Protein kinase C enhances myosin light-chain kinase effects on force development and ATPase activity in rat single skinned cardiac cells

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Many neurohormones alter the force of cardiac contraction by variations in the intracellular Ca^{2+} concentration. α_1 -Adrenergic and muscarinic stimulations, rather, modify the sensitivity of contractile proteins to Ca^{2+} ions. Measuring the force developed by rat single skinned cardiac cells, the present study demonstrates that the Ca^{2+} -calmodulin–myosin light-chain kinase (MLCK) complex induces a large increase in Ca^{2+} sensitivity (0.14 pCa unit) of these easily accessible myofilaments. This increase is further enhanced by up to 0.19 pCa unit when protein kinase C (PKC) is added together with MLCK. Similarly, the Ca^{2+} ATPase activity of skinned cells in suspension is increased in the presence of MLCK and further in the presence of both kinases. ^{32}P -labelling and SDS/PAGE show that these changes are associated with light-chain 2 (LC_2) phosphorylation together with phosphorylation of troponin I and troponin T when PKC is added. Although to a smaller extent than in smooth muscle, phosphorylation of cardiac myosin LC_2 may be involved in the modulation of heart contractility.

INTRODUCTION

Several neurohormones modulate the force of contraction of cardiac muscle. Most of the positive inotropic neuromediators exert their effects by increasing Ca^{2+} mobilization in cardiac myocytes [1,2]. Stimulation by α_1 -adrenergic and muscarinic agonists, which both accelerate phosphatidylinositol turnover [3], does not share this mechanism; rather, these agonists cause an increase in sensitivity of myofilaments to Ca^{2+} in single cells. Such an effect cannot be attributed to the increase in intracellular inositol trisphosphate concentration. Indeed, we and others have reported that inositol trisphosphate was unable to modify properties of contractile proteins [4,5]. Therefore protein kinase C (PKC) remains a good candidate to account for the rise in myofilament Ca^{2+} sensitivity induced by α_1 -adrenergic and muscarinic agonists. The role of this kinase in the modulation of cardiac inotropism is still controversial. We previously demonstrated that PKC activators, namely phorbol 12-myristate 13-acetate and 1,2-dioctanoylglycerol, applied to intact cells were able to enhance myofilament Ca^{2+} sensitivity [4]. Capogrossi *et al.* [6] also observed this effect in intact cardiomyocytes, and others reported a phorbol-ester-induced decrease in myofilament Ca^{2+} sensitivity in permeabilized cardiac muscle [7].

Among contractile proteins, subunit I and particularly T of cardiac troponin have been reported to be phosphorylated by PKC *in vivo* [8]. Myosin light-chain 2 (LC_2) of smooth muscle is also a good substrate for PKC [9,10]. In this regard, α_1 -adrenergic and muscarinic stimulation triggers a contraction which is mediated by the Ca^{2+} -dependent phosphorylation of myosin LC_2 by the myosin light-chain kinase (MLCK) [11–14]. Furthermore, an increase in Ca^{2+} sensitivity of smooth-muscle myofilaments after stimulation by phenylephrine or carbachol was recently reported [15,16]. In this tissue also, phorbol esters induced a contraction mediated by calmodulin-dependent MLCK [12,13,17]; this occurs despite the fact that PKC phosphorylation of MLCK *in vitro* decreases the enzyme's affinity for calmodulin [18]. Moreover, the activation of PKC by phorbol

ester leads to the phosphorylation of smooth-muscle LC_2 [19,20] and to a rise in tension in intact arterial muscle after the addition of KCl [21].

The myosin LC_2 of cardiac muscle can also be specifically phosphorylated by MLCK, the activation of which was shown to occur by reversible binding of Ca^{2+} -calmodulin at Ca^{2+} concentrations similar to those required for activation of myofibrillar ATPase [22,23]. The phosphorylation of this protein increases tension in cardiac muscle [24,25]. In intact mammalian fast-twitch skeletal muscle, a correlation was found between the phosphate content of myosin LC_2 and the extent of isometric twitch tension potentiation [26]. However, a direct Ca^{2+} -calmodulin modulation of MLCK activity after α_1 -adrenergic and muscarinic stimulation is unlikely in cardiac tissues. Indeed, most studies have demonstrated that the Ca^{2+} transients do not greatly increase under these conditions [27–29].

The purpose of this work was to examine the effects of PKC and MLCK on Ca^{2+} sensitivity of cardiac contractile proteins by recording the force developed by the myofilaments of a single skinned cell and by measuring actomyosin ATPase activity in skinned cells in suspension. The effects of the kinases on the Ca^{2+} sensitivity of the myofilaments were compared with the phosphorylation of the contractile proteins using ^{32}P labelling and SDS/PAGE. We found that PKC, which phosphorylates cardiac troponin I and T, did not increase the sensitivity of myofilaments to Ca^{2+} , and that MLCK, which phosphorylates cardiac LC_2 , caused a rise in sensitivity to Ca^{2+} for both tension and ATPase activity at submaximal levels. The sensitivity of the contractile proteins to Ca^{2+} was further increased by adding MLCK together with PKC.

MATERIALS AND METHODS

Preparation of rat skinned cardiac cells

Ventricular myocytes were dissociated from hearts of adult rats using a collagenase method previously described by Pucéat *et al.* [4]. To evaluate the Ca^{2+} sensitivity of myofilaments,

isolated rat myocytes were skinned by incubation, with shaking, at 20 °C in a relaxing solution containing Triton X-100 (0.3 %, v/v) for 6 min. This protocol allowed us to obtain skinned cells in which only myofilaments were preserved, as previously described [4].

Treatment with protein kinases

To investigate the effects of PKC on the sensitivity of myofilaments to Ca^{2+} , the kinase purified from bovine brain (0.3 $\mu\text{g}/\text{ml}$; specific activity 12 pmol/min per μg of protein) was used to treat skinned cells in the presence of 150 μg of phosphatidylserine/ml and 50 μM -1,2-dioctanoylglycerol. Other batches of skinned cells were incubated with either chicken gizzard MLCK (0.2 μM) in the presence of calmodulin (0.8 μM) and 1 μM - Ca^{2+} or PKC-treated MLCK under the same conditions. In the latter case, MLCK (2 μM) was previously incubated for 20 min at room temperature with PKC (0.3 $\mu\text{g}/\text{ml}$), phosphatidylserine (150 $\mu\text{g}/\text{ml}$) and dioctanoylglycerol (50 μM) in Ca^{2+} -containing solution {pCa ($-\log [\text{Ca}^{2+}]$) 6}. This solution was then diluted tenfold with the solution at pCa 6 containing 200 000 skinned cells/ml to which calmodulin (0.8 μM) was added. Some skinned cells were treated with 620 μg of PKA/ml from bovine heart (specific activity 1 pmol/min per μg of protein) in the presence of 5 μM -cAMP. At different Ca^{2+} concentrations, ATPase activity and force were measured on the cells after 30 min in these conditions. All incubating solutions contained (mM): phosphocreatine, 12; potassium acetate, 78.4; sodium acetate, 30.6; magnesium acetate, 3.5; imidazole, 30; EGTA, 10; dithiothreitol, 0.3; Na_2ATP , 3.5; CaCO_3 , 7.8.

Measurement of tension

Tension developed by the Ca^{2+} -activated myofilaments was measured as described in detail by Pucat *et al.* [4]. Briefly, one extremity of a single skinned cell was glued to a thin glass rod attached to a transducer (AME, Horten, Norway) with an optical adhesive (Norland Products Inc., North Brunswick, N.J., U.S.A.) which polymerized in u.v. light. The other end of the cell was fixed by sucking it into a glass pipette. The sarcomere length was adjusted to 2.1 μm . The mechanical properties of single skinned cells attached to the transducer have previously been characterized [4]. Bandwidth of the transducer and rod is 500 Hz; however, for the illustrations, bandwidth was lowered to 0.2 Hz to decrease noise. The isolated skinned myocyte was then superfused with solutions at different pCa values. Activating and relaxing solutions were as in Ventura-Clapier *et al.* [30]. They contained (mM): phosphocreatine, 12; potassium acetate, 78.4; sodium acetate, 30.6; magnesium acetate, 3.5; imidazole, 30; EDTA, 10; dithiothreitol, 0.3; Na_2ATP , 3.5; CaCO_3 , 35 μM (pCa 9) or 10 mM (pCa 4.5). pH was adjusted to 7.1 at room temperature with acetic acid. Intermediate Ca^{2+} concentrations were obtained by mixing relaxing solution (pCa 9) and maximal activating solution (pCa 4.5). At each pCa, we measured the force developed by skinned cells after treatment with kinases as described above. Tension/pCa relations are conveniently described by the Hill equation: $\%T = S^h/(K + S^h)$, where $\%T$ is the relative tension, S is the Ca^{2+} concentration, K is the apparent affinity constant and h the Hill coefficient. The curve permits calculation of pCa_{50} , the concentration of Ca^{2+} required to elicit half-maximal tension [$\text{pCa}_{50} = (-\log K)/h$]. Experiments were performed at room temperature (22 ± 2 °C).

Measurement of actomyosin ATPase

ATPase activity was measured in suspensions of skinned cells using a fluorimetric coupled enzyme assay. The oxidation of NADH was measured at 25 °C in Ca^{2+} -containing solutions with

lactate dehydrogenase (1.5 units/ml), pyruvate kinase (1.5 units/ml), ATP (1 mM), phosphoenolpyruvate (30 μM) and NADH (20 μM). The NADH excitation and emission wavelengths were 340 and 460 nm respectively. Consumption of NADH was measured in the fluorimeter cuvette containing solutions with different Ca^{2+} concentrations after addition of either control or kinase-treated myofilaments. At least three separate samples were tested at each pCa. One unit of enzyme activity is defined as 1 μmol of substrate consumed per min. Maximal ATPase activity was measured at pCa 4.5 and allowed to normalize the results. The ATPase/pCa relationships were fitted according to the Hill equation. The basal activity of the K^+ ATPase measured at pCa 9 was subtracted from all the activities measured at different pCa values; thus the relationship was a function of the Ca^{2+} -dependent actomyosin ATPase only.

Identification of rat cardiac LC_2 and troponin subunits I and T

One-dimensional gel electrophoresis was carried out using a mini-Protean apparatus (Bio-Rad) according to the method of Laemmli [31]. Briefly, skinned cells prepared as described above were lysed in a denaturing solution [0.125 M-Tris/HCl (pH 6.8), 20 % (v/v) glycerol, 5 mM-dithiothreitol, 1 mM-EDTA, 5 % SDS, 0.05 % Bromophenol Blue]. Samples were loaded on the top of 15 % acrylamide slab gels with a 5 % acrylamide stacking gel, both containing SDS. The running buffer was the same as described by Laemmli [31]. Electrophoresis was carried out at constant current (30 mA) for 10 min and at constant voltage (150 V) for 1 h. Slabs were stained in a solution containing 0.1 % Coomassie Blue in ethanol/acetic acid/water (5:1:4, by vol.) for 1 h, then destained in ethanol/acetic acid/water (3:1:6, by vol.) and ethanol/acetic acid/water (1:0.5:8.5, by vol.) for 2 h. For immunoblotting, immediately after electrophoresis, proteins were transferred (150 mA, 90 min) from the gel to nitrocellulose membrane. The nitrocellulose sheets were incubated overnight with a rabbit antiserum raised against bovine cardiac myosin light chains. After several washes, the blot was incubated for 1 h with anti-rabbit IgG coupled to peroxidase and revealed with Nitro Blue Tetrazolium.

Some immunoblots were performed using a mouse monoclonal antibody raised against cardiac troponin I or a mouse monoclonal antibody raised against troponin T. Consecutively an anti-mouse IgG coupled to peroxidase was used as second antibody.

Phosphorylation and separation of contractile proteins

Myofilaments of single skinned cells were phosphorylated in a solution at pCa 6 containing 25 μM - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10 Ci/mmol) and the different kinases with their activators. After incubation for 30 min at room temperature, proteins were precipitated with trichloroacetic acid. After centrifugation, pellets were washed twice with ice-cold acetone and lysed in 0.125 M-Tris/HCl (pH 6.8)/20 % glycerol/5 mM-dithiothreitol/1 mM-EDTA/5 % SDS/0.05 % Bromophenol Blue. Samples were subjected to one-dimensional SDS/PAGE as described above to permit characterization of all the labelled proteins. After staining and drying the gels, phosphoprotein bands were identified by autoradiography using Amersham Hyper-MP film with two intensifying screens. In some experiments, gels and autoradiograph bands were quantified by scanning densitometry.

Materials

Molecular-mass protein standards (kDa) used were β -lactalbumin (14.4), trypsin inhibitor (20.1), carbonic anhydrase (30), ovalbumin (43), BSA (67) and phosphorylase *b* (94) and were purchased from Pharmacia. $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was from Dupont de Nemours (France). Electrophoresis reagents were provided by

Table 1. Effects of protein kinases on sensitivity to Ca²⁺ of force developed by rat single skinned cells

MLCK, 0.2 μ M; calmodulin, 0.8 μ M; PKC, 0.3 μ g/ml [bovine brain; specific activity 12 pmol/min per μ g of protein with phosphatidylserine (150 μ g/ml) and dioctanoylglycerol (50 μ M)]; MLCK (PKC) refers to MLCK which has been pretreated with PKC as described in the legend of Fig. 2. pCa₅₀ refers to the Ca²⁺ concentration required to elicit half-maximal tension, *h* to the Hill coefficient and *n* to the number of cells used under the different experimental conditions. Values for a given condition were obtained from at least two hearts. Statistical analysis was performed using Student's *t* test for unpaired observations. Values are means $\pm$ S.E.M.

	Control	PKC	Calmodulin	MLCK	MLCK + calmodulin	MLCK (PKC)	MLCK (PKC) + calmodulin	MLCK + PKC + calmodulin
pCa ₅₀	5.78 $\pm$ 0.01	5.82 $\pm$ 0.01	5.77 $\pm$ 0.02	5.76 $\pm$ 0.05	5.92 $\pm$ 0.01*	5.78 $\pm$ 0.01	5.97 $\pm$ 0.02*	5.96 $\pm$ 0.02*
<i>h</i>	2.76 $\pm$ 0.12	3.10 $\pm$ 0.12*	2.70 $\pm$ 0.30	2.51 $\pm$ 0.14	2.36 $\pm$ 0.13*	2.85 $\pm$ 0.39	2.77 $\pm$ 0.16	2.80 $\pm$ 0.30
<i>n</i>	14	6	3	4	8	6	8	3

* pCa₅₀ and *h* significantly different from control at *P* $\leq$ 0.01.

Bio-Rad. Anti-(troponin T) monoclonal antibody and all other chemicals were from Sigma.

RESULTS

Effects of kinases on the force developed by skinned cardiac cells

The direct activation of PKC in a cardiac cell by its permeant activators dioctanoylglycerol and phorbol esters increased the sensitivity to Ca²⁺ when the force developed by the myofilaments was measured [4]. To investigate further the involvement of PKC in this effect, we applied PKC (in the presence of phosphatidylserine and dioctanoylglycerol) directly to skinned cells for 30 min. Despite different batches of PKC purified from bovine brain being tested, the kinase failed to reproduce the effects of dioctanoylglycerol and phorbol esters observed in intact cells on the Ca²⁺ sensitivity of contractile proteins, as indicated by the unchanged pCa₅₀ compared with control (Table 1). However, it is noteworthy that the Hill coefficient, *h*, was significantly increased.

The application of Ca²⁺-calmodulin-dependent MLCK to a single skinned cardiac cell led to a slow increase in force at pCa 6, which reached a plateau within 9 min. After this treatment, the force developed at pCa 5.75 was much larger than previously (Fig. 1). The kinase effect was stable and remained for several hours after washout of the MLCK. However, this effect could be removed by the addition of alkaline phosphatase (results not shown). Fig. 2(a) illustrates a typical recording of tension developed by a single skinned control cell superfused with solutions of decreasing pCa. Pretreatment of skinned cells with MLCK in the presence of Ca²⁺ and calmodulin increased the Ca²⁺ sensitivity of the myofilaments (Fig. 2b). This is also indicated by the shift

of the tension/pCa curve to the left (Fig. 2d) and by the increased value of pCa₅₀ compared with control cells (Table 1). It should be noted that a greater increase in Ca²⁺ sensitivity occurred at the lower concentration of Ca²⁺ as shown by the decrease in the slope of the tension/pCa curve (Fig. 2d). Maximal tension was not changed by MLCK treatment (results not shown). We generally used MLCK purified from chicken gizzard, which is known to phosphorylate cardiac LC₂ [23]. In some experiments we used pig ventricular cardiac MLCK. When activated by Ca²⁺-calmodulin, the effects of cardiac MLCK on the tension/pCa curve (*n* = 3) were very similar to those of gizzard MLCK. Neither MLCK nor calmodulin alone altered the tension/pCa relation, indicating that neither of them remained bound to the myofilaments of a single skinned cell (Table 1).

The simultaneous application of MLCK and PKC to a skinned cell markedly enhanced Ca²⁺ sensitivity of the contractile response (Table 1) without altering maximal force. The tension/pCa relation was shifted by 0.18 and 0.04 pCa unit when compared with the tension/pCa relation recorded in control and in Ca²⁺-calmodulin-MLCK conditions respectively. The slope of the tension/pCa relation in the presence of MLCK and PKC was the same as in the control (Fig. 2d; Table 1). Similar results were obtained when MLCK had been pretreated with PKC; in this case, about one-tenth of the previous PKC concentration was added to the skinned cells as a contaminant of treated MLCK. The PKC-pretreated MLCK induced a shift to the left by 0.19 and 0.05 pCa unit when compared with control and non-pretreated MLCK conditions respectively (Table 1). A typical case is depicted in Fig. 2(c). The incubation of contractile proteins with PKC-treated MLCK (in the absence of calmodulin) was ineffective (Table 1).

Effects of kinases on actomyosin ATPase activity

The actin-activated myosin ATPase activity measured on isolated skinned cells treated with Ca²⁺-calmodulin-MLCK was increased at each pCa value without a change in the slope of the ATPase activity/pCa relation compared with control conditions (Fig. 3). We generally used chicken gizzard MLCK in these experiments. In a few cases, we also tested pig ventricular cardiac MLCK; its effects on the Ca²⁺-dependent ATPase activity (*n* = 3) were very similar to those of the smooth-muscle MLCK. Treatment of skinned cells with PKC in the presence of phosphatidylserine and dioctanoylglycerol affected neither the Ca²⁺ sensitivity of the actomyosin ATPase nor the maximal enzyme activity (0.056 unit/mg of protein and 0.054 unit/mg of protein in control and PKC-treated cells respectively). Similar results were obtained with three different skinned cell preparations.

PKC-pretreated MLCK appeared more potent than native

Fig. 1. Effects of MLCK on tension developed by a single skinned cell

During continuous recording, the application of chicken gizzard MLCK (0.2 μ M) in the presence of calmodulin (0.8 μ M) increases the force developed at pCa 6 by a single skinned cell isolated from a rat heart. This effect is confirmed by comparing the force developed at pCa 5.75 before and after treatment with the kinase. Relaxing solution is pCa 9. Different pCa values were obtained by mixing relaxing solution (pCa 9) and maximal activating solution (pCa 4.5).

Fig. 2. Effects of MLCK and PKC on the sensitivity of contractile proteins to Ca^{2+} .

Continuous recordings of tension developed by skinned cells before (a) and after incubation with either chicken gizzard MLCK ($0.2 \mu\text{M}$) in the presence of calmodulin ($0.8 \mu\text{M}$) at pCa 6 (b) or with PKC-treated MLCK under the same conditions (c). In the latter case, MLCK ($2 \mu\text{M}$) was preincubated for 20 min with PKC ($0.3 \mu\text{g/ml}$), phosphatidylserine ($150 \mu\text{g/ml}$) and dioctanoylglycerol ($50 \mu\text{M}$) in Ca^{2+} -containing solution (pCa 6). (d) Tension/pCa relationships established in control conditions (■), after treatment by MLCK (▲) or by MLCK and PKC added simultaneously (●), with the concentrations described above. Each symbol represents the mean $\pm$ S.E.M. for at least three experiments at a given pCa.

MLCK (both stimulated by Ca^{2+} -calmodulin) in enhancing myofilament ATPase activities at different pCa values. The shift to the left was larger than in the presence of MLCK alone (Fig. 3). The slopes of the three ATPase/pCa curves were not significantly different. Under these conditions, the amounts of PKC added to the cells together with MLCK were the same as during the force-measurement experiments.

Identification of rat cardiac myosin LC_2

Fig. 4 illustrates the Coomassie-Blue-stained electrophoresis profile of rat ventricular myofilaments (lanes a and b). Myosin light chains 1 and 2 were first identified by their apparent molecular mass [32,33]. The immunoblot revealed that the antibody raised against cardiac light chains recognized two bands at 27 and 20 kDa, which further confirmed the identity of these proteins (Fig. 4, lane c).

Fig. 3. Effects of MLCK and PKC on the sensitivity of actomyosin ATPase to Ca^{2+} .

ATPase activity/pCa relationships of skinned cells in suspension in control conditions (■) and after incubation with either chicken gizzard MLCK ($0.2 \mu\text{M}$) and calmodulin ($0.8 \mu\text{M}$) at pCa 6 (▲) or with PKC-pretreated MLCK (●) under similar experimental conditions to those in Fig. 2. Each symbol represents the mean $\pm$ S.E.M. for at least three experiments at a given pCa.

Fig. 4. Characterization of rat cardiac myosin light chains

One-dimensional gel electrophoresis of rat cardiac myofilaments: SDS/PAGE, 15% acrylamide; $20 \mu\text{g}$ of proteins were electrophoresed (lane b). Standard proteins are shown in lane a. Lane c is the immunoblot analysis after electrophoretic transfer of proteins to nitrocellulose and determination of immunoreactive light chains with an antiserum.

Phosphorylation of the myofilaments

To investigate whether the increase in myofilament Ca^{2+} sensitivity could be related to the phosphorylation of cardiac myosin LC_2 , skinned cells were incubated with gizzard MLCK in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ under the same conditions as in the experiments described above. We investigated the effects of the cyclic AMP-dependent protein kinase, protein kinase A (PKA), as a control, and also those of PKC to check whether the PKC concentration used in the mechanical experiments was sufficient to phosphorylate the contractile proteins. Skinned cells, treated with MLCK in the presence of calmodulin, were subjected to SDS/PAGE on 15% (w/v) polyacrylamide gels. The autoradiograph revealed only one labelled band which appeared with

Fig. 5. Pattern of contractile proteins phosphorylated with PKA or MLCK

Autoradiographs of SDS/polyacrylamide gels after electrophoresis of cardiac myofilaments phosphorylated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (20 μg per lane). Lane a, myofilaments phosphorylated by MLCK in the presence of calmodulin. Lane b, myofilaments phosphorylated by PKA in the presence of cyclic AMP. Proteins with molecular masses of 29 kDa (lane b) and 20 kDa (lane a) appear to be phosphorylated. Lane c is the immunoblot performed by using an antibody raised against cardiac troponin I (TNI).

Fig. 6. Pattern of contractile proteins phosphorylated with PKC and MLCK

Autoradiographs of SDS/polyacrylamide gels after electrophoresis of proteins phosphorylated by PKC in the presence of phosphatidylserine and dioctanoylglycerol (lane a) or by PKC and MLCK in the presence of calmodulin (lane b). ^{32}P is incorporated into 40 and 29 kDa contractile proteins after phosphorylation with PKC and into 20 kDa LC_2 after treatment with MLCK and PKC. Lane c is the immunoblot analysis of cardiac myofilaments. Immunoreactive troponin T (TNT), troponin I (TNI), troponin I.

a molecular mass of 20 kDa, typical of the myosin LC_2 (Fig. 5, lane a). As expected, PKA phosphorylated the different isoforms of troponin I (28–30 kDa), identified by immunoblotting with an anti-(troponin I) monoclonal antibody (Fig. 5, lanes b and c). The band that appeared on the autoradiograph with a high molecular

mass is thought to be the autophosphorylated regulatory subunit of the type-II PKA [34].

In the presence of its activators, PKC essentially phosphorylated two proteins (Fig. 6, lane a). The lower labelled band appeared with a molecular mass of 29 kDa and had the same location as the protein phosphorylated by PKA and identified as troponin I. This suggests that PKC phosphorylates troponin I. The other labelled protein appeared at a molecular mass around 40 kDa; the anti-(troponin T) monoclonal antibody recognized this protein on the immunoblot (Fig. 6, lane c), indicating that PKC phosphorylates troponin T. It should be noted that bovine brain PKC did not phosphorylate cardiac LC_2 . In the presence of PKC, myosin LC_2 was also phosphorylated after treatment with MLCK (Fig. 6, lane b). However, the amount of LC_2 labelling was lower after treatment with MLCK in the presence of PKC than in its absence. The extent of LC_2 phosphorylation was decreased by 60%. The autoradiograph also revealed two labelled bands at 29 and 40 kDa, troponin I and T, as being phosphorylated by PKC (Fig. 6, lane b). A high-molecular-mass protein appeared to be strongly labelled; this could be the smooth-muscle MLCK, which is known to be phosphorylated by PKC [18].

DISCUSSION

The results described above demonstrate, on easily accessible myofilaments from single skinned cardiac cells, that the Ca^{2+} -calmodulin-MLCK complex induces a large increase in Ca^{2+} sensitivity of the tension developed by contractile proteins. This increase is markedly enhanced by MLCK treatment of skinned cells in the presence of activated PKC. Likewise, the sensitivity to Ca^{2+} of the actomyosin ATPase of a skinned cell suspension is increased by MLCK and is further enhanced by MLCK in the presence of PKC. These changes are associated with myosin LC_2 phosphorylation alone or together with phosphorylation of troponin I and T when PKC is present.

Treatment of skinned cardiac cells with activated MLCK induces cardiac myosin LC_2 phosphorylation; the protein was clearly identified by its molecular mass and by immunoblot analysis. Thus the MLCK-induced increase in Ca^{2+} sensitivity of the myofilaments, apparent on tension and ATPase activity, can be ascribed to the phosphorylation of cardiac myosin LC_2 . The slope of the tension/pCa relationship is much shallower after MLCK treatment than in control conditions with an up to twofold increase in the force developed at pCa 6 and a much greater increase at the lower Ca^{2+} concentrations expected to be reached during a single heart beat. In addition, there is no change in the slope of the ATPase activity/pCa relationships. Consequently, the decrease in h might be attributed to alterations in thin filament co-operativity rather than to limited activation of calmodulin-MLCK at high Ca^{2+} concentration. A slight shift of the tension/pCa relationship to the left at levels below 50% maximal activation, together with a decrease in slope, has previously been described in permeabilized cardiac muscle [24]. More recently, in pig ventricular preparations, phosphorylation, but not thiophosphorylation, of LC_2 was shown to increase Ca^{2+} sensitivity by 0.1 pCa unit, with no change in slope [25,35]. In agreement with previous results obtained in skeletal muscle [25,36], we can speculate that the phosphorylation of LC_2 by its specific kinase increases the rate constant of force generating cross-bridge attachment, and consequently this would account for the observed increase in Ca^{2+} sensitivity.

The effects of phosphorylation of myosin LC_2 on ATPase activity are more controversial. It was previously shown that phosphorylation of myosin LC_2 increased activity of dog reconstituted actin-activated Mg^{2+} ATPase and of Ca^{2+} ATPase prepared

from untrained or trained rats [37,38]. However, more recent reports suggest that Ca^{2+} ATPase activity, as well as V_{max} (the unloaded shortening velocity), are decreased or unchanged [39,40].

PKC treatment had no effect on ATPase activity. The fact that the pCa_{50} of ATPase activity was not changed after treatment of skinned cells by PKC is in good agreement with the results of Noland & Kuo [41]; however, these authors reported a 40% decrease in maximal activity. This discrepancy might result from the different experimental protocols. Noland & Kuo [41] first phosphorylated isolated troponin T or I and then measured the actomyosin ATPase activity in reconstituted myofilaments, whereas we treated the skinned cells directly with the kinase. The tension pCa_{50} was also unchanged in PKC-treated skinned cells. This finding strengthens the result obtained by measuring ATPase activity.

Application of PKC, together with MLCK in the presence of calmodulin at pCa 6 to single skinned cells markedly enhances Ca^{2+} sensitivity of both the force developed by the skinned cells and the actomyosin ATPase activity, without altering their maximal values. The shifts to the left are larger than with application of the Ca^{2+} -calmodulin-MLCK complex alone. The effects of PKC cannot be attributed to a simple activation of apoenzyme MLCK, since the incubation of contractile proteins with PKC-treated MLCK in the absence of calmodulin was ineffective. We also verified that dioctanoylglycerol and phosphatidylserine were not responsible for these effects. Unfortunately, we could not distinguish whether some of the effects of PKC in the presence of MLCK were related to alterations in MLCK properties. Similar results were obtained with PKC-pretreated MLCK; however, they could be attributed to the low amount of PKC which was added together with MLCK. Our attempts to inhibit PKC by using a pseudosubstrate inhibitor [42] were misleading; indeed, the peptide used at a concentration as low as 7 nM also prevented the shift to the left induced by the Ca^{2+} -calmodulin-MLCK complex alone (results not shown).

Under the above experimental conditions, the phosphorylated proteins of cardiac skinned cells incubated with MLCK and PKC are LC_2 and troponin I and T. MLCK is shown to phosphorylate myosin LC_2 specifically (Fig. 5, lane a). Phosphorylation of the troponin subunits can be totally attributed to PKC activity, since the amount of ^{32}P incorporated in this last experimental condition was similar to the amount of phosphate incorporated after PKC treatment alone. Besides, the phosphorylation of LC_2 was decreased. A decrease in the affinity of MLCK for calmodulin after its PKC-dependent phosphorylation previously demonstrated in smooth muscle could account for such an effect [18]. Thus the effect of PKC cannot be related to either a direct phosphorylation of LC_2 or the facilitation of MLCK-induced phosphorylation of this contractile protein. We cannot totally rule out the possibility that LC_2 phosphorylation by MLCK on specific sites allowed the PKC to phosphorylate LC_2 on other sites. Rather, we propose that the PKC-dependent phosphorylation of at least one other contractile protein could be responsible for the synergistic effects of MLCK and PKC on the sensitivity of the myofilament to Ca^{2+} . In this regard, troponin I and T subunits represent good candidates, since PKC phosphorylates these proteins to an extent that does not depend on the presence of MLCK.

The above kinase treatments induce increases in myofilament Ca^{2+} sensitivity estimated by both their ATPase activity and tension development; however, these increases were not identical. This could be related to the different experimental conditions used to measure the two parameters. Tension was estimated under quasi-isometric conditions, whereas ATPase activity was determined on myofilaments from skinned cells allowed to

shorten under unloaded conditions. This latter condition is known to facilitate greatly ADP release by myosin ATPase [43], whereas, under isometric conditions, ADP slows cross-bridge turnover as indicated by the kinetics of tension recovery after quick length changes [30]. This might explain why ATPase is activated at higher pCa values than is tension development, and might account for the larger slope of tension/ pCa curves. Furthermore, both under control conditions and after MLCK treatment, the addition of activated PKC increases the slope of the tension/ pCa curves. This may be related to the fact that cooperativity expressed by the Hill coefficient exists between troponin T and tropomyosin and may be modified after phosphorylation of troponin T [44,45]. However, these effects were seen only under isometric conditions, but not on ATPase activity/ pCa curves; we have as yet no clear interpretation.

These results do not allow a definite conclusion about the origin of the synergistic effects of MLCK and PKC on cardiac myofilaments. Further investigations are required. However, we demonstrate that the complex Ca^{2+} -calmodulin-MLCK increases the Ca^{2+} sensitivity of the contractile proteins in skinned cardiac myocytes and that this increase is even larger when both MLCK and PKC are added together. Phosphorylation by these two kinases may account for the increase in Ca^{2+} sensitivity of the myofilaments induced by the neuromediators which stimulate phosphatidylinositol turnover and may be a major pathway to account for their inotropic effects. The PKC- and MLCK-involved pathway might be specific to the agonists accelerating the phosphatidylinositol turnover, since phosphorylation of LC_2 was previously shown not to occur during perfusion of rat heart with adrenaline or isoprenaline, two preferentially β -adrenoceptor agonists [46,47]. In fact, an increase in the ^{32}P content of the light chains was reported in papillary muscle stimulation by noradrenaline [48], whereas in another study the incorporation of ^{32}P was correlated with both negative and positive inotropy [49]. Although not as essential in the control of contraction as in smooth muscle, phosphorylation of cardiac myosin LC_2 may play a role in the neurohormonal modulation of inotropy. Owing to the significant increase in Ca^{2+} sensitivity induced by light-chain phosphorylation, the role of such a mechanism during each cardiac cycle may have to be reconsidered even if MLCK and protein phosphatase activities are reported to be low [50].

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