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Signaling pathways regulating murine cardiac CREB phosphorylation

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Abstract

Using the mouse Langendorff heart perfusion model, the signaling pathways that regulate cardiac CREB-S133 phosphorylation have been defined. In mouse hearts stimulated with isoproterenol (ISO) (10^{-8} M), endothelin-1 (ET-1) (10^{-8} M), and phorbol 12-myristate 13-acetate (TPA) (10^{-7} M), CREB-S133 phosphorylation was attained only by TPA-treatment. Activation of protein kinase A (PKA) was achieved by ISO. ISO- and ET-1-stimulation activated Ca^{2+}/calmodulin-dependent kinase II (CaMKII). Protein kinase C (PKC) and $p90^{RSK}$ were activated with all three stimuli. Inhibition of ERK1/2 with PD98059 (10^{-5} M) completely inhibited the activation of $p90^{RSK}$, but did not block CREB-S133 phosphorylation in TPA-perfused heart, indicating that PKA, CaMKII, and $p90^{RSK}$ do not phosphorylate CREB-S133 in the murine heart. PKC activation is signal specific. Analyses of PKC isoforms suggest that CREB phosphorylation is mediated by PKCε translocating into nucleus only with TPA stimulation. These results, unlike those reported in other tissues, demonstrate that cardiac CREB is not a multi-signal target.

Keywords: CREB phosphorylation; Signaling pathways; Langendorff heart perfusion; PKA; CaMKII; $p90^{RSK}$; PKC; Murine

Many normal physiological challenges, such as rigorous exercise and pregnancy, increase the workload of the cardiovascular system. The myocyte responds by increasing cellular dimensions, contractile function, and the synthesis of contractile proteins. This adaptive "physiological" hypertrophy is beneficial and reversible. Pathological conditions including obesity, diabetes, atherosclerosis, and cardiovascular injury also place increased demand on the cardiovascular system. However, in these circumstances, the heart responds by initiating an inappropriate, chronic hypertrophic response that leads to dilated myopathy, multiple organ failure, and death.

Numerous intracellular signaling cascades including PKA, CaMKII, MAPK, and PKC are activated in cardiac hypertrophy and heart failure (reviewed in Refs. [1–3]). In many tissues, activation of these pathways in the cytoplasm ultimately affects nuclear transcription factors. One critical stimulus-induced transcription factor is the cyclic AMP response element (CRE)-binding protein, CREB [4]. Transcriptional activity of CREB is positively regulated by phosphorylation of a critical serine residue, Ser133. Once phosphorylated, CREB promotes assembly of the transcriptional apparatus through recruitment of the coactivator paralogues CREB-binding protein (CBP) and P300 to the promoter of a CREB target gene then induces the assembly of an active polymerase II transcription compound thus leading to target gene activation [4].

CREs are present in several cardiovascular gene promoter regions including β-adrenergic receptors (β-AR) [5], phospholamban (PLN) [6], protein phosphatase 2Aα [7], Kv1.5 [8], and atrial natriuretic peptide (ANP) [9], which suggest a role for CREB in cardiac hypertrophy and heart failure. In fact, when CREB function is blocked in mice with heart-specific overexpression of a non-phosphorylatable dominant-negative CREB-mutant, S133A, the transgenic mice develop four-chamber dilation and severe heart failure within a few weeks after birth [10].

Phosphorylation at Ser133 of CREB was initially attributed to cAMP-dependent PKA [11,12]. Numerous studies

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in many tissues have shown, however, that CREB is activated in response to a vast array of physiological stimuli that activate several kinases including CaMKII [13], $p90^{RSK}$ [14], Akt [15], and PKC [16]. Despite, the extensive understanding of CREB phosphorylation in many tissues [4], knowledge of its activation in the murine heart is incomplete. We have used agents including isoproterenol (ISO), endothelin-1 (ET-1), and phorbol 12-myristate 13-acetate (TPA), in isolated perfused mouse hearts to define the signaling pathways that regulate cardiac CREB phosphorylation.

Materials and methods

Chemicals and antibodies. Isoproterenol (ISO), endothelin-1 (ET-1), and phorbol 12-myristate 13-acetate (TPA), PD98059, proteinase inhibitors, phosphatase inhibitors, and other chemicals used in the experiments were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-phospho-CREB (Ser133), total-CREB, phospho-$p90^{RSK}$ (Thr359/Ser363), and phospho-(Ser) PKC substrate antibodies were obtained from Cell Signaling (Beverly, MA, USA). Anti-phospholamban Thr17 and Ser16 were purchased from Badrilla Ltd. (Leeds, LS179WA, England). Anti-total PKCα, β, δ, and ε antibodies were purchased from Santa Cruz (Santa Cruz, CA, USA). Anti-GAPDH antibody was purchased from Calbiochem (La Jolla, CA, USA). Secondary antibodies were purchased from Bio-Rad (Hercules, CA, USA). The Western blotting chemiluminescence reagent kit was purchased from Amersham Biosciences (Piscataway, NJ, USA).

Heart isolation and Langendorff perfusion. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the IACUC of the University of Cincinnati.

Three- to four-month-old FVB/N wild type mice were anesthetized and their hearts rapidly excised. The aorta was cannulated with a 20-gauge cannula and perfused retrogradely at a pressure of 80 mm Hg with Krebs–Henseleit bicarbonate-buffered saline (25 mM $NaHCO_3$, 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.28 mM $MgSO_4$, and 0.5 mM EDTA, pH 7.3–7.4, at 37 °C) supplemented with 10 mM glucose and equilibrated with 95 % O_2, 25% CO_2. The buffer was filtered through a 1 μm micro-filter (Radnoti, Colorado Springs, CO, USA). The temperature of the perfusates and hearts was maintained at 37 °C by the use of a water-jacketed apparatus, monitored, and recorded on the 4S PowerLab (AD Instruments, Colorado Springs, CO, USA). A polyethylene (PE-50) catheter was inserted through the left atrium and pulled through the left ventricle at the apex. The distal end was connected to the pressure transducer and the left ventricle pressure recorded on the 4S PowerLab System. Immediately above the aortic cannula, a pressure transducer was connected to record the perfusion pressure on the 4S PowerLab System.

All hearts were perfused for a 20-min equilibration period before ISO (10^{-8} M), ET-1 (10^{-8} M), or TPA (10^{-7} M) was infused for a period of 10 min. Saline solution was used in the control group. All drugs were added by infusion pump through an injection port directly above the aortic cannula. Three hearts were used for each condition. At the end of the perfusion, hearts were quickly frozen in dry ice and stored at −80 °C.

Cytoplasmic and nuclear protein fraction isolation. Nuclear and cytoplasmic protein fractions of perfused hearts were obtained using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL) according to the manufacturer's protocol. Briefly, frozen hearts were homogenized for 10 s on ice (repeated four times) in ice-cold CER I buffer (at ratio of 50 mg/ml) containing protease and protein phosphotase inhibitors. The homogenates were incubated on ice for 10 min, and then CER II (55 μl/ml CRE I buffer used) were added and mixed by vortexing for 5 s. Samples were centrifuged for 5 min at 4 °C at 14,000*g*, and the supernatants were collected as the cytoplasmic extracts. The pellets were resuspended in nuclear extraction reagent, NER (500 μl/ml CRE I buffer used) containing protease and phosphotase inhibitors, and incubated on ice for a total of 40 min with vortexing at 10-min intervals. The sample was then centrifuged for 10 min at 4 °C at 16,000*g* and supernatants collected as nuclear extracts. The cytoplasmic and nuclear fractions were stored at −70 °C.

Western blot. SDS sample buffer was added to the cytoplasmic or nuclear fractions, which were then denatured for 5 min at 95 °C. An equal volume from each sample was resolved on an SDS–polyacrylamide gel by electrophoresis and then transferred electrophoretically onto nitrocellulose membranes. Western blots were performed according to the manufacturer's instructions. The bands were detected using ECL with X-ray films.

Results and discussion

CREB-S133 phosphorylation was observed in TPA-, but not ISO- or ET-1-stimulated perfused mouse hearts

CREB-S133 is located in a domain that contains the phosphorylation motifs for several serine–threonine kinases including PKA, CaMKII, $p90^{RSK}$, and PKC (Fig. 1A). To examine if these kinases are involved in CREB phosphorylation, mouse hearts were stimulated with ISO (10^{-8} M), ET-1 (10^{-8} M), and TPA (10^{-7} M). Western blot analyses of nuclear extracts using anti-phospho-CREB-S133 antibody

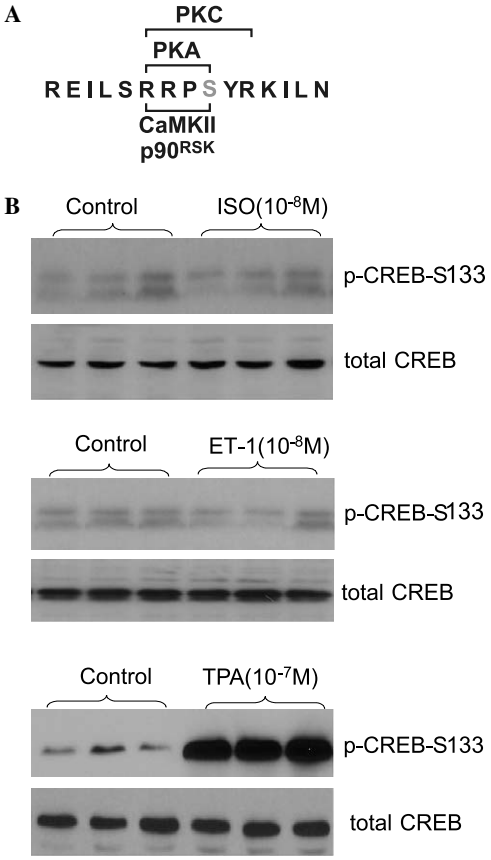

Fig. 1. (A) CREB kinase motifs. CREB-S133 resides in a kinase-inducing domain that contains the phosphorylation motifs of PKA, CaMKII, PKC, and $p90^{RSK}$. (B) Western blot analyses of CREB phosphorylation. Nuclear extracts from control, ISO-, ET-1-, and TPA-stimulated hearts were subjected to SDS–PAGE and immunoblotted with anti-phospho-CREB-S133 or anti-total CREB antibodies.

revealed that CREB-S133 phosphorylation was strongly induced with TPA-perfusion, but not induced in either ISO- or ET-1-stimulated hearts (Fig. 1B). The total levels of CREB protein expression, as detected by an antibody against total CREB, were unchanged (Fig. 1B).

PKA, nuclear CaMKIIδ_B, and p90^{RSK} do not phosphorylate cardiac CREB in perfused mouse heart

Activation of protein kinases in the relevant signaling cascades is essential for target protein phosphorylation; thus, we next investigated if the protein kinases, PKA, CaMKII, and p90^{RSK}, were activated in the perfused mouse hearts stimulated with ISO, ET-1, and TPA.

Phosphorylation of PLN at Ser16 (a substrate of PKA [17]), Thr17 (a substrate of CaMKII [2]), and p90^{RSK} at Thr359/Ser363 was used as an indicator of PKA, CaMKII, and p90^{RSK} activation. As shown in Fig. 2A, PLN-Ser16 was phosphorylated with ISO-stimulation only and PLN-Thr17 was phosphorylated by ISO and ET-1, but not TPA, indicating the cytosolic activation of these two protein kinases with ISO- or ET-1-stimulation. However, neither ISO nor ET-1 signal leads to CREB phosphorylation (Fig. 1B). On the other hand, PKA and CaMKII were not activated (Fig. 2A), but CREB was phosphorylated in TPA-perfused hearts (Fig. 1B). Therefore, PKA and CaMKII do not participate in CREB phosphorylation in perfused mouse hearts treated with ISO, ET-1, or TPA.

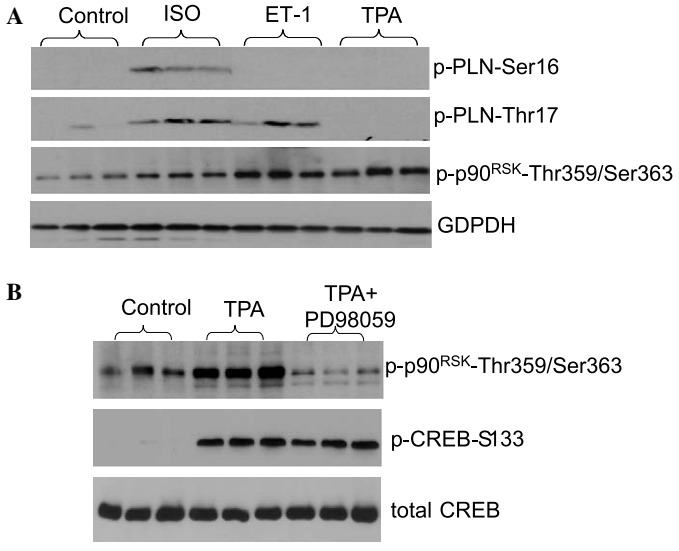

Fig. 2. Western blot analyses of PKA, CaMKII, and p90^{RSK} activation. (A) Cytoplasmic extracts from control and ISO-stimulated hearts were subjected to SDS–PAGE and immunoblotted with phospho-PLN-Ser16, -Thr17, anti-phospho-p90^{RSK}-Thr359/Ser363, or GAPDH. (B) Mouse hearts were perfused with PD98059 (10^{-5} M) for 5 min after a 20-min equilibration, then stimulated with TPA in the presence of PD98059 for 10 min. Nuclear extracts from control, TPA-, and TPA-plus PD98059-stimulated hearts were subjected to SDS–PAGE and immunoblotted with anti-phospho-P90^{RSK} -Thr359/Ser363, anti-phospho-CREB-S133, or anti-total CREB antibodies.

Our finding that PKA does not phosphorylate CREB-S133 has precedence. Seternes et al. [18] reported that dibutyryl cAMP is able to activate PKA, but fails to induce CREB-S133 phosphorylation in NIH 3T3 cells. Pende et al. [19] showed that activation of PKA in glial cell progenitors with forskolin (50 mM) did not lead to CREB-S133 phosphorylation. Steinberg and Brunton [20] stated that the spatial relationship of the components of the response pathway (adenylyl cyclase, cAMP, PKA, and PKA substrates) plays an important role in the ultimate response. Our result implies a subcellular compartmentation of the components of cyclic AMP action in the heart. The spatial separation of PKA and CREB may prevent CREB-S133 phosphorylation in ISO-perfused mouse hearts.

There are four isoforms of CaMKII, α, β, δ, and γ. The δ isoforms are the prominent cardiac isoform and there are two splice variants of CaMKIIδ, δ_B, and δ_C. The δ_B isoform contains an 11-amino acid nuclear localization signal and localizes to the nucleus and the δ_C isoform localizes to the cytoplasm [2]. Although CREB has been shown to be a CaMKII target in other tissues [13], it has only been considered a possible candidate target for CaMKII in the heart. Phosphorylation of PLN-Thr17 with ISO- and ET-1-stimulation (Fig. 2A) indicated cytoplasmic CaMKIIδ_C activation in our study. ET-1 activation of nuclear CaMKIIδ_B in cardiac myocytes was demonstrated by Wu et al. [21]. ET-1-stimulates phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol (DAG). $InsP_3$ then triggers Ca^{2+} release from an $InsP_3$R-dependent store that is located in the nuclear envelop, which in turn activates CaMKIIδ_B in the nucleus. Therefore, it is conceivable that mouse hearts perfused with ET-1 activated CaMKIIδ_B in our study, but such activation was not directed to CREB-S133 phosphorylation. A recently identified cardiac nuclear CaMKII target is histone deacetylase 4 (HDAC4) [22], which upon phosphorylation by CaMKII translocates from the nucleus to the cytoplasm and releases its inhibitory effect on MEF2 transcription. The induced transcription of MEF2 causes hypertrophic changes in cardiac myocytes.

Western blot analyses showed strong phosphorylation of p90^{RSK} at Thr359/Ser363 (Fig. 2A) with ISO, ET-1, and TPA-stimulation, indicating the activation of p90^{RSK}. However, ISO and ET-1 perfusion did not induce CREB-S133 phosphorylation (Fig. 2A). The results suggest that, p90^{RSK}, though activated by ISO or ET-1, does not phosphorylate cardiac CREB-S133.

Since TPA induced CREB-S133 phosphorylation (Fig. 1B) and p90^{RSK} activation (Fig. 2A), the participation of p90^{RSK} in CREB-S133 phosphorylation in TPA-stimulated hearts was further investigated. PD98059, an inhibitor of activated ERK1/2 signaling, was added to the TPA-stimulation protocol. Mouse hearts were first perfused with PD98059 (10^{-5} M) for 5 min after equilibration and then stimulated with TPA in the presence of the inhibitor for 10 min. As shown in Fig. 2B, phosphorylation of

p90^{RSK} at Thr359/Ser363 was completely blocked by PD98059, whereas CREB-S133 phosphorylation was not reduced (Fig. 2B). These results demonstrate that TPA induced CREB phosphorylation is not through protein kinase p90^{RSK}.

The PKCε isoform mediates CREB phosphorylation in TPA-stimulated mouse hearts

Using anti-phospho-(Ser)-PKC substrate antibody [antigen motif: (R/K)X(S*)(Hyd) (R/K)], PKC activation in ISO-, ET-1-, and TPA-stimulated hearts was analyzed. Phosphorylation of PKC-Ser substrates was achieved with all three stimuli as shown in Fig. 3A. This result implies that CREB-S133 phosphorylation could be mediated through PKC since PKA, CaMKII, and p90^{RSK} were eliminated earlier. The puzzle though, is why only TPA-induced CREB-S133 phosphorylation in mouse hearts. Close examination of the results reveals that different PKC-Ser substrates were phosphorylated when stimulated with ISO, ET-1, or TPA (Fig. 3A), which suggests that PKC isoform activation is signal specific. The PKC family comprises 12 structurally related serine–threonine kinase. The prominent cardiac isoforms are PKCα, β, δ, and ε [23]. Activation of PKC by ISO, ET-1, or TPA is through different pathways (ISO through Gs-coupled receptor, ET-1 through Gq-protein-coupled receptor, and TPA directly), which could lead to specific PKC isoform activation. In fact, several studies have shown distinct PKC isoform activation through stimuli ET-1, norepinephrine (NE), angiotensin II (AgII), and TPA [24–28]. The results shown in Fig. 3A suggest that different PKC isoforms were activated in ISO-, ET-1-, and TPA-perfused hearts. Only the PKC isoform(s) stimulated through the TPA-signal directed CREB phosphorylation.

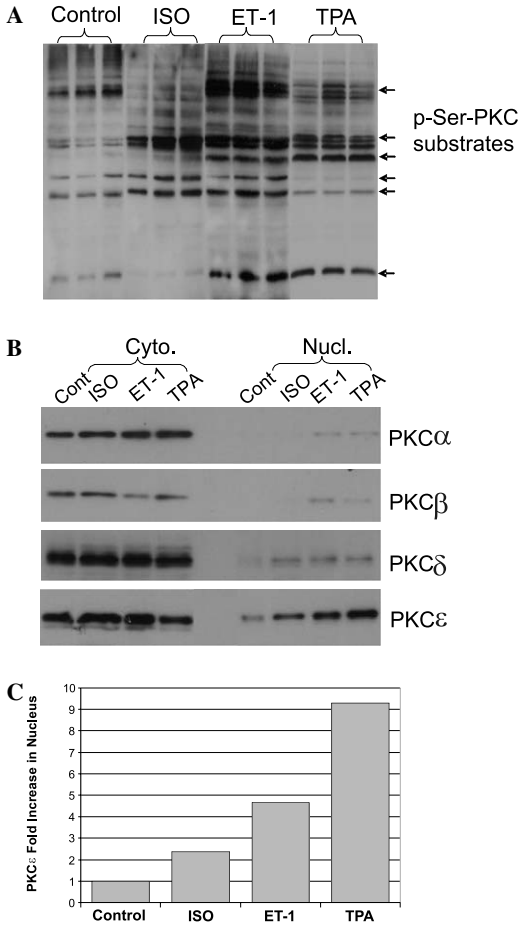

Fig. 3. PKC isoform activation analyses. (A) Western blot analyses of PKC activation. Cytoplasmic extracts from control, ISO-, ET-1-, and TPA-stimulated hearts were subjected to SDS–PAGE and immunoblotted with anti-phospho-(Ser)-PKC substrate antibody. Arrows indicate distinct substrates phosphorylated by PKC stimulated with ISO, ET-1, or TPA. (B) Western blot analyses of PKCε translocation. Cytoplasmic and nuclear extracts from control, ISO-, ET-1-, and TPA-stimulated hearts were subjected to SDS–PAGE and immunoblotted with anti-PKCα, β, δ, and ε antibodies. (C) Quantitative analyses of PKCε in the nucleus. Bands were scanned and quantified using AlphaEaseFC software [Alpha Innotech Co., (San Leandro, CA)].

We next dissected which PKC isoform is responding to TPA signal and then mediates CREB-S133 phosphorylation. Upon activation, PKCs translocate from the cytosol to multiple cellular compartments, including the plasma membrane, endosomes, Golgi, and the nucleus, to phosphorylate downstream target proteins [29]. The translocation of α, β, δ, and ε to the nucleus was analyzed since CREB resides in the nucleus [4]. Western blot analyses of cytoplasmic and nuclear fractions (Fig. 3B) show that ISO, ET-1, and TPA induced mostly PKCε but little α, β, and δ translocation to the nucleus. These results suggest that PKCε is the isoform involved in CREB-S133 phosphorylation. Although all three signals activated PKCε translocation, quantitative examination revealed significant differences in the amounts of PKCε translocated to the nucleus. PKCε in the nuclear fraction of TPA-stimulated heart was four and two times more than in ISO- and ET-1-treated hearts, respectively (Fig. 3C). The quantitative difference in nuclear PKCε could be the key element that decides CREB-S133 phosphorylation. Studies have shown that the activity of any given PKC isoform is dependent upon its expression level, its localization within the cell, and its phosphorylation state [30]. Clearly, it is the TPA signal, not ET-1 or ISO, leading to PKCε activation, which then phosphorylated CREB in the nucleus. In contrast, ISO and ET-1 activated different PKC isoforms that apparently have different physiological roles other than the phosphorylation of CREB-S133.

In summary, our study has revealed a unique aspect of CREB regulation of gene expression in the murine heart. Unlike other tissues studied, CREB phosphorylation does not respond to multiple signals in mouse heart. TPA, not ISO or ET-1 signals, specifically induces CREB-S133 phosphorylation. Treatment of perfused mouse heart with ISO, ET-1, and TPA is able to activate multiple protein kinases including PKA, CaMKII, and p90^{RSK}. However, ISO and ET-1 signals do not lead to CREB-S133 phosphorylation. Cardiac CREB-S133 phosphorylation is restricted to TPA-stimulation acting via PKCε (Fig. 4). The stimulus-specific

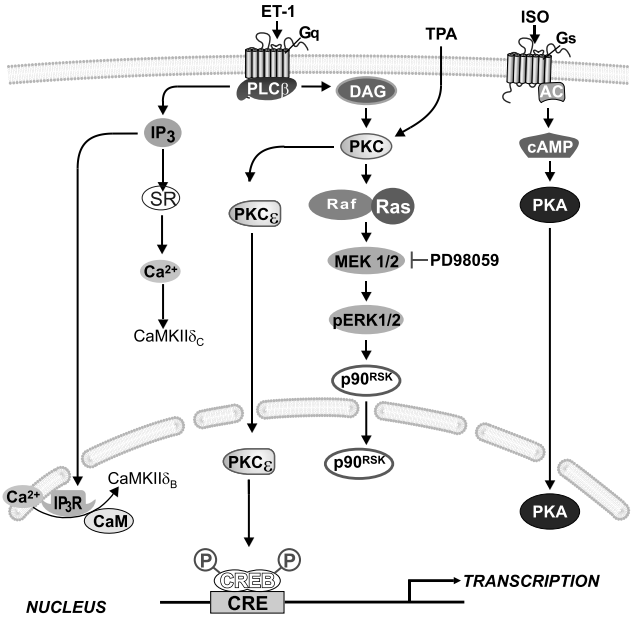

Fig. 4. Kinases involved in CREB phosphorylation in murine heart. Diverse agonists act through G-protein-coupled receptors and other effectors to activate PKA, CaMKII, PKC, and ERK1/2. CREB is specifically phosphorylated by PKCε only in TPA-stimulated murine heart. AC, adenylyl cyclase; IP_3, inositol 1,4,5-trisphosphate; IP_3R, IP_3 receptor.

response in CREB-S133 phosphorylation could be mediated through the compartmentation of kinases and substrates, the level of kinase expression, and specific kinase isoform activation. Our results imply that changes in gene expression associated with cardiac hypertrophy and heart failure induced by the intracellular signaling pathways such as PKA, CaMKII, and ERK1/2 are not CREB-activation associated. Identification of CREB targeted genes will provide further information on the role of CREB-S133 phosphorylation by PKCε in the TPA-stimulated isolated perfused murine heart.

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