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Highly Specific Modulators of Protein Kinase C Localization: Applications to Heart Failure

Nir Qvit and Daria Mochly-Rosen *

Department of Chemical and Systems Biology, Stanford University, School of Medicine, Stanford CA 94305-5174

Abstract

Heart failure (HF) in which the blood supply does not match the body's needs, affects 10% of the population over 65 years old. The protein kinase C (PKC) family of kinases has a key role in normal and disease states. Here we discuss the role of PKC in HF and focus on the use of specific PKC regulators to identify the mechanism leading to this Pathology and potential leads for therapeutics.

Introduction

Although there are many etiologies for heart failure (HF), they are all manifested as inability of the heart to deliver sufficient blood to the body. HF is a major cardiovascular disease. In 2008 were about 5 million people had HF, there were 300,000 HF-related deaths and the estimated costs for HF management were over \$34 billion in the US alone. Current treatment is insufficient and multiple studies examining new targets to modulate the disease are underway. Here we focus on the signaling events regulated by protein kinase C (PKC). Notably, the levels of at least one PKC isozyme are highly elevated in failed human hearts [1], suggesting that it plays a role in this pathology (see in the following).

Protein Kinase C

Protein kinase C (PKC) isozymes regulate multiple signaling events in normal and disease states. Initially identified by Nishizuka and coworkers more than 30 years ago [2], the highly homologous isozymes are divided into three categories, depending on their mode of activation: classical PKCs (cPKC) include α , β _I, β _{II}, and γ isoforms; novel PKCs (nPKC) include δ , ϵ , η , and θ isoforms; and atypical PKCs (aPKC) include ζ and ι/λ isoforms (Figure 1). nPKCs are activated by diacylglycerol (DAG) only, whereas the cPKCs are activated by DAG and Ca^{2+} , and aPKCs are dependent on other lipid-derived second messengers [3]. Upon activation, PKC isozymes translocate from the soluble to the particulate cell fraction [4], including plasma membrane [5,6], nucleus [7], ER/Golgi [7,8] and mitochondria [9]. The PKC isozymes contain a highly conserved (C) regions separated by a number of variable (V) regions. The C-terminal half of PKC contains the catalytic region and the N-terminal half contains the regulatory domains, both regions are separated by a flexible hinge

Corresponding author: Mochly-Rosen, D., mochly@stanford.edu, Tel: 650-725-7720, Fax: (650) 723-4686.

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region (Figure 1). The homology in the catalytic region between various members of the PKC family is approximately 70%, whereas the homology in the regulatory domain is much more limited. The common C1 region in the regulatory domain binds second messengers and the C2 region mediates a number of inter- and intramolecular protein-protein interactions between individual PKC isozymes and their anchoring proteins, Receptors for Activated C Kinase (RACK) [10]. Binding of a specific activated PKC to its RACK provides access to and phosphorylation of their substrates (Figure 2i). Therefore, anchoring to RACKs is a required step for PKC function.

Relevant to heart disease, PKC isozymes play a key role in regulating cell proliferation [11-13], heart failure [14], heart attack [15], angiogenesis [16] and regulation of the immune response [17]. Academic researchers and the pharmaceutical industry identified the PKC family as an attractive target for therapeutic purposes. However, the majority of the available pharmacological agents acts on many protein kinases by targeting the conserved catalytic site and do not show sufficient selectivity for a specific PKC isozyme [18]. Many studies demonstrated that individual PKC isozymes have unique and even opposing roles, for example in the heart [19], and the vasculature [20], demonstrating the need for isozyme-selective regulators. Driven by the importance of identifying selective regulators for the various PKC isozymes, we set out to discover such specific PKC activators and inhibitors and apply them to different diseases such as HF. Our approach was to focus on regulating selective protein-protein interactions that govern PKC signaling using short peptides [13,21]. The first intra-molecular protein-protein interaction site that was identified is the pseudosubstrate site; it mediates binding of the regulatory domain to the catalytic domain thus maintaining PKC in an inactive conformation (Figure 2A; [3]). Other inter- and intra-molecular sites in PKC are described below.

Identification of peptide inhibitors of protein-protein interactions: Random and rational approaches

Protein-protein interactions (PPI) play a pivotal role in the functional selectivity of enzymes participating in cellular signal transduction cascades. Peptides that regulate protein-protein interactions can be identified by screening large libraries, using systematic and random methodologies, or by rational approaches. The large screen approaches include: **(Ia)** *Systematic search of large domain involved in protein-protein interactions*: The systematic search is based on fine mapping of the interaction site within the protein-protein interaction domain and extensive screening of small fragments [22]. **(Ib)** *Random search of large domain involved in protein-protein interactions*: Random search uses random libraries consisting of many possible peptides of a certain length that are synthesized or expressed in virus and tested for their bioactivity [23]. **(Ic)** *Search of key amino acid residues involved in protein-protein interactions*: Performed by screening a library in which certain position in the peptides include specific amino acids, for example a phosphorylated tyrosine [24]. All of the above approaches were used successfully to identify peptides that modulate protein-protein interactions. However, these methods require the use of large libraries, up to billions of peptides, which are intensive and costly. Further, only limited use in biology was made with peptides discovered using these methods [25-27].

We developed simple rational approaches to identify short peptides (6-13 amino acids) that inhibit protein-protein interaction (Review [28]). These peptides were used by many laboratories for *in vitro* and *in vivo* testing of a variety of species including humans and resulted in over 250 publications. Several rational approaches for the design were used.

(IIa) Identification of sequences shared by non-related proteins that interact with a common protein

Some signaling enzymes interact with multiple unrelated proteins; short homologous sequences between these unrelated proteins could represent the binding site for the enzyme. Peptides that are derived from these sequences may regulate the protein-protein interaction and thereby regulate the enzyme function. For example, a short sequence of homology between two PKC-binding proteins, 14-3-3 and annexin I, was noted in a short note to Nature [29]. We suggested that this short homologous region in these two unrelated proteins might be the PKC-binding site and showed that a peptide based on that short sequence, peptide I, is a β PKC-specific translocation inhibitor (Figure 3a; [30,31]).

(IIb) Identification of conserved sequences in homologous domains of otherwise non-related proteins

Conserved sequences within homologous domains of multiple signaling enzymes may be essential for a unique function. One example is the C2 domain, which is found in a large number of proteins. 3D structures of several C2 domains revealed their structure as a beta sandwich that is composed of four anti-parallel beta strands [69,73–77]. We showed that peptides derived from homologous sequences in the C2 domain of β PKC and synaptotagmin are effective β PKC inhibitors; β C2-1, β C2-2 and β C2-4, bind to RACK1, the specific RACK of β PKC, and inhibit the binding of β PKC to RACK1 and further signaling (Figure 3b;[32]).

(IIc) Identification of evolutionarily conserved sequences

Such conserved sequences in proteins from evolutionarily distant organisms are expected to serve the same function. For example, the homologous sequence in the ϵ PKC C2 domain from *Aplysia* and *Homo sapiens* were the basis for the design of the $\psi\epsilon$ RACK peptide, an ϵ PKC-specific agonist, and ϵ V1-2 peptide, an ϵ PKC-specific antagonist (Figure 3c; [33]).

(IId) Identification of sequences involved in intra-molecular interactions

Intra-molecular interactions within a variety of enzymes keep them inactive. Peptides that correspond to these sequences can serve as agonists, because they interfere with the inhibitory intramolecular interaction. For example, the β PKC agonist peptide corresponds to a six amino acid sequence of homology between β PKC and its RACK, RACK1. This sequence was chosen, in part, because it differs in one charged amino acid, suggesting that the affinity of the peptide from β PKC that mediates the intra-molecular interaction is not as good as that from the partner protein, RACK1; indeed, the PKC-derived peptide is an agonist, whereas the RACK1-derived peptide is an antagonist (Figure 3d;[34]).

β PKC has two alternatively spliced forms, β I PKC and β II PKC, that vary only in their last 50 amino acids in the V5 region of the enzyme. Therefore, C2-derived peptides could not be used to selectively inhibit only one of the isozymes. However, short peptides derived from the V5 region were isozyme selective (Figure 3e;[32]).

Cell-Penetrating Peptides (CPP)

Are used to enable the delivery of a variety of cargoes, including other peptides across biological membranes. CPP are short, amphipathic, net positively charged peptides. Their penetration into cells is rapid and small cargos do not affect this rate. Some of the most common CPP are antennapedia₄₃₋₅₈ homeodomain transcriptional factor from *Drosophila* and TAT₄₇₋₅₇, derived from the transcription activating factor of the HIV. They can be conjugated to carrier using a permanent or a labile bond and they effectively deliver peptides even into the CNS (Review [35]).

Heart failure and PKC

Various PKC isozymes regulate cardiac hypertrophy, which may lead to left ventricle dilation and impaired cardiac function. α PKC was shown to be necessary and sufficient to induce cardiomyocyte hypertrophy in cell culture. Knock-out of α PKC protected mice from pressure overload-induced HF [36]. Dysfunction of remodeled myocytes obtained from rats with post-MI HF was improved by adding α PKC to permeabilized cells, suggesting a role for this isozyme in myocyte contractility [37]. However, α PKC levels in human patient samples with end-stage HF were found to be low compared with normal subjects [1], suggesting that α PKC may not be critical in HF, in humans.

There is a strong evidence for role for β PKC in HF in both model systems and in humans. Both β PKC isozymes are required for PMA-induced cardiomyocyte hypertrophy in cell culture [32]. Yet in mice models, over-expression of β IIPKC leads to hypertrophy with myocardial dysfunction similar to HF [38,39] whereas, a knockout β PKC model demonstrates no role for β PKC in HF [40]. In rats with hypertension-induced HF, the levels of β PKC are elevated [41] and selective inhibition of β IIPKC by β IIV5-3, the selective peptide inhibitor of β IIPKC [32], improves cardiac function and prolongs survival of the rats (Figure 3e, Table 1; [32]). Samples of human hearts with HF show, in two independent studies, a major increase in activation and level of β PKC [1,14].

A potential role of ϵ PKC has also been described. Mice that express the ϵ V1 domain, the ϵ PKC-selective translocation inhibitor, developed from birth dilated eccentric cardiomyopathy and HF, whereas, transgenic mice expressing $\psi\epsilon$ RACK, the specific peptide activator of ϵ PKC, had normal cardiac function, suggesting that ϵ PKC activation may cause hyperplasia and its signaling may be part of a compensatory pro-proliferative pathway, at least during early postnatal development (Table 1; [42]). These early developmental roles of ϵ PKC related to its role in pathological remodeling was tested in rat models of HF. Sustained inhibition of ϵ PKC using ϵ V1-2, the specific peptide inhibitor of ϵ PKC [43], prolonged rat survival, reduced hypertrophy and corrected cardiac dysfunction (Table 1; [41,44]). The opposing role of ϵ PKC in these two studies can be explained by species differences or because the mouse model of post-natal expression does not recapitulate the human disease.

With pathological stressors such as ischemia and hypertension, fibroblasts are known to migrate to the injury site leading to the accumulation of collagen, causing an excess of *cardiac fibrosis*, which interferes with myocardial metabolism and decreases cardiac elasticity. A number of PKC isozymes that are present in cardiac fibroblasts were shown to control the deposition of collagen. Both δ PKC and ζ PKC isozymes are activated during TGF β -induced fibroblast proliferation. However, while, δ PKC selective inhibition increased TGF- β 1-induced proliferation of cardiac fibroblast, a selective inhibitor of δ PKC blocked TGF- β 1-induced cardiac fibroblast proliferation (Table 1; [13]). ϵ PKC regulates the adhesion and migration of cardiac fibroblasts as a result of Ang II treatment and fibroblast and cardiac myofibroblasts from ϵ PKC knockout mice show impaired adhesion and migration [45]. HF in hypertensive rats treated with the ϵ PKC selective inhibitor is associated with inhibition of increased interstitial fibrosis (Table 1; [46]). All of the above data corroborate the important role of different PKC isozymes in cardiac fibrosis.

An increase in *inflammatory* cells in the myocardium participates in the pathogenesis of HF. Selective inhibition of ϵ PKC with ϵ V1-2 causes infiltration of inflammatory cells into the myocardium in the hypertension-induced HF model in rats (Table 1; [44]). The inhibition of ϵ PKC isozyme was also associated with a decreased degranulation of cardiac mast cells, which resulted in inhibition of the pro-inflammatory, pro-hypertrophic, and pro-fibrotic mast

cells effects. Therefore, dysregulation of PKC in inflammatory cells and in cardiac mast cells may contribute to the development of HF.

Summary and Conclusions

The evidence for a role for PKC in HF is substantial. However, because PKC isozymes have different and sometimes opposing effects, studies that use non-selective pharmacological tools may be inconclusive. Further, studies that rely on genetic manipulation of PKC cannot distinguish between the role the enzymes play during development and those that are critical for the etiology of HF. A special issue may relate to the study of HF in mice, as an emerging body of work demonstrates that mouse models are not as predictive of human disease as models in rats [47]. Regardless of these limitations, PKC plays a pivotal role in HF with strong evidence for a negative role for a member of the classical PKC isozymes, likely β IIIPKC and for the novel isozyme, ϵ PKC.

We have described here the use of regulators of protein-protein interactions as pharmacological tools in the study of HF. Although traditionally, protein-protein interactions were thought to be difficult to regulate with pharmacological agents, we showed that peptides derived from the interaction surfaces provide superb pharmacological tools to regulate PKC-mediated signaling. Based on rational design approaches, we developed isozyme-specific inhibitors and activators that helped us identify the role of different PKC isozymes in HF. Some of these peptides were already shown to be safe in humans when used acutely [48], and animal studies suggest that they are safe also when delivered in a sustained manner. Whether peptide regulators will be useful in the treatment of HF remains to be determined, but the above studies illustrate that specific PKC isozymes may serve as specific targets for future drug development for HF.

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Figure 1. PKC isozymes and domains

A schematic diagram of the structure of PKC isozymes indicating the regulatory and catalytic domains of each isozyme. PKC isozymes are classified into three subsets based on their activation requirements. The classical isozymes (α , β I, β II and γ) bind calcium and diacylglycerol (DAG). The atypical isozymes (ζ , and λ/ι) that are not sensitive to either calcium or DAG and the novel isozymes (ϵ , δ , η and θ) activated by binding only DAG. Scheme is not drawn to scale.

Figure 2. Model of peptide regulators of protein-protein interactions between PKC isozymes and their anchoring proteins, RACKs

(A) Shown are Receptors for Activated C Kinase (RACK) - specific anchoring proteins for a PKC isozyme (marked in orange) and corresponding to PKC isozyme (marked in grey). The PKC isozyme is in equilibrium between active and inactive conformations. Four intra-molecular interactions sites are depicted: pseudo substrate site, pseudo RACK site, the RACK-binding site and the substrate-binding site.

(i) Upon activation, PKC isozymes bind to their RACKs, enabling the phosphorylation of their respective substrates that are localized near the RACK (marked in blue).

(iia) Competitive antagonist - peptide inhibitor (marked in red), corresponding to a sequence in PKC, binds to the PKC-binding site in the **RACK**, competing with the protein-protein interaction between PKC and RACK and thereby inhibiting phosphorylation of the substrate.

(iib) Allosteric agonist - (1) peptide activator (marked in green) is derived from the PKC pseudo-RACK site. It binds to a **PKC** isozyme at the RACK-binding site, tilting the equilibrium between the active and inactive conformations towards the active conformation.

(2) The affinity of the pseudo-RACK peptide to the PKC is lower than the affinity of RACK, allowing PKC to bind to its RACK and phosphorylate the substrate. The schemes are not drawn to scale.

Aplysia ϵ 6 M - V F N G S V K I K V C E A V D L K P T
 Human ϵ 1 M V V F N G L L K I K I C E A V S L K P T
 Human η 1 M S S G T M K F N G Y L R V R I G E A V G

ϵ V1-2 (antagonist)

Aplysia ϵ 26 D F S L R L Q K G S T K E K A S Q M I E
 Human ϵ 22 A W S L R H A V G P R P Q T F L L D -
 Human η 22 L Q P T R W S L R H S L F K K G H Q

Aplysia ϵ 88 N L T V F H D A A I P P D E F V A N C T I
 Human ϵ 81 E L A V F H D A P I G Y D D F V A N C T I
 Human η 81 H L E L A V F H E T P L G Y D H F V A N C

$\psi\epsilon$ RACK (agonist)

Aplysia ϵ 6 743
 Human ϵ 1 737
 Human η 1 683

β PKC 230 Q L K E S D K D R R L S V E I W D W D L T
 RACK1 246 N R Y W L C V A Y G P S I K I W D L A C K

ψ RACK1

β PKC 1 671
 RACK1 1 318

Figure 3.

Figure 3a. Identification of sequences shared by non-related proteins that interact with a common protein. A sequence corresponding to the only homologous domain between two PKC-binding proteins: Annexin I (gi: 197692249; residues 332-346) and member of the 14-3-3 family protein (gi: 192988247; residues 124-138) has been identified. We reasoned that this homologous sequence corresponds to the PKC-binding site on these proteins. A peptide corresponding to the homologous sequence, peptide I (KGDYEKILVALCGG), is a PKC translocation inhibitor [31]. Amino acids are represented by the one-letter code; full line boxes indicate identical amino acids and dashed boxes indicate conserved amino acid substitutions. Scheme is not drawn to scale.

Figure 3b. Identification of conserved sequences in homologous domains of otherwise non-related proteins. The C2 domain (conserved domain 2) is found in a large number of proteins and mediates important protein-protein interactions. Therefore homologous sequences within the C2 domains should mediate a unique function. The C2 homology domain between βPKC (*Rattus norvegicus*; gi: 288806594, residues: 186-229) and synaptotagmin 1 (*Rattus norvegicus*; gi: 149067023, residues: 173-212) was aligned. Based on that sequence alignment four peptides were designed; three of the peptides were found to be specific bioactive βPKC antagonists and the fourth peptide was design as negative control. Amino acids are represented by the one-letter code; full line boxes indicate identical amino acids and dashed boxes indicate conserved amino acid substitutions. Scheme is not drawn to scale.

Figure 3c. Identification of evolutionarily conserved sequences. Two peptides within the V1 region of εPKC were designed based on the most conserved sequences between two distant species: *Aplysia californica* (gi: 155792; residues: 6-64) and *Homo sapiens* (gi: 80476652; residues: 1-58). The first peptide, ψεRACK, has agonist activity, and the second peptide, εV1-2, acts as an antagonist. The KIK residues (amino acids 9-11 in εPKC) were not included in the peptide to reduce possible non-specific interactions of the positive charge with the negatively charged PKC activator, phosphatidylserine. Also aligned in the figure, the ηPKC isozyme from *Homo sapiens* (gi: 28557781; residues: 1-58); ηPKC is the closest PKC isozyme to εPKC from an evolutionarily point of view. Based on that alignment, it is obvious how the two PKC human isoforms are much less homologous compared to the two

εPKC isozymes from the two distant species. Amino acids are represented by the one-letter code; full line boxes indicate identical amino acids. Scheme is not drawn to scale.

Figure 3d. Identification of sequences involved in intra-molecular interactions. Peptides that correspond to the regulatory domains of the enzyme may mediate intra-molecular interactions through binding to the catalytic site. A sequence alignment was done between βPKC (*Rattus norvegicus*; gi: 288806594, residues: 230–250) and Receptor for Activated C Kinase 1 (RACK1, *Glossina morsitans morsitans*, gi: 289741695; residues: 245–265), the specific RACK for βPKC. Based on that alignment, a sequence that differs in one amino acid was identified. This sequence includes a difference of one charged residue (marked in red square) and led to the design of the specific βPKC activator. Amino acids are represented by the one-letter code; full black line boxes indicate identical amino acids. Scheme is not drawn to scale.

Figure 3e. Design of βPKC-specific isozyme peptides. A sequence alignment was done between the V5 domain of the two βPKC isozymes from human (βPKCI: gi: 47157322, residues: 621–673 and βPKCII: gi: 20127450, residues: 621–671). Based on the difference between the two isozymes, three peptides were designed. βIIV5-1 partially inhibits βIIPKC binding to RACK1, and βIV5-3 and βIIV5-3 are isozyme-selective translocation inhibitors of βIPKC and of βIIPKC, respectively [32]. Amino acids are represented by the one-letter code; full line boxes indicate identical amino acids and dashed boxes indicate conserved amino acid substitutions. Scheme is not drawn to scale.

The sequences of βPKC in the database are a little confusing. At the NCBI database (<http://www.ncbi.nlm.nih.gov>) there are two βPKC isozymes from human (βPKCI: gi: 47157322, and βPKCII: gi: 20127450) and two βPKC isozymes from rat (βPKCI: gi: 288806592, and βPKCII: gi: 288806594). However, the human βPKCI is more similar to rat βPKCII and the other way around. Opposing that, in the Swiss-Port database (<http://ca.expasy.org/sprot>) the two βPKC isozymes from human (βPKCI: P05771-1, and βPKCII: P05771-2) and from rat (βPKCI: P68403-1, and βPKCII: P68403-2) are matching (human βPKCI is similar to rat βPKCI and the same for βPKCII isozymes).

Table 1

PKC-derived peptides and their application in HF

Peptide	PKC isozyme	Model	Treatment	response	Ref
β IIIV5-3 ^a	β PKC	Neonatal rat cardiac myocytes	Cells were permeabilized with peptide and PMA for 5 min	Inhibited cardiac myocyte hypertrophy	[32]
δ V1-1 ^b	δ PKC	Neonatal rat cardiac fibroblast	TGF- β 1 and isozyme specific inhibitor	Increased TGF- β 1-induced proliferation	[13]
ζ -pseudo substrate ^c	Atypical PKC isozymes	Neonatal rat cardiac fibroblasts	TGF- β 1 and isozyme specific inhibitor	Stimulates proliferation of neonatal rat cardiac fibroblasts	[13]
ϵ -V1 ^d	ϵ PKC	Transgenic mice	Cardiomyocyte-specific expression	Lethal dilated cardiomyopathy	[42]
$\psi\epsilon$ RACK ^e	ϵ PKC	Transgenic mice	Cardiomyocyte-specific expression	Decreased cardiomyocyte size	[42]
ϵ V1-2 ^f	ϵ PKC	Dahl rats	Sustained treatment with ϵ V1-2	Attenuated cardiac Mast cells degranulation	[41,44]
ϵ V1-2	ϵ PKC	Hypertensive Dahl rats	Sustained treatment with ϵ V1-2	Decreased cardiac fibrosis	[46]
ϵ V1-2	ϵ PKC	Murine heterotopic transplantation	Sustained treatment with ϵ V1-2	Suppress inflammation and prolong graft survival	[49,50]

^a β IIIV5-3- β PKC isozyme-selective peptide translocation inhibitor.

^b δ V1-1- δ PKC isozyme-selective peptide translocation inhibitor.

^c ζ -pseudosubstrate – a selective peptide inhibitor for the atypical PKC isozymes.

^d ϵ -V1 - ϵ PKC-isozyme-selective translocation inhibitor.

^e $\psi\epsilon$ RACK - ϵ PKC-isozyme-selective peptide translocation activator.

^f ϵ V1-2 - ϵ PKC- isozyme-selective peptide translocation inhibitor.