Protein kinase C (PKC) isozymes translocate to unique subcellular sites following activation. We previously suggested that translocation of activated isozymes is required for their function and that in addition to binding to lipids, translocation involves binding of the activated isozymes to specific anchoring proteins (receptors for activated protein kinase C). Using cultured cardiomyocytes we identified inhibitors, the V1 fragment of ePKC (eV1), and an 8-amino acid peptide derived from it that selectively inhibited the translocation of ePKC. Inhibition of ePKC translocation but not inhibition of δ or βPKC translocation specifically blocked phorbol ester- or norepinephrine-mediated regulation of contraction. These isozyme-selective translocation inhibitors provide novel tools to determine the function of individual PKC isozymes in intact cells.

Activation of protein kinase C (PKC) isozymes is associated with translocation of the enzymes from the cell soluble to the cell particulate fraction (1). These isozymes are activated by binding to lipid-derived second messengers and negatively charged phospholipids present in the cell particulate fraction (2, 3). In addition to binding to lipids, specific anchoring proteins participate in binding the activated PKC isozymes to this fraction (4–9). We collectively termed these proteins RACKs, for receptors for activated protein kinase C (7, 10).

In cultured neonatal cardiomyocytes, immunofluorescence studies demonstrated isozyme-specific subcellular localization following activation with either 4-β phorbol 12-myristate-13-acetate (PMA) or with norepinephrine (NE) via an α1-adrenergic receptor (11, 12). Similar isozyme-specific localization was found in other cells following PKC activation (e.g. (13)). This isozyme-specific localization suggests that unique sequences in each isozyme (14) contain at least part of the recognition site for the anchoring molecules, the isozyme-specific RACKs.

Here, we focus on the ePKC unique region, eV1, which is the largest variable region in this isozyme. Some homology between eV1 and the C2 region of the classical PKCs, α, β, and γ, was noted (15). Because C2 contains at least part of the RACK-binding site of classical PKCs (16, 17), an ePKC specific RACK-binding site may reside within eV1. In that case, an eV1 fragment should bind to the ePKC-specific RACK when introduced into cells and thus inhibit PMA- or hormone-induced ePKC translocation and binding to that RACK. Translocation of other PKC isozymes should not be affected by eV1. The following study confirms these predictions and demonstrates the use of translocation inhibitors to determine the role of specific isozymes in regulating cardiac contraction.

EXPERIMENTAL PROCEDURES

Peptides and Reagents—Peptides eV1–1 (EAVSLKPT; ePKC (14–21)), scrambled eV1–2 (LSETKPAV), eV1–3 (LAVFHDHA; ePKC (81–87)), eV1–2 (EAVGLQPT; ePKC (18–25)), and βC2–4 (SLNPENWNE; βPKC (218–226)) were synthesized at the Beckman Center Protein and Nucleic Acid Facility at Stanford. All the peptides used in this study were over 90% pure. 4-β PMA was from LC Laboratories Inc, and diisoyerylcar and phosphatidylycerine were purchased from Avanti Inc. PKC was partially purified from rat brain and assayed as described previously (18).

Expression and Purification of the V1 Regions—The V1 regions of e and δPKC (amino acids 2–144) were amplified by polymerase chain reaction from a rat cDNA library (Stratagene) and tagged with a FLAG epitope (DYKDDDDK) at the 5′ end of the fragments, and the polymerase chain reaction fragments were subcloned into pMAL-c2 vector (New England BioLabs) for overexpression as fusion proteins with maltose binding protein in Escherichia coli. Protein purification and factor Xa proteolysis of the fusion proteins were as before (19), and the resulting V1 fragments were >90% pure.

Permeabilization of Neonatal Rat Cardiomyocytes Culture and Immunolocalization of PKC Isozymes—Primary neonatal rat cardiomyocytes were cultured on chamber slides as described previously (12) and transiently permeabilized with saponin (50 μg/ml) with or without 150 μg/ml of rat recombinant PKC fragment, eV1 or δV1.2 PKC isozyme localization was determined by immunofluorescence in cells fixed with methanol and acetone (12). The anti-δ and ePKC antisera (Research and Diagnostic Antibodies, Inc.) do not recognize the corresponding V1 fragments. Multiple randomly selected microscopic fields were monitored for each study to determine the percentage of cells having the tested isozymes at the activated site. There was no bias in scoring; in an additional blind study, essentially identical results were obtained.

The following criteria were used to confirm that the immunostaining is specific for the monitored isozymes. No immunostaining is obtained by antisera preadsorbed with the corresponding immunizing peptide or by the corresponding sf9-expressed isozyme (Ref. 12 and data not shown.) We also showed that there was a complete correlation between translocation from the cell soluble to the cell particulate fraction as measured by Western blot analysis (using antibodies from Life Technologies, Inc.) and translocation measured by immunofluorescence using the same antibodies as in this study (12, 21, 22). Furthermore, the results obtained with the commercial anti-ePKC are indistinguishable from those that we previously obtained with our own monoclonal antibody CK 1.4 (11). Finally, localization of PKC to cross-striated structures in heart was reported by Kuo and collaborators using another antiseraum (23).

Monitoring of Cardiomyocyte Contraction Rates—Cells were cultured and permeabilized with saponin as above in the absence or the presence of 150 μg/ml δV1 fragment, eV1 fragment, or the indicated concentration of various PKC-derived peptides. Basal contraction rates were then monitored for 10 min to ensure stable contraction rates following permeabilization. (The rates of contraction after permeabilization with
FIG. 1. The \( \epsilon V1 \) fragment specifically inhibits 4-\( \beta \) PMA-induced translocation of \( \epsilon \)PKC in neonatal rat cardiomyocytes. \( A \), translocation of \( \epsilon \)PKC after a 20-min incubation with 3 nM 4-\( \alpha \) (open bar) or 4-\( \beta \) PMA (hatched bar) in cells permeabilized in the absence (--) or the presence of \( \epsilon V1 \). \( B \)–\( F \), as in \( A \), except cells were incubated for 5 min with 100 nM PMA and translocation was determined for \( \epsilon \) (\( B \)), \( \delta \) (\( C \)), \( \beta \) (\( D \)), and \( \alpha \)PKC (\( E \)) after permeabilization in the absence (--) or the presence of \( \epsilon V1 \) or \( \delta V1 \) fragments, as indicated. Data are percentage of cells having the tested isozyme at the activated site. The data in \( A \) are the means \( \pm \) S.E. of three independent experiments. In \( B \)–\( E \) are the means \( \pm \) S.E. obtained by scoring 27 random microscopic fields from a single experiment, with greater than 700 cells scored for each treatment group in each experiment. Similar data on translocation were obtained by several experimenters. 

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vehicle, δV1, or eV1 fragment were 49 ± 5, 50 ± 6, and 40 ± 6 contractions per 15 s (mean ± S.E. of 12, 6, and 12 experiments, respectively). The cells were then treated with 4-β PMA, and the rate of contraction was monitored at the indicated times as described previously (22).

**RESULTS**

To determine whether the first variable region of ePKC, eV1, contains a specific anchoring site of the enzyme, cardiomyocytes were transiently permeabilized by saponin with or without eV1, and the subcellular localization of different PKC isozymes following PMA stimulation was determined (Fig. 1). Transient permeabilization alone did not affect many cellular functions including cell viability, spontaneous and stimulated contraction rates (see Figs. 2 and 3), gene expression, and hypertrophy. The intracellular concentration of the fragment was ~10% of that applied. As in a previous study (12), we found that in cardiomyocytes, activated αPKC is localized to the nuclear boundary (Fig. 1, F, j), activated βIPKC is localized inside nuclei (not shown), activated δPKC is localized to perinuclear structures (Fig. 1, F, f), and activated ePKC is localized to cross-striated structures (Fig. 1, F, h). Introduction of eV1 caused selective inhibition of translocation of ePKC (Fig. 1A) but not of α, βI, or δPKC (not shown) to their corresponding subcellular sites following treatment with 3 nM 4-β PMA. eV1 also caused partial inhibition of ePKC (see Fig. 1, F, c versus b) but not α, δ or βIPKC translocation (Fig. 1, B–F) when the isozymes were fully activated (22) with 100 nM 4-β PMA. Therefore, eV1 fragment appears to compete with endogenous activated ePKC and selectively prevents its translocation and binding to cross-striated structures. As a control, we used cells that were transiently permeabilized with the first variable region of δPKC, the δV1 fragment. We found that δV1 selectively inhibited 4-β PMA-induced translocation of δPKC (Fig. 1, F, h versus f) but not the translocation of α, βI, or ePKC, to their respective subcellular sites (Fig. 1, B–F). Therefore, eV1 and δV1 probably contain a corresponding PKC-specific RACK-binding site and can be used as isoyme-selective inhibitors of activation-induced translocation.

If stimulation-induced translocation of PKC isozymes is required for their unique functions, then introduction of the above isozyme-specific translocation inhibitors into cardiomyocytes should inhibit these functions. We focused on the effect of eV1 on 4-β PMA and α1-adrenergic regulation of contraction, because activated ePKC and the contractile apparatus in cardiomyocytes are both located in cross-striated structures (10) and because correlation studies using 4-β PMA implicated ePKC in this function (22). Cardiomyocytes in culture contract spontaneously at ~200 beats/min and incubation with 4-β PMA reduces this rate (22). We determined the effect of eV1 on this 4-β PMA-induced negative chronotropy. eV1 or δV1 fragments were introduced into the cells by transient permeabilization with saponin as above. Following removal of saponin, a stable rate of spontaneous contraction resumed within a few minutes. In cells permeabilized with vehicle alone or with δV1, 4-β PMA inhibited the basal contraction rate by an average of 70–85% (Fig. 2, A and B). In contrast, following permeabilization in the presence of eV1, the 4-β PMA-induced negative chronotropy was abolished even 60 min after PMA addition (3–10 nM; Fig. 2C). This effect was dependent on PMA dose, with an average of 50% inhibition by eV1 in cells treated with 100 nM 4-β PMA for 60 min (n = 3). Of interest, eV1 did not affect the catalytic activity of partially purified ePKC (~100 units/mg) in vitro; phosphorylation of a purified 55-kDa ePKC substrate derived from cardiomyocyte sarcoplasmic reticulum in the presence of eV1 was 118 ± 17% of that in its absence (mean ± S.E., n = 3). Furthermore, because eV1 specifically inhibited 4-β PMA-induced translocation of ePKC but not the translocation of δ, βI or αPKC (Fig. 1, A and B versus C, D, and E), these data suggest that eV1 antagonizes 4-β PMA regulation of contraction by inhibiting translocation of ePKC to its site of action.

We next determined whether eV1 also inhibits hormone-induced regulation of contraction rate in cardiomyocytes. These cells contain α1- and β1-adrenergic receptors (ARs), which are coupled to activation of PKC and cAMP-dependent protein kinase, respectively. Stimulation with NE, which activates both ARs, resulted in a transient increase in contraction rate (Fig. 3A). Inhibition of both ARs with prazosin and propranolol followed by stimulation with NE inhibited the effect of NE (Fig. 3B). Inhibition of the β1-AR by propranolol allowed the effect of α1-AR activity to be manifested and hence caused a 4-β PMA-like reduction of contraction rate (albeit to a smaller extent, Figs. 3C versus 2A). In contrast, specific inhibition of the α1-AR by prazosin caused a greater increase in the immediate rise in NE-induced contraction rate and a longer period of elevated rate than that observed with NE alone (e.g. see 40 min after drug addition; Fig. 3, D versus A). Therefore, α1-AR activation, which results in translocation of all the PKC isozymes in these cells (12), causes negative chronotropic effects and attenuates the positive chronotropic effect of β1-AR stimulation.

If these α1-AR-stimulated effects on contraction are mediated by ePKC, then cells containing eV1 should respond to NE alone as though prazosin were present. Indeed, NE caused a greater and a more sustained increase in the rate of contraction in cells permeabilized in the presence of eV1 as compared with cells permeabilized in the absence of any fragment or with cells permeabilized in the presence of δV1 (Fig. 3E). These data indicate that α1-AR effects on contraction rate are me-

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1 M. M. Rodriguez and D. Mochly-Rosen, unpublished data.
vehicle, respectively, and four, three, and four independent experiments for panels A in independent experiments for A described for A inhibited 4-
effect on translocation and function of peptides (Fig. 4C).

Diomyocytes selectively inhibits sus B indicated times. Time of NE addition is indicated by panel 150
thesized based on the sequences conserved between Aplysia 14–21, 81–87, 92–100, and 116–125, respectively, were syn-
e diomyocytes. The contraction rates following exposure to 1 μM NE (A); NE, 1 μM prazosin (PRAZ), and 25 nM propranolol (PROP) (B); NE and 25 nM propranolol (C); or NE and 1 μM prazosin (D) were monitored for the indicated times. Time of NE addition is indicated by arrows in each panel. In E, cells were first permeabilized in the presence of vehicle ( ), 150 μg/ml of V1 fragment ( ), or V1 fragment ( ) and then treated as described for A. The results are the means ± S.E. of four and five independent experiments for panels A and B and panels C and D, respectively, and four, three, and four independent experiments for vehicle, V1, and V1-treated cells in E, with monitoring of four cells in each experiment.

diated by εPKC and that this εPKC-selective translocation inhibitor also blocks hormone-induced regulation of function in cardiomyocytes.

Finally, short peptides derived from the C2 region of βPKC (e.g. βC2–4) selectively inhibit translocation and function of C2-containing isozymes (17, 24). We synthesized several short peptides derived from the εV1 region and determined their effect on translocation and function of εPKC. The peptides εV1–1, -2, -3, -4, and -5 corresponding to amino acids 5–11, 14–21, 81–87, 92–100, and 116–125, respectively, were syn-
thesized based on the sequences conserved between Aplysia and rat εPKC (15). We reasoned that conserved sequences between the evolutionarily remote species are likely to be functionally important. Of the five peptides tested, only εV1–2 inhibited 4-β PMA-induced εPKC translocation (Fig. 4, D versus B). Translocation of βI or βPKC was not inhibited by this peptide (Fig. 4C). Therefore, at least part of the RACK-binding site on εPKC is localized between amino acids 14–21 in the V1 region. Furthermore, εV1–2 but not βC2–4 (a translocation inhibitor specific for C2-containing isozyme (17)) inhibited the 4-β PMA-induced negative chronotropic effect in cardiomyocytes (Fig. 4E) at intracellular concentrations as low as 10 nM (not shown). No inhibition of the 4-β PMA effect on contraction was observed in the presence of 1–10 μM of the other εV1-derived peptides, a γPKC-derived peptide that is highly homologous to εV1–2 (EAVgLqPT; lower case for amino acid substitution), or a scrambled εV1–2 (not shown). Therefore, an εV1-derived octapeptide selectively inhibits εPKC translocation and function.

DISCUSSION

Although PKC has been a focus of research for many years, understanding of the role of individual members of this family of isozymes was hindered by the lack of isozyme-selective inhibitors. As Wilkinson and Hallam commented in a recent review (25), “A full understanding of the role of the specific
PKC isotypes in physiological and pathophysiological processes awaits the development of yet more specific activators and isoenzyme-selective inhibitors.” The work described above directly addresses this issue.

Neonatal cardiomyocytes contain at least six different PKC isozymes (12, 22, 26, 27) that translocate following NE or 4-βPMA treatment (11, 12). Because 4-βPMA and NE induce a variety of physiological responses including hypertrophy, modulation of contraction rate, gene expression, and organization of contractile elements (22, 28–32), it is likely that each isozyme mediates a different function. Our data indicate that translocation of ePKC to the cross-striated structure but not the translocation of other isozymes such as α, βI, or δPKC to their subcellular sites is required for 4-βPMA and NE modulation of spontaneous contraction rate. Therefore, as we predicted earlier (10, 11), the subcellular localization of the activated isozymes influences the specific function and presumably the substrates of each PKC isozyme.

Recently, the C1 and V3 regions of ePKC were suggested to determine subcellular localization of inactive ePKC. Nonactivated overexpressed ePKC was localized to multiple subcellular sites including the Golgi apparatus, whereas a C1-containing fragment, for example, was localized only to Golgi (33, 34). The effect of the C1 fragment on the localization and function of either the overexpressed full-length ePKC or the endogenous enzyme were not determined. In addition, an ePKC-unique C1-derived pentapeptide representing an actin-binding sequence (20) was found to inhibit ePKC binding to actin in vitro. However, the function of this binding in vivo has not yet been determined. In the present work, we showed that the V1 region is required for anchoring of activated ePKC and that inhibition of the anchoring by the V1 region or by amino acids 14–21 (eV1–2) are sufficient to inhibit translocation and function of the activated ePKC in intact cells. Therefore, PKC translocation is required for its function, and translocation inhibitors can be used to obtain isozyme-selective inhibition of PKC-mediated functions.

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