Proteomic Analysis of Pharmacologically Preconditioned Cardiomyocytes Reveals Novel Phosphorylation of Myosin Light Chain 1

D. Kent Arrell, Irina Neverova, Heather Fraser, Eduardo Marbán, Jennifer E. Van Eyk

Abstract—Proteomic analysis of rabbit ventricular myocytes revealed a novel posttranslational modification to myosin light chain 1 (MLC1), consisting of phosphorylation at two sites. Subproteomic extraction to isolate myofilament-enriched fractions enabled determination of the extent of phosphorylation, which increased from 25.7±1.6% to 34.0±2.7% (mean±SE, n=4; P<0.05) after adenosine treatment at levels sufficient to pharmacologically precondition the myocytes (100 μmol/L). Mass spectrometry of MLC1 tryptic digests identified two peptide fragments modified by phosphorylation. These two phosphopeptides were characterized by peptide mass fingerprinting to determine the phosphorylation sites within rabbit ventricular MLC1, which correspond to Thr69 and Ser200 of rat MLC1, and to Thr64 and Ser194 or 195 of human MLC1. This proteomic analysis of preconditioned myocardium has revealed a previously unsuspected in vivo posttranslational modification to MLC1. (Circ Res. 2001;89:480-487.)

Key Words: proteomics  ■  myosin light chain  ■  phosphorylation  ■  adenosine  ■  preconditioning

Myocardial ischemic preconditioning (PC), a phenomenon that exists in all species examined, including humans,1-3 is a form of cardioprotection whereby a brief ischemic episode reduces the extent of damage from subsequent prolonged ischemia.4 PC may also be recruited pharmacologically, with one of many activators being adenosine.5 Both ischemic and pharmacological PC trigger two windows of protection, the first (classical PC) becoming manifest within 15 minutes and lasting 1 to 3 hours.1,6-8 The rapid onset and short duration of protection afforded by classical PC are likely the result of posttranslational protein modifications, because 15 minutes does not suffice to recruit de novo transcription and translation.

Although the cardioprotective effect of PC is well established, the molecular mechanisms remain elusive. Although much of the current research into effectors of classical PC focuses on the opening of the inner mitochondrial ATP-sensitive potassium (mitoKATP) channel in response to activation of complex kinase signaling cascades, there may well be other effectors of PC. It is known that ischemia-induced release of adenosine, bradykinin, opioids, and free radicals leads to receptor-mediated activation of protein kinase C (PKC).9,10 In addition, other kinases that have been implicated in PC include a tyrosine kinase and a number of mitogen-activated protein kinases (MAPKs), the most likely candidates of which are in the c-Jun N-terminal kinase (JNK) and p38 MAPK families.9 Although these kinases are known to be activated during PC, their intracellular substrates during this process have yet to be determined. Until these substrates are identified, potential mechanisms by which these signaling cascades combine to foster cardioprotection will remain obscure.

Proteomics is an ideal approach by which to elucidate posttranslational protein modifications associated with kinase activation. The sheer complexity of any proteome, however, precludes detection and characterization of potentially subtle protein modifications. By focusing the effort on various subproteomes in isolation, essentially peeling away individual layers of the proteome, this needle-in-a-haystack task is simplified. Herein, we describe the use of such an approach to identify a novel phosphorylation of myosin light chain 1 (MLC1) in a myofilament-enriched subproteome of rabbit ventricular myocytes, the extent of which increased significantly after adenosine treatment.

Materials and Methods

Isolation and Preconditioning of Rabbit Ventricular Myocytes

Rabbits were used in compliance with the Animals for Research Act (Province of Ontario), the Canadian Council on Animal Care, and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1985).

Rabbit ventricular myocytes were isolated by collagenase dissociation from New Zealand White rabbits (weighing 1 to 2 kg), as
described previously.11 Hearts were perfused with collagenase (1.0 mg/mL, Worthington type II) for 14 minutes at a maintained perfusion pressure of 75 mm Hg on a Langendorff apparatus, yielding >50% Ca2+-tolerant ventricular myocytes. Cell isolation was followed directly by pharmacological preconditioning, which was carried out by treatment with 100 μmol/L adenosine (Sigma) for 60 minutes in a 37°C water bath, as described previously.12 Untreated cells were prepared concurrently as drug-free controls. Equivalent 25-μL aliquots of cells (containing ~30 mg of protein as determined by Lowry assay13) were frozen in a dry ice/ethanol bath and stored at −80°C until analysis.

Protein Extraction and Protein Dephosphorylation
All steps in the IN Sequence protein extraction protocol were carried out at 4°C, and all centrifugations were conducted at 16,000g for 2 minutes at 4°C. Each aliquot of myocyte proteins was first extracted by two rounds of homogenization in 100 μL of HEPES extraction buffer, consisting of (in mmol/L) HEPES 25 (pH 7.4), NaF 50, Na3VO4 0.25, phenylmethylsulfonyl fluoride 0.25, EDTA 0.5, and (in μmol/L) leupeptin 1.25 and pepstatin A 1.25. After homogenization and centrifugation, the supernatants were pooled and saved as the cytosolic extract. The remaining pellet was subjected to further extraction by two rounds of homogenization in 50 μL of acid extraction buffer, consisting of 1% vol/vol trifluoroacetic acid (TFA) and 1 mmol/L Tris (2-carboxyethylphosphine) hydrochloride (pH 8.0). Supernatants were again pooled and saved as the acid extract. The two extracts and remaining pellet were frozen and stored at −80°C.

Proteins were dephosphorylated using the following protocol. An aliquot of myocytes in cell medium was mechanically homogenized and divided into two equal volumes (~375 μg of protein each). Alkaline phosphatase reaction buffer (2.5 μL of 10× stock) consisting of (in mmol/L) NaCl 1100, MgCl2 100, dithiothreitol (DTT) 10, and Tris–HCl 500 (pH 7.9 at 25°C) was added to each sample, then 10 μL of calf intestinal alkaline phosphatase (10 U/μL, New England Biolabs) in 50% glycerol and (in mmol/L) KCl 50, MgCl2 1, ZnCl2 0.1, and Tris–HCl 10 (pH 8.2) was added to one sample, while 10 μL of the same solution lacking alkaline phosphatase was added to the other. Samples were incubated at 37°C for 15 minutes and the reaction stopped by addition of 20 volumes (500 μL) of isoelectric focusing (IEF) buffer (8 μL/mL urea, 2% wt/vol 3-[3-cholamidopropyl]-1-propane-sulfonate, 0.5% ampholines (either pH 4 to 7 or 3 to 10), 50 mmol/L DTT, and 0.01% wt/vol bromophenol blue). Samples were frozen and stored at −80°C until analyzed by two-dimensional gel electrophoresis (2-DE).

Two-Dimensional Gel Electrophoresis
IEF was carried out using a Protean IEF cell (Bio-Rad) according to the manufacturer’s protocol. Immobilized pH gradient (IPG) Ready Strips (170 mm, pH 4 to 7 or pH 3 to 10 linear gradient, Bio-Rad) were activated for 2 hours in water solution, then rehydrated at 50 V for 10 hours to enhance protein uptake, then subjected to the following conditions using a rapid-voltage ramping method: 100 V for 25 Volt-hours (Vh), 500 V for 125 Vh, 1000 V for 250 Vh, and 8000 V for 85 kVh. A Peltier temperature control platform maintained gels at 4°C throughout the experiment. Focused gels were stored at −20°C before sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

For SDS-PAGE, IPG strips were incubated for 10 minutes in equilibration buffer (50 mmol/L Tris–HCl [pH 8.8], 6 mol/L urea, 30% vol/vol glycerol, 2% wt/vol SDS) supplemented with 10 mg/mL DTT, followed by a 10-minute incubation in equilibration buffer supplemented with 25 mg/mL iodoacetamide, then rinsed once with SDS-PAGE buffer (25 mmol/L Tris, 192 mmol/L glycine [pH 8.3], 0.1% wt/vol SDS). IEF strips were then embedded in a 5% acrylamide stacking gel, and the proteins were resolved by 12.5% SDS-PAGE (10% for Figure 1B) using a Protein II XL system (Bio-Rad). Electrophoresis was carried out at 50 V for 30 minutes, followed by 150 V for 7.5 hours.

Protein Transfer and Western Blotting
After 2-DE, gels were equilibrated in SDS-PAGE buffer supplemented with 20% vol/vol methanol for 10 minutes, then transferred in the same buffer to nitrocellulose at 200 mA constant current for 2 hours. Nitrocellulose membranes were then rinsed with phosphate-buffered saline/Tween-20 (PBS/T), consisting of (in mmol/L) NaCl 137, KCl 2.7, NaHPO4 10.1, and KH2PO4 1.8 (pH 7.4), supplemented with 0.1% vol/vol Tween-20, then blocked overnight at 4°C with 1% vol/vol blocking reagent (Roche Diagnostics) in PBS/T. Western blotting for MLC1 was carried out using monoclonal antibody (mAb) 39-121 (Spectral Diagnostics) at 1 μg/mL and detected by chemiluminescence with an alkaline phosphatase–conjugated secondary antibody.

Silver Staining of Two-Dimensional Gels
Two-dimensional gels were silver-stained according to the protocol of Shevchenko et al.14 for compatibility with subsequent analysis of protein by mass spectrometry. Gels were fixed overnight in 50% vol/vol methanol, 5% vol/vol acetic acid, followed for 10 minutes by 50% vol/vol methanol, then 10 minutes in deionized distilled (dd) H2O. Gels were sensitized for 1 minute in 0.02% wt/vol sodium thiosulfate, followed by two 1-minute ddH2O washes, then incubated in chilled (4°C) 0.1% wt/vol silver nitrate for 20 minutes, followed again by two 1-minute ddH2O washes. Proteins were then visualized by several washes with developing solution (2% wt/vol sodium carbonate, 0.04% vol/vol formalin) until a desired level of staining was achieved, after which development was stopped with 5% vol/vol acetic acid.

Image Analysis and Quantification
Silver-stained 2-D gels were digitized at a resolution of 150 dpi (pixels per inch) using a PowerLook II scanner (UMAX Data Systems, Inc) on a Sun Ultra5 computer (Sun Microsystems, Inc). Protein spots were then located, quantified, and matched to spots on other gels using Investigator HT Proteome Analyzer 1.0.1 software (Genomic Solutions, Inc). Fifteen manually defined spots were selected as anchors for triangulation of remaining spots. Composite images were then prepared by matching spots from four gel images for each treatment group (adenosine and control) and normalized using a match ratio method to compensate for any variation in protein loading and level of silver stain development between gels (see online Figure 1, available in the data supplement at http://www.circresaha.org). Gels used for MLC1 quantification were all within an optimal staining range for MLC1 (see online Figure 2). Quantification of MLC1 phosphorylation in each silver-stained gel was carried out using Proteome Analyzer in the following manner. Integrated intensities of the three MLC1 spots were obtained with Proteome Analyzer, then summed to give an overall integrated intensity of MLC1 for each gel, and these values were converted to percentages of the total MLC1 for each of the three spots. Thus, determination of the extent of phosphorylation (mean ± SE) was conducted within individual gels, and never between gels, to minimize error associated with gel-to-gel variability. Data were analyzed by one-way ANOVA, with a value of P < 0.05 considered to be significant.

Mass Spectrometry
Protein spots extracted from 2-D gels were destained according to the protocol of Gharahdaghi et al.,15 then dried under vacuum before enzymatic digestion with sequence-grade modified trypsin (Promega). Tryptic peptides were extracted with 50% acetonitrile (ACN)/5% TFA, dried under vacuum, and reconstituted with 3 μL of 50% ACN/0.1% TFA. Reconstituted extract (0.5 μL) was mixed with 0.5 μL of matrix (10 mg/mL α-cyano-4-hydroxy-trans-cinnamic acid in 50% ACN, 0.1% TFA), spotted on a stainless steel 100-well mass spectrometry plate, and air-dried. Samples were analyzed using a Voyager DE-Pro matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometer (PerSeptive Biosystems) operated in the delayed extraction/reflector mode with an accelerating voltage of 20 kV, grid voltage...
setting of 72%, and a 50-ns delay. Five spectra (50 to 100 laser shots/spectrum) were obtained for each sample. External calibration was performed using a Sequazyme Peptide Mass Standard kit (PerSeptive Biosystems) containing the following standards: des-Arg-bradykinin, angiotensin-1, and Glu-fibrinopeptide B.

Bioinformatic Data Analysis
Peptide mass fingerprinting (PMF) was conducted with the database search tool MS-Fit in the program Protein Prospector (version 3.2.1), available at http://prospector.ucsf.edu/. A number of restrictions were applied to the initial search: species=mammals, isoelectric point (pI) range=4 to 7, mass range=0 to 30 kDa (1 Da mass tolerance), with a minimum of 4 peptides to match, and a maximum of one missed tryptic cleavage. Top candidate proteins identified by MS-Fit were then analyzed for theoretical tryptic peptide fragments using MS-Digest, taking into account phosphorylation of Ser, Thr, and Tyr residues, then compared with the observed spectra, allowing a mass tolerance of 0.2 Da. MLC1 sequence alignments were prepared with the program Clustal W (version 1.81) provided online at the European Bioinformatics Institute (http://www2.ebi.ac.uk/clustalw/).

Results
Silver-stained 2-D gels containing 750 µg of protein were able to resolve as many as 1500 protein spots from rabbit ventricular myocyte whole-cell homogenates (Figure 1A). Locating posttranslational modifications within this complex
proteome was initially facilitated by the use of narrower (pH 4 to 7) IEF gradients, with protein loading reduced to 250 μg (Figure 1B). Two examples of adenosine-mediated changes located within this gel (labeled regions 1 and 2) are enlarged adjacent to their corresponding regions from 2-D gels of drug-free controls to illustrate these differences (Figure 1B, right panels). There were, however, limitations to the identification of these proteins from whole-cell homogenates. For example, the low abundance of the protein in region 1 precluded its identification by mass spectrometry, whereas at increased loads this region was obscured by other proteins. Although abundance was no longer an issue with the proteins in region 2, the spot pattern was very complex. To reduce this complexity, two sequential subproteomes were isolated (Figure 2).

Numerous lower-abundance cytosolic proteins were enriched in the first subproteome, after extraction at physiological pH (Figure 2A, cytosolic extracts). This process reduced the complexity in these extracts to ~800 protein spots and enabled visualization of additional protein changes not evident in whole-cell homogenates. Adenosine-mediated changes located within this gel (labeled regions 3 through 5) are enlarged adjacent to corresponding regions from control 2-D gels to depict these changes (Figure 2A, right panels). The second subproteome was derived from acid extraction of the pellet remaining after cytosolic extraction (Figure 2B, acid extracts). This subproteome greatly enriched a number of high-abundance proteins (which all proved to be myofilament proteins; data not shown), including protein spots in the same position as those of region 2 in Figure 1B. Proteins visible by silver staining of these extracts were clustered around pH 5; thus 2-DE resolution was improved by separation within the narrower pH 4 to 7 IEF gradient, yielding ~55 detectable spots. The most highly reproducible difference between the acid extracts of adenosine-treated and control myocytes was again observed in region 2 (see Figure 2B, right panel), with a trail of only four protein spots remaining from the original proteome in this particular extract (compare to region 2 of Figure 1B). In this case, however, the extent of the acidic shift of protein was less prominent than that observed in the whole-cell homogenate. When compared with known 2-D gel databases, at least one or possibly more of these four spots (relative mobility [M] ~26 kDa and pI ~4.7 to 5.0) were suspected to be ventricular MLC1, although posttranslational modification of MLC1 has never previously been demonstrated.

Protein identity and the nature of its posttranslational modification were initially confirmed by Western blotting with a ventricular MLC1 monoclonal antibody to probe 2-DE–separated acid extracts (data not shown) and whole-cell homogenates (Figure 3A). Three of the four spots were confirmed to be MLC1 in both the myofilament-enriched subproteome and the entire proteome (compare region 2 in Figures 1B, 2B, and 3A). The three-spot pattern was no longer evident in samples dephosphorylated prior to 2-DE. Instead, only a single spot remained (Figure 3A, bottom panel). Together, the Western blots indicated that (1) MLC1 was posttranslational modified and (2) MLC1 modification resulted from phosphorylation. Thus, MLC1 was present in rabbit ventricular myocytes as a mixed population of unphosphorylated, monophosphorylated, and diphosphorylated proteins.

Composite images representing the normalized average of four rabbits for each experimental group showed subtle differences visible in the extent of MLC1 phosphorylation (Figure 3B, composite images), with increased phosphorylation after adenosine treatment. Quantification of the exact extent of MLC1 phosphorylation was carried out within each of the gels used to derive these composite images. For adenosine-treated rabbits (n=4), 66.1±2.7% of the total MLC1 was unphosphorylated, 28.9±2.4% was monophosphorylated, and 5.1±1.0% was diphosphorylated. For drug-free controls (n=4), the corresponding values were 74.3±1.6%, 20.6±1.7%, and 5.2±0.5%, respectively. Overall, there was a significant increase in MLC1 phosphorylation, from 25.7±1.6% in controls, to 34.0±2.7% after treatment with adenosine (P<0.05). Of note, adenosine treatment increased specifically the monophosphorylated MLC1 form, with virtually no change in the proportion of diphosphorylated-MLC1.

Further confirmation of protein identity and, importantly, the identification of the phosphorylated amino acid residues was achieved by tryptic peptide fingerprinting using MALDI-TOF mass spectrometry. Tryptic peptide fragments isolated from each of the three spots yielded highly consistent mass spectra (Figure 3A). PMF analysis of these fragments identified strong matches to two proteins, rat ventricular MLC1 and human ventricular MLC1, further supporting protein identity. Important differences were observed, however, in spectra obtained from unphosphorylated versus phosphorylated MLC1 spots. Fragments with masses of 558.667 and 790.845 Da present in unphosphorylated MLC1 spectra (Figures 4B and 4C, upper spectra) were conspicuously absent from phosphorylated samples. Meanwhile, mass spectra from phosphorylated MLC1 samples contained two other fragments not present in unphosphorylated samples, with masses of 638.601 and 870.764 Da (Figures 4B and 4C, lower spectra). Addition of a phosphate moiety (79.9797 Da) to each of the two unique fragments detected in unphosphorylated MLC1 mass spectra would result in phosphopeptides varying in mass by less than 0.1 Da from the two unique fragments identified in phosphorylated MLC1 mass spectra, indicating that the differences in mass spectra arose from phosphorylation of one amino acid residue within each of these two tryptic peptide fragments.

Although no rabbit MLC1 sequence was available, PMF matched these fragments to theoretical tryptic peptide fragments of rat ventricular MLC1. Rat MLC1 amino acids 196 to 200 matched the smaller 558.667-Da fragment, whereas amino acids 69 to 75 matched the larger 790.845-Da fragment. Clustal W sequence alignments indicated that these peptides aligned with human ventricular MLC1 amino acid peptide fragments 191 to 195 and 64 to 70, respectively (see rat and human ventricular MLC1 entries, Table). Importantly, each of the two rat and two human MLC1 fragments contained phosphorylatable residues. Thus, the potential MLC1 phosphorylated residues correspond to Thr69 and
Ser200 in rat (Thr64 and Ser194 or Ser195 in human). These amino acids were located in highly conserved regions of MLC1 and were consistently present in various species and striated muscle types (Table).

**Discussion**

Despite extensive studies of MLC1, the protein has never previously been shown to be phosphorylated in vivo (see MLC review16). The only successful phosphorylation of

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**Figure 2.** Adenosine-induced changes to cytosolic and myofilament-enriched subproteomes. Cytosolic and myofilament-enriched subproteomes obtained by sequential extraction (described in Materials and Methods) were separated by 2-DE to identify adenosine-induced protein modifications. Panel A (pH 3 to 10 linear gradient) shows a silver-stained gel of adenosine-treated cytosolic extract (90% of the protein extracted from 750 μg of whole-cell homogenate). Adenosine-induced protein modifications observed in these extracts are indicated by dashed boxes, designated 3 (~28 kDa, pI ~5.5), 4 (~37 kDa, pI ~8.5), and 5 (~36 kDa, pI ~6.0). Enlargements of these regions from 2-D gels of adenosine-treated (Ado) and control (Ctrl) samples are shown in the side panels for comparison. Panel B (pH 4 to 7 linear gradient) shows a silver-stained gel of adenosine-treated myofilament-enriched extract (containing 10% of the TFA-solubilized protein isolated from cytosolic extract pellets). An adenosine-induced protein modification observed in these extracts is indicated by a dashed box, designated 2, as it corresponds to region 2 of Figure 1B (~26 kDa, pI ~4.7 to 5.0). Enlargement of this region from 2-D gels of adenosine-treated (Ado) and control (Ctrl) samples is shown in the side panel for comparison. The positions of SDS-PAGE molecular weight standards are indicated on the left for each gel.
MLC1 was carried out in vitro, using MLC kinase. This could only be accomplished using ATPγS, and the same could not be achieved in the presence of [32P]-γATP. In these studies, neither the extent of MLC1 phosphorylation nor the phosphorylated amino acid residues of MLC1 were identified.

MLC1 amino acid sequences share an extremely high degree of identity, and the proposed MLC1 phosphorylation sites are also extremely conserved throughout known striated muscle MLC1 sequences (Table). Based on the crystal structure of the myosin head region, it can be inferred that both residues reside in exposed regions of this protein near the myosin heavy chain (MHC) lever arm. The serine site is located directly adjacent to the MHC lever arm extending through MLC1, while the threonine site is located in the region of closest proximity between MLC1 and myosin light chain 2 (MLC2), a protein that is also associated with the MHC lever arm adjacent to MLC1. Therefore, MLC1 phosphorylation in this region might also influence MLC2. Phosphorylation of MLC2 results in movement of crossbridges out from the myosin filament into closer proximity to actin, increasing not only the likelihood of attachment and force generation but also Ca2+ sensitivity and ATPase activity.

What effect might MLC1 phosphorylation have on its own function? Although there is no prior evidence of any MLC1 posttranslational modification to give us a clue, there are several reported instances of genetic modification of MLC1 ventricular expression. Interestingly, a single Met149Val point mutation in human ventricular MLC1 was found to increase in vitro filament translocation velocity by 41%. In addition, a number of cases of altered MLC1 ventricular expression in chronic cardiovascular disease states may be associated with significant functional consequences. Hypertrophied left ventricles of patients with ischemic, dilated, and hypertrophic cardiomyopathy express small amounts of atrial MLC1, not normally present in the adult ventricle. Postsurgical return to a normal hemodynamic state decreased this ventricular expression of atrial MLC1. Importantly, partial substitution of atrial for ventricular MLC1 in human...
ventricles was accompanied by increased maximal shortening velocity, rate of tension redevelopment, isometric force generation, and Ca\(^{2+}\) sensitivity, thereby improving cardiac contractility. In fact, atrial MLC1 expression at only 3% of total MLC1 in ventricles increased isometric tension from 352 to 660 kN/m at saturating Ca\(^{2+}\) concentrations in skinned fibers with fully dephosphorylated MLC2.

These functional changes between atrial and ventricular MLC1 may relate to the proposed MLC1 function as an actin/myosin tether. The N-terminus of MLC1 is known to interact with the C-terminus of actin. Inhibition of this interaction by competitive inhibition with synthetic N-terminal MLC1 peptides at low peptide-to-actin ratios also increased force production, shortening velocity, and myofibrillar ATPase activity.

Taken together, these examples clearly illustrate that minute changes in ventricular MLC1 composition, even as little as 3%, can have a dramatic impact on myocyte function and heart contractility at the gene expression level. Nevertheless, the functional implications of this novel phosphorylation are unknown, and its possible contribution to the preconditioned phenotype remains to be dissected. Perhaps MLC1 phosphorylation provides a potential avenue for influencing contractility in a similar fashion at the posttranslational level.

### Alignment of Sequences Surrounding Proposed MLC1 Phosphorylation Sites†

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<th>Ventricular</th>
<th>Atrial</th>
<th>Skeletal</th>
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<tbody>
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<td>Rat vMLC1</td>
<td>51-FTPQEQEYFKEALFDR</td>
<td>44-FADQDEKFEEALFDR</td>
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<td>Human vMLC1</td>
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<td>41-FSEKQOSQEVDFEALFDR</td>
</tr>
<tr>
<td>Mouse aMLC1</td>
<td>44-FADQDEKFEEALFDR</td>
<td>41-FSEKQOSQEVDFEALFDR</td>
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</table>

| Rat A2sMLC1 | 45-FSEKQOSQEVDFEALFDR |
| Human A2sMLC1 | 47-FSEKQOSQEVDFEALFDR |
| Mouse A2sMLC1 | 41-FSEKQOSQEVDFEALFDR |
| Bovine fMLC1 | 21-FSKQQDEKFEEALFDR   |
| Chick A2sMLC1 | 43-FSEKQOSQEVDFEALFDR |

<table>
<thead>
<tr>
<th>C-Terminal Sequence</th>
<th>Ventricular</th>
<th>Atrial</th>
<th>Skeletal</th>
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</thead>
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<td>Rat vMLC1</td>
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<td>127-YEDFVGLYVEKSMGGYR</td>
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<td>Rat aMLC1</td>
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<td>Human aMLC1</td>
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<td>122-YEDFVGLYVEKSMGGYR</td>
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<tr>
<td>Mouse aMLC1</td>
<td>128-YEDFVGLYVEKSMGGYR</td>
<td>124-YEDFVGLYVEKSMGGYR</td>
<td></td>
</tr>
</tbody>
</table>

| Rabbit A2sMLC1      | 127-YEDFVGLYVEKSMGGYR   | 123-YEDFVGLYVEKSMGGYR   |
| Rabbit fMLC1        | 85-YEDFVGLYVEKSMGGYR   | 120-YEDFVGLYVEKSMGGYR   |
| Rat A2sMLC1         | 124-YEDFVGLYVEKSMGGYR   | 121-YEDFVGLYVEKSMGGYR   |
| Rabbit fMLC1        | 86-YEDFVGLYVEKSMGGYR   | 122-YEDFVGLYVEKSMGGYR   |
| Rabbit fMLC1        | 86-YEDFVGLYVEKSMGGYR   | 123-YEDFVGLYVEKSMGGYR   |
| Mouse A2sMLC1       | 129-YEDFVGLYVEKSMGGYR   | 125-YEDFVGLYVEKSMGGYR   |
| Mouse A2sMLC1       | 129-YEDFVGLYVEKSMGGYR   | 126-YEDFVGLYVEKSMGGYR   |
| Bovine fMLC1        | 103-YEDFVGLYVEKSMGGYR   | 127-YEDFVGLYVEKSMGGYR   |
| Chick A2sMLC1       | 126-YEDFVGLYVEKSMGGYR   | 128-YEDFVGLYVEKSMGGYR   |

†All sequences are full-length MLC1, except for rabbit A2 and rat A2 fast skeletal isoforms (MLC3) and bovine fast skeletal MLC1 (only partial sequence is available). Shaded regions correspond to proposed phosphorylation sites. Identical (*) amino acid residues and conserved (: or semiconserved (.) substitutions are indicated below the aligned sequences. Numbers before and after sequences indicate the amino acid position of the first and last amino acid, respectively.
modification level, but this generalization must be couched as speculative.

In conclusion, exposure of isolated rabbit ventricular myocytes to adenosine at levels that have been established by Sato et al. to confer cardioprotection produced a small but significant increase in the levels of ventricular MLC1 phosphorylation. Since adenosine accumulation during ischemia leads to a complex signaling cascade and the involvement of many kinases, it was not surprising to identify a protein modified by phosphorylation. The surprise was that the protein was MLC1. The ability to observe a fully preconditioned, homogeneous cell population by a subproteomic protein was MLC1. The ability to observe a fully preconditioned, homogeneous cell population by a subproteomic approach increases the likelihood of locating and identifying such subtle protein modifications associated with pharmacological PC, changes that might otherwise be overlooked.

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References

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Online Figure 1. Production of composite gel images.
TFA extracts from ventricular myocytes of four separate rabbits were resolved by 2-DE (pH 4-7 linear gradient) and silver-stained (panel A, adenosine-treated extracts). Gels were digitized, then analyzed with Proteome Analyzer. All identified spots were quantified and matched, then matched spots were normalized by a match ratio method. Finally, data from the four gels were grouped to create a composite image (panel B, adenosine composite image). The positions of SDS-PAGE molecular weight standards are indicated on the left for each gel. For details of composite image construction, see methods section.

Online Figure 2. Protein quantification considerations from silver stained gels.
Quantification of total MLC1 and the relative proportions of each modification within a sample were not trivial, as suggested by surface profile plots of MLC1 from three separate silver-stained 2-D gels (A-C). As relative quantities of each MLC1 spot differ dramatically from one another, total protein load was critical for obtaining quantifiable spot intensities. Too little protein resulted in the faintest MLC1 spot remaining almost imperceptible above background (panel A). Too much protein resulted in oversaturation of the major MLC1 spot, resulting in decreased intensity at the centre of the spot (panel C). Thus, in these cases, subsequent quantification by peak integration would underrepresent the faintest and most intense spots, respectively. It was essential, therefore, to ensure that the faintest spot was accurately represented, and that the most intense spot did not become saturated, as observed in panel B. Background intensity of the three images was also noticeably different, as the development time in panel A was extended in an effort to see the faintest spot, while development time in panel C was shortened in response to the saturation of the most intense spot. Once protein load was optimized, quantification of MLC1 was carried out as described in the methods section.