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Protein kinase C and phenylephrine induce comparable decrease in calcium sensitivity in skinned adult rat cardiac myocytes

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Kulcsszavak:
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Aims: *The in vivo regulation of cardiomyocyte function by α_1-adrenergic receptor agonist phenylephrine (PE) has not been clarified yet. It has been suggested that PE acts partly via protein kinase C (PKC)-mediated pathway. We aimed to compare the mechanism of action of PE and PKC on myofilament function.*
Methods: *Isometric force and rate of force redevelopment (K_{tr}) were studied in adult rat single permeabilized cardiomyocytes at various Ca^{2+} concentrations before and after treatment with the catalytic subunit of PKC (PKC-M; 0.1 and 0.2 U/ml 40 min) and after stimulation with PE. In order to reveal PKC-dependent phosphorylation of contractile proteins two-dimensional gel electrophoresis was performed.*
Results: *PKC-M induced a dose-dependent decrease in Ca^{2+} sensitivity with ΔpCa_{50}= 0.049±0.003 at low and ΔpCa_{50}=0.089±0.005 at high activity. Respectively, similar decreasing tendency was observed in maximal force (ΔF_{max}=16.5±2.8 and 21.2±2.3%), in passive force (ΔF_{pas}=7.6±6.6 and 38.6±6.6%), in myofilament cooperativity (ΔnHill= 0.40±0.06 and 0.96±0.19) and in K_{tr} (ΔK_{tr}-Forcesslope=3.86±0.85 and 4.03±0.69). PE treated cardiomyocytes also possessed decreased Ca^{2+} sensitivity (pCa_{50}=5.68±0.01) compared to controls (pCa_{50}=5.77±0.02), but unlike PKC-M treated cells, myofilament cooperativity was higher in the PE group (nHill=3.23±0.06) versus control group (nHill=2.89±0.10). There was no significant change in maximal force, in passive force and in K_{tr}. Neither PKC-M nor PE altered the phosphorylation status of a potential phosphorylation target, myosin light chain 2 (MLC-2).*
Conclusion: *The decrease in Ca^{2+} sensitivity following PE treatment is comparable to the change observed after PKC-M administration. However the overall PKC-M effects were more pronounced, probably because PKC-M mimics the effects of a wider range of PKC isoforms than are activated during PE stimulation. MLC-2 phosphorylation plays no potential role in this process.*

A protein-kináz-C és a phenylephrin hasonló Ca^{2+}-érzékenység csökkenéshez vezet izolált patkány szívizomsejteken
Célkitűzés: *A szelektív α_1-adreneg receptor agonista phenylephrin (PE) myocardiumra gyakorolt hatásáról keveset tudunk. Egyes feltételezések szerint a PE hatásai részben protein-kináz-C (PKC)-mediált szignáltranszdukciós útvonalon keresztül valósulnak meg. Célkitűzésünk az volt, hogy összefüggést keressünk a PE és PKC miofilamentumokon megvalósuló hatásmechanizmusa között.*
Módszerek: *PKC katalitikus alegységgel (PKC-M; 0,1 és 0,2 U/ml 40 perc), illetve PE-vel kezelt felnőtt patkány izolált permeabilizált szívizomsejteken vizsgáltuk a különböző Ca^{2+}-koncentráció jelenlétében generált izometrikus erőt és az erőgenerálódás kinetikáját (K_{tr}). A kontraktilis fehérjék PKC-függő foszforilációjának vizsgálatára 2-dimenziós gélelektroforézist végeztünk.*
Eredmények: *A PKC-M aktivitás-függő módon csökkentette a Ca^{2+}-szenzitivitást, ΔpCa_{50}=0,049±0,003 volt alacsony, illetve ΔpCa_{50}=0,089±0,005 magas aktivitás mellett. Hasonlóan, aktivitásfüggő csökkenést mutatott a maximális erő (ΔF_{max}=16,5±2,8, illetve 21,2±2,3%), a passzív erő (ΔFpas=7,6±6,6, illetve 38,6±6,6%), a miofilament kooperativitás (ΔnHill=0,40±0,06, illetve 0,96±0,19) valamint a K_{tr} is (ΔK_{tr}-Forcesslope=3,86±0,85, illetve 4,03±0,69). A cardiomyocyták PE-vel történő kezelése is csökkent Ca^{2+}-érzékenységet (pCa_{50}=5,68±0,01) eredményezett a kontrollhoz képest (pCa_{50}= 5,77±0,02), azonban a PKC-M hatástól eltérően, PE hatására a miofilament kooperativitás növekedett (nHill=3,23±0,06) a kontrollhoz viszonyítva (nHill=2,89±0,10). Ellenben a maximális generált erő, a passzív erő és a K_{tr} nem változott szignifikánsan. Sem a PKC-M sem a PE nem volt hatással a miozin könnyű lánc 2 (MLC-2) foszforilációs állapotára, mely a PKC egyik potenciális foszforilációs targetje.*
Következtetések: *A PE hatására kialakuló Ca^{2+}-érzékenység csökkenés hasonló ahhoz, amit a PKC-M-kezelés indukál. Azonban a PKC-M hatásai összességében sokkal kifejezettebbek. Ennek egyik lehetséges magyarázata, hogy a PKC-M egyszerre több PKC izoforma hatását utánozza, mint amelyek PE stimulációkor aktiválódnak. Ebben a folyamatban a MLC-2 foszforilációnak nincs szerepe.*

Introduction

Protein kinase C (PKC) in the heart has been implicated in the regulation of force development via phosphorylation of cardiac contractile proteins and proteins involved in calcium homeostasis and ion conductance (1, 2). Mammalian PKC is a large family of Ser/Thr protein kinases consisting of several isotypes encoded by various genes (3). It has been hypothesized that the individual isozymes may subserve distinct biological function; however the precise mechanism by which PKC affects cardiac myofibrillar function is ambiguous. Our knowledge on PKC-mediated contractile changes in the heart is mainly based on in vitro experiments using direct, non-specific cardiac PKC activator phorbol esters. 12-deoxyphorbol 13 isobutyrate 20 acetate (DPBA) treatment led to a decrease in isometric twitch force, in Ca^{2+} sensitivity and in nHill in human trabeculae carneae (4), phorbol 12-myristate 13-acetate (PMA) decreased the contractility of rat papillary muscle (5), but bovine brain PKC and 12-O-tetradecanoylphorbol-13-acetate (TPA) induced a decrease in maximal MgATPase activity without change in Ca^{2+} sensitivity (6). *Ward et al.* (7) found both, positive and negative inotropic effect after PMA treatment depending on the PMA concentration in Langendorff-perfused guinea pig hearts. *Pi et al.* (8) reported increased Ca^{2+} sensitivity of isometric tension and of ATPase activity with concomitant inhibition of maximum ATPase rate in mouse heart. In human skinned cardiac myocytes *van der Velden et al.* (9) have shown decreased Ca^{2+} sensitivity after treatment with the catalytic subunit of PKC (PKC-M), while maximal force was not significantly altered.

PKC activation has been directly demonstrated to occur in response to α_1-adrenergic stimulation (1, 10–15) and it has been observed that the enzyme is translocated to the myofibrils (16). However, the role of PKC in mediating the contractile changes of cardiac muscle during alpha1-adrenergic stimulation presently appears also controversial. α_1-adrenoceptor stimulation (i.e. phenylephrine (PE)) has been repeatedly shown to exert positive inotropic effect (5, 12, 17–21) and to increase myofilament Ca^{2+} sensitivity in skinned single cardiac cells (22–25) and in intact muscle strips of rabbit (18). In contrast, others (26–29) found negative inotropic effect in adult rat and mouse ventricular cardiomyocytes due to PE stimulation. *Pyle et al.* (30) reported decrease in maximal isometric tension and stiffness in mouse papillary fiber boundles but no change in Ca^{2+} sensitivity or nHill was observed. *Suematsu et al.* (31) also failed to detect change in Ca^{2+} sensitivity in isolated canine cardiomyocytes. Furthermore, in skinned rat cardiomyocytes *Strang et al.* (32) detected no change in maximal and resting tension in addition to unchanged Ca^{2+}sensitivity due to α_1-adrenergic stimulation. It is tempting to speculate that upon PE exposition, changes in myofibrillar function are attributable, at least in part, to PKC activation and consecutive contractile protein phosphorylation. Cardiac Troponin I (cTnI), cardiac Troponin T (cTnT) (9, 33, 34), ventricular myosin light chain-2 (MLC-2) (34), and Myosin Binding Protein C (MyBPC) (6, 35) are among the putative intracellular PKC targets but their cue in regulating myofibrillar Ca^{2+} responsiveness is obscure.

Based on the observations above, we designed a study to compare the effects of the active PKC and a specific receptor-mediated PKC activator PE on myofibrillar Ca^{2+} responsiveness. PKC activity was mimicked by treating single permeabilized cardiomyocytes from rat hearts with the catalytic subunit of PKC (PKC-M). PKC-M is no longer associated with the autoinhibitory regulatory domain therefore is persistently active (36). On the other hand, we tested whether consecutive changes in myofibrillar protein phosphorylation takes place during cell treatment; that is believed to participate in the translation of signals upon PKC activation.

Methods

Myocyte isolation

Adult male Wistar rats were used for the experiments. The protocols were approved by the Committee of the Ethics of Animal Experiments. To obtain mechanically isolated left ventricular single cardiomyocytes the animals were anaesthetised with sodium pentobarbital (80 mg/kg ip.) and 1000 U heparine was added intravenously. The heart was quickly removed and perfused with 2,3-butanedione monoxime (BDM)-containing Ringer's solution (in mM): $MgCl_2$ 1.2, $CaCl_2$ 1.0, KCl 4.8, NaCl 118, KH_2PO_4 2, pyruvate 5.0, glucose 11, insuline 1, HEPES 25 (pH7.4), BDM 30. The left ventricle was dissected and processed immediately as described previously (37).

During the isolation the tissue was kept on ice. To remove all membranous structures and endogenous kinases/phosphatases the myocytes were skinned at room temperature by means of 0.3% Triton X-100 (5 minutes) and kept on ice until further use in isolating relaxing solution containing (in mM): free Mg^{2+} 1, KCl 145, EGTA 2, ATP 4, imidazole 10 (pH 7.0). In case of enzymatically isolated myocytes, cells were washed in oxygenated Ringer's solution to get rid of the Medium. 50% of the cells were saved for protein analysis the rest was divided into two aliquots. The cells were resuspended in oxygenated Ringer's solution (control group) or phenylephrine (10 μmol/l: Sigma) and atenolol (1 μmol/l: ICN, ß-blocker) -containing

oxygenated Ringer's solution (PE-stimulated group). Both groups were incubated for 5 minutes at 37 °C then quickly skinned at room temperature in 0.5% Triton X-100-containing isolating relaxing solution (5 minutes) to preserve myofibrillar protein phosphorylation level. Cells were kept on ice until further use.

Experimental protocol

Permeabilized cardiomyocytes were mounted in the experimental set-up built on a Zeiss inverted microscope as described (37). Briefly, a single myocyte was attached between a force transducer and a piezoelectric motor with silicon adhesive glue. Average sarcomere length (SL) was 2.23±0.01 μm. The myocyte was transferred from the mounting area to the stage of the microscope and isometric force was measured by transferring the cell from relaxing solution to activating solutions at 15 °C. The composition of relaxing and activating solutions was calculated as described by *Papp et al.* (38). The pCa, i.e. $-\log_{10}$ [Ca^{2+}] of the relaxing and maximal [Ca^{2+}]activating solution were 9 and 4.5, respectively. Solutions with submaximal Ca^{2+} concentration were obtained by mixing the maximal [Ca^{2+}] activating and relaxing solutions. Maximal active isometric force per crossectional area was calculated from difference between the zero force level and the peak isometric force following correction for passive force. Submaximal force values obtained at pCa>4.5 were normalised to the interpolated control (F_0) values measured at pCa4.5. Experiments were discarded if the F_0 at the end of the measurement was less than 80% of F_0 at the beginning of the experiment, assuming a linear decrease in maximal force with every activation. Cells with a starting F_0<20 kN/m^2 or a change in sarcomere length during the experiment >0.2 μm were also discarded. The rate constant of force redevelopment (K_{tr}) was estimated using the slack test method as described previously (39). Briefly, when the steady level of force was developed in activating solution the myocyte was rapidly slackened and re-stretched by 30% of its length. Due to slackening the force drops to zero and upon re-stretch force redevelopment occurs to the initial steady level. The force redevelopment was fitted with a single exponential at pCa values from 4.5 to 5.6 At low Ca^{2+} concentration (pCa>5.6) force redevelopment could not be fitted accurately due to the low signal-noise ratio.

The protocol was performed on 25 enzymatically-isolated cells (control group n=11, PE group n=14) and on 20 mechanically isolated cells (control and PKC-M). After a first force-pCa series the mechanically isolated cells were incubated on the set-up for 40 minutes at 20 °C in 6 mM DTT-containing relaxing solution with 0.1 or 0.2 U/ml PKC-M (Sigma P-1909 batch No.50K1178) or without PKC-M (served as time control), n=7, n=9 and n=4 respectively. After incubation the force measurement protocol was repeated.

In vitro protein phosphorylation and Two-dimensional gel electrophoresis

Small pieces of rat left ventricular tissue (about 5 mm^3) were separated from the rat hearts used for mechanical isolation and quickly frozen in liquid nitrogen and freeze-dried at –80 °C. Tissue homogenate was made in relaxing solution containing 500 μg/ml Pefabloc and 10 μl/ml Triton X-100, with 10 mg/ml total protein concentration and incubated overnight at 4 °C. Following centrifugation at 15 000 r/m for 10 minutes at room temperature the pellet was resuspended in 500 μg/ml Pefabloc-containing relax and was centrifuged again. The pellet was resuspended in relaxing solution containing 500 μg/ml Pefabloc and 500 μg/ml Creatine Phosphokinase, and was incubated together with 1 μl/500 μg protein Phosphatase Inhibitor Coctail I in the presence of 2 or 4U/ml PKC or in the absence of PKC at 20 °C for 60 minutes or at 37 °C for 120 minutes. The reaction was stopped with adding 2D Sample buffer. Samples (15 μg) were loaded on immobiline strips with pH gradient of 4.5 to 5.5 (Amersham Pharmacia Biotech). In the second dimension proteins were separated by sodium dodecyl sulfate (SDS)-PAGE (40) on gels with 12% total acrylamide concentration. Proteins were visualised by silver staining (41). Gels were analysed using Image Quant Software (Molecular Dynamics) (42).

The phosphorylation status of MLC-2 of the control and PE-stimulated enzymatically isolated cells was also determined. Before loading the immobiline strips the cells were treated with TCA to fixate the phosphorylation status of the myofibrillar proteins immediately after the stimulation protocol.

Untreated left ventricular rat tissue (directly frozen in liquid nitrogen) served as reference sample.

Data analysis

Force-pCa relations were fitted by a non-linear fit procedure to a modified Hill equation:

$$F(Ca^{2+})/F_0 = [Ca^{2+}]^{nH}/(Ca_{50}^{nH} + [Ca^{2+}]^{nH})$$

F is the steady state force, F_0 denotes the steady force at saturating [Ca^{2+}], nH reflects the steepness of the relationship, and Ca_{50} represents the midpoint of the relation.

All values are presented as means±S.E.M. of n experiments. Differences were tested by means of paired Student's t-test, unpaired Student's t-test and Mann–Whitney-test when applicable, P<0.05 was chosen to indicate statistical significance.

Results

Effect of PKC on force production

Force recordings at maximal (pCa 4.5) and at submaximal (pCa 5.4) Ca^{2+} concentrations from permeabilized cardiomyocytes before and after PKC treatment are shown in *Fig.1.* Initial maximal isometric tension (F_{max}), obtained at saturating Ca^{2+} concentration was 41.9±3.5 kN/m^2. Passive force per cross-sectional area (F_{pas}), obtained in relaxing solution (pCa 9) amounted to 2.1±0.1 kN/m^2 (n=20). PKC treatment (0.1 U/ml, n=7; 0.2 U/ml, n=9) resulted in a decrease in F_{max} and F_{pas} in an activity-dependent manner. As illustrated in *Fig. 2B,* relative force development at maximal Ca^{2+} dropped to 83.5±2.8% and 78.8±2.3% after low dose PKC (0.1 U/ml, closed circles) and high dose PKC (0.2 U/ml, closed squares), respectively. PKC treatment decreased F_{pas} with 7.6±6.6% (0.1 U/ml) and 38.6±6.6% (0.2 U/ml). The decrease was significant only at the higher PKC activity.

Force-pCa relations were obtained by exposing the cells to different Ca^{2+} concentrations. Data were fitted with a modified Hill equation. The average force-pCa relationship obtained before PKC incubation is shown in panel A of *Fig. 2*. The steepness of the relation (nHill) was amounted to 3.67±0.10. Ca^{2+} sensitivity of force (pCa_{50}) was 5.63±0.01. The recordings in *Fig. 1* show that after PKC treatment the decline in force development was even larger at submaximal Ca^{2+} than at maximal Ca^{2+} concentration. In addition the steepness was found to be decreased following incubation with PKC, both at 0.1 and 0.2 U/ml PKC activity (ΔnHill=0.40±0.06 and 0.96±0.20, respectively). Indeed, upon 0.1 U/ml PKC incubation the curve was significantly shifted to higher [Ca^{2+}] (i.e. lower pCa) (ΔpCa_{50}=0.049±0.003), which indicates a decrease in myofibrillar Ca^{2+} sensitivity. This decrease in Ca^{2+} sensitivity was even more pronounced at higher PKC activity (0.2 U/ml; ΔpCa_{50}=0.089±0.005).

Since F_{max} was decreased after PKC treatment, force development at submaximal Ca^{2+} concentration should be scaled for the decrease in F_{max}. The resulting force-pCa curves *(Fig 2; panel B)* reveal a marked decline in Ca^{2+} responsiveness upon PKC.

The K_{tr} values obtained at pCa values ranging from 4.5 to 5.6 before and after PKC incubation are summarized in panel C of *Fig. 2.* The low dose of PKC (0.1 U/ml) had no influence on the K_{tr} value measured at pCa 4.5 and 5.6 but a decrease in K_{tr} was observed at 5.0, 5.2 and 5.4 ($P<0.05$). The high dose of PKC (0.2 U/ml) caused an overall decrease in K_{tr} regardless of pCa. When plotted K_{tr} as a function of force at each measured cell, an approximately linear relation was found between K_{tr} and relative force (graph not shown). Statistical analyses revealed a significant decrease in the regression line of 0.1 U/ml PKC treated myocytes (3.86±0.85). This decrease was even more prominent at higher PKC activity (4.03±0.69).

In control experiments (n=4), when PKC was omitted from the incubation medium, no significant change was observed in any of the measured parameter (ΔF_{max}=90.11±2.32%, ΔFpas=101.25±3.64%, ΔpCa_{50}= 0.011±0.0025, ΔnHill=0.40±0.08, ΔK_{tr} regr.= 1.24±0.51).

Effect of PE on force production

Enzymatically-isolated rat cardiomyocytes incubated in Ringer without PE (control) or with PE (PE) were

Figure 1. Isometric force development of mechanically isolated myocytes at maximal (A) and at submaximal (B) Ca^{2+} concentration before and after incubation with PKC (0.2 U/ml). The abrupt changes in force are due to the cell transfer from relaxing solution into activating solution and back. The reduction in force when the steady state level had been reached is caused by the slack test (see Methods) performed to measure the rate of force redevelopment (K_{tr})

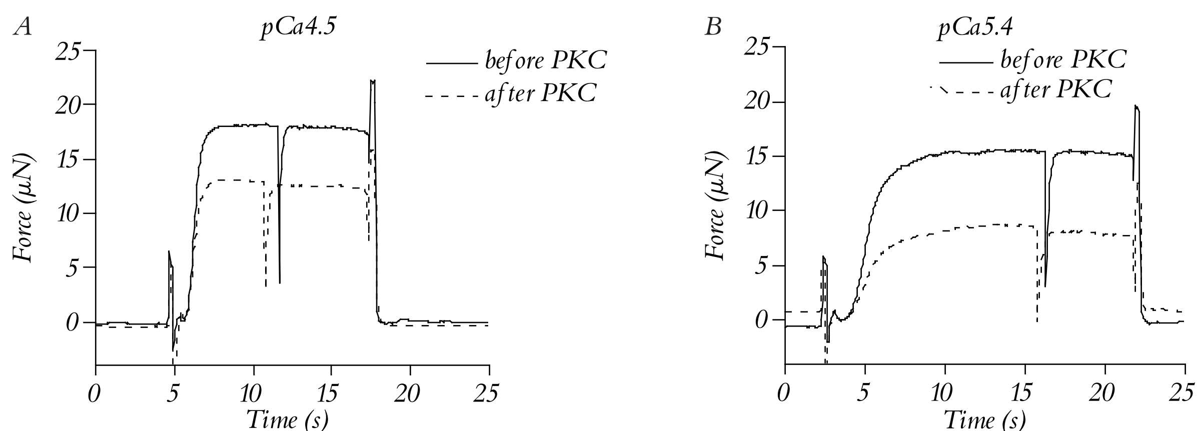
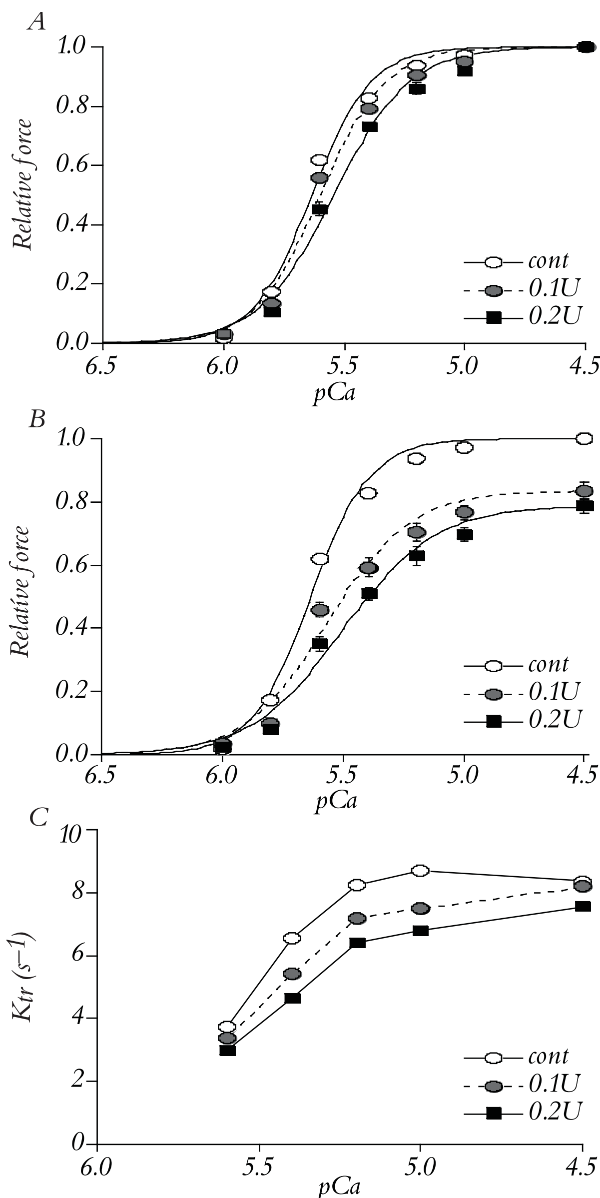


Figure 2. (A) Isometric force-pCa relations of mechanically isolated myocytes before (control; n=20) and after treatment with PKC (0.1 and 0.2 U/ml, n=7 and 9 respectively). Data were fitted to the mean values. Changes in Ca^{2+}-sensitivity of force arose 0.049±0.003 at low dose and 0.089±0.005 at high dose of PKC, the decrease in steepness was 0.40±0.06 and 0.96±0.19, respectively. (B) Force values of treated cells are given as fractions of the last recorded maximal force at saturating (Ca^{2+}) immediately before PKC treatment and of the maximal force obtained immediately after PKC treatment. Absolute forces were significantly lower following PKC treatment in an activity-dependent manner (P<0.05). K_{tr} values before and after PKC incubation are indicated as a function of pCa (C). Error bars visible only when larger than symbol size



permeabilized, and force measurement and slack test were performed (n=14 and 11; respectively).

F_{max} did not differ significantly between the control (40.7±4.5 kN/m^2) and PE (41.2±3 kN/m^2) group. In addition no difference was found in Fpas between the two groups (2.5±0.3 and 2.0±0.1 kN/m^2 in control and PE, respectively).

Average force-pCa curves are presented in the panel A of *Fig. 3.* PE treatment shifted the force-pCa curve to the right (pCa_{50}=5.68±0.01), indicating that Ca^{2+} sensitivity of the PE myocytes was decreased compared to controls (pCa_{50}=5.77±0.02). The steepness of the curve was slightly, though significantly higher in the PE treated myocytes (3.23±0.06) than in the control cells (2.89±0.10). It was shown previously that beta-blocker alone does not alter Ca^{2+} sensitivity of isometric force (32, 43).

K_{tr} at high Ca^{2+} concentration tended to be lower in PE treated than in the control group, though not significantly. However, at lower Ca^{2+} concentration (pCa 5.4 and 5.6) K_{tr} values were significantly lower in the PE group (P<0.05) in comparison to the control group.

PKC and PE effect on protein phosphorylation

2D-gel electrophoresis and silver staining were performed on rat tissue homogenates (untreated reference sample, Relax incubated and PKC treated samples) and on a suspension of cardiomyocytes from the control and the PE group.

Ventricular MLC-2 in the rat heart consists of two isoforms (LC-2 and LC-2*), with the dominancy of LC-2. Analyses of immediately TCA treated tissue (reference sample) indicated that both LC-2 isoforms were partly phosphorylated (LC-2P and LC-2*P) *(panel A and D of Fig. 4)* under basal conditions. The distribution of the isoforms was in %: LC-2: 48.1, LC-2*: 10.8, LC-2P: 38.2 and LC-2*P: 2.9. The homogenate we used for the PKC experiments was pretreated with Triton in relaxing solution overnight, which shifted the isoform composition towards the dephospho forms, LC-2*P was completely, while LC-2P was almost completely dephosphorylated. PKC treatment (2 U/ml) of the homogenate at 20 °C for 1 hour did not alter phosphorylation of the LC-2 isoforms *(panel C and D)* compared to control incubation *(panel B)*. Even if we increased PKC activity to 4 U/ml, temperature to 37 °C and prolonged the time of incubation to two hours, the phosphorylation pattern was similar to the control incubations (panel B and D).

Densitometric analyses of the 2D-gels from the Ringer incubated and the PE treated cells are shown in panel D. In the enzymatically isolated rat myocytes, incubated only in Ringer solution a low level of LC-2 phosphorylation remained as compared to basal LC-2

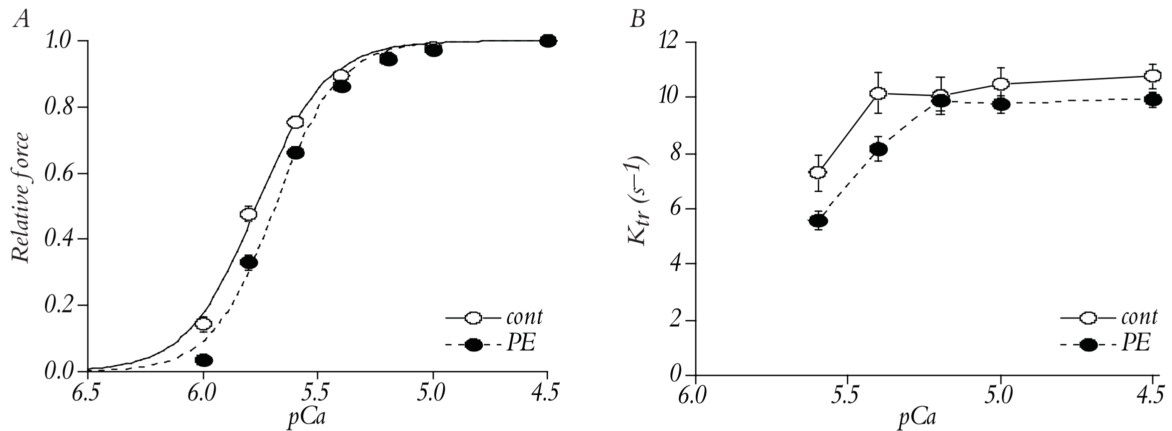


Figure 3. (A) Force-pCa relations of control (Ringer, n=11) and PE treated (n=14) cells. Submaximal forces were normalised to the maximal force measured at saturating [Ca^{2+}] PE treatment significantly decreased Ca^{2+} sensitivity (pCa_{50}=5.69±0.01) versus control (pCa_{50}=5.77±0.02) in enzymatically isolated rat cardiomyocytes and caused a slight increase in nHill. pCa dependence of K_{tr} in the Ringer and the PE group is shown in panel B. Values are expressed as Mean±S.E.M. Error bars are shown only when larger than symbol size.

phosphorylation. PE stimulation did not induce LC-2 phosphorylation.

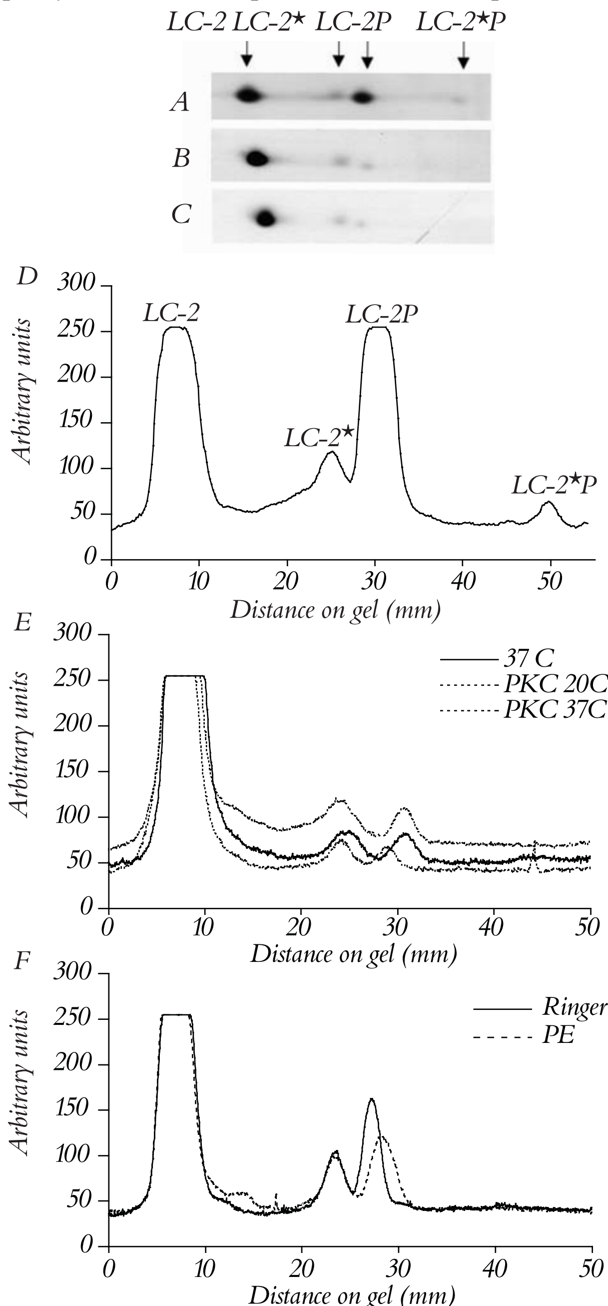
Discussion

Despite the increasing number of publications discussing the effect of PKC on myocardial function, our knowledge in molecular understanding is incomplete. In the present study we provide evidence that in rat cardiomyocytes both PKC-M and the PKC activator PE decrease the Ca^{2+} sensitivity of isometric force, to similar extent. Since PKC-M and PE results in similar decrease in Ca^{2+} sensitivity of force, we assume that PE effects are mediated through PKC activation. PKC effects rely on protein phosphorlyation. In our experimental setting, theoretically the active catalytic PKC subunit directly can phosphorylate the accessible substrates that can alter myocyte function, while upon PE stimulation, PKC gets translocated to incompletely defined intracellular target(s) also leading to protein phosphorylation and change in myocyte function. The phosphorylation of the following substrates is considered.

In many studies, PKC-mediated phosphorylation of MLC-2 increased the Ca^{2+} sensitivity of force production (44, 45), while others found no change in Ca^{2+} sensitivity (6, 46) or even a decrease in Ca^{2+} sensitivity (24, 47). Since we showed by the analysis of 2D-gels, that MLC-2 phosphorylation by PKC-M or during PE stimulation does not take place, the contribution of MLC-2 phosphylation to the decreased Ca^{2+} sensitivity of force can be ruled out in our experimental settings. In a similar model of PKC treated human cardiomyocytes, Ca^{2+} sensitivity was decreased without the phosphorylation of MLC-2 (9). *Holroyde et al.* (48), *High et al.* (49) *and Jeacocke et al.* (50) also failed to detect MLC-2 phosphorylation in beating hearts following treatment with inotropic agents. We have to discuss briefly a theory by *Andersen* (5) *and Suematsu* (31), who suggested that the PE effects are independent from PKC activation and depend on MLC kinase and Rho kinase. The unchanged phosphorylation level of MLC-2 indirectly proves that the participation of MLCK or Rho kinase in the PE-induced signal transduction pathway is not very likely.

In additon to MLC-2, cTnI and cTnT are also putative PKC targets. Exclusive (in vitro) cTnI phosphorylation by PKC has been shown to inhibit maximal MgATPase activity, or MgATPase Ca^{2+} sensitivity or it had dual action depending on the PKC isoform and on the phosphorylated site(s) of the substrate (6, 51, 52). Others also demonstrated decrease in myofilament Ca^{2+} sensitivity or in maximal tension (53). According to *Pi et al.* (8) PKC itself decreases maximal ATPase activity, increases Ca^{2+} sensitivity of ATPase activity and of isometric tension but the functional consequences of cTnI phosphorylation by PKC is dependent on the phosphorylation status of the PKA sites. Exclusive cTnT phosphorylation caused a decrease in maximal tension, in Ca^{2+} sensitivity, in ATPase activity and in cross-bridge cycling kinetics (46, 53–56). Furthermore, cTnT seems to have a modulating role on the functional effect of PKC-mediated phosphorylation of cTnI when they are cophosphorylated (57). cTnI-cTnT cophosphorylation inhibits maximal MgATPase activity but has no effect on actomyosin ATPase Ca^{2+} sensitivity (6, 44, 46, 55, 56). *Van der*

Figure 4. Silver stained 2D-gels (pH range 4.5-5.5, 12% acrylamide gel) illustrate ventricular myosin light chain 2 (LC-2) composition in untreated rat tissue (A), in tissue incubated without and with PKC (B, no PKC at 37 °C for 2 hours and C, 2 U/mL PKC at 20 °C for 1 hour). Ventricular myosin light chain 2 isoforms (LC-2 and LC-2*), which are both partly phosphorylated (LC-2P and LC-2*P, respectively) in untreated tissue as shown in A and the corresponding densitometric scan in the upper panel of D. The densitometric scans shown in panel E indicate LC-2 phosphorylation level of tissue incubated in relaxing solution for 1 hour at 20 °C or 2 hours at 37 °C (B) and in Ringers' solution (gel not shown). Incubation of rat tissue with PKC did not increase LC-2 phosphorylation (C and E panel) nor did PE (F panel)



Velden et al. found cTnI and cTnT cophophorylation in skinned PKC-M treated cardiomyocytes. We did not check cTnI and cTnT phospho status, but it is possible that the phosphylation of these two proteins took place in our model as well, leading to the decreased Ca^{2+} sensitivity.

MyBPC phosphorylation also has to be taken into account. There is evidence that MyBPC does not affect actomyosin ATPase activity (58), however *Venema et al.* (6) came to the conclusion that MyBPC has a dominant role in decreasing MgATPase actity. In our model MyBPC phosphorylation was not tested, its role in modifying myocyte function cannot be excluded.

Our data is discordant considering the change in maximal force, passive force, nHill and K_{tr} when comparing PKC and PE groups. The catalytic subunit of PKC developed decrease in maximal force, in passive force, in nHill and in K_{tr}, whereas PE increased nHill, the rest of the parameters were unchanged. This difference does not mean that PE effects are not PKC-mediated. We cannot expect general PKC effect and specific, receptor–mediated PKC activation to be equivalent. Adult cardiomyocytes express a set of PKC isoforms, such as PKCα, PKCδ, PKCε, and PKCζ(13, 15, 59–61). The isofoms are believed to subserve distinct biological functions. PE may preferentially activate certain PKC isoforms (14, 15, 62) with different substrate specificity, while the catalytic subunit of PKC we used in our study, lacks specificity of the different PKC isoforms (36). PE has restriction in activating PKC isoforms (53), eg. PKCα, PKCδ and ε phosphorylates cTnI more than cTnT, while PKCζ works just opposite. PKCα phosphorylates $Ser^{43/45}$ of cTnI which leads to the decrease in maximal ATPase activity while PKCδ phosphorylates $Ser^{23/24}$ and $Ser^{43/45}$ at the same time as a hibrid of PKCα and PKA and have dual actions, decreases maximal ATPase activity via $Ser^{43/45}$ and decreases Ca^{2+} sensitivity via $Se^{23/24}$ (52). PE may preferentially activate PKCε and PKCδ (14, 15, 62).

We also have to keep in mind that the functional consequences of cTnI phosphorylation by PKC is dependent on the phosphorylation status of the PKA sites (8). PKC alone increases Ca^{2+} sensitivity of force via phosphorylating its specific sites $Ser^{43/45}$ and Thr^{144}, but if PKC crossphosphorylates the PKA sites ($Ser^{23/24}$), it can mask PKC effects on myofilament Ca^{2+} sensitivity and the increase is abolished or a decrease in Ca^{2+} sensitivity occurs. It is generally known that during the procedure of enzymatic cell isolation the proteins become dephosphorylated as we found with LC-2 *(Fig. 3)*. (Ringer group versus reference sample). If cTnI is poorly phosphorylated, the PKA sites are accessible for PKC.

Considering the possible variations, we can speculate that the overall PKC effect we found is the result of a combined action of different PKC isoforms on various substrates. This is supported by the fact that we found similar changes in myocyte's function as *Gwathmey et al.* (4) did in human trabeculae carneae as a result of PKC panactivation by DPBA. It is known that non-physiological PKC activation does not mimic the effect of DAG on PKC activation, which is generated endogenously by α-stimulation (63). In deed, after PE stimulation only the decrease in Ca^{2+} sensitivity was consistently similar to the PKC incubation data.

Conclusion

Our data suggests that PE-induced changes in cardiomyocyte function are mediated via PKC activation since the decrease in Ca^{2+} sensitivity following PE stimulation was comparable to the change observed after PKC-M treatment. However, the overall PKC-M effects were more pronounced, probably because PKC-M mimics the effects of a wider range of PKC isoforms with distinct substrate specificity than are activated during PE stimulation. MLC-2 phosphorylation plays no potential role in this process.

Irodalom

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