Role of protein kinase C in the phosphorylation of cardiac myosin light chain 2

Richard C. VENEMA,* Robert L. RAYNOR, Thomas A. NOLAND, JR. and J. F. KUO

Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

The role of protein kinase C (PKC) in the phosphorylation of myosin light chain 2 (MLC2) in adult rat heart cells has been investigated. PKC-mediated phosphorylation of MLC2 in adult rat cardiac myofibrils *in vitro* occurs with a stoichiometry (0.7 mol of phosphate/mol of protein) similar to that mediated by myosin light chain kinase (MLCK). Two-dimensional tryptic phosphopeptide mapping of MLC2 following phosphorylation by PKC or MLCK *in vitro* yields the same major phosphopeptides for each protein kinase. These sites are also ^{32}P -labelled *in situ* when isolated cardiomyocytes are incubated with ^{32}P PPi. ^{32}P labelling of MLC2 in cardiomyocytes is increased by 5-fold in 10 min upon incubation with the phosphatase inhibitor calyculin A, demonstrating the existence of a rapidly turning over component

of MLC2 phosphorylation in these cells. ^{32}P label is completely removed from MLC2 when myocytes are exposed to 2,3-butanedione monoxime, an inhibitor of cardiac contraction known to desensitize the myofilaments to activation by Ca^{2+} . ^{32}P labelling of MLC2 is also decreased by 50–100% following exposure to the PKC-selective inhibitors calphostin C and chelerythrine, suggesting that PKC, and not MLCK, is primarily responsible for incorporation of rapidly turning over phosphate into MLC2 *in situ*. Taken together, these data implicate PKC in the phosphorylation of MLC2 in heart cells and support the hypothesis that phosphorylation of cardiac MLC2 has a role in determining myofibrillar Ca^{2+} sensitivity.

INTRODUCTION

The 20 kDa light chain of myosin in vertebrate non-muscle and smooth muscle cells is phosphorylated by a specific Ca^{2+} /calmodulin-dependent myosin light chain kinase (MLCK) [1,2]. MLCK-mediated phosphorylation in these tissues is associated with an increase in the actin-activated MgATPase activity of myosin of greater than 100-fold [3,4] and is a primary mechanism for initiation of contraction. Furthermore, in these cell types, myosin light chain is also known to be a substrate for the Ca^{2+} /phospholipid-dependent protein kinase C (PKC). PKC incorporates phosphate into smooth muscle or non-muscle myosin light chain *in vitro* [5,6] and *in situ* [7,8] at sites that are distinct from the MLCK sites. PKC-mediated phosphorylation inhibits the increase in MgATPase activity associated with phosphorylation by MLCK [9]. The physiological significance of this phosphorylation, however, has not been clearly defined [10].

In contrast to the case in smooth muscle, myosin phosphorylation in cardiac (and other striated) muscle is not an obligatory or initiating step in the activation of contraction [11]. Ca^{2+} activation of contraction in these tissues is, instead, mediated by the thin filament regulatory protein troponin. Cardiac myosin light chain (MLC2), however, is known to be phosphorylated in heart cells and a cardiac form of MLCK is also known to be present [12,13]. In recent years, evidence has accumulated that MLC2 phosphorylation in myocardial cells may have a modulatory (as opposed to initiating) role in the regulation of contraction. For example, addition of exogenous MLCK and calmodulin to chemically skinned heart cells results in an increased Ca^{2+} sensitivity of force development [14–17] and actomyosin MgATPase activity [17], presumably due to phosphorylation of MLC2. Whether and when levels of light chain phosphorylation are altered in heart cells, however, has been a subject of controversy [18,19]. Because MLCK is a Ca^{2+} -activated enzyme,

one might expect that increased phosphorylation of MLC2 would occur following elevation of cytoplasmic Ca^{2+} induced by positive inotropic agents. However, despite some reports that this does occur [20,21], many investigators have failed to observe any change in light chain phosphorylation upon perfusion of beating hearts with inotropic agents [22–24]. Thus it has been proposed that there may be insufficient MLCK activity in heart to cause a rapid rise in the overall level of light chain phosphorylation, even under conditions of increased cytoplasmic Ca^{2+} [25].

For many years it has been assumed that MLCK is exclusively responsible for MLC2 phosphorylation in heart cells [12–25]. Recently, however, we have found that PKC incorporates phosphate stoichiometrically into MLC2 in cardiac myofibrils *in vitro* [26]. This observation raises the possibility that, by analogy with the case in smooth muscle, light chain phosphorylation in cardiac cells is mediated, not only by MLCK, but also by PKC. In the present study, therefore, we have examined the PKC-mediated phosphorylation of cardiac MLC2 in greater detail. Phosphorylation by PKC *in vitro* has been compared with that by MLCK in terms of its stoichiometry and site specificity. Evidence that PKC contributes to MLC2 phosphorylation *in situ* (i.e. in intact cardiomyocytes) is also presented and the role of this phosphorylation in determining myofibrillar Ca^{2+} sensitivity is discussed.

MATERIALS AND METHODS

Materials

Collagenase B and calmidazolium were obtained from Boehringer-Mannheim; phosphate-free Dulbecco's minimum essential medium was purchased from Gibco; chelerythrine and calyculin A came from LC Services Corp.; trypsin (diphenyl-carbamyl chloride-treated), phosphatidylserine, diolein,

Abbreviations used: MLCK, myosin light chain kinase; PKC, protein kinase C; MLC2, myosin light chain 2; BDM, 2,3-butanedione monoxime; TnT, troponin T; TnI, troponin I; MLC1, myosin light chain 1; Tm, tropomyosin.

* To whom all correspondence should be addressed.

phosphoserine, phosphothreonine, 2,3-butanedione monoxime (BDM), kaempferol, A23187, ionomycin and calmodulin were purchased from Sigma Chemical Co.; calphostin C and KT5926 were obtained from Kamiya Biomedical Co.; [γ - 32 P]ATP were from ICN Radiochemicals. Skeletal muscle MLCK and cardiac myosin light chains were kindly provided by Dr. James T. Stull, University of Texas Southwestern Medical Center at Dallas.

Preparation of cardiac myofibrils and phosphorylation of myofibrils *in vitro*

Cardiac myofibrils were prepared from adult rat hearts by homogenization and extraction with Triton X-100 as described by Murphy and Solaro [27]. PKC was purified from bovine brain as described previously and represents a mixture of PKC isoenzymes found in brain [28]. Myofibrils (20 μ g) were incubated at 30 °C for the times indicated in a reaction mixture (0.1 ml) containing 50 mM Tris/HCl, pH 7.5, at 30 °C, 30 mM 2-mercaptoethanol, 10 mM MgCl₂, 0.5 mM EGTA, 0.2 mM [γ - 32 P]ATP (1000 c.p.m./pmol) and 1 mM sodium orthovanadate (to inhibit ATP depletion by the myofibrillar MgATPase activity). Phosphorylation with PKC (0.5 μ g) was carried out in the presence or absence of 0.9 mM CaCl₂, 50 μ g/ml phosphatidylserine and 2 μ g/ml diolein. Phosphorylation with skeletal muscle MLCK (0.1 μ g) was carried out in the presence or absence of 0.9 mM CaCl₂ and 20 μ g/ml calmodulin. Phosphorylation was terminated by the addition of SDS sample buffer. 32 P-labelled proteins were analysed by SDS/PAGE (5–15% gradient gels) and autoradiography. In some experiments, radiolabelled MLC2 was excised from the gel and the 32 P incorporated was quantified by liquid scintillation counting.

Isolation of cardiomyocytes and labelling of myofibrils *in situ* with [32 P]_i

Cardiomyocytes were isolated from adult rat hearts by the collagenase perfusion method described by Kaku *et al.* [29] with minor modifications [26]. The viability of isolated cells was assessed by microscopic inspection and only cell suspensions of greater than 70% viability were used in the experiments. Myocytes from a single heart were suspended in 10 ml of phosphate-free Dulbecco's minimum essential medium containing [32 P]_i (0.5 mCi/ml). Cells were incubated for 2 h at 37 °C in 5% CO₂ humidified incubator. After a 2 h preincubation, aliquots (1 ml) of 32 P-labelled cell suspension were further incubated for the times indicated in the presence of protein kinase inhibitors (chelerythrine, calphostin C or KT5926), a protein phosphatase inhibitor (calyculin A), or a chemical dephosphorylating agent (BDM). Control incubations were performed by adding the reagent solvent (dimethyl sulphoxide) only. At the end of the incubation period, myofibrils were prepared as described previously [26] and solubilized in SDS sample buffer, and incorporation of 32 P label into protein was analysed by SDS/PAGE and autoradiography. In some experiments, 32 P label in MLC2 was quantified by liquid scintillation counting.

Analysis of site specificity and stoichiometry of MLC2 phosphorylation

A comparison of the sites phosphorylated in MLC2 *in situ* or *in vitro* by PKC and MLCK was carried out by two-dimensional tryptic phosphopeptide mapping under conditions described

previously [26,30]. 32 P incorporation into specific peptides was quantified by autoradiography or by scraping of spots from t.l.c. plates and liquid scintillation counting. Two-dimensional phosphoamino acid analysis of phosphorylated MLC2 was performed as described by Cooper *et al.* [31]. The stoichiometry of phosphate incorporation into MLC2 *in vitro* was determined based on a known specific radioactivity of [γ - 32 P]ATP; mol of phosphate was calculated based on this value and on the amount of 32 P present in excised MLC2 bands from gels. The amount of protein (mol) was determined by densitometric scanning of Coomassie Blue-stained gels and comparison with a cardiac light chains standard of known protein concentration. Protein concentration was determined by the method of Bradford [32]. Calculation of mol of protein was based on a molecular mass for MLC2 of 19 kDa.

RESULTS AND DISCUSSION

Phosphorylation of MLC2 in cardiac myofibrils by PKC and MLCK *in vitro*

Cardiac myofibrils, prepared by homogenization and Triton X-100 extraction of adult rat hearts, contained proteins identified on Coomassie Blue-stained SDS/polyacrylamide gels (Figure 1) as myosin heavy chain (205 kDa), C-protein (150 kDa), actin (45 kDa), troponin T (TnT) (43 kDa), tropomyosin (Tm) (35 kDa), troponin I (TnI) (30 kDa), myosin light chain 1 (MLC1) (27 kDa) and (MLC2) (19 kDa). Myofibrils were

Figure 1 Phosphorylation of isolated adult rat cardiac myofibrils *in vitro* by PKC and MLCK

Myofibrils were incubated with [γ - 32 P]ATP and either PKC or MLCK for 60 min at 30 °C. Incubations with PKC were carried out in the presence or absence of phosphatidylserine/diolein/Ca²⁺ (PS/DO/Ca) and incubations with MLCK were carried out in the presence or absence of Ca²⁺/calmodulin (Ca/Cam). Following incubation, myofibrillar proteins were solubilized in SDS sample buffer and analysed by SDS/PAGE and autoradiography. Myofibril proteins were identified as myosin heavy chain (MHC), C-protein (C-prot), actin, TnT, Tm, TnI, MLC1 and MLC2. The results shown are representative of three experiments.

Figure 2 Time course of phosphorylation of cardiac myofibrillar MLC2 by PKC and MLCK *in vitro*

Myofibrils were incubated at 30 °C with [γ - 32 P]ATP and either PKC/phosphatidylserine/diolelin/ Ca^{2+} , MLCK/calmodulin/ Ca^{2+} , or both. At the times indicated, proteins were solubilized in SDS sample buffer and analysed by SDS/PAGE. MLC2 was excised from the gel and the amount of 32 P incorporated into the protein was determined by liquid scintillation counting. Phosphorylation stoichiometry was calculated as described in the Materials and methods section. Similar results were obtained in three different experiments.

examined as a substrate for purified PKC (brain) and purified MLCK (skeletal muscle) *in vitro*. In agreement with our previous report [26], PKC incorporated phosphate in a Ca^{2+} - and phospholipid-dependent manner into four different cardiac myofibrillar proteins: C-protein, TnT, TnI and MLC2 (Figure 1). MLCK, by comparison, selectively phosphorylated only MLC2. Phosphorylation by MLCK was completely dependent on the presence of Ca^{2+} and calmodulin. The time course and stoichiometry of myofibrillar light chain phosphorylation by PKC *in vitro* was examined and compared with that catalysed by MLCK (Figure 2). The maximal stoichiometry of phosphorylation by PKC (0.7 mol/mol) was slightly higher than that by MLCK (0.6 mol/mol). However, MLCK-catalysed phosphorylation occurred at a faster rate, reaching a maximum within 10 min at 30 °C. In contrast, phosphorylation by PKC required 60 min to reach a maximum. Kinetic constants for phosphorylation of myofibrillar MLC2 by PKC and MLCK under these *in vitro* conditions were also determined. K_m ($4.8 \pm 0.3 \mu\text{M}$; mean $\pm$ S.D.) and V_{max} ($128 \pm 8 \text{ pmol/min per mg}$; mean $\pm$ S.D.) values for MLCK also reflected the faster rate of phosphorylation by this enzyme as compared with that by PKC ($K_m = 10.3 \pm 3 \mu\text{M}$; $V_{\text{max}} = 76 \pm 15 \text{ pmol/min per mg}$). V_{max} values for phosphorylation of MLC2 using mixed light chains as substrate were more than 100-fold higher for both PKC and MLCK than when myofibrils were used. It is possible, therefore, that ATP depletion by the intrinsic myofibrillar MgATPase reduces the rate of myofibrillar MLC2 phosphorylation under *in vitro* conditions. The maximal phosphorylation stoichiometry reached when myofibrils were incubated with both PKC and MLCK together was no greater than that reached upon incubation with PKC alone (0.7 mol/mol), suggesting that PKC may phosphorylate the MLCK sites in MLC2 *in vitro*. This hypothesis was supported by two-dimensional tryptic phosphopeptide mapping. Phosphorylation of myofibrillar MLC2 by MLCK *in vitro* resulted in the detection of two phosphopeptides

Figure 3 Two-dimensional phosphopeptide mapping of MLC2 phosphorylated by PKC and MLCK *in vitro*

Myofibrils were phosphorylated *in vitro* by either PKC or MLCK. Myofibrillar proteins were separated by SDS/PAGE, and MLC2 was excised from the gel, trypsin-digested and subjected to two-dimensional phosphopeptide mapping. The origins are indicated by arrows. (a) MLCK, (b) PKC, (c) 1:1 mixture of MLCK and PKC. Reproducible results were obtained in four separate experiments.

(spots 1 and 3) on two-dimensional maps (Figure 3a). Trypsin digestion and mapping of the protein following phosphorylation by PKC *in vitro* generated what appear to be the same two phosphopeptides (Figure 3b). Approx. 85% of the 32 P label incorporated into MLC2 by PKC was present in the MLCK phosphopeptides (spots 1 and 3), with a lesser incorporation of label (15%) into one or more additional sites (spots 5 and 6). A composite map (Figure 3c) of a 1:1 mixture of the samples mapped in 3(a) and 3(b) further suggested that the major sites of phosphorylation *in vitro* may be the same for both MLCK and PKC. Phosphoamino acid analysis showed that incorporation of phosphate into MLC2 under these conditions by either MLCK or PKC occurred exclusively at phosphoserine residues (results not shown).

Effects of protein kinase inhibitors and protein phosphatase inhibitors in *in situ* phosphorylation of MLC2 in isolated cardiomyocytes

In a previous investigation of MLC2 phosphorylation in perfused rat heart, Herring and England [25] found evidence for a very

Figure 4 Effects of calyculin A, calphostin C and chelerythrine on phosphorylation of myofibrillar proteins in isolated cardiomyocytes

Isolated cardiomyocytes were incubated for 2 h with [32 P] P_i and for a further 10 min with no addition (control), 10 nM calyculin A (CL-A), 5 μ M calphostin C (Cal C) or 10 μ M chelerythrine (Chel.). The 32 P content of myofibrillar proteins was analysed by SDS/PAGE and autoradiography. Representative autoradiograms from two different reproducible experiments (a and b) are shown. The apparent increase in basal (control) 32 P labelling in experiment (b) is due to longer exposure time of the dried gel to X-ray film.

rapidly turning over component of light chain phosphorylation which, these authors suggested, could have a significant regulatory role in cardiac contraction. In the present study, therefore, we have examined further the rapidly turning over phosphate of cardiac MLC2 and the possible involvement of PKC in the phosphorylation/dephosphorylation cycle. Isolated adult rat cardiac myocytes were metabolically labelled with [32 P] P_i and then briefly exposed (10 min) to various protein phosphatase inhibitors and protein kinase inhibitors. Analysis of myofibrillar proteins by SDS/PAGE and autoradiography showed that four proteins, identified as TnT, Tm, TnI and MLC2, were phosphorylated under basal (control) conditions (Figures 4a and 4b). Inhibition of dephosphorylation in myocytes with 10 nM calyculin A, a highly potent inhibitor of type 1 (IC_{50} 2 nM) and type 2A (IC_{50} 1 nM) protein phosphatases [33], resulted in a rapid and marked build-up of 32 P label in two myofibrillar proteins identified as TnI and MLC2 (Figure 4a). Within 10 min, the 32 P content of TnI was increased by 3.3 ± 0.8 -fold (mean $\pm$ S.D., $n = 5$) and that of MLC2 was increased by 5.4 ± 0.8 -fold ($n = 5$), suggesting a rapid turnover of phosphate in both of these proteins *in situ*. However, the rapid build-up of phosphate that occurred in MLC2 did not appear to be mediated by the Ca^{2+} -activated enzyme, MLCK, since the Ca^{2+} ionophores A23187 (0.01, 0.1 or 1.0 μ M) and ionomycin (1.0 or 10.0 μ M) had no effect on phosphorylation (results not shown).

In order to identify whether PKC might be responsible for incorporating the rapidly turning over phosphate in MLC2 *in situ*, the PKC-selective protein kinase inhibitors calphostin C and chelerythrine were used. Calphostin C [34] and chelerythrine [35] have been reported to be more selective for inhibition of PKC than are other commonly used inhibitors such as

staurosporine [36]. Staurosporine, for example, inhibits ATP binding to PKC but inhibits binding of ATP to other protein kinases as well. In contrast, calphostin C and chelerythrine are highly selective for PKC because they interact specifically with either the regulatory domain or the active site of the enzyme [34,35]. Brief exposure of cardiomyocytes (10 min) to calphostin C (5 μ M) or chelerythrine (10 μ M) significantly decreased 32 P labelling of MLC2 (Figure 4b). The 32 P content of MLC2 was reduced by $54 \pm 9\%$ (mean $\pm$ S.D.; $n = 3$) and $98 \pm 8\%$ ($n = 3$) respectively, indicating that PKC, and not MLCK, was primarily responsible for the incorporation of rapidly turning over phosphate into cardiac MLC2. This interpretation was supported by the results of *in vitro* phosphorylation experiments in which the selectivity of the two inhibitors for PKC over MLCK was confirmed. Phosphorylation of MLC2 in cardiac myofibrils by PKC *in vitro* was almost completely inhibited by 5 μ M calphostin C and by 10 μ M chelerythrine. Phosphorylation by MLCK *in vitro* was unaffected by either of these two inhibitors but was significantly inhibited *in vitro* under the same conditions by the MLCK-selective inhibitors kaempferol [37] and KT5926 [38]. The effects of KT5926 and of the calmodulin antagonist calmidazolium [39] on *in situ* phosphorylation of MLC2 in cardiomyocytes were also examined. The selective inhibition of MLCK appeared not to be achieved in these experiments, however. Neither KT5926 nor calmidazolium inhibited MLC2 phosphorylation *in situ*, except when used at concentrations high enough to also inhibit TnI phosphorylation (suggesting non-selective effects of the inhibitors at these higher concentrations).

The site specificity of MLC2 phosphorylation *in situ* was analysed by two-dimensional tryptic phosphopeptide mapping. Basal (control) phosphorylation occurred almost exclusively in phosphopeptides 1 and 3 (Figure 5a). These sites correspond to the major sites of phosphorylation by PKC *in vitro* (Figure 3b) and to the only sites of phosphorylation by MLCK *in vitro* (Figure 3a). However, in view of the significant inhibition of phosphorylation by the PKC-selective inhibitors chelerythrine and calphostin C (Figure 4), we conclude that this basal site-specific phosphorylation was catalysed primarily by PKC. Furthermore, phosphorylation by PKC *in situ* appears to occur almost exclusively at the sites phosphorylated *in vitro* by both PKC and MLCK (Figure 3). A faint spot (spot 6) was also detected for phosphorylation *in situ* which corresponds to a minor site of phosphorylation by PKC *in vitro*. This phosphopeptide was never detected for MLC2 phosphorylated by MLCK *in vitro*. Calyculin A (10 nM) treatment of myocytes increased the 32 P labelling of both major phosphopeptides (spots 1 and 3; Figure 5b), presumably as a result of blocking dephosphorylation at sites in MLC2 phosphorylated by PKC. However, it is conceivable that phosphatase inhibition by calyculin A could also have had an indirect effect on MLC2 phosphorylation through an inhibition of dephosphorylation of the PKC (or MLCK) enzyme itself, with a concomitant increase in activity of the protein kinase. Phosphoamino acid analysis of MLC2 phosphorylated in myocytes showed that all phosphate was contained in phosphoserine; no phosphothreonine was detected for the protein from either control or calyculin-A-treated myocytes (results not shown).

Role of MLC2 phosphorylation in determining myofibrillar Ca^{2+} sensitivity

It has been shown previously that addition of exogenous MLCK and calmodulin to skinned cardiac muscle fibres results in increased myofibrillar Ca^{2+} sensitivity, an effect that has been attributed to MLCK-mediated phosphorylation of MLC2

Figure 5 Two-dimensional phosphopeptide mapping of MLC2 phosphorylated *in situ* in isolated cardiomyocytes

Myofibrils were phosphorylated *in situ* in ^{32}P -labelled cardiomyocytes by incubation for 2 h with $[\text{}^{32}\text{P}]\text{P}_i$ followed by a further 10 min incubation with either no addition (control) or 10 nM calyculin A (CL-A). Equal amounts of myofibrillar protein from each condition were analysed by SDS/PAGE, then MLC2 was excised from the gel, trypsin-digested and subjected to two-dimensional phosphopeptide mapping. Arrows indicate the origins. (a) Control, (b) calyculin A. Results were confirmed in four experiments.

[14–17]. An important implication of the present study is that PKC-mediated phosphorylation of MLC2 may have the same effect on sensitivity, since the same sites in MLC2 are phosphorylated by both MLCK and PKC. A further possibility suggested by these findings is that addition of exogenous MLCK to skinned cells may mimic an effect that is actually mediated in heart by PKC. Whether mediated by PKC or by MLCK, however, phosphorylation does appear to be linked to increased Ca^{2+} sensitivity. Previous investigations into this link have relied on the use of chemically skinned cells and exogenously added protein kinase [14–17]. Whether a relationship exists between endogenous phosphorylation and Ca^{2+} sensitivity in intact (unskinned) heart cells has not been established. In the present study, we have found evidence for this relationship in intact cells by using the Ca^{2+} -desensitizing contractile inhibitor BDM [40]. Treatment of ^{32}P -labelled myocytes for 10 min with 5 mM BDM resulted in significant dephosphorylation of myofibrillar proteins (Figure 6), consistent with the known effect of the nucleophilic agent to act as a 'chemical phosphatase' [41]. Interestingly, TnI phosphorylation was not affected by BDM treatment, suggesting that phosphate incorporated into this protein was not accessible to nucleophilic attack by the agent. In contrast, ^{32}P -labelled phosphate was clearly removed from C-protein, TnT and Tm. The most pronounced effect of BDM, however, was to promote the complete removal of labelled phosphate from MLC2. The negative inotropic effect of BDM on cardiac contraction is known to involve a decreased Ca^{2+} responsiveness of the myofilaments [42–44]. It has, therefore, been suggested that BDM exerts its Ca^{2+} desensitizing effect through binding to saturable sites on myofibrillar proteins [43]. The results presented

Figure 6 Effects of BDM on the phosphorylation of myofibrillar proteins in isolated cardiomyocytes

Isolated cardiomyocytes were incubated for 2 h with $[\text{}^{32}\text{P}]\text{P}_i$ and for a further 10 min with either no addition (control) or 5 mM BDM. Phosphorylation of myofibrillar proteins was analysed by SDS/PAGE and autoradiography. Proteins are identified by Coomassie Blue staining as myosin heavy chain (MHC), C-protein (C-prot), actin, TnT, Tm, TnI, MLC1 and MLC2. These results were observed in three different experiments.

here suggest an alternative explanation, namely desensitization through dephosphorylation of MLC2.

General discussion

The results of the present study demonstrate that cardiac MLC2 (or a subpopulation thereof) is subject to rapid cycles of phosphorylation and dephosphorylation *in situ*. We have also provided evidence that PKC, and not MLCK, is primarily responsible for incorporating this rapidly turning over phosphate into MLC2 in heart cells. A role for PKC in MLC2 phosphorylation was also suggested in our previous investigation [26] in which MLC2 was shown to be phosphorylated by PKC *in vitro* and in phorbol ester-stimulated cardiomyocytes. The relatively modest effects of phorbol esters on the phosphorylation of MLC2 in this previous study (0.2 mol of phosphate incorporated/mol of protein) may have been due to the rapid rate of PKC-mediated MLC2 phosphorylation shown here to occur even under basal (non-phorbol-ester-stimulated) conditions. Whereas the present results implicate PKC in MLC2 phosphorylation, they also implicate protein phosphatases 1 and/or 2A in MLC2 dephosphorylation in heart cells. Previous studies [45,46] have shown that both of these phosphatases effectively dephosphorylate cardiac MLC2 *in vitro*. The rapid increase in the amount of ^{32}P label in MLC2 that we have observed (due to phosphatase inhibition *in situ*) suggests that the rate of dephosphorylation by protein phosphatases and/or 2A is very fast. In contrast, phosphorylation by PKC *in vitro* and *in situ* [26] is much slower. Thus dephosphorylation rather than

phosphorylation may be more important in determining the state of phosphorylation of cardiac MLC2 *in situ*.

Examination of the stoichiometry and site specificity of MLC2 phosphorylation *in vitro* shows that PKC and MLCK phosphorylate the same sites to similar extents. Phosphorylation by either kinase, therefore, is likely to have the same consequence of increasing myofibrillar Ca^{2+} sensitivity. However, the overall effect of PKC-catalysed phosphorylation of myofibrils *in vitro* (where C-protein, TnT, TnI and MLC2 are all phosphorylated) does not involve a detectable change in Ca^{2+} sensitivity [47]. The dominant effect that is observed under these conditions of phosphorylation is an inhibition of maximal actomyosin MgATPase activity, most likely as a result of phosphorylation of TnI and TnT [48]. Any effect of PKC-catalysed phosphorylation of MLC2 in increasing Ca^{2+} sensitivity appears to be over-ridden in this case by the inhibitory effects of phosphorylation of TnI and TnT. It remains possible, however, that selective phosphorylation by PKC of MLC2 only could alter the Ca^{2+} sensitivity in the same way as does MLCK-mediated phosphorylation. Studies designed to directly test this hypothesis are currently under way in our laboratory. Such studies could have relevance *in vivo* if different PKC-mediated signalling pathways exist in the heart which involve selective phosphorylation of certain (but not necessarily all) PKC substrates. For example, different signalling pathways could be coupled to different PKC isoenzymes having different substrate specificities. This possibility has been suggested recently by Pucat et al. [49] as an explanation for why phorbol esters and α -adrenergic agonists, both activators of PKC, can have opposite effects on cardiac contractility. These authors hypothesize that phorbol esters may activate all PKC isoenzymes present in heart, whereas α -adrenoreceptor agonists may activate only a specific subtype. In this regard, it is tempting to speculate that the known effect of α -adrenergic agonists in increasing cardiac myofibrillar Ca^{2+} sensitivity [50,51] may occur, at least in part, through PKC-mediated phosphorylation of MLC2.

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