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CaMKII and at least two unidentified kinases phosphorylate regulatory light chain in non-contracting cardiomyocytes

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ABSTRACT

In cardiac tissue, regulatory light chain (RLC, myosin light chain 2) phosphorylation (Ser^{15}) leads to modulation of muscle contraction through Ca^{2+}-sensitization. To elucidate which kinases that are involved in the basal (diastolic phase) RLC phosphorylation, we studied non-contracting adult rat cardiomyocytes. RLC kinase activities *in situ* were unmasked by maximally inhibiting myosin light chain phosphatase (MLCP) by calyculin A in the absence and presence of various protein kinase inhibitors. Surprisingly MLCK did not contribute to the phosphorylation of RLC in the non-contracting cardiomyocytes. Two kinase activity groups were revealed by different sensitivities to staurosporine. The fraction with the highest sensitivity to staurosporine was inhibited by KN-93, a selective CaMKII inhibitor, producing a 23% ± 7% reduction in RLC phosphorylation. Calmodulin antagonism (W7) and reduction in Ca^{2+} (EGTA) combined with low concentration of staurosporine caused a larger decrease in RLC phosphorylation than staurosporine alone. These data strongly suggest that in addition to CaMKII, there is another Ca^{2+}/calmodulin-dependent kinase and a Ca^{2+}/calmodulin-independent kinase phosphorylating RLC. Thus the RLC phosphorylation seems to be ensured by redundant kinase activities.

1. Introduction

Regulation of cardiac contraction is well understood in terms of the activating role of Ca^{2+} on the troponin-tropomyosin complex, but less in terms of modulation by thick filament accessory proteins. Regulatory light chain (RLC, myosin light chain-2) is a 20 kDa protein located in the hinge region of the myosin heavy chain. In cardiac muscle, increased RLC phosphorylation (RLC-P) at Ser^{15} modulates contraction by increasing force without increase of $[Ca^{2+}]_i$ [1–5]. Thus, the degree of basal RLC-P without receptor stimulation influences the basal contractile function. Positive inotropic effects of stimulating α_1-adrenoceptors (α_1-AR) [6,7], FP prostanoid receptors [8] and endothelin receptors [9] in normal hearts, and 5-HT_{2A} [10] and muscarinic M_2 receptors [11] in failing hearts, are thought to be dependent on increased RLC-P. RLC has been identified as an essential component of sarcomere assembly and contractility in the vertebrate heart [12,13]. Therefore, understanding the signalling elements controlling RLC-P state may help identify attractive targets of therapeutic intervention in association with cardiac muscle dysfunction.

The RLC-P status in beating myocardium is dependent upon the opposing effects of myosin light chain kinase (MLCK) [6] and myosin light chain phosphatase (MLCP) [14]. We have recently published that during phosphatase inhibition in contracting cardiomyocytes CaMKII in addition to MLCK is phosphorylating RLC and that both are Ca^{2+}/calmodulin dependent [15]. As RLC is a crucial regulator of cardiac contractility [16], its basal phosphorylation state is important. We hypothesize that such an important basal mechanism is dependent on more than one protein kinase. The aim of the present study was to characterize the *in situ* basal kinase activities involved in RLC-P in non-contracting cardiomyocytes. We elucidated the role of CaMKII, MLCK and other potential kinases by applying various kinase inhibitors in combination with phosphatase inhibition. Surprisingly, we found no contribution from MLCK to RLC-P in non-contracting

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cardiomyocytes. Our data suggest at least three active kinase activities contributing to RLC-P in non-contracting cardiomyocytes: CaMKII, another Ca^{2+}/calmodulin-dependent kinase activity and a Ca^{2+}/calmodulin-independent kinase activity.

2. Materials and methods

2.1. Animals

Animal care was according to the Norwegian Animal Welfare Act, which conforms to the *European Convention for the protection of Vertebrate animals used for Experimental and other Scientific Purposes* (Council of Europe no. 123, Strasbourg 1985). The animals were housed in a temperature-regulated room with a 12 h:12 h day/night cycle.

2.2. Isolation of rat cardiomyocytes

Ventricular cardiomyocytes were isolated from male adult Wistar rat hearts by the collagenase perfusion method described previously [17]. Cardiomyocytes were plated on laminin-coated dishes and incubated overnight before use. The final cell population contained >95% elongated cardiomyocytes even after the longest incubation times (125 min).

2.3. In situ kinase activities phosphorylating RLC

The degree of RLC-P *in situ* is the result of a dynamic equilibrium between kinase and phosphatase activities. By fully inhibiting MLCP *in situ*, kinase activities are unmasked and changes in the rate of RLC-P will reflect changes in kinase activity only. Thus calyculin A (a phosphatase 1 and 2A inhibitor) was added during treatment with various kinase inhibitors to identify kinases involved. We earlier found calyculin A (0.1 μM) to inhibit 93% of the phosphatase activity [18]. Basal RLC-P level was defined as the phosphorylation level before phosphatase inhibition by calyculin A.

Cardiomyocytes, incubated in the absence or presence of the relevant kinase inhibitor throughout the experiments, were exposed to calyculin A (0.1 μM unless otherwise indicated) after 45 min to obtain time-response curves (0–80 min). The reactions were stopped at 0, 10, 20, 30, 40 and 80 min after addition of calyculin A, and the degree of RLC-P was determined. The kinase inhibitors were used in the following concentrations: staurosporine (broad spectrum protein kinase inhibitor; 0.03–100 μM; Sigma, St. Louis, MO, USA), ML-7 (MLCK inhibitor; 10 μM;Sigma), ML-9 (MLCK and Rho kinase inhibitor; 60 μM;Tocris, Ellisville, MO, USA), Y27632 (Rho kinase inhibitor; 50 μM;Tocris), bisindolylmaleimide I (BIM; Rho kinase inhibitor; 10 μM;Sigma), W7 (Ca^{2+}/calmodulin inhibitor; 50 μM;Tocris) and KN-93 (CaMKII-selective inhibitor; 50 μM;Sigma). The samples were prepared for determination of RLC-P as previously described [15]. The solutions used for preparing the samples for electrophoresis, contained phosphatase inhibitor (20 mM NaF).

2.4. Determination of phosphorylated RLC

The urea solubilised samples (6 μg/lane) were subjected to electrophoresis as described earlier [8,10] separating RLC and RLC-P based on charge. RLC-P was determined using mouse anti-ventricular MLC-2 monoclonal antibody (Alexis Biochemicals, Lausen, Switzerland). Each lane contained two separate bands: RLC and RLC-P. Percentage of RLC-P was calculated from densitometric values of the bands: RLC-P (%) = (RLC-P/(RLC + RLC-P)) × 100. Various dilutions of the samples gave similar results (data not shown). The rate of RLC-P between 0 and 30 min after Calyculin A addition was expressed as the slope (%/min) calculated by linear regression of the time-response relationship.

2.5. Statistical analysis

Linear mixed-effect model fit on paired observations was used to analyze the time-dependent effect of the various kinase inhibitors, and paired *t*-test for comparison at specific time points. Data are presented as mean ± SEM with linear regression plots and/or percentile plots. The percentile plot was obtained by normalizing the RLC-P level from each experiment between 0 and 100, and the resulting values were used to calculate the percentile effect at each actual point of measurement (=x-axis, time or drug concentration). The curves were based on the x,y pairs corresponding to defined percentiles of the effector variable (for every 10% up to 100%) and plotted with the resulting curve, for illustration. This means that the x value of the 50 percentile indicates the time to half maximal effect [18]. $P < 0.05$ was considered to reflect statistically significant differences.

3. Results and discussion

3.1. Effect of phosphatase inhibition

In order to unmask the effect of kinases phosphorylating RLC, we examined the concentration-response relationship between the phosphatase inhibitor calyculin A and RLC-P (Fig. 1A). Calyculin A (40 min) increased the RLC-P concentration-dependently from 16% ± 2% (0 M) to 46% ± 4% (0.1 μM), $p < 0.001$, $n = 6$ with a $-\log EC_{50} = 8.0 \pm 0.1$. Thus, we routinely used 0.1 μM calyculin A to rapidly and almost fully inhibit MLCP and unmask and estimate the *in situ* agonist-independent kinase activities phosphorylating RLC in non-contracting adult cardiomyocytes. Treating the cardiomyocytes with calyculin A (0.1 μM) increased RLC-P from 20% ± 2% to 56% ± 2% during 80 min (Fig. 1B). These levels are close to those found previously in non-contracting cardiomyocytes [18]. The basal RLC-P level (before calyculin A) was in correspondence with what had earlier been found in beating cardiomyocytes [15], muscle strips [8,10,11] and left ventricular tissue [19]. However, the RLC-P level after 80 min with calyculin A was lower than found in our previous work in contracting cardiomyocytes (about 80% after 80 min) [15].

3.2. Effect of Rho kinase inhibition

Rho kinase plays a pivotal role in inhibiting MLCP by phosphorylating myosin phosphatase targeting protein (MYPT) [20]. Since the RLC-P level under phosphatase inhibition in non-contracting cardiomyocytes was lower than that found in contracting cardiomyocytes [15], we elucidated if 0.1 μM calyculin A inhibited MLCP efficiently by using the Rho kinase inhibitor Y27632. If MLCP were still activatable in the presence of calyculin A, RLC-P should be lowered by Y27632. Treating cardiomyocytes with Y27632 did not, however, alter the phosphorylation characteristics of RLC compared to control (Fig. 1B) as also indicated by the slope of the linear regression curves (slope = 0.7% ± 0.1%/min, $slope_{Y27632} = 0.6\% \pm 0.1\%$/min, NS, $n = 7$). Thus 0.1 μM calyculin A exerted an essentially complete inhibition of MLCP.

3.3. Effect of MLCK inhibition

MLCK has so far been thought to play a major role in RLC-P in the vertebrate heart [21]. Recently, we showed that cardiac MLCK (cMLCK) phosphorylated RLC in electrically stimulated (contracting) cardiomyocytes [15]. Thus, also in the present study the