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ORIGINAL ARTICLE

The β1-adrenergic receptor mediates extracellular signal-regulated kinase activation via Gαs

Junfang Zheng · Hui Shen · Ying Xiong · Xiaomei Yang · Junqi He

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Abstract β-Adrenergic receptors can activate extracellular signal-regulated kinases (ERKs) via different mechanisms. In this study, we investigated the molecular mechanism of β1-adrenergic receptor (β1AR)-mediated ERK activation in African green monkey kidney COS-7 cells. Treatment of cells with isoproterenol (ISO), a β1AR selective agonist, induced phosphorylation of ERK1/2 in a dose-dependent manner. ISO-stimulated ERK phosphorylation was not influenced by the Gβγ inhibitor, βAR kinase carboxyl terminal (βARKct) or by the Gi inhibitor, pertussis toxin (PTX), but it was clearly abolished via inhibition of protein kinase A (PKA) with H89, or of mitogen-activated protein kinase kinase (MEK1) with PD98059, revealing that the Gαs subunit is involved in ERK regulation through the PKA/MEK1 pathway. We also tested the effect of the adenylate cyclase activator forskolin on ERK activation, and the result was identical to that of ISO stimulation. Moreover, pretreatment with the epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor AG1478 or with the Src tyrosine kinase inhibitor PP2 did not affect ERK activation. These observations suggest a mechanism of β1AR-mediated ERK activity that involves the Gαs subunit, but not EGFR or Src tyrosine kinase.

Keywords Src-related kinase · G protein · Protein kinase A · Epidermal growth factor receptor · Mitogen-activated protein kinase

Abbreviations

COS-7	African green monkey kidney cells
DMEM	Dulbecco's modified Eagle's medium
ISO	Isoproterenol
β1AR	β1-Adrenergic receptor
GPCR	G protein-coupled receptor
G protein	GTP binding regulatory protein
MAP	Mitogen-activated protein
ERK	The mitogen-activated protein kinases extracellular signal-regulated kinase
βARK	β-Adrenergic receptor kinase
CT	Carboxyl terminal
FSK	Forskolin
AC	Adenylate cyclase
PTX	Pertussis toxin
cAMP	Cyclic AMP
PKA	Protein kinase A
PKC	Protein kinase C
H89	*N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide
PP2	4-Amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d]pyrimidine
MEK1	Mitogen-activated ERK kinase 1
PD98059	2-(2-Amino-3-methoxyphenol)-oxanaphthalen-4-one
EGFR	Epidermal growth factor receptor
AG1478	2-(2-Amino-3-methoxyphenyl)-oxanaphthalen-4-one, and 4-(3-chloroanilino)-6,7-dimethoxyquinazoline
SDS	Sodium dodecyl sulfate

J. Zheng and H. Shen contributed equally to this work.

J. Zheng · H. Shen · Y. Xiong · X. Yang · J. He (✉)
Department of Biochemistry and Molecular Biology,
Capital Medical University, No. 10 Xitoutiao, You An Men Wai,
100069 Beijing, People's Republic of China
e-mail: jq_he@ccmu.edu.cn

PAGE	Polyacrylamide gel electrophoresis
PSD-95	Postsynaptic density-95
MAGI-2	Membrane-associated guanylate kinase inverted-2
CNrasGEF	cAMP-dependent guanine nucleotide exchange factor
GIPC	GAIP-interacting protein carboxyl terminus
CAL	Cystic fibrosis transmembrane conductance regulator-associated ligand
MAGUK	Membrane-associated guanylate kinase

Introduction

At present, three β-adrenergic receptor (AR) subtypes are recognized: β1, β2, and β3. All three receptors modulate peripheral vascular tone. These receptors are prototypic Gs coupled receptors, whose signaling properties are largely mediated by generation of intracellular cyclic AMP (cAMP) and the subsequent activation of protein kinase A (PKA). In addition to stimulating production of cAMP, the β2AR and β3AR can also influence the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) pathway by interacting with tyrosine kinase receptors, and mediate ERK activation through several mechanisms (Crespo et al. 1995; Lazou et al. 1994; Maudsley et al. 2000; Soeder et al. 1999; Wang and Bachrach 2002). However, the relationships between the β1AR signaling system, tyrosine kinase receptors and ERK activation remain largely undefined.

The physiological activity of β1AR is largely mediated by the classical Gs/adenylate cyclase (AC)/protein kinase A (PKA) pathway (Jans and Pavo 1995; Steinberg 1999; Xiao 2001; Xiao et al. 1999), although the phosphorylated receptor may also engage the cyclase-negative Gαi subunits (Martin et al. 2004). Sustained β1AR activation may also be implicated in a number of adverse effects including cardiomyocyte hypertrophy and eventually apoptosis (Ahmet et al. 2005). The activation of β1AR also contributes to ischemia–reperfusion damage, and has a role in ischemic preconditioning (Spear et al. 2007). This receptor is therefore the target for treatment of several common diseases, including congestive heart failure, asthma and benign prostatic hyperplasia. Blockade of β1AR improves survival in left ventricular systolic dysfunction (Ahmet et al. 2005; de Groote et al. 2005). However, the precise mechanisms of β1AR activity in these processes need to be clarified.

Activation of the mitogen-activated protein kinase (MAPK) kinase (MEK)/ERK1/2 pathway is involved in the development of cardiac hypertrophy (Massey et al. 1998; Molkentin and Dorn 2001; Yanagawa and Nagaya 2007) and elevated ERK activity has been reported in failing human hearts exhibiting dilated cardiomyopathy (Takeishi et al. 2002). Several studies have shown that G protein-coupled receptors (GPCRs) can directly or indirectly influence these processes by modulating the activity of the ERK1/2 pathway. It is also clear that βARs are key regulators of MAPK signaling, especially in the context of heart disease (Xiao et al. 2006). However, the mechanism of β1AR-activated MAPK signaling is still not well understood. Therefore, we first explored the relationship between stimulation of β1AR and ERK activation, and further investigated ERK activation and signaling molecules involved in GPCR cascades and MEK1 activity.

G protein-coupling receptors (GPCRs) affecting tyrosine kinase signaling pathways are mostly activated by members of the superfamily of tyrosine kinase receptors, and modulate long-term cellular responses associated with adaptations to growth or stress factors. We therefore also explored whether the epidermal growth factor receptor (EGFR) and Src family kinase are involved in ERK activation.

We found that stimulation of the human β1AR expressed in COS-7 (African green monkey kidney) cells specifically activates ERK1/2. This was mediated by coupling of the receptor to G protein Gs α subunit, and thus is dependent upon activation of adenylate cyclase. We failed to observe a role for Src family tyrosine kinases or EGFR in the ERK/MAPK activation mediated by β1AR.

Materials and methods

Cell culture, transfection and cell treatments

COS-7 cells (American Type Culture Collection, Manassas, VA, USA) were grown in complete medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and 1% penicillin/streptomycin) in a 37°C/5% CO_2 incubator. The carboxyl terminal portion of βARK1 (βARKct) were kindly provided by Dr. Robert J. Lefkowitz (Duke University). For transfections, 1 μg of cDNA coding for human β1AR in the pcDNA3 expression vector, or 1 μg each of β1AR and βARKct (which is an inhibitor of G$\beta\gamma$ subunit) was mixed with Lipofectamine 2,000 (10 μl) (Invitrogen, Carlsbad, USA) and added to 5 ml of incomplete medium in 10-cm tissue culture plates containing cells at 50–70% confluence. Following a 4 h incubation, fetal bovine serum was added to the medium to 10%.

In order to dissect the signal transduction pathways underlying the β1AR-mediated activation of ERK, COS-7 cells were serum starved overnight, then treated with (−)-isoproterenol (ISO, 5 min at 37°C; Sigma Chemical Corp., St Louis, MO, USA), and/or forskolin (FSK, Adenylate

cyclase activator, 5 min at 37°C; Sigma) (Lumbreras et al. 2006), after the cells were pre-incubated with *Bordetella pertussis* toxin (PTX, Gi inhibitor, 100 ng/ml, 16 h; Sigma), *N*-[2-((*p*-bromocinnamyl)amino)ethyl]-5-isoquinolinesulfonamide (H89, a PKA inhibitor, 20 μM, 45 min; Calbiochem Corp., La Jolla, CA, USA), 2-(2-amino-3-methoxyphenol)-oxanaphthalen-4-one (PD 98059, MEK1 inhibitor, 50 μM, 30 min; Calbiochem) (Yuan et al. 2007), 2-(2-amino-3-methoxyphenyl)-oxanaphthalen-4-one, and 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (tyrphostin AG1478, EGFR inhibitor, 100 nM, 30 min; Calbiochem) or 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl) pyrazolo [3,4-d]pyrimidine (PP2, a Src tyrosine kinase inhibitor, 10 μM, 15 min; Calbiochem).

Western blotting and antibodies

Western blotting was performed as described previously (Cheon et al. 2003). Briefly, sample aliquots corresponding to 25 μg of protein were resolved using 4–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Invitrogen) for 1 h at 150 V and then transferred to nitrocellulose. The blots were blocked in the blot buffer (2% nonfat dry milk, 0.1% Tween 20, 50 mM NaCl, 10 mM Hepes, pH 7.4) for at least 30 min and then incubated with primary antibody in the blot buffer for 1 h at room temperature. The blots were then washed three times with 10 ml of the blot buffer each and incubated for 30 min at room temperature with a horseradish peroxidase-conjugated secondary antibody (GE/Amersham Biosciences, Buckinghamshire, UK) in the blot buffer. Finally, the blots were washed three more times with 10 ml of the blot buffer each and visualized by enzyme-linked chemiluminescence as described above. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from GE/Amersham Biosciences.

Phospho-extracellular signal-regulated kinase assay

This assay was performed essentially as previously described (Flamigni et al. 2007). Briefly, 24 h after transfection the cells were split into 6-well dishes and incubated in serum-free medium overnight prior to experiments. Agonist stimulation was performed at 37°C in serum-free media for 5 min. The medium was removed, and the cells were harvested in 1× SDS-PAGE sample buffer (10 mM Tris–HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 2 mM Na_3VO_4, 10 mM NaF and protease inhibitors). The samples were sonicated briefly and analyzed by SDS-PAGE. The levels of p42/44 ERK phosphorylation were visualized by Western blotting using an anti-phospho-ERK1/2(Thr 202/Tyr 204) antibody (Cell Signaling Technology, Beverly, MA, USA), whereas the levels of total ERK in the same lysates were assessed using an anti-ERK antibody (Cell Signaling Technology). Immunoreactive bands were visualized by chemiluminescence and quantified using the US National Institutes of Health Image 1.62 program. For each sample, the level of phospho-ERK immunoreactivity was normalized to the total ERK immunoreactivity.

Statistics

The data are presented as the means ± SE. Statistical significance was determined by one-way ANOVA, followed by Tukey's multiple comparison test.

Results

The mitogen-activated protein kinase pathway is activated by stimulation of β1-adrenergic receptor

In order to examine β1 adrenergic receptor-mediated extracellular signal-regulated kinase activation, COS-7 cells were transiently transfected with either pcDNA3 vector or FLAG-β1AR. Following isoproterenol (ISO, 10 μM) stimulation, cells were collected and sonicated, and then subjected to Western blotting to detect the ERK activity. The data showed that ISO stimulation of β1AR induced a robust ERK phosphorylation/activation compared with non-stimulated cells, which was approximately four fold that cells transfected with vector alone. These results suggested that ERK phosphorylation in receptor-transfected cells is mostly β1AR stimulation-dependent (Fig. 1a). We further explored β1AR-stimulated ERK activation over a range of agonist concentrations. The dose response curve for ISO-stimulated ERK activation is shown in Fig. 1b. The results indicated that ERK phosphorylation could be detected with 1 nM ISO stimulation. Increasing concentrations of ISO activated ERK accordingly. These results demonstrated that ERK1/2 in COS-7 cells could be activated by β1AR.

Extracellular signal-regulated kinase stimulation by β1-adrenergic receptor is not mediated by the G$\beta\gamma$ complex

Signal transduction via G protein-coupled receptors triggers the activation of heterotrimeric ($\alpha\beta\gamma$) G proteins, resulting in the separation of Gα and G$\beta\gamma$ subunits. There is evidence that both Gα and G$\beta\gamma$ subunits transduce upon receptor activation. A single receptor type can therefore activate more than one effector by complementary transduction via α and $\beta\gamma$ subunits of the cognate G protein. β1AR might use either Gα or G$\beta\gamma$

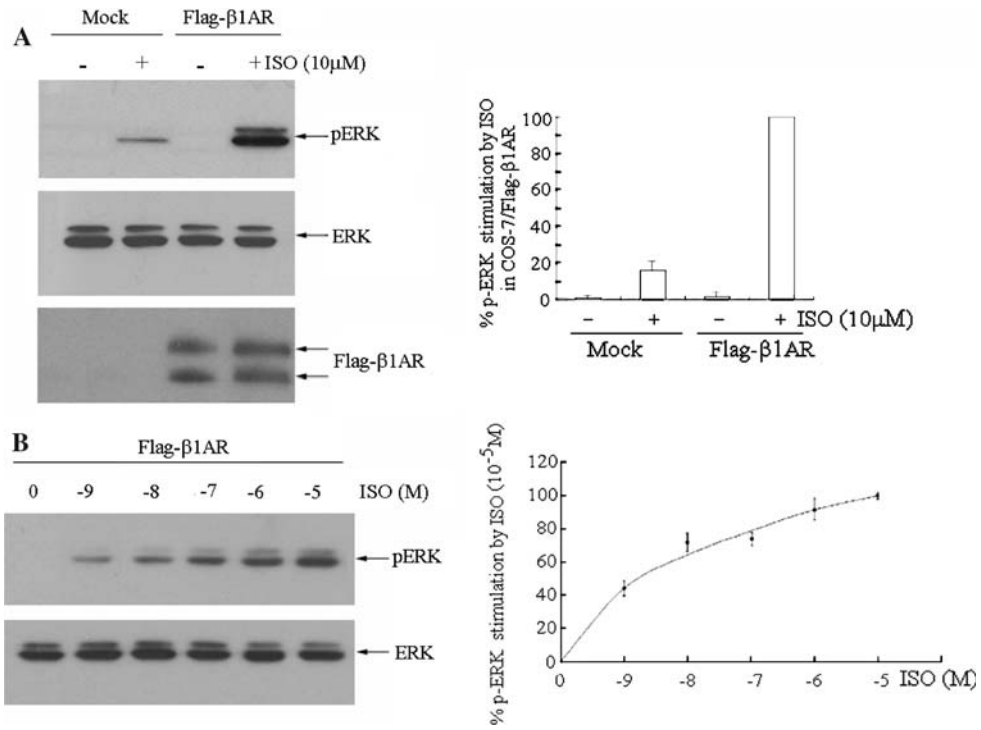

Fig. 1 Extracellular signal-regulated kinase is activated by stimulation of β1-adrenergic receptor. **a** β1AR stimulation enhanced ERK phosphorylation. COS-7 cells were transiently transfected with β1AR. Twenty-four hours after transfection, cells were treated with serum-free medium overnight. Serum-starved COS-7 cells were stimulated for 5 min with 10 μM ISO at 37°C. The cells were solubilized in 1× SDS-PAGE sample buffer. Phosphorylation of ERK in the whole cell lysates was detected by Western blot analysis using an anti-phospho-ERK1/2 antibody. The data presented is representative of a minimum of three independent experiments. **b** β1AR-mediated ERK activation is dose-dependent. COS-7 cells were transiently transfected with β1AR. Serum-starved cells were stimulated with the indicated doses of ISO for 5 min at 37°C. Phosphorylation of ERK was detected and ERK activation was quantified

subunit to activate ERK. To investigate the possible role of the G$\beta\gamma$ complex in the activation of ERK by β1AR, we cotransfected β1AR and the G$\beta\gamma$ inhibitor βARKct into COS-7 cells. There was no difference in the activation of ERK by ISO between COS-7 cells expressing β1AR in the presence or absence of βARKct co-expression (Fig. 2a, $P > 0.05$), indicating that ERK activation was independent of the G$\beta\gamma$ subunits, and was mediated by the Gαs subunit.

Extracellular signal-regulated kinase stimulation by β1-adrenergic receptor is through adenylate cyclase pathway

To determine whether the extracellular signal-regulated kinase activation was a consequence of the classic β1AR-mediated increase in intracellular cAMP concentration, we directly stimulated adenylate cyclase with 1 μM forskolin (FSK), and found that the extent of ERK activation with FSK was very similar to that with ISO (Fig. 2b, $P > 0.05$), indicating that AC activity is involved in the ERK stimulation mediated by β1AR.

Extracellular signal-regulated kinase stimulation by β1AR is independent of the functional status of Gi/o α subunits

The above results indicated, as expected, that extracellular signal-regulated kinase activation was mediated via the Gs α subunit stimulating adenylate cyclase. Then, we further investigated whether ERK activation was mediated by Gi/o α subunits, and found that preincubation with PTX (catalyzing ADP ribosylation of Gi/o α subunits and blocking their function) did not produce a significant change in the stimulation of ERK activity by either ISO (10 μM) or FSK (100 nM) (Fig. 3, $P > 0.05$). This result confirmed that the activation of ERK was independent of Gi/o subunits.

Extracellular signal-regulated kinase stimulation by the β1-adrenergic receptor requires mitogen-activated protein kinase-kinase and protein kinase A activity

To investigate whether the activation of extracellular signal-regulated kinase by the β1-adrenergic receptor was mediated

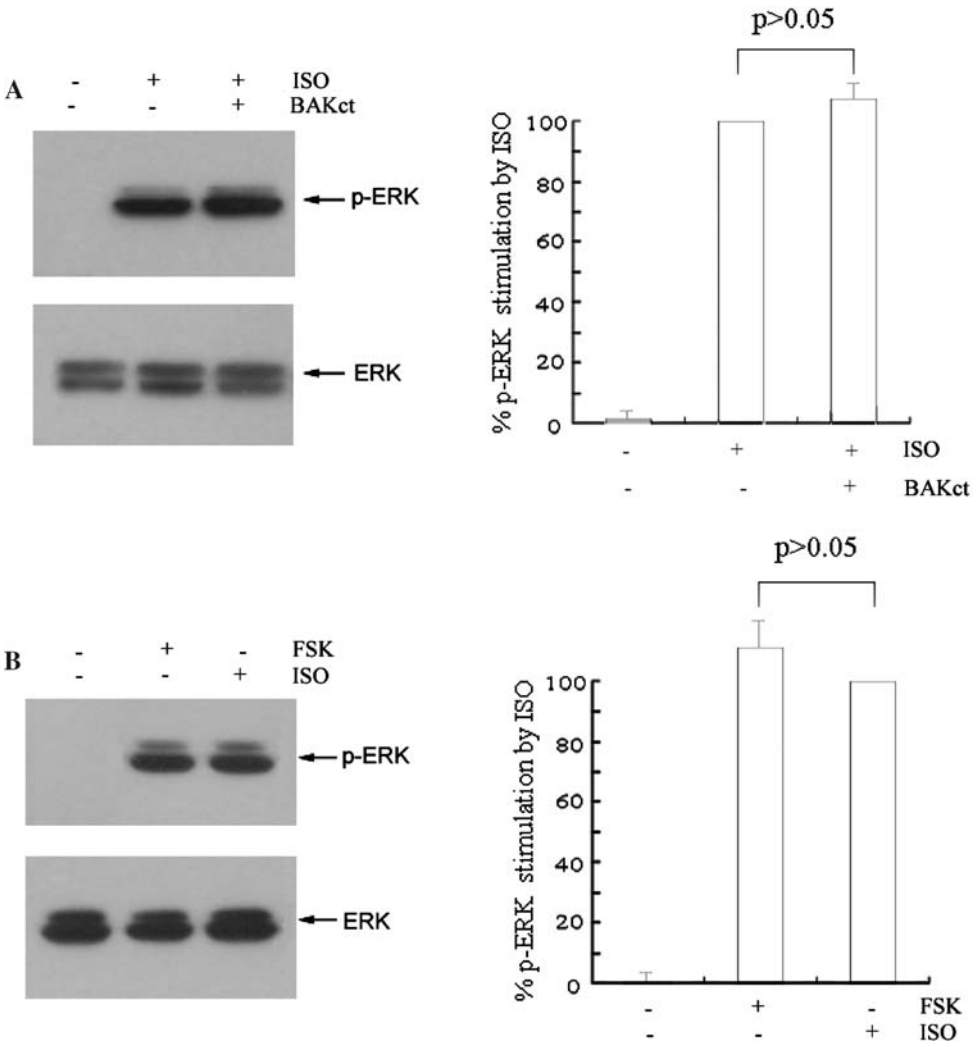

Fig. 2 Extracellular signal-regulated kinase stimulation by β1-adrenergic receptor occurs through Gα but not the Gβγ complex. **a** Inhibition of Gβγ with βARKct does not alter β1AR-mediated p44/42 MAPK activation. COS-7 cells were transiently transfected with the β1AR with or without βARKct co-expression. Cells were serum starved overnight and stimulated with ISO (100 nM) for 5 min. Quantification of ERK activation did not show the difference between COS-7 cells expressing only β1AR and those co-expressing β1AR and βARKct ($P > 0.05$). **b** β1AR-mediated ERK activation by forskolin (FSK). COS-7 cells were transiently transfected with β1AR. Serum-starved COS-7 cells were stimulated with 1 μM FSK for 5 min or 100 nM ISO for 5 min at 37°C. There is no difference in ERK activation with FSK or ISO stimulation ($P > 0.05$)

by activation of MAPK kinase (MEK), the cells were incubated with PD98059, an inhibitor of MEK, for 30 min before activation of the β1AR, and it was shown (Fig. 4, $*P < 0.01$) that pretreatment with PD98059 led to a complete inhibition of the β1AR-mediated activation of ERK by ISO. Similarly, inhibition of PKA by preincubation with the PKA antagonist H89 completely inhibited the activation of ERK (Fig. 4, $*P < 0.01$). These two results revealed that PKA and MEK participated in the activation of ERK via Gs mediation, since PKA is known to be stimulated through Gs activity.

Protein tyrosine kinases of EGFR and Src families are not involved in extracellular signal-regulated kinase stimulation mediated by the β1-adrenergic receptor

It is well established that G protein-coupled receptor signaling systems can network with tyrosine kinase receptors by several mechanisms. We decided to investigate whether β1AR could transmit its signal in cooperation with tyrosine kinase receptors. The involvement of protein tyrosine kinases in the activation of ERK by the β1AR was tested by incubating the cells with AG1478 (an EGFR tyrosine kinase inhibitor) or PP2 (an inhibitor of Src family tyrosine kinases). Neither of these agents significantly changed the activation of ERK, indicating that EGFR and Src family tyrosine kinase are not involved in ERK stimulation by β1AR (Fig. 4, $P > 0.05$).

Discussion

GPCRs activate the extracellular signal-regulated kinases ERK/MAPK pathways via diverse signaling pathways depending on the receptors, the cell types and the types of agonists (Daaka et al. 1997; Galandrin and Bouvier 2006; Galandrin et al. 2008; Gesty-Palmer et al. 2006; Soeder et al. 1999). The β1AR, perhaps by virtue of its ability to couple with multiple signaling pathways, exhibits remarkable cell and ligand specificity regarding the mechanism of ERK stimulation.

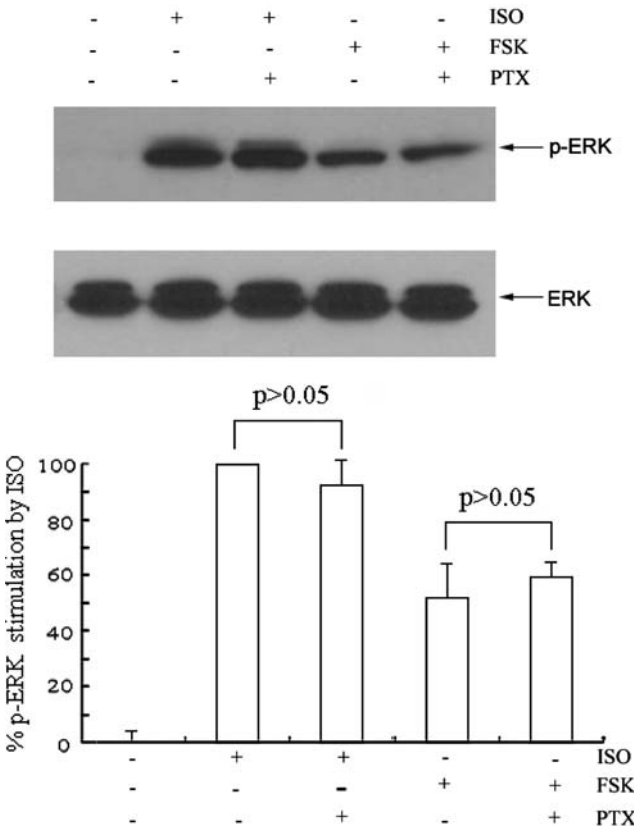

Fig. 3 Inhibition of Gi signaling with pertussis toxin treatment does not alter β1-adrenergic receptor-mediated extracellular signal-regulated kinase activation. COS-7 cells were transiently transfected with β1AR. Serum-starved cells were pre-treated with PTX overnight before stimulation by ISO (10 μM for 5 min) or FSK (100 nM for 5 min). Quantification of ERK activation showed a similar response in the presence or absence of PTX treatment ($P > 0.05$)

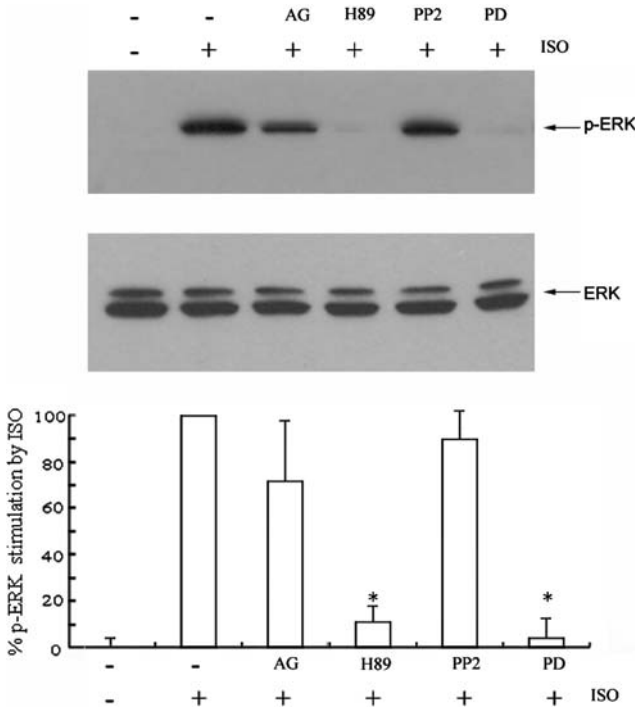

Fig. 4 Protein kinase A and mitogen-activated protein kinase kinase1 but not epidermal growth factor receptor or Src family tyrosine kinases are required for β1-adrenergic receptor stimulation of ERK/MAPK in African green monkey kidney COS-7 cells. COS-7 cells were transiently transfected with the β1AR. Serum-starved cells were pre-incubated with 20 μM H89 for 45 min, 50 μM MEK inhibitor PD 98059 (PD) for 30 min, 100 nM EGFR inhibitor AG1478 (AG) for 30 min, or 10 μM Src tyrosine kinase inhibitor PP2 for 15 min, respectively, before stimulation by ISO (100 nM for 5 min). Quantification of the effect of protein kinase A and mitogen-activated protein kinase kinase1 on ERK activation is also shown

Both the β1AR and β2AR subtypes are expressed in the heart and appear to regulate cardiac function through similar intracellular signaling pathways (Brodde 1991). β2AR has been extensively studied in receptor-mediated ERK activation (Daaka et al. 1997; Luttrell 2002; Luttrell et al. 1997), and β2AR-stimulated ERK1/2 activation has been reported in cultured HEK-293 and COS-7 cells, and in isolated cardiac myocytes (Crespo et al. 1995; Daaka et al. 1998; Lazou et al. 1994). It has been clearly demonstrated that stimulation of β2AR activates the MAP kinase ERK in a manner mediated by the $\beta\gamma$ subunits of PTX-sensitive G proteins (Gi) through a pathway involving the non-receptor tyrosine kinase c-Src, the small G protein Ras and Raf-1 kinase (Luttrell 2002; Luttrell et al. 1997). The mechanism(s) of β1AR-stimulated ERK activation is somewhat more controversial. A number of groups have reported that the β1AR is unable to stimulate ERK activation, a conclusion based on the perceived inability of β1AR to couple to Gi (Kilts et al. 2000; Lavoie et al. 2002). However, recent data from cardiac myocytes suggest that β1AR can activate ERK and p38 in a Gi-dependent manner, even though β1AR is less potent at stimulating ERK activation than β2AR (Chesley et al. 2000; Communal et al. 2000). The involvement of Gs in isoproterenol-stimulated ERK1/2 activation has also been proposed for β1AR in HEK293 cells, cardiac myocytes, COS-7 cells and rat adipocytes (Chaudhry et al. 1994; Gauthier et al. 1996; Kobayashi et al. 2005; Tutor et al. 2007). Other results have indicated that activation of β1AR by ISO triggers different signal transduction pathways in order to stimulate ERK1/2 (Galandrin et al. 2008; Kim et al. 2008; Wisler et al. 2007). For example, ISO caused ERK activation in a src-dependent manner through both G$\beta\gamma$- and G protein-independent pathways in HEK293 cells (Galandrin et al. 2008). But so far, the mechanisms behind the observed differential activation of ERK are still unknown.

The objective of this study was to further elucidate the mechanism of β1AR mediated ERK activation. We found that ERK can be activated by β1AR stimulation in a dose-dependent way (Fig. 1) and that inhibiting the G$\beta\gamma$ subunit by cotransfection with βAR kinase carboxyl terminal

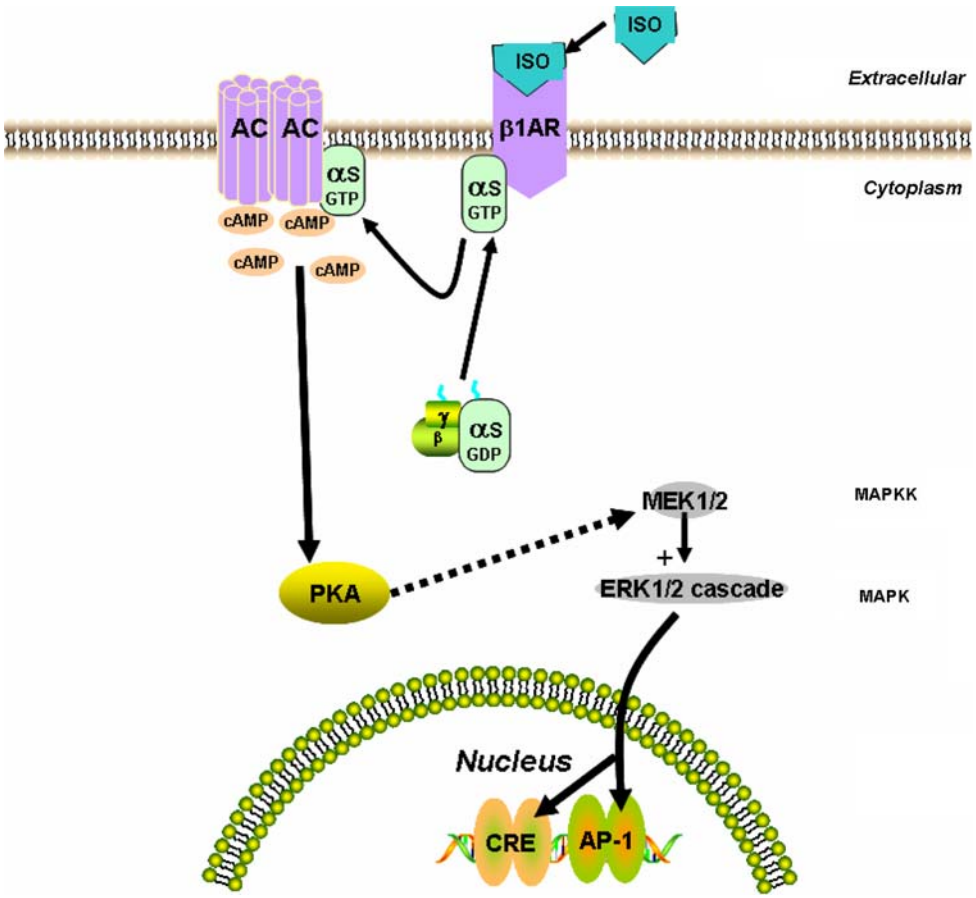

Fig. 5 Proposed model for isoproterenol-induced extracellular signal-regulated kinase activation via β1-adrenergic receptor. β1AR agonist ISO induces a potent ERK1/2 stimulation by the classic Gs/AC/PKA cascade and not by the Src family tyrosine kinase. Following the binding of agonist and activation of heterotrimeric G proteins (step 1), Gαs and AC mediate the production of the cAMP. Subsequently, PKA, MEK1 and other ERK proteins were activated. That is, β1AR signaling pathway was involved in linearly regulating ERK1/2 activation via the classic Gαs/AC/cAMP/PKA pathway in COS-7 cells, and PTX-sensitive Gi protein, Src family tyrosine kinase and EGFR were not required for ERK activation

(βARKct, Gβγ inhibitor) did not influence ISO-stimulated ERK phosphorylation (Fig. 2). When we stimulated β1AR with forskolin (FSK), an adenylate cyclase activator, the ERK activation level was similar to that obtained with ISO stimulation. Blocking the Gi pathway using pertussis toxin (PTX, Gi inhibitor) also did not change the activation level of ERK (Fig. 3). Inhibiting protein kinase A or mitogen-activated protein kinase kinase MEK1 with H89 (protein kinase A inhibitor) or PD98059 (MEK1 inhibitor), respectively, almost completely abolished the ERK activation mediated by ISO (Fig. 4). These results indicate that β1AR stimulation can specifically activate ERK signaling mediated by a Gαs-dependent pathway. We also found that AG1478 (epidermal growth factor receptor EGFR tyrosine kinase inhibitor) or PP2 (Src tyrosine kinase inhibitor) pretreated cells did not change the activation of ERK by β1AR stimulation (Fig. 4), indicating that β1AR activated ERK signaling pathway is independent of EGFR or Src related kinase.

Like most GPCRs, β1AR exhibits marked variation in its behavior in distinct cell types, with substantial differences in the rate and extent of agonist-promoted internalization being especially notable (Green and Liggett 1994; Shiina et al. 2000; Suzuki et al. 1992; Tang et al. 1999). Such differences in β1AR behavior in distinct cells may be explained in large part by the differential expression of β1AR-interacting proteins such as PDZ scaffolds. It has been shown that several PDZ proteins associate with β1AR, including PSD-95 (postsynaptic density-95) (Hu et al. 2000, 2002; Xu et al. 2001), membrane-associated guanylate kinase inverted-2 (MAGI-2) (Xu et al. 2001), cAMP-dependent guanine nucleotide exchange factor, also known as PDZ-GEF1 (CNrasGEF) (Pak et al. 2002), GAIP-interacting protein, carboxyl terminus (GIPC) (Hu et al. 2003), and cystic fibrosis transmembrane conductance regulator-associated ligand, also known as GOPC or FIG (CAL) (He et al. 2004). MAGI-2 and PSD-95 are structurally related PDZ proteins of the membrane-associated guanylate kinase-like (MAGUK) family, but nonetheless they exhibit diametrically opposing effects on agonist-induced β1AR internalization; MAGI-2 strongly promotes β1AR internalization (Xu et al. 2001), whereas PSD-95 markedly inhibits it (Hu et al. 2000; Xu et al. 2001). In contrast, CNrasGEF and GIPC have no obvious effects on β1AR endocytosis but rather regulate various aspects of β1AR signaling (Hu et al. 2003; Pak et al. 2002), whereas the Golgi-associated protein CAL directs β1AR anterograde trafficking through the endoplasmic reticulum–Golgi complex to the plasma membrane (He et al. 2004). These PDZ scaffolds are not expressed uniformly across all

tissues but instead tend to exhibit profound differences in expression levels between different tissues and cell types (Hung and Sheng 2002). Thus, interactions of GPCRs such as β1AR with PDZ scaffolds that exhibit distinctive patterns of expression across different tissues may account for many examples of cell-type specific regulation of GPCR signaling such as ERK activation (He et al. 2006).

The difference in the ERK signaling pathway in COS-7 cells may also be explained by the difference between isolates, such as expression level of signaling molecules and sensitivity of the different assay systems. It has been appreciated that many receptors, including the β2AR, can exist in multiple ''active'' conformations after ligand binding (Ghanouni et al. 2001; Granier et al. 2007; Kenakin 1995; Swaminath et al. 2005). These variable conformations may lead to widely differing cellular outcomes and may help explain the diverse signaling profiles in different reports. In this study, we showed that β1AR-mediated ERK activation was not sensitive to the EGFR inhibitor and Src family protein inhibitor PP2. Our result was consistent with previous reports that tyrosine kinases were not essential for β1AR signaling to ERK/MAPK in some cell types (Tutor et al. 2007), suggesting that β1AR mediated a different ERK activation pathway from β2AR and β3AR (Cao et al. 2000; Kursula 2008; Luttrell et al. 1999; Maudsley et al. 2000; Robidoux et al. 2006), and that the ERK activation by β1AR might be a direct consequence of Gs-mediated ERK activation. The proposed model of β1AR-mediated ERK activation with our findings is presented in Fig. 5.

It has been demonstrated that βAR stimulation of ERK1/2 may play a role in the development of cardiac hypertrophy (Zou et al. 1999). Activation of the MEK/ERK1/2 pathway is involved in the development of cardiac hypertrophy (Molkentin and Dorn 2001), and elevated ERK activation has been reported in failing human hearts with dilated cardiomyopathy (Takeishi et al. 2002). The evidence of adverse, pro-apoptotic effects of β1-adrenergic receptor (β1AR) stimulation in an isolated cardiac myocyte model has provided a mechanistic basis for the selective blockade of β1AR in chronic heart failure (Bristow 1997). As such, the elucidation of the mechanism of ERK activation is helpful for finding new drug targets. Knowledge of the molecules involved in the signal transduction pathway activated by β1AR, therefore, could have important therapeutic implications, guiding the development of new drugs for conditions associated to impaired vascular responses such as hypertension, heart failure and coronary disease. However, some details of this model remain to be determined. GPCR-activated ERK is generally translocated to the nucleus, where it phosphorylates and regulates transcription factors (Pierce et al. 2001). Therefore, potential downstream targets of β1-ERK signaling in COS-7 remain to be identified. Also, we should detect if the difference of ERK activation mediated by β1AR mechanism among different researchers is because of differential expression of PDZ proteins.

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