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Identification of εPKC targets during cardiac ischemic injury

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Abstract

Background—Activation of ε protein kinase C (εPKC) protects hearts from ischemic injury. However, some of the mechanism(s) of εPKC mediated cardioprotection are still unclear. Identification of εPKC targets may aid to elucidate εPKC–mediated cardioprotective mechanisms. Previous studies, using a combination of εPKC transgenic mice and difference in gel electrophoresis (DIGE), identified a number of proteins involved in glucose metabolism, whose expression was modified by εPKC. These studies, were accompanied by metabolomic analysis, and suggested that increased glucose oxidation may be responsible for the cardioprotective effect of εPKC. However, whether these εPKC-mediated alterations were due to differences in protein expression or phosphorylation was not determined.

Methods and Results—Here, we used an εPKC-specific activator peptide, ψεRACK, in combination with phosphoproteomics to identify εPKC targets, and identified proteins whose phosphorylation was altered by selective activation of εPKC most of the identified proteins were mitochondrial proteins and analysis of the mitochondrial phosphoproteome, led to the identification of 55 spots, corresponding to 37 individual proteins, which were exclusively phosphorylated, in the presence of ψεRACK. The majority of the proteins identified were proteins involved in glucose and lipid metabolism, components of the respiratory chain as well as mitochondrial heat shock proteins.

Conclusion—In summary the protective effect of εPKC during ischemia involves phosphorylation of several mitochondrial proteins involved in glucose, lipid metabolism and oxidative phosphorylation. Regulation of these metabolic pathways by εPKC phosphorylation may lead to εPKC-mediated cardioprotection induced by ψεRACK.

Keywords

εPKC; ischemia; phosphorylation; mitochondria

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Competing interests: D.M.R. is the founder of KAI Pharmaceuticals Inc, a company that aims to bring PKC regulators to the clinic. None of the research performed in her laboratory is in collaboration with or supported by the company. The other authors declare that they have no competing interests.

Authors' contributions: G.B., H.M.C.J., A.T.D.F., J.P. and J.C.B.F. performed all experiments. D.S. and D.M-R designed the study. D.S. directed the study. D.S. and J.E.K. wrote the manuscript.

Introduction

We previously developed and used an εPKC isoenzyme- selective activator peptide and found that εPKC activation reduces cardiac cell death induced by ischemia [1, 2]. To provide insight into εPKC-mediated cytoprotective mechanisms, we used a proteomic approach combining antibodies that specifically recognize proteins phosphorylated at the PKC consensus phosphorylation site and an εPKC activator peptide [3]. This approach led to the identification of mitochondrial aldehyde dehydrogenase 2 (ALDH2) as an εPKC substrate, whose phosphorylation and activation is necessary and sufficient to induce cardioprotection during an ischemic injury [3]. We also demonstrated that the cytoprotective mechanism of εPKC is mediated, at least in part, by ALDH2-mediated detoxification of reactive aldehydes, such as 4-hydroxy-2-nonenal (4-HNE), that accumulate in the heart during ischemia [3-5]. Studies by others, using transgenic and dominant negative εPKC mice, identified other εPKC signaling complexes, composed of proteins involved in glucose and lipid metabolism, and proteins related to transcription/ translation, suggesting that εPKC-mediated cytoprotection involves regulation of other cellular processes [6-8]. A study using difference in gel eletrophoresis (DIGE) comparing hearts of mice overexpressing catalytically active and dominant negative εPKC identified alterations in the levels of proteins involved in glucose metabolism. Metabolomic studies confirmed that during ischemia/ reperfusion glucose is metabolized faster in animals expressing constitutively active εPKC [9]. However, these studies did not clarify whether the differences in the identified proteins were due to differential expression or phosphorylation levels. Overexpression of εPKC lead to its mislocalization [10] and a compensatory effect observed by δPKC overexpression [9]. Therefore, some of the targets identified in this study could possibly have been phosphorylated by overexpressed δPKC or mis-localized εPKC. Nevertheless, these studies suggested that the cardioprotective mechanism of εPKC is also due to the regulation of glucose and lipid metabolism.

In the present study we used an adult heart Langendorff coronary perfusion system, and treated isolated hearts with an εPKC specific activator peptide (ψεRACK) prior to ischemia, to determine phosphorylation events, following selective activation of εPKC. Proteins whose phosphorylation increased in the presence of ψεRACK were detected in 2D Gels with a phospho-specific dye. Mass spectrometry of the phosphorylated proteins demonstrated that most of the proteins identified in total heart lysates that were differentially phosphorylated upon εPKC activation were mitochondrial proteins. Isolation of mitochondria from ψεRACK treated and control hearts confirmed that εPKC activation led to an increase inphosphorylation levels of proteins involved in, the electron transport chain as well as lipid metabolism.

Materials and Methods

Ex vivo rat heart model of cardiac ischemia

Animal protocols were approved by the Stanford University Institutional Animal Care and Use Committee. Rat hearts (Wistar, 250-300g), each group consisting of three rats, were perfused *via* the aorta at a constant flow rate of 10 ml/min with oxygenated Krebs-Henseleit buffer (120 mM NaCl, 5.8 mM KCl, 25 mM NaHCO3, 1.2 mM NaHCO3, 1.2 mM MgSO4, 1.2 mM CaCl2, and 10 mM dextrose, pH 7.4) at 37°C. After a 20 min. equilibration period, hearts were subjected to 35 min global, no-flow ischemia. The εPKC-selective agonist ψεRACK peptide [HDAPIGYD [11] fused to the cell permeable Tat protein transduction domain peptide, amino acids 47-57 [12] (1mM) was perfused for 10 min immediately prior to ischemia onset.

Preparation of heart lysates and sub-cellular fractionation

At the end of ischemia, hearts were removed from the cannnula and immediately homogenized on ice to obtain total and mitochondrial fractions. To obtain the total lysate fraction, heart ventricles were homogenized in BufferA [7M urea, 2M tiourea, 4% CHAPS, 5mM magnesium acetate, 17μg/mL PMSF and phosphatase inhibitor cocktail diluted 1:300 (Sigma # P8340 and Sigma # P5726)]. To obtain the mitochondrial fraction, heart ventricles were homogenized in ice-cold mannitol-sucrose (MS) buffer [210 mM mannitol, 70 mM sucrose, 5 mM MOPS and 1mM EDTA containing Protease) and phosphatase Inhibitors as above]. The homogenate was centrifuged at 700g for 10 minutes (to pellet the cytoskeletal fraction), the resultant supernatant was filtered through gauze, and centrifuged at 10,000g for 10 minutes (to pellet the mitochondrial fraction). The mitochondrial pellet was washed 3x in MS buffer before the pellet was resuspended in DIGE buffer.

Two-Dimensional Gel Electrophoresis

Protein samples (300μg for analytic gels and 500 μg for preparative gels of total heart lysate and 250 μg for analytic/ preparative gels of mitochondrial fraction) were applied onto 3-10 linear immobilized pH gradient strips (13cm, GE, Healthcare, Life Science). Strips were rehydrated for 16 hours at room temperature. Isoelectric focusing (IEF) was performed on an IPGphor III apparatus (GE Healthcare Life Science) at 17 KVh. For the second dimension strips were incubated at room temperature, for 20 min in equilibration buffer [6 M urea, 2% (w/v) SDS, 50 mM Tris-HCl pH 6.8, 30% (v/v) glycerol, 0.001% (w/v) bromophenol blue] with 2% (w/v) DTT, followed by incubation with 4% (w/v) iodoacetamide in equilibrium buffer, for 20 min. The second dimension was separated using vertical SDS-PAGE. Experiments were performed in triplicates. Phospho-proteins were detected by staining with Pro-Q Diamond (Invitrogen) per manufacturer's instructions. Gels were scanned using a Typhoon TRI scanner (Healthcare Life Science), stained with Coomassie Brilliant Blue G250 (CBB) [13] and scanned using a UTA-1100 scanner and Labscan v 5.0 software (GE Healthcare Life Science).

Image analysis was performed using Image Master Software v.5.01 (GE Healthcare Life Science). For each pair of samples analyzed, individual spot volumes of replicate gels were determined in Pro-Q Diamond stained gels (phospho-proteins), followed by normalization (individual spot volume/ volume of all spots × 100). Spots (of treated samples) that appeared or showed a change in spot volume of least 1.5 fold as compared to samples of hearts submitted to ischemia alone were excised from CBB-stained preparative gels and identified by mass spectrometry. Differences between experimental groups were evaluated by the Mann-Whitney t-test for proteomic analysis. A * p value < 0.05 was considered statistically significant.

"In-gel" protein digestion and MALDI-TOF/TOF MS

Digestion of selected spots was performed as previously described [14]. Matrix-Assisted Laser Desorption ionization Time-of-Flight/Time-of-Flight Mass Spectrometry) as analysis executed aspreviously described [15]. MASCOT MS/MS Ion Search (www.matrixscience.com) software was used to blast sequences against the SwissProt and NCBInr databanks. Combined MS-MS/MS searches were conducted with parent ion mass tolerance at 50 ppm, MS/MS mass tolerance of 0.2 Da, carbamidomethylation of cysteine (fixed modification) and methionine oxidation (variable modification). According to MASCOT probability analysis only hits with significant $P < 0.05$ were accepted Spots from total lysates were identified at the Mass Spectrometry Facility at Stanford University (mass-spec.stanford.edu).

Results

Identification of phosphoproteins

Hearts were exposed to global, no-flow ischemia (35 min) in the presence or absence of ψεRACK (1μM) applied for normoxia 10 min prior to an ischemic onset, with no wash-out, as previously described [16]. Both groups had a 20 min equilibration period, after which hearts were subjected to 30 min global, no-flow ischemia. To one of the groups the εPKC-selective agonist peptide, ψεRACK, was perfused for 10 min (1μM), immediately prior to ischemia onset and kept throughout ischemia. Total lysate of 3 hearts, from 3 independent experiments, were prepared, and run individually on 2D gels. Considering phosphorylated spots that had at least a 1.5X increase, we compared phosphorylated spots from hearts of animals subjected to, ischemia and ψεRACK + ischemia. The phosphorylation of 20 spots increased only in ischemic hearts treated with ψεRACK. Of these, 18 spots were identified by mass spectrometry (Figure 1, 2 and Table 1).

Since the majority of the proteins (~70%) identified were mitochondrial proteins and since a number of previous studies demonstrated that εPKC can interact with and phosphorylate mitochondrial proteins [8, 17-20] we set out to analyze the εPKC phosphoproteome in isolated mitochondria.

Identification of phosphoproteins in mitochondrial fractions

Mitochondria from, ischemia and ψεRACK+ ischemia treated hearts were isolated as described in materials and methods. In a previous study we verified the purity of our mitochondrial preparation by electron microscopy and Western blot analysis of specific mitochondrial proteins [20]. Mitochondrial proteins were separated by 2-D gel electrophoresis and phosphoproteins stained with Pro-Q Diamond. Of the 183 spots that appeared or were increased in gels of mitochondria from hearts of animals treated with ψεRACK + ischemia, 62 spots were visible by Coomassie Brilliant Blue and 56 spots corresponding to 38 different proteins were identified by in-gel excision followed by mass spectrometry (Figures 3, 4 and Table 2). Twenty seven proteins were mitochondrial proteins. Nine proteins were mitochondrial inner membrane proteins and one outer membrane protein. Proteins involved in fatty acid oxidation, electron transport chain (complexes I-IV), heat shock proteins as well as structural proteins were also identified. Interestingly, protein disulfide-isomerase A3 precursor, oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide), tubulin alpha 1A, mitochondrial aconitase, creatine kinase, mitochondrial 2, acyl-Coenzyme A dehydrogenase very long chain, 3-oxoacid CoA transferase 1, carnitine palmitoyltransferase II, electron transfer flavoprotein-ubiquinone oxidoreductase, succinate dehydrogenase complex, subunit A, flavoprotein (Fp), glyceraldehyde 3-phosphate-dehydrogenase, desmin, ubiquinol-cytochrome c reductase core protein I and Coq9 protein had a change in more than one phospho-spot indicative of multiple phosphorylation sites.

Recently we showed that translocation of εPKC to the mitochondria is mediated by HSP90, therefore the identified substrates can be direct targets of εPKC [20]. Using scansite (http://scansite.mit.edu/) we predicted PKC phosphorylation sites of the mitochondrial proteins whose phosphorylation increased upon treatment with ψεRACK. All identified mitochondrial proteins had putative PKC phosphorylation some which matched phosphorylation sites deposited in http://www.phosphosite.org/ (Table 4).

Discussion

Several lines of evidence suggest that selective εPKC activation reduces cardiac damage due to ischemic injury. Activation of εPKC reduces infarct size and improves functional recovery of the heart [1-3]whereas εPKC inhibition or knockout negates the infarct-sparing

effect of ischemic preconditioning [1, 3, 9, 21, 22]. A number of mechanisms have been proposed for εPKC mediated cardioprotection, including regulation of sarcolemmal and/or mitoK$_{ATP}$ channels [17, 23], regulation of gap-junction permeance through phosphorylation of connexin 43 [24], modulation of proteasomal activity [16] or regulation of mitochondrial permeability transition pore (MPTP) opening through direct phosphorylation of MPTP components [8]. We recently identified mitochondrial ALDH2 as a direct εPKC substrate whose phosphorylation and activation is essential for εPKC-mediated cardioprotection [3]. The cytoprotective mechanism of ALDH2 activation by εPKC is due to the increased metabolism of reactive aldehydes, such as 4-Hydroxy-2-nonenal (4-HNE), which are produced as a by-product of ROS-induced lipid peroxidation, and accumulate, in the ischemic/ reperfused heart [25]. In the present study, we used the Pro-Q Diamond phospho-specific staining method to label proteins whose phosphorylation increased by ψεRACK during ischemia. The majority (~70%) of the εPKC phosphoproteins identified in total heart homogenates treated with ψεRACK during ischemia were mitochondrial proteins. The observation that εPKC activation and cytoprotection results in phosphorylation of mitochondrial proteins and is consistent with other studies reporting that εPKC-mediated cardioprotection is mediated by phosphorylation of mitochondrial proteins [1, 3, 9, 17, 18, 22].

To provide a more extensive analysis of the εPKC mitochondrial phosphoproteome, we repeated the Pro-Q Diamond analysis on the cardiac mitochondrial-enriched subfraction. In the presence of ψεRACK we saw the appearance of 182 phosphorylated spots, suggesting that εPKC activation results in phosphorylation of a number of mitochondrial proteins. We identified novel mitochondrial εPKC phosphoproteins involved in lipid oxidation, glycolysis, electron transport chain (including proteins from complexes I-IV), ketone body metabolism, and heat shock proteins.

We found an increase in the phosphorylation of inner-mitochondrial protein components of the respiratory chain, (complexes I, II and III); NADH dehydrogenase (ubiquinone) Fe-S protein, electron transfer flavoprotein-ubiquinone oxidoreductase, succinate dehydrogenase complex, subunit A, flavoprotein (Fp) and ubiquinol-cytochrome c reductase core protein I. Our results are in agreement with a number of biochemical and functional analyses which found εPKC to interact with, and phosphorylate inner-mitochondrial proteins involved in mitochondrial respiration [7-9, 26]. Further, the presence of εPKC in a highly purified inner mitochondrial membrane preparation has already been previously demonstrated [23]. An increase in the activity of the electron transport chain and activation of cytochrome c oxidase subunit IV (COX) by direct εPKC phosphorylation has also been previously demonstrated [27]. COX activation was suggested to be one of the cardioprotective mechanisms of εPKC, possibly due to increased electron flux through the electron transport chain, resulting in enhanced ATP generation and reduced ROS generation [22, 27, 28]. An εPKC-mediated increase in cytochrome c oxidase activity was also shown to protect lens from ischemic damage [29]. Selective activation of εPKC with ψεRACK increased the phosphorylation and activity of complexes I, III and IV in synaptic mitochondria, indicating that other components of the electron transport chain are also regulated by εPKC phosphorylation [30], and εPKC activation led to a decrease in mitochondrial ROS generation of neuronal mitochondria [30]. In agreement with a role for εPKC in mitochondrial respiration, hearts of constitutively active εPKC transgenic mice demonstrate preserved coupling of oxidative phosphorylation, maintained mitochondrial membrane potential and decreased cytochrome c release induced by ischemic reperfusion [31]. The εPKC transgenic mice used have a mutation of Ala^{159} to Glu in the εPKC resulting in constitutively active εPKC and increased resistance to cardiac ischemic reperfusion [8]. Interestingly, in constitutively active εPKC transgenic mice, mitochondrial PKC expression is preferentially increased over cytosolic expression, suggesting that the active form of PKC results in its mitochondrial translocation [8]. Taken together, these data suggest that phosphorylation of

intra-mitochondrial targets is crucial for εPKC-mediated cytoprotection. In the present study we identify other components of the respiratory chain and inner mitochondrial phosphorylated proteins. However, whether there is a direct physical association between εPKC and each of the inner mitochondrial εPKC phosphoproteins identified here, and whether these are direct or indirect εPKC substrates remains to be determined. Nevertheless future studies can, be directed by the results obtained here.

We did not detect ALDH2, however this may be due to the fact that different methods of detecting protein phosphorylation have different sensitivities. Some of the εPKC targets identified can be indirect targets whose phosphorylation may be activated upon ALDH2 activation.

Using difference in gel eletrophoresis (DIGE) of cardiac mitochondria from transgenic mice expressing constitutively active or dominant negative εPKC it was found that the majority of spots unique to constitutively active εPKC corresponded to proteins involved in glucose metabolism [9]. These studies were combined with metabolomic studies which detected an increase in glucose metabolites in hearts expressing constitutively active εPKC subjected to ischemia/ reperfusion [9]. The authors proposed that activating glycolytic pathways during ischemia is a novel mechanism for the cardioprotective role of εPKC. In the present study we used a phospho-specific dye and ψεRACK to investigate direct protein phosphorylation events mediated by εPKC. Despite the different methods and methodology used to activate εPKC, (constitutively active transgenic vs. dynamic activation) we identified many of the same proteins, previously described in the DIGE study, including; isocitrate dehydrogenase, oxoglutarate (alpha-ketoglutarate) dehydrogenase, pyruvate dehydrogenase, succinate dehydrogenase. [[6, 7, 9] and Table 4]. We also identified additional εPKC substrates involved in glycolysis, and Krebs cycle such as: aldolase A, ATP-specific succinyl-CoA synthase beta subunit, dihydrolipoamide dehydrogenase (E3), mitochondrial aconitase and aconitase 2, confirming that εPKC activation leads to phosphorylation of proteins involved in glycolysis and the Krebs cycle. Our identification of aconitase as an εPKC target suggests that regulation of the TCA cycle is mediated by εPKC. Aconitase has been previously identified as a PKCβII substrate in diabetic rats, however, aconitase phosphorylation by PKCβII impaired TCA cycle since there was an increase in reverse activity of aconitase (isocitrate to aconitase) [32]. While we identified some proteins identified previously, others were not detected in the present study, such as proteins involved in the Malate/Aspartate shuttle. This could be explained by the different methodology or the sensitivity of the methods (DIGE vs ProQ Diamond) and that we only identified the more abundant phosphorylated proteins. Alternatively, some of the proteins previously detected could have their expression and not phosphorylation status altered [9]. In a study identifying εPKC complexes it has been suggested that εPKC may also play a role in regulating transcription and translation processes [6]. Accordingly, the phosphorylation of Coq9, a key regulator of coenzyme Q synthesis [33], was also regulated by εPKC in the present study. Further studies should be performed to determine the specific regulation of glycolytic pathways by εPKC phosphorylation and whether different isoenzymes can phosphorylate different sites.

εPKC could also have a direct or indirect role in mitochondrial protein assembly, folding, and import since we identified three mitochondrial heat shock proteins that play a role in the import and folding of proteins inside the mitochondria, and sorting and assembly machinery component 50 (SAM50), homolog of a protein involved in the assembly of outer mitochondrial membrane proteins [34].

Cardioprotective signals from G protein coupled receptors (GPCRs), activated for example by bradykinin, propagating from the plasma membrane to the mitochondria through signalosomes, vesicular multimolecular complexes derived from caveoli have been

previously proposed [35]. In fact εPKC was found in signalosomes and inhibition of εPKC by εV1-2 blocks signalosome stimulation of mitoK_{ATP} [35]. We found two proteins that are found in caveoli, Annexin A2 and PTRF also known as Cavin [36], these proteins could be part of the signalosome probably co-purified with our mitochondrial fraction. PTRF phosphorylation has been shown to be important in caveoli formation [36].

Conclusions

A number of mechanisms have been proposed for εPKC-mediated cardioprotection by preconditioning. In the present study we identified several εPKC phosphoproteins which may be responsible for the cardioprotective effect of εPKC. The εPKC targets identified are in line with many of the previously proposed mechanisms for εPKC mediated cardioprotection. We identified components of the signalosome contributing to the idea that εPKC-mediated cardioprotection involves transduction of GPCR signaling to the mitochondria [35]. We also found components of lipid and carbohydrate oxidation pathways consistent with the idea that lipid and carbohydrate metabolism is modulated by εPKC [9]. Activation of the respiratory chain and increase in oxygen consumption have also been proposed to be protective mechanisms of εPKC during preconditioning, to this end we identified components of Krebs cycle, and respiratory chain, whose phosphorylation was modulated by εPKC [27, 29, 30]. The exact mechanisms by which εPKC phosphorylation leads to these different cardioprotective pathways still needs to be elucidated. The data obtained in the present study can therefore direct further studies to characterize the specific role of individual mitochondrial protein phosphorylation in εPKC-mediated cardioprotection. Taken together, our data suggest that εPKC-mediated phosphorylation events in the mitochondria are important for the maintenance of metabolic activity and cardioprotection during ischemic injury.

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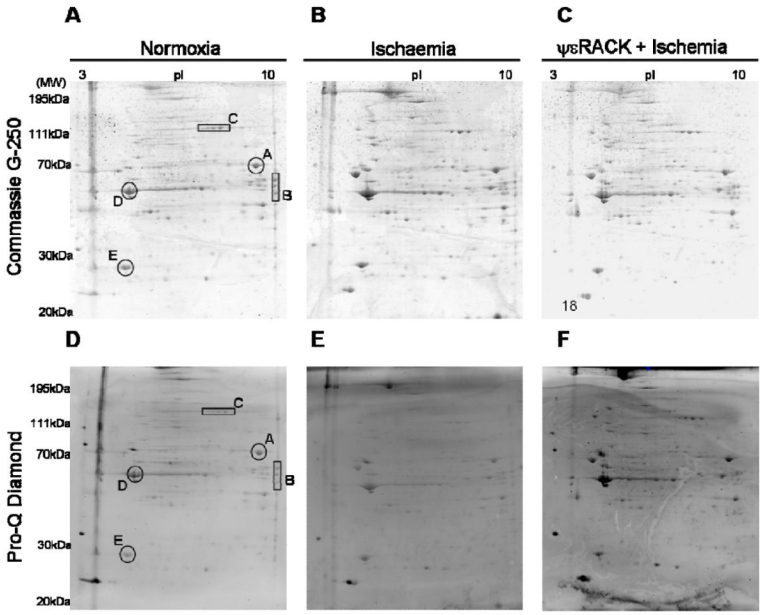

Figure 1.

Detection of direct and indirect εPKC substrates in total rat heart lysates. Representative 2DE gels (n= 3 hearts of individual animals) of lysates from control hearts (A and D), hearts subjected to, ischemia alone (B and E) and Ischemia + ψεRACK (C and F) as indicated. Coommassie blue G250 stained gels (A-C) and gels stained with phospho-specific dye Pro-Q Diamond (D-F). Spots used to align gels are labeled (A and D).

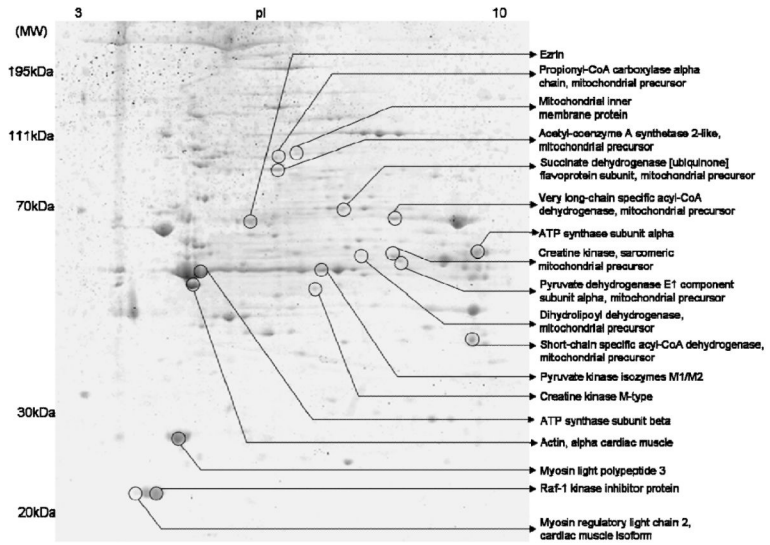

Figure 2.

Coomassie blue G250 stained gel of total heart lysate treated with ψεRACK+ ischemia indicating the spots identified by mass spectrometry whose phosphorylation significantly increased in hearts from rats treated with ψεRACK + ischemia as compared to hearts subjected to ischemia alone. For the annotation of the proteins identified see Table 1.

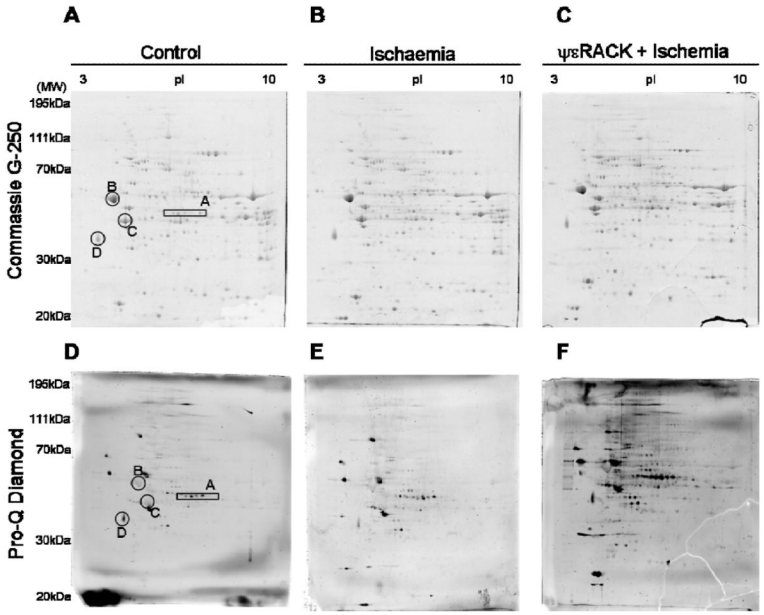

Figure 3.

Detection of direct and indirect εPKC substrates in isolated rat heart mitochondria. Representative 2DE gels (n=3 of mitochondria isolated from individual animals) of lysates from control hearts (A and D) and hearts subjected to, Ischemia (B and E) and ψεRACK+ ischemia (C and F) as indicated. Coommassie blue G250 stained gels (A-C) and gels stained with phospho-specific dye Pro-Q Diamond (D-F). Spots used to align gels are labeled (A and D).

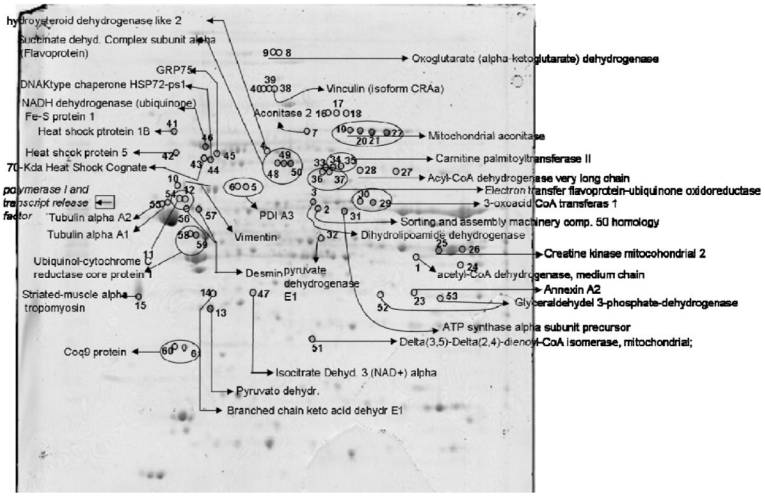

Figure 4.

Detection of direct and indirect εPKC substrates in isolated rat heart mitochondria. Representative 2DE gels (n=3 of mitochondria isolated from individual animals) of lysates from hearts subjected to, Ischemia and ψεRACK+ ischemia as indicated in figure 1. Coommassie blue G250 stained gels upper panels and gels stained with phospho-specific dye Pro-Q Diamond, lower panel

Table 1

Proteins identified by mass spectrometry whose phosphorylation increased in total heart lysates of hearts subjected to ψεRACK+ ischemia relative to ischemia alone. Identified proteins indicated in figure 2 together with Uniprot accession number, number of peptides identified, Mascot score, theoretical and experimental molecular weight (M.W.) and isoeletric point, % 24 volume of ischemia where ischemia = normoxia (average of three experiments) and p-values as determined by Whitney t-test where *P<0.05 are indicated.

Spot No.	Protein	Accession No.	Peptide count	Mascot prot. score	Theorical		Experimental		% Vol	Location	P value
					MW	pI	MW	pI			
1	ATP synthase subunit beta, mitochondrial precursor	P10719	16	589	56kDa	5.19	42kDa	5.23	2.3	mitochondria	0.02*
2	Myosin light polypeptide 3	P16409	12	495	22kDa	5.03	23kDa	4.94	2.6	cytosol	0.02*
3	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial precursor	Q920L2	29	693	71kDa	6.75	65kDa	7.32	2.0	mitochondria	0.03*
4	Creatine kinase, sarcomeric mitochondrial precursor	P09605	15	309	47kDa	8.76	45kDa	7.99	2.2	mitochondria	0.04*
5	Short-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	P15651	11	280	44kDa	8.47	31kDa	9.06	2.5	mitochondria	0,08
6	Very long-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	P45953	23	300	70kDa	9.01	59kDa	8.00	1.8	mitochondria	0.01*
7	Mitochondrial inner membrane protein	Q8CAQ8	17	225	83kDa	6.18	75kDa	6.35	5.1	mitochondria	0.02*
8	Propionyl-CoA carboxylase alpha chain, mitochondrial precursor	P14882	12	78	77kDa	6.33	69kDa	6.41	5.1	mitochondria	0.02*
9	Dihydrolipoyl dehydrogenase, mitochondrial precursor	Q6P6R2	13	207	54kDa	7.96	48kDa	7.33	4.1	mitochondria	0.01*
10	ATP synthase subunit alpha, mitochondrial precursor	P15999	20	694	59kDa	9.22	45kDa	9.14	3.0	mitochondria	0.04*
11	Creatine kinase M-type	P00564	14	568	43kDa	6.58	39kDa	6.87	4.0	mitochondria	0.01*
12	Pyruvate dehydrogenase E1 component subunit alpha, mitochondrial precursor	P26284	13	266	43kDa	8.49	44kDa	8.06	3.3	mitochondria	0.03*
13	Actin, alpha cardiac muscle 1	P68035	18	1030	41kDa	5.23	40kDa	5.14	6.5	cytosol	0.04*
14	Ezrin	P31977	12	93	69kDa	5.83	55kDa	5.80	3.5	cytosol	0.03*
15	Acetyl-coenzyme A synthetase 2-like, mitochondrial precursor	Q99NB1	10	91	74kDa	6.51	66kDa	6.40	5.8	mitochondria	0,09
16	Pyruvate kinase isozymes M1/M2	P11980	26	790	57kDa	6.63	46kDa	7.01	1.7	mitochondria	0.01*
17	Phosphatidylethanolamine-binding protein 1	P31044	5	326	20kDa	5.48	19kDa	4.65	6.0	cytosol	0.01*
18	Myosin regulatory light chain 2, ventricular/cardiac muscle isoform	P08733	15	409	18kDa	4.86	19kDa	4.35	2.7	cytosol	0.01*

Table 2

Proteins identified by mass spectrometry whose phosphorylation increased in mitochondria isolated from hearts subjected to ψεRACK+ ischemia relative to ischemia alone. Identified proteins indicated in Figure 6 are shown together with Uniprot accession number, number of peptides identified and, Mascot score, theoretical and experimental molecular weight (M.W.) and 26 isoeletric point. %volume of control (average of three experiments). * P<0.05, as determined by Whitney t-test.

Spot No.	Protein	Accession No.	Peptide Count	Ion Score	Theorical		Experimental		Coverage	Vol (% Ischemia)
					M.W.	pI	M.W.	pI	(%)	
1	acetyl-CoA dehydrogenase, medium chain	Gi: 8392833	9	214	46kDa	8.63	39kDa	7.53	13	appeared
2	sorting and assembly machinery component 50 homolog	gi:51948454	4	57	52kDa	6.34	59KDa	6.51	9	appeared
3	dihydrolipoamide dehydrogenase	gi:40786469	5	102	54kDa	7.96	61KDa	6.43	9	appeared
4	hydroxysteroid dehydrogenase like 2 [Rattus norvegicus]	gi|71043858	3	49	58KDa	5.85	85KDa	6.2	6	appeared
5	protein disulfide-isomerase A3 precursor	gi:1352384	8	116	57kDa	5.88	66KDa	5.92	11	appeared
6	protein disulfide-isomerase A3 precursor	gi|1352384	10	329	57KDa	5.88	66KDa	5.95	23	appeared
7	aconitase 2	gi|18079339	8	163	85KDa	8.05	105KDa	6.4	8	appeared
8	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	gi|62945278	6	171	12KDa	6.3	174KDa	5.83	8	appeared
9	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	gi|62945278	6	44	12KDa	6.3	174Kda	5.93	8	appeared
10	vimentin	gi|14389299	5	147	54KDa	5.06	67KDa	4.85	5	appeared
11	tubulin alpha 1A	gi:38328248	4	32	50kDa	4.94	64KDa	5.24	10	appeared
12	tubulin alpha 1A	gi:38328248	4	36	50kDa	4.94	64KDa	5.31	11	appeared
13	pyruvate dehydrogenase (lipoamide) beta	gi|56090293	5	247	39KDa	6.2	40KDa	5.47	20	appeared
14	branched chain keto acid dehydrogenase E1, beta polypeptide	gi|158749538	4	267	43KDa	6.41	42KDa	5.48	13	appeared
15	striated-muscle alpha tropomyosin	gi|207349	9	95	37KDa	4.71	38KDa	4.07	13	appeared
19	mitochondrial aconitase	gi|10637996	9	196	85KDa	7.87	105KDa	7.16	12	appeared
20	mitochondrial aconitase	gi|10637996	9	190	85KDa	7.87	105KDa	7.29	12	appeared
21	mitochondrial aconitase	gi|10637996	8	229	85KDa	7.87	104KDa	5.23	12	appeared
22	mitochondrial aconitase	gi|10637996	8	325	85KDa	7.87	104KDa	7.71	13	appeared
23	annexin A2	gi|9845234	8	442	39KDa	7.55	48KDa	7.1	30	appeared
24	aldolase A	gi|202837	4	125	40KDa	8.3	39KDa	8.04	22	appeared
25	creatine kinase, mitochondrial 2	gi|38259206	6	326	47kDa	8.64	46KDa	7.57	21	appeared

Spot No.	Protein	Accession No.	Peptide Count	Ion Score	Theorical		Experimental		Coverage	Vol (% Ischemia)
					M.W.	pI	M.W.	pI	(%)	
26	creatine kinase, mitochondrial 2	gi|38259206	9	442	47kDa	8.64	45KDa	8.65	26	appeared
27	acyl-Coenzyme A dehydrogenase, very long chain	gi|6978435	7	125	71KDa	9.01	71KDa	7.62	18	appeared
28	acyl-Coenzyme A dehydrogenase, very long chain	gi|6978435	5	181	71KDa	9.01	71KDa	7.42	13	appeared
29	3-oxoacid CoA transferase 1	gi|189181716	8	463	57kDa	8.7	61KDa	7.52	23	appeared
30	3-oxoacid CoA transferase 1	gi|189181716	4	238	57kDa	8.7	61KDa	7.35	13	appeared
31	ATP synthase alpha subunit precursor	gi|203055	8	327	59KDa	9.22	59KDa	8.25	20	appeared
32	pyruvate dehydrogenase E1 alpha form 1 subunit	gi|57657	5	211	43KDa	8.32	66KDa	6.48	7	appeared
33	carnitine palmitoyltransferase II	gi|1850592	8	257	74KDa	7.02	74KDa	7.1	7	appeared
34	carnitine palmitoyltransferase II	gi|1850592	7	125	74KDa	7.02	74KDa	7.12	15	appeared
35	carnitine palmitoyltransferase II	gi|1850592	7	174	74KDa	7.02	74KDa	7.27	13	appeared
36	Electron transfer flavoprotein-ubiquinone oxidoreductase	gi|52000614	6	158	61KDa	7.33	70KDa	7.11	15	appeared
37	Electron transfer flavoprotein-ubiquinone oxidoreductase	gi|52000614	6	321	61KDa	7.33	70KDa	7.18	14	appeared
38	vinculin (predicted), isoform CRA_a	gi|149031250	5	41	123KDa	5.54	146KDa	5.84	5	appeared
41	heat shock protein 1, beta (HSP90)	gi|40556608	7	268	83KDa	4.97	105KDa	4.65	9	appeared
42	heat shock protein 5 (HSP70 ptn5) glucose regulated protein	gi|25742763	9	296	72KDa	5.07	84KDa	4. 7	14	appeared
43	70-Kda Heat Shock Cognate Protein	gi|178847300	9	309	60KDa	5.91	72KDa	5.13	20	appeared
44	DNAK-type molecular chaperone hsp72-ps1	gi|347019	8	369	71KDa	5.43	73KDa	5.21	16	appeared
45	grp75	gi|1000439	8	414	74KDa	5.87	79KDa	5.24	16	appeared
46	NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa	gi|53850628	7	283	80KDa	5.65	81KDa	5.14	12	appeared
47	isocitrate dehydrogenase 3 (NAD+) alpha	gi|16758446	7	221	40KDA	6.47	41KDa	6.3	26	appeared
48	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	gi|18426858	10	249	72KDa	6.75	72KDa	6.45	20	appeared
49	succinate dehydrogenase complex, subunit A, flavoprotein (Fp	gi|18426858	10	364	72KDa	6.75	71KDa	6.56	19	appeared
50	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	gi|18426858	10	355	72KDa	6.75	71KDa	6.82	21	appeared
51	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase: Precursor	gi|6015047	8	219	36kDa	8.13	33KDa	6.47	35	appeared
52	glyceraldehyde 3-phosphate-dehydrogenase	gi|56188	4	265	36KDa	8.43	47KDa	7.43	3	appeared
53	glyceraldehyde 3-phosphate-dehydrogenase	gi|56188	3	74	36kDa	8.43	47KDa	7.65	17	appeared

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Spot No.	Protein	Accession No.	Peptide Count	Ion Score	Theorical		Experimental		Coverage	Vol (% Ischemia)
					M.W.	pI	M.W.	pI	(%)	
54	ATP synthase beta subunit	gi|1374715	6	238	51KDa	4.92	65KDa	4.87	20	appeared
55	tubulin, beta, 2	gi|5174735	6	110	50kDa	4.79	66KDa	4.75	11	appeared
56	Desmin	gi|11968118	27	65	53kDa	5.21	64KDa	4.87	44	appeared
57	Desmin	gi|11968118	28	72	53kDa	5.21	64Kda	5.12	53	appeared
58	ubiquinol-cytochrome c reductase core protein I	gi|51948476	22	38	53kDa	5.57	51KDa	5.43	32	appeared
59	ubiquinol-cytochrome c reductase core protein I	gi|51948476	7	385	53kDa	5.57	52KDa	5.59	21	appeared
60	Coq9 protein	gi|51259441	2	62	35KDa	5.5	30KDa	4.87	10	appeared
61	Coq9 protein	gi|51259441	8	ND	35KDa	5.5	30KDa	5.09	25	19,5*
62	polymerase I and transcript release factor	gi:6679567	3	46	44kDa	5.43	64KDa	3.31	7	19,5*

Table 3

Summary of the function and localization of proteins whose phosphorylation was unique or increased 1.5X (in two out of three gels, of independent samples) in mitochondria from hearts treated with ψεRACK + ischemia relative to ischemia. The biological process, mitochondrial compartment and references to previous descriptions of protein phosphorylation or expression modulated by PKC_ε are indicated in the table.

Function	Protein	Localization	Reference
Fatty Acid oxidation	carnitine palmitoyltransferase II	mitochondrial inner membrane	
	delta(3,5)-delta(2,4)-dienoyl-CoA isomerase: precursor	mitochondrial matrix	
Glycolysis/ Gluconeogenesis	aldolase A	mitochondrial matrix	
Krebs cycle	aconitase 2	mitochondrial matrix	
	ATP-specific succinyl-CoA synthase beta subunit	mitochondrial matrix	
	isocitrate dehydrogenase 3 (NAD+) alpha	mitochondrial matrix	6, 9
	dihydrolipoamide dehydrogenase (E3)	mitochondrial matrix	
	mitochondrial aconitase	mitochondrial matrix	
	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	mitochondrial matrix	9
	pyruvate dehydrogenase (lipoamide) beta	mitochondrial matrix	9
	pyruvate dehydrogenase E1 alpha form 1 subunit	mitochondrial matrix	9
	glyceraldehyde 3-phosphate-dehydrogenase	mitochondrial matrix	6
Electron transport chain	electron transfer flavoprotein-ubiquinone oxidoreductase	mitochondrial inner membrane	
Complex I	NADH dehydrogenase (ubiquinone) Fe-S protein	mitochondrial inner membrane	
	electron transfer flavoprotein-ubiquinone oxidoreductase	mitochondrial inner membrane	
Complex II	succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	mitochondrial inner membrane	6
	electron transfer flavoprotein-ubiquinone oxidoreductase	mitochondrial inner membrane	
Complex III	ubiquinol-cytochrome c reductase core protein I	mitochondrial inner membrane	
	electron transfer flavoprotein-ubiquinone oxidoreductase	mitochondrial inner membrane	
ATP Synthase	ATP synthase alpha subunit precursor	mitochondrial inner membrane	6
	ATP synthase beta subunit	mitochondrial inner membrane	6, 9
Ketone body metabolism	3-oxoacid CoA transferase 1	mitochondrial matrix	
	branched chain keto acid dehydrogenase E1, beta polypeptide	mitochondrial matrix	
	vimentin	Cytosol	6, 7
	tubulin alpha 1A	Cytosol	
Cytoskeletal elements	tubulin, beta, 2	Cytosol	
	desmin	Cytosol	6, 7
	vinculin, isoform CRA_a	Cytosol	6, 7
	heat shock protein 1, beta (HSP90)	Cytosol	
Heat Shock Protein	heat shock protein 5 (HSP70 ptn5) glucose regulated protein	Mitochondria	
	dnaK-type molecular chaperone hsp72-ps1	Mitochondria	6, 7
	grp75	Mitochondria	
Caveoli	polymerase I and transcript release factor (PTRV)	Caveolin	
	annexin A2	membranes (Caveolin)	6, 7

Function	Protein	Localization	Reference
	sorting and assembly machinery component 50 homolog	mitochondrion outer membrane	
	hydroxysteroid dehydrogenase like 2 [Rattus norvegicus]	mitochondrial inner membrane	
Other	protein Coq9 protein	mitochondrial inner membrane	
	protein disulfide-isomerase A3 precursor	endoplasmic reticulum	
	striated-muscle alpha tropomyosin	Sarcomere	

Table 4

Predicted PKC Phosphorylation sites and validated sites of the mitochondrial proteins phosphorylated upon ischemia and ψεRACK. The phosphorylated residue is underlined.

protein	predicted p-site	peptide sequence[1]	PKC isoenzyme	Validated[2]
sorting and assembly machinery component 50 homolog	-			
	T160	LGRAEKV<u>T</u>FQFSYGT	**PKCδ/ζ**	
	S164	EKVTFQF<u>S</u>YGTKETS	**cPKC**	
	S171	SYGTKET<u>S</u>YGLSFFK	**PKCε/δ**	
	S189	GNFEKNF<u>S</u>VNLYKVT	**PKCζ**	
	S203	TGQFPWS<u>S</u>LRETDRG	**cPKC**	
	S216	RGVSAEY<u>S</u>FPLCKTS	**PKCζ**	
	T225	PLCKTSH<u>T</u>VKWEGVW	**cPKCε/δ**	
	S243	GCLARTA<u>S</u>FAVRKES	**cPKC/ζ**	
	S312	NKPLVLD<u>S</u>VFSTSLW	**PKCε**	
	S332	PIGDKLS<u>S</u>IADRFYL	**PKCε**	
dihydrolipoamide dehydrogenase	-			
	S10	SWSRVYC<u>S</u>LAKKGHF	cPKC/ζ	
	T165	GKNQVTA<u>T</u>TADGSTQ	**PKCε**	
	S170	TATTADG<u>S</u>TQVIGTK	**PKCδ**	
	S208	VSSTGAL<u>S</u>LKKVPEK	**cPKC**	
	T279	FKLNTKV<u>T</u>GATKKSD	**cPKC/ζ**	
	T282	NTKVTGA<u>T</u>KKSDGKI	**cPKC**	
	S502	REANLAA<u>S</u>FGKPINF	**cPKC**	
hydroxysteroid dehydrogenase like 2	-			
	T12	TGKLAGC<u>T</u>VFITGAS	**PKCδ**	
	T53	RHPKLLG<u>T</u>IYTAAEE	**PKCδ/ζ**	yes
	T169	FKQHCAY<u>T</u>IAKYGMS	**cPKC/ δ/ ζ**	
	S237	SIFKRPK<u>S</u>FTGNFII	**PKCs/ δ/ ζ**	
	S426	TFRIVKD<u>S</u>LSDEVVR	**PKCε**	
	S476	DRADVVM<u>S</u>MATEDFV	**PKCε**	
	T493	FSGKLKP<u>T</u>MAFMSGK	**cPKC/ζ/ δ/ ε**	
protein disulfide-isomerase A3 precursor	-			
	S239	IKKFIQE<u>S</u>IFGLCPH	PKCζ	
	T228	AYTEKKM<u>T</u>SGKIKKF	**PKCζ**	
	S229	YTEKKMT<u>S</u>GKIKKFI	**cPKC**	
	S239	IKKFIQE<u>S</u>IFGLCPH	**PKCδ/ζ**	
	S303	KLNFAVA<u>S</u>RKTFSHE	**cPKC**	
	T306	FAVASRK<u>T</u>FSHELSD	**PKCδ/ε**	yes

protein	predicted p-site	peptide sequence[1]	PKC isoenzyme	Validated[2]
	T452	YEVKGFPTIYFSPAN	**PKCε**	
	T463	SPANKKLTPKKYEGG	**cPKC**	
aconitase 2	-			
	T64	KRLNRPLTLSEKIVY	**PKCζ**	
	T366	HPVADVGTVAEKEGW	**PKCζ**	
	T415	LKCKSQFTITPGSEQ	**PKCδ/ε**	
	T467	IKKGEKNTIVTSYNR	**PKCε/ ζ**	
	T504	TALAIAGTLKFNPET	**cPKC/δ**	
	S690	GRAIITKSFARIHET	**PKCζ**	
	S770	IEWFRAGSALNRMKE	**PKCζ**	
oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	-			
	T19	RPLTASQTVKTFSQN	**cPKC/e/d**	
	S71	AWLENPKSVHKSWDI	**cPKC**	
	S103	PLSLSRSSLATMAHA	**PKCε/χ/δ**	yes
	T106	LSRSSLATMAHAQSL	**PKCδ**	
	S112	ATMAHAQSLVEAQPN	**PKCδ**	
	T190	DKVFHLPTIIFIGGQ	**PKCδ**	
	T191	KVFHLPIMFIGGQE	**PKCδ**	
	T262	LARLVRSTRFEEFLQ	**PKCε**	
	S663	AEYMAFGSLLKEGIH	**PKCζ**	
	S273	EFLQRKWSSEKRFGL	**PKCζ**	
	S274	FLQRKWSSEKRFGLE	**cPKC/δ**	
	S405	TEGKKVMSILLHGDA	**PKCζ**	
	T437	PSYTTHGTVHVVVNN	**PKCδ**	
	S861	LIVFTPKSLLRHPEA	**PKCζ**	
aldolase A	-			
	S39	AADESTGSIAKRLQS	**PKCδ/ζ**	yes
	S46	SIAKRLQSIGTENTE	**PKCε**	yes
	T227	HHVYLEGTLLKPNMV	**PKCζ**	
	S309	YGRALQASALKAWGG	**cPKCδ**	
	S336	IKRALANSLACQGKY	**cPKCδ**	
acyl-Coenzyme A dehydrogenase, very long chain	-			
	S60	ETLSSDASTREKPAR	**cPKC/ε**	
	S72	PARAESKSFAVGMFK	**PKC8/ε**	
	T194	KGILLYGTKAQKEKY	**PKCζ**	
	S227	SSGSDVASIRSSAVP	**cPKCδ**	

protein	predicted p-site	peptide sequence[1]	PKC isoenzyme	Validated[2]
	S287	TAFVVERSFGGVTHG	**PKCδ**	
	T347	GRFGMAATLAGTMKA	**PKCζ**	
	S423	AISKIFGSEAAWKVT	**PKCζ**	
	S517	RRRTGIGSGLSLSGI	**PKCζ**	
3-oxoacid CoA transferase 1	-			
	S16	SGLRLCASARNSRGA	**cPKC**	
	S35	CACYFSVSTRHHTKF	**cPKC**	
	T58	KDIPNGATLLVGGFG	**PKCδ**	
	T140	VELTPQGTLAERIRA	**PKCζ**	
	T163	YTSTGYGTLVQEGGS	**PKCε**	
	S179	IKYNKDGSVAIASKP	**PKCε/ζ/δ**	
	S253	EEIVDIGSFAPEDIH	**PKCε**	
	S283	EKRIERLSLRKEGEG	**cPKC/ε/δ/ζ**	
	T397	RGGHVNLTMLGAMQV	**PKCζ**	
	T440	SKTKVVVTMEHSAKG	**cPKC/ε**	
	T457	HKIMEKCTLPLTGKQ	**cPKCδ**	
ATP synthase alpha subunit precursor	-			
	T102	ITPETFSTISVVGLI	**PKCδ**	
pyruvate dehydrogenase E1 alpha form 1 subunit	-			
	T35	RNFANDATFEIKKCD	**PKCζ**	
	T70	KYYRMMQTVRRMELK	**cPKC/ε**	
	T124	AYRAHGFTFNRGHAV	**PKCδ**	
	T139	RAILAELTGRRGGCA	**PKCδ**	
	S152	CAKGKGGSMHMYAKN	**PKCδ/ζ**	
	T266	ILCVREATKFAAAYC	**PKCδ**	
	S293	TYRYHGHSMSDPGVS	**PKCε**	yes
carnitine palmitoyltransferase II	-			
	S15	RAWPRCPSLVLGAPS	**PKCδ**	
	T60	PIPKLEDTMKRYLNA	**cPKC**	
	T156	LTRATNLTVSAVRFL	**PKCδ**	
	S320	ETLKKVDSAVFCLCL	**PKCζ**	
	S411	AATNSSASVETLSFN	**PKCδ**	
	S416	SASVETLSFNLSGAL	**PKCδ**	
	T428	GALKAGITAAKEKFD	**PKCζ**	
	T437	AKEKFDTTVKTLSID	**PKCε/δ/χ**	
	S462	FLKKKQLSPDAVAQL	**PKCδ**	
	T491	ATYESCSTAAFKHGR	**PKCζ**	

protein	predicted p-site	peptide sequence[1]	PKC isoenzyme	Validated[2]
	T501	FKHGRTE<u>T</u>IRPASIF	**cPKC**	
	S513	SIFTKRC<u>S</u>EAFVRDP	**PKCζ**	
Electron transfer flavoprotein-ubiquinone oxidoreductase	-			
	T46	PQITTHY<u>T</u>IHPREKD	**cPKC**	
	T229	KDGAPKT<u>T</u>FERGLEL	**PKCδ**	
	T241	LELHAKV<u>T</u>IFAEGCH	**PKCε/δ**	
	S306	DRHTYGG<u>S</u>FLYHLNE	**PKCζ**	
	S347	QRWKHHP<u>S</u>IRPTLEG	**cPKC/δ**	
	T401	PKIKGTH<u>T</u>AMKSGSL	**PKCε/δ/ζ**	
	S407	HTAMKSG<u>S</u>LAAEAIF	**PKCε/δ**	
	S490	WTLKHKG<u>S</u>DSEQLKP	**cPKC/ε**	
	S550	IPVNRNL<u>S</u>IYDGPEQ	**PKCζ**	yes
NADH dehydrogenase (ubiquinone) Fe-S protein 1, 75kDa	-			
	S69	RPLTTSM<u>S</u>LFIIAPT	**PKCε/ζ**	
	S110	PFILATS<u>S</u>LSVYSIL	**PKCε**	
	S128	WASNSKY<u>S</u>LFGALRA	**PKCε/δ**	
	T139	ALRAVAQ<u>T</u>ISYEVTM	**PKCδ**	
	S258	YPELYST<u>S</u>FMTETLL	**PKCε**	
	S276	TFLWIRA<u>S</u>YPRFRYD	**cPKC**	
	T297	WKNFLPL<u>T</u>LAFCMWY	**PKCζ**	
isocitrate dehydrogenase 3 (NAD+) alpha	-			
	S340	ATIKDGK<u>S</u>LTKDLGG	**PKCδ/ζ**	
	T334	IEAACFA<u>T</u>IKDGKSL	**cPKC/δ**	
succinate dehydrogenase complex, subunit A, flavoprotein (Fp)	-			
	S28	ATRGFHF<u>S</u>VGESKKA	**cPKC**	
	S36	VGESKKA<u>S</u>AKVSDAI	**PKCδ**	
	T118	WRWHFYD<u>T</u>VKGSDWL	**cPKC**	
	S169	QRAFGGQ<u>S</u>LKFGKGG	**cPKC/δ/ζ**	
	S206	RSLRYDT<u>S</u>YFVEYFA	**PKCε/ζ**	
	T244	HRIRAKN<u>T</u>IIATGGY	**cPKC/ε/δ/ζ**	
	S462	FGRACAL<u>S</u>IAESCRP	**cPKC/δ**	
	S466	CALSIAE<u>S</u>CRPGDKV	**cPKC**	
	S484	KANAGEE<u>S</u>VMNLDKL	**PKCδ**	
	S497	KLRFADG<u>S</u>VRTSELR	**PKCε/χ/δ/ζ**	
	S506	RTSELRL<u>S</u>MQKSMQS	**cPKC**	

protein	predicted p-site	peptide sequence[1]	PKC isoenzyme	Validated[2]
	S510	LRLSMQK<u>S</u>MQSHAAV	**PKCδ/ζ**	
	S522	AAVFRVG<u>S</u>VLQEGCE	**PKCδ/ζ**	yes
	T618	AEHWRKH<u>T</u>LSYVDTK	**PKCε/δ/ζ**	
	S620	HWRKHTL<u>S</u>YVDTKTG	**cPKC/ζ**	
	T630	DTKTGKV<u>T</u>LDYRPVI	**PKCε**	
	T640	YRPVIDK<u>T</u>LNEADCA	**PKCε**	
Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase: Precursor	-			
	S30	RQLYFNV<u>S</u>LRSLSSS	**cPKC/ζ**	
	T153	SRYQKTF<u>T</u>VIEKCPK	**PKCε/ζ**	
	T225	RSLVNEL<u>T</u>FTARKMM	**PKCδ**	
glyceraldehyde 3-phosphate-dehydrogenase	-			
	T57	THGKFNG<u>T</u>VKAENGK	cPKC/ε	yes
	T185	AITATQK<u>T</u>VDGPSGK	**PKCδ**	yes
	T292	NSNSHSS<u>T</u>FDAGAGI	**PKCε/δ**	
ubiquinol-cytochrome c reductase core protein I	-			
	S107	TKSSKES<u>S</u>EARKGFS	**PKCε/δ**	
	T120	FSYLVTA<u>I</u> IIVGVAY	**PKCδ**	
	T122	YLVTAII<u>I</u>VGVAYAA	**PKCε**	
	T180	PLFVRHR<u>T</u>KKEIDQE	**cPKC**	
pyruvate dehydrogenase (lipoamide) alpha	-			
	T35	RNFANDA<u>T</u>FEIKKCD	**PKCζ**	
	T70	KYYRMMQ<u>T</u>VRRMELK	**cPKC/ε**	
	T124	AYRAHGF<u>T</u>FNRGHAV	**PKCδ**	
	T139	RAILAEL<u>T</u>GRRGGCA	**PKCδ**	
	S152	CAKGKGG<u>S</u>MHMYAKN	**PKCδ/ζ**	
	T266	ILCVREA<u>T</u>KFAAAYC	**PKCδ**	
	S293	TYRYHGH<u>S</u>MSDPGVS	**PKCε**	
pyruvate dehydrogenase (lipoamide) beta	-			
	S16	RGPLRQA<u>S</u>GLLKRRF	**PKCζ**	
	T112	RPICEFM<u>T</u>FNFSMQA	**PKCζ**	
	T235	AKIERQG<u>T</u>HITVVAH	**PKCζ**	
	S282	DIEAIEA<u>S</u>VMKTNHL	**PKCδ**	
ATP synthase beta subunit	-			
	S51	RDYAAQS<u>S</u>AAPKAGT	**PKCζ**	
	S231	AKAHGGY<u>S</u>VFAGVGE	**PKCζ**	

protein	predicted p-site	peptide sequence[1]	PKC isoenzyme	Validated[2]
	T288	RVALTGLTVAEYFRD	**PKCζ**	
	S353	IIIIKKGSITSVQAI	**PKCδ/ε/χ**	
Branched chain keto acid dehydrogenase E1, beta polypeptide	-			
	T105	FGGVFRCTVGLRDKY	**cPKC**	
	S177	GDLFNCGSLTIRAPW	**cPKC**	

[1] Predicted by Scansite (http://scansite.mit.edu).

[2] Valic ated sites reported in phosphosite (http//www.phosphosite.org).