in cultured NIH3T3 cells (Hernandez et al., 2001). F-actin is certainly not in short supply in cardiac myofibrils, as it constitutes roughly 30% of the protein content by weight. Moreover, associated with the actin filament in cardiac myocytes are the well characterized PKC substrates troponin I, troponin T and tropomyosin, so F-actin was considered a strong candidate for a PKC anchoring site in myocytes. Our results confirmed that ε-PKC bound with high affinity to F-actin and supported the suggestion that the ε-PKC sequence motif LKKQET (residues 223-228 located between the first and second cysteine rich regions on the C_1 domain of ε-PKC) defines at least part of the interface (Prekeris et al., 1996). The six-amino-acid peptide LKKQET inhibited ε-PKC binding to purified F-actin in a concentration-dependent manner, but was without effect on ε-PKC binding to cardiac myofilaments. Conversely, the eight-amino-acid peptide EAVSLKPT (Johnson et al., 1996) inhibited ε-PKC binding to cardiac myofilaments in a concentration-dependent manner, but had no effect on the ε-PKC/F-actin interaction. The results strongly suggest that the binding interface between ε-PKC and cardiac myofilaments is mainly on the V1 region of ε-PKC and the interface between ε-PKC and F-actin is mainly on the C1 region of ε-PKC. In addition, lipid activators of PKC were not required for the ε-PKC/F-actin interaction as indicated by binding being indistinguishable in the absence and presence of activating concentrations of AA or PMA. By contrast, the ε-PKC/cardiac myofilament interaction was greatly stimulated by AA or PMA under the same conditions. These data further demonstrate an important difference between myofilament anchoring of ε-PKC through its C1 region and through its V1 region. Binding to F-actin through the C1 domain does not require preactivation of ε-PKC by lipids; however it is presently unclear whether ε-PKC bound to F-actin without activators is catalytically active or inactive. Overall, we conclude that F-actin is not a major anchoring site in cardiac tissues. A probable explanation for why ε-PKC does not bind to cardiac sarcomeric actin is the presence of other actin binding proteins such as troponin, tropomyosin, tropomodulin and myosin that could mask sites on F-actin to which ε-PKC binds.

A number of putative PKC anchoring proteins were removed from consideration by the relatively strong skinning conditions used here to prepare the cardiac myofibrils. Among the proteins that could be demonstrated to be extracted by the up to 1% Triton X-100 treatment were the highly expressed proteins Cypher-1, ryanodine receptors and dihydropyridine

receptors. We assume therefore that the vast majority of membrane proteins and loosely associated myofilament proteins were removed. One tightly associated protein that remained was the signature Z-line protein α-actinin. Overlay experiments with blotted α-actinin and with purified non-denatured α-actinin failed to detect a direct interaction between ε-PKC and α-actinin (not shown).

The selective activation of ε-PKC by arachidonic acid in cardiac myocytes is likely to be physiologically relevant because in the heart *cis*-unsaturated fatty acids are released early in ischemia (low blood flow) and possibly play a part in the phenomenon of ischemic preconditioning (Starkopf et al., 1998). It is well documented that ischemia causes degradation of membrane phospholipids resulting in accumulation of unesterified fatty acids including arachidonic acid (Chien et al., 1984). Several studies have demonstrated selective translocation of ε-PKC in ischemic preconditioning and suggested the involvement of ε-PKC in this form of cardioprotection (Ping et al., 1997; Ping et al., 1999; Ping et al., 2002; Cross et al., 2002). A role for ε-PKC in mediating cardioprotection was also indicated in isolated adult rabbit myocytes subjected to ischemic preconditioning. An eight-amino-acid peptide (EAVSLKPT) designed to block ε-PKC translocation was introduced into rabbit myocytes by using an Antennapedia peptide delivery system. In the presence of the ε-PKC translocation inhibitor, the protective effect of preconditioning was abolished. Peptide inhibitors of β- and δ-PKC had no effect, suggesting that ε-PKC was the isoform necessary for the cardioprotective effect of preconditioning in this model (Ping et al., 1999; Liu et al., 1999). The precise roles of arachidonic acid and myofilament anchoring of ε-PKC are not yet clear.

In summary, arachidonic acid is capable of activating purified recombinant ε-PKC and promoting its binding to cardiac myofibrils with an apparent K_d of less than 100 nM. Other PKC isoforms highly expressed in cardiac muscle, α- and δ-PKC, did not bind under these conditions. With the application of two different peptides, our experimental results demonstrate that the unique V1 domain of ε-PKC is critical for the anchoring of activated ε-PKC and that inhibition of this domain by εV_{1-2} peptide is sufficient to prevent ε-PKC/myofilament association. While the C1 domain of ε-PKC has been revealed to have a different properties in protein-protein interaction, it can bind to purified F-actin with no need for lipid activators on binding affinity. The identity of the RACK(s) mediating ε-PKC binding to myofilaments remains unknown, but at least in this system F-actin and cypher-1 have been ruled out. A peptide derived from the interface between the regulatory domain of ε-PKC and RACK2 competed with the binding interaction, suggesting specific ε-PKC anchoring to myofibrils through a similar interface.

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