Peptides Derived from the C2 Domain of Protein Kinase Cε (εPKC) Modulate εPKC Activity and Identify Potential Protein-Protein Interaction Surfaces

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Peptides derived from protein kinase C (PKC) modulate its activity by interfering with critical protein-protein interactions within PKC and between PKC and PKC-binding proteins (Souroujon, M. C., and Mochly-Rosen, D. (1998) Nat. Biotechnol. 16, 919–924). We previously demonstrated that the C2 domain of PKC plays a critical role in these interactions. By focusing on εPKC and using a rational approach, we then identified one C2-derived peptide that acts as an isozyme-selective activator and another that acts as a selective inhibitor of εPKC. These peptides were used to identify the role of εPKC in protection from cardiac and brain ischemic damage, in prevention of complications from diabetes, in reducing pain, and in protecting transplanted hearts. The efficacy of these two peptides led us to search for additional C2-derived peptides with PKC-modulating activities. Here we report on the activity of a series of 5–9-residue peptides that are derived from regions that span the length of the C2 domain of εPKC. These peptides were tested for their effect on PKC activity in cells in vivo and in an ex vivo model of acute ischemic heart disease. Most of the peptides acted as activators of PKC, and a few peptides acted as inhibitors. PKC-dependent myristoylated alanine-rich C kinase substrate phosphorylation in εPKC knock-out cells revealed that only a subset of the peptides were selective for εPKC over other PKC isozymes. These εPKC-selective peptides were also protective of the myocardium from ischemic injury, an εPKC-dependent function (Liu, G. S., Cohen, M. V., Mochly-Rosen, D., and Downey, J. M. (1999) J. Mol. Cell. Cardiol. 31, 1937–1948), and caused selective translocation of εPKC over other isozymes when injected systemically into mice. Examination of the structure of the C2 domain from εPKC revealed that peptides with similar activities clustered into discrete regions within the domain. We propose that these regions represent surfaces of protein-protein interactions within εPKC and/or between εPKC and other partner proteins; some of these interactions are unique to εPKC, and others are common to other PKC isozymes.

The protein kinase C (PKC)3 family of serine/threonine protein kinases is involved in normal cell functions such as apoptosis (3, 4), cell proliferation (5–7), and secretion (8), as well as in disease states such as ischemic heart disease (9–12) and stroke (13, 14). PKC activation is associated with binding to the negatively charged phospholipids, phosphatidylserine, and different PKC isozymes have varying sensitivities to Ca2+ and lipid-derived second messengers such as diacylglycerol (15). Upon activation, PKC isozymes translocate from the soluble to the particulate cell fraction (16), including cell membrane, nucleus (17), and mitochondria (18).

PKC primary sequence can be broadly separated into two domains as follows: the N-terminal regulatory domain and the conserved C-terminal catalytic domain. The regulatory domain of PKC is composed of the C1 and C2 domains that mediate PKC interactions with second messengers, phospholipids, as well as inter- and intramolecular protein-protein interactions. (The C2 domain in one subfamily of PKC isozymes was termed V1 until the homology to the C2 domain was identified (19).) Differences in the order and number of copies of signaling domains, as well as sequence differences that affect binding affinities, result in the distinct activity of each PKC isozyme (15, 20).

We previously reported on the role of the C2 domain in protein-protein interactions (21–23) and showed that some peptides derived from that domain act as competitive inhibitors of these interactions (review in Ref. 1). Relevant to εPKC, a peptide interfering with protein-protein interactions between the εPKC isozyme and its anchoring protein (εRACK) inhibits its function, e.g. regulation of the contraction rate of heart muscle cells (22) or protection from cell death because of ischemia (24). Conversely, a peptide interfering with inhibitory protein-protein interactions, for example the intramolecular autoinhibitory interactions in PKC, causes εPKC activation and induced protection from ischemic damage (12). Similar 6–10-amino acid-long peptide inhibitors and activators for each of the classical and novel PKC isozymes have been identified (11, 22, 25–28). These rationally designed peptides were shown to be selective and effective in regulating the biological activities of the corresponding isozymes. The peptide agonists and antagonists were used to study the physiologic role of PKC isozymes (7, 13, 18, 29–41) and may have potential as drug leads.

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2 The founder of KAI Pharmaceuticals, Inc., a company that plans to bring PKC regulators to the clinic. However, none of the work described in this study is based on or supported by the company. To whom correspondence should be addressed: Dept. of Chemical and Systems Biology, Stanford University School of Medicine, CCSR, Rm. 3145A, 269 Campus Dr., Stanford, CA 94305-5174. Tel.: 650-725-7720; Fax: 650-723-2253; E-mail: mochly@stanford.edu.
3 The abbreviations used are: PKC, protein kinase C; MARCKS, myristoylated alanine-rich C kinase substrate; CHO, Chinese hamster ovary; PMA, phorbol 12-myristate 13-acetate; TTC, triphenyltetrazolium chloride; WT, wild type; KO, knock-out.
For ePKC, the following two peptides were identified rationally: eV1–2, an ePKC inhibitor derived from a sequence in the C2 region that binds to the anchoring protein eRACK (β COP) (22), and ψeRACK, an ePKC allosteric activator derived from a sequence implicated in autoinhibitory interactions (12). Both peptides represent regions in eC2 that were well conserved across species, including Aplysia and rat ePKC, whereas it is only 36% identical between rat ePKC and rat δPKC. Furthermore, the ψeRACK peptide, derived from eC2, is homologous to a sequence within its cognate receptor, eRACK (12), a characteristic that indicates a site of intramolecular interaction (1, 25). The finding of peptides corresponding to short sequences within the C2 domain with isozyme-selective activities (42) suggests that other short peptides derived from that domain may have such activities. To test the hypothesis, we designed a series of 5–9-residue peptides derived from the C2 domain of ePKC. These additional 13 peptides, spanning the majority of the ePKC C2/V1 domain (Table 1 and Fig. 1), were tested in four biological assays. As before, these peptides (“cargo”) were introduced into cells by conjugating them to the cell-penetrating “carrier” peptide, TAT-(47–57) (43, 44).

**EXPERIMENTAL PROCEDURES**

Peptide Synthesis—Peptides were synthesized and conjugated to TAT carrier peptide (residues 47–57) via cysteine S–S bond by Anaspec, San Jose, CA. Peptides are 5–8 residues and represent sequences in the C2/V1 domain of ePKC. Three control peptides have also been synthesized as follows: scrambled eV1–2, scrambled ψeRACK, and TAT carrier peptide alone. See Table 1 for a summary of peptides synthesized, including sequences.

PKC Translocation in Cells—ePKC translocation from the soluble to the particulate fraction in CHO cells was used to assess the relative amount of activated (membrane-bound) ePKC, an assay that has been described previously (42). Evidence of translocation is either by an increase in the amount of PKC in the particulate fraction or by a decrease in the soluble fraction. Briefly, after stimulation, cells were washed with cold phosphate-buffered saline, scraped in homogenization buffer, passed through a syringe needle (25-gauge 5/8-inch), and spun at 100,000 g for 30 min at 4 °C. The pellet was then resuspended in homogenization buffer with 1% Triton X-100. Where applicable, the cells were preincubated with 500 nM peptide for 15 min prior to stimulation. PKC was stimulated with submaximal levels of the general PKC activator phorbol 12-myristate 13-acetate (PMA, 3 nM). The samples were then analyzed by Western blot. Antibodies against ePKC were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and used at 1:500 dilution. Antibodies against actin, used as a loading control, were obtained from Sigma and used at 1:1000 dilution.
PKC Substrate Phosphorylation in Cells—Phosphorylation of myristoylated alanine-rich C kinase substrate (MARCKS), a general PKC substrate, was monitored by Western blot of cell lysates. To assess PKC activation by the peptides and their specificity for ePKC, MARCKS phosphorylation in wild-type primary skeletal muscle cells was compared with MARCKS phosphorylation in primary skeletal muscle cells isolated from ePKC knock-out mice (45), as we described previously (46). For the ePKC inhibition assay, WT cells were treated with 1 μM peptide 7 for 15 min, and ψeRACK (500 nm) was then added for 30 min prior to cell lysis. Antibodies against phosphorylated MARCKS were obtained from Cell Signaling (Danvers, MA) and used at a 1:500 dilution. Antibodies against actin, used as a loading control, were obtained from Sigma and used at 1:1000 dilution.

Ex Vivo Cardiac Protection—Activation of ePKC prior to ischemia mediates cardiac protection in an ex vivo model of acute ischemic heart damage (2), an assay that has been described previously (47). Briefly, Wistar rats (300–350 g) were heparinized (1000 units/kg intraperitoneally) and then anesthetized with sodium pentobarbital (100 mg/kg intraperitoneally). Hearts were rapidly excised and then perfused with an oxygenated Krebs-Henseleit buffer containing NaCl (120 mmol/liter), KCl (5.8 mmol/liter), NaHCO3 (25 mmol/liter), NaH2PO4 (1.2 mmol/liter), MgSO4 (1.2 mmol/liter), CaCl2 (1.0 mmol/liter), and dextrose (10 mmol/liter) at pH 7.4 and 37 °C in a Langendorff coronary perfusion system. A constant coronary flow rate of 10 ml/min was used. Hearts were submerged into a heat-jacketed organ bath at 37 °C. Coronary effluent was collected to determine creatine phosphokinase release. After 10 min of equilibration, the hearts were subjected to 40 min of global ischemia and 60 min of reperfusion. The hearts were perfused with 1 μM TAT-conjugated peptide for 10 min prior to ischemia. In addition to the relative amount of creatine phosphokinase released, a measure of cardiac myocyte lysis, tissue damage was assessed by triphenyltetrazolium chloride (TTC) staining of heart cross-sections to quantify the amount of infarcted (dead) tissue, as we described previously (36).

Isozyme Selectivity in Vivo—To determine that activator peptides causing MARCKS phosphorylation and decreasing ischemia-reperfusion damage in heart also induce ePKC translocation in vivo, we injected the respective peptides at 20 nmol in 200 μl of saline into the peritoneum of 15–20 g mice, as reported previously (48). Fifteen minutes later, the mice were sacrificed, and heart and brain were collected. Soluble and particulate fractions from mouse tissue were prepared as described previously (48). α-, ε-, ζ-, and δPKC translocations were determined by Western blot analysis using selective anti-PKC antibodies from Santa Cruz Biotechnology and used at a 1:500 dilution. Sarcomeric actin (1:1000, Sigma) was used as a loading control for all fractions.

RESULTS

We synthesized 13 new 5–9-residue peptides derived from sequences that span the C2 domain (also known as the V1

**TABLE 1**

| Peptide | Primary sequence | Origin of sequence |
|---------|------------------|--------------------|
| eV1–2 | CFNGLLKI | eC2/V1-(4–12) |
| Scrambled eV1–2 | LSETKPA | Scrambled eC2/V1-(14–21) |
| 2 | CGVPRQPT | eC2/V1-(28–35) |
| 3 | CPDLLPI | eC2/V1-(36–41) |
| 4 | CPILLYVD | eC2/V1-(40–47) |
| 5 | CIRGQ | eC2/V1-(49–53) |
| 6 | CTATKQT | eC2/V1-(54–60) |
| 7 | CPAWHD | eC2/V1-(62–67) |
| 8 | CEPYTDV | eC2/V1-(68–73) |
| 9 | CNGRKI | eC2/V1-(74–79) |
| 10 | CIELAVY | eC2/V1-(79–84) |
| ψeRACK | CHDAPIGYD | eC2/V1-(85–92) |
| Scrambled ψeRACK | CPYHDAGI | Scrambled eC2/V1-(85–92) |
| 11 | CHFEDWID | eC2/V1-(112–118) |
| 12 | CLEPEK | eC2/V1-(119–124) |
| 13 | CVVVIDL | eC2/V1-(125–131) |
| TAT carrier | CGRRKKRRRRR | TAT-(47–57) |

*Peptides were conjugated by Cys (underlined) S–S bond to TAT-(47–57) carrier peptide.

*Data were published previously as ePKC-specific inhibitor eV1–2 (22).

*This was used as a control peptide.

*Data were published previously as ePKC-specific activator ψeRACK (12).
domain (19)) of ePKC and tested them for their effect on PKC activation. Fig. 1 shows the β-sandwich revealed in the x-ray crystal structure of the domain (49), with peptide regions colored in ROYGBV order by primary sequence, to ease their identification. In Fig. 1, gray represents regions from which peptides were not analyzed. Residues in the top loop region that are not resolved in the crystal structure appear as a gap in connectivity. Three peptides were synthesized as controls as follows: scrambled versions of a previously established peptide activator (ψRACK) and inhibitor (eV1–2), and the TAT carrier peptide alone. Table 1 lists all the peptides tested in this study.

We hypothesized that peptides that represent regions involved in intramolecular interactions with an inhibitory domain or in intermolecular interactions with an inhibitory protein will act as agonists of ePKC as they will disrupt these inhibitory interactions. We also hypothesized that peptides that correspond to the binding sites for activating proteins such as ψRACK will act as antagonists. Finally, we predicted that peptides corresponding to unique interactions for ePKC will show isoform selectivity, whereas other peptides represent regions of intra- or intermolecular interactions that are common for more than one PKC isoform and therefore will affect many PKC isozymes. Four assays were used to investigate the effect of the TAT-conjugated peptides on ePKC as follows: ePKC translocation in cultured cells (16), phosphorylation of PKC substrate MARCKS in cultured cells (39, 50), cardiac protection assay, a known ePKC-mediated function (12, 24, 32) that is carried out using intact heart ex vivo, and in vivo translocation of PKC after a single intraperitoneal injection (48).

**ePKC Translocation in Cells**—Upon activation, PKC translocates from the soluble to the particulate cell fraction, which can be monitored by Western blot analysis (16). We previously found that the effect of peptides on PKC translocation is better observed when the cells are treated with a submaximal concentration of PMA (11, 42). Larger concentrations of PMA, a non-physiological activator, stimulate a greater degree of PKC translocation over translocation stimulated by peptide activators alone (51). PKC translocation upon peptide treatment, although less than that seen with large amounts of PMA, has been shown to have important physiological consequences (e.g., this work and see Refs. 11, 12, and 32). Pretreatment with most peptides prior to PMA stimulation resulted in an increase in the amount of ePKC in the pellet fraction and a decrease in the amount of ePKC in the soluble fraction, indicating that these peptides stimulated ePKC translocation. Unexpectedly, all the peptides altered ePKC translocation when assayed on cells in culture; the majority of the peptides increased PMA-induced ePKC translocation. Pretreatment with four peptides, 4, 7, 12, and eV1–2 (previously reported to be an ePKC-selective inhibitor), before PMA stimulation results in a decrease in the amount of ePKC in the pellet fraction relative to control, indicating that these peptides may act as inhibitors of ePKC translocation. Furthermore, and not corroborated by any of the other assays (see in the following), the control peptide scrambled eV1–2 (LSETKPAV) also stimulated ePKC translocation in CHO cells. Pretreatment with TAT carrier peptide alone (500 nM) had no effect on PKC translocation. Data for two representative peptides are shown as follows: peptide 7 inhibits translocation, and peptide 2 enhances translocation (Fig. 2A). A summary on the effect of each peptide on ePKC translocation in cultured CHO cells is provided in Fig. 2B and Table 2. (Note that translocation data provide evidence of changes in PKC activity but are not quantitative enough to assess the relative strength of the effects.)

**PKC Substrate Phosphorylation in Cells**—Although translocation is indicative of PKC activation (16), a more direct measure of PKC activation is PKC substrate phosphorylation in cells. Because there are no known ePKC-specific substrates, we used

### TABLE 2

| Peptide | ePKC translocation in cells | MARCKS phosphorylation in WT cells | MARCKS phosphorylation in eKO cells | Ischemic heart injury, ex vivo | PKC translocation, in vivo |
|---------|-----------------------------|-----------------------------------|------------------------------------|-----------------------------|-----------------------------|
| c        | Activator                    | Activator                         | No effect                          | Cardioprotective           | NT*                         |
| 2        | Activator                    | Activator                         | No effect                          | Cardioprotective           | NT*                         |
| 5        | Activator                    | Activator                         | No effect                          | Cardioprotective           | NT*                         |
| 7        | Activator                    | Activator                         | No effect                          | Cardioprotective           | NT*                         |
| 12       | Activator                    | Activator                         | No effect                          | Cardioprotective           | NT*                         |
| 11       | Activator                    | Activator                         | No effect                          | Cardioprotective           | NT*                         |
| 10       | Activator                    | Activator                         | No effect                          | Cardioprotective           | NT*                         |
| 13       | Activator                    | Activator                         | No effect                          | Cardioprotective           | NT*                         |
| eV1–2    | Inhibitor                    | Inhibitor                         | No effect                          | No effect                  | NT*                         |
| 4        | Inhibitor                    | Inhibitor                         | No effect                          | No effect                  | NT*                         |
| 7        | Inhibitor                    | Inhibitor                         | No effect                          | No effect                  | NT*                         |
| 12       | Inhibitor                    | Inhibitor                         | No effect                          | No effect                  | NT*                         |
| Scrambled eV1–2 | Activator | No effect | No effect | No effect | No effect |
| Scrambled ψRACK  | NT* | NT* | NT* | NT* | NT* |
| TAT carrier | No effect | No effect | No effect | No effect | NT* |

* NT indicates not tested.

a Data are from a previous study (48).
MARCKS is a widely distributed actin cross-linking protein, and upon PKC activation, MARCKS is highly phosphorylated on serine residues (50, 52). We determined the level of MARCKS phosphorylation after incubation of primary mouse muscle cells in culture with each peptide for 30 min. This assay has been used before to show that integrin-induced PKC activation leads to MARCKS phosphorylation (39).

Eleven of the peptides tested increased MARCKS phosphorylation relative to no peptide treatment or TAT control treatment (Fig. 3A and Table 2), indicating that these peptides were PKC activators. Importantly, no change in MARCKS phosphorylation was observed following treatment with the control peptides, scrambled εV1–2, or scrambled ψεRACK. Only peptide 8 had no conclusive effect on MARCKS phosphorylation (Fig. 3A and Table 2).

We next determined whether the peptides that increased MARCKS phosphorylation are selective for εPKC. We reasoned that because activation of several PKC isozymes leads to MARCKS phosphorylation (50), lack of increased MARCKS phosphorylation in cells derived from εPKC knock-out (KO) mice can identify peptides that are selective for εPKC. We found that most of the peptides (6 out of 11 activators: peptides 1, 2, 3, 5, 9, and ψεRACK; Fig. 3, B versus A, and Table 2) increased MARCKS phosphorylation in WT but not in KO muscle cells and are therefore εPKC-selective activators. A few peptides increased MARCKS phosphorylation in both cell types and are therefore not specific for εPKC (e.g. peptides 11 and 13; Fig. 3, B versus A, and Table 2). As expected, in no case did the peptides affect MARCKS phosphorylation in KO and not WT cells (Fig. 3; Table 2). In this study, we focused on gain of function peptides and did not further corroborate the potential inhibitory activity of the peptides that resulted in a decline in MARCKS phosphorylation (i.e. peptides 4, 7, and 12). Fig. 3C shows one example of εPKC inhibitory activity. It demonstrates the effect of a 15-min pretreatment with a representative peptide inhibitor, peptide 7 (1 μM), on WT cells stimulated with the previously identified peptide activator, ψεRACK. MARCKS phosphorylation is decreased in the presence of the peptide inhibitor relative to peptide activator alone. Thus, MARCKS phosphorylation can be used to assess both activation and inhibition of PKC function. Together, this assay suggested that several peptides derived from the C2 domain of εPKC affect the catalytic activity of the enzyme in cells and that a subset of these peptides is isozyme-selective.
Ex Vivo Cardiac Protection—To further assess the functional relevance of ePKC modulation as well as to demonstrate the potential for these newly identified peptides as drug leads, an ex vivo model of ischemia and reperfusion was used, as described previously (47). Work by several laboratories, including our own, has demonstrated that activation of ePKC in hearts prior to an ischemic event leads to reduced damage of the myocardium (12, 24, 53−55). We showed that pretreatment of hearts subjected to ischemia and reperfusion ex vivo (using a Langendorff apparatus) with the $\psi$eRACK peptide leads to reduced damage (infarction) (12, 43). Because other PKC isozymes have no effect on cardiac protection (e.g. $\beta$PKC (43)) or even have opposing effects to ePKC activation (e.g. $\delta$PKC activation increases damage (11)), cardiac protection following perfusion of the $\epsilon$C2-derived peptides will further support their identification as ePKC activators. Assuming that the TAT-dependent delivery of each peptide provides similar access to the tissue (as indicated by our previous studies (48)), lack of an effect on cardiac damage will indicate either that the peptide is an ePKC inhibitor or that it affected other isozymes, including those with opposing roles in this response.

Fig. 4 provides a representative subset of the peptides tested for this study. Note that none of the peptides that appear to have inhibitory activity based on translocation and MARCKS phosphorylation (peptides 4, 7, and 12), except for $\epsilon$V1–2, were tested in this assay. Representative cross-sections of control and treated hearts stained with TTC after ischemia and reperfusion are shown in the top panels of Fig. 4; dead (infarcted) tissue remains white and live tissue is stained red. According to the level of damage observed by TTC staining (Fig. 4A, top histogram) and release of creatine phosphokinase (a cytosolic enzyme that is released from the myoblasts when the muscle is damaged; Fig. 4B), we concluded that peptides 2, 5, 6, and 9, as well as the previously characterized ePKC-selective agonist, $\psi$eRACK, were cardioprotective (Fig. 4 and Table 2), indicating that they are likely ePKC-selective activators. Peptides 1 and 3 showed a trend toward cardioprotection, but this trend was not statistically significant (Fig. 4 and Table 2). The four peptides that did not induce MARCKS phosphorylation in wild-type and eKO skeletal muscle cells, peptide 8, and the three control peptides (scrambled $\epsilon$V1–2, scrambled $\psi$eRACK, and TAT) (Fig. 3 and Table 2) did not cause any cardiac protection (Fig. 4; Table
PKC translocation in hearts and other organs after a single intraperitoneal injection (48). By using this assay, we found here that \( \psi'eRACK \), peptide 1, and peptide 5 caused translocation of ePKC but not \( \delta' \) or \( \alphaPKC \) in hearts (Fig. 5, A–C), indicating that these peptides are selective ePKC activators. Because peptides 11 and 13 caused MARCKS phosphorylation in myocytes from the ePKC knock-out mice (Fig. 3), we concluded that they activated both the cardiac protective isozyme, ePKC (11, 12, 56), and the isozyme that mediates cardiac damage, \( \deltaPKC \) (11, 36). We therefore tested these peptides directly in mice. As expected, we found that injection of peptide 11 caused a substantial translocation of \( \deltaPKC \), \( \alphaPKC \), and ePKC (Fig. 5C). Atypical \( \deltaPKC \), present only in the soluble cell fraction, did not translocate in response to any peptide tested (Fig. 5D). Finally control peptide, scrambled eV1–2, did not cause an increase in either ePKC or \( \deltaPKC \) (Fig. 5C). (To limit animal use, other peptides have not been tested in vivo.)

**DISCUSSION**

We have previously used a rational approach to identify two peptides that selectively modulate ePKC activity; one peptide interferes with ePKC interaction with its anchoring protein, eRACK (eV1–2 peptide) (22), and the other interferes with PKC autoinhibitory intramolecular interactions (\( \psi'eRACK \) peptide) (12). Because these peptides were both derived from the C2 domain, we set out here to determine whether there are other C2-derived peptides that regulate PKC activity and whether a peptide-scan method can be used to identify regions within the C2 domain of ePKC that are functionally important.

The peptides tested in this study covered almost all the \( \beta \)-sheet regions and parts of the top and bottom loop regions of the C2 domain (Fig. 1). We found that all of the peptides derived from the C2 domain of ePKC modulated the activity of ePKC in the two cell-based assays (translocation and MARCKS phosphorylation). Ten C2-derived peptides were ePKC activators (1, 2, 3, 5, 6, 9, 10, \( \psi'eRACK \), 11, and 13) as shown by an increase in ePKC translocation in CHO cells and an increase in MARCKS phosphorylation in primary muscle cells. Five of these peptides that were found to be ePKC activators in the two cell-based assays were also cardioprotective (2, 5, 6, 9, and \( \psi'eRACK \)), and of these, four were selective for...
**ePKC C2 Domain Peptides**

A.

![Diagram of ePKC C2 Domain Peptides]

B.

- **ePKC-specific protein-protein interactions** (corresponding peptide activators are marked in green, and inhibitors in red)
- **selectivity of protein-protein interactions** (unknown, yellow)
- **non-specific PKC protein-protein interactions** (blue)
C. ePKC 2C Domain Peptides

| Peptide | Sequence | Notes |
|---------|----------|-------|
| etal | MVVGTVKLKICAVS/LKPAASSRHLAVGVPRQFGFLLLPTALWQD | |
| delta | MSSLTNNKFGNYLVR/IGAVGLOP5KRSLRHHLFPKG-WQJLDPYT/LTVSVQD | |
| alpha | TTEKSSLKNLKEVTD-EKMYVT/DRKSLNPMLE N | |
| beta | SMMRGRQYQAHDRE-EVLYVVDRDKNLPMDESSL | |

**FIGURE 6.** A and B, surface mapping of the C2 domain of ePKC. The activity of the peptides is mapped back to the C2/V1 domain of ePKC, colored in blue for PKC activators (not selective for ePKC), green for ePKC-selective activators, yellow for inconclusive results about specificity, and red for ePKC inhibitors. Both ribbon (A) and space-filled (B) models are provided. ePKC-selective activators are peptides that cause MARCKS phosphorylation in WT cells and not in KO cells and protect cardiac tissue from ischemia and reperfusion damage. Most of the peptides were found to be activators (green and blue regions). Three of the four PKC inhibiting peptides cluster in three-dimensional space (red regions). However, their selectivity for ePKC has not yet been fully characterized. C, multiple sequence alignment of the C2 of five PKC isozymes. Alignment is based on structure (β-strands are aligned) and sequence. Peptide activity is colored (red for inhibitors, green for selective activators, and blue for isoyme nonselective activators). Peptides (indicated in color) from other isozymes are as follows: PKC agonist S6-RACK (SVFWD) activates all the classical PKC isozymes (25) and PKC antagonists ρC2–4 (SLNPEWNET) and C2–2 (MPDNGLSFYVXL) are inhibitors of all the classical PKC isozymes (25). ρPKC agonist (S6-RACK, MRAEDPVM) and antagonist (SVI–1, 5FNSVYGLG) are selective for ρPKC (1). The sequences in 1PKC (EAVGLOPT for inhibitor and HETPLGYD for activator) are as in Ref. 24. Surface alignment of C2 domains of several PKC isoforms predicts regions from which potential peptide regulators (selective and nonselective) for other PKC isoforms can be generated.

ePKC over other PKC isoforms (2, 5, 9, and ρeRACK). Selectivity was determined by observing phosphorylation of MARCKS in WT, and not in cells lacking ePKC. A trend suggesting cardiac protection from ischemia was obtained following treatment with the two other selective peptides (peptides 1 and 3), which may reflect either low EC50 values and/or some effect on other PKC isoforms (Fig. 5 and Table 2). Two of the peptides (peptides 11 and 13) activated PKC, as evidenced by increased translocation and MARCKS phosphorylation, but their effect was not selective for ePKC; treatment with these peptides increased MARCKS phosphorylation also in cells lacking ePKC (Fig. 3). As we expected, these two peptides did not induce cardiac protection from ischemia and reperfusion (Fig. 4), supporting the interpretation that they are interfering with intramolecular interactions to render the enzyme more active, but they are likely to affect similarly other PKC isoforms, e.g. ρPKC that increases cardiac damage by ischemia (11). Indeed, translation studies in vivo confirmed that peptide 11 activated δ- and ePKC as well as ρPKC (Fig. 6, A–C). Four peptides (4, 7, 12, and the previously identified ePKC inhibitor εV1–2) appear to act as ePKC inhibitors (Fig. 2), and peptide 7, for example, inhibited ρeRACK-induced MARCKS phosphorylation (Fig. 3C). However, further characterization and isoyme selectivity of the peptide inhibitors was not studied further. Importantly, in no case did one assay indicate activity for a particular peptide (e.g. ePKC activation) and another assay indicate the opposite activity for the same peptide (e.g. ePKC inhibition). Together, this work demonstrated the ability to generate many ρ–9 amino acid peptides, derived from the C2 domain, that exert biological activity. Furthermore, the majority of these peptides appear to be selective for ePKC. These data suggest that most of the protein-protein interactions that are mediated by the C2 domain of ePKC are unique.

eC2 derived peptides with similar activity clustered in three-dimensional space when mapped back to the C2/V1 domain structure from which they were derived (Fig. 6A). Most activating peptides were specific for ePKC and clustered on one face of the β-sandwich (green in Fig. 6). Three of the four inhibiting peptides clustered in three-dimensional space (red in Fig. 6). Based on this peptide scan analysis, we suggest that the C2 participates in critical protein-protein interactions and that peptides derived from these regions are useful tools to probe for such regions; these interaction surfaces likely mediate intramolecular interactions or intermolecular interactions with ePKC partners.

Because the fold of the C2 domain from other PKC isoforms is structurally similar, the data also suggest that peptides derived from homologous positions in the other isoforms will have similar biological activities. We predict that as in ePKC, many peptides derived from the C2 domain of other PKC isoforms may also act as isoyme-selective PKC activators and inhibitors and may also reveal protein-protein interaction surfaces. A multiple sequence alignment based on structure (49, 57, 58) and sequence reveals homologous regions in other PKC isoforms (Fig. 6C). A similar peptide scan approach in other PKC isoforms will allow for better characterization of the C2 domain in the PKC family; similarities will reveal common PKC regulation themes, and differences will highlight how each isoyme plays its specific role.

Because homologs of the C2 domain are present in over different proteins (59), many of which are signaling proteins, the information obtained in this study is likely to provide new means to affect the functions of these other C2-containing proteins and therefore serve as useful pharmacological tools or even drug leads for human diseases. The approach described here may also provide a useful means to map potential interaction surfaces in other β-sandwich domains,
ePKC C2 Domain Peptides

such as that in 5-lipoxygenase (60), pleckstrin (61), and the tumor necrosis factor family (62), to identify drug leads that selectively regulate these proteins, and to predict more reliably their quaternary structures.

Characterizing protein-protein interaction surfaces helps further our understanding of key events in signaling, including assembly of macromolecular complexes and networks (63). As these surfaces can be slow to characterize, novel techniques such as computational tools (63) and peptide scanning such as done in this study can provide complementary information toward learning about what these surfaces look like and how they function, including specificity.

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