Naunyn-Schmied Arch Pharmacol (2009) 380:443–450
DOI 10.1007/s00210-009-0444-6

ORIGINAL ARTICLE

Reciprocal regulation between M_3 muscarinic acetylcholine receptor and protein kinase C-ε in ventricular myocytes during myocardial ischemia in rats

Peng-zhou Hang · Jing Zhao · Yu-ping Wang · Li-hua Sun · Yong Zhang · Li-li Yang · Na Zhao · Zhi-dan Sun · Yu-ying Mao · Zhi-min Du

Received: 25 June 2009 / Accepted: 24 July 2009 / Published online: 15 August 2009
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Abstract We have studied the association between M_3 muscarinic acetylcholine receptors (M_3-mAChR) and protein kinase C-ε (PKC-ε) during ischemic myocardial injury using Western blot analysis and immunoprecipitation technique. Myocardial ischemia (MI) induced PKC-ε translocation from cytosolic to membrane fractions. This translocation participated in the phosphorylation of M_3-mAChR in membrane fractions, which could be abolished by the inhibitor of PKC, chelerythrine chloride. On the other hand, M_3-mAChR could also regulate the expression of PKC-ε in ischemic myocardium. Choline (choline chloride, an M_3 receptor agonist, administered at 15 min before occlusion) strengthened the association between PKC-ε and M_3-mAChR. However, blockade of M_3-mAChR by 4-diphenylacetoxy-*N*-methylpiperidine methiodide (an M_3 receptor antagonist, administered at 20 min before occlusion) completely inhibited the effect of choline on the expression of PKC-ε. We conclude that the translocation of PKC-ε is required for the phosphorylation of M_3-mAChR; moreover, increased PKC-ε activity is associated with M_3-mAChR during MI. This reciprocal regulation is likely to play a role in heart signal transduction during ischemia between ventricular myocytes.

P.-z. Hang · Y.-p. Wang · L.-l. Yang · N. Zhao · Z.-d. Sun · Y.-y. Mao · Z.-m. Du (✉)
Institute of Clinical Pharmacology of Second Hospital, Harbin Medical University,
Key Laboratory of Heilongjiang Province,
Baojian Road 157, Nangang District,
Harbin, Heilongjiang Province 150081,
People's Republic of China
e-mail: dzm1956@126.com

J. Zhao · L.-h. Sun · Y. Zhang
Department of Pharmacology, Harbin Medical University,
Harbin 150081, People's Republic of China

Keywords Protein kinase C-ε · M_3 muscarinic acetylcholine receptor · Myocardial ischemia · Western blot

Introduction

It has been well documented that the main reason of higher morbidity and mortality is fatal arrhythmias and sudden cardiac death accompanied with myocardial ischemia (MI; Cascio et al. 2005; Yang et al. 2007). Recent studies have paid close attention to several important kinases under ischemic conditions in mammalian hearts (Robinet et al. 2005). During these kinases, the protein kinase C (PKC) family has been the focus of many researchers (Mayr et al. 2004; Melling et al. 2009). To date, 12 isozymes of PKC have been recognized according to the calcium dependency and regulation by lipid modulators (Cain et al. 1999). Among them, PKC-δ and PKC-ε have been studied generally in cardiovascular diseases, especially MI and hypertrophy (Chen et al. 2001; Lawrence et al. 2005). Translocation of PKC isoforms after activation from cytosolic to particulate fractions such as plasma membrane, the Golgi apparatus, and mitochondria is a necessary action (Goodnight et al. 1995; Gordon et al. 1997; Shirai et al. 1998). An abundance of information was available concerning the potential therapeutic role of PKC-ε for the ischemic heart diseases (Inagaki et al. 2006). However, there are still controversies that exist about translocation of PKC-ε to membrane fractions and its exact mechanisms (Ooie et al. 2003; Simkhovich et al. 1996), which will be examined in detail in our study.

Meanwhile, some evidence has indicated the expression of multiple subtypes of muscarinic acetylcholine receptors

(mAChR), including M_1–M_5, in the myocardium (Wang et al. 2001). The M_3-mAChR is a G-protein-coupled receptor that associates with a delayed rectifier K^+ channel named I_{KM3} and participates in cardiac repolarization, resulting in negative chronotropic actions and antiarrhythmic effects (Liu et al. 2009; Shi et al. 1999a, b; Shi et al. 2004; Wang et al. 1999). The potential protective role of M_3-mAChR in the heart has been examined by several researchers. For example, M_3-mAChR offers obvious cytoprotection against myocardial ischemic injury (Yang et al. 2005) through multiple mechanisms, including the interaction with gap junction channel connexin (Cx)43 to maintain cell-to-cell communication (Yue et al. 2006), activation of antiapoptotic signal molecules Bcl-2 and p38 mitogen-activated protein kinase (MAPK), and decrease of intracellular Ca^{2+} overload of cardiac myocytes (Tobin and Budd 2003; Yang et al. 2005). The M_3-mAChR has been known to improve cardiac contraction and hemodynamics by activating intracellular phosphoinositide hydrolysis via a G_q pathway (Wang et al. 1999; Shi et al. 2004). Moreover, previous studies by our group and others (Shi et al. 1999a, b; Wang et al. 2007, 2009) have shown that phospho-M_3-mAChR played an important role in the activity in the myocardium, and therefore the regulator of phospho-M_3-mAChR should be a key point in the signaling pathway of M_3-mAChR.

Based on these findings, both PKC-ε and M_3-mAChR were involved in the process of MI. However, the relationship between M_3-mAChR and PKC-ε was not fully understood. The present study was therefore designed to investigate their association and further detect the role of PKC-ε in the regulation of phosphorylation of M_3-mAChR and the part of M_3-mAChR in the PKC-ε signaling pathway in ventricular myocytes during MI.

Materials and methods

Materials

Antibodies against PKC-ε (polyclonal) and phosphoserine (monoclonal) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-M_3-mAChR antibody (polyclonal) was obtained from Alomone Biolab (Jerusalem, Israel). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; anti-GAPDH antibody) was provided by Kangcheng (Shanghai, China). Chelerythrine chloride (CHE) was purchased from Sigma (St. Louis, MO, USA).

Global MI model

Wistar rats (250±20 g, male; Experimental Animal Center of Harbin Medical University, Harbin, China) were used. All rats were kept at a room with 23±1°C, humidity of 55±5%, and 12 h dark/light cycle and allowed unlimited food and water. All experimental procedures were approved by the Experimental Animal Ethic Committee of Harbin Medical University, China. The rats were anesthetized with chloral hydrate (300 mg/kg) and heparin (500 IU/kg) i.p. The procedures for making the global MI model have been described in detail elsewhere (Ooie et al. 2003; Zhang et al. 2007). Briefly, the heart was rapidly excised after thoracotomy and placed in cold heparinized Tyrode's solution (4°C). The aorta was immediately cannulated, and the hearts was perfused retrograde in the Langendorff perfusion apparatus at 75 mmHg constant perfusion pressure with modified Krebs-Henseleit (KH) buffer of the following composition (in mM): NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and glucose 11. The rat hearts were randomly divided into three groups: (1) control (rats were perfused with KH buffer for 15 min); (2) ischemia (rats were subjected to 30-min no-flow global ischemia after 15-min KH buffer perfusion); (3) CHE (the procedure was similar to that for ischemia group, except that 2 μM CHE, a PKC inhibitor, was administered during the 15-min perfusion).

Acute MI model

To assess the potential regulation effect of M_3-mAChR on the PKC-ε, 16 Wistar rats were randomly divided into four groups, namely control, ischemic, choline-treated, and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) + choline-treated groups. The procedures for making the acute MI model have been described in detail elsewhere (Yang et al. 2005). Briefly, the left anterior descending coronary artery was occluded, and the chest was then closed. Electrocardiographic ST segment elevation was used as an index to evaluate MI. At given times during the experiments, the right ventricles, which had no ischemia-induced tissue damage, were immediately removed and prepared for subsequent immunoblotting analysis. All treatments were administered via the tail vein with doses of choline and 4-DAMP used in previous studies (Yang et al. 2005). In the ischemic group, rats were subjected to 20-min MI. In the choline group, rats were injected with choline chloride (5 mg/kg, i.v.) 15 min prior to the 20-min period of MI. In the 4-DAMP + choline-treated group, rats were injected with 4-DAMP (0.5 μg/kg, i.v.), followed, 5 min later, by choline injection and then, 15 min later, by the 20-min period of MI.

Cytosolic and membrane protein preparation

The procedures for cytosolic and membrane protein preparation have been described previously (Ooie et al. 2003). The preparations included mincing and washing

with ice-cold phosphate-buffered saline (PBS) buffer. The tissues were then homogenized in ice-cold lysis buffer (buffer A) pH7.4, which contained (in mM): 320 sucrose, 10 Tris HCl, 1 ethylene glycol tetraacetic acid, 5 NaN_3, 10 β-mercaptoethanol, 0.02 leupeptin, 0.00015 pepstatin A, 0.2 phenylmethylsulfonyl fluoride, and 50 NaF. The homogenate was centrifuged at 2,000×*g* for 15 min at 4° C. The supernatants (containing cytosolic and membrane fractions) were centrifuged at 100,000×*g* for 1 h. The 100,000×*g* supernatant from this step was the cytosolic fractions, and the pellet from this 100,000×*g* spin (containing the membrane fractions) was stirred in 0.3% Triton-X-100 containing buffer A (buffer B) for 1 h at 4°C and then centrifuged at 100,000×*g* for 1 h. Supernatant from this step was the membrane fractions. Protein concentrations were measured spectrophotometrically using a BCA kit (Universal Microplate Spectrophotometer; Bio-Tek Instruments, Winooski, VT, USA).

Immunoprecipitation and Western blot

Protein samples (100μg) were added to 600-μL radio-immunoprecipitation assay lysis buffer (Beyotime Biotechnology, China) containing 3-μL antibody preparations and 6-μL protease inhibitors. The mixture was rotated at 4°C for 6 h, followed by incubation overnight with 18μL protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sample was centrifuged, and the pellet was resuspended in 12μL of 2×sodium dodecyl sulfate (SDS) sample buffer; the mixture was then boiled for 5 min. Proteins were separated by electrophoresis on 10% SDS–polyacrylamide gels and transferred moist to polyvinylidene difluoride membranes. Membranes were blocked by 5% nonfat dry milk in PBS and incubated overnight at 4°C. Membranes were washed three times, for 15 min each time, with PBS containing 0.5% Tween 20 (PBS-T) and then incubated with primary antibody for 1.5 h, washed three times for 15 min each time with PBS-T, and incubated with secondary antibody for 1 h. The images were captured on the Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Western blot bands were quantified using Odyssey v1.2 software by measuring the band intensity (area×OD) in each group and normalizing to GAPDH (anti-GAPDH antibody) as an internal control.

Statistical analyses

All data were expressed as mean±SEM and analyzed using SPSS 13.0 software. Statistical comparisons among multiple groups were performed using analysis of variance. A two-tailed $P<0.05$ was considered to be statistically significant.

Results

Effect of MI on the expression of PKC-ε in cytosolic and membrane fractions

Western blotting analysis was performed to verify the presence of PKC-ε proteins in the heart and to determine the effect of MI on the expression of PKC-ε in the cytosolic and membrane fractions of cardiomyocytes. PKC-ε (90 kDa) was detected in both the left and right ventricles in control group (Fig. 1a, c). The expression of PKC-ε in cytosolic fractions in both left and right ventricles was significantly lower in MI group compared with control group. In addition, the expression of PKC-ε in CHE group was also obviously decreased compared with MI groups (Fig. 1a, c). For example, PKC-ε protein levels in the MI group were 35% lower than those in control group (Fig. 1b); moreover, PKC-ε protein expression of the left ventricle in the CHE group was 30% lower than that in MI group (Fig. 1b). Similar results were obtained from the right ventricle (Fig. 1d). In contrast, the expression of PKC-ε in membrane fractions was significantly higher in MI group compared with control group. Figure 2a, c shows that PKC-ε levels increased in both left and right ventricles after ischemia compared with control group. In addition, the expression of PKC-ε in CHE group was diminished markedly compared with MI groups (Fig. 2a, c). For example, PKC-ε protein levels in MI group were 32% higher than those in control group, whereas PKC-ε protein expression in the left ventricle of the CHE group was 26% lower than that in MI group (Fig. 2b). Similar results were obtained from the right ventricle (Fig. 2d).

Quantitation of redistribution of PKC-ε after MI

MI significantly decreased the abundance of PKC-ε in the cytosolic fractions, while it increased its abundance in the membrane fractions. These results suggested that ischemia induced PKC-ε translocation from cytosolic to membrane fractions. For example, the normalized ratio of cytosolic/membrane fractions in MI group was 50% lower than control group in left ventricle (Fig. 3a). Similar results were obtained from the right ventricle (Fig. 3b).

Regulation of PKC-ε translocation on the phosphorylation of M_3-mAChR

Having confirmed the expression of PKC-ε after ischemia, we further verified the expression of M_3-mAChR. The increase of M_3-mAChR expression was observed after MI for about 20%. Meanwhile, no differences were found in expression of M_3-mAChR between myocardial ischemic group and CHE groups (Fig. 4a, b). To confirm the

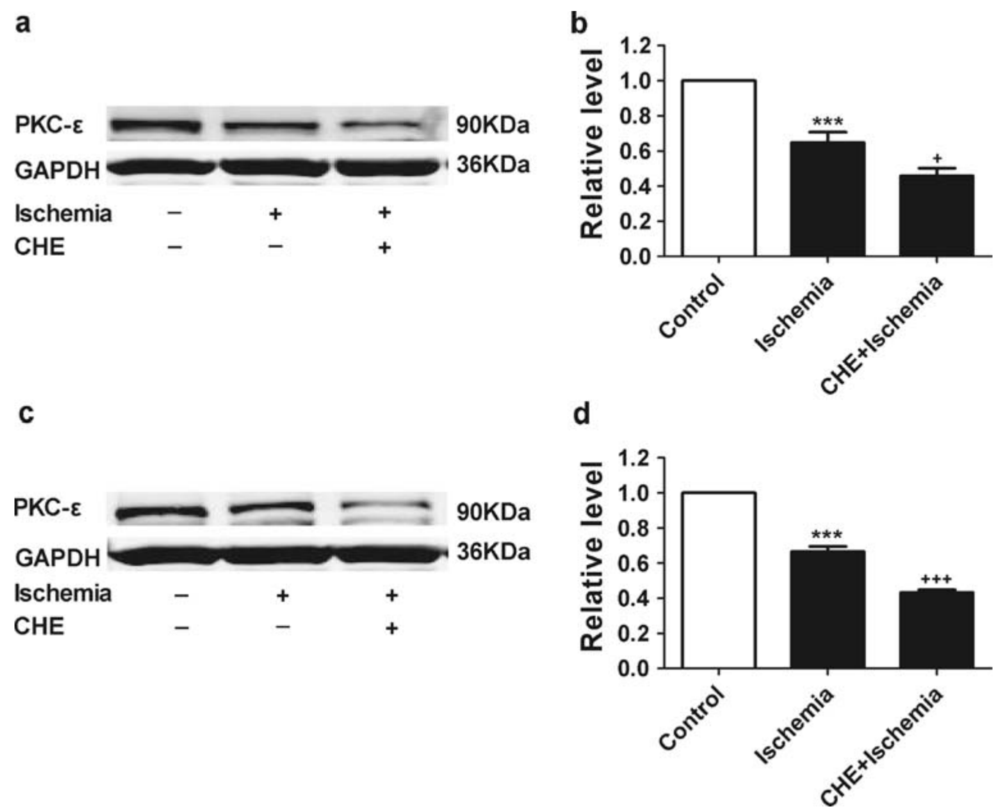

Fig. 1 Effect of myocardial ischemia on the expression of protein kinase C-ε (*PKC-ε*) protein in cytosolic fractions. **a**, **c** Western blot results for PKC-ε expression in the left and right ventricles. Myocardial ischemia decreased the expression of PKC-ε in cytosolic fractions in both left (**a** and **b**) and right ventricles (**c** and **d**). Values given are normalized to band intensity of GADPH (anti-GAPDH antibody) used as internal control. All values are expressed as mean±SEM (n=4 independent experiments). ***P<0.001 compared with control, +P<0.05, +++P<0.001 compared with ischemia

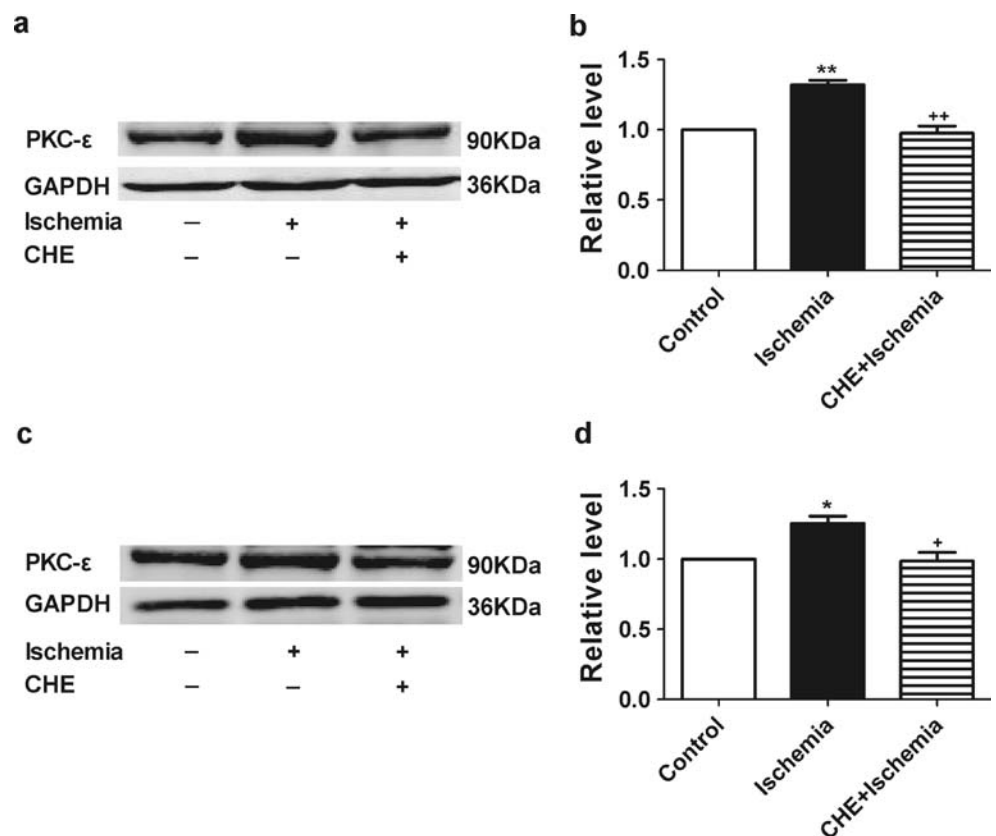

Fig. 2 Effect of myocardial ischemia on the expression of protein kinase C-ε (*PKC-ε*) protein in membrane fractions. **a**, **c** Western blot results for PKC-ε expression in the left and right ventricles. Myocardial ischemia enhanced the expression of PKC-ε in membrane fractions in both left (**a** and **b**) and right ventricles (**c** and **d**). Values given are normalized to band intensity of GADPH (anti-GAPDH antibody) used as internal control. All values are expressed as mean±SEM (n=4 independent experiments). *P<0.05, **P<0.01 compared with control, +P<0.05, ++P<0.01 compared with ischemia

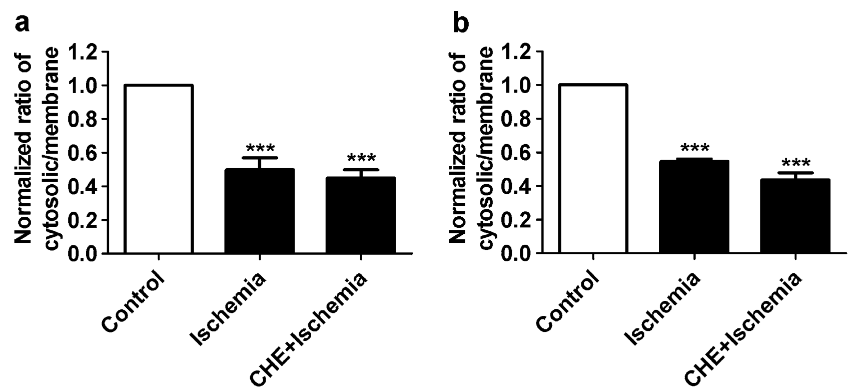

Fig. 3 Quantitation of redistribution of protein kinase C-ε (*PKC-ε*) induced by myocardial ischemia. Data were normalized to the ratio of cytosolic/membrane fractions of each group in left (**a**) and right (**b**) ventricles. The subcellular distribution of PKC-ε was altered after ischemia in left and right ventricles (**a** and **b**). All values are expressed as mean±SEM (n=4 independent experiments). ***P<0.001 compared with control

potential regulation of phospho-M_3-mAChR by PKC-ε, we used the immunoprecipitation approach to detect the level of phospho-M_3-mAChR in both groups. The phosphorylation part of M_3-mAChR on serine was decreased up to 20% after inhibiting the PKC-ε by CHE compared with MI group, which means PKC-ε took part in the phosphorylation on serine of M_3-mAChR (Fig. 4d).

Regulation of choline on the expression of PKC-ε

After testing the effect of PKC-ε on M_3-mAChR, the effect of choline, an agonist of M_3-mAChR, on the expression of PKC-ε in acute MI was investigated. Protein levels of PKC-ε and M_3-mAChR both significantly increased after application of choline (Fig. 5a, c). The expression of PKC-ε in choline group was 27% greater than that in MI group, however, 4-DAMP, an M_3-mAChR antagonist, attenuated the effect of choline on PKC-ε protein levels (Fig. 5d).

Discussion

The main finding of the present study is that M_3-mAChR is closely associated with PKC-ε. This notion is strongly supported by the following evidence: (1) ischemia induced PKC-ε translocation to membrane fractions to participate in the phosphorylation of M_3-mAChR. (2) M_3-mAChR also regulated the expression of PKC-ε after ischemia.

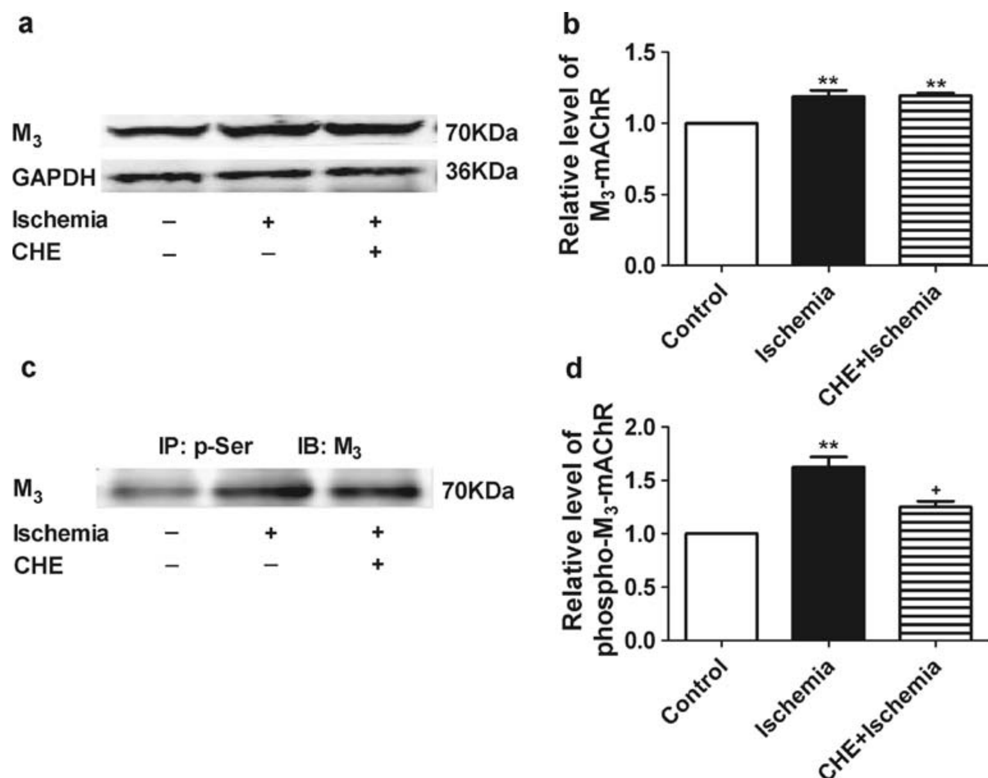

Fig. 4 Effect of protein kinase C-ε (*PKC-ε*) on the expression of M_3 muscarinic acetylcholine receptor (M_3-mAChR). **a**, **b** Western blot bands representing M_3-mAChR after 30-min global ischemia or CHE perfusion. **c**, **d** Immunoprecipitation showing the effects of PKC-ε on the phosphor-M_3-mAChR. All values are expressed as mean± SEM (n=4 independent experiments). **P<0.01 compared with control, +P<0.05 compared with ischemia. IP, immunoprecipitation; IB, immunoblot

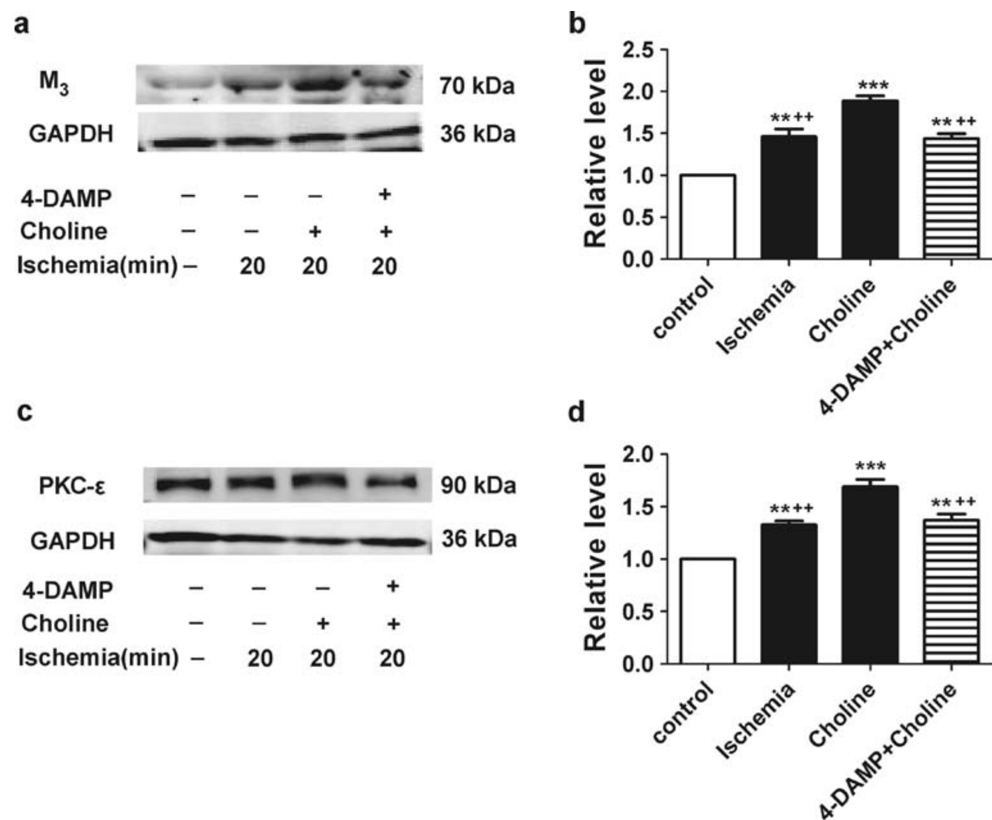

Fig. 5 Effect of choline on the expression of M_3 muscarinic acetylcholine receptor (M_3-mAChR) and protein kinase C-ε (PKC-ε). **a**, **b** Western blotting results showing the expression of M_3-mAChR induced by choline and 4-diphenylacetoxy-*N*-methylpiperidine methiodide (*4-DAMP*) during myocardial ischemia. **c**, **d** Western blotting results showing the effects of choline and 4-DAMP pretreatment on the expression of PKC-ε. Values given are normalized to band intensity of GADPH (anti-GAPDH antibody) used as internal control. All values are expressed as mean±SEM (n=4 independent experiments). **P<0.01, ***P<0.001 compared with control, ++P<0.01 compared with choline

Although many groups have examined the translocation of PKC-ε in several models (Goodnight et al. 1995; Gordon et al. 1997; Shirai et al. 1998), Simkhovich et al. (1996) found that ischemic preconditioning did not cause PKC translocation in rabbit heart, and Ooie et al. (2003) found that PKC-ε persistently translocated from the cytosolic to the particulate fractions in the diabetic heart but only transiently in the normal heart after ischemia in rat. Based on these controversies, our results confirmed that PKC-ε could be activated after ischemia and persistently translocate up to 30 min to membrane fractions in rat heart (Fig. 3).

In addition, it has been recently shown that increased phospho-M_3-mAChR further indicated the potential protective function of M_3-mAChR during ischemic injury (Wang et al. 2009). Our results showed that the redistribution of PKC-ε from cytoplasm to membrane increased the expression of phospho-M_3-mAChR (Fig. 4c). Our data firstly demonstrated that PKC-ε also could regulate the expression of M_3-mAChR in MI. Therefore, we could speculate that the regulation of M_3-mAChR by PKC-ε may be another mechanism of the preconditioning effect of PKC-ε in MI. Moreover, except those molecules such as Bcl-2 and p38 MAPK induced by activating M_3-mAChR (Yang et al. 2005; Luo et al. 2008), we found PKC-ε to be another one regulated by M_3-mAChR, which may be another potential mechanism of cardioprotection of M_3-mAChR (Fig. 5c).

Previous studies have shown that PKC-ε plays a cardioprotective role during ischemic preconditioning through several mechanisms, such as reduction of the intracellular Ca^{2+} (Zhang et al. 2007), activation of the mitochondrial cytochrome c oxidase, and phosphorylation of Cx43 (Budas and Mochly-Rosen 2007; Doble et al. 2000; Guo et al. 2007). Among them, the activation and migration of PKC-ε to membrane Cx43 gap junctions or to mitochondria are essential for its protection from ischemia. (Kabir et al. 2006). Interestingly, it has been shown from our findings that activated PKC-ε could also contact M_3-mAChR besides Cx43. Multiple phosphorylation sites of Cx43 on serine, potential targets of several groups of kinases, suggest that it may serve as a discriminating sensor of change in the cellular environment (Doble et al. 2000). Meanwhile, considering the colocalization between Cx43 and M_3-mAChR, the interaction between PKC-ε and M_3-mAChR is probably relevant to Cx43. However, whether the M_3-mAChR is phosphorylated directly as a substrate of PKC-ε or indirectly via intermediate like Cx43 is not clear and interesting to study in the future.

It should be noted that CHE is a nonselective inhibitor of PKC. However, previous studies have confirmed that the calcium-independent PKC-ε has been reported to localize to intercalated disk-like sites on stimulation (Disatnik et al. 1994; Doble et al. 2000), which is also the location of M_3-mAChR and Cx43 in cardiomyocytes (Yue et al. 2006),

while no evidence indicates that any other isoforms could also translocate like PKC-ε. Moreover, ischemia induced the increased association of PKC-ε with the cardiac myocyte membrane fractions and the increased phosphorylation of Cx43 (Doble et al. 2000), which was colocalized with M_3-mAChR (Yue et al. 2006). We thus considered PKC-ε as a potential candidate for mediating M_3-mAChR phosphorylation. Our data provide evidence that a functional link between PKC-ε and M_3-mAChR and a protective role during MI may exist.

In fact, it has been thought that the main location of M_3-mAChR is in the smooth muscle such as gastrointestinal tract and bladder. However, recently, researchers have confirmed its existence and significant effect in the heart. During these two decades, researchers did several works around the effect of M_2 and M_3-mAChR in the heart (Shi et al. 1999a, b; Wang et al. 2007, 2009). Among them, a group examined it using knockout M_2 and M_3 technology. They found that acetylcholine-induced vasodilation in aorta and coronary circulation is mediated predominantly by M_3 receptors and not by M_2 receptors (Lamping et al. 2004). In addition, our previous data also supported the important role of M_3-mAChR in heart diseases; thus, we got to know the significance of M_3-mAChR (Wang et al. 2009). Moreover, many investigations have also demonstrated the pivotal role of the phosphorylation form of receptors and ion channels (Tobin and Nahorski 1993; Doble et al. 2000). Therefore, the phosphorylation of M_3-mAChR by PKC-ε in the heart could not be ignored.

Taken together, these findings of the present study indicated that the recognized cytoprotective effects of PKC-ε and M_3-mAChR in MI were conferred by reciprocal regulation between these two distinct proteins. Furthermore, administration of choline before ischemia strengthened facilitation of the association between PKC-ε and M_3-mAChR induced by MI. The interaction may be a new therapeutic target in the treatment of myocardial ischemic diseases.

Acknowledgement This study was supported by grants from the National Natural Science Foundation of China (no. 30672462) and the Specialized Research Fund for the Doctoral Program of Higher Education (no. 20050226010).

Conflict of interest None declared.

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