

Selective Phosphorylation Inhibitor of Delta Protein Kinase C—Pyruvate Dehydrogenase Kinase Protein–Protein Interactions: Application for Myocardial Injury *in Vivo*

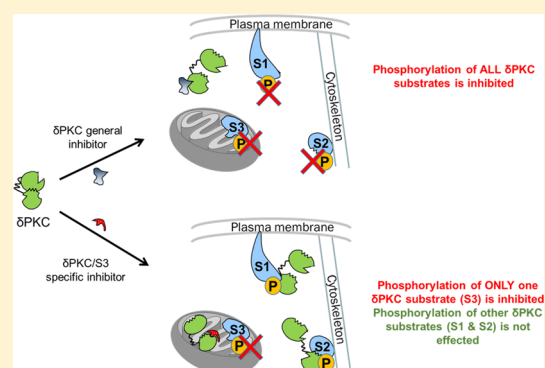
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S Supporting Information

ABSTRACT: Protein kinases regulate numerous cellular processes, including cell growth, metabolism, and cell death. Because the primary sequence and the three-dimensional structure of many kinases are highly similar, the development of selective inhibitors for only one kinase is challenging. Furthermore, many protein kinases are pleiotropic, mediating diverse and sometimes even opposing functions by phosphorylating multiple protein substrates. Here, we set out to develop an inhibitor of a selective protein kinase phosphorylation of only one of its substrates. Focusing on the pleiotropic delta protein kinase C (δ PKC), we used a rational approach to identify a distal docking site on δ PKC for its substrate, pyruvate dehydrogenase kinase (PDK). We reasoned that an inhibitor of PDK's docking should selectively inhibit the phosphorylation of only PDK without affecting phosphorylation of the other δ PKC substrates. Our approach identified a selective inhibitor of PDK docking to δ PKC with an *in vitro* K_d of ~ 50 nM and reducing cardiac injury IC_{50} of ~ 5 nM. This inhibitor, which did not affect the phosphorylation of other δ PKC substrates even at $1 \mu\text{M}$, demonstrated that PDK phosphorylation alone is critical for δ PKC-mediated injury by heart attack. The approach we describe is likely applicable for the identification of other substrate-specific kinase inhibitors.



INTRODUCTION

The protein kinases superfamily accounts for approximately 2% of the eukaryotic genes, and about 518 protein kinases are predicted in the human kinome.¹ Protein kinases catalyze phosphorylation, the transfer of the γ -phosphoryl group from adenosine triphosphate (ATP) to the hydroxyl group of a defined amino acid, which regulates many biological processes, including metabolism, transcription, cell cycle progression, and differentiation. Phosphorylation is the most widespread type of post-translational modification in signal transduction, with over 500 000 potential phosphorylation sites for any given kinase in the human proteome and 25 000 phosphorylation events described for 7000 human proteins.^{2,3} Phosphorylation is mediated by the catalytic domain that consists of a small N-terminal lobe of β -sheets, a larger C-terminal lobe of α -helices, and the ATP binding site in a cleft between the two lobes.⁴ Many kinase inhibitors target the highly conserved ATP-binding pocket.⁵ However, since the catalytic domain of most eukaryotic kinases is structurally similar, developing specific protein kinase inhibitors that target the conserved ATP-binding pocket in a selective manner remains a significant challenge, and targeting different sites in addition to the conserved ATP-binding site to increase selectivity is a promising approach.

One way to achieve specificity between a kinase and its specific substrate involves targeting interactions between docking motifs on the substrate with interaction domains on the kinase, termed docking sites. The interaction site between the substrate and the kinase involves a binding surface for the substrate that is distinct from the catalytic active site on the kinase, and a binding surface on the substrate that is separate from the phosphorylation motif that is chemically modified by the kinase.^{2,6} Distinct docking sites were identified for different substrates, and these sites do not compromise the stereochemical requirements for efficient catalysis by the kinase's active site.⁷ Docking has been characterized for a number of protein kinase families, including c-Jun N-terminal kinases (JNKs), A cyclin-dependent kinase complex (CDKC), and mitogen-activated protein (MAP) kinases.^{8–15} For example, Lee et al. identified a six-amino-acid substrate-docking site on the C-terminal Src kinase (Csk), and a peptide mimicking the docking site inhibits Csk phosphorylation of Src ($IC_{50} = 21 \mu\text{M}$), but only moderately inhibits its general kinase activity.¹⁶

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Protein kinase C (PKC) is a multigene family of related serine/threonine kinases that regulate many cellular processes, including cell cycle, homeostatic control, stress response, and programmed cell death.¹⁷ There are 10 different isoforms within the PKC family, divided into three subfamilies according to the nature of their regulatory domain. All PKCs comprise a C-terminal catalytic domain that is very similar for the different isoforms, linked through a variable domain to a regulatory domain, which is highly divergent among the different isoforms. The uniqueness of the regulatory domains of each PKC, mainly at the C2 domains, plays a critical role for the specific activity of each isoform. Each PKC isoform phosphorylates multiple protein substrates, and selectivity is achieved in part by their subcellular location and the mode of their activation.^{18–20} Previously, we developed inhibitors of protein–protein interactions that elucidated the functions of each PKC isoform. These inhibitors are 6–10-amino-acid long peptides that mimic part of one surface of the interacting proteins, thereby inhibiting the resulting signaling pathways of the given PKC isoform, in a highly specific manner.^{21,22} The ability to modulate PKC signaling in an isoform-specific manner provided an advantage over the isoform-nonspecific PKC inhibitors.²³ However, each PKC isoform phosphorylates many different substrates in the same cell.²⁴ A tool that will selectively inhibit the phosphorylation of one substrate at a time will be highly valuable in identifying how the PKC isoform regulates a particular cellular function.

Delta protein kinase C (δ PKC), cloned over 25 years ago,²⁵ is a pleiotropic kinase that phosphorylates many protein substrates, including heat shock protein 27 (HSP),²⁶ myristoylated alanine-rich C-kinase substrate (MARCKS),²⁷ signal transducer and activator of transcription (STAT),²⁸ glyceraldehyde 3-phosphate dehydrogenase (GAPDH),²⁹ troponin I,³⁰ and pyruvate dehydrogenase kinase (PDK).³¹ The importance of δ PKC signaling was demonstrated in several models of human diseases, such as cancer,³² stroke,³³ sepsis,³⁴ diabetes,^{35,36} neurodegenerative diseases,^{37,38} and ischemic heart disease (heart attack).^{39,40} Previously, a correlation between δ PKC-mediated PDK phosphorylation and cell death following cardiac ischemia was demonstrated.³¹ However, since δ PKC phosphorylates many substrates, the extent to which PDK phosphorylation mediates cardiac injury could not be determined.

Using a rational design, we developed a pharmacological tool to selectively inhibit only δ PKC-mediated PDK phosphorylation. We developed a peptide corresponding to the PDK substrate docking site on δ PKC, ψ PDK peptide. ψ PDK peptide, derived from the regulatory C2 domain of δ PKC, selectively inhibited δ PKC-mediated phosphorylation of only PDK, without affecting the phosphorylation of other δ PKC substrates under the same conditions. We demonstrated that ψ PDK effectively minimized cardiac injury induced by ischemic events *ex vivo* and *in vivo*. Thus, kinase–substrate selective interactions can be useful drug targets, and our rational approach can help identify them.

RESULTS AND DISCUSSION

Rational Design of an Inhibitor of PDK Phosphorylation by δ PKC. Mitochondrial PDK phosphorylation and activation by δ PKC following cardiac ischemia correlates with a large infarct size and inhibition of δ PKC reduces cardiac injury.³¹ However, in addition to PDK, many other substrates are phosphorylated by the pleiotropic enzyme δ PKC. To

determine if inhibition of PDK phosphorylation alone is sufficient to prevent cardiac injury, we developed a selective inhibitor of δ PKC-mediated PDK phosphorylation. To develop such a PDK-selective inhibitor, we reasoned that in addition to binding of δ PKC's catalytic site to the phosphoacceptor site on PDK, a selective PDK-docking site secures the anchoring of this substrate to δ PKC. Such substrate docking sites were previously described for some other kinases.²

We hypothesized that PDK binding to δ PKC should occur only after δ PKC activation, and when δ PKC is inactive, its PDK-docking site may be occupied by a pseudo-PDK (ψ PDK) site, a PDK-like sequence that mimics the δ PKC-binding site on PDK (Figure 1A). However, following δ PKC activation, a

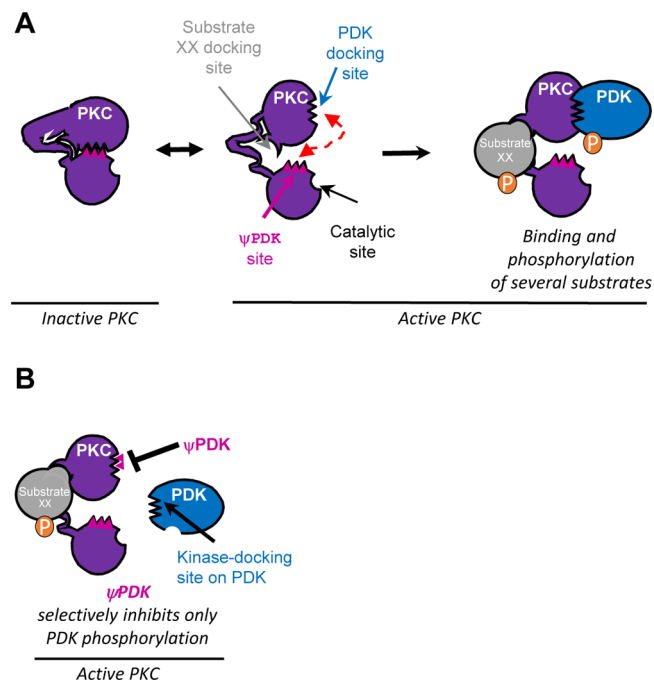


Figure 1. Development of an inhibitor that selectively inhibits only one substrate phosphorylation. (A) An inhibitor that selectively inhibits the docking and phosphorylation of PDK by the multi-substrate (pleiotropic) kinase, δ PKC. Intramolecular interactions within δ PKC are disrupted by PKC activation, exposing the catalytic site as well as selective substrate-docking sites (red arrow, shown are docking sites for PDK and substrate XX on δ PKC). Docking of these substrates to the kinase, concomitantly or one substrate at a time, increases the access of the catalytic site for the substrates, leading to their phosphorylation (P). In the inactive δ PKC (left), the PDK-docking site interacts with a PKC sequence, ψ PDK site, which mimics the kinase-docking site on PDK (blue). (B) A peptide corresponding to this ψ PDK site is a competitive inhibitor for docking to and phosphorylation of PDK by δ PKC, without affecting docking and phosphorylation of other δ PKC substrates (e.g., substrate XX).

conformational change in δ PKC will dissociate the PDK docking site from the ψ PDK site, and will expose the PDK docking site, making it available for protein interaction between the kinase and the substrate (Figure 1A, right panel). A similar concept led to the identification of a pseudo-phosphorylation sequence on many kinases that mimics the phosphoacceptor sequence on substrates.⁴¹ Since a peptide corresponding to pseudosubstrate is a competitive phosphorylation inhibitor of all the substrates of a given kinase, a peptide corresponding to the ψ PDK site should selectively inhibit only the PDK docking

and phosphorylation (Figure 1B). However, this peptide should not affect the binding of other δ PKC substrates (e.g., substrate XX; Figure 1B).

Using Align, we searched for a PDK-like sequence in δ PKC and identified ALSTE (δ PKC_{36–40}), which is highly similar to ALSTD in the four isoforms of PDK (PDK_{391–395}; Figure 2A–C). We reasoned that if the ALSTE/D sequence is required for PDK docking and phosphorylation by δ PKC, it should be conserved across species. Indeed, ALSTE/D is conserved in both δ PKC and PDK, in the species that have δ PKC (Figure 2D,E). Importantly, in species that lack δ PKC, the PDK sequence is missing altogether (e.g., worm and yeast; Figure 2F). The ALSTE/D sequence is also found in a number of other human proteins (Figure 2G). However, this sequence was only 100% conserved in PDK and δ PKC across species (Figure 2G, arrows), suggesting that ALSTE/D is functionally important only in δ PKC and PDK. ALSTE is also absent from other novel PKC isozymes (Figure 2H,I), together suggesting a selective role for ALSTE/D (the ψ PDK site) for δ PKC and PDK interaction. Note that if the overall sequence similarity between the kinase and its substrate is high, it will be challenging to identify a selective docking site using the method described here. Nevertheless, since there are many examples of kinases and substrates with low homology between them, this method will likely be of general use.

Activity and Selectivity of ψ PDK Peptide *in Vitro*. We synthesized a peptide corresponding to ψ PDK site in δ PKC (Chart 1 and Supporting Information, Supplementary Scheme 1 and Supplementary Table 2, for peptide characterization) and including an additional amino acid from δ PKC (ALSTER, Figure 2J), because several peptide inhibitors of protein–protein interactions that we identified previously are at least six amino acids long.^{21,22} ψ PDK peptide blocked δ PKC binding to PDK, as determined by inhibition of co-immunoprecipitation (Figure 3A), and inhibited δ PKC-mediated PDK phosphorylation by over 65%, *in vitro* as compared to ψ PDK analogue with the Thr changed to an Ala (ALSAER, Chart 1; Figure 3B,C). However, ψ PDK peptide did not affect the phosphorylation of other δ PKC substrates, such as GAPDH (Supplementary Figure 1). Next, we determined δ PKC binding to ψ PDK *in vitro*. δ PKC bound to ψ PDK peptide *in vitro* in a time-dependent manner (Figure 3D) with $K_d = 53 \pm 19$ nM (Figure 3E); ϵ PKC, another novel PKC isozyme, did not bind to ψ PDK under the same experimental conditions (Figure 3D). There was a significantly higher K_d measured for the ψ PDK analogue with Thr changed to Ala (ALSAER, Chart 1), which was 1.25 μ M or about 25-fold higher than K_d for δ PKC.

Selectivity of ψ PDK1 Peptide for δ PKC Substrates *ex Vivo*. To test the biological activity of ψ PDK, the peptide was conjugated to the TAT-derived cell-permeating peptide (ψ PDK1, Chart 1, Supplementary Scheme 1, and Supplementary Table 2 for peptide characterization), TAT_{47–57},⁴² which enables safe and effective delivery of peptides into cells in culture, *in vivo*^{43,44} and even in humans.^{45–49} We next determined the effect of ψ PDK1 peptide in a model of myocardial infarction, in which an intact perfused and beating heart is subjected to no-flow (ischemia) followed by reperfusion, as an *ex vivo* model of heart attack. Using this model of ischemic attack (ischemia/reperfusion), we found that ψ PDK1 completely inhibited ischemia/reperfusion-induced increase in phosphorylation of PDK (Figure 4B,C). This effect was similar to δ V1-1 effect (Figure 4B,C), which inhibits translocation and access of δ PKC to all its substrates.³⁹ (Note

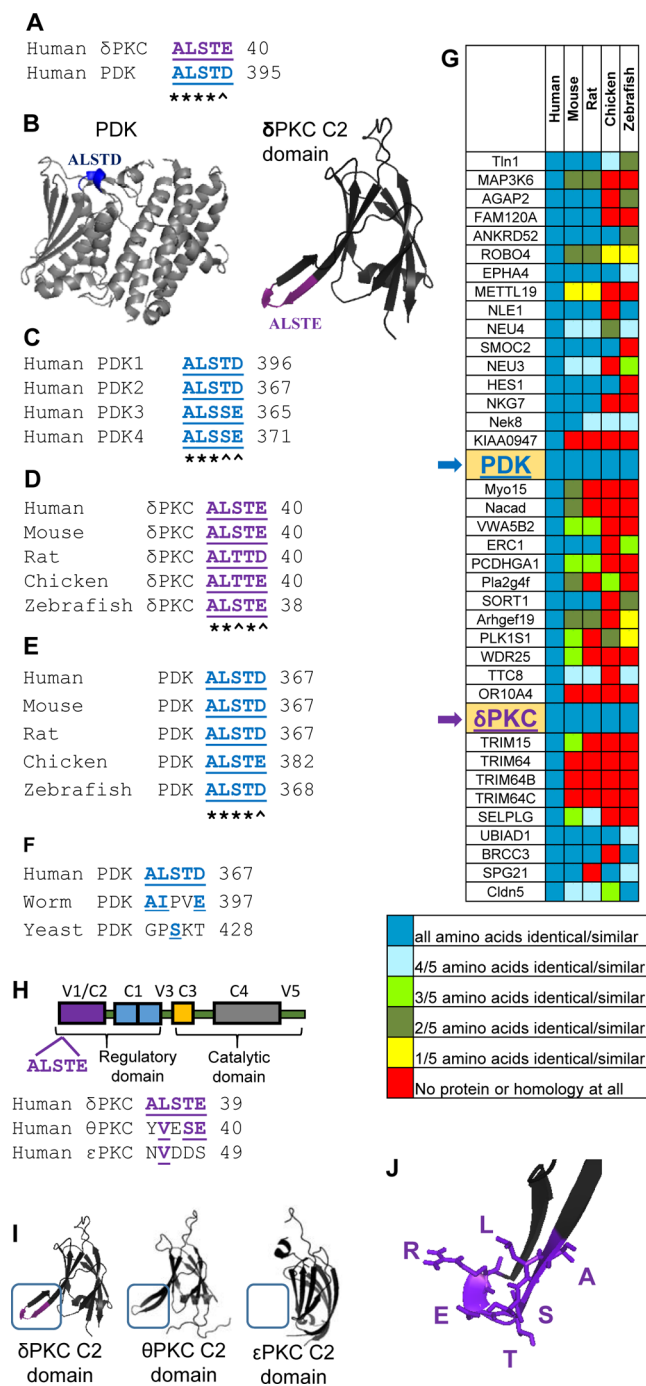
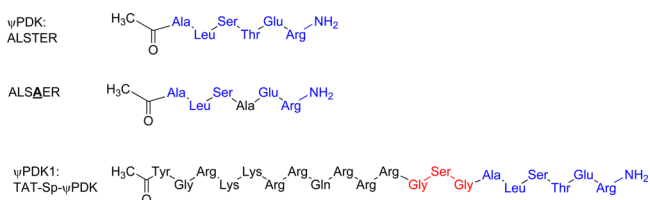


Figure 2. Rational design of an inhibitor of PDK phosphorylation by δ PKC. (A) Sequence alignment of human δ PKC and PDK identified a short sequence of homology, ALSTE/ALSTD. (B) ALSTD in PDK (PDB: 1JM6) and ALSTE in the C2 domain of δ PKC (PDB: 1BDY) are exposed and are available for protein–protein interactions (see colored structures). (C) Conservation of ALSTD sequence in the four PDK isoforms. (D) Conservation of ALSTE sequence in δ PKC and (E) conservation of ALSTD in PDK in a variety of species. (F) Lack of ALSTD conservation in orthologues of PDK in worm or yeast, species that lack δ PKC. (G) ALSTE/ALSTD sequences are found in 39 human proteins. Heat-map of the ALSTE/D conservation in orthologues of these proteins shows ALSTE/D conservation only in PDK and δ PKC. Note that although three proteins [EPHA4 (Ephrin type-A receptor 4), Nek8 (Never in mitosis A-related Kinase 8), and UBIAD1 (Transitional Epithelial Response Protein 1)] exhibit at least four or five amino acids similarity to ALSTE/D in multiple species, all five amino acids are conserved only in PDK and δ PKC and a non-

Figure 2. continued

homologous substitution of any one amino acid in ALSTE/D is sufficient to cause a loss of activity of this peptide (see Figure 5G). (Further information about all of the proteins is given in the Supporting Information, Supplementary Table 1). (H,I), ALSTE sequence is not present in the C2 domains of other members of the novel PKC isozymes, ϵ PKC and θ PKC, to which δ PKC belongs.³⁹ (J) The ψ PDK site, ALSTE (including the adjacent R; see below), in the C2 domain of δ PKC (PDB: 1BDY). * denotes identity, and ^ denotes homology.

Chart 1. Chemical Structure of the ψ PDK, ψ PDK Analogue, and ψ PDK1 Peptides^a



^a ψ PDK peptide, an analogue of ψ PDK with an Ala substitution for the Thr (ALSAER) and ψ PDK with TAT₄₇₋₅₇ carrier peptide, using GSG as a spacer (ψ PDK1).

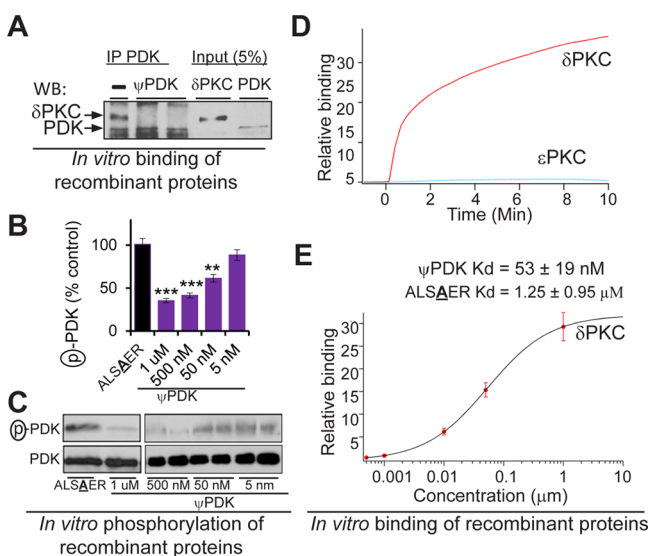


Figure 3. Activity and selectivity of ψ PDK peptide *in vitro*. (A) ψ PDK (1 μ M) inhibited PDK/ δ PKC interaction *in vitro*, determined by co-precipitation and Western blot analysis ($n = 3$). (B,C) δ PKC-mediated PDK phosphorylation *in vitro* was inhibited by ψ PDK (5 nM to 1 μ M) relative to control peptide analogue of ψ PDK, in which one amino acid (Thr) was changed for an alanine (ALSAER) ($n = 3$). (D) Binding curves of δ PKC and ϵ PKC, at ~ 75 μ g/mL (~ 1 μ M), to ψ PDK peptide. ψ PDK selectively binds to δ PKC as compared with another novel PKC, ϵ PKC. (E) Binding assay of increasing amounts of δ PKC to ψ PDK or to ALSAER, an analogue of ψ PDK, in which one amino acid (Thr) was substituted for an alanine. ψ PDK selectively binds to δ PKC ($IC_{50} = 53$ nM) compared with ALSAER ($IC_{50} = 1.25$ μ M). Data presented as mean \pm SEM, ** $p < 0.01$, *** $p < 0.005$, compared to TAT control.

that two-dimensional polyacrylamide gel electrophoresis (PAGE) allows the separation of PDK phosphorylation states from the lowest, spot 4, to the highest, spot 1; see Figure 4B.) Quantitation of spots 1 and 2 is provided in Figure 4C. For the analysis, we focused on spots 1 and 2, because only these two

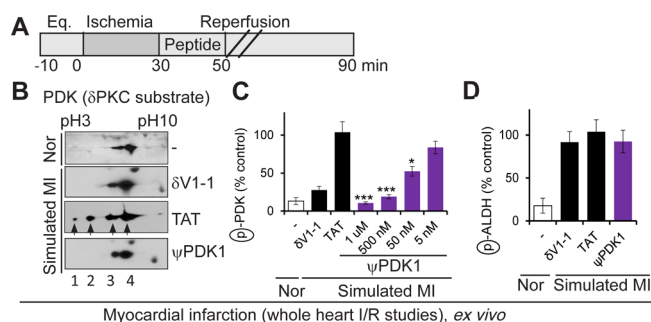


Figure 4. ψ PDK1 peptide selectivity for δ PKC substrates: measurement in whole heart subjected to simulated myocardial infarction *ex vivo*. (A) Protocol of myocardial infarction model using isolated hearts subjected to ischemia and reperfusion (a model of simulated myocardial infarction; MI) or normoxia (Nor). Bars indicate the length (in minutes) of each treatment (eq = equilibration). Rat hearts were subjected to 30 min ischemia followed by 60 min reperfusion without or with peptide treatment for the first 20 min only. (B) Phosphorylation of PDK in heart extracts after ischemia and reperfusion in the presence of ψ PDK1 or control peptide (1 μ M). (C) Dose-dependent effect of ψ PDK1 peptide treatment on the phosphorylation of PDK spots 1 and 2 (B) is expressed as percent change from phosphorylation in the presence of control peptide, TAT ($n = 6$). (D) As a further indication for the selectivity of ψ PDK1 peptide, we showed that phosphorylation of another mitochondrial protein, aldehyde dehydrogenase 2 (ALDH2), that is also phosphorylated under ischemic conditions,⁵¹ was unaffected by ψ PDK1 peptide treatment ($n = 4$). Data presented as mean \pm SEM, * $p < 0.05$, *** $p < 0.005$, compared to TAT control.

spots were inhibited by δ PKC phosphorylation, as seen when using the general δ PKC inhibitor, δ V1-1; PDK basal level of phosphorylation (spots 3 and 4) is not mediated by δ PKC. PDK activation leads to phosphorylation and inhibition of the mitochondrial pyruvate dehydrogenase (PDH), thus inhibiting ATP generation by the mitochondria.⁵⁰ As expected, ψ PDK1 treatment inhibited PDK activation, as demonstrated by inhibition of PDH phosphorylation (Supplementary Figure 2). To determine whether ψ PDK1 inhibition was selective for δ PKC-mediated phosphorylation inside the mitochondria, we also examined the phosphorylation state of aldehyde dehydrogenase 2 (ALDH2), an ϵ PKC-selective mitochondrial substrate.⁵¹ As expected for isozyme-specific peptide inhibitor, ψ PDK1 peptide did not affect ALDH2 phosphorylation (Figure 4D), demonstrating the selective effect of ψ PDK1 for δ PKC-mediated phosphorylation.

It is formally possible that a given substrate uses the same interface to interact with and be phosphorylated by several different protein kinases and thus a protein–protein interaction substrate inhibitor identified by our method may affect more than one kinase of that substrate. This possibility could not be examined directly for PDK and ψ PDK, as the identity of the other PDK kinases are not known. Nevertheless, our data in Figure 4 demonstrate that only the δ PKC-dependent phosphorylation (δ V1-1 sensitive spots, 1 and 2) were also inhibited by ψ PDK. Spots 3 and 4, which are product of phosphorylation by another kinase, were not inhibited by ψ PDK treatment, suggesting that the above possibility is unlikely, at least for this protein–protein interaction.

Treatment with ψ PDK1 at Reperfusion (after the Ischemic Period) Decreases Cardiac Injury. As discussed above, PDK phosphorylation inhibits PDH, thereby shutting down ATP generation through the tricarboxylic acid (TCA)

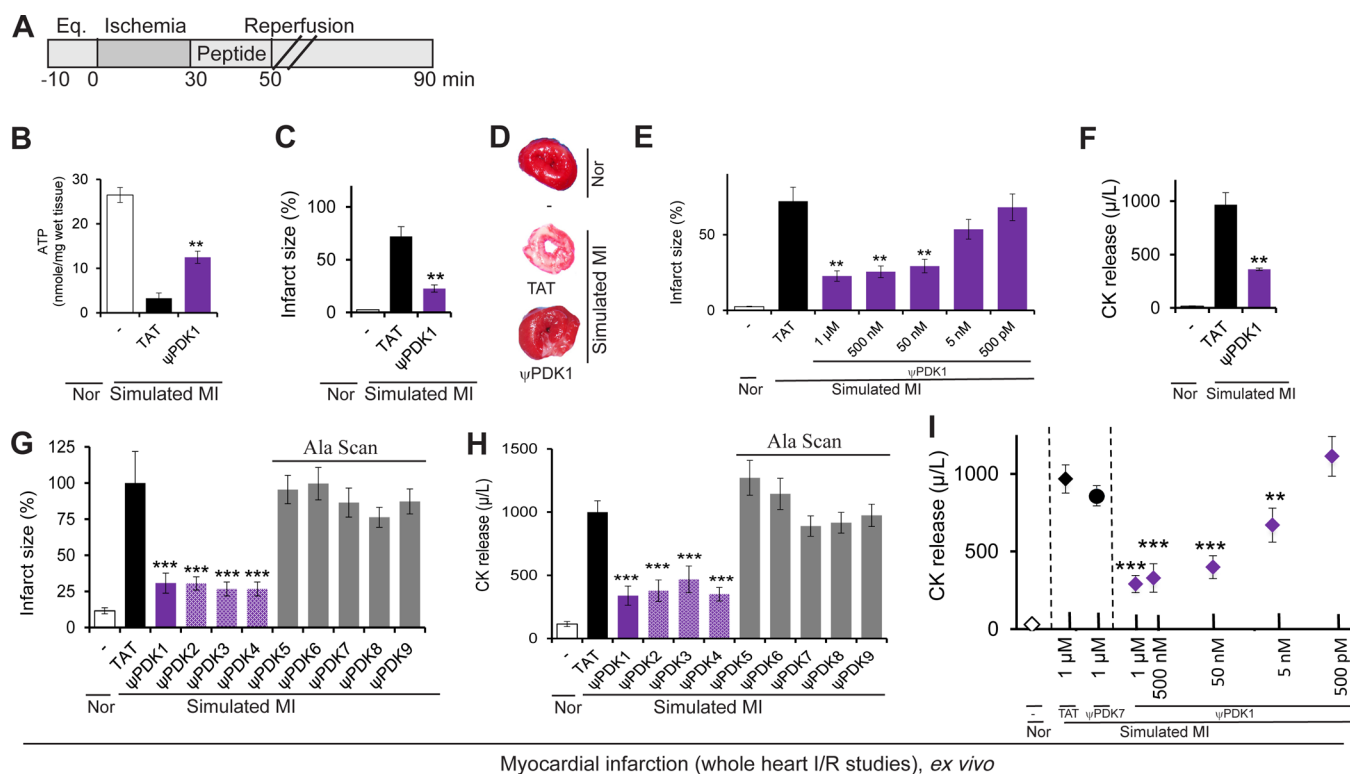
Myocardial infarction (whole heart I/R studies), *ex vivo*

Figure 5. ψ PDK1 peptide cardioprotective activity and structure activity studies; measurement in whole heart subjected to simulated myocardial infarction *ex vivo*. (A) Protocol of myocardial infarction model using isolated hearts subjected to ischemia and reperfusion (a model of simulated myocardial infarction, MI) or normoxia (Nor). Bars indicate the length (in minutes) of each treatment (eq = equilibration). Rat hearts were subjected to 30 min ischemia followed by 60 min reperfusion without or with peptide treatment for the first 20 min only. (B) Protection from myocardial injury was determined by analyses of the levels of tissue ATP, expressed as nmol ATP per cardiac wet weight ($n = 3$); (C,D) 2,3,5-Triphenyltetrazolium chloride (TTC) staining (red indicates live tissue and white indicates dead; $n = 6$ /hearts per treatment), and (E) dose-dependent effect of ψ PDK1 peptide treatment on TTC staining. (F) Protection from myocardial injury was determined by analyses of the levels of release of CK ($n = 6$) via structure–activity relationship (SAR) studies. We tested the effect of the relative positions of the cargo (ALSTER) and the carrier (TAT) on the bioactivity. Protection from myocardial injury was determined by infarct size and CK levels following simulated myocardial infarction (G,H, purple columns). We also performed alanine scanning of ψ PDK1 demonstrated that substitution of any of the amino acids with alanine (A) caused a reduction or a complete loss of bioactivity (G,H, gray columns) ($n = 6$ hearts per treatment). (I) Dose-dependent effect of ψ PDK1 peptide treatment on total CK release following simulated MI during 30 min reperfusion demonstrates an $IC_{50} \approx 5$ nM ($n = 6$ hearts/dose). Data are presented as mean \pm SEM, $**p < 0.01$, $***p < 0.005$, compared to TAT control.

cycle. As expected, and using the above model of heart attack *ex vivo* (Figure 5A), ψ PDK1, which inhibits δ PKC-mediated PDK phosphorylation (Figure 4B,C), increased ATP levels (Figure 5B). Following ischemia and reperfusion ATP levels were $\sim 15\%$ of the levels under normoxic conditions and following treatment with ψ PDK1, ATP levels increased by three folds (Figure 5B).

Importantly, ψ PDK1 treatment decreased infarct size by $\sim 70\%$, as compared to treatment with control peptides when delivered only during reperfusion (Figure 5C–E). ψ PDK1 treatment also decreased the release levels of creatine kinase (CK; Figure 5F), a marker that is used extensively as an indication for myocardial damage in heart attacks in humans, as well as reduced levels of JNK phosphorylation, a marker of cell death (Supplementary Figure 3).

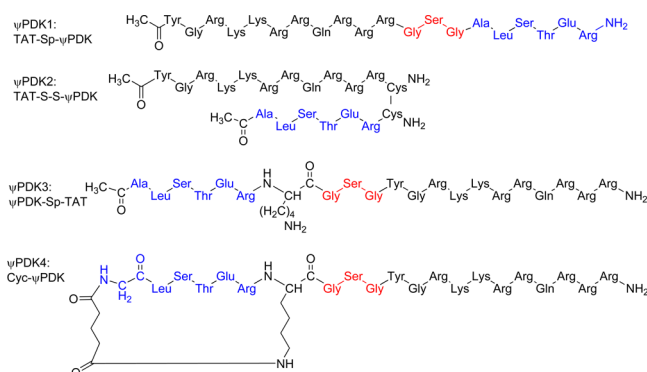
Structure–Activity Relationship (SAR) Studies. There was no significant change in activity of ψ PDK1 when the cargo was linked at the C-terminus of TAT (ψ PDK1), or at the N-terminus of TAT (ψ PDK3) as one polypeptide. In addition, there was no difference in activity if the cargo was conjugated to TAT by disulfide bridge (ψ PDK2). In some cases cyclization can improve the bioactivity properties of linear peptides, here we tested one cyclic peptide (ψ PDK4) with preliminary linker,

which also did not improve the bioactivity of the linear peptide (ψ PDK1). (Figure 5G,H; see Chart 2 for peptide structure and Supplementary Table 2 for peptide characterization). In addition, we determined the contribution of all the amino acids of ψ PDK to the biological activity of this peptide using Ala scan of the cargo by substituting each amino acid with an alanine. Confirming our binding studies (Figure 3E), we found that changing the Thr (ALSAER) or any other amino acid with an alanine abolished the biological activity of the peptide (Figure 5G,H, ψ PDK5-9; for peptide structure see Supplementary Chart 1, and for peptide characterization see Supplementary Table 2). This supports our results examining evolutionary conservation of this sequence (Figure 2G). A dose–response study demonstrated that ψ PDK1 is highly active; the IC_{50} for ψ PDK1 peptide effect in reducing cardiac injury *ex vivo* was ~ 5 nM (Figure 5I), as measured by cardiac CK release, a clinical biomarker for heart attack.

Cardioprotective Effect of ψ PDK1 Peptide *in Vivo*. Treatment with ψ PDK1 peptide (2 mg/kg) *in vivo*, immediately after 30 min ischemia reduced infarct size by $\sim 50\%$ (Figure 6B,C), indicating the efficacy of the peptide.

We also confirmed that ψ PDK1 is safe. A 6-week sustained treatment of mice with 2 mg/kg/day by implanting an osmotic

Chart 2. Chemical Structure of the ψ PDK1 Peptide Analogues^a



^aThe peptides comprise TAT₄₇₋₅₇, a short positively charged peptide that is used as a carrier for the delivery of the peptides into the cell (black); a spacer, composed of three amino acids used as spacers between TAT and the cargo (red); and the cargo (blue). A cyclic peptide analogue with conformational constraints was also prepared; this analogue has the same amino acids as the linear peptide with extra alkyl chains that were used for cyclization.

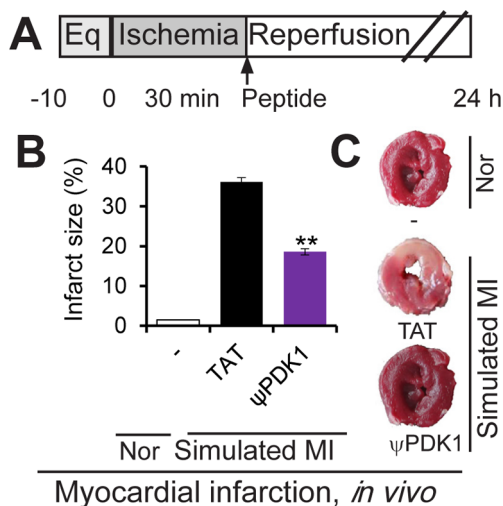


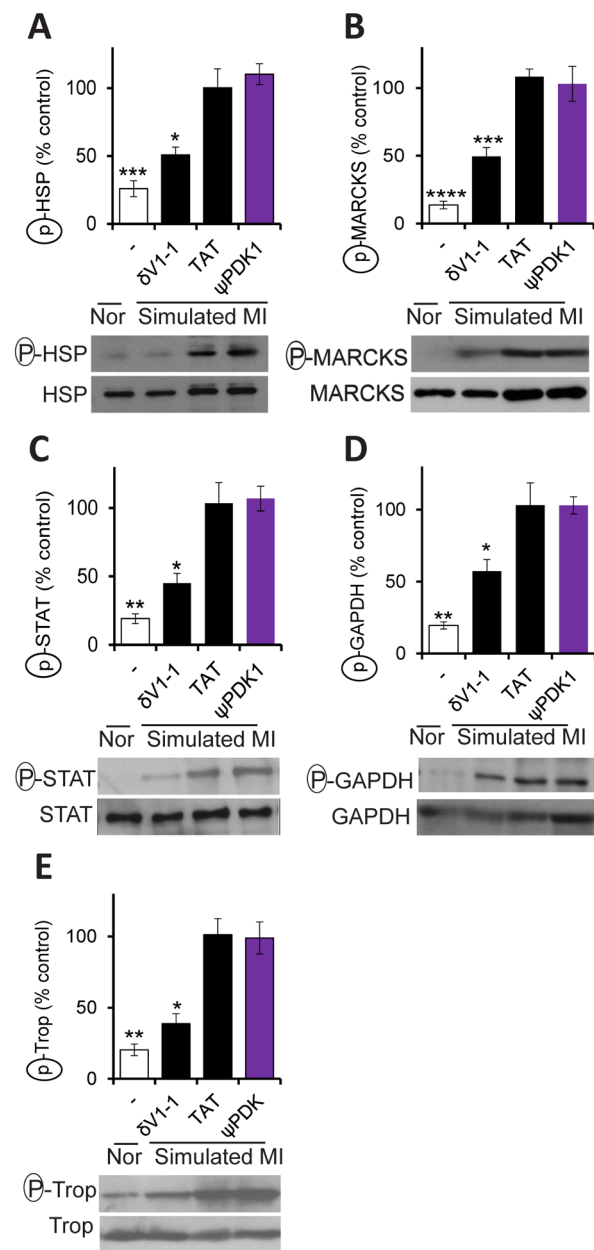
Figure 6. Activity of ψ PDK1 peptide *in vivo*. (A) Protocol of myocardial infarction model *in vivo*. Rats were subjected to 30 min ischemia and 24 h reperfusion without or with peptide treatment (2 mg/kg intraperitoneal injection). Shown are the effects of ψ PDK1 and TAT control peptides on infarct size ($n = 6$) (B), and examples of TTC staining (C). Data are presented as mean \pm SEM, ** $p < 0.01$, compared to TAT control.

pumps subcutaneously on their back, which provide slow and sustained delivery of the peptide,⁵² caused no changes in behavior, weight gain, and other toxicity measures (Supplemental Figure 4).

Selectivity of ψ PDK1 Peptide for Different δ PKC Substrates *ex Vivo*. Since δ PKC is a pleiotropic kinase, phosphorylating many protein substrates,⁵³ we next determined the selectivity of ψ PDK1 peptide for PDK phosphorylation by measuring the phosphorylation of five other δ PKC substrates. At 1 μ M, a concentration that is 200-fold higher than its IC₅₀ (Figure S1), ψ PDK1 peptide inhibited the phosphorylation of PDK (Figure 4B,C), but not the phosphorylation of HSP,²⁶ MARCKS,²⁷ STAT,²⁸ GAPDH,²⁹ and troponin I³⁰ (Figure 7A–E). These data demonstrate the high specificity of ψ PDK1

as a selective inhibitor of only one substrate of δ PKC, the phosphorylation of PDK.

Finally, as most protein kinases have pleiotropic roles by phosphorylating multiple protein substrates, we designed additional inhibitors using the same rational approach. Table 1 lists three of these potential inhibitors. One derived from δ PKC and another substrate, annexin V (ANXAS).⁵⁴ Another

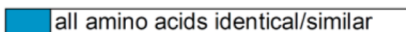


Myocardial infarction (whole heart I/R studies), *ex vivo*

Figure 7. Selectivity of ψ PDK1 peptide as an inhibitor of PDK phosphorylation; phosphorylation of five other δ PKC substrates following simulated myocardial infarction was not inhibited. Phosphorylation of HSP27 (A), MARCKS (B), STAT (C), GAPDH (D), or troponin I (E) in heart extracts after ischemia and reperfusion in the presence of control or ψ PDK1 peptide (1 μ M). Phosphorylation is expressed as percent change from control (TAT)-treated hearts. Data are representative of at least four independent experiments and presented as mean \pm SEM, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, **** $p < 0.001$, compared to ψ PDK1 peptide-treated hearts.

Table 1. Rational Design of Potential Inhibitors of Three Ser/Thr Kinases for One of Their Substrates^a

Proteins	Sequences	Human	Mouse	Rat	Chicken	Zebrafish
PKC						
PKC δ	VLMRAAE ***^** .					
ANXA5	VLLQANR					
PKA						
PKA	DGQKIVV ***^**					
CREB	DGQQILV					
PKB						
AKT1	IKITDFG ^***^**					
ASK1	LKISDFG					

 all amino acids identical/similar

^a * denotes identity, ^ denotes homology, and . denotes opposite charge.

one derived from a substrate of the cyclic AMP-dependent protein kinase (PKA),⁵⁵ and the last one from a substrate of protein kinase B or Akt (PKB/Akt).⁵⁶ Identifying the particular substrate that mediates a given function involves extensive mutagenesis, which is time-consuming and expensive. We suggest that our rational approach can provide a quick path to generate highly selective and effective inhibitors of pleiotropic protein kinases, to be used in basic research and as leads for novel drugs.

Sequence alignment of δ PKC, cAMP-dependent protein kinase (PKA), and protein kinase B (PKB) with one of their substrates identifies short sequences of homology that likely represent a docking site for that kinase on the corresponding substrate. For δ PKC we identified another substrate, annexin V (ANXA5). For PKA we identified the docking site on cyclic AMP-responsive element-binding protein 1 (CREB).⁵⁵ For PKB we identified the docking site on the apoptosis signal-regulating kinase 1 (ASK1).⁵⁶ Shown is a heat-map (Table 1) of the conservation of these sequences in evolution and color code for conservation. Peptides corresponding to each of these short sequences should be effective inhibitor of the corresponding kinases and therefore useful pharmacological tools to determine what is the functional consequence of phosphorylation of that substrate by the given kinase.

CONCLUSION

In this study, we describe a rational approach to develop a selective inhibitory peptide for a kinase phosphorylation of one of its many substrates as a mean to determine the functional contribution of this one substrate. This approach is fast and inexpensive as compared to screening of big chemical libraries to identify an inhibitor, and is not as laborious as mutating each phosphorylation site on a given protein substrate. To date, over 50 peptides have been approved for clinical use, resulting in many therapeutic products, such as cyclosporin A, tyrocidine A, gramicidin S, and somatostatin,⁵⁷ and in 2013, their market value was estimated to be \$15 billion.⁵⁸ Although not examined in details in this study, peptide modification, such as N-methylation, the use of D-amino acids and cyclization was

shown to improve peptides stability and bioavailability. Therefore, the use of rational approach to identify inhibitory peptides is a promising approach to develop novel therapeutics.

In addition to the potential acute use of ψ PDK analogues for the treatment of heart attack, PDK activation contributes to cardiac dysfunction and heart failure,^{59,60} suggesting that PDK inhibition may be an effective target for treating chronic heart disease. However, although small molecule PDK inhibitors can increase the recovery rate of cardiac function following simulated myocardial infarction *in vitro*,^{61,62} these PDK inhibitors cause myocardial steatosis and sometimes death within a few days of treatment *in vivo*.⁶³ In contrast, ψ PDK1 peptide selectively inhibited only excessive activation of PDK by δ PKC, but not basal PDK activity. Therefore, ψ PDK1 peptide will likely have a therapeutic advantage over other inhibitors of PDK activity.⁶⁴

Finally, because δ PKC mediates different and sometimes opposing effects,⁶⁵ depending on the substrates that it phosphorylates, ψ PDK1 peptide has also an advantage relative to other existing δ PKC kinase inhibitors (e.g., the ATP competitive inhibitor, rottlerin,⁶⁶ and the anchoring inhibitory peptide, δ V1-1^{39,47}). Since it does not affect other potentially protective δ PKC-mediated functions, ψ PDK1 peptide can be used in cases when it is necessary to target only this particular substrate of δ PKC, such as following myocardial infarction.

EXPERIMENTAL METHODS

Peptide Synthesis. In brief, peptides were synthesized on solid support using a fully automated microwave peptide synthesizer (Liberty, CEM Corporation). The peptides were synthesized by solid-phase peptide synthesis (SPPS) methodology⁶⁷ with a fluorenylmethoxycarbonyl (Fmoc)/*tert*-Butyl (tBu) protocol. The lysine side chain was protected with *N*-methyltrityl (Mtt), a protection group that can be deprotected selectively using acid labile conditions.⁶⁸ After completion of the synthesis of the linear peptide, an anhydride spacer was coupled to the N-terminal amino group and cyclization was performed using amide bonds between the moiety linker at the backbone N-terminus and an epsilon amino on the side chain of a C-terminal Lys residue.^{69,70} The final cleavage and side chain deprotection was done manually without microwave energy. Peptides were analyzed by analytical reverse-phase high-pressure liquid chromatography (RP-HPLC) (Shimadzu, MD, USA) and matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and purified by preparative RP-HPLC (Shimadzu, MD, USA). For full details, see Supporting Information.

Sequence Alignments. Sequences from different species were aligned using Lalign server, using δ PKC proteins [*Homo sapiens* (Q05655), *Mus musculus* (P28867), *Rattus norvegicus* (P09215), *Gallus gallus* (gil57524924), and *Danio rerio* (gil47550719)], PDK proteins [*Homo sapiens* (Q15119), *Mus musculus* (Q9JK42), *Rattus norvegicus* (Q64536), *Gallus gallus* (gil315583003), *Danio rerio* (gil41055902), *Ascaris suum* (worm) (O02623), and *Saccharomyces cerevisiae* (yeast) (P40530)]; ϵ PKC protein [human (Q02156)]; and θ PKC protein [human (Q04759)]. Details about additional proteins used for sequence alignments are in Supplementary Table 1.

ψ PDK Inhibits PDK/ δ PKC Interaction *in Vitro*. First, 200 ng of recombinant δ PKC (Invitrogen, CA, USA) was incubated with or without the indicated peptides (1 μ M) for 10 min, prior to adding 300 ng of recombinant PDK2-GST (Abnova, Taiwan) for 20 min at 37 °C. PDK was immunoprecipitated using anti-PDK (AP9827a, Abgent, CA, USA), and δ PKC binding to PDK was determined using rabbit anti- δ PKC antibodies (C-17, Santa Cruz Biotechnology, CA, USA). The intensity of the spots was measured using NIH ImageJ.⁷¹

ψ PDK Inhibits PDK Phosphorylation by δ PKC *in Vitro*. First, 200 ng of recombinant δ PKC protein (Invitrogen, CA, USA) was incubated with or without the peptides (5 nM to 1 μ M) for 10 min, and then 200 ng of recombinant PDK2 (Abcam, UK) was added for

10 min at 37 °C in kinase buffer (40 μ L), Tris-HCl (20 mmol/L), $MgCl_2$ (20 mmol/L), DTT (1 μ mol/L), ATP (25 μ mol/L), and $CaCl_2$ (1 mmol/L) in the presence of the PKC activators, phosphatidylserine (PS, 1.25 μ g) and 1,2 dioleoyl-*sn*-glycerol (DG, 0.04 μ g). The kinase assay was terminated by adding loading Laemmli buffer containing 5% SDS, and the samples were loaded on a 10% PAGE-SDS polyacrylamide gel, and the levels of phosphorylated PDK2 protein were determined using anti-phosphothreonine (9381S and 2351S, Cell Signaling, MA, USA) and anti-phosphoserine PKC substrate (2261L, Cell Signaling, MA, USA) antibodies. The nitrocellulose was also reprobed using anti-PDK (AP9827a, Abgent, CA, USA) to confirm that equal amounts of PDK were used. The intensity of the spots was measured using NIH ImageJ.⁷¹

ψ PDK Binding to δ PKC and ϵ PKC *In Vitro*. Binding data of δ PKC and ϵ PKC to immobilized ψ PDK or control peptide (ALSAER) *in vitro* was gathered using an AGILE R100 label-free binding assay (Nanomemedical Diagnostics Inc., CA, USA), following their standard protocol. 1-Ethyl-3-(3-(dimethylamino)propyl)carbodiimide (EDAC) (2 mg) and sulfo-*N*-hydroxysuccinimide (sNHS) (6 mg) from Sigma-Aldrich (MO, USA) were used in MES buffer (pH 6.0, 5 mL) for 15 min to covalently attach the amine of the peptide the carboxyl on the chip. Peptide solution (6 μ M) was incubated with the chip for 15 min. Next, an amine-terminated short chain polyethylene glycol (3 mM) followed by ethylamine (1 M) were applied serially for 15 min each to quench remaining unoccupied binding sites on the chip. After a rinse in PBS, baseline current levels for the chip were recorded for at least 2 min. Next, the PBS was aspirated and a 30 μ L droplet of the tested protein (75 μ g/mL, recombinant δ PKC, Invitrogen, CA, USA) was applied to the chip and the change in the sensor chip readout was recorded for 15 min. Additional measurements were performed using varying concentrations or using recombinant ϵ PKC (75 μ g/mL, GenWay, CA, USA), as a control. After data were gathered, the responses of 25 sensors on a single assay chip were averaged, and any background drift recorded in PBS was subtracted. A Hill equation fit was used to determine K_d . K_d values were also calculated by measurement of the Kon and Koff values at a single concentration. This was done by fitting the binding curve to a double exponential function and the first rinse to a single exponential using a single concentration for the tested peptides and values obtained by these calculation methods were almost identical.

Animal Studies. Based on our previous experience, a minimum of six rodents per group is required to obtain statistically meaningful data.⁴⁰ An experimental group size of six or more animals is necessary to achieve at least a 20% minimal difference for a power of 95% with $\alpha < 0.05$ and $\beta < 20\%$. All treatments were performed between 9:00 a.m. and 4:00 p.m. by observers blinded to the treatment groups. Rodents were housed in a temperature- and light-controlled room for at least 3 days before use. All animals were randomized and assigned to testing groups to generate biological replicates for each group.

Animal Care. Animal care and husbandry procedures were in accordance with established institutional and National Institutes of Health guidelines. The animal protocols were approved by the Stanford University Institutional Animal Care and Use Committee, and by the Jinan University Institutional Animal Care and Use Committee.

***Ex Vivo* Rat Heart Model of Myocardial Infarction-Induced Ischemia and Reperfusion Injury.** An *ex vivo* model of acute ischemic heart injury was carried out as previously described.³⁹ Briefly, Wistar male rats (250–275 g) 4–6 weeks old, purchased from Charles River (MA, USA), were heparinized (1000 units/kg; intraperitoneal injection), anesthetized with Beuthanasia-D (100 mg/kg intraperitoneal injection), and then treated with different peptides. Hearts were rapidly excised and then perfused with an oxygenated Krebs–Henseleit buffer containing NaCl (120 mmol/L), KCl (5.8 mmol/L), $NaHCO_3$ (25 mmol/L), NaH_2PO_4 (1.2 mmol/L), $MgSO_4$ (1.2 mmol/L), $CaCl_2$ (1.0 mmol/L), and dextrose (10 mmol/L) 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. After 10 min of equilibration, the hearts were subjected to 30 min of global ischemia and 60 min of

reperfusion. The hearts were perfused with 500 pM to 1 μ M peptides for 20 min immediately following the ischemic period. Normoxic control hearts were subjected to 90 min of perfusion in the absence of ischemia. Coronary effluent was collected to determine CK release during the first 30 min of the reperfusion period. At the end of the reperfusion period, hearts were sliced into 1-mm-thick transverse sections and incubated in triphenyltetrazolium chloride solution (TTC, 1% in phosphate buffer, pH 7.4) at 37 °C for 15 min. Infarct size, expressed as a percentage of the risk zone (equivalent to total muscle mass), CK release, and JNK phosphorylation were used to assess cardiac damage, as described previously.^{39,72} Hearts were excluded from the study if they met one of the following criteria: (1) time to perfusion was over 3 min, (2) coronary flow was outside the range of 9–15 mL/min, or (3) heart rate was below 240 beats/min or appearance of severe arrhythmia.

***In Vivo* Rat Acute Ischemia and Reperfusion (Acute Myocardial Infarction) Model.** An open chest model was carried out using Sprague–Dawley male rats (200–230 g) from Jinan University. After inducing anesthesia with isoflurane (2.5% in air), artificial respiration was set via cannulation (rate, 120 breaths/min; volume, 2 mL/time; body temperature maintained at 37 °C). Left thoracotomy was performed between the fourth and fifth ribs to expose the heart. After opening the pericardial cavity and 10 min equilibrium, the left anterior descending coronary artery (LAD) was ligated with 3–0 silk suture at the middle part of the left coronary artery. Occlusion was determined by observation of immediate pallor of the left ventricular free wall. Thirty minutes after artery ligation, the suture was released and flow was recovered in the coronary artery. Where indicated, peptides (2 mg/kg) were injected intraperitoneally at reperfusion. The normoxia control animals (sham) were exposed to the same procedure with no ligation. The chest was then closed in layers with 2–0 silk suture, and 24 h later the animals were euthanized with an overdose of pentobarbital (100 mg/kg) delivered by intraperitoneal injection. The chest was opened and the heart was isolated for further infarct size analysis.

***In Vivo* Peptide Toxicity Assay.** BALB/c mice (23–26 g, 2–8 weeks old), were treated with the peptides (6–8 mice per group) for 6 weeks to assess their toxicity. Osmotic pumps (#2002, 0.5 μ L/h, Alzet, CA, USA) filled with ψ PDK1 (2 mg/kg/day) or control peptide were implanted subcutaneously on the back of the mice following anesthesia using a standard surgical procedure as recommended by the manufacturer.

Western Blot Analysis and 2D Gel Analysis. Rat hearts were homogenized in a buffer A [mannitol (210 mmol/L), sucrose (70 mmol/L), MOPS (5 mmol/L), and ethylenediaminetetraacetic acid (EDTA) (1 mmol/L)] in the presence of protease inhibitor and phosphatase inhibitor mixtures (Sigma-Aldrich, MO, USA) followed by isolation of the mitochondrial fraction. Tissue extract was centrifuged at 700g to pellet nuclei and unbroken cellular debris, followed by centrifugation at 10000g to collect mitochondrial-enriched fractions, as described.⁷³

For protein phosphorylation, fractions were resuspended in buffer A. For 2D IEF/SDS polyacrylamide gel electrophoresis, the samples were homogenized in buffer consisting of urea (7 mol/L), thiourea (2 mol/L) and CHAPS (4%) in the presence of protease inhibitor and phosphatase inhibitor mixtures (Sigma-Aldrich, MO, USA). Supernatants were subjected to a first dimensional separation by an IPGphor isoelectric focus power supply using pre-cast Immobiline DryStrip pI 3–10 strips according to the manufacturer's instruction manual (Amersham Biosciences, NJ, USA). 10% SDS gel electrophoresis and Western blotting were carried out using anti-PDK2 (AP9827a, Abgent, CA, USA), anti-PDH (456600, Invitrogen, CA, USA), anti-ALDH2 (48837, Santa Cruz Biotechnology, CA, USA), anti-phosphothreonine (9381S and 2351S, Cell Signaling, MA, USA), and anti-phosphoserine PKC substrate (2261L, Cell Signaling, MA, USA) antibodies. Phosphatase treatment confirmed that the leftward shift in PDK mobility is due to phosphorylation.³¹ The intensity of the spots was measured using NIH ImageJ.⁷¹

Phosphorylation of HSP27, MARCKS, STAT, Troponin I, and JNK in the *ex vivo* model was determined on 1D SDS PAGE, using anti-

phospho-HSP27 (04-447, EMD Millipore, MA, USA) and anti-HSP27 (ADI-SPA-800-F, Enzo Life Sciences, NY, USA), anti-phospho-MARCKS (2741, Cell Signaling, MA, USA) and anti-MARCKS (6455, Santa Cruz Biotechnology, CA, USA), anti-phospho-STAT (8826S, Cell Signaling, MA, USA) and anti-STAT (346, Santa Cruz, CA, USA), anti-phospho-Troponin I (4004, Cell signaling, MA, USA) and anti-Troponin I (31655, Santa Cruz Biotechnology, CA, USA), anti-phospho-SAPK/JNK (9251, Cell Signaling, MA, USA), and anti-SAPK/JNK (9252, Cell Signaling, MA, USA) antibodies. The levels of phosphorylated substrates were normalized for total substrate and presented as a ratio to the material from hearts subjected to ischemia/reperfusion in the presence of treatment. The intensity of the spots were measured using NIH ImageJ.⁷¹

Phosphorylation of GAPDH in the *ex vivo* model was determined after immunoprecipitation. Samples were incubated with anti-GAPDH antibody (Mab6C5, Advanced Immunochemical, CA, USA) in buffer containing Tris-base, pH 7.4 (10 mM), NaCl (150 mM), Triton X-100 (0.1%), EDTA, pH 8 (5 mM), and protease inhibitor overnight at 4 °C with gentle agitation. Protein A/G beads were then added, and the mixture was incubated for 2 h at 4 °C. The mixture was centrifuged for 1 min at 800g, and the immunoprecipitates were washed three times with buffer and analyzed by 10% SDS-PAGE and followed by Western blot using anti-phosphothreonine (9381S and 2351S, Cell Signaling, MA, USA), anti-phosphoserine PKC substrate (2261L, Cell Signaling, MA, USA) and anti-GAPDH antibodies (Mab6C5, Advanced Immunochemical, CA, USA). The levels of phosphorylated substrates were normalized for total substrate and presented as a ratio to the material from hearts subjected to ischemia/reperfusion in the presence of control peptide. The intensity of the spots was measured using NIH ImageJ.⁷¹

ATP Level Determination. Following the *ex vivo* ischemia/reperfusion experiment, 100 mg heart tissue was homogenized in 1% TCA (500 μ L). The lysate was spun to remove debris and the pH was adjusted to pH 7.4. A total of 10 μ L of the lysate was used in a 200 μ L assay for quantitative determination of ATP levels with recombinant firefly luciferase and its substrate, D-luciferin, according to the manufacture's protocol (Invitrogen, NY, USA).

Statistical Analysis. Data are provided as means \pm SEM; the number of independent experiments performed is provided in each data set. Data were tested for significance by using the two-tailed unpaired Student *t* test. Differences were considered statistically significant when *P* values were <0.05. Sample sizes were estimated on the basis of previous experience with similar assays and the size effect observed in preliminary experiments. All samples were identical prior to allocation of treatments, and the observer was blinded to the experimental conditions.

■ ASSOCIATED CONTENT

● Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b02724.

Experimental details; additional figures and data (PDF)

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Notes

The authors declare no competing financial interest.

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