Protein Kinase C Contributes to the Maintenance of Contractile Force in Human Ventricular Cardiomyocytes*

Andrea Molnár‡, Attila Borbély‡, Dániel Czuriga‡, Siket M. Ivetta‡, Szabolcs Szilágyi‡, Zita Hertelendi‡, Enikő T. Pásztor‡, Ágnes Balogh‡, Zoltán Galajda§, Tamás Szerafin§, Kornelia Jaquet§, Zoltán Papp¶, István Édes‡, and Attila Tóth‡1,2

From the 1Division of Clinical Physiology and the 2Center of Cardiac Surgery, Institute of Cardiology, University of Debrecen, Debrecen H-4032, Hungary, and the 3Forschungslabor Molekulare Kardiologie, St. Josef Hospital/Bergmannsheil, Kliniken der Ruhr-Universität, Bochum 44791, Germany

Prolonged Ca2+ stimulations often result in a decrease in contractile force of isolated, demembranated human ventricular cardiomyocytes, whereas intact cells are likely to be protected from this deterioration. We hypothesized that cytosolic protein kinase C (PKC) contributes to this protection. Prolonged contracture (10 min) of demembranated human cardiomyocytes at half-maximal Ca2+ resulted in a 37 ± 5% reduction of active force (p < 0.01), whereas no decrease (2 ± 3% increase) was observed in the presence of the cytostol (reconstituted myocytes). The PKC inhibitors GF 109203X and Go6976 (10 μmol/liter) partially antagonized the cytosol-mediated protection (15 ± 5 and 9 ± 2% decrease in active force, p < 0.05). Quantitation of PKC isoform expression revealed the dominance of the Ca2+-dependent PKCα over PKCβ and PKCe (189 ± 31, 7 ± 3, and 7 ± 2 ng/mg protein, respectively). Ca2+ stimulations of reconstituted human cardiomyocytes resulted in the translocation of endogenous PKCα, but not PKCβ1, δ, and ε from the cytosol to the contractile system (PKCα association: control, 5 ± 3 arbitrary units; +Ca2+, 39 ± 8 arbitrary units; p < 0.01, EC50,Ca = 645 nmol/liter). One of the PKCα-binding proteins were identified as the thin filament regulatory protein cardiac troponin I (TnI). Finally, the Ca2+-dependent interaction between PKCα and TnI was confirmed using purified recombinant proteins (binding without Ca2+ was only 28 ± 18% of that with Ca2+). Our data suggest that PKCα translocates to the contractile system and anchors to TnI in a Ca2+-dependent manner in the human heart, contributing to the maintenance of contractile force.

Protein kinase C (PKC)3 is a family of serine/threonine kinases (1). Multiple PKC isozymes are often expressed in the same cell, mediating specific functions. Conventional and novel PKCs can be activated by lipids, like the endogenous diacylglycerol (DAG) or the exogenous phorbol ester PMA. It was reported decades ago that PMA activation of PKC leads to the translocation of PKC from the soluble to the particulate fraction (2). This observation has been confirmed by later works, and some of the binding proteins for activated PKC isozymes were identified (receptors for activated C kinases) (3, 4). Binding to its respective receptors for activated C kinase localizes each PKC isozyme in the vicinity of a subset of substrates and away from others, and hence this spatial organization may well explain the specificity of PKC isozymes in their intracellular signaling.

It is of interest that from the many PKC isozymes expressed in the heart (5), PKCα is the single isozyme that translocates to the contractile system upon Ca2+ stimulation in the rat heart (6), suggesting a unique physiological role for PKCα in the Ca2+-dependent regulation of myofibrillar contractility. As a matter of fact, PKCα has been implicated in models of ischemic heart failure, myocardial hypertrophy, hypertension, and atherosclerosis (7). In addition, PKC-dependent phosphorylation of myofibrillar proteins such as desmin (8), myosin light chain (9), troponin I (TnI), and troponin T (TnT) (10) has been documented with suggested functional consequences ranging from changes in mechanical integrity of the cardiac sarcomere to decreased actin–myosin ATPase activity and force generation.

Long term activation of PKC is an essential step in ischemic preconditioning (11), although it is also well established that PKC overexpression contributes to heart failure (12–18) at least in part by enhanced phosphorylation of troponin proteins resulting in decreased cardiac contractility (12, 19–25) in various animal models. In contrast, limited information is available on the possible role of PKC under physiological conditions in human heart and the possible PKC-binding proteins responsible for myofibrillar targeting of PKC.

Here we show that endogenous PKCα participates in the maintenance of contractile force upon long term Ca2+ stimulations (a condition associated with ischemia–reperfusion) and identify the sarcomeric regulatory protein TnI as a PKCα targeting protein. Our data suggest a new physiological role for PKCα and pinpoints its intermolecular interaction with TnI as a possible pharmacological target to regulate in vivo PKCα activity in a sarcomere-specific manner.
Experimental Targeting of PKCα to the Contractile System

Experimental Procedures

Tissue Source—Healthy human hearts were obtained from five general organ donor patients (two men and three women with a mean age of 39.2 years) whose hearts were explanted to obtain pulmonary and aortic valves for valve replacement surgery (donor hearts). The donors did not show any sign of cardiovascular abnormalities and did not receive any medication except plasma volume expanders, dobutamine, and furosemide. The causes of death included cerebral contusion caused by accidents and cerebral hemorrhage and subarachnoidal hemorrhage caused by stroke. These experiments complied with the Helsinki Declaration of the World Medical Association and were approved by the Hungarian Ministry of Health (number 323/8/2005-1018EKU). Left ventricular wall samples were frozen in liquid nitrogen and stored at −80 °C.

Myocyte Preparation and Measurement of the Mechanical Properties—Left ventricular tissue samples (about 0.2 g of wet weight) were treated and isolated as described earlier (26). In short, the tissue was mechanically disrupted in isolating solution (containing 4 mmol/liter Na2ATP, 1 mmol/liter Mg2+, 145 mmol/liter KCl, 2 mmol/liter EGTA, 10 mmol/liter imidazole, pH 7.0), and the particulate fraction was separated from the cytosol by centrifugation (1,000 × g, 5 min; supernatant is referred to as cytosol). The mechanically isolated cardiac myocytes in the particulate fraction were demembranated by Triton X-100 (0.3%, stirring on ice for 5 min) containing isolation solution. Then the demembranated myocytes were separated by centrifugation (1,000 × g, 5 min; pellet: demembranated cardiomyocytes). The cytosol fraction was kept on ice until further experiments, and the myocytes were washed three times in cell isolation solution without Triton X-100 before measurements. Protein concentrations were determined by the BCA method (Sigma-Aldrich) using bovine serum albumin as a standard.

Measurement of Physiological Properties—Demembranated single cardiomyocytes were mounted between two thin needles with silicone adhesive (Dow Corning, Midland) while being viewed under an inverted microscope (Axiovert 135, Zeiss, Germany). One needle was attached to a force transducer element (SensoNor, Horten, Norway), and the other was attached to an electromagnetic motor (Aurora Scientific Inc., Aurora, Canada). The measurements were performed at 15 °C, and the average sarcomere length was adjusted to 2.2 μm as described previously (26). A mounted cardiomyocyte is shown on Fig. 1A from both vertical and horizontal directions. The pCa (−log[Ca2+]) values of the relaxing and activating solutions (pH 7.2) were 9 and 4.75, respectively. Solutions with intermediate free Ca2+ levels were obtained by mixing activating and relaxing solutions. All of the solutions for force measurements contained 1 mmol/liter Mg2+, 5 mmol/liter MgATP, 15 mmol/liter phosphate, and 100 mmol/liter BES. The ionic equivalent was adjusted to 150 mmol/liter with KCl. Isometric force was measured after the preparation had been transferred from the relaxing solution to a Ca2+-containing solution. When a steady force level was reached, the length of the myocyte was reduced by 20% within 2 ms and then quickly restretched (release-restretch maneuver). As a result, the force first dropped from the peak isometric level to zero (difference = total peak isometric force) and then started to redevelop. The passive force component was determined in relaxing solution following the Ca2+-contractures. The Ca2+-activated isometric force was calculated as the difference of the passive and maximal active isometric force. Representative recordings are shown on Fig. 1 (B and C).

After the first maximal activation at pCa 4.75, resting sarcomere length was readjusted to 2.2 μm, if necessary. Then cells were subsequently exposed to a series of solutions with intermediate pCa to construct a force-pCa relationship. After obtaining the first (control) force-pCa, the myocytes were incubated in the presence of a Ca2+-concentration, which evoked ~50% of maximal force for 10 min and then the functional parameters (force-pCa relationship and passive tension) were measured again to reveal the effects of the Ca2+-stimulation (representative experiments are shown on Fig. 1).

Ca2+-force relations were fitted to a modified Hill equation as described earlier (26),

\[ F = F_0 [Ca^{2+}]^{nHill}/(pCa_{50}^{Hill} + [Ca^{2+}]^{nHill}) \]  

where \( F \) is the steady-state force, \( F_0 \) is the steady isometric force at saturating Ca2+-concentration, the Hill coefficient \( nHill \) is a measure of the steepness of the relationship, and \( pCa_{50} \) is the mid-point of the relation. The values are given as the means ± S.E. for \( n \) myocytes. The differences were tested by means of Student’s paired \( t \) test comparing the values before and after treatments. The level of significance was \( p < 0.05 \).

Reconstitution of Human Cardiomyocytes—Human cardiomyocytes were reconstituted by adding the cytosol to the demembranated myocytes at a dilution of ~1 mg of myocyte protein/ml. In case of stimulation, reconstituted cardiomyocytes were incubated in the presence or absence of PMA (10 μmol/liter), Ca2+ and PKC inhibitors GF109207X and Gö6976 (10 μmol/liter) for 10 or 30 min (physiological and biochemical assays, respectively). For the biochemical assays, the myocytes were pelleted and subsequently washed three times (centrifuged at 1,000 × g for 2 min) in 450 μl of cell isolation solution, containing the same concentration of Ca2+ and PMA as during the incubations mentioned above. The pellets were then resuspended in 60 μl of SDS-PAGE sample buffer (Sigma-Aldrich), and 30 μl was subjected to SDS-PAGE and Western immunoblot analysis.

In Vitro Phosphorylation—Human ventricular heart muscle homogenates were incubated with PKCs α, γ, δ, ε, and η (Biomol, Plymouth Meeting, PA) for 60 min at 37 °C in 50 μl of reaction mixture containing: for PKCα and PKCγ: 20 mmol/liter HEPES, pH 7.4, 10 mmol/liter MgCl2, 200 μg/ml 1-phosphatidylinositol-1-serine, 20 μg/ml 1.2 DAG, 100 μmol/liter [γ-32P]ATP, and 100 μmol/liter CaCl2; for PKCδ, PKCε, and PKCη: 20 mmol/liter HEPES, pH 7.4, 10 mmol/liter MgCl2, 200 μg/ml 1-phosphatidylinositol-1-serine, 20 μg/ml DAG, 100 μmol/liter [γ-32P]ATP, and 100 μmol/liter EGTA (chemicals were from Sigma, except [γ-32P]ATP, which was from ICN, Costa Mesa, CA). In parallel to the phosphorylation of myocardial proteins, kinase activities were measured using 1 mg/ml histone IIIS (Sigma) substrate. The kinase activities on this control substrate were 14 ± 5, 24 ± 5, 30 ± 9, 23 ± 9, and 23 ± 4 pmol
phosphate incorporation/min for PKCs α, γ, δ, ε, and η, respectively. The reactions were initiated by adding the radioactive ATP. After the phosphorylation, the reaction mixtures were boiled with 50 μl of 2× SDS sample buffer (Sigma) for 5 min. The proteins were then subjected to polyacrylamide gel electrophoresis. After electrophoresis, the gels were stained with Coomassie and dried. The stained, dried gels were then subjected to autoradiography. In parallel, 5 μl of reaction mixture was dropped onto P81 phosphocellulose papers (Whatman, Fairfield, NJ). After drying, the papers were washed with 0.5% phosphoric acid three times each for 5 min and with acetone. After drying the radioactivity bound to the papers was measured by a TriCarb scintillation counter (PerkinElmer Life Sciences).

Western Immunoblot—Human left ventricular tissue samples were homogenized in radioimmunoprecipitation assay buffer containing 50 mmol/liter Tris–Cl, pH 7.4, 150 mmol/liter NaCl, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholate. Protein concentrations were determined by BCA assay (Sigma) using bovine serum albumin as standard, and the concentration of the homogenates were adjusted to 4 mg/ml. Then the homogenates were mixed with equal volumes of 2× SDS sample buffer (Sigma) and were boiled for 10 min before electrophoresis. 50 μg of proteins was loaded onto 10% SDS-polyacrylamide gels and after separation transferred to nitrocellulose membranes. The membranes were probed with antibodies against PKCα (Sigma-Aldrich; dilution, 1:5 000) PKCβ and PKCe (both from Santa Cruz Biotechnology; dilution, 1:1 000), and the signal was detected by a peroxidase-conjugated anti-rabbit IgG-specific antibody (Sigma-Aldrich; dilution, 1:50 000). For the assessment of PKC isozyme expression in the human heart, recombinant human PKC isozymes were loaded on the same gels/membranes as control proteins (Sigma-Aldrich). Then the bands were visualized by ECL and evaluated by Image J software. Only bands on the same membrane were used.

**Functional Targeting of PKCα to the Contractile System**

In Vitro Binding of Recombinant Human PKCα to Recombinant Cardiac TnI or Troponin Complex—Recombinant, purified PKCα (0.1 μg; Sigma-Aldrich) was incubated with recombinant, purified TnI (0.1 μg/ml TnI) or reconstituted troponin complex (0.3 μg/ml; 1:1:1 stoichiometry of TnI, TnC, and TnT) in the absence or presence of Ca2+ (5 mmol/liter) for 2 h at room temperature. TnI was precipitated with an anti-TnI antibody (clone 16A11; Research Diagnostics Inc., Flanders, NJ) or with mouse IgG (control, Zymed Laboratories Inc., San Francisco, CA) and then pulled down with a protein A-Sepharose CL-4B resin (Amersham Biosciences). The precipitates were washed five times with TBS containing 0.1% Triton X-100 and the respective concentration of Ca2+ (Fig. 7B). The precipitates were analyzed by Western immunoblot for PKCα as detailed above.

**RESULTS**

Effects of Endogenous Protein Kinase C on the Ca2+-activated Contractile Force of Human Ventricular Cardiomyocytes—Incubation of demembranated human cardiac myocytes with a Ca2+ concentration evoking about half-maximal force production (pCa 5.8, active force during the incubation: 62 ± 1% of the maximum) for 10 min resulted in a 37 ± 5% reduction in the force.
In parallel, the structural effects of the maintained contractions were also recorded as a means of light microscopy. Apparently, the maintained contractions evoked some deterioration of the cross-striation pattern of the myocytes, both in the presence and in the absence of cytosol (Fig. 1, B and C).

Next, the possible contribution of cytosolic proteins (probably with Ca\(^{2+}\)-regulated activity) was investigated. From the many candidates, our attention has been attracted to PKC because of its ability (i) to regulate contractility, (ii) to translocate from the soluble fractions to the particulate fractions, and (iii) to be activated by Ca\(^{2+}\) (classical PKC isoforms).

The administration of the general PKC activator PMA (10-min application) apparently affected neither the contractility (maximal active force: 96 ± 1% of the maximal active force obtained before the treatment; Fig. 3, n = 5), nor the cytosol-mediated protection upon prolonged Ca\(^{2+}\) contractions (maximal active force: 98 ± 1% of the maximal active force obtained before the treatment; Fig. 3, n = 10).

In contrast, inhibition of PKC by GF 109203X and by Gö 6976 (10 \(\mu\)mol/liter) partially antagonized the cytosol mediated protection (a decrease of active force by 15 ± 5%, \(p < 0.05\), n = 6 and by 9 ± 2%, \(p < 0.05\), n = 8, respectively, as shown on Fig. 4). In addition, control experiments with GF 109203X showed no effects on the active force in the absence of Ca\(^{2+}\) incubations (2 ± 3% increase in the maximal active force, \(n = 5\); Table 1).

**Effects of Endogenous Protein Kinase C on the Passive Tension of Human Ventricular Cardiomyocytes**—Isolated cardiac myocytes possess a Ca\(^{2+}\)-independent passive tension (force) at a sarcomere length of 2.2 \(\mu\)m. In contrast to the Ca\(^{2+}\)-dependent (active) force, the passive force of the myocytes was elevated (passive force: 302 ± 46%, \(p < 0.05\), \(n = 6\); Fig. 5) after prolonged Ca\(^{2+}\) contractions. The addition of cytosol significantly antagonized this elevation (passive force: 146 ± 9%, \(p < 0.05\), \(n = 5\); Fig. 5). As the matter of the contribution of PKC to these changes, PKC activation by PMA or inhibition by GF 109203X and by Gö 6976 was without effect on this parameter both in the absence and in the presence of Ca\(^{2+}\), as summarized in Table 1.

**Effects of Endogenous Protein Kinase C on the Ca\(^{2+}\)-Sensitivity of Force Development in Human Ventricular Cardiomyocytes**—There was no apparent relationship between PKC activation (PMA) or inhibition (GF 109203X and Gö 6976) and the \(p\text{Ca}_{50}\) or Hill coefficient (summarized in Table 1). In general, \(p\text{Ca}_{50}\)
values tended to be higher after the treatments, whereas the Hill coefficients were not affected.

Expression of Protein Kinase C Isozymes in Human Ventricular Cardiomyocytes—The expression levels of three PKC isoforms (α, δ and ε) were quantified in human left ventricular tissue samples. These assays suggested that PKCα is an abundant isoform in the human heart with an expression level about 20 times higher than those for PKCδ or PKCe (expression was 189 ± 31, 7 ± 3, and 7 ± 2 ng/mg protein for PKCα, PKCδ, and PKCe, respectively; n = 4 for PKCδ and n = 3 for PKCδ and PKCe; data not shown).

Phosphorylation of Human Ventricular Proteins by Protein Kinase C Isozymes—The ability of PKC isoforms to phosphorylate myofibrillary regulatory proteins was checked by in vitro phosphorylation experiments. The Ca^{2+}-dependent PKCα and γ showed similar activity (4.3 ± 0.9 and 4.4 ± 2.1 pmol/min, respectively) and substrate specificity, whereas the Ca^{2+}-independent isoforms produced a rather unique pattern. PKCβ possessed the highest activity (6.6 ± 3.3 pmol/min) and selectively phosphorylated a protein with a molecular mass of 26 kDa. PKCe showed the lowest overall activity (1.6 ± 0.3 pmol/min) and seemed to be specific to phosphorylate a protein with a molecular mass of 60 kDa. PKCγ (activity, 2.7 ± 0.8 pmol/min) selectively phosphorylated two proteins with molecular masses of >200 and 48 kDa (data not shown). Overall, the expression and in vitro phosphorylation data suggest the dominance of PKCα over PKCδ and ε in the human heart (calculated relative activities (expression multiplied by the activity) are: 813, 46, and 11, respectively).

Intracellular Targeting of Protein Kinase Ca in the Human Ventricular Cardiomyocytes—As a matter of in vivo regulation, experiments were performed to investigate the possible translocation of endogenous PKCα, β1, δ, and ε from the cytosol to the contractile protein machinery. In accordance with a predominantly cytosolic localization under unstimulated conditions, only a low level of association of PKC isoforms were found in the absence of Ca^{2+} (Fig. 6A, control). Importantly, in the presence of Ca^{2+}, the level of PKCα bound to the contractile system was selectively increased (Fig. 6A). Interestingly, the widely employed PKC activator PMA alone was without significant effects on the interaction between PKC isoforms and myofilaments, although it evoked significant translocation when applied together with Ca^{2+} (PKCα and ε), suggesting a decisive role for Ca^{2+} in the regulation of translocation. When the Ca^{2+} dependence of PKCα translocation was assayed, an EC_{50} = 645 nmol/liter for the free [Ca^{2+}] was obtained (Fig. 6B).

Ca^{2+}-regulated Interaction of Human Cardiac TnI and Protein Kinase Ca—The interactions of PKCα and its putative anchoring proteins were further explored by overlay assays (Fig. 7A). Five potential PKCα-binding proteins were found in the myofilibrillar system of human cardiomyocytes. Moreover, one of the most prominent bands migrated with the thin filament regulatory protein TnI. In vitro binding assays, using purified, recombinant human TnI and purified recombinant PKCα verified the existence of the Ca^{2+}-dependent interaction between these two molecules (Fig. 7, B and C). In the cardiac myocytes, TnI functions as a member of the troponin complex, consisting

### TABLE 1
Catalogue of values determined in the mechanical measurements

There are four parameters determined in the mechanical measurements: active force (force evoked by increased Ca^{2+} concentrations), passive force (passive tension of the cardiomyocytes at 2.2-µm sarcomere length), pCa_{50} value (Ca^{2+} sensitivity of the contraction), and Hill value (representing the cooperativity in the development of force upon Ca^{2+} stimuli). These values are shown in respect to the applied treatments. The number of individual experiments are also shown. Absolute force values stand for the measured contractile force (in millinewtons) divided by the cross-sectional area (in mm²) of the cardiomyocytes, whereas relative force values are the percentage of the response after treatments compared with the measured values before treatments.

| Treatment | n | Active force before | Active force (relative) after | Passive force before | Passive force (relative) after | pCa_{50} Before | pCa_{50} After | Hill Before | Hill After |
|-----------|---|---------------------|-----------------------------|---------------------|-----------------------------|----------------|----------------|-------------|------------|
| +Ca^{2+}  | 6 | 24.17 ± 3.79        | 63 ± 5²                   | 1.89 ± 0.20         | 302 ± 46                   | 5.92 ± 0.04     | 5.97 ± 0.03     | 2.06 ± 0.12 | 1.94 ± 0.34 |
| Cytosol + Ca^{2+} | 5 | 19.80 ± 2.84        | 102 ± 2.3                 | 1.61 ± 0.24         | 146 ± 9²                   | 5.89 ± 0.02     | 5.97 ± 0.02     | 2.03 ± 0.21 | 2.10 ± 0.10 |
| Cytosol + PMA | 5 | 20.12 ± 4.20        | 96 ± 1                    | 2.05 ± 0.51         | 115 ± 11                   | 6.00 ± 0.03     | 6.05 ± 0.01     | 2.06 ± 0.16 | 2.58 ± 0.20 |
| Cytosol + GF  | 5 | 25.96 ± 4.72        | 102 ± 2.3                 | 1.58 ± 0.41         | 92 ± 6                     | 5.96 ± 0.06     | 6.03 ± 0.04     | 1.65 ± 0.16 | 2.14 ± 0.16 |
| Cytosol + PMA + Ca^{2+} | 10 | 21.67 ± 3.04 | 98 ± 1                   | 2.07 ± 0.24         | 176 ± 20                   | 5.90 ± 0.04     | 6.04 ± 0.05     | 2.10 ± 0.28 | 2.09 ± 0.19 |
| Cytosol + Ca^{2+} + G6 | 8 | 28.87 ± 2.90        | 91 ± 2.2                  | 2.57 ± 0.57         | 165 ± 19                   | 5.94 ± 0.03     | 6.04 ± 0.03     | 1.82 ± 0.20 | 1.97 ± 0.13 |
| Cytosol + PMA + Ca^{2+} + G6 | 6 | 22.48 ± 3.24        | 85 ± 5                    | 2.58 ± 0.23         | 147 ± 11                   | 5.92 ± 0.03     | 6.01 ± 0.05     | 1.84 ± 0.12 | 2.08 ± 0.29 |

* Significant differences were found (p < 0.05, paired t-test) between the values obtained before and after treatments.
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of TnT, TnC, and TnI in equimolar concentrations. Hence, the association of PKCα to TnI may be influenced by other constituents of the troponin complex. In vitro reconstitution of the troponin complex, however, did not affect the Ca^{2+}-dependent interaction between TnI and PKCα (Fig. 7B).

Colocalization of Human Cardiac TnI and Protein Kinase Cα in the Human Ventricle—In accordance with the biochemical data, we found significant colocalization of PKCα with TnI in human ventricular tissue samples, although the majority of PKCα was expressed in the cytosol (Fig. 8), consistent with its cytosolic location under low Ca^{2+} conditions.

DISCUSSION

PKC is involved in the regulation of force generation of human ventricular cardiomyocytes (27) and implicated in the pathomechanism of various cardiovascular disorders, including ischemic heart disease, congestive heart failure, myocardial hypertrophy, hypertension, and atherosclerosis (7). PKC can phosphorylate several amino acid residues in TnI, TnT, myosin light chain 2, myosin-binding protein C, and desmin (8, 28–30). As a result of these phosphorylations, decreased Ca^{2+} sensitivity of the MgATPase activity (31, 32), prolongation of isovolumetric relaxation time, increased afterload (33), decreased myosin sliding speed (20), decreased contractility (12, 20, 22, 24, 25, 34–36), and reduced Ca^{2+} sensitivity of contraction (12, 20, 37) have been observed.

Our data add to the range of these effects, suggesting that PKC is also involved in the maintenance of contractile force in case of prolonged increased intracellular Ca^{2+} concentrations, a condition that generally occurs upon ischemia-reperfusion (38). This beneficial effect of PKC was totally unexpected in light of the wealth of evidence for decreased contractility upon PKC activation. In particular, Belin et al. recently reported (12) that heart failure is accompanied by increased PKC-dependent myofibrillar protein phosphorylation and decreased contractility in the rat. Moreover, this effect of PKC was probably mediated by increased phosphorylation of troponin proteins (19). The differences in the experimental set-ups provide a plausible explanation for the controversial findings (increase versus decrease in contractility). In the earlier reports the effects of PKC were tested under conditions, where PKC activity was several times higher than the control, like heart failure or transgenic models (16, 19, 21, 34), or phosphorylation of myofibrillar proteins by in vitro kinase treatments (12, 20, 37), or target proteins altered by site directed mutagenesis (20, 22, 24, 25, 35, 36). In contrast we used reconstituted cardiomyocytes containing physiological levels of PKC isoforms, in addition to the endogenous mixture of myofibrillar substrates and the respective targeting proteins. It is therefore possible that the development of heart failure is characterized by a dysregulation of PKC pathway, leading to the disruption of its physiological targeting and pathological phospho-
In addition to the differences in the experimental conditions, a striking difference was found in the PKC isoform expression between rodent and human hearts. For example, whereas PKCe seems to be the predominant isoform in rodent hearts (6, 7, 13, 14, 16, 39–43), PKCo expression is the highest in human heart samples (17). In particular, we found about 20-fold higher PKCa expression than that of PKCδ and PKCe in donor human hearts. Moreover, although an increase in PKC expression is a hallmark of heart failure independently of the species, significant differences were found in the isoform expression pattern in human ventricular samples in end stage dilated cardiomyopathy and in severe aortic stenosis (44), again highlighting the complexity of PKC pathway in the heart.

In this context, PKC isoform selectivity can be achieved by at least three ways, namely (i) by selective expression, (ii) by different substrate specificities (iii), or by different targeting of the enzyme. Here an effort was made to investigate all of these possibilities. As a matter of substrate selectivity, in vitro phosphorylation assays were performed on human ventricular myocytes and confirmed earlier data (4, 31), suggesting a pivotal role for intracellular targeting in the determination of the apparent PKC isoform selectivity, in vivo. In particular, although differences in kinase activities and specificities were clearly recognized, all of the studied PKC isoforms were able to phosphorylate the main myofibrillar targets of PKC, such as TnI and TnT, in vitro.

In accordance, the intracellular targeting of PKC isoforms and in particular PKCa was investigated in detail. First of all, increase in Ca2+ concentration selectively induced the translocation of PKCa to the particulate fraction in the rat heart (6), which was confirmed in this study using human ventricular tissue samples. This pinpointed PKCa as the most possible mediator of the sarcomeric effects, resulting in depression of contractility (12, 19) or in protection of contractile force (this report), depending on the conditions.

Moreover, PKCa appears to be a promising therapeutic target to improve cardiac contractility (12, 14, 17). To exploit this potential, the ubiquitous expression and the importance of PKCa in various physiological processes should be taken into account. The optimal drug candidate would act selectively on the heart muscle located PKCa and specifically modulate its effects on Ca2+ handling (14, 17) or on thin filament-mediated regulation of sarcomeric sensitivity to Ca2+ (12).
We made an effort to reveal the molecular mechanism of PKCα targeting to sarcomeric protein machinery. The Ca^{2+} concentration required for half-maximal translocation of PKCα to the contractile system (645 nmol/liter) was in the physiological range. Next, the sarcomeric PKCα-anchoring proteins were investigated. The thin filamentous regulatory TnI was identified as a potential binding protein. Importantly, the interaction between TnI and PKCα was modulated by Ca^{2+} and was not affected by interaction of TnI with the other members (TnT and TnC) of the troponin complex. Moreover, immunohistochemical analysis revealed PKCα and TnI colocalization in the human ventricle. These data suggest that pharmacological modulation of TnI-PKCα interaction may be a strategy to regulate PKCα effects selectively on the sarcomeric proteins.

In the biochemical point of view, PKC-binding proteins are classified as substrates that interact with protein kinase C, receptors for inactive protein kinase C, and receptors for activated protein kinase C (4). In general, PKC activation involves the binding of DAG (or experimentally its stable analogue PMA) to the soluble PKC, which then anchors to membranous structures. Although TnI is apparently one of the substrates that interact with protein kinase C in the human heart, the interaction between TnI and PKCα seems to be regulated by Ca^{2+} alone, independently of lipids. One of the proteins with similar properties is the sdr protein, which targets PKCα to the caveolae in a Ca^{2+}-dependent manner, in the absence of DAG or its analogues (45). This finding suggested that Ca^{2+} evokes a conformation change in PKC, revealing new interaction sites for nearby proteins. Analysis of the binding of PKCα to sdr suggested that although Ca^{2+} facilitates the sdr−PKC interaction, it is stabilized by phosphatidyl serine. In contrast, we found an apparently stable interaction between TnI and PKCα in the absence of lipids, although it may be further stabilized by phosphatidyl serine, in vivo.

It was suggested that temporal and spatial changes in intracellular free Ca^{2+} concentrations regulate the localization of PKCα in vascular smooth muscle cells (46). The same phosphorylation of sarcomeric proteins and to a decrease in contractility (12, 19).

In summary, our data suggest that PKC plays a role in the maintenance of contractile force in human ventricular cardiomyocytes. The proposed mechanism of the PKC-mediated protection is that PKCα translocates to the contractile protein machinery in a Ca^{2+}-dependent manner, where it is anchored to the TnI. A practical application of these findings may be the pharmacological modulation of PKCα targeting in ischemia-reperfusion to improve human cardiac contractility.

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