Novel determinant of PKC-ε anchoring at cardiac Z-lines

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Robia, Seth L., Misuk Kang, and Jeffery W. Walker. Novel determinant of PKC-ε anchoring at cardiac Z-lines. Am J Physiol Heart Circ Physiol 289: H1941–H1950, 2005. First published June 17, 2005; doi:10.1152/ajpheart.01111.2004.—The Z-line represents a critical link between the transverse tubule network and cytoskeleton of cardiac cells with a role in anchoring structural proteins, ion channels, and signaling molecules. Protein kinase C-ε (PKC-ε) regulates cardiac excitability, cardioprotection, and growth, possibly as a consequence of translocation to the Z-line/T tubule region. To investigate the mechanism of PKC-ε translocation, fragments of its NH2-terminal 144-amino acid variable domain, εV1, were fused with green fluorescent protein and evaluated by quantitative Fourier image analysis of decorated myocytes. Deletion of 23 amino acids from the NH2-terminus of εV1, including an EAVSLKPT motif important for binding to a receptor for activated C kinase (RACK2), reduced but did not abolish Z-line binding. Further deletions of up to 84 amino acids from the NH2-terminus of εV1 also did not prevent Z-line decoration. However, deletions of residues 85–144 from the COOH-terminus strongly reduced Z-line binding. COOH-terminal deletions caused 2.5-fold greater loss of binding energy (ΔΔG) than did NH2-terminal deletions. Synthetic peptides derived from these regions modulated εV1 binding and cardiac myocyte function, but also revealed considerable heterogeneity within populations of adult cardiac myocytes. The COOH-terminal subdomain important for Z-line anchoring maps to a surface in the εV1 crystal structure that complements the eight-amino acid RACK2 binding site and two previously identified membrane docking motifs. PKC-ε anchoring at the cardiac Z-line/T tubule appears to rely on multiple points of contact probably involving protein-lipid and protein-protein interactions.

protein kinase C-ε

THE PROTEIN KINASE C (PKC) family of serine-threonine kinases is taxonomically divided into three isoform categories based on differential regulation by various cofactors (28, 30). In the heart, PKC mediates signaling from several G protein-coupled receptors, including α1-adrenergic, endothelin, and ANG II receptors (6). PKC substrate specificity has been determined in vitro to be quite general, both in terms of the wide variety of protein substrates phosphorylated and the low degree of isoform selectivity of a particular substrate (28, 29). This belies the apparent need for a diversity of isoforms and suggests that substrate specificity in vivo arises from other recognition mechanisms. In this regard, much attention has been focused upon “translocation,” the physical redistribution of PKC to specific locations within the cell in response to second messenger activators such as Ca2+ and/or diacylglycerol (21). Despite dynamic reversibility, translocation and anchoring significantly reduce the apparent diffusion coefficient of PKC (42), and may help confer specificity by constraining PKC to act on substrates within defined regions of the cell. The molecular mechanisms underlying this process have not been fully elucidated.

This study focused on PKC-ε, one of the calcium-insensitive novel isoforms, which has been specifically implicated in L-type channel regulation (18), regulation of thin filament Ca2+ sensitivity (11, 41), and ischemic preconditioning (12, 23, 34, 35). PKC-ε has been shown to bind through its first variable domain (εV1) to the coatomer protein β-COP [also designated receptor for activated C kinase (RACK2)] when activated by phosphatidylserine and diacylglycerol (4). PKC-ε-RACK2 binding, and thus kinase translocation, were proposed to be mediated by a discrete 8-residue RACK-binding sequence, EAVSLKPT, near the NH2-terminus of this εV1 domain (7).

The crystal structure of the εV1 domain was recently solved by Ochoa et al. (32), who proposed a different mode of εV1-mediated anchoring of PKC-ε to membranes. In their model, key hydrophobic and electrostatic interactions occur between surface loops 1 and 3 of εV1 and the phospholipid membrane (3, 24, 32). Mg2+ is also hypothesized to play a critical role in coordinating charged residues at the binding interface. This model is more analogous to Ca2+-dependent membrane binding of the homologous C-2 domain of classical PKCs (30) and contrasts with the RACK model in its supposition of a distributed binding interface, rather than a discrete eight-amino acid binding site.

We have previously described decoration of saponin-permeabilized cardiac myocytes with PKC-ε green fluorescent protein (GFP) fusion constructs as an assay for investigating the localization and kinetics of PKC-ε anchoring (36). This method provides the ability to simultaneously measure dynamic anchoring properties while observing the spatial distribution of the anchoring sites, all under conditions of defined solution composition, pH, ionic strength, and probe concentration. Using this assay, we have now evaluated the relative binding efficacy of a series of deletion mutants of the εV1 regulatory domain of PKC-ε to determine contributions of specific sequences to anchoring. The constructs were designed to explicitly test the role of previously identified motifs in anchoring of PKC-ε in cardiac myocytes and therefore contained various combinations of the eight-amino acid RACK binding site and membrane docking loops 1 and 3. The data reveal a new segment of PKC-ε important for Z-line anchoring in cardiac myocytes. Complementary experiments with synthetic peptides were generally consistent with the deletion analysis and provided insight into the physiological role of PKC-ε translocation in myocytes. Overall, the data suggest important refinements to existing models of PKC-ε anchoring and translocation.
which are discussed in the context of the crystal structure of the eV1 regulatory domain.

MATERIALS AND METHODS

Molecular biology and protein expression. Amplification of the desired fragments of eV1 was accomplished with PCR. Oligonucleotides flanking the desired regions of the eV1 domain were designed with integral restriction sites for fragment subcloning. Forward-oriented oligonucleotides were engineered with 5′ BamHI sites, and reverse oligonucleotides were engineered with 5′ XhoI sites. The oligonucleotide sequences for forward and reverse primers used for PCR cloning were as follows: for fragment 1–144: 5′-gagctctggactggtgatctcc-3′ and 5′-ggctcggacgactggagcct-3′; for fragment 145–164: 5′-ggctcgagtctcttcattgtctttaggtgc-3′ and 5′-gagctctggactggtgatctcc-3′; for fragment 1–84: 5′-gagctctggactggtgatctcc-3′ and 5′-ggctcggacgactggagcct-3′; for fragment 85–144: 5′-gagctctggactggtgatctcc-3′ and 5′-ggctcggacgactggagcct-3′; for fragment 145–94–144: 5′-gagctctggactggtgatctcc-3′ and 5′-ggctcggacgactggagcct-3′; for fragment 1–93: 5′-ggctcggacgactggagcct-3′ and 5′-gagctctggactggtgatctcc-3′. Custom synthetic DNA was purchased from Operon (Alameda, CA). The gene encoding encoded green fluorescent protein (EGFP) was excised from phosphorylated EGFP (Clontech) and inserted in the histidine-tag vector pTrehisB (Invitrogen) out of frame to prevent EGFP expression. To engineer eV1 fragment protein constructs with EGFP fused to their COOH-termini, cDNA sequences encoding eV1 truncation mutants were inserted in this vector at the 5′-end of the EGFP start codon. This restored the proper reading frame and founded fluorescent green colonies, which facilitated screening. Histidine-tagged GFP-fusion constructs were expressed in DH5α cells and purified with a Ni2+-nitriloacetic acid (NTA) column (Qiagen, Valencia, CA) as described (36). Concentrations of purified GFP-fusion proteins were determined by fluorimetric comparison with standard preparations of GFP. In addition, purified fusion proteins were subjected to 12% PAGE, stained with Coomassie blue, and quantified by visual comparison with BSA standards.

Identification and purities of fusion protein bands were further confirmed by staining with InVision Hs-tag protein stain and by running partially denaturing gels that permitted GFP fluorescence of gel bands to be monitored. Preparations were further purified or discarded if quantification by fluorimetry and PAGE gave mismatched concentration estimates.

Preparation of unlabeled eV1. eV1 was subcloned into pGEX-4T-1 (Amersham Biosciences, Piscataway, NJ) and expressed as a GST fusion protein in Escherichia coli. The protein was purified with glutathione-Sepharose 4B and cleaved from the support with thrombin, according to the manufacturer’s instructions. Purity and concentration were estimated by SDS-PAGE.

Alexa Fluor-568 succinimidyl ester labeling of eV1. Unlabeled eV1 was dialyzed for 3 h against PBS, pH 8.1, to remove Tris and diithiothreitol before labeling. Lyssine-reactive Alexa Fluor-568 succinimidyl ester (Molecular Probes, Eugene, OR) was dissolved in dimethyl sulfoxide at 10 mg/ml and added to dialyzed eV1 solution at a dye-to-protein weight ratio of 1.75. The labeling reaction proceeded for 2 h at room temperature. Unincorporated dye was removed using a Sephadex G50 spin column (Amersham Biosciences). Spectrophotometric analysis of product indicated a protein-to-dye molar ratio of 25:1.

Electron microscopy. Adult rat ventricular myocytes were prepared as described (15), pelleted at 800 g for 45 s, and fixed with 4% paraformaldehyde and 0.5% glutaraldehyde. Additional sample preparation and imaging were performed per standard protocols at the University of Wisconsin Electron Microscopy Facility.

Myocyte decoration. Adult rat ventricular myocytes were permeabilized with 100 μg/ml saponin in relaxing solution (in mM: 100 KCl, 1 MgCl2, 2 EGTA, 4.5 ATP, 10 imidazole, and 1 diithiothreitol, pH 7.0) as described (36). In some cases, cells were permeabilized in fresh 0.5% Triton X-100 (Pierce Biotechnology, Rockford, IL). Permeabilized myocytes were washed extensively in Relaxing Solution and blocked with 2% BSA to prevent nonspecific binding. eV1 fragment GFP protein constructs were applied at a final concentration of 200–800 nM, and Alexa-568-labeled eV1 was applied at 90 nM. Confocal microscopy was performed using 488 nm illumination and 522 nm acquisition for GFP-fusion proteins and 568 nm illumination and 605 nm acquisition for Alexa-568-labeled eV1. The intensity of striations was quantified by two-dimensional fast-Fourier transform (FFT) analysis using the public domain software Image J or a custom script that runs in Origin (Microcal Software, Northampton, MA). First-order peaks in the transformed power spectrum real image were manually circumscribed, and the peak volume was integrated for each image. Integrated volumes were normalized to cell sectional area. Results were similar to those obtained with lineout peak height analysis (36). Striation pattern intensities for fragments of eV1 were normalized to the intensity obtained for an equal concentration of full-length eV1-GFP (1–144) on the same experimental day.

Reversibility of binding was tested by dilution of decorated myocytes with buffer and observing the loss of myocyte fluorescence, as well as by measuring the recovery of fluorescence after regional photobleaching (36). For peptide-blocking experiments, permeabilized myocytes were preincubated with 100 μM EAVSLKPT or 4 μM HDPAGYD for 15 min at room temperature and then decorated with 200 nM eV1-GFP.

Myocyte twitch measurements. Adult rat ventricular myocytes were evaluated within 6 h after enzymatic isolation as described (19). Briefly, myocytes were subjected to electrical field stimulation at 0.5 Hz, 40 volts, and 21–23°C in 1 mM Ca2+ Ringer solution (in mM: 125 NaCl, 5 KCl, 2 NaH2PO4, 5 sodium pyruvate, 1.2 MgSO4, 11 glucose, 0.5 CaCl2, and 25 HEPES, pH 7.4) after setting on the glass floor of a custom perfusion chamber. Twitches were digitized at 30 Hz using a Video Edge detector (Crescent Electronics, Sandy, UT). Peptides were dissolved at a final concentration of 500 nM in 1 mM Ca2+ Ringer and perfused in the stimulation chamber at 0.4–0.5 ml/min. Peptides with an NH2-terminal cysteine (side chain protecting group: 3-nitro-2-pyridinesulfenyl) were prepared on an ABI 432a solid-phase synthesizer with 9-fluorenylmethoxycarbonyl (Fmoc) amino acids and a Wang resin. Peptides were conjugated to antenopedia peptide (5) containing an unprotected NH2-terminal cysteine by amine linking in a 1:2 molar ratio in water under N2 gas for 24 h. The disulfide-conjugated peptides were purified by reverse-phase HPLC using a BioCAD Vision system (PerSeptive Systems), and peptide identities were confirmed by MALDI-TOF mass spectrometry.

RESULTS

Myocyte decoration with eV1-GFP. Decoration of saponin-skinned rat ventricular myocytes with eV1-GFP resulted in a pronounced striated staining pattern that colocalized with the Z-line of the sarcomere (Fig. 1A and Ref. 36). This striation pattern was further characterized here with an emphasis on demonstrating specificity of eV1 binding. 8V1-GFP, an analogous GFP construct derived from PKC-6 (rather than e), gave no striation staining, suggesting that the interaction between eV1 and the Z-line of myocytes was isoform specific (Fig. 1B). A fluorescent eV1 construct was prepared without the use of GFP by covalent modification of lysine side chains with Alexa-568. This fluorescent eV1 construct gave rise to a similar striation pattern to eV1-GFP (Fig. 1C), indicating that COOH-terminal GFP did not contribute significantly to binding. The Alexa-568 probe complements eV1-GFP by being labeled in a completely distinct manner and location, but also

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has advantages over GFP, including brighter fluorescence and an emission that is more spectrally separated from tissue autofluorescence. Specificity in PKC-V1 binding was supported by the observation that PKC-V1-GFP and PKC-V1-Alexa-568 bound reversibly, as demonstrated by both washout and fluorescence recovery after photobleaching (FRAP) experiments (see below). The PKC-V1 domain has previously been shown to bind to phospholipid bilayers (3, 32), so we treated myocytes with Triton X-100 to remove cell membranes. Figure 2 shows a comparison of PKC-V1-GFP staining of saponin-skinned myocytes vs. Triton X-100-skinned myocytes. Saponin is thought to permeabilize membranes (17) by reacting with cholesterol to form 12- to 60-nm pores (39) with minimal disruption of plasma membrane ultrastructure or internal membrane integrity, whereas Triton X-100 causes large-scale membrane solubilization (effects on myocyte ultrastructure are shown in Fig. 3 and are described below). Despite this pronounced difference in the two skinning procedures, PKC-V1-GFP stains both types of skinned myocytes with a marked striation pattern (Fig. 2, A and B). In saponin-permeabilized myocytes, two other types of staining were observed, particularly at high concentrations of fluorescent PKC-V1. One we refer to as longitudinal streaking, which may correspond to labeling of intracellular membranous organelles (e.g., Figs. 1A and 2A). These longitudinal streaks somewhat confounded the regular sarcomeric striations at the highest probe concentrations and placed a practical upper limit on concentrations used for quantitative analyses. The other type of staining was at the intercalated disc, which was particularly prominent with the Alexa-568-PKC-V1 probe (Fig. 1C). Triton X-100-skinned myocytes revealed decoration of sarcomeres only at the Z-line, without detectable longitudinal streaks or intercalated disc staining (Fig. 2B).

Electron micrographs of skinned myocytes showed near-intact mitochondria and T tubules after 100 μg/ml saponin skinning (Fig. 3B), with a trend toward more disruption at 150 μg/ml saponin such as larger-diameter T tubules (Fig. 3C). In contrast, skinning with 0.5% Triton X-100 caused large-scale solubilization of membranes and virtually eliminated all membranes and organelles (Fig. 3D). The presence of a striated binding pattern with both skinning procedures indicated that Triton-insoluble cytoskeletal structures play a major role in PKC-ε anchoring near the cardiac Z-lines.

An important test of specific PKC-V1 binding is to demonstrate competition by unlabeled probes. Preincubation with an unlabeled form of PKC-V1 (containing no GFP or Alexa-568) reduced striated staining of both saponin-skinned and Triton X-100-skinned myocytes (Fig. 4), consistent with competition between PKC-V1 and PKC-V1-GFP for the same sites. This reduction in staining was variable from myocyte to myocyte and was typically incomplete (Fig. 4, summarized in Table 1). It is

![Fig. 1. Representative confocal images of isolated permeabilized myocytes decorated with fluorescent PKC domains.](image1)

![Fig. 2. Comparison of myocyte decoration patterns with two skinning procedures.](image2)
likely that a minor component of εV1-GFP binding to the Z-line/T tubule region may involve association in a nonsaturable, nonblockable manner with phospholipid membranes.

**Myocyte decoration with εV1 fragments.** A systematic deletion strategy was used to define regions within the 144-amino acid domain of εV1 responsible for striation staining of myocytes. A schematic diagram of deletion mutants and a structural representation showing areas of εV1 affected by truncations are shown in Fig. 5. The goal was to create constructs lacking one or more subdomains previously proposed to mediate protein or membrane anchoring of PKC-ε, including EAVSLKPT (residues 14–21), HDAPIGYD (residues 85–92; see Ref. 7), and membrane docking loops 1 and 3 (32). The probes were expressed in *Escherichia coli* and purified to >75% purity as assessed by SDS-PAGE with Coomassie staining (Fig. 5C). In partially denaturing gels, GFP fluorescence was restricted to a single fluorescent band in each lane (data not shown), indicating that minor contaminating bands were nonfluorescent species. InVision His-tag protein stain also identified a single major band at the expected molecular weight for each construct (data not shown). Only construct 94–144 was difficult to express and purify in large quantities, possibly because of poor folding. Constructs were administered over a range of concentrations to saponin- and Triton X-100-skinned myocytes to identify regions of εV1 necessary for binding.

Confocal images showing the effects of deletions on the nature and intensity of staining are shown in Fig. 6. Unexpectedly, deleting the RACK-binding sequence 14-EAVSLKPT-21 did not abolish the striated decoration of saponin-permeabilized rat cardiac myocytes. Construct GFP-(24–144) still labeled myocytes in a striated pattern, although the intensity of the striations was reduced (Fig. 6B). Also surprising was the observation that construct GFP-(1–84) showed only a low level of binding (Fig. 6C) even though it contained the putative 14-EAVSLKPT-21 RACK binding sequence and was lacking the autoinhibitory pseudoRACK sequence 85-HDAPIGYD-92. Similar results were obtained with Triton X-100-skinned myocytes (data not shown).

One rationalization of these results is that high-affinity PKC-ε anchoring might require the presence of both

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Fig. 3. Electron micrographs of isolated myocytes subjected to various skinning methods. A: intact (not skinned) myocyte. B: myocyte skinned with 100 μg/ml saponin. C: myocyte skinned with 150 μg/ml saponin. D: myocyte skinned with 0.5% Triton X-100. Black and white arrowheads indicate T tubules in cross section; Z, Z-line. Scale bars = 1 μm.

Fig. 4. Competition between εV1-GFP and unlabelled εV1. A and B: saponin-permeabilized myocytes stained with 800 nM εV1-GFP for 15 min. C and D: Triton-skinned myocytes stained with 800 nM εV1-GFP for 10 min. In B and D, myocytes were preincubated with 4 μM unlabelled εV1 for 15 min before εV1-GFP. Scale bars = 10 μm.
### Table 1. Effects of translocation inhibitor and activator peptides on eV1-GFP binding

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Control</th>
<th>+ Peptide</th>
<th>P</th>
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<tbody>
<tr>
<td>EVASLKPT (100 μM)</td>
<td>100±21 (9)</td>
<td>89±18 (9)</td>
<td>0.40</td>
</tr>
<tr>
<td>HDAPIGYD (4 μM)</td>
<td>100±14 (21)</td>
<td>126±21 (21)</td>
<td>0.07</td>
</tr>
<tr>
<td>eV1 (unlabelled) (1 μM)</td>
<td>100±19 (15)</td>
<td>41±24 (15)</td>
<td>0.01</td>
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</table>

Data are means ± SE; n. of cells analyzed in parentheses. Saponin-permeabilized myocytes preincubated with vehicle (control) or with peptide (+peptide) for 15 min before 15 min incubation with 150 nM first variable domain of PKC-ε (eV1)-green fluorescent protein (GFP). Striation patterns were quantified by two-dimensional fast-Fourier transform analysis and normalized to controls determined on the same experimental day. P values from a Student’s t-test, with significance taken at P < 0.05.

**EAVSLKPT and HDAPIGYD sequences.** This was tested by studying fragments 85–144 and 94–144, which lacked one or both of these sequences. eV1 fragments missing 14-EAVSLKPT-21 (Fig. 6D) or both 14-EAVSLKPT-21 and 85-HDAPIGYD-92 (Fig. 6E) bound in striated distributions with similar intensity. This observation demonstrates that even a peptide segment (i.e., 94–144) lacking both EAVSLKPT and HDAPIGYD sequences displayed significant decoration at the Z-line and directed our attention to this COOH-terminal aspect of eV1. However, poor expression limited the usefulness of the 94–144 construct (Fig. 5C). A complementary construct containing both 14-EAVSLKPT-21 and 85-HDAPIGYD-92 segments but lacking the COOH-terminus, namely fragment 14–93, expressed very well but bound most poorly of all GFP constructs tested. The GFP-(14–93) fragment appeared to preferentially decorate myocytes in longitudinal streaks (Fig. 6F), a pattern indicative of general membrane localization. Other GFP constructs, but not GFP itself (36), also showed decoration of longitudinal membrane structures when applied at high concentration. At lower concentrations, however, the clear striated pattern was the dominant visible feature for all constructs except fragment GFP-(14–93).

**FFT analysis of striation patterns.** To facilitate quantification of staining intensities for GFP constructs, we performed two-dimensional (2D)-FFT analysis of images of the type shown in Fig. 6. A quantitative comparison of striation intensities of all probes analyzed by 2D-FFT image analysis is given in Fig. 7A. The regular sarcomeric autofluorescence pattern of cardiac myocytes results in a background signal in the power spectrum at the same sarcomere length as the additional fluorescence signal of bound probe. This autofluorescence background places a lower limit on the detection of low-intensity signals, and is listed in Fig. 7A as “no probe.” A statistical analysis of differences was carried out by a Student’s t-test with significance taken as P < 0.05. In general, the data showed that the full-length probe bound more effectively than any of the truncated constructs, whereas GFP-(14–93) bound most poorly. Other probes gave intermediate quantitative binding values consistent with visual impressions of the intensities of the striated patterns in the image data. Binding of fragments of eV1-GFP did not appear to be hampered by nonspecific binding at concentrations up to 500 nM, as striation patterns were readily and completely reversed by washout or by FRAP with the exception of GFP-(14–93), see below. Integrated first-order peak volumes associated with the different fragments were not systematically correlated to fragment length (R² = 0.61; Fig. 7B), further arguing against nonspecific binding.

**Estimates of affinity and binding energy.** The observed differences in binding of the various fragments held across a range of concentrations. Figure 7C shows the concentration dependencies for the NH₂-terminal fragment [GFP-(1–84)] and two COOH-terminal fragments [GFP-(21–144) and GFP-(85–144)]. The values shown are means ± SE minus autofluorescence background. Because the experiments were performed at different microscope settings to maintain quantitative linearity, each data point was normalized to a reference measurement of eV1-GFP on the same experimental day. Data from all experiments were then scaled to a previously determined eV1-GFP binding isotherm (36) and plotted as in Fig. 7C. Hyperbolic fits of the form y = ax/(b + x), where “a” was constrained to maximal GFP-(1–144) binding, gave estimates of dissociation constant (Kd) as follows: 230 nM for GFP-(1–144); 750 nM for GFP-(24–144); 880 nM for GFP-(85–144); and 4.8 μM for GFP-(1–84). Thus the data suggest that the COOH-terminal fragments GFP-(24–144) and GFP-(85–144) possess ~3-fold lower affinity than the full-length eV1 domain, whereas the NH₂-terminal GFP-(1–84) is ~20-fold lower in affinity than full length. These changes in affinity were translated into changes in free energy using the following relationship: ΔΔG = −RT ln(Kd/Kd2), where R is the universal gas constant, T is temperature, Kd is the dissociation constant, T is temperature, Kd2 is the dissociation constant corresponding to 20-fold lower affinity.
constant for GFP-(1–144), and \( K_d \) is the dissociation constant for the test fragment. The losses of binding energy, or \( \Delta \Delta G_s \), resulting from various deletions were estimated to be 1.2 RT for deletion of NH\(_2\)-terminal sequences up to residue 23 vs. 3.0 RT for deletion of COOH-terminal sequences beyond residue 85. Thus, in this myocyte decoration assay, elimination of COOH-terminal segments of the εV1 domain had a greater impact on affinity (by 7-fold) and on binding energy (by 2.5-fold) than did elimination of NH\(_2\)-terminal segments.

Reversibility. Z-line decoration by GFP constructs was generally reversible, with complete washout occurring within several minutes (data not shown). Moreover, dilution of equilibrated probes caused relaxation to a new steady state on a time scale compatible with previously determined kinetics (half-time = 2–5 min; Ref 36). The one εV1 fragment that failed to demonstrate reversibility was GFP-(14–93). At high concentrations, this probe showed conspicuous general membrane binding, little Z-line decoration, and incomplete washout, even after 30 min. Otherwise, all GFP-tagged εV1 fragments washed out of myocytes fully over the course of 5–10 min.

Peptide modulators of PKC translocation. Competition studies were performed with synthetic peptides widely used to block or stimulate PKC-ε translocation (7). The inhibitory peptide EAVSLKPT only modestly reduced binding of full-length εV1-GFP, even at the relatively high concentration of 100 μM peptide. Cell-to-cell variability prevented this effect from achieving statistical significance (summarized in Table 1). This may be an indication that membrane binding contributes significantly to εV1 binding in this system or that EAVSLKPT represents only a portion of the binding interface, as suggested by the deletion analysis described above. Interestingly, the loop 3 peptide HDAPIGYD also did not reduce binding but tended to enhance the intensity of εV1-GFP striation staining, consistent with the notion that this peptide may stimulate PKC-ε translocation and anchoring under some conditions (7). Cell-to-cell variability was also large for these
experiments (Table 1). These peptide competition experiments generally supported the conclusions of the deletion analysis that neither 14-EAVSLKPT-21 nor 85-HDAPIGYD-92 behaved as exclusive anchoring interfaces for PKC-ε binding to cardiac Z-lines.

Functional studies with these peptide modulators provided insight into a possible cause of cell-to-cell variability. Exposure of isolated rat ventricular myocytes to a cell-permeant form of EAVSLKPT (at 500 nM) was without effect on myocyte twitches (data not shown), whereas a cell-permeant form of HDAPIGYD (at 500 nM) gave rise to a strong enhancement of the myocyte twitch amplitude and a prolongation of the twitch time course (Fig. 8). This observation is consistent with activation and translocation of PKC-ε by HDAPIGYD in this cell system (19). It is important to note that only 35% of myocytes tested showed this response to HDAPIGYD, with the remainder showing normal twitches and no inotropic response was observed in 14 out of 40 myocytes (35%), with mean ± SE increases of 42 ± 13% in twitch amplitude and 19 ± 6% in twitch duration. The 26 nonresponding cells showed no change in twitch amplitude or time course.

DISCUSSION

Two models have been advanced to account for anchoring of PKC-ε to membranes and other cellular structures via its unique 144- amino acid εV1 domain. The work of Dorn and Mochly-Rosen (7) identified an eight-amino acid sequence within the εV1 domain that interacted with a cloned RACK2. This sequence [EAVSLKPT at residues 14–21 of εV1 (see Fig. 5 for location of residues in a ribbon structure)], was hypothesized to be the binding interface responsible for PKC-ε translocation and anchoring. The work of Mochly-Rosen and coworkers (7, 8, 26, 38) also provided evidence for the existence of an intramolecular interaction with another sequence (HDAPIGYD at residues 85–92) that sequestered the anchoring interface when PKC-ε was inactive. From this one might predict that deletion of 14-EAVSLKPT-21 would eliminate εV1 binding, whereas deletion of 85-HDAPIGYD-92 would activate binding by relieving autoinhibition.

An alternative model has been proposed in conjunction with a recent report of the crystal structure of the εV1 domain (3, 32). Several charged and hydrophobic amino acids were identified in the structure that could participate in binding of εV1 to phospholipid membranes (3, 24, 32). In this model, loop 1 (residues 17–30) and loop 3 (residues 85–92), which are well separated in the εV1 primary sequence, cooperate in a manner analogous to membrane binding of conventional Ca2+-sensitive C-2 domains. Although the ε isoform does not bind Ca2++, a coordinated Mg2+ may serve a similar purpose of mediating interactions with charged phospholipid head groups. If this mechanism was responsible for the observed εV1 binding in myocytes, deletions affecting loops 1 and 3 should reduce myocyte decoration.

Taken together, the anchoring models of Mochly-Rosen et al. (26) and Ochoa et al. (32) focus primarily on two regions of the NH2-terminus of the variable domain of PKC-ε (Fig. 5 for a ribbon structure). First, loop 1, comprised of amino acids 17–30, contains most of the putative RACK2 interaction interface, 14-EAVSLKPT-21, and putative membrane docking residues within 22–30. Second, amino acids 85–92 represent both the pseudoRACK sequence (85-HDYPGYD-92) and additional membrane docking residues (loop 3). Here, we tested these models by making NH2- and COOH-terminal deletions in the εV1 variable domain and then quantifying their ability to bind Z-lines of cardiac myocytes.

Several important conclusions emerge from the data. First, the discrete RACK-binding sequence 14-EAVSLKPT-21 identified by Johnson et al. (16) is not likely to be the sole mediator of binding in this experimental context. Deletion of this sequence in the probe GFP-(24–144) significantly reduced but did not abolish binding. Second, dissection of εV1 into two complementary subdomains, an NH2-terminal [GFP-(1–84)] and a COOH-terminal (85–144-GFP) fragment, revealed that both NH2- and COOH-terminal fragments decorated cardiac cells in a striated pattern, albeit less intensely than when they were integrated as full-length GFP-(1–144). It seems, therefore, that rather than being mediated by a small, discrete binding sequence, the anchoring interaction may involve multiple binding regions distributed along the εV1 primary structure.

Such a “distributed interface” for εV1 binding is compatible with the Ochoa et al. model involving surface loops 1 and 3, which align at one end of the molecule in the crystal structure. These loops appear to play a critical role in membrane docking via phosphatidic acid and phosphatidylycerine head groups both in vitro (3) and in vivo (24). However, such a membrane docking model is an incomplete explanation of the present results, since it does not account for apparent saturation of binding of the εV1 probe, nor does it explain the involvement of regions COOH-terminal to loop 3. Consistent with this general view of εV1 anchoring in cardiac myocytes, we also found that the EAVSLKPT peptide blocker widely used to disrupt RACK2/PKC-ε interactions (7, 12, 13, 16, 26) sponsored modest inhibitory effects, although these did not achieve statistical significance. The loop 3 peptide HDAPIGYD also
did not inhibit but tended to enhance εV1-GFP binding in this system, consistent with its proposed role as a translocation activator (7, 8). We also observed a positive inotropic effect of the loop 3 peptide in a subset of myocytes, consistent with the expected physiological effects of stimulating PKC-ε translocation and anchoring in adult myocytes (19).

Importantly, transgenic mice expressing the same translocation inhibitor peptide (EAVSLKPT) or translocation activator peptide (HDAPIGYD) revealed profound physiological consequences in vivo despite only a 20% change in the distribution of PKC between cytosolic and particulate compartments (8, 26). Such small equilibrium shifts may not be easily resolvable in the in vitro binding assay described here. Thus a weakness of single myocyte imaging studies may be that relatively modest changes in binding are masked by relatively large variability in binding from cell to cell. In previous experiments using myocyte populations and Western blotting, we did observe inhibition of full-length PKC-ε binding to the cardiac Z-line by EAVSLKPT (albeit only 60% blockade at 50 μM peptide; see Ref. 13). This partial inhibition by the EAVSLKPT peptide is also generally compatible with the results of the present study suggesting the existence of additional determinants of Z-line anchoring.

In the myocyte decoration assay described here, fluorescent ligands may encounter many potential binding surfaces, so specificity is an important consideration. Extra caution may be necessary given the highly regular sarcomeric structure and unique composition of the Z-line/T tubule region of myocytes, which may conspire to exaggerate regular patterns in nonspecific binding or in tissue autofluorescence. A number of observations argue for the selectivity of the observed Z-line decoration. Striation pattern intensity was not correlated to fragment length \( R^2 = 0.61 \), and Z-line decoration of GFP constructs was readily reversible [with the exception of GFP-(85–144)]. Binding of εV1-GFP was saturable, with a concentration dependence well described by the hyperbolic function \( y = ax/(b + x) \) (see Refs. 13 and 36). Binding of εV1-GFP was also significantly reduced by preincubation with unlabeled εV1 protein. In both Triton X-100- and saponin-skinned myocytes, the striated binding pattern was not observed for GFP alone at concentrations beyond 1 μM (36). εV1 labeled with Alexa-568 on lysine side chains (instead of a COOH-terminal GFP) decorated the Z-line much like εV1-GFP. A fluorescent protein fusion of residues 1–130 from the NH₂-terminal variable domain of PKC-δ, δV1-GFP, did not decorate cardiac Z-lines at concentrations up to threefold higher than used for εV1-GFP. This isoform specificity is consistent with the finding that activated PKC-ε bound much more effectively to Z-lines of cardiac myofilaments than did activated PKC-δ (13, 14). Thus the weight of evidence is consistent with a specific binding site on the cardiac Z-line for the V1 domain of PKC-ε.

With regard to the likely involvement of both lipids and proteins in the mechanism of PKC-ε translocation, Souroujon et al. (40) recently described a monoclonal antibody that recognizes an epitope on the εV1 domain that is exposed only transiently, after lipid activation but before RACK anchoring. This result suggests the existence of an intermediate state in which activated and membrane-associated PKC searches for a receptor (i.e., RACK) along the plane of the membrane (25, 27). Of these two “translocated states” of PKC-ε, the RACK-bound state but not the lipid membrane-associated state would be expected to be blocked by competitive inhibitors. Overall, the data presented here are consistent with the existence of both a specific “blockable” (perhaps anchoring protein-mediated) and a minor “nonblockable” (perhaps lipid membrane-mediated) component of εV1-GFP binding at the cardiac Z-line/T tubule.

Strong binding of GFP-(85–144) provides evidence that a critical determinant of anchoring lies in a COOH-terminal stretch of sequence. A similar observation was reported by Lehel et al. (22), who deleted specific domains of full-length PKC-ε and examined the effects on translocation in NIH 3T3 cells. A 33-amino acid subdomain just upstream of the C-1 domain was found that strongly biased PKC-ε translocation to (unknown) cytoskeletal elements. The region identified by Lehel et al. overlaps the final 10 amino acids of the COOH-terminus of εV1 investigated here. Clearly, models of εV1 and PKC-ε anchoring need to be refined to account for the observation that probes encompassing either the COOH-terminus or the NH₂-terminus of εV1 decorate the Z-line of permeabilized myocytes. One possibility is that the COOH-terminal and NH₂-terminal segments of εV1 anchor to sites that are physically separate on a “saturable receptor,” whereas full-length εV1 is capable of binding these sites simultaneously. In this way, the εV1 domain could communicate with separate and distinct aspects of the anchoring protein, or even bind to multiple members of a signaling complex (1, 43).

The crystal structure of εV1 was used to incorporate most of the available data in a more complete model of anchoring (Fig. 9). Most importantly, Fig. 9 projects on the crystal structure

![Model of PKC-ε anchoring at the cardiac Z-line/T tubule.](http://ajpheart.physiology.org/)

Fig. 9. Model of PKC-ε anchoring at the cardiac Z-line/T tubule. Ribbon structure: A, interaction of 14-EAVSLKPT-21 (red) with receptor for activated C kinase (RACK2; see Refs. 4 and 6); B, interaction of loop 1 with membranes (3, 32) via insertion of hydrophobic 23Trp residue in the bilayer; C, interaction of loop 3 (green) with membranes (3, 32) via insertion of hydrophobic residues 89Ile,91Tyr in the bilayer while 86Asp and 92Asp coordinate an Mg²⁺ that in turn interacts with phospholipid head groups; D, point of convergence between 85-HDAPIGYD-92 (green; equivalent to loop 3), 14-EAVSLKPT-21 (red), and COOH-terminal residues 94–144 (orange). The site of interaction between PKC-ε and the cardiac Z-line is hypothesized to be composed of all or part of the red/orange surface. Space-filling structure: E, prominent side chain interaction between 122Glu (orange) and 144Lys (red) and F, prominent side chain interaction between 93Asp (orange) and 26Arg (gray).
how the COOH-terminal aspect of εV1 (orange) contributes to a surface that is contiguous with the EAVSLKPT sequence (red) identified by Mochly-Rosen and colleagues (12, 16, 26). We propose that the COOH-terminal aspect of εV1 (residues 94–144) participates in PKC-ε anchoring, either by contributing to a RACK2 interaction interface, by binding to other unidentified anchoring proteins, and/or by stabilizing the degree of the εV1 domain and preventing inappropriate intramolecular interactions between 14-EAVSLKPT-21 and 85-HDAPIGYD-92.

It is of interest to know precisely how the HDAPIGYD peptide promotes translocation of PKC-ε. The suggestion by Mochley-Rosen and coworkers (8, 38, 40) was that it disrupted an autoinhibitory interaction between loop 3 and EAVSLKPT. The present results can be interpreted in the context of such an autoinhibitory interaction. In particular, the finding that the 14–93 construct does not bind cardiac Z-lines is generally compatible with enhanced interactions between 14-EAVSLKPT-21 and 85-HDAPIGYD-92 in the absence of residues 1–13 and 94–144. However, 14-EAVSLKPT-21 (red) and 85-HDAPIGYD-92 (green) do not appear to interact directly in the crystal structure of 1–144 (32), although, at one residue away, 93Asp forms a prominent ionic interaction with 26Arg farther along in loop 1 (Fig. 9F). The fact that both binding and twitch amplitudes were enhanced by exogenous application of the negatively charged HDAPIGYD peptide suggests that conformational changes induced by its binding to the εV1 domain promote a structural reorganization that facilitates anchoring. One possibility is that endogenous loops 1 and 3 move away from the core structure, permitting these loops to interact with membranes. Such conformational changes may also stabilize or make more accessible the putative anchoring interface formed by juxtaposition of 14-EAVSLKPT-21 (red) and side chains from the COOH-terminal domain (orange). In the surface rendering of εV1, a number of interactions between these regions are quite prominent, including a salt bridge between residues 19Lys (red) and 122Glu (orange in Fig. 9E), H-bonds, and hydrophobic interactions (data not shown).

It is also of interest to know precisely what the εV1 regulatory domain binds to at the cardiac Z-line. We consider it unlikely that εV1-GFP binds exclusively to the T tubule lipid bilayer as the models of Corbalan-Garcia (3) and Ochoa et al. (32) might suggest. The minimum sequence that decorated Z-lines is 94–144, a segment located on the opposite side of the εV1 domain from the Ochoa et al. membrane interface. T-tubular membrane staining typically appears punctate in the confocal microscope because of the "discontinuous" nature of the T tubule network when sampled by optical sectioning. On this basis, εV1-GFP and its subfragments appear to bind to a more continuous cytoskeletal structure such as the Z-line itself. Moreover, εV1-GFP decorated Z-lines of Triton X-100-skinned myocytes despite the loss of membrane ultrastructure visible by electron microscopy. Protein–protein interactions that mediate Z-line anchoring of PKC-ε remain to be fully elucidated, but early indications tend to rule out F-actin and Cypher-1 as candidate anchoring proteins at this location (13).

In conclusion, evaluation of Z-line binding of truncated fragments of the εV1 domain (labeled at their COOH-termini with GFP) leads us to suggest refinements to current models of PKC-ε anchoring. The NH2-terminal region containing the 14-EAVSLKPT-21 RACK binding sequence probably contributes to but is not solely responsible for PKC-ε binding to Z-lines. Our data support a model in which multiple regions of the εV1 domain dictate anchoring in myocytes, including a novel segment within residues 94–144 at the COOH-terminus of the first variable regulatory region of PKC-ε. In the crystal structure of εV1, the 14-EAVSLKPT-21 sequence forms a contiguous surface with the 94–144 subdomain, extending tens of angstroms away from the proposed plane of the inner bilayer leaflet (32). This surface provides a cytoplasmic interaction interface through which PKC-ε can bind to adjacent proteins or protein complexes. Importantly, the previously proposed autoinhibitory 85-HDAPIGYD-92 sequence forms no obvious contacts with 14-EAVSLKPT-21 in the crystal structure, but both of these peptide segments form side chain interactions with COOH-terminal side chains. This proposed COOH-terminal anchoring interface is well-positioned to complement Mg2+–dependent interactions with phospholipids and is compatible with additional membrane interactions via diacylglycerol binding to the adjacent C-1 domain of full-length PKC-ε.

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REFERENCES


