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The MLCK-mediated α_1-adrenergic inotropic effect in atrial myocardium is negatively modulated by PKCε signaling

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1 The present study examined the role of myosin light chain kinase (MLCK), PKC isozymes, and inositol 1,4,5-trisphosphate (IP_3) receptor in the positive inotropic effect of α_1-adrenergic stimulation in atrial myocardium.

2 We measured inotropic effects of phenylephrine (0.3–300 μM) in isolated left atrial preparations (1 Hz, 37°C, 1.8 mM Ca^{2+}, 0.3 μM nadolol) from male 8-week FVB mice ($n=200$). Phenylephrine concentration-dependently increased force of contraction from 1.5 ± 0.1 to 2.8 ± 0.1 mN (mean ± s.e.m., $n=42$), which was associated with increased MLC-2a phosphorylation at serine 21 and 22 by 67% and translocation of PKCε but not PKCα to membrane (+30%) and myofilament (+50%) fractions.

3 MLCK inhibition using ML-7 or wortmannin right-shifted the concentration–response curve of phenylephrine, reducing its inotropic effect at 10 μM by 73% and 81%, respectively.

4 The compound KIE1-1 (500 nM), an intracellularly acting PKCε translocation inhibitor peptide, prevented PKCε translocation and augmented the maximal inotropic effect of phenylephrine by 40%. In contrast, inhibition of Ca^{2+}-dependent PKC translocation (KIC1-1, 500 nM) had no effect. Chelerythrine, a PKC inhibitor, decreased basal force without changing the inotropic effect of phenylephrine.

5 The IP_3 receptor blocker 2-APB (2 and 20 μM) concentration-dependently decreased basal force, but did not affect the concentration–response curve of phenylephrine.

6 These results indicate that activation of MLCK is required for the positive inotropic effect of α_1-adrenergic stimulation, that the Ca^{2+}-independent PKCε negatively modulates this effect, and that PKCα and IP_3 receptor activation is not involved.
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Abbreviations: 2-APB, 2-aminoethoxydiphenyl borate; DAG, 1,2-diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; MLC-2, regulatory myosin light chain; MLCK, myosin light chain kinase; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate

Introduction

Several studies have suggested that in the mammalian heart, activation of myosin light chain kinase (MLCK) is causally related to the positive inotropic responses to Gq-coupled receptor agonists such as phenylephrine, endothelin, and angiotensin-II (Rossmanith *et al*., 1997; Aoki *et al*., 2000; Andersen *et al*., 2002; Chu & Endoh, 2005; Grimm *et al*., 2005). Support for a critical role of myosin light chain phosphorylation in heart function comes from other lines of investigation: in transgenic mice, exchange of MLC-2 by a non-phosphorylatable isoform was associated with dramatic functional and morphological changes of the atria (Sanbe *et al*., 1999), suggesting that phosphorylation of MLC-2 is of particular importance in the atrial compartment. It is known that α_1-adrenergic signaling plays an important role in the modulation of normal atrial systolic function and cardiac output, especially under pathophysiological conditions (Hoit & Gabel, 2000; McCloskey *et al*., 2003; Korzick *et al*., 2004). Yet, the molecular mechanisms underlying the positive inotropic effect of α_1-adrenergic agonists in atrial myocardium remain incompletely understood.

The α_1-adrenergic agonist phenylephrine accelerates phosphoinositide hydrolysis to generate the second messengers 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG activates protein kinase C (PKC) isozymes. Depending on experimental conditions, PKC either potentiates the effect of MLC-2 phosphorylation on myofibrillar Ca^{2+} sensitivity (Clement *et al*., 1992) or decreases Ca^{2+} sensitivity *in vitro* (van der Velden *et al*., 2006). Functional studies on intact myocardium led to the hypothesis that PKC activation is necessary for the inotropic effect of α_1-AR stimulation (Endoh *et al*., 1993; Deng *et al*., 1997), but they are in contrast to other studies (Endou *et al*., 1991; Andersen *et al*., 2002). Although PKCα is the most abundant (>80%) conventional PKC in

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adult mouse heart (Hahn *et al*., 2003), a number of other PKC isozymes with distinct functions have been found in mammalian hearts, most consistently PKCδ and PKCε (Das, 2003). The PKC family consists of more than 12 isozymes, and the PKC isozymes activated by α_1-adrenergic agonists and involved in their inotropic effects are not clearly identified. As PKC isozymes not only exhibit agonist selectivity but could also exert opposing effects such as the Ca^{2+}-independent δ- and ε-isozymes (Chen *et al*., 2001a), it is reasonable to assume that isozyme selective modulation of PKC function is necessary to identify the roles of PKC isozymes. The present study was designed specifically to investigate the roles of PKCα and PKCε in the contractile response of atrial myocardium to α_1-adrenergic stimulation. We performed experiments with novel intracellularly acting PKC-modulating peptides to modify PKC translocation in atrial muscle. The peptides have been shown to enter cardiomyocytes of intact mouse hearts where they are released from a carrier peptide and selectively affect PKC activity within 10 min after application (Schwarze *et al*., 1999; Chen *et al*., 2001a, b; Inagaki *et al*., 2003; Begley *et al*., 2004; Inagaki *et al*., 2005).

The role of IP_3 and the IP_3-receptor-mediated Ca^{2+} release in the α_1-adrenergic inotropic effect is controversial (Zima & Blatter, 2004; Wang *et al*., 2005). In our present study, we investigated the role of IP_3 receptor using the membrane-permeable compound 2-APB that has been used successfully to inhibit IP_3 receptor in isolated heart (Gysembergh *et al*., 1999) as well as isolated cardiac myocytes (Li *et al*., 2005).

Our data demonstrate that α_1-adrenoceptor-mediated activation of the Ca^{2+}-independent PKCε negatively modulates the MLCK-dependent α_1-adrenergic positive inotropic effect in atrial myocardium. We present evidence that IP_3-induced Ca^{2+} release from sarcoplasmic reticulum does not contribute to the positive inotropic effect of α_1-AR stimulation.

Methods

Animals

Male FVB/N mice at 8 weeks were employed in the study. The investigation conforms with the University Medical Center, Hamburg guidelines for the use of laboratory animals.

Reagents

All standard reagents, if not otherwise stated, were obtained from Sigma (Toufkirchen, Germany). Wortmannin, ML-7, and ML-9 were supplied by Alexis Biochemicals (Lausen, Switzerland). Ro-31-8220 was supplied by Calbiochem (La Jolla, CA, U.S.A.). Cariporide was provided by Aventis Pharma, Frankfurt, Germany. KB-R7943 was purchased from Tocris, Bristol, U.K. Selective peptide modulators of PKC isozymes were provided by KAI Pharmaceuticals, South San Francisco, CA, U.S.A.

Quantification of MLC-2a phosphorylation

Phosphorylation of MLC-2a was measured in mouse left atria by Western blot as described (Grimm *et al*., 2005). The polyclonal antibody against MLC-2a was provided by the University of Texas Southwestern Medical Center at Dallas, TX, U.S.A. The polyclonal antiserum MLC-2a-P was custom made by Eurogentec, Seraing, Belgium. It was raised against the peptides CTKQAQRGpSSNVFS, CTKQAQRGSpSNVFS, and CTKQAQRGpSpSNVFS. The antibody recognizes mono- or diphosphorylated MLC-2a of human (Ser-22 and/or Ser-23) and mouse (Ser-21 and/or Ser-22) origin. Proteins were detected using SuperSignal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL, U.S.A.), and densitometric band intensities were evaluated using the ChemiGenius^2 system (Syngene, U.K.), using total MLC2a as denominator.

Muscle strip preparation and force measurement

Experiments were performed as previously described for human atrial trabeculae (Grimm *et al*., 2005). Briefly, the heart was quickly removed from the mouse after CO_2 narcosis and cervical dislocation, and the excised left atrium was kept at room temperature in continuously oxygenated (95% O_2 + 5% CO_2) modified Tyrode's solution containing 119.8 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 0.42 mM NaH_2PO_4, 22.6 mM $NaHCO_3$, 0.05 mM Na_2EDTA, 0.5 mM ascorbic acid, 10 mM glucose, and 5 mM pyruvate. Muscle stretch was adjusted to maximal twitch force (i.e. the maximum of its length–tension relationship). Nadolol at 0.3 μM was added after stabilization of contractile force. Inotropic responses to phenylephrine (0.3–300 μM) were measured in both the absence and presence of pharmacological inhibitors or peptide modulators of PKC isozyme translocation. We used translocation activator peptides selective for Ca^{2+}-dependent PKC isozymes (KAC1-1) and for PKCε (KAE1-1). Translocation inhibitor peptides used were selective for Ca^{2+}-dependent PKC isozymes (KIC1-1) and for PKCε (KIE1-1). The carrier peptide alone (C1) served as control. All experiments were performed after 10 min incubation with 500 nM peptide.

Subcellular fractionation of atrial muscle samples

To test the translocation of PKC isoforms, electrically stimulated mouse left atria were subjected to isometric contraction experiments as indicated and freeze clamped in liquid N_2. Later homogenization buffer (20 mM Tris, 5 mM EDTA, 5 mM $MgCl_2$, 50 mM NaF, 2 μg ml^{-1} aprotinin) was added to the samples, which were then homogenized on ice using a glass/glass potter homogenizer. After 10 min incubation on ice, the particulate fraction (pellet) was separated from the soluble fraction (supernatant) by centrifugation at 100,000 $\times g$ for 30 min. In some experiments, immunoblot analysis was carried out on each of three subcellular fractions: cytosol, membranes, and myofilaments. A myofilament fraction was separated from the particulate fraction by resuspension in lysis buffer supplemented with 1% Triton X-100. The samples were spun again at 14,000 $\times g$ for 10 min at 4°C producing a Triton X-100-insoluble pellet (myofilament fraction) and a supernatant that was designated the membrane fraction. Samples were adjusted to a final protein concentration of 1–2 mg ml^{-1} in standard Laemmli sample buffer, boiled for 5 min, and then subjected to SDS–PAGE and immunodetection. Immunoreactive bands were detected using the ECL Plus Detection System (Amersham Biosciences, Freiburg, Germany) ChemiGenius^2 system (Syngene Europe, Cambridge, U.K.).

Statistical analysis

Data were calculated as mean ± s.e.m. Differences between mean values were evaluated by Student's *t*-test. Repeated measures analysis of variance (ANOVA) and Bonferroni *post hoc* test were performed where appropriate using StatView version 5.0 for Windows, SAS Institute Inc., Cary, NC, U.S.A.

Results

Effect of MLCK inhibition on the α_1-adrenergic inotropy in mouse left atrium

To test whether the positive inotropic effect of α_1-adrenergic agonists in mouse left atrium is accompanied by increased phosphorylation of MLC-2a, we performed immunoblotting of shock-frozen mouse left atrial muscles after conventional and 2D gel electrophoresis. We generated a phospho-specific antibody against MLCK target sites of MLC-2a, serine 21, and 22. The antibody detects mono- and diphosphorylated MLC-2a, but does not crossreact with dephosphorylated MLC-2a (Figure 1a). Stimulation of contracting atria with phenylephrine (100 μM) for 10 min increased phosphorylation of MLC-2a by a mean of 67% (Figure 1b). Preincubation of atria with the MLCK inhibitor ML-7 (10 μM) reduced the phenylephrine-induced increase of MLC-2a phosphorylation to 23% (Figure 1b) and inhibited the inotropic effect of phenylephrine (Figure 1c). Maximal inhibition of the inotropic effect was 73% at 10 μM phenylephrine. In contrast, ML-7 did not affect the maximal contractile force exerted by addition of 10 μM isoprenaline to the same preparations (ML-7: 5.3 ± 0.4 vs Ctr: 5.5 ± 0.5 mN) or the concentration response to isoprenaline when studied alone (Figure 1d). Another MLCK inhibitor, ML-9, completely blocked the positive inotropic effect of phenylephrine (force at 100 μM 0.6 ± 0.1 mN vs 1.2 ± 0.2 mN at baseline; $n = 13$), but did not affect the force elicited by addition of 10 μM isoprenaline (ML-9: 3.0 ± 0.4 vs Ctr: 3.3 ± 0.3 mN). ML-7 and ML-9 decreased basal twitch tension relative to control by 13 and 29%, respectively. A third

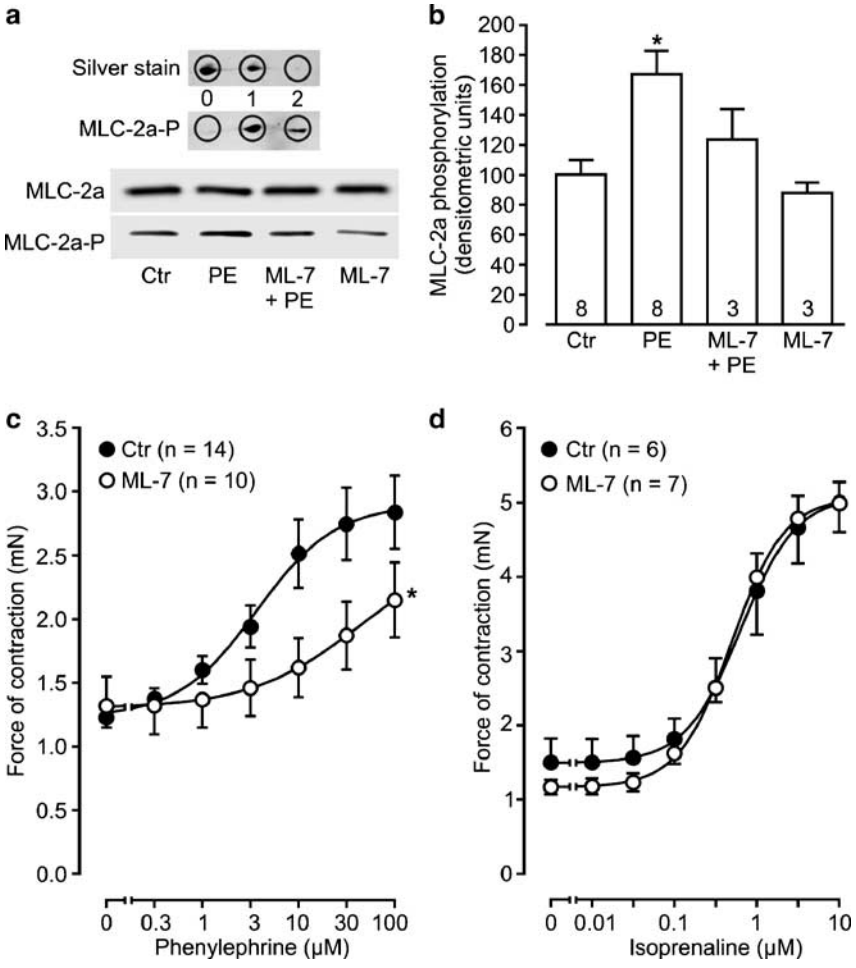

Figure 1 Effect of phenylephrine (PE) on MLC-2a phosphorylation and force of contraction in mouse atrium. (a) Top: silver stain and Western blot from the same region of a pH 4.5–5.5 IPG 2D gel with magnification of region of interest (~20 kDa) showing three spots separated from mouse left atrial preparation corresponding to the MLC-2a (0) and their mono- (1) and diphosphorylated forms (2). Bottom: representative 1D Western blot from mouse left atrial myofilament fraction shows phosphorylation of MLC-2a in the presence and absence of 100 μM PE after 10-min incubation (lower bands) and in the presence and absence of ML-7 (10 μM, 15 min incubation). Total MLC-2a protein (upper bands) served as loading control. (b) Summary of densitometric data (MLC-2a-P normalized to total MLC2a). Effect of MLCK inhibitor ML-7 (10 μM, 15 min incubation) on the positive inotropic effect of adrenergic agonists in isolated mouse left atria in the presence of 0.3 μM nadolol. (c) Concentration–response curve of PE. (d) Concentration–response curve of isoprenaline. *$P < 0.05$, significantly different from ctr.

and structurally different MLCK inhibitor, wortmannin (10 μM), had a similar effect as ML-7 and ML-9 on the positive inotropic effect of phenylephrine (-81% at 10 μM phenylephrine), but induced trigger-independent spontaneous beats and/or beat-to-beat changes in contraction amplitude in 33% of the atria (Supplement Figure 1). Phenylephrine concentration-dependently increased these irregularities to 72% at 100 μM. Only regularly beating atria were included in mean steady-state force calculations and statistics. No irregularities were noted in the presence of the vehicle DMSO.

Differential inotropic effects of PMA and α_1-AR stimulation in mouse left atrium

We examined the response of mouse left atria to the cell-permeable phorbol ester PMA. PMA mimicks the action of the second messenger DAG that is produced by α_1-adrenergic activation of phospholipase C (PLC). DAG is an endogenous activator of most PKC isozymes (Newton, 2004). PMA at 10 μM caused a triphasic inotropic response, with a small negative phase, a larger positive phase reaching a maximum after about 15 min, and a decline to baseline values over another 15 min (Figure 2a). The inotropic effect was completely absent in the presence of the PKC inhibitor chelerythrine (10 μM; 20 min incubation), indicating that the positive inotropic effect of PMA was indeed due to activation of PKC. Chelerythrine alone decreased basal force of contraction by 34%, but had no effect on the positive inotropic effects of phenylephrine or isoprenaline (Figure 2b and c). In mouse atrium, PMA has been shown previously to stimulate the release of noradrenaline (Musgrave & Majewski, 1989). As shown in Figure 2d, α- and β-adrenoceptor antagonists (prazosin at 1 μM and nadolol at 1 μM) completely abolished the positive inotropic effect of phenylephrine and right-shifted

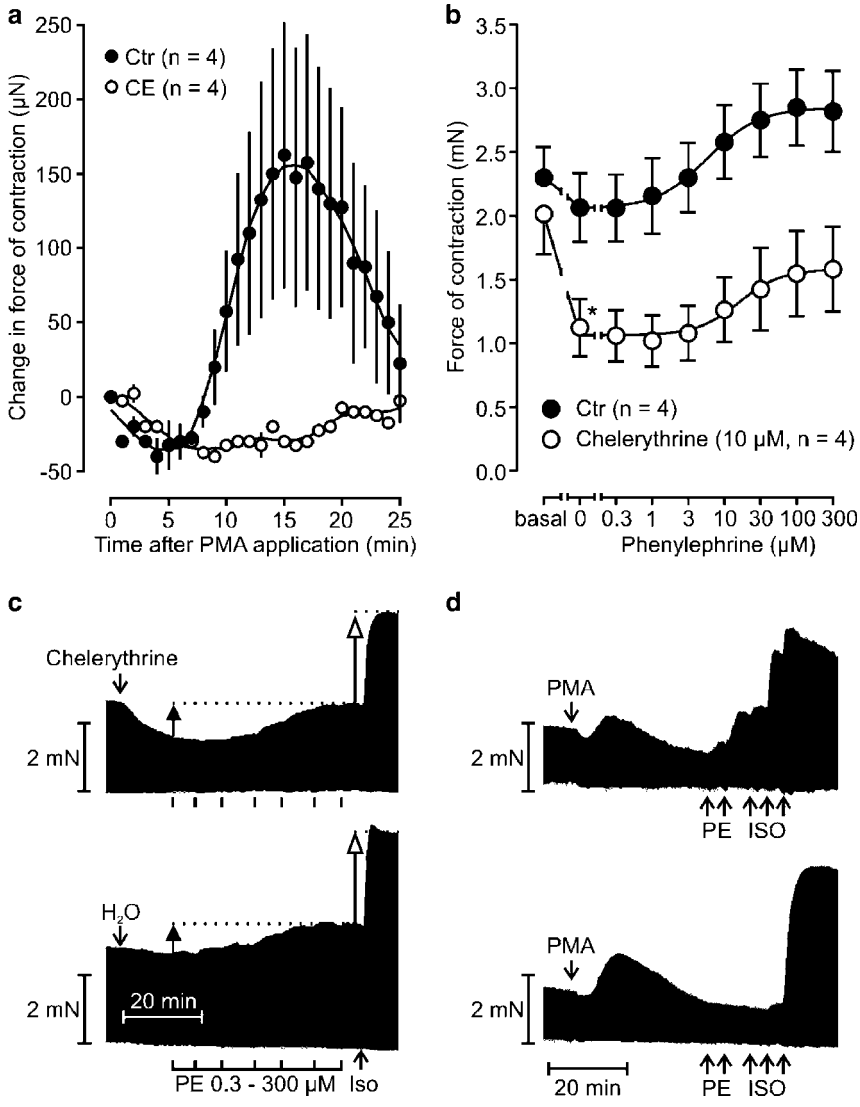

Figure 2 Effect of the PKC inhibitor chelerythrine (CE, 10 μM, 15 min incubation) on basal force and on inotropic effects of PMA and phenylephrine in isolated mouse left atria. (a) Comparison of twitch tension time courses after addition of PMA (10 μM at 0 min). Absolute values of basal force were 2.1 ± 0.3 mN (Ctr) and 1.1 ± 0.2 mN (CE). (b) Effect of chelerythrine on basal force and on the concentration–response curve of PE. (c) Representative original recordings. Dashed lines indicate maximal inotropic response of PE at 300 μM (closed arrowhead) and 10 μM isoprenaline on top of PE (open arrowhead). (d) Representative original recordings in the absence (top) and presence of 1 μM nadolol and 1 μM prazosin (bottom). Arrows indicate application of PMA, phenylephrine (PE, 10 and 100 μM), and isoprenaline (ISO, 0.1, 1, and 10 μM).

the concentration–response curve of isoprenaline, but did not affect the transient positive inotropic effect of PMA, suggesting that noradrenaline does not mediate the inotropic actions of PMA.

Role of PKCε in α_1-adrenergic inotropy

Stimulation of Gq-coupled receptors in human atrium leads to translocation of PKCε from the cytosol to myofilament structures and membrane (Kilts *et al*., 2005). To test whether phenylephrine activates PKCε in mouse atrium, we analyzed subcellular fractions by Western blot. In the membrane fraction, two immunoreactive PKCε bands were visible at almost identical intensity, both of which were slightly increased by the PKCε translocation activator peptide (1.2 ± 0.2-fold, $n=4$) and by phenylephrine (1.3 ± 0.2-fold; $n=4$). The phenylephrine-mediated increase of PKCε membrane expression was completely prevented by the PKCε translocation inhibitor (1.0 ± 0.2, $n=4$). Under control conditions, a single weak immunoreactive band was observed for PKCε in the myofilament fraction (Figure 3a). The translocation activator peptide did not reproducibly increase myofilament levels of PKCε (1.1 ± 0.1, $n=5$). In contrast, phenylephrine at 100 μM induced a robust translocation of PKCε to the myofilament fraction (1.5 ± 0.4-fold, $n=5$). Preincubation with the translocation inhibitor peptide blocked phenylephrine-induced translocation (1.1 ± 0.2, $n=3$). Importantly, inhibition of PKCε translocation enhanced the positive inotropic effect of phenylephrine by 40%, whereas the PKCε translocation activator had no effect (Figure 3b). These results indicate a negative regulatory role for PKCε in the inotropic effect of α_1-adrenergic agonists.

Role of the Ca^{2+}-dependent PKCα in α_1-adrenergic inotropy

Activation of cPKC by agonists that activate PLC-DAG signaling triggers translocation of cPKC from the cytosol to target structures. Western blot analyses were performed from the soluble (S) and the particulate (P) fraction of snap-frozen mouse left atria using a polyclonal PKCα antibody. As shown in Figure 4a, the P/S ratio increased from 1.0 ± 0.2 ($n=3$) in control samples to 1.7 ± 0.4 ($n=3$) upon incubation with cPKC translocation activator for 10 min. In contrast, phenylephrine at 100 μM did not induce PKCα translocation (1.0 ± 0.1, $n=3$). Not surprisingly therefore, inhibition of cPKC translocation had no effect on the concentration–response of phenylephrine (Figure 4b). However, activation of cPKCs by incubation with a cPKC activator peptide significantly decreased the inotropic response to the α_1-agonist (Figure 4b), but had no effect on basal force.

IP_3-induced Ca^{2+} release is not necessary for α_1-AR-mediated force of contraction in atrial trabeculae

Incubation of electrically paced mouse left atria with the IP_3 receptor antagonist 2-aminoethoxydiphenyl borate (2-APB) had no significant effect on basal force when compared to time-matched controls at 2 μM (Figure 5a), but decreased it at 20 μM (28 vs 11% in Ctr; Figure 5b). However, the compound had no effect on phenylephrine-stimulated force of contraction. Phenylephrine at 100 μM increased force to 175 ± 15% in the absence and to 185 ± 21% in the presence of 20 μM 2-APB. Thus, 2-APB concentration-dependently decreased basal force, but not the phenylephrine effect (Figure 5c).

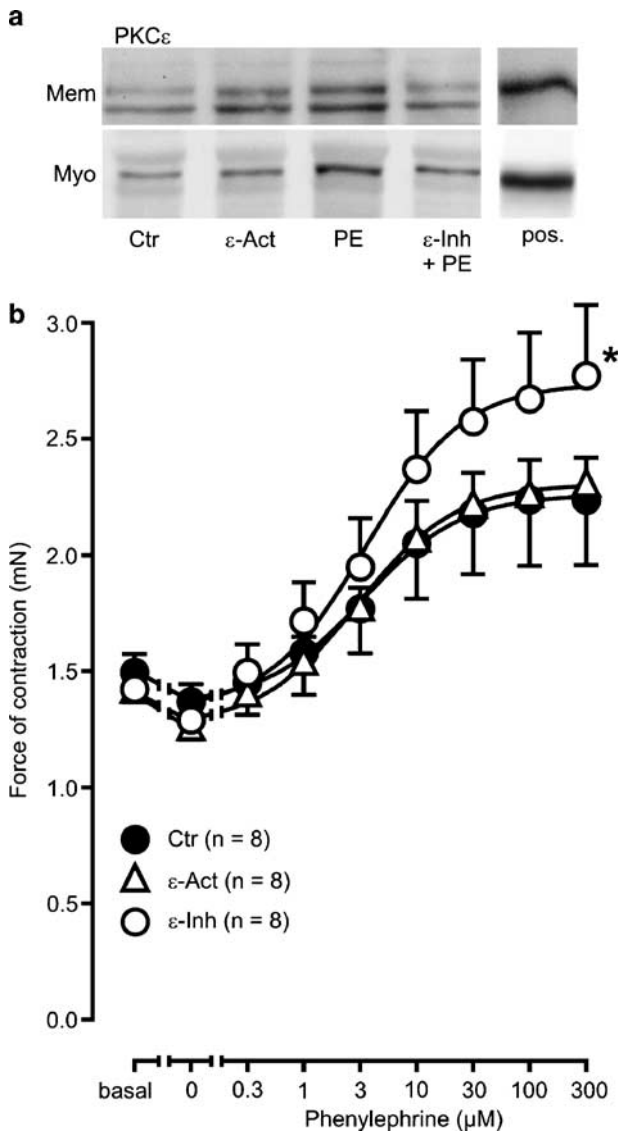

Figure 3 (a) Representative Western blots of membrane and myofilament fractions from mouse left atria after 10-min stimulation with 500 nM carrier peptide (Ctr), 500 nM PKCε translocation activator peptide (ε-Act), 100 μM PE), and 100 μM PE in the presence of 500 nM PKCε translocation inhibitor peptide (ε-Inh + PE) probed with anti-PKCε. Mouse brain homogenate (1 : 5 dilution) served as positive control. (b) Effect of PKCε activator and inhibitor peptides on basal force of contraction and on the concentration–response curve of PE. Basal value was taken before 10-min incubation with carrier peptide alone (Ctr, 500 nM), PKCε translocation activator (ε-Act, 500 nM), or PKCε translocation inhibitor (ε-Inh, 500 nM). *$P<0.05$ (ANOVA, % of predrug) concentration–response curve significantly different from Ctr.

Discussion

Based on data from this study in mouse (Figure 1) and from previous work by our group in human atria (Grimm *et al*., 2005) and by others in rat and rabbit preparations (Aoki *et al*., 2000; Andersen *et al*., 2002; Rajashree *et al*., 2005), it is now

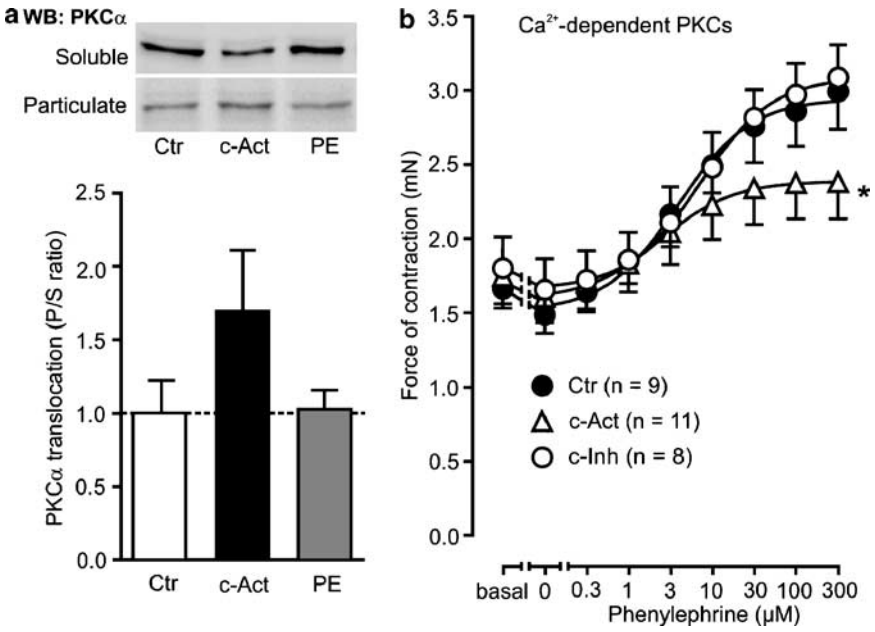

Figure 4 (a) Representative Western blot showing PKCα translocation from soluble to particulate fraction in left atria 10 min after stimulation with 500 nM carrier peptide alone (Ctr), translocation activator peptide of Ca^{2+}-dependent PKC isozymes (c-Act, 500 nM), or 100 μM PE. (b) Effect of translocation activator and inhibitor peptides of Ca^{2+}-dependent PKC isozymes on basal force of contraction and on the concentration–response curve of PE. Basal value was taken before 10-min incubation with carrier peptide alone (Ctr, 500 nM), translocation activator peptide (c-Act, 500 nM), or translocation inhibitor peptide (c-Inh, 500 nM). $*P<0.05$ (ANOVA, % of predrug) concentration–response curve significantly different from Ctr.

clear that a primary downstream target of α_1-AR/Gq signaling that mediates positive inotropy is MLC-2. The observations that levels of phosphorylated MLC-2 match protein concentrations of MLCK (Davis *et al*., 2001) and that phenylephrine-induced phosphorylation of MLC-2 is Ca^{2+}-dependent (Aoki *et al*., 2000) are consistent with the idea that modulation of MLCK activity by α_1-AR/Gq signaling is a major determinant of MLC-2 phosphorylation in cardiac muscle. In the present study, phosphorylation of MLC-2a was quantified by Western blot with an antibody that was specific for phosphorylated MLCK target sites, as confirmed by MALDI-TOF MS of proteolytic fragments of the three MLC-2a spots resulting from 2D electrophoresis (own unpublished observation). Inhibition of MLC phosphatase through the α_1-AR–RhoA–Rho-kinase pathway also modulates MLC-2 phosphorylation (Andersen *et al*., 2002; Grimm *et al*., 2005; Rajashree *et al*., 2005).

The α_1-AR–Gq pathway activates PLC (Gorelik *et al*., 1988), which produces DAG, an endogenous PKC activator. Earlier data showed that the effect of MLC-2 phosphorylation on myofibrillar Ca^{2+} sensitivity was potentiated in the presence of PKC (Clement *et al*., 1992), indicating that both MLCK and PKC could act as positive force modulators. Indeed, activation of endogenous PKC by PMA, a DAG analogue, or addition of purified PKC was associated with increased phosphorylation of cardiac MLC-2 (Noland & Kuo, 1993a; Venema *et al*., 1993; Damron *et al*., 1995) and a positive inotropic effect (MacLeod & Harding, 1991; Ward & Moffat, 1992). Others have shown that the positive inotropic response, observed after stimulation with phenylephrine or arachidonic acid, correlated in time with translocation of PKC to the myofilaments (Deng *et al*., 1997; Huang *et al*., 1997), and that pharmacological inhibition of PKC reduced the α_1-adrenergic positive inotropic effect in neonatal rat and rabbit myocardium (Endoh *et al*., 1993; Deng *et al*., 1997). However, our present data (Figure 2b) and data by others (Endou *et al*., 1991; Andersen *et al*., 2002) do not support a general role of PKC isoforms as mediators of the sustained positive inotropic effect of α_1-adrenergic stimulation. PMA had an acute positive inotropic effect in mouse atrium that could be blocked by a non-selective PKC inhibitor (Figure 2a), but this effect was not sustained. The acute effect of PMA is even more pronounced in rat atrium under the same experimental conditions (Supplementary Figure 2a), but it is absent in human atrial myocardium where phenylephrine has a pronounced and sustained positive inotropic effect (own unpublished data). Interestingly, when force of contraction returned to pre-drug values (30 min after application of PMA), the inotropic effect of phenylephrine was inhibited in the presence of PMA (Supplementary Figure 2b). In fact, selective activation of PKCα, the most abundant (>80%) Ca^{2+}-dependent PKC in adult mouse heart (Hahn *et al*., 2003), reduced the maximal phenylephrine-induced inotropic effect as well (Figure 4b), supporting previous data that PKCα mediates *negative* inotropic responses (Deng *et al*., 1997; Braz *et al*., 2004). However, it is important to note that we did not observe PKCα activation at 300 μM phenylephrine (Figure 4a), suggesting that signaling through this isozyme can be disregarded when studying inotropic effects of α_1-adrenergic agonists in mouse atrium.

In addition to PKCα, PKCδ and PKCε have been found most consistently in mammalian hearts (Das, 2003). Different PKC isozymes respond differently to endogenous activators, and activation of PKCδ and PKCε in response to phenylephrine has been reported under conditions that did not cause activation of PKCα (Puceat *et al*., 1994). It appears that also in human atrium only novel PKCs (δ and ε) are activated by acute α_1-adrenergic stimulation (Kilts *et al*., 2005). A principal

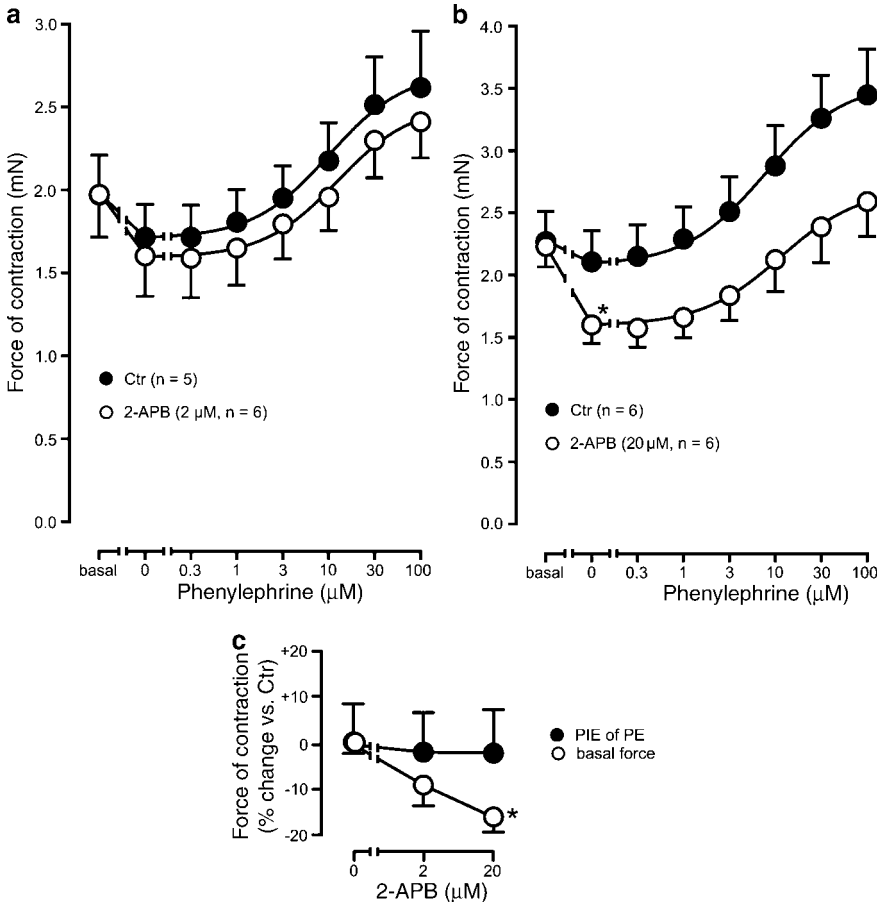

Figure 5 Comparison of basal force and inotropic effect of phenylephrine in isolated mouse left atria in the presence and absence of 2-APB. (a) Effect of 2-APB at 2 μM on the concentration–response curve of PE. (b) Effect of 2-APB at 20 μM on the concentration–response curve of PE. (c) 2-APB-induced change in basal force and the positive inotropic effect (PIE) of PE. $^*P<0.05$ (unpaired *t*-test, effect on basal force).

finding of the present study is that PKCε is translocated, that is, activated, by α_1-adrenergic stimulation and that PKCε negatively modulates the α_1-adrenergic inotropic effect in mouse atrium, because inhibition of PKCε increased the maximal effect of phenylephrine by 40% (Figure 3b). One may have expected that, conversely, activation of PKCε decreases the effect of phenylephrine, but this was not the case. This suggests that either the activating effect of phenylephrine on PKCε was already maximal or the effect of the activator peptide on PKCε translocation was too weak (Figure 3a). Another explanation could be that the activator peptide and phenylephrine activate PKCε in different intracellular compartments. Given that PKCδ and PKCε, but not conventional PKCs, are activated by phenylephrine, and considering that selective PKCε inhibition (Figure 3b), but not unselective PKC inhibition (Figure 2b), facilitated the inotropic response of phenylephrine, then opposing effects of PKCδ and PKCε on α_1-adrenergic inotropy could explain these findings. Indeed, opposing effects of PKCδ and PKCε in the heart have been reported (Chen *et al*., 2001a). Recently, the first report demonstrating a positive inotropic effect of PKCδ has been published (Kang & Walker, 2005): The phorbol ester PDBu caused a robust and sustained positive inotropic response in ventricular myocytes expressing PKCδ-GFP, whereas PDBu caused a sustained negative inotropic response (not significant) in myocytes expressing PKCε-GFP at a comparable expression level (about three-fold over endogenous PKCε). Interestingly, the negative inotropic response was paralled by PKCε translocation to the surface plasma membrane, whereas the positive inotropic effect was associated with PKCδ translocation to the Golgi apparatus. The Golgi has been suggested as an intracellular Ca^{2+} store in some cell types (Wuytack *et al*., 2003), but evidence for a role in cardiac contraction is lacking. In mouse right ventricular strips, both phenylephrine and PDBu produce a negative inotropic effect that seems to be mediated by activation of PKCδ (Varma *et al*., 2003). In this context, it is interesting to note that α_1-adrenergic stimulation exerts a negative inotropic response in isolated mouse ventricular muscle strips, but an increase in force in the intact mouse heart (Turnbull *et al*., 2003), similar to the response in isolated atria. A limitation of our study is that the selective PKCδ inhibitor peptide was not available for investigational use during the time of our experiments. Further studies are needed to clarify the role of PKCδ in α_1-adrenergic inotropy and to identify the phenylephrine-activated PKC target(s).

What are the mechanisms of the negative modulation of the phenylephrine effect by PKCε? Phenylephrine induced translocation of PKCε to the myofilament and membrane fractions of atrial muscle (Figure 3a). A very attractive PKCε target in the membrane fraction is the sarcolemmal L-type

Ca^{2+} channel. Phenylephrine increases Ca^{2+} influx through L-type Ca^{2+} channels (Liu & Kennedy, 1998), and activation of PKCε can inhibit L-type Ca^{2+} currents in the heart (Hu *et al*., 2000). A possible target in the myofilament fraction is troponin I, which contains PKC phosphorylation sites and whose phosphorylation leads to reduced contractility (Noland & Kuo, 1993a, b). Several groups have demonstrated phosphorylation of cardiac troponin I and other myofibrillar proteins by α_1-adrenergic stimulation (Clement *et al*., 1992; Damron *et al*., 1995; Huang *et al*., 1997; Noland & Kuo, 1993b; Kilts *et al*., 2005). Finally, the Ca^{2+}/calmodulin-dependent activity of smooth muscle MLCK decreases upon phosphorylation by PKC, at least *in vitro* (Nishikawa *et al*., 1985). The only known target of MLCK is MLC-2 (Gallagher *et al*., 1997) and smooth muscle MLCK is the only classic MLCK detected in purified cardiac myocytes (Herring *et al*., 2000).

The precise signaling pathway by which α_1-AR/Gq mediates activation of MLCK in atrial myocardium remains unidentified. MLCK activation requires Ca^{2+}. Miyamoto *et al*. (2000) reported that inhibition of IP_3 receptors by xestospongin C shifted the positive inotropic effect of phenylephrine in guinea-pig papillary muscles to the right and reduced it by about 60%. The inhibitory effect of xestospongin C was abolished in the presence of ryanodine, indicating that the IP_3-induced Ca^{2+} release derives from the same Ca^{2+} pool as the Ca^{2+}-induced Ca^{2+} release through ryanodine receptors. Whereas these data indeed suggest a contribution of IP_3 to the positive inotropic effect of phenylephrine, they also show an important IP_3-independent component. As mentioned above, functional differences exist between atrial and ventricular myocytes. In this context, it should be pointed out that there is evidence that atrial IP_3 receptors are co-located to ryanodine receptors at the sarcoplasmic reticulum (Mackenzie *et al*., 2002), whereas ventricular IP_3 receptors have been identified primarily at the nuclear envelope (Bare *et al*., 2005). In our study on atrial preparations, we observed that 2-APB at a very high concentration of 20 μM decreased basal force and shifted the concentration–response curve of phenylephrine downward, resulting in a decrease of maximal force at 100 μM phenylephrine (Figure 5). However, 2-APB did not change the relative (normalized to basal force) inotropic effect of phenylephrine (Figure 5c). Taken together, the present data suggest that IP_3-mediated Ca^{2+} release is not essential for the inotropic effect of α_1-adrenergic agonist in the heart.

A recent report using an IP_3 receptor 2 (IP_3-R2) knockout mouse model convincingly showed that the stimulatory effects of endothelin-1 on SR Ca^{2+} release and spark frequency were completely absent in IP_3-R2-deficient atrial myocytes (Li *et al*., 2005). In fact, IP_3-R2 deficiency mimicked the presence of 2-APB, which was used as a membrane-permeable pharmacological inhibitor of the IP_3-R that does not affect Ca^{2+} release from the ryanodine-sensitive Ca^{2+} store (Maruyama *et al*., 1997). These apparently discrepant data could have several possible explanations: (i) The mechanisms of action of phenylephrine and endothelin-1 differ. An argument for this assumption is that endothelin-1 exerts a prominent positive inotropic effect in mouse atria when applied on top of a saturating concentration of phenylephrine (Supplement Figure 3b), and *vice versa* (Supplement Figure 3c). (ii) A highly compartmentalized, 2-APB-insensitive, phenylephrine- and endothelin-induced Ca^{2+} increase exists that is sufficient to increase force of contraction *via* MLCK, but too small to be detected as a change in global $[Ca^{2+}]_i$ transients as determined in the study by Li and colleagues. Contraction experiments in the IP_3-R2-deficient mouse could answer this question, but have not been reported yet. (iii) 2-APB may not permeate whole heart tissue at a sufficient concentration to block IP_3-R. However, the strong effects of 2-APB at 20 μM on basal force and the fact that 2-APB has been used successfully to inhibit IP_3 receptor in isolated heart (Gysembergh *et al*., 1999) would argue against this idea. Finally, reduction of basal force could result from a nonspecific leak of Ca^{2+} from intracellular stores (Missiaen *et al*., 2001), inhibition of SERCA activity (Bilmen *et al*., 2002), or other nonspecific effects of 2-APB.

In conclusion, the results presented herein extend previous studies by providing evidence that MLCK-mediated phosphorylation of MLC-2a is also required for the positive inotropic effect of phenylephrine in mouse atrium. The major new finding is that PKCε negatively modulates this effect, whereas Ca^{2+}-dependent PKC isozymes appear not to be involved. The results also argue against a major role of the IP_3 receptor in this pathway.

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