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## Effects of contractile protein phosphorylation on force development in permeabilized rat cardiac myocytes

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■ **Abstract** The phosphorylation status of myofibrillar proteins influences the  $\text{Ca}^{2+}$  responsiveness of the myofilaments, but the contribution of and the interaction between the individual components is poorly characterized. Therefore, in Langendorff perfused rat hearts ( $n = 30$ ), the phosphorylation levels of cardiac myosin binding protein-C (cMyBP-C), troponin I and T (cTnI, cTnT) and myosin light chain 1 and 2 (MLC-1, MLC-2) were determined by 1- and 2-dimensional gel electrophoresis. Isometric force development, its  $\text{Ca}^{2+}$ -sensitivity, the rate of tension redevelopment ( $k_{tr}$ ) and passive force ( $F_{pas}$ ) were studied at optimal sarcomere length ( $2.2 \mu\text{m}$ ) in mechanically isolated, permeabilized cardiomyocytes at  $15^\circ\text{C}$ . Protein phosphorylation was varied by: 1) blocking spontaneous cardiac activity by lidocaine ( $0.35 \text{ mM}$ ; Quiescence); 2) electrical stimulation of the hearts at  $5 \text{ Hz}$  (Contraction) and 3. treatment of contracting hearts with Isoprenaline ( $1 \mu\text{M}$ ). MLC-2 phosphorylation was increased in the Contraction group almost 2-fold, relative to the Quiescence group, whereas cMyBP-C and cTnI phosphorylation remained the same. Isoprenaline resulted in 3.7-fold increases in both cMyBP-C and cTnI phosphorylation, but did not result in a further increase in MLC-2 phosphorylation. No significant differences were found in maximum force and  $k_{tr}$  between groups, both before and after protein kinase A (PKA) treatment.  $\text{Ca}^{2+}$ -sensitivity in the Contraction and Isoprenaline groups was significantly reduced in comparison to the Quiescence group. These differences were largely abolished by PKA and  $F_{pas}$  was reduced. These results highlight the impact of PKA-dependent phosphorylation on  $\text{Ca}^{2+}$ -sensitivity and provide evidence for an interaction between the effects of TnI and MLC-2 phosphorylation.

■ **Key words** contractile function –  $\text{Ca}^{2+}$ -sensitivity – cardiac – myocyte – phosphorylation

### Introduction

The activity of the contractile apparatus in cardiac myocytes is determined by the intracellular  $\text{Ca}^{2+}$  concentration and the sensitivity of the myofilaments to  $\text{Ca}^{2+}$ . The  $\text{Ca}^{2+}$ -sensitivity of the myofilaments is modulated by the delicate balance between phosphorylation by kinases, e. g. protein kinase A and C (PKA and PKC),

myosin light chain kinase (MLCK) and the  $\text{Ca}^{2+}$  calmodulin dependent kinase II (CaMK II), and dephosphorylation by phosphatases, e. g. protein phosphatase 1 and 2A (PP-1 and PP-2A). For recent overviews see [2, 8, 22]. In the last decades, the impact of phosphorylation on  $\text{Ca}^{2+}$ -sensitivity of only a limited number of contractile proteins has been resolved, whereas the interactions between the various proteins are largely unclear.

One of the proteins whose role is not completely un-

derstood is myosin binding protein C (cMyBP-C). This approximately 150 kDa protein, discovered more than 30 years ago, is part of the thick (myosin) filament [30] and shows up as the third (= C) band in an SDS gel from crude myosin [35]. The interest in the functional role of cMyBP-C has risen with the discovery of frequently occurring mutations in the *MYBPC3* gene that result in the phenotype of hypertrophic cardiomyopathy [e. g. 29]. It has been found that the C terminal part of cMyBP-C binds to titin and to the light meromyosin region of myosin [9, 21], while the N terminus binds to the myosin S2 region [13, 20]. The N terminal region of rat cardiac cMyBP-C contains three serine phosphorylation sites (S279, S288, S308), phosphorylatable with different stoichiometry by PKA and PKC [26]. Recently, results have been presented indicating the existence of additional phosphorylation sites [45]. Several lines of evidence suggest that phosphorylation of S288 by CaMK II is required before S279 and S308 can be phosphorylated by PKA [12, 24, 33]. On the other hand, cMyBP-C can be dephosphorylated in vitro both by PP-1 and PP-2A [34]. As a consequence of the presence of these multiple phosphorylation sites and the complex signal transduction routes involved, the functional effects of cMyBP-C phosphorylation are largely unclear.

It has been proposed that cMyBP-C phosphorylation modulates the interaction between cMyBP-C and the S2 region of myosin [13, 20] resulting in an increase in the proximity of myosin to actin. This would enhance the probability of crossbridge formation [15, 37, 43]. Accordingly, the PKA-mediated phosphorylation of cMyBP-C might contribute to the increased cardiac output in response to  $\beta$ -adrenergic stimulation [37].

The phosphorylation of myosin light chain 2 (MLC-2), cardiac troponin T (cTnT) and troponin I (cTnI) has considerable impact on contractile function as such, but could also modulate the effects of cMyBP-C phosphorylation and vice versa. For instance, recently it has been suggested from a study using cMyBP-C knockout mice that myofilament desensitisation induced by PKA requires the presence of cMyBP-C [4]. MLC-2 phosphorylation by the  $\text{Ca}^{2+}$ -calmodulin-dependent MLCK results in an increase in  $\text{Ca}^{2+}$ -sensitivity [39]. In human tissue, our laboratory has presented evidence that the effects of MLC-2 dephosphorylation depend on the level of cTnI phosphorylation, i. e. an interaction between the effect of the phosphorylation of a thin (actin) filament protein and that of a thick (myosin) filament protein [42].

Therefore, in the present study we induced differences in the degree of phosphorylation of the myofilaments to explore the role of phosphorylation of cMyBP-C in isolated rat ventricular myocytes under physiological conditions, taken into account the phosphorylation levels of the other contractile proteins such as MLC-2, cTnT and cTnI.

## Methods

A thoracotomy was performed on Wistar rats ( $300 \pm 7$  g) anaesthetised with ketamine 0.2 ml/kg i. m. and pentobarbital 60 mg/kg i. p. The heart was quickly excised and placed in ice cold Tyrode solution (in mM: NaCl: 120, KCl: 5,  $\text{MgCl}_2$ : 1.2,  $\text{NaH}_2\text{PO}_4$ : 2,  $\text{NaHCO}_3$ : 27, d-glucose: 10 saturated with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ ) supplemented with 30 mM 2,3-butanedione monoxime (BDM). The "Principles of laboratory animal care" (NIH publication No 86-23, revised 1985) were followed and all protocols were approved by the Animal Experimental Welfare Committee of the VU University Medical Center. The  $\text{CaCl}_2$  concentrations in the Tyrode solution were either 0.2 mM, 1.36 mM or 2.5 mM, as specified in each sub-protocol. The aorta was cannulated and the complete heart was mounted on a Langendorff apparatus in which coronary perfusion was maintained at 15 ml/min using 95%  $\text{O}_2$ -5%  $\text{CO}_2$  equilibrated Tyrode solution without BDM at 37 °C (pH 7.4). The atria were removed and the ventricles could be stimulated electrically via two platinum electrodes.

We aimed at different cMyBP-C phosphorylation levels and used different experimental conditions as tools to achieve this. Phosphorylation of cMyBP-C will be low in quiescent hearts perfused with lidocaine at a relatively low  $\text{Ca}^{2+}$  concentration [32]. In theory, higher levels of phosphorylation can be obtained by  $\beta$ -adrenergic stimulation, enhancement of the CaMK II activity by an increase in the time averaged intracellular  $\text{Ca}^{2+}$  concentration, and/or by blockade of phosphatase activity [11, 28]. These considerations led to the following experimental groups. Hearts ( $n = 30$ ) were perfused according to Langendorff for 90 min at 37 °C with Q1: 0.2 mM  $\text{Ca}^{2+}$  and 0.35 mM lidocaine (Lido) to attain quiescence ( $n = 4$ ), Q2: physiological  $\text{Ca}^{2+}$  (1.36 mM) + Lido ( $n = 5$ ), C1: 1.36 mM  $\text{Ca}^{2+}$ , stimulation at 5 Hz ( $n = 8$ ), C2: 5 Hz stimulation at a high  $\text{Ca}^{2+}$  (2.5 mM) + okadaic acid (OA: 0.1  $\mu\text{M}$ ) ( $n = 5$ ), Iso1: 1.36 mM  $\text{Ca}^{2+}$ , 5 Hz stimulation followed by 2 min of Isoprenaline (1  $\mu\text{M}$ ) ( $n = 4$ ) and Iso2: 1.36 mM  $\text{Ca}^{2+}$ , 5 Hz stimulation + OA followed by 2 min of Isoprenaline + OA ( $n = 4$ ).

Immediately after interruption of the Langendorff perfusion the heart was flash frozen in liquid nitrogen. The left ventricle (LV) plus septum were separated from the rest of the heart and stored at -80 °C until further analysis.

### ■ Myocyte measurements

A small piece (~25 mg) of LV tissue was defrosted in cold relaxing solution, containing in mM free  $\text{Mg}^{2+}$ : 1, KCl: 100, EGTA: 2, Mg-ATP: 4, imidazole: 10 (pH 7.0, adjusted with KOH). This piece was cut into small pieces with scissors and homogenized in 5-10 s, using a tissue ho-

mogenizer. The resultant suspension of small clumps of myocytes, single myocyte-sized preparations and cell fragments was treated with 0.3% Triton X-100 in relaxing solution (5 min), in order to permeabilize the cardiomyocytes. The suspension was washed two times in relaxing solution without Triton and kept at 0 °C for 6–8 h [41].

A single myocyte was attached with silicon adhesive to thin stainless steel needles while viewed by an inverted microscope. One needle was attached to a force transducer (SensoNor, Horten, Norway) and the other to a piezoelectric motor (Physike Instrumente, Waldbrunn, Germany), both connected to joystick-controlled micromanipulators. During cell attachment and subsequent force measurements myocytes were viewed at 320X magnification. Images were captured by means of a CCD camera and stored on a personal computer. See Fig. 1 for a typical example. Average sarcomere length was determined by means of a spatial Fourier transform as described previously and adjusted to approximately 2.2  $\mu\text{m}$  [6]. The diameters of the preparation were measured microscopically, in two almost perpendicular directions. Cross-sectional area was calculated assuming an elliptical cross-section.

After curing of the glue for 50 min, the preparation was transferred from the mounting area to a small temperature controlled well (15 °C; volume 79  $\mu\text{l}$ ), containing relaxing solution, from which the myocyte could be transferred to a similar temperature controlled well containing activating solution. Relaxing and activating solutions for force measurements contained, respectively (in mM):  $\text{MgCl}_2$ : 6.48 and 6.28,  $\text{Na}_2\text{ATP}$ : 5.89 and 5.97, EGTA: 7.0 and 0, CaEGTA: 0 and 7.0. In addition, both contained 14.5 mM phosphocreatine and 60 mM BES (pH:7.1, adjusted with KOH). The ionic strength of the solutions was adjusted to 180 mM with K-propionate. The pCa, i. e.  $-\log_{10} [\text{Ca}^{2+}]$ , of the relaxing and activating solution was, respectively, 9 and 4.5. Calculated free  $\text{Mg}^{2+}$  and  $\text{MgATP}$  concentrations were 1 and 5 mM, respectively. Solutions with lower free  $\text{Ca}^{2+}$  concentrations

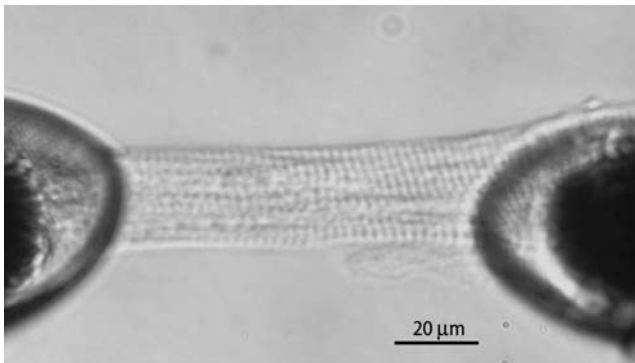
were obtained by appropriate mixing of the activating and relaxing solutions, assuming an apparent stability constant of the Ca-EGTA complex of  $10^{6.56}$ .

Isometric force was measured after the preparation was transferred from relaxing to activating solution, by moving the stage of the inverted microscope. When steady force was reached, the myocyte was reduced in length by 20% within 2 ms using the piezoelectric motor and restretched after 30 ms (slack test). As a result of this intervention, force first dropped to zero and then quickly redeveloped to the original steady-state level. A single exponential was fitted to force redevelopment to estimate the rate constant of force redevelopment ( $k_{tr}$ ). Subsequently, the myocyte was returned to the relaxing solution, and a second slack test (10 s duration) was performed allowing the determination of passive force ( $F_{pas}$ ). Active isometric force was calculated from the force level in activating solution –  $F_{pas}$ . Force and length signals were monitored using a pen-recorder and stored on a personal computer. Sampling rate during the experiment was 20 Hz, and 500 or 1000 Hz during the first slack-test.

$\text{Ca}^{2+}$ -sensitivity ( $\text{pCa}_{50}$ ) was determined using different pCa values (5.0, 5.2, 5.4, 5.6, 5.8 and 6.0) in a random order, bracketed by control activations at pCa 4.5. The initial control force at pCa 4.5 was used to calculate the maximal force per cross-sectional area. The force values at submaximal  $[\text{Ca}^{2+}]$  were normalized to the interpolated control values. Thereafter, the myocytes were incubated for 40 min at 20 °C in relaxing solution containing 6 mM dithiothreitol (DTT) (time control) or 6 mM DTT and 100 U/ml of the catalytic subunit of PKA (Sigma, P2645 from bovine heart) and the  $\text{Ca}^{2+}$ -sensitivity measurements were repeated. Experimental series where maximum force value during the control activations at pCa 4.5 declined more than 20%, were not used for analysis.

### ■ Time dependent phosphorylation in vitro

Homogenized tissue samples (400  $\mu\text{g}$  dry weight) were incubated in 0.1 ml of relaxing solution with PKA (Calbiochem, Cat. No. 539481, 1250 U/ml), protease inhibitor cocktail 10  $\mu\text{l}/\text{ml}$  (Sigma, P8340), phosphatase inhibitor cocktail 1 and 2 (10  $\mu\text{l}/\text{ml}$  each, Sigma, P2850 and P3750, respectively) and 2.5  $\mu\text{l}/\text{ml}$  10% Triton for 0, 5, 10, 15, 20, 30 and 60 min at 30 °C. The reaction was stopped by addition of cold (–20 °C) trichloroacetic acid dissolved in acetone (TCA; 10% v/v). Phosphorylation was determined from ProQ diamond stained 1D gradient gels (see below).



**Fig. 1** Single cardiomyocyte, isolated from left ventricular tissue from the Quiescence group, glued between force transducer and piezoelectric motor

## ■ Two-dimensional gel electrophoresis

Part of the LV was treated with TCA to maintain the phosphorylation status of the contractile proteins. Freeze dried samples were homogenized in 1D sample buffer (62.5 mM TRIS (pH 6.8), 15% glycerol, 1% SDS and 1.5% hydroxyethyl disulfide), and diluted [1:17] in 2D sample buffer (7 M urea, 2.5 M thiourea, 4% CHAPS, 0.5% ampholytes pH 4–7, 10% glycerol and 10% isopropanol). 2D-gel electrophoresis of the samples (200 µg dry weight) was performed using immobiline strips with a pH gradient of 4–7 as described previously [42] to determine MLC-1, MLC-2 and cTnT phosphorylation from the Coomassie stained gels.

## ■ ProQ diamond staining

TCA-treated tissue was washed 3 times in acetone and dissolved in 1D sample buffer with 100 mM DTT, heated (5 min at 80 °C), centrifuged (20 min, 12000 g at 20 °C) and a sample (~35 µg dry weight in 25 µl) was applied on a 4–15% gradient gel (Criterion, BioRad). The gel was run at 100 V for 30 min followed by 200 V for 50 min. The gel was stained for one hour with ProQ Diamond (Molecular Probes, Eugene OR, USA) according to the manufacturer's instructions. Staining was analysed with a LAS-3000 system (Fuji Science Imaging Systems) and AIDA Image analyser software (Isotopenmeßgeräte GmbH, Staubenhardt, Germany). Subsequently, the gel was washed and stained with Sypro Ruby (Molecular Probes) according to the manufacturer's instructions, and analysed.

## ■ Data analysis

The force–pCa relation was fitted using a modified Hill equation

$$F(\text{Ca}^{2+})/F_{\max} = [\text{Ca}^{2+}]^{nH}/(\text{Ca}_{50}^{nH} + [\text{Ca}^{2+}]^{nH})$$

where  $F$  is steady state force.  $F_{\max}$  denotes the steady isometric force at saturating  $\text{Ca}^{2+}$  concentration, the Hill coefficient  $nH$  is a measure of the steepness of the relationship, and  $\text{Ca}_{50}$  (or  $\text{pCa}_{50}$ ) represents the mid-point of the relation. Values are given as means  $\pm$  S. E. M. of  $n$  experiments. Differences between the groups were tested by means of an ANOVA followed by Bonferroni, differences due to PKA treatment were analysed in the main groups using a paired Student's  $t$ -test. A  $P$ -value of  $< 0.05$  was considered significant.

## Results

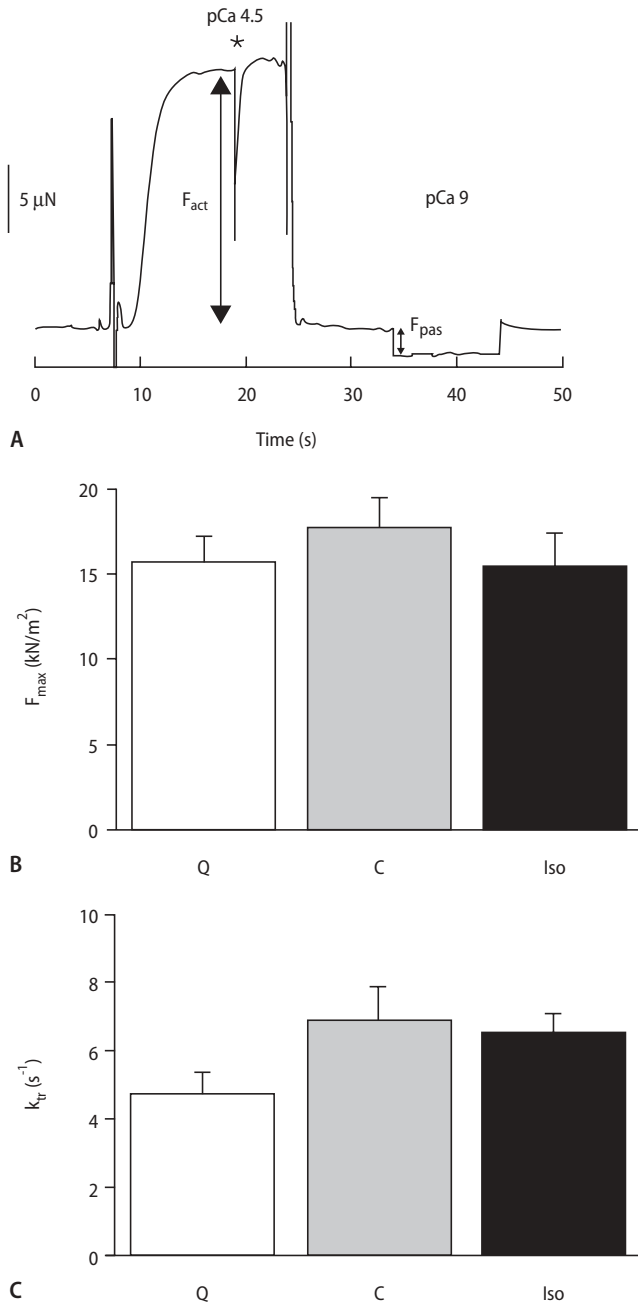
The experimental conditions during Langendorff perfusion were varied in order to attain different levels of contractile protein phosphorylation as described in Methods. As can be seen from the overviews of the results in Tables 1 and 2, there were no significant differences in the averaged data of the Quiescence (Q1, Q2), Contraction (C1, C2) and Isoprenaline subgroups (Iso, Iso + OA). Therefore, for the ease of presentation in the figures and discussion, the data within each of the three main groups were pooled, but the data of the subgroups are given in Tables 1 and 2.

## ■ Force measurements

An example of the force recording in a single permeabilized cardiomyocyte is shown in Fig. 2. From each animal 2–4 myocytes were analysed in order to calculate the mean force parameter values per animal. Subsequently, the means of the values of all animals in each (sub)group were calculated to allow a comparison with the contractile protein phosphorylation status of the tissue. The maximal active force per cross-sectional area ( $F_{\max}$ ) was measured at saturating  $\text{Ca}^{2+}$ -concentration (pCa 4.5) and 2.2 µm sarcomere length. No significant differences were found between the groups (Fig. 2B; Table 1). The overall averaged value amounted to  $16.5 \pm 1.1$  kN/m<sup>2</sup>. Passive force ( $F_{\text{pas}}$ ) also did not differ between groups. The overall averaged value amounted to  $2.7 \pm 0.3$  kN/m<sup>2</sup>, i. e.  $15 \pm 2\%$  of the total ( $F_{\text{tot}} = F_{\max} + F_{\text{pas}}$ ) force. The rate of force redevelopment ( $k_{\text{tr}}$ ) at saturating  $\text{Ca}^{2+}$ -concentration did not differ between groups (Fig. 2C; Table 1).

The relation obtained between active force ( $F_{\text{act}}$ ) and the free  $\text{Ca}^{2+}$ -concentration is shown in Fig. 3. For each cardiomyocyte the data were fitted to the modified Hill equation and for each animal the mean parameter values of  $F_{\max}$ ,  $\text{pCa}_{50}$  and  $nH$  were obtained and group means were calculated (Fig. 3D; Table 1). These data indicate that  $\text{Ca}^{2+}$ -sensitivity ( $\text{pCa}_{50}$ ) in the Quiescence group was significantly higher than in the Contraction and Isoprenaline groups ( $P < 0.05$ ). The steepness of the  $\text{Ca}^{2+}$ -sensitivity relations ( $nH$ ) did not differ between groups (Table 1). The rate of force redevelopment ( $k_{\text{tr}}$ ) decreased significantly when the pCa in the activating solutions was increased from 4.5 to 5.4 with  $19.4 \pm 2.4\%$ . However, its  $\text{Ca}^{2+}$ -dependence did not differ between groups.

The impact of saturating PKA-sensitive phosphorylation of cMyBP-C, cTnI and (possibly) titin on force development was studied by repeating the  $\text{Ca}^{2+}$ -sensitivity measurements after incubation of the cardiomyocytes with the active subunit of PKA. The effect of PKA treatment on the  $\text{Ca}^{2+}$ -sensitivity of  $F_{\text{act}}$  is illustrated in Fig. 3A, B and C for the Quiescence, Contraction and Iso-



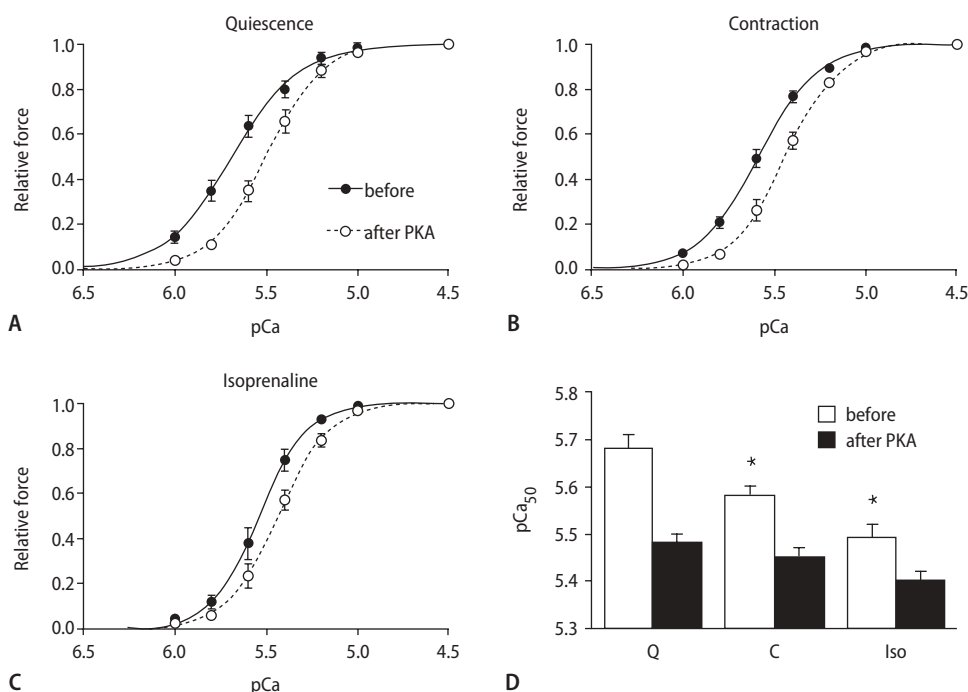
**Fig. 2** **A** Contraction-relaxation sequence recorded from a cardiomyocyte from the Quiescence group at maximally activating Ca<sup>2+</sup> concentration (pCa 4.5). \*Slack test used to determine the rate of force redevelopment ( $k_{tr}$ ).  $F_{act}$  represents the active force development in Ca<sup>2+</sup>-containing activating solution.  $F_{pas}$  represents the passive force development measured in relaxing solution (pCa 9). **B** Averaged values ( $\pm$  SEM) of the active force development at maximally activating Ca<sup>2+</sup>-concentration normalized for cross-sectional area ( $F_{max}$ ) in the Quiescence (Q), Contraction (C) and Isoprenaline (Iso) group. **C** Averaged values ( $\pm$  SEM) of  $k_{tr}$  at maximally activating Ca<sup>2+</sup> concentration in the Quiescence, Contraction and Isoprenaline group. Both  $F_{max}$  and  $k_{tr}$  did not vary significantly between groups (ANOVA)

**Table 1** Overview of the results of the force measurements

Group	$F_{max}$ (kN/m <sup>2</sup> )	$F_{max} + PKA$ (kN/m <sup>2</sup> )	pCa <sub>50</sub>	pCa <sub>50</sub> + PKA	$F_{pas}$ (kN/m <sup>2</sup> )	$F_{pas} + PKA$ (kN/m <sup>2</sup> )	nH	nH + PKA	$k_{tr}$ (s <sup>-1</sup> )	$k_{tr} + PKA$ (s <sup>-1</sup> )
Q1 0.2 mM Ca <sup>2+</sup> (n = 4)	16.3 $\pm$ 2.0	16.3 $\pm$ 2.5	5.69 $\pm$ 0.04	5.51 $\pm$ 0.04	3.9 $\pm$ 0.9	2.7 $\pm$ 0.7	3.19 $\pm$ 0.14	3.16 $\pm$ 0.42	4.56 $\pm$ 0.49	4.51 $\pm$ 0.47
Q2 1.36 mM Ca <sup>2+</sup> (n = 5)	15.2 $\pm$ 2.4	15.3 $\pm$ 3.6	5.67 $\pm$ 0.05	5.46 $\pm$ 0.03	3.5 $\pm$ 0.6	2.3 $\pm$ 0.4	2.76 $\pm$ 0.20	3.35 $\pm$ 0.19	5.29 $\pm$ 1.4	5.00 $\pm$ 0.94
<b>Quiescence (9)</b>	<b>15.7 <math>\pm</math> 1.5</b>	<b>15.8 <math>\pm</math> 1.7</b>	<b>5.68 <math>\pm</math> 0.03<sup>a,b</sup></b>	<b>5.48 <math>\pm</math> 0.02</b>	<b>3.7 <math>\pm</math> 0.5</b>	<b>2.5 <math>\pm</math> 0.4<sup>c</sup></b>	<b>2.95 <math>\pm</math> 0.14</b>	<b>3.27 <math>\pm</math> 0.20</b>	<b>4.74 <math>\pm</math> 0.62</b>	<b>4.75 <math>\pm</math> 0.51</b>
C1 1.36 mM Ca <sup>2+</sup> (n = 8)	19.0 $\pm$ 1.7	17.2 $\pm$ 2.1	5.60 $\pm$ 0.03	5.47 $\pm$ 0.03	3.3 $\pm$ 0.7	2.2 $\pm$ 0.3	2.61 $\pm$ 0.14	3.32 $\pm$ 0.16	4.81 $\pm$ 0.45	4.70 $\pm$ 0.43
C2 2.5 mM Ca <sup>2+</sup> + OA (n = 5)	15.3 $\pm$ 3.6	9.6 $\pm$ 2.3	5.55 $\pm$ 0.02	5.41 $\pm$ 0.03	1.0 $\pm$ 0.3	1.1 $\pm$ 0.3	4.08 $\pm$ 0.40	4.32 $\pm$ 0.67	9.77 $\pm$ 1.79	7.79 $\pm$ 1.04
<b>Contraction (13)</b>	<b>17.7 <math>\pm</math> 1.7</b>	<b>14.3 <math>\pm</math> 1.9</b>	<b>5.58 <math>\pm</math> 0.02</b>	<b>5.45 <math>\pm</math> 0.02</b>	<b>2.4 <math>\pm</math> 0.5</b>	<b>1.8 <math>\pm</math> 0.3<sup>c</sup></b>	<b>3.18 <math>\pm</math> 0.27</b>	<b>3.70 <math>\pm</math> 0.29</b>	<b>6.86 <math>\pm</math> 1.01</b>	<b>5.98 <math>\pm</math> 0.64</b>
Iso (n = 4)	15.4 $\pm$ 3.0	11.7 $\pm$ 2.2	5.50 $\pm$ 0.06	5.42 $\pm$ 0.02	2.3 $\pm$ 0.7	1.8 $\pm$ 0.4	4.5 $\pm$ 0.39	5.31 $\pm$ 1.04	6.49 $\pm$ 1.11	6.58 $\pm$ 1.08
Iso + OA (n = 4)	15.5 $\pm$ 2.2	11.3 $\pm$ 1.0	5.48 $\pm$ 0.04	5.38 $\pm$ 0.04	1.7 $\pm$ 0.5	1.5 $\pm$ 0.5	3.93 $\pm$ 0.29	3.90 $\pm$ 0.50	6.56 $\pm$ 0.55	6.88 $\pm$ 0.41
<b>Isoprenaline (n = 8)</b>	<b>15.4 <math>\pm</math> 1.7</b>	<b>11.5 <math>\pm</math> 1.1</b>	<b>5.49 <math>\pm</math> 0.03</b>	<b>5.40 <math>\pm</math> 0.02</b>	<b>2.0 <math>\pm</math> 0.4</b>	<b>1.6 <math>\pm</math> 0.3</b>	<b>4.22 <math>\pm</math> 0.25</b>	<b>4.60 <math>\pm</math> 0.60</b>	<b>6.52 <math>\pm</math> 0.57</b>	<b>6.73 <math>\pm</math> 0.53</b>

<sup>a</sup> P < 0.05 vs Isoprenaline group; <sup>b</sup> P < 0.05 vs Contraction group; <sup>c</sup> P < 0.05 vs before PKA

**Fig. 3 A, B, C** Isometric force normalized to the force at saturating  $\text{Ca}^{2+}$  concentration plotted as a function of the pCa of the activating solution, before and after PKA (100 U/ml) incubation for 40 min at 20 °C, in the Quiescence, Contraction and Isoprenaline group, respectively. **D** Bar graph illustrating the differences in  $\text{Ca}^{2+}$ -sensitivity ( $\text{pCa}_{50}$ ) in the three groups. At baseline  $\text{pCa}_{50}$  was significantly lower in the Contraction and Isoprenaline groups than in the Quiescence group (\*  $P < 0.05$  vs Quiescence group; Bonferroni post hoc test). After PKA treatment  $\text{Ca}^{2+}$ -sensitivity decreased, and the  $\text{pCa}_{50}$  values attained were fairly similar



prelinaline group, respectively. PKA caused a decrease in  $\text{Ca}^{2+}$ -sensitivity, which was larger in the Quiescence group than in the Contraction and Isoprenaline group. After PKA, the  $\text{pCa}_{50}$  values in the three groups were comparable (Fig. 3D). PKA incubation resulted in a small but significant reduction in  $F_{\text{pas}}$  in the Quiescence and in the Contraction group but did not alter  $F_{\text{max}}$  (Table 1). Control incubations without PKA did not have any effect on the force parameters or their  $\text{Ca}^{2+}$ -sensitivity (data not shown).

To assess the effect of PKA on  $k_{\text{tr}}$ , the  $k_{\text{tr}} - \text{pCa}$  and the  $k_{\text{tr}} - F_{\text{act}}$  relations were studied before and after PKA treatment. Accurate determination of  $k_{\text{tr}}$  was only possible at relatively high forces, i. e. in the pCa range between 4.5 and 5.4. In general, it appeared that  $k_{\text{tr}}$  at submaximal pCa values before PKA treatment was somewhat less than the corresponding values before PKA treatment.

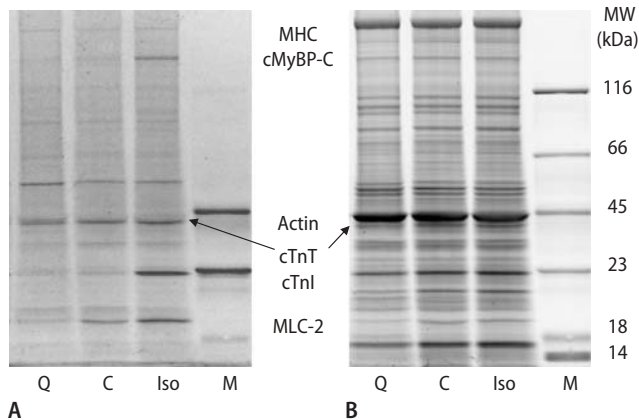
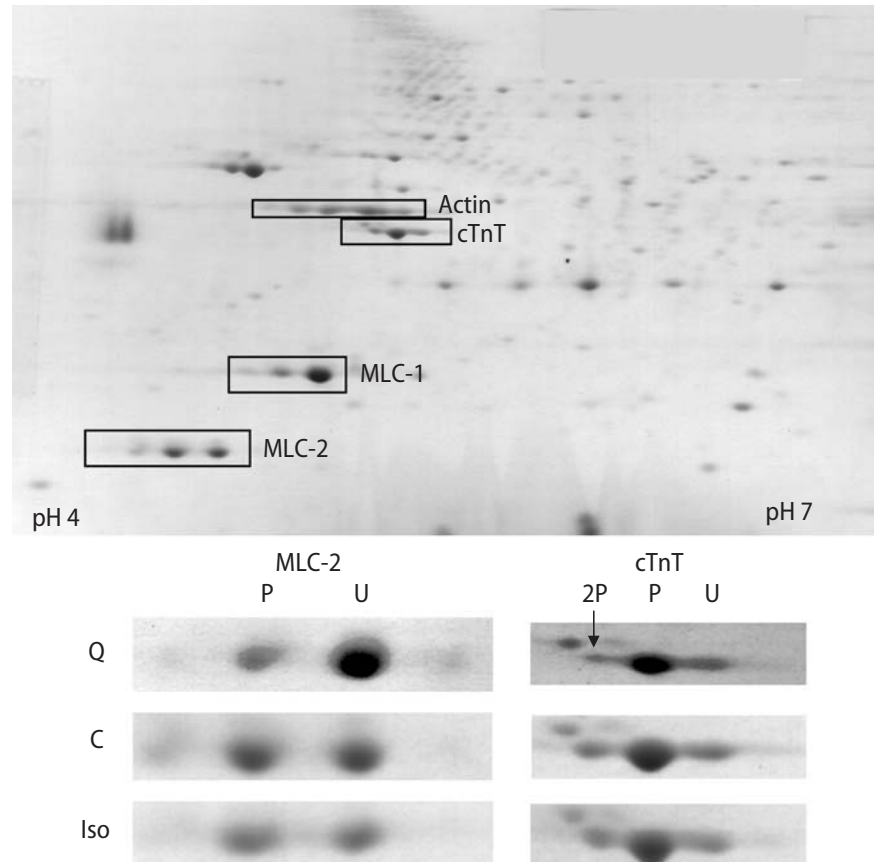
### ■ Protein analysis

The endogenous phosphorylation levels of contractile proteins in TCA-treated tissue from the flash frozen hearts were determined from 2D-gels (cTnT, MLC-1 and MLC-2) and from ProQ Diamond stained 1D-gels (cMyBP-C, cTnT, cTnI and MLC-2). Typical examples of the gels obtained are shown in Figs. 4 and 5. The 2D-gels (pH range 4–7) allow the distinction between different phosphorylated forms of cTnT, MLC-1 and MLC-2 and permit the quantification of the relative distribution of these forms of each protein. The ProQ Diamond stained

1D-gels are convenient to determine the overall degree of phosphorylation of high molecular weight proteins such as cMyBP-C, and very basic proteins such as cTnI. There was good agreement in the results of protein phosphorylation in the samples of the various groups, where comparisons could be made (cTnT and MLC-2). In both cases cTnT phosphorylation, expressed as a percentage for 2D-gels or normalized for cMyBP-C protein content in each sample derived from Sypro Ruby stained 1D-gels, did not vary between groups. Therefore normalization of the phosphostaining of other protein bands on the ProQ Diamond gels could be performed relative to the staining intensity of the corresponding cTnT band in each lane.

2D-analysis by means of Sypro Ruby and ProQ Diamond staining and Western Immunoblotting using a monoclonal cTnT antibody (2G3, RDI Research Diagnostics) clearly indicated that the group labeled as cTnT in Fig. 4 consists of a predominant lower molecular weight form, which is mainly mono-phosphorylated and slightly bis-phosphorylated. In addition, a less prominent group with a slightly higher molecular weight at a slightly more acidic pH can be seen, included in the cTnT box, which possesses a very similar phosphorylation pattern. In addition, the ProQ Diamond stained gel confirmed the identity and distribution of the MLC-2 phospho-species. These images also provided evidence for the presence of a bis-phosphorylated form of MLC-2 with very low abundance. The intensity of this spot was negligible relative to the intensity of the mono-phosphorylated spot.

**Fig. 4** Coomassie blue stained 2D-gel of left ventricular tissue (200 µg dry weight) of the Quiescence group. On the horizontal axis proteins are separated by isoelectric point (pH range: 4–7) and vertically proteins are separated based on molecular weight (top: ~200 kDa, bottom: 15 kDa). Abbreviations used: troponin T (cTnT), myosin light chain 1 (MLC-1), myosin light chain 2 (MLC-2). The relative expression of the unphosphorylated (U), mono (P) and bis (2P) phosphorylated forms of a number of proteins can be quantitated from these 2D-gels. The lower panels illustrate the MLC-2 and cTnT regions in 2D-gels from the Quiescence (Q), Contraction (C) and Isoprenaline (Iso) group (approximately 200 µg dry weight of sample). MLC-2 was more phosphorylated in the Contraction and Isoprenaline group in comparison to the Quiescence group. The degree of cTnT phosphorylation was very similar as can be seen from the correspondence in the relative distribution of the intensities in the U, P and 2P spots

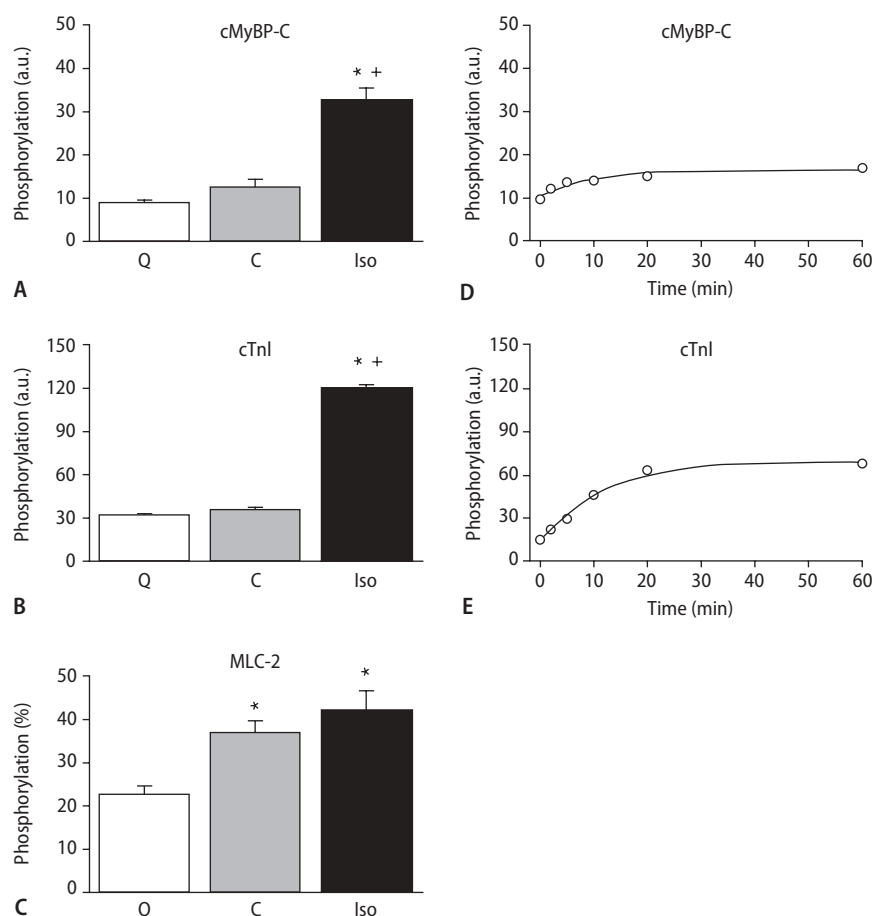


**Fig. 5** **A** Representative 4–15 % gradient gel stained with Pro-Q Diamond from samples (approximately 35 µg dry weight) of the Quiescence (Q), Contraction (C) and Isoprenaline (Iso) group. **B** The same gel was subsequently stained with Sypro Ruby to visualize all proteins. *M* molecular weight PeppermintStick Phosphoprotein marker in which ovalbumin (45 kDa) and  $\beta$ -casein (23 kDa) are phosphorylated. MHC myosin heavy chain. The degree of phosphorylation of myosin binding protein C (cMyBP-C), troponin I (cTnI) and myosin light chain 2 (MLC-2) was higher in the Isoprenaline group than in the Quiescence and Contraction group, while the phosphorylation status of troponin T (cTnT) did not vary between groups

From the results presented in Table 2, a comparison can be made with regards to the MLC-2 phosphorylation between the results obtained using ProQ diamond staining of 1D gels and the Coomassie stained 2D-gels. It can be noted that the correspondence between the results of the two different techniques is good and that the outcome of the statistical analysis of the differences in MLC-2 phosphorylation between experimental groups does not depend on the quantitation method used.

An overview of differences in phosphorylation of cMyBP-C, cTnI and MLC-2 in the Quiescence, Contraction and Isoprenaline group is shown in Fig. 6A, B and C, respectively. A complete summary of the data is given in Table 2. Significant differences were found in the phosphorylation of cMyBP-C, cTnI, MLC-1 and MLC-2 in the three groups (ANOVA), while cTnT phosphorylation (approximately 80 % of total cTnT) remained the same. Cardiac MyBP-C and cTnI phosphorylation were markedly increased (3.7-fold) in the Isoprenaline group relative to the Quiescence group ( $P < 0.01$ ). The average cMyBP-C phosphorylation in the Contraction group was somewhat elevated (1.4-fold) but the difference between the Quiescence and Contraction group was not significant (Bonferroni post-hoc test). MLC-2 phosphorylation increased significantly from 22.4% in the Quiescence

**Fig. 6 A, B** Quantitative analysis of cMyBP-C and cTnI phosphorylation from 1D-gels by Pro-Q Diamond and Sypro Ruby staining. Pro-Q Diamond intensity signals of cMyBP-C and cTnI were normalized to cTnT staining intensity in the same lane. Averaged values ( $\pm$  SEM) values are shown in the Quiescence (Q), Contraction (C) and Isoprenaline (Iso) group. cMyBP-C and cTnI phosphorylation in the Isoprenaline group were significantly increased relative to the Quiescence and Contraction group. **C** MLC-2 phosphorylation obtained by 2D-gel electrophoresis expressed as percentage of mono-phosphorylated MLC-2 relative to total MLC-2 intensity. MLC-2 phosphorylation in the Contraction and Isoprenaline groups was significantly increased relative to the Quiescence group. **D, E** Effect of incubation of tissue from the Quiescence group with the active subunit of protein kinase A. cMyBP-C and cTnI phosphorylation increased with the duration of the incubation and saturated after approximately 30 min. The final levels attained are smaller than the corresponding levels reached in the Isoprenaline group, indicating that Isoprenaline phosphorylated other phosphorylation sites in addition to the classical PKA-sites. \*  $P < 0.05$  vs Quiescence group and +  $P < 0.05$  vs Contraction group, in a Bonferroni post hoc test



**Table 2** Overview of the endogenous phosphorylation levels from 1D- (expressed in arbitrary units) and 2D-gel electrophoresis (expressed as percentage of total protein)

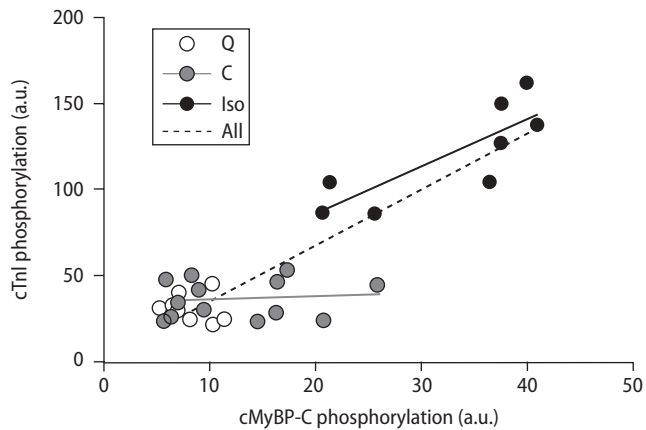
Group	cMyBP-C (a. u.)	cTnI (a. u.)	MLC-2 (a. u.)	cTnT (%)	MLC-1 (%)	MLC-2 (%)
Q1 (0.2 mM Ca <sup>2+</sup> )	7.5 $\pm$ 1.3	31.8 $\pm$ 3.2	22.9 $\pm$ 2.6	81.3 $\pm$ 3.4	13.9 $\pm$ 1.1	23.7 $\pm$ 3.6
Q2 (1.36 mM Ca <sup>2+</sup> )	9.8 $\pm$ 0.8	32.3 $\pm$ 4.6	27.1 $\pm$ 4.2	79.6 $\pm$ 2.9	13.2 $\pm$ 0.8	21.3 $\pm$ 2.8
<b>Quiescence</b>	<b>8.8 <math>\pm</math> 0.8<sup>a</sup></b>	<b>32.1 <math>\pm</math> 2.8<sup>a</sup></b>	<b>25.2 <math>\pm</math> 2.6<sup>a, b</sup></b>	<b>80.3 <math>\pm</math> 1.8</b>	<b>13.5 <math>\pm</math> 0.6<sup>a</sup></b>	<b>22.4 <math>\pm</math> 2.1<sup>a, b</sup></b>
C1 (1.36 mM Ca <sup>2+</sup> )	12.9 $\pm$ 2.4	39.8 $\pm$ 4.3	67 $\pm$ 15	81.4 $\pm$ 2.4	13.8 $\pm$ 1.6	35.9 $\pm$ 3.7
C2 (2.5 mM Ca <sup>2+</sup> + OA)	12.0 $\pm$ 4.4	30.6 $\pm$ 5.7	60 $\pm$ 11	75.5 $\pm$ 0.9	19.0 $\pm$ 1.7	37.9 $\pm$ 5.2
<b>Contraction</b>	<b>12.6 <math>\pm</math> 1.8<sup>a</sup></b>	<b>35.6 <math>\pm</math> 3.0<sup>a</sup></b>	<b>64 <math>\pm</math> 10</b>	<b>79.1 <math>\pm</math> 1.7</b>	<b>15.8 <math>\pm</math> 1.3</b>	<b>36.7 <math>\pm</math> 2.9</b>
Iso1	30.9 $\pm$ 4.6	115 $\pm$ 18	92 $\pm$ 12	75.2 $\pm$ 2.2	17.5 $\pm$ 2.4	41.9 $\pm$ 6.9
Iso2 (+ OA)	34.1 $\pm$ 4.3	124 $\pm$ 12	84 $\pm$ 22	77.6 $\pm$ 1.5	20.5 $\pm$ 2.6	42.2 $\pm$ 7.4
<b>Isoprenaline</b>	<b>32.5 <math>\pm</math> 3.0</b>	<b>120 <math>\pm</math> 10</b>	<b>88 <math>\pm</math> 12</b>	<b>76.4 <math>\pm</math> 1.3</b>	<b>19.0 <math>\pm</math> 1.7</b>	<b>42.0 <math>\pm</math> 4.7</b>

<sup>a</sup>  $P < 0.05$  vs Isoprenaline group; <sup>b</sup>  $P < 0.05$  vs Contraction group

group to 36.7% in the Contraction group and to 42% in the Isoprenaline group. MLC-2 phosphorylation did not differ significantly between the Contraction group and Isoprenaline group, indicating that 2 min of 1  $\mu$ M Isoprenaline did not change MLC-2 phosphorylation. MLC-1 was mainly in the dephosphorylated form; the MLC-1 phosphorylation was increased slightly ( $P < 0.05$ ) from 13.5% in the Quiescence group to 19%

in the Isoprenaline group (Table 2). Although the effect of okadaic acid was not significant, this increase might – at least partly – be caused by okadaic acid, which blocks PP-1 and PP-2 activity.





**Fig. 7** Relation between cTnI and cMyBP-C phosphorylation. The overall regression line (interrupted) was calculated on the basis of the individual data points in the Quiescence (Q, white), Contraction (C, gray) and Isoprenaline (Iso, black) group ( $R^2 = 0.78$ ,  $P < 0.0001$ ). Regression lines are also shown for the Contraction ( $R^2 = 0.02$ ,  $P > 0.05$ ) and Isoprenaline group ( $R^2 = 0.66$ ,  $P < 0.02$ )

### ■ Time-dependent phosphorylation by PKA

To assess the time course and magnitude of PKA-induced phosphorylation of cMyBP-C and cTnI, suspensions of cardiomyocytes from a sample of the Quiescence group, where phosphorylation was low, were incubated with recombinant PKA as described in the Methods. The data and the single exponential curve fittings are shown in Fig. 6 (panels D and E). In both cases, the time constant amounted to approximately 10 min, indicating that with the activity used, PKA-induced phosphorylation saturated within 60 min. Comparison of the final levels reached with the corresponding levels in Fig. 6A and B clearly illustrates that cMyBP-C and cTnI phosphorylation was not complete in the Contraction group, while Isoprenaline treatment resulted in phosphorylation levels that were higher than those attained after PKA treatment. This latter finding suggests that Isoprenaline also caused phosphorylation of other sites on cMyBP-C and cTnI, distinct from the PKA-sites (cTnI: Ser23/24; cMyBP-C: S279/288/308).

### ■ Relation between force development and contractile protein phosphorylation

As expected from the absence of any difference in  $F_{\max}$ ,  $F_{\text{pas}}$ , and  $k_{\text{tr}}$  between groups, no significant correlations were observed between these parameters and the endogenous levels of cMyBP-C, cTnI and MLC-2 phosphorylation in tissue from the different animals. On the other hand, significant positive correlations were observed between the  $p\text{Ca}_{50}$  values from the different animals and endogenous phosphorylation levels.  $R^2$  values ranged between 0.20 and 0.37 ( $P < 0.02$ ). After PKA, the

slopes of the regression lines were reduced but the relations remained significant with  $R^2$  values of 0.22 and 0.23, except for MLC-2. ( $R^2 = 0.03$ ,  $P > 0.05$ ).

The overall correlation between the endogenous cTnI and cMyBP-C phosphorylation levels (Fig. 7) turned out to be positive and highly significant. The relation within the Isoprenaline group was also significant and rather similar to the overall relation. The relations within the Quiescence and Contraction group were not significant. There was some variability in the cMyBP-C phosphorylation levels within the Contraction and Isoprenaline group. This variability, however, did not appear to be associated with the differences in the  $\text{Ca}^{2+}$ -concentration in the Contraction group nor with the presence of okadaic acid in the Contraction and Isoprenaline subgroups.

## Discussion

The phosphorylation levels of the contractile proteins varied considerably with the experimental conditions used. Pacing the Langendorff perfused hearts at 5 Hz resulted in an almost 2-fold increase in MLC-2 phosphorylation (relative to the Quiescence group) and, in ~50% of the cases, in an increase in cMyBP-C phosphorylation, while cTnI phosphorylation remained constant. Addition of  $\beta$ -agonist Isoprenaline to the electrically paced hearts resulted in 3.7-fold increases, relative to Quiescence, in both cMyBP-C and cTnI phosphorylation. These alterations in phosphorylation had a large impact on  $\text{Ca}^{2+}$ -sensitivity but not on maximum force and  $k_{\text{tr}}$ .

### ■ Alterations in contractile protein phosphorylation

The phosphorylation status of contractile proteins is determined by the kinase-phosphatase balance inside the cardiomyocytes. In quiescent Langendorff-perfused hearts MLC-1, MLC-2, cMyBP-C and cTnI were mainly dephosphorylated, indicating that the relevant balance had been shifted in favour of phosphatase activity. MLC-1 phosphorylation increased significantly from  $13.5 \pm 0.6\%$  to  $19.0 \pm 1.7\%$  in the Isoprenaline group. The kinase involved in MLC-1 phosphorylation is unknown: it could be phosphorylated *in vitro* by MLCK, but only in the presence of ATP $\gamma$ S and not in the presence of  $^{32}\text{P}$ -labeled  $\gamma$ ATP [27] and, *in vivo*, by 0.1 mM adenosine [1]. The increase in MLC-2 phosphorylation in the Contraction group with respect of the Quiescence group indicates that a marked increase in intracellular  $\text{Ca}^{2+}$  by pacing is required to activate the  $\text{Ca}^{2+}$ -calmodulin dependent MLCK. The averaged levels of cMyBP-C and cTnI phosphorylation in the Contraction group were not significantly increased. However, the variability in cMyBP-C phosphorylation in this group suggests

that the activity of CaMK II may have been increased near to the threshold for suprabasal cMyBP-C phosphorylation.

The correspondence between the cMyBP-C and cTnI phosphorylation levels in the Quiescence hearts perfused with 0.2 mM and 1.36 mM CaCl<sub>2</sub> was unexpected. McClellan et al. [24] reported a large decrease in the level of cMyBP-C phosphorylation when the Ca<sup>2+</sup> concentration in the incubation solution for the cardiac trabeculae was reduced from 2.5 mM to 1.25 mM. This difference could be explained by a temperature-dependent shift in the kinase-phosphatase balance (e. g. [25]) since our experiments in Langendorff-perfused hearts were carried out at 37 °C, and those of McClellan et al. at 23 °C.

Addition of Isoprenaline to the electrically paced (5 Hz) heart caused marked elevations of the cMyBP-C and cTnI phosphorylation levels, but did not result in a further significant increase in MLC-2 phosphorylation. The increases in cMyBP-C and cTnI phosphorylation are consistent with literature data (e. g. [5]) and can be attributed to the increased activity of PKA but, because of the associated rise in the time-averaged intracellular Ca<sup>2+</sup> concentration [2], altered activities of the Ca<sup>2+</sup>-dependent kinases CaMK II and classical PKC isoforms and, indirectly, of phosphatases such as PP-1 [22] may be involved as well. The notion that Isoprenaline phosphorylates other sites on cTnI and cMyBP-C is supported by the observation that the phosphorylation levels attained exceeded those obtained in the myocyte suspension after PKA treatment. On the other hand, the finding that the phosphorylation level of MLC-2 was not increased may be attributed by substrate specificity of the (Ca<sup>2+</sup>-dependent) kinases and phosphatases involved.

### ■ Phosphorylation and force development

The maximum force generating capacity ( $F_{\max}$ ) in the different experimental groups studied was very similar. Moreover, as was observed in previous studies (e. g. [16, 40]),  $F_{\max}$  was not influenced by PKA treatment. This indicates that phosphorylation by PKA of cMyBP-C and cTnI did not modulate  $F_{\max}$ . These observations are at variance with the results of McClellan et al. obtained in cardiac trabeculae where cMyBP-C phosphorylation resulted in an increase of  $F_{\max}$ . The reason for this difference is unclear and previous data are equivocal: a large body of evidence has accumulated from transgenic animals [18, 38] and by adding peptide fragments of cMyBP-C [14, 23] as well as extraction of cMyBP-C [15] that cMyBP-C is able to modulate the isometric force and kinetics of force generation in cardiac tissue at submaximal levels of activation but not at saturating Ca<sup>2+</sup>-concentrations. However, other reports indicate that cMyBP-C is able to influence  $F_{\max}$  [19, 20].

The  $k_{tr}$  at saturating Ca<sup>2+</sup>-concentration and Ca<sup>2+</sup>-dependence of  $k_{tr}$  in the different experimental groups did not differ significantly. The absence of an effect of cMyBP-C phosphorylation on maximum  $k_{tr}$  is in agreement with the results of Stelzer et al. [36, 38]. Their results at submaximal activation indicate that ablation of murine cardiac cMyBP-C and PKA phosphorylation of cMyBP-C both accelerate the rate of force generation. Our measurements at submaximal activation suggested a small reduction in  $k_{tr}$  after PKA treatment, i. e. a slowing of force generation, in particular in the Contraction group. It should be noted that we studied the Ca<sup>2+</sup>-dependence of  $k_{tr}$  in the pCa range between 4.5 and 5.4, where the Ca<sup>2+</sup>-dependent reduction in  $k_{tr}$  was quite modest.

The Ca<sup>2+</sup>-sensitivity in the Quiescent group was larger than in the Contraction and Isoprenaline groups ( $P < 0.05$ ). The origin of this difference is not clear, but since the effect of PKA was larger in the Quiescent group than in the Contraction and Isoprenaline groups, we consider it likely that it is a consequence of differences in cAMP-dependent phosphorylation. If this would be the case, minor differences in phosphorylation at low cAMP levels may have a considerably larger impact on Ca<sup>2+</sup>-sensitivity than at moderate or high levels.

The passive force decreased significantly after PKA treatment. This observation is similar to the findings in our Laboratory in cardiomyocytes isolated from biopsies from heart failure patients with diastolic dysfunction [3]. Recent studies indicate that the reduction in passive force could be caused by the phosphorylation of titin [10, 44].

### ■ Effects of phosphorylation on Ca<sup>2+</sup>-sensitivity

PKA phosphorylation desensitised the myofilaments in all groups. The magnitude of this effect depended on the endogenous phosphorylation levels since similar pCa<sub>50</sub> values were attained after PKA treatment. These experiments indicate that the PKA-sensitive phosphorylation sites on cMyBP-C and/or cTnI were not saturated under basal conditions even in the Isoprenaline group. Additional evidence to support this notion originates from the observations that the final phosphorylation levels of cMyBP-C and cTnI in the cardiomyocyte suspension after PKA-treatment exceeded the average values in the Quiescence and Contraction group. The effects of PKA in the Isoprenaline group also indicate that, although the dose used was in the high range of values reported in the literature, Isoprenaline did not result in complete saturation of the PKA sites. Since in preliminary experiments pressure development stabilised within 1 min following Isoprenaline administration, these observations could be caused by a lag in phosphorylation of contractile proteins relative to that of Ca<sup>2+</sup>-handling proteins

and/or a delayed onset of dose-dependent activation of phosphatases.

Since cMyBP-C and cTnI phosphorylation are strongly associated (Fig. 6D), it is not possible from these experiments to distinguish directly between effects of cMyBP-C and cTnI phosphorylation on Ca<sup>2+</sup>-sensitivity of force development. A number of studies in transgenic animals, however, indicated that cMyBP-C phosphorylation has considerable impact on cardiac contractility, but not directly on Ca<sup>2+</sup>-sensitivity of isometric force [7, 16, 17].

Olsson et al. [31] have shown that an increase in MLC-2 phosphorylation from 7 to 58% resulted in an increase in Ca<sup>2+</sup>-sensitivity corresponding to 0.06 pCa units. Our data provided evidence for an even stronger, *inverse* association between MLC-2 phosphorylation and pCa<sub>50</sub>. Regression analysis indicated that this association was

lost after PKA treatment. The cMyBP-C and cTnI phosphorylation levels in the Quiescence and Contraction group were very similar. Therefore our results indicate the presence of a strong interaction between the effects of phosphorylation of cTnI and MLC-2 on Ca<sup>2+</sup>-sensitivity. Since the intrinsic positive effect of MLC-2 phosphorylation did not become visible after PKA treatment, this interaction might be the consequence of the coordinated action of different kinases and phosphatases, resulting in a specific pattern of phosphate incorporation on the interacting proteins.

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