Original Paper

Signaling Pathway for Endothelin-1- and Phenylephrine-Induced cAMP Response Element Binding Protein Activation in Rat Ventricular Myocytes: Role of Inositol 1,4,5-Trisphosphate Receptors and CaMKII

Krishna P. Subedi^{a,b} Min-Jeong Son^a Bojjibabu Chidipi^a Seong-Woo Kim^a
Jun Wang^a Kyeong-Hee Kim^a Joon-Chul Kim^a Sun-Hee Woo^a

^aCollege of Pharmacy, Chungnam National University, Yuseong-gu, Daejeon, South Korea; ^bcurrent address: Secretory Physiology Section, Molecular Physiology and Therapeutics Branch, National Institute of Dental and Craniofacial Research, NIH, Bethesda, MD, USA

Key Words

cAMP response element binding protein • Inositol 1,4,5-trisphosphate receptor • Protein kinase C • Ca²⁺-calmodulin-dependent protein kinase II • Ventricular myocytes

Abstract

Background/Aims: Endothelin-1 (ET-1) and the α_1 -adrenoceptor agonist phenylephrine (PE) activate cAMP response element binding protein (CREB), a transcription factor implicated in cardiac hypertrophy. The signaling pathway involved in CREB activation by these hypertrophic stimuli is poorly understood. We examined signaling pathways for ET-1- or PE-induced cardiac CREB activation. **Methods:** Western blotting was performed with pharmacological and genetic interventions in rat ventricular myocytes. **Results:** ET-1 and PE increased CREB phosphorylation, which was inhibited by blockade of phospholipase C, the extracellular-signal-regulated kinase 1/2 (ERK1/2) pathway, protein kinase C (PKC) or Ca²⁺-calmodulin-dependent protein kinase II (CaMKII). Intracellular Ca²⁺ buffering decreased ET-1- and PE-induced CREB phosphorylation by $\geq 80\%$. Sarcoplasmic reticulum Ca²⁺ pump inhibitor, inositol 1,4,5-trisphosphate receptor (IP₃R) blockers, or type 2 IP₃R (IP₃R2) knock-out abolished ET-1- or PE-induced CREB phosphorylation. ET-1 and PE increased phosphorylation of CaMKII and ERK1/2, which was eliminated by IP₃R blockade/knock-out or PKC inhibition. Activation of CaMKII, but not ERK1/2, by these agonists was sensitive to Ca²⁺ buffering or to Gö6976, the inhibitor of Ca²⁺-dependent PKC and protein kinase D (PKD). **Conclusion:** CREB phosphorylation by ET-1 and PE may be mainly mediated by IP₃R2/Ca²⁺-PKC-PKD-CaMKII signaling with a minor contribution by ERK1/2, linked to IP₃R2 and Ca²⁺-independent PKC, in ventricular myocytes.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Neurohumoral hormones such as endothelin-1 (ET-1) or the α_1 -adrenoceptor (AR) agonist phenylephrine (PE) stimulate hypertrophic growth of cardiac myocytes [1]. PE and ET-1 commonly activate phospholipase C (PLC) via G_q -coupled receptors to produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol from phosphatidylinositol 4,5-bisphosphates, thereby inducing IP_3 receptor (IP_3R)-mediated Ca^{2+} release and protein kinase C (PKC) activation [2, 3]. It has been shown in ventricular myocytes that type 2 IP_3Rs (IP_3R_2), the major IP_3R subtype, are localized in the nuclear envelope [4, 5], intercalated discs [6], and in the dyadic junctional sarcoplasmic reticulum [7]. Nuclear IP_3Rs -mediated Ca^{2+} signal has been proposed to modulate Ca^{2+} -calmodulin-dependent protein kinase (CaMK) II (CaMKII), histone deacetylases (HDAC), and calcineurin-NFATc signaling in ventricular myocytes [1, 5, 8]. These mechanisms are implicated in transcriptional regulation of hypertrophy-specific genes [5, 9-12]. IP_3R_2 proteins are up-regulated in cardiac hypertrophy and failure [13] via positive feedback regulation of the calcineurin-NFATc signaling pathway [14].

The adenosine 3',5'-monophosphate (cAMP) response element-binding protein (CREB) functions as a Ca^{2+} - and cAMP-regulated transcription factor for certain genes [15, 16]. Phosphorylation of Ser¹³³ in CREB by different kinases, including protein kinase A, CaMKs, p90 ribosomal S6 kinases and mitogen- and stress-activated protein kinases (MSKs), results in recruitment of the co-activator CREB-binding protein, thus augmenting CREB-induced gene transcription [15-20]. It has been shown that PE and ET-1 promote CREB phosphorylation in rat cardiac myocytes [21, 22], but not in mouse perfused heart preparations [23]. CREB phosphorylation is implicated in the regulation of expression of genes involved in cardiac hypertrophic changes [21, 22]. ET-1- or PE-induced CREB phosphorylation was shown to be mediated by either extracellular signal-regulated kinase1/2 (ERK1/2) or p38 mitogen activated protein kinase (MAPK) signaling in rat ventricular myocytes [21, 22, 24]. Because the ERK1/2 cascade involved in the ET-1- or PE-induced cardiac CREB phosphorylation was suppressed by a PKC inhibitor [24], it was postulated that CREB activation by ET-1 or PE is mediated by PKC-ERK1/2 signaling. However, the role of PKC in regulating CREB phosphorylation, as well as its interacting partner molecules in such hormonal signaling, has been unknown. Although IP_3R or CaMKII has been known to modulate CREB phosphorylation in other tissues, such as skeletal muscle [25], neurons [26], and cholangiocytes [27], whether IP_3Rs and CaMKII regulate cardiac CREB phosphorylation has been unclear.

Therefore, in this study, we explored signal transduction pathways responsible for CREB phosphorylation in rat ventricular myocytes under the exposure to ET-1 or PE. We examined the potential roles of intracellular Ca^{2+} , IP_3Rs , PKC, CaMKII, and ERK1/2 using western blotting combined with pharmacological and genetic interventions. We found that CREB phosphorylations stimulated by either ET-1 or PE was mainly mediated by CaMKII, that, in turn, was activated by IP_3R_2 -mediated Ca^{2+} increase and Ca^{2+} -dependent PKC/protein kinase D (PKD) signaling in these myocytes. Unlike this signaling pathway, the ERK1/2 activation by ET-1 and PE was mediated by IP_3R_2 and Ca^{2+} -independent PKC, in a manner resistant to Ca^{2+} buffering.

Materials and Methods

Single cell isolation and treatment

Ventricular myocytes were enzymatically isolated [28] from male Sprague Dawley rats (200-300 g) and from wild-type (WT) and IP_3R_2 knock-out (KO) mice [29] (C57/B6 background, 3-4 months of age, 24-26 g). This study conforms with the Guiding Principles for the Care and Use of Experimental Animals published by the Korean Food and Drug Administration and Animal and Plant Quarantine Agency in South Korea. The experiments were carried out according to the guidelines laid down by the Chungnam National University Animal Care and Use Committee (Approval No. CNU-00368). Rats or mice were deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.), the chest cavity was opened, and the hearts were excised. This

surgical procedure was carried out in accordance with the university's ethical guidelines. The excised hearts were retrogradely perfused at 7 ml/min through the aorta, first for 3 min with a Ca^{2+} -free Tyrode solution composed of (in mM): 137 NaCl, 5.4 KCl, 10 HEPES, 1 MgCl_2 , 10 glucose, and pH 7.3, at 36.5°C; and then with a Ca^{2+} -free Tyrode solution containing collagenase (1.4 mg/ml, Type 1, Roche) and protease (0.14 mg/ml, Type XIV, sigma) for 12 min; and finally with Tyrode solution containing 0.2 mM CaCl_2 for 8 min. The ventricles of the digested heart were then cut into several sections and subjected to gentle agitation to dissociate the cells. The Ca^{2+} concentration of Tyrode solution was gradually increased to 2 mM. The cells were then gently resuspended in the Tyrode solution containing ET-1 (100 nM) or PE (100 μM) or other inhibitors and incubated at 37°C for indicated durations.

Western blotting

After stimulation for the desired time periods, cells were solubilized in SDS lysis buffer containing 10 mM Tris-HCl, pH 7.4, 1% (w/v) SDS, 1 mM phenylmethanesulfonyl fluoride, 1 mM Na_3VO_4 and complete protease inhibitor mixture (Roche Molecular Biochemicals) for 10 min at 70°C, and then triturated several times by passing through a 1-ml syringe and centrifuged at 12,000 g for 10 min. The supernatant was combined with 2 × Laemmli sample buffer (Bio-Rad, Hercules, CA, USA) and heated for 10 min at 70°C. Protein samples were separated by 10% SDS-PAGE and electroblotted on to nitrocellulose membranes. Total CREB, phospho-CREB, CaMKII, phospho-CaMKII, ERK1/2 and phospho-ERK1/2 were detected with antibodies that specifically recognize the proteins (CREB: Cell signaling technology, #9197S, rabbit monoclonal Ab; Ser¹³³-phosphorylated CREB: Cell signaling technology, #9198S, rabbit monoclonal Ab; p44/42 MAPK (ERK1/2): Cell signaling technology, #9102, rabbit polyclonal Ab; Thr²⁰²/Tyr²⁰⁴-phosphorylated p44/42 MAPK (ERK1/2): Cell signaling technology, #9101S, rabbit polyclonal Ab; CaMKII: Abcam, #ab52476, rabbit monoclonal Ab; Thr²⁸⁶-phosphorylated CaMKII: Cell signaling technology, #3361S, rabbit polyclonal Ab) using a standard Western blot protocol. All blots were imaged and quantified using a ChemiDoc XRS densitometer (Bio-Rad).

Statistics

Results are mean ± SEM with significance ($P < 0.05$) determined using unpaired 2-tailed Student's *t* test. *n* indicates the number of Western blotting experiments.

Results

ET-1 and PE stimulate CREB phosphorylation through ET_A receptor and α₁-adrenoceptor, respectively

Enhancement of CREB phosphorylation at Ser¹³³ by ET-1 and PE was demonstrated, respectively, in primary cultures of neonatal rat cardiac myocytes [21] and in adult rat ventricular myocytes, respectively [22]. We first confirmed, by western blotting, whether CREB phosphorylation is increased in isolated adult rat ventricular myocytes treated with these agonists, and further investigated hormonal receptors that mediate these responses. Myocytes were incubated with ET-1 (100 nM) or PE (100 μM) for 15 min, because these agonists maximally affected Ca^{2+} transients and CREB phosphorylation under these conditions [21, 22]. CREB phosphorylation was significantly enhanced by ET-1 and PE (Fig. 1A). To estimate the effects of the agonists on phosphorylating potency, the level of the phosphorylated CREB was normalized to the signal for total CREB and then the ratios for agonist-treated samples were normalized again to the ratio for the control (upper graphs in the figures). CREB activation by ET-1 was almost completely suppressed by pre-treating cells with the ET_A receptor blocker BQ123 (1 μM , 30 min) (Fig. 1A). The ET_B receptor blocker BQ788 did not affect the ET-1-induced CREB activation (Data not shown). Similarly, CREB phosphorylation by PE was suppressed by ~80% by the α₁-adrenoceptor antagonist prazosin (5 μM , 10 min) (Fig. 1A). The residual small increase in CREB phosphorylation after PE stimulation, in the presence of prazosin, may have been mediated by the β-adrenergic receptor [22]. This result indicates that both ET-1 and PE can stimulate CREB phosphorylation in ventricular myocytes, primarily through ET_A receptors and α₁-adrenoceptors, respectively.

Fig. 1. Enhancement of CREB phosphorylation by ERK1/2 signaling, CaMKII and PLC under the stimulation of ET_A receptors and α₁-adrenoceptors in rat ventricular myocytes. Upper panels show comparison of quantitative levels of phosphorylated CREB relative to the total CREB (normalized to control) shown in the Western blot analysis below. Fifty-μg cell extracts were subjected to SDS/PAGE and immunoblotted for phosphorylated CREB (middle panels) and total CREB (lower panels). (A) Ventricular myocytes were unstimulated or exposed to endothelin-1 (ET-1) (100 nM, 15 min) or phenylephrine (PE) (100 μM, 15 min) without and with ET_A receptor antagonist BQ123 (1 μM, 30 min; left) or α₁-adrenoceptor inhibitor prazosin (5 μM, 10 min; right). Four series of experimental results were quantified. **P* < 0.05 vs. each control (untreated). #*P* < 0.05 vs. ET-1 (left) or PE (right). (B and C) Ventricular myocytes were either not exposed to inhibitors (control) or pretreated for 15 min with H89 (25 μM), PD98059 (10 μM), or 20 min with KN93 (1 μM), U73122 (5 μM). Then the pretreated cells were exposed to 100 nM ET-1 (B) or 100 μM PE (C) for 15 min. Four series of experimental results were quantified. **P* < 0.05 vs. control (untreated). #*P* < 0.05 vs. ET-1 (B) or PE (C).

Role of ERK1/2-MSK1 signaling, PLC and CaMKII in ET-1- and PE-induced CREB phosphorylation

It has been previously demonstrated that PE and ET-1 cause MSK1 phosphorylation via PKC-ERK1/2 signaling and p38 MAPKs in adult rat ventricular myocytes [24]. In addition, the ERK1/2-MSK1 signaling cascade mediates the CREB phosphorylation induced by ET-1 in neonatal rat cardiac myocytes [18] and by PE in adult rat ventricular myocytes [22]. We first confirmed in rat ventricular myocytes whether ERK1/2-MSK1 signaling mediates both ET-1- and PE-induced CREB phosphorylation in rat ventricular myocytes. We used PD98059, a drug inhibiting activation of MAPK kinase-1 (also called MEK1 or ERK kinase) and, hence, the activation of MAPKs/ERKs. We also used H89, a drug suppressing several kinases and a potent MSK1 inhibitor [30]. In cells exposed to 100 nM ET-1 or 100 μM PE for 15 min, CREB phosphorylation was increased, and in both cases this was completely inhibited by pre-treatment with H89 (25 μM, 15 min) or PD98059 (10 μM, 10 min) (Fig. 1B and C). These results suggest that both ET-1 and PE activate CREB via MEK1-ERK1/2-MSK1 signaling, consistent with the previous reports in neonatal and adult rat ventricular myocytes [21, 22].

Fig. 2. Roles of global Ca^{2+} increase and SR Ca^{2+} release in the enhancement of CREB phosphorylation by ET-1 and PE. Ventricular myocytes were either not exposed to interventions (control) or pretreated for 30 min with BAPTA-AM (10 μM ; A) or for 10 min with thapsigargin (1 μM ; B), and then treated with ET-1 or PE for 15 min. Upper panels show comparison of quantitative levels of phosphorylated CREB relative to the total CREB (normalized to control) shown in the Western blot analysis below. Fifty- μg cell extracts were subjected to SDS/PAGE and immunoblotted for phosphorylated CREB (middle panels) and total CREB (lower panels). Four and five series of experiments were quantified in BAPTA and thapsigargin, respectively. * $P < 0.05$ vs. control (untreated). # $P < 0.05$ vs. ET-1 (A) or thapsigargin (B). † $P < 0.05$ vs. PE.

Receptors for ET-1 and PE are G-protein coupled and commonly activate PLC signaling, thereby activating IP_3 Rs and PKC and mobilizing intracellular Ca^{2+} in cardiac myocytes [2, 3, 31-33]. Both ET-1 and PE were reported to activate CaMKII in cardiac myocytes, thereby regulating excitation-contraction coupling and gene transcription [5, 34, 35]. However, the role of CaMKII in the enhancement of CREB phosphorylation by ET-1 and PE is not known. We tested its involvement using the CaMKII inhibitor KN93 (1 μM , 20 min). Stimulation of CREB phosphorylation by ET-1 was suppressed to 10%-15% in cells pretreated with KN93 (Fig. 1B), while the response to PE was inhibited by KN93 to approximately 30% (Fig. 1C). Stimulation of CREB phosphorylation by ET-1 or PE was eliminated by the PLC inhibition (U73122, 5 μM , 20 min; Fig. 1B and C). These results suggest that CaMKII and PLC, in addition to the ERK1/2 signaling, may mediate ET-1- and PE-stimulated CREB phosphorylation.

Role of SR Ca^{2+} release and $\text{IP}_3\text{R}2$ in the enhancement of CREB phosphorylation by ET-1 and PE

We next examined whether cytosolic Ca^{2+} increase plays a role in the enhancement of CREB phosphorylation by α_1 -adrenoceptor and ET_A receptor stimulation. When cytosolic Ca^{2+} was buffered by loading the cells with BAPTA-AM (10 μM) for 30 min, CREB phosphorylation was only slightly increased, by about 10%, by subsequent stimulation with ET-1. Under this condition, PE-induced CREB phosphorylation was suppressed by about 70%-80% with cytosolic Ca^{2+} buffering (Fig. 2A). To further examine whether Ca^{2+} release from the sarcoplasmic reticulum (SR) plays a role in ET-1- and PE-induced CREB phosphorylation, Ca^{2+} in the SR lumen was depleted using the SR Ca^{2+} pump inhibitor, thapsigargin (1 μM , 10 min). Exposure to thapsigargin alone increased CREB phosphorylation (Fig. 2B). This effect was similar to that of ET-1 or PE on CREB phosphorylation and might be stimulated by increased Ca^{2+} levels caused by decreased cytosolic Ca^{2+} uptake into the SR. This result was consistent with previous reports of the Ca^{2+} -dependence of CREB phosphorylation

Fig. 3. Role of IP₃R2 in ET-1- and PE-induced CREB phosphorylation. Ventricular myocytes were either not exposed to interventions (control) or pretreated for 45 min with 10 μ M xestospongine C (XeC; A; $n = 4$) or for 30 min with 3 μ M 2-APB (B; $n = 4$), and then treated with ET-1 or PE for 15 min. * $P < 0.05$ vs. control (untreated). # $P < 0.05$ vs. ET-1. † $P < 0.05$ vs. PE. Upper panels show comparison of quantitative levels of phosphorylated CREB relative to the total CREB (normalized to control) shown in the Western blot analysis below. Fifty- μ g cell extracts were subjected to SDS/PAGE and immunoblotted for phosphorylated CREB (middle panels) and total CREB (lower panels). (C) Western blotting analysis for phosphorylated CREB and CREB in wild-type (WT; $n = 3$) and IP₃R2 knock-out (KO; $n = 3$) mouse ventricular myocytes without and with ET-1 or PE. * $P < 0.05$ vs. control (untreated). # $P < 0.05$ vs. WT ET-1. † $P < 0.05$ vs. WT PE.

Fig. 4. Role of PKC in ET-1 and PE-induced CREB phosphorylation. Ventricular myocytes were either not exposed to interventions (control) or pretreated for 10 min with PKC inhibitor GF109203X (5 μ M; $n = 4$; A and B), for 45 min with PI3K inhibitor LY294002 (50 μ M; $n = 4$; A and B), or for 30 min with PKA blocker KT5720 (5 μ M; $n = 3$; C). Then the cells were treated with ET-1 or PE for 15 min. Upper panels show comparison of quantitative levels of phosphorylated CREB relative to the total CREB (normalized to control) shown in the Western blot analysis below. Fifty- μ g cell extracts were subjected to SDS/PAGE and immunoblotted for phosphorylated CREB (middle panels) and total CREB (lower panels). * $P < 0.05$ vs. control (untreated). # $P < 0.05$ vs. ET-1 (A) or PE (B).

[16]. Application of ET-1 to the cells pre-exposed to thapsigargin did not further increase CREB phosphorylation. PE treatment decreased the CREB phosphorylation levels that had already been increased by thapsigargin (Fig. 2B). These data indicate that increase in CREB phosphorylation by ET-1 and PE may be mainly caused by intracellular Ca^{2+} increase due to SR Ca^{2+} release.

To identify the Ca^{2+} release pathway responsible for ET-1- or PE-stimulated CREB phosphorylation, we investigated the role of IP₃Rs, activated by ET_A/ α_1 -adrenergic receptor signaling. Both ET-1- and PE-induced increases in the CREB phosphorylation were inhibited by treatment of either xestospongine C (10 μ M) or 2-APB (3 μ M), the IP₃R blockers (Fig. 3A and B). Because these inhibitors are not specific for IP₃Rs [36, 37], we confirmed the role

Fig. 5. Activation of CaMKII by ET-1 and PE via IP₃R, intracellular Ca²⁺ increase, and Ca²⁺-dependent PKC/PKD. Ventricular myocytes were either not exposed to interventions (control) or pretreated for 30 min with 3 μ M 2-APB (A; *n* = 4) or 10 μ M BAPTA (B; *n* = 4). Then the cells were additionally treated with ET-1 (100 nM) or PE (100 μ M) for 15 min. Inhibition of IP₃Rs or Ca²⁺ buffering inhibited ET-1- and PE-induced CaMKII phosphorylation. Upper panels show comparison of quantitative levels of phosphorylated CaMKII relative to the total CaMKII (normalized to control) shown in the Western blot analysis below. (C) Pretreatment of chelerythrine (5 μ M, 30 min) or Gö6976 (10 μ M, 15 min) significantly suppressed increase in CaMKII phosphorylation by ET-1 or PE (*n* = 5). Fifty- μ g cell extracts were subjected to SDS/PAGE and immunoblotted for phosphorylated CaMKII (middle panels) and total CaMKII (lower panels). **P* < 0.05 vs. control (untreated). #*P* < 0.05 vs. ET-1. †*P* < 0.05 vs. PE.

of IP₃Rs in these hormonal responses by using ventricular myocytes from IP₃R2 KO mice. In WT myocytes, ET-1 or PE treatment consistently increased CREB phosphorylation (Fig. 3C). In sharp contrast, neither ET-1 nor PE increased the CREB phosphorylation in the IP₃R2 KO myocytes (Fig. 3C). In the KO cells, CREB phosphorylation was, instead, decreased by ET-1 or PE (Fig. 3C, right). This was consistent with the observation on decrease in the level of phosphorylated CREB in the presence of 2-APB alone (Fig. 3B). These results suggest that IP₃R2 plays a key role in the increase of CREB phosphorylation under the stimulation of ET_A receptors or α_1 -adrenoceptors in ventricular myocytes.

Role of PKC in ET-1- and PE-induced CREB phosphorylation

Previous papers have shown that ERK1/2-MSK1 signaling is an important mediator of the CREB phosphorylation in the presence of ET-1 or PE [21, 22]. In addition, MSK1 activation by these agonists was inhibited by a PKC inhibitor [24]. We also found that increase in CREB phosphorylation by ET-1 and PE was eliminated by inhibitors for ERK1/2 and MSK1 (Fig. 1B and C). Activation of PKC during stimulation of α_1 -adrenoceptors and ET_A receptors could be facilitated by a Ca²⁺ increase via activation of IP₃Rs. Therefore, we examined the potential roles of PKC and other important kinases in CREB activation during these hormonal stimulations. Inhibition of PKC by GF109203X (5 μ M, 10 min) completely suppressed CREB phosphorylation induced by ET-1 or PE (Fig. 4A). However, blockade of protein kinase A (5 μ M KT5720, 30 min) or phosphoinositide 3-kinase (50 μ M LY294002, 45 min) did not alter ET-1- or PE-induced CREB phosphorylation (Fig. 4). These results indicate that PKC may be an important mediator for CREB phosphorylation during the stimulation of α_1 -adrenoceptors and ET_A receptors.

Fig. 6. Enhancement of ERK1/2 phosphorylation through PLC/ Ca^{2+} -independent PKC, but not CaMKII. (A) Quantitative comparison of the level of phosphorylated ERK1/2 normalized to total ERK1/2 in the absence and presence of ET-1 (100 nM, $n = 3$) or PE (100 μM , $n = 3$) in untreated myocytes and in the cells pre-exposed to PKC inhibitor (GF109203X, 5 μM , 15 min). (B) comparison of the level of phosphorylated ERK1/2 normalized to total ERK1/2 in the absence and presence of ET-1 ($n = 4$) or PE ($n = 3$) in untreated myocytes and in the cells pre-incubated with CaMKII inhibitor KN93 (1 μM , 20 min), MEK1 inhibitor PD98059 (10 μM , 10 min), or PLC inhibitor U73122 (5 μM , 20 min). (C) Comparison of the level of phosphorylated ERK1/2 normalized to total ERK1/2 showing no change in the level of ERK1/2 phosphorylation by ET-1 or PE in the presence of G66976 (10 μM , 15 min; $n = 3$). Fifty- μg cell extracts were subjected to SDS/PAGE and immunoblotted for phosphorylated ERK1/2 (middle panels) and total ERK1/2 (lower panels). * $P < 0.05$ vs. control (untreated). # $P < 0.05$ vs. ET-1 or PE.

Activation of CaMKII by ET-1 and PE via IP_3 Rs and Ca^{2+}

After finding evidence for suppression of the ET-1- and PE-induced CREB phosphorylation by inhibition of either the CaMKII or the IP_3 Rs (Figs. 1 and 3), we monitored the phosphorylated form of CaMKII to directly assess its activation in cells treated with these agonists. Both ET-1 and PE significantly increased CaMKII phosphorylation (Fig. 5A). We further tested whether ET-1- or PE-stimulated CaMKII phosphorylation requires activation of the IP_3 Rs, using their inhibitor 2-APB. Pre-incubation of the ventricular myocytes with 2-APB (3 μM , 30 min) fully suppressed ET-1-induced CaMKII phosphorylation and suppressed PE-induced CaMKII phosphorylation by 70%-80% (Fig. 5A). Activation of CaMKII by ET-1 and PE was not observed in the cells pre-loaded with BAPTA (10 μM , 30 min) (Fig. 5B), suggesting role of Ca^{2+} increase in CaMKII activation by these agonists. This result indicates that ET-1 and PE may activate CaMKII mainly via an increase in intracellular Ca^{2+} through IP_3 Rs activation, and that PE can also cause CaMKII activation, in part, via an IP_3 R-independent pathway. This result is also consistent with a previous report that ET-1 induces CaMKII phosphorylation via IP_3 Rs in rat ventricular myocytes [5]. However, it has also been reported that PE-induced CaMKII phosphorylation is eliminated by PKC blockade in ventricular myocytes [34]. Because our data showed that CREB phosphorylation by ET-1 and PE was sensitive to inhibitors of either PKC or CaMKII (Figs. 1 and 4), we tested whether PKC was involved in CaMKII activation in cells treated with ET-1 or PE. Pre-incubation of cells with the PKC inhibitor chelerythrine (5 μM , 30 min) abolished CaMKII phosphorylation by ET-1 or PE (Fig. 5C). Because inhibition of either IP_3 Rs or PKC suppressed most of the CaMKII activation caused by ET-1 and PE,

Fig. 7. Enhancement of ERK1/2 phosphorylation by ET-1 and PE via IP_3R_2 , but not global Ca^{2+} increase. (A) Both ET-1 and PE failed to increase ERK1/2 phosphorylation in the presence of IP_3R inhibitors 2-APB ($n = 3$) or XeC ($n = 3$). * $P < 0.05$ vs. control (untreated). # $P < 0.05$ vs. ET-1. † $P < 0.05$ vs. PE. (B) ERK1/2 phosphorylation was increased in WT mouse ventricular myocytes by ET-1 (100 nM) or PE (100 μ M) ($n = 3$), but slightly decreased in IP_3R_2 KO myocytes by these agonists ($n = 3$). * $P < 0.05$ vs. control (untreated). # $P < 0.05$ vs. WT ET-1 or WT PE. (C) No change in the ERK1/2 phosphorylation by 100 nM ET-1 and 100 μ M PE in Ca^{2+} -buffered myocytes using BAPTA-AM. Quantitative comparison of the level of phosphorylated ERK1/2 normalized to total ERK1/2 in the absence and presence of ET-1 or PE in untreated myocytes and in the BAPTA-AM (10 μ M, 30 min) pre-incubated myocytes ($n = 4$). Fifty- μ g cell extracts were subjected to SDS/PAGE and immunoblotted for phosphorylated ERK1/2 (middle panels) and total ERK1/2 (lower panels). * $P < 0.05$ vs. control (untreated).

PKC may be the downstream molecule that is activated by IP_3R - Ca^{2+} signaling. To test this hypothesis, we repeated our experiments in the presence of the Ca^{2+} -regulated PKC (PKC α , β , and μ [PKD]) inhibitor Gö6976 (10 μ M, 15 min) [38]. In cells treated with Gö6976, ET-1 or PE did not increase CaMKII phosphorylation (Fig. 5C). These results support the hypothesis that CaMKII is activated by the Ca^{2+} -dependent PKC/PKD via an IP_3R -mediated Ca^{2+} increase after stimulation of either α_1 -adrenoceptors or ET_A receptors.

Role of Ca^{2+} -independent PKC and IP_3R_2 in ERK1/2 activation by ET-1/PE

Because CREB phosphorylation, stimulated by ET-1 or PE, was prevented either by an ERK inhibitor or by PKC inhibitors (Figs. 1 and 4), we next examined whether ET-1 and PE activate ERK1/2 and the role of PKC in this activation. To estimate ERK1/2 activation, its phosphorylation was measured by western blotting. Application of ET-1 or PE significantly increased levels of phosphorylated ERK1/2 (Fig. 6A). Inhibition of PKC using GF109203X (5 μ M, 10 min) suppressed ET-1-induced ERK1/2 phosphorylation by 60%-70%, while abolishing the effects of PE (Fig. 6A). Because CaMKII activation by ET-1 and PE was sensitive to blockade of Ca^{2+} -dependent PKC/PKD (Fig. 5C), we further examined whether ERK1/2 phosphorylation under these hormonal stimulations is also affected by Gö6976. Pre-exposure of cells to Gö6976 (10 μ M, 15 min) did not alter the enhancement of ERK1/2 phosphorylation by ET-1 or PE (Fig. 6B), suggesting no role of Ca^{2+} -dependent PKC/PKD in ERK1/2 activation by ET-1 or PE. Consistently, inhibition of CaMKII using KN93 (1 μ M, 20 min) did not suppress ET-1- or PE-induced ERK1/2 phosphorylation (Fig. 6C). As we hypothesized, ET-1- or PE-mediated ERK1/2 phosphorylation was suppressed by PLC inhibition (U73122; Data not shown for ET-1) or by the ERK1/2 pathway inhibitor PD98059 (10 μ M, 15 min) (Fig. 6C).

Fig. 8. Proposed signal transduction pathway for CREB activation under the stimulation of ET_A receptor and α_1 -adrenoceptor. Both receptors activate IP_3R2 -mediated Ca^{2+} releases, which, in turn, activate Ca^{2+} -dependent PKC/PKD-CaMKII signaling, thereby activating CREB. This pathway may be responsible for BAPTA-sensitive (≥ 80) component of CREB phosphorylation. A Ca^{2+} -independent PKC-ERK1/2 signaling pathway also mediates a smaller portion of CREB phosphorylation under the stimulation of these receptors. IP_3R2 may play a critical role in the regulation of ERK1/2 activity and the interaction between IP_3R2 and ERK1/2 appears to occur in a Ca^{2+} -independent manner. CaMKII may, in turn, phosphorylate IP_3R2 , thereby negatively regulating its opening [4].

These results suggest that ET-1 and PE activate ERK1/2 via PLC and Ca^{2+} -independent PKC signaling, but not via CaMKII and PKD.

In the next series of experiments, we examined whether IP_3 Rs and Ca^{2+} increase regulate ERK1/2 activation under these hormonal stimulations. When IP_3 Rs were suppressed using 2-APB or XeC, ET-1- or PE-induced ERK1/2 phosphorylation was significantly decreased (Fig. 7A). To know whether the enhanced ERK1/2 phosphorylation in the presence of ET-1 or PE was caused by IP_3R2 , the level of phosphorylated ERK1/2 was compared in agonist-stimulated ventricular myocytes from WT and IP_3R2 KO mice. In the WT cells, phosphorylated ERK1/2 was higher in the presence of ET-1 or PE (Fig. 7B). In contrast, ET-1 and PE caused a slight decrease in the level of phosphorylated ERK1/2 in the IP_3R2 KO cells (Fig. 7B). When the cytosolic Ca^{2+} increase was suppressed by preincubating cells with the Ca^{2+} buffer BAPTA-AM (10 μ M), ET-1 and PE continued to increase ERK1/2 phosphorylation to levels similar to those without BAPTA (Fig. 7C). This result suggests that ERK1/2 signaling may be regulated either directly by IP_3R2 or through local Ca^{2+} signaling in a compartment inaccessible to BAPTA.

Discussion

In this study, we demonstrated that ET-1 and PE stimulate CREB phosphorylation in rat ventricular myocytes by activation of ET_A receptors and α_1 -adrenoceptors, respectively, through two general effector molecules, CaMKII and ERK1/2, and that CaMKII and ERK1/2 are regulated by IP_3 Rs and PKC through PLC (Fig. 8). The CREB activation under these agonists was almost completely eliminated by intracellular Ca^{2+} buffering using BAPTA or by SR Ca^{2+} depletion (Fig. 2). Interestingly, of the two major effectors activated by ET-1 and PE, the activity of CaMKII, but not ERK1/2, was sensitive to the Ca^{2+} buffers (Figs. 5B and 7C), suggesting that Ca^{2+} -dependent activation of CaMKII may play a major role in CREB phosphorylation stimulated by ET-1 or PE. Contrasting effects of Gö6976, the inhibitor of Ca^{2+} -dependent PKC/PKD, on the activation of CaMKII and ERK1/2 by these agonists further indicate that only CaMKII activity is regulated by Ca^{2+} -dependent PKC/PKD (Figs. 5C, 6B and 8). The sensitivity of ERK1/2 phosphorylation to IP_3R2 KO and its resistance to internal BAPTA suggest that ERK1/2 signaling during the stimulation of ET_A receptors and α_1 -adrenoceptors may occur in a separate compartment that is more closely localized with IP_3R2 and different PKC isozymes. These data also suggest that ERK1/2 signaling is responsible for the portion (10%-20%) of CREB phosphorylation resistant to Ca^{2+} buffering during agonist-induced stimulation (Fig. 8).

Our data provide evidence for the possible role of IP_3 Rs and CaMKII in the regulation of gene expression during the stimulation of ET_A receptor and α_1 -adrenoceptor via activation of CREB. So far, there has been no report showing the role of IP_3R and CaMKII in CREB

Fig. 9. Non-specific effects of PD98059 on phosphorylation of CREB and CaMKII. (A) Application of PD98059 (10 μ M, 10 min), but not U73122 (5 μ M, 20 min), significantly reduced the level of CREB phosphorylation ($n = 4$). * $P < 0.05$ vs. control (untreated). (B) Ventricular myocytes were un-stimulated or exposed to PD98059 (10 μ M, 15 min), or endothelin-1 (ET-1) (100 nM) or PE (100 μ M) without and with PD98059 ($n = 4$). * $P < 0.05$ vs. control (untreated). # $P < 0.05$ vs. PD98059 alone. Upper panels show comparison of quantitative levels of phosphorylated CREB (A) or CaMKII (B) relative to the total CREB (A) or CaMKII (B) (normalized to control) shown in the western blot analysis below.

phosphorylation in the heart. It is assumed that Ca^{2+} release through the IP_3R_2 occurs around the nucleus, t-tubules and intercalated disks in ventricular myocytes, where IP_3R_2 molecules are localized [4, 6, 7]. Nuclear Ca^{2+} , increased via IP_3R_2 , is likely to directly regulate nearby CaMKII, known to co-localize with IP_3R_2 in the nuclear envelope in rat ventricular myocytes [4, 5]. Pre-dominant peri-nuclear Ca^{2+} signal or loss of inner-nuclear envelope Ca^{2+} during the activation of IP_3Rs using the direct IP_3R activator, IP_3BM or adenophostin, has been shown in ventricular myocytes [5, 39, 40]. Thus, it may be assumed that PE and ET-1 increase Ca^{2+} in the perinucleus more significantly than in other regions in rat ventricular myocytes. In addition, IP_3R -mediated Ca^{2+} releases can also facilitate Ca^{2+} spark occurrence via Ca^{2+} -induced Ca^{2+} release [29, 41], and via other downstream signaling molecules such as CaMKII [4, 42]. Such cytosolic Ca^{2+} increases are supposed to be more sensitive to BAPTA than nuclear Ca^{2+} changes and, thus, may be pertinent to the Ca^{2+} -dependent (: BAPTA-sensitive) PKC/PKD-CaMKII-CREB signaling axis. Activation of CaMKII can phosphorylate the IP_3R_2 and decrease its open probability, which may feed back to shut off further Ca^{2+} release ([4]; Fig. 8). ERK1/2 phosphorylation was resistant to BAPTA, suggesting that ERK1/2 and IP_3R_2 may interact directly or communicate in a restricted microdomain via local Ca^{2+} . In the mouse cerebellum, type 1 IP_3Rs directly interact with ERK1/2 and ERK1/2 reduces IP_3R_1 activity by phosphorylation [43]. However, how ERK1/2 interacts with IP_3R_2 in cardiac myocytes requires further investigation.

In the present study, we demonstrated, for the first time, that CaMKII is critical for CREB activation under stimulation of α_1 -adrenoceptors or ET_A receptors in cardiac myocytes. Because CREB activation is implicated in expression of hypertrophic marker genes [5, 35], our data provide a possible mechanism for CaMKII-mediated cardiac hypertrophy. It was previously reported that CaMKII is activated by both ET-1 [5] and PE [34]. It should be noted, however, that there is some controversy in the activation mechanisms for CaMKII in the presence of ET-1 and PE. ET-1-induced CaMKII activation was removed by IP_3R blockade [5], whereas that by PE was suppressed by PKC inhibition [34]. We found that activation of CaMKII by both agonists was suppressed by inhibition of either PKC or IP_3Rs (Fig. 5A and C). Because treatment with either Ca^{2+} buffers or an inhibitor of Ca^{2+} -dependent PKC/PKD eliminated both ET-1- and PE-induced CaMKII phosphorylation (Fig. 5B and C), it is plausible to think that Ca^{2+} increase due to the IP_3R -triggered Ca^{2+} releases may facilitate the activity of PKC/PKD, thereby stimulating CaMKII activation in the presence of ET-1 or PE. Recent evidence suggests activation of PKD by ET-1 and PE and role of PKC-PKD signaling in cardiac hypertrophy [44, 45]. Possible role of PKD and/or PKC isozyme(s) in the signaling pathway

involving CaMKII-CREB under stimulation of α_1 -adrenoceptors and ET_A receptors needs to be investigated.

We found a complete blockade of ET-1- or PE-induced CREB phosphorylation by PD98059, the ERK1/2 pathway inhibitor (Fig. 1B and C), which appears to be consistent with the previous reports [21, 22]. However, it should be noted that PD98059 itself reduced levels of the phosphorylated forms of both CREB and CaMKII (Fig. 9). Blockade of CaMKII phosphorylation by PD98059 may suggest that the ERK1/2 pathway may be an upstream signaling for CaMKII activation. However, this is unlikely during stimulation of ET_A receptors and α_1 -adrenoceptors, because ET-1- or PE-induced CaMKII phosphorylation, but not that of ERK1/2, was blocked by BAPTA or Gö6976 (Figs. 5-7). In addition, ET-1- or PE-induced CaMKII phosphorylation in the presence of PD98059 was still higher than CaMKII phosphorylation under PD98059 only (Fig. 9). Therefore, the pharmacological effects by PD98059 on ET-1- or PE-induced CREB phosphorylation may have been overestimated because of its nonspecific suppression of CaMKII that also mediates CREB phosphorylation. Important new finding in our study is that ERK1/2 activation under the stimulation of ET_A receptors and α_1 -adrenoceptors is caused by the IP₃R2 and Ca²⁺-independent PKC (Figs. 6B and 7B). This ERK1/2 signaling upon activation of ET_A receptors or α_1 -adrenoceptors appears to involve different upstream molecules than CaMKII signaling, such as Ca²⁺ and PKC isozymes. In addition, ERK1/2 appears to contribute significantly less than CaMKII to CREB activation under prolonged (15 min) stimulation of these hormonal receptors. Considering the previous report showing that ET-1- or PE-induced ERK1/2 phosphorylation reaches a maximum at 1-2 min after application of these agonists, with a gradual decay after the maximum effects [24], the role of ERK1/2 may be more significant immediately after exposure to ET-1 or PE than it is later.

Although ET-1 and PE have been thought to induce ventricular hypertrophy [1, 5, 9-12, 46], signal transduction pathways for their activation of transcriptional regulators were poorly understood. Our study provide new evidence that the transcription factor CREB is activated mainly via IP₃R2-mediated Ca²⁺ release and subsequent PKC/PKD-CaMKII signaling in ventricular myocytes (Fig. 8). In addition, our data suggest that activation of CREB by ET-1 and PE is also partly regulated by Ca²⁺-independent PKC- and IP₃R2-dependent ERK1/2 signaling in a separate compartment (Fig. 8). We propose that IP₃Rs-PKC-CaMKII-CREB signal transduction may be an important mechanism by which ET-1 and α_1 -adrenergic agonist regulate gene expression of ventricular myocytes.

Acknowledgements

We thank Prof. Ju Chen at University of California, San Diego for IP₃R2 KO mice. The work was supported by the National Research Foundation of Korea (NRF) grants funded by the Korea Government (MEST) (2015R1A2A2A01002625, 2009-0093815).

Disclosure Statement

The authors declare that there are no conflicts of interest.

References

- 1 Sugden P, Clerk A: Cellular mechanisms of cardiac hypertrophy. *J Mol Med* 1980;76:725-742.
- 2 Takanashi M, Endoh M: Concentration- and time-dependence of phosphoinositide hydrolysis induced by endothelin-1 in relation to the positive inotropic effect in the rabbit ventricular myocardium. *J Pharmacol Exp Ther* 1992;262:1189-1194.
- 3 Terzic A, Pucéat M, Vassort G, Vogel SM: Cardiac alpha1-adrenoceptors: an overview. *Pharmacol Rev* 1993;45:147-175.

- 4 Bare DJ, Kettlun CS, Liang M, Bers DM, Mignery GA: Cardiac type 2 inositol 1,4,5-trisphosphate receptor: interaction and modulation by calcium/calmodulin-dependent protein kinase II. *J Biol Chem* 2005;280:15912-15920.
- 5 Wu X, Zhang T, Bossuyt J, Li X, McKinsey TA, Dedman JR, Olson EN, Chen J, Brown JH, Bers DM: Local InsP₃-dependent perinuclear Ca²⁺ signaling in cardiac myocyte excitation-transcription coupling. *J Clin Invest* 2006;116:675-682.
- 6 Kijima Y, Saito A, Jetton TL, Magnuson MA, Fleischer S: Different intracellular localization of inositol 1,4,5-trisphosphate and ryanodine receptors in cardiomyocytes. *J Biol Chem* 1993;268:3499-3506.
- 7 Mohler PJ, Davis JQ, Bennett V: Ankyrin-B coordinates the Na/K ATPase, Na/Ca exchanger, and InsP₃ receptor in a cardiac T-tubule/SR microdomain. *PLoS Biol* 2005;3:2158-2167.
- 8 Rinne A, Blatter LA: Activation of NFATc1 is directly mediated by IP₃ in adult cardiac myocytes. *Am J Physiol Heart Circ Physiol* 2010;299:H1701-H1707.
- 9 Ramirez MT, Zhao XL, Schulman H, Brown JH: The nuclear deltaB isoform of Ca²⁺/calmodulin-dependent protein kinase II regulates atrial natriuretic factor gene expression in ventricular myocytes. *J Biol Chem* 1997;272:31203-31208.
- 10 Zhu W, Zou Y, Shiojima I, Kudoh S, Aikawa R, Hayashi D, Mizukami M, Toko H, Shibasaki F, Yazaki Y, Nagai R, Komuro I: Ca²⁺/calmodulin-dependent kinase II and calcineurin play critical roles in endothelin-1-induced cardiomyocyte hypertrophy. *J Biol Chem* 2000;275:15239-15245.
- 11 Colomer JM, Means AR: Chronic elevation of calmodulin in the ventricles of transgenic mice increases the autonomous activity of calmodulin-dependent protein kinase II, which regulates atrial natriuretic factor gene expression. *Mol Endocrinol* 2000;14:1125-1136.
- 12 Zhang T, Maier LS, Dalton ND, Miyamoto S, Ross J Jr, Bers DM, Brown JH: The δ_c isoform of CaMKII is activated in cardiac hypertrophy and induces dilated cardiomyopathy and heart failure. *Circ Res* 2003;92:912-919.
- 13 Harzheim D, Movassagh M, Foo RS, Ritter O, Tashfeen A, Conway SJ, Bootman MD, Roderick HL: Increased InsP₃Rs in the junctional sarcoplasmic reticulum augment Ca²⁺ transients and arrhythmias associated with cardiac hypertrophy. *Proc Natl Acad Sci USA* 2009;106:11406-11411.
- 14 Sankar N, deTombe PP, Mignery GA: Calcineurin-NFATc regulates type 2 inositol 1,4,5-trisphosphate receptor (InsP₃R2) expression during cardiac remodeling. *J Biol Chem* 2014;289:6188-6198.
- 15 Gonzalez GA, Montminy MR: Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. *Cell* 1989;59:675-680.
- 16 Sheng M, Thompson MA, Greenberg ME: CREB: a Ca²⁺-regulated transcription factor phosphorylated by calmodulin-dependent kinases. *Science* 1991;252:1427-1430.
- 17 Tan Y, Rouse J, Zhang A, Cariati S, Cohen P, Comb MJ: FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J* 1996;15:4629-4642.
- 18 Deak M, Clifton AD, Lucocq JM, Alessi DR: Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO J* 1998;17:4426-4441.
- 19 Wiggin GR, Soloaga A, Foster JM, Murray-Tait V, Cohen P, Arthur JS: MSK1 and MSK2 are required for the mitogen- and stress-induced phosphorylation of CREB and ATF-1 in fibroblasts. *Mol Cell Biol* 2002;22:2871-2881.
- 20 Kobrinsky E, Duong SQ, Sheydina A, Soldatov NM: Microdomain organization and frequency-dependence of CREB-dependent transcriptional signaling in heart cells. *FASEB J* 2011;25:1544-1555.
- 21 Li B, Kaetzel MA, Dedman JR: Signaling pathways regulating murine cardiac CREB phosphorylation. *Biochem Biophys Res Commun* 2006;350:179-184.
- 22 Harrison JG, Sugden PH, Clerk A: Endothelin-1 promotes phosphorylation of CREB transcription factor in primary cultures of neonatal cardiac myocytes: implications for the regulation of c-jun expression. *Biochim Biophys Acta* 2004;1644:17-25.
- 23 Markou T, Hadzopoulou-Cladaras M, Lazou A: Phenylephrine induces activation of CREB in adult rat cardiac myocytes through MSK1 and PKA signaling pathways. *J Mol Cell Cardiol* 2004;37:1001-1011.
- 24 Markou T, Lazou A: Phosphorylation and activation of mitogen- and stress-activated protein kinase-1 in adult rat cardiac myocytes by G-protein-coupled receptor agonists requires both extracellular signal-regulated kinase and p38 mitogen-activated protein kinase. *Biochem J* 2002;365:757-763.
- 25 Cárdenas C, Liberona JL, Molgó J, Colasante C, Mignery GA, Jaimovich E: Nuclear inositol 1,4,5-trisphosphate receptors regulate local Ca²⁺ transients and modulate cAMP response element binding

- protein phosphorylation. *J Cell Sci* 2005;118:3131-3140.
- 26 Müller M, Cárdenas C, Mei L, Cheung KH, Foskett JK: Constitutive cAMP response element binding protein (CREB) activation by Alzheimer's disease presenilin-driven inositol trisphosphate receptor (InsP3R) Ca^{2+} signaling. *Proc Natl Acad Sci U S A* 2011;108:13293-13298.
- 27 Francis H, Glaser S, DeMorrow S, Gaudio E, Ueno Y, Venter J, Dostal D, Onori P, Franchitto A, Marzioni M, Vaculin S, Vaculin B, Katki K, Stutes M, Savage J, Alpini G: Small mouse cholangiocytes proliferate in response to H1 histamine receptor stimulation by activation of the IP3/CaMK I/CREB pathway. *Am J Physiol Cell Physiol* 2008;295:C499-C513.
- 28 Kim JC, Woo SH: Shear stress induces a longitudinal Ca^{2+} wave via autocrine activation of P2Y_1 purinergic signaling in rat atrial myocytes. *J Physiol* 2015;593.23:5091-5109.
- 29 Li X, Zima AV, Sheikh F, Blatter LA, Chen J: Endothelin-1-induced arrhythmogenic Ca^{2+} signaling is abolished in atrial myocytes of inositol-1,4,5-trisphosphate(IP3)-receptor type 2-deficient mice. *Circ Res* 2005;96:1274-1281.
- 30 Caivano M, Cohen P: Role of mitogen-activated protein kinase cascades in mediating lipopolysaccharide-stimulated induction of cyclooxygenase-2 and IL-1 β in RAW264 macrophages. *J Immunol* 2000;164:3018-3025.
- 31 Woo SH, Lee CO: Role of PKC in the effects of alpha1-adrenergic stimulation on Ca^{2+} transients, contraction, and L-type Ca^{2+} current in guinea-pig ventricular myocytes. *Pflügers Arch* 1999;437:335-344.
- 32 Woo SH, Lee CO: Effects of endothelin-1 on Ca^{2+} signaling in guinea-pig ventricular myocytes: role of protein kinase C. *J Mol Cell Cardiol* 1999;31:631-643.
- 33 Kockskämper J, Zima AV, Roderick HL, Pieske B, Blatter LA, Bootman MD: Emerging roles of inositol 1,4,5-trisphosphate signaling in cardiac myocytes. *J Mol Cell Cardiol* 2008;45:128-147.
- 34 O-Uchi J, Komukai K, Kusakari Y, Obata T, Hongo K, Sasaki H, Kurihara S: α 1-adrenergic stimulation potentiates L-type current through Ca^{2+} /calmodulin-dependent PK II (CaMKII) activation in rat ventricular myocytes. *Proc Natl Acad Sci U S A* 2005;102:9400-9405.
- 35 Anderson ME, Brown JH, Bers DM: CaMKII in myocardial hypertrophy and heart failure. *J Mol Cell Cardiol* 2011;51:468-473.
- 36 Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM: 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca^{2+} entry but an inconsistent inhibitor of InsP_3 -induced Ca^{2+} release. *FASEB J* 2002;16:1145-1150.
- 37 Hu HZ, Gu Q, Wang C, Colton CK, Tang J, Kinoshita-Kawada M, Lee LY, Wood JD, Zhu MX: 2-Aminoethoxydiphenyl borate is a common activator of TRPV1, TRPV2, and TRPV3. *J Biol Chem* 2004;34:35741-35748.
- 38 Zugaza JL, Sinnott-Smith J, Van Lint J, Rozengurt E: Protein kinase D (PKD) activation in intact cells through a protein kinase C-dependent signal transduction pathway. *EMBO J* 1996;15:6220-6230.
- 39 Luo DL, Gao J, Lan XM, Wang G, Wei S, Xiao RP, Han QD: Role of inositol 1,4,5-trisphosphate receptors in α 1-adrenergic receptor-induced cardiomyocyte hypertrophy. *Acta Pharmacol Sin* 2006;27:895-900.
- 40 Zima AV, Blatter LA: Inositol-1,4,5-trisphosphate-dependent Ca^{2+} signaling in cat atrial excitation-contraction coupling and arrhythmias. *J Physiol* 2004;555:607-615.
- 41 Domeier TL, Zima AV, Maxwell JT, Huke S, Mignery GA, Blatter LA: IP_3 receptor-dependent Ca^{2+} release modulates excitation-contraction coupling in rabbit ventricular myocytes. *Am J Physiol Heart Circ Physiol* 2008;294:H596-H604.
- 42 Wehrens XH, Lehnart SE, Reiken SR, Marks AR: Ca^{2+} /calmodulin-dependent protein kinase II phosphorylation regulates the cardiac ryanodine receptor. *Circ Res* 2004;94:e61-e70.
- 43 Bai GR, Yang LH, Huang XY, Sun FZ: Inositol 1,4,5-trisphosphate receptor type 1 phosphorylation and regulation by extracellular signal-regulated kinase. *Biochem Biophys Res Commun* 2006;348:1319-1327.
- 44 Haworth RS, Goss MW, Rozengurt E, Avkiran M: Expression and activity of protein kinase D/protein kinase C μ in myocardium: evidence for α 1-adrenergic receptor- and protein kinase C-mediated regulation. *J Mol Cell Cardiol* 2000;32:1013-1023.
- 45 Bossuyt J, Chang CW, Helmstadter K, Kunkel MT, Newton AC, Campbell KS, Martin JL, Bossuyt S, Robia SL, Bers DM: Spatiotemporally distinct protein kinase D activation in adult cardiomyocytes in response to phenylephrine and endothelin. *J Biol Chem* 2011;286:33390-33400.
- 46 Wang YS, Zhou J, Hong K, Cheng XS, Li YG: MicroRNA-223 displays a protective role against cardiomyocyte hypertrophy by targeting cardiac troponin I-interacting kinase. *Cell Physiol Biochem* 2015;35:1546-1556.