Quantitative measurements of Ca^{2+}/calmodulin binding and activation of myosin light chain kinase in cells

Ramaz Geguchadze[a,1], Gang Zhi[a], Kim S. Lau[a], Eiji Isotani[a,2], Anthony Persechini[b], Kristine E. Kamm[a], James T. Stull[a,*]

[a] *Department of Physiology, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9040, USA*
[b] *Division of Molecular Biology and Biochemistry, University of Missouri at Kansas City, 5007 Rockhill Road, Room B412, Kansas City, MO 64110-2499, USA*

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Abstract Myosin II regulatory light chain (RLC) phosphorylation by Ca^{2+}/calmodulin (CaM)-dependent myosin light chain kinase (MLCK) is implicated in many cellular actin cytoskeletal functions. We examined MLCK activation quantitatively with a fluorescent biosensor MLCK where Ca^{2+}-dependent increases in kinase activity were coincident with decreases in fluorescence resonance energy transfer (FRET) in vitro. In cells stably transfected with CaM sensor MLCK, increasing $[Ca^{2+}]_i$ increased MLCK activation and RLC phosphorylation coincidently. There was no evidence for CaM binding but not activating MLCK at low $[Ca^{2+}]_i$. At saturating $[Ca^{2+}]_i$ MLCK was not fully activated probably due to limited availability of cellular Ca^{2+}/CaM.

Key words: Myosin light chain kinase; Calmodulin; Calcium; Phosphorylation; Fluorescence resonance energy transfer

1. Introduction

Calcium is crucial for numerous biological functions [1] and calmodulin (CaM) serves as its intracellular receptor mediating its second messenger role [2,3]. CaM is an abundant protein present in cells at 10^{-6} M to 10^{-5} M. The local intracellular availability of CaM is of biological significance because different CaM binding proteins are regulated over a wide range of free CaM concentrations as well as by the amplitude and frequency modulation of $[Ca^{2+}]_i$ [2–4].

Ca^{2+}/CaM-dependent myosin light chain kinase (MLCK) phosphorylates Ser-19 and Thr-18 of the regulatory light chain (RLC) of myosin II which is implicated in many actin cytoskeletal-dependent functions in smooth and non-muscle cells including contraction, cytokinesis, stress fiber formation and motility [5–7]. Using a fluorescence resonance energy transfer (FRET)-based biosensor in transiently transfected cells, it was recently shown that MLCK was activated in lamellae of migrating cells and at the cleavage furrow during cell division [8]. However, quantitative information on the extent of MLCK activation relative to RLC phosphorylation was lacking.

Recent studies have shown that only a small fraction of the total CaM may be free [9–12]. In endothelial cells Ca^{2+}/CaM binding to nitric oxide synthase decreased the activities of other CaM targets through reduction in the size of a limiting pool of available Ca^{2+}/CaM [13]. Thus, when $[Ca^{2+}]_i$ increases in response to a stimulus, there may be a limiting amount of Ca^{2+}/CaM for MLCK activation. CaM may also be tethered to the MLCK under resting conditions in cells in a way that does not activate the kinase but would be available for a rapid and complete activation with increased $[Ca^{2+}]_i$ [14,15]. Based on these proposed mechanisms, we prepared a cell line stably transfected with a biosensor MLCK that measures Ca^{2+}/CaM binding and kinase activation by a decrease in FRET to examine its cell regulation properties.

2. Materials and methods

2.1. Construction and expression of CaM sensor MLCK

The plasmid pCDIC-35 which encodes enhanced yellow and cyan fluorescent proteins (EYFP and ECFP) linked by the smooth muscle MLCK CaM binding sequence under a CMV promoter was used [11]. The rabbit smooth muscle MLCK cDNA encoding residues 1–1038 with a *Bam*HI site at the 3′ end was inserted downstream of the CMV promoter and fused with the EYFP and ECFP coding region in pCDIC-35. The sites of insertion were confirmed by DNA sequencing. Plasmids containing the cDNA for CaM sensor MLCK (pCMVKCS35) were transfected into HEK-293 cells and protein expression was measured by kinase activity and Western blot procedures.

2.2. Cell culture and transfection

HEK-293 human embryonic kidney cells (obtained from ATCC, CRL-1573) were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4 mM L-glutamine, 1.5 g/l sodium bicarbonate,

*Corresponding author. Fax: (1)-214-648 2974.
E-mail addresses: ramaz.geguchadze@utsouthwestern.edu (R. Geguchadze), gang.zhi@utsouthwestern.edu (G. Zhi), kim.lau@utsouthwestern.edu (K.S. Lau), isoensrg@tmd.ac.jp (E. Isotani), persechinia@umkc.edu (A. Persechini), kristine.kamm@utsouthwestern.edu (K.E. Kamm), james.stull@utsouthwestern.edu (J.T. Stull).

[1] Present address: Department of Biochemistry, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9038, USA.
[2] Present address: Department of Neurosurgery, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyoku, Tokyo 113-8519, Japan.

Abbreviations: CaM, calmodulin; DIFP, diisopropylfluorophosphate; DTT, dithiothreitol; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane; FRET, fluorescence resonance energy transfer; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; RLC, regulatory light chain; MLCK, myosin light chain kinase; EYFP, enhanced yellow fluorescent protein; ECFP, enhanced cyan fluorescent protein; GFP, green fluorescent protein; BFP, blue fluorescent protein

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4.5 g/l glucose, 1 mM sodium pyruvate, 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO_2. HEK-293 cells were plated in 100 mm dishes 1 day before transfection and DNA was transfected by a liposome-mediated method according to the manufacturer's instructions (FuGENE™ 6, Roche). Single or mixed clonal populations of stably transfected cells were grown under G418 selection for at least 6 weeks.

2.3. Western blot

The cells were collected and lysed on ice in a buffer containing 20 mM 3-(*N*-morpholino)-propanesulfonic acid (MOPS) at pH 7.5, 1% Nonidet P-40, 0.5 mM ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 50 mM $MgSO_4$, 10% glycerol, 10 mM dithiothreitol (DTT), 0.25 mM *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane (E64), 0.25 mM diisopropylfluorophosphate (DIFP). The lysates were centrifuged at 7000×*g* for 5 min at 4°C to remove the insoluble fraction. Aliquots of the cell lysates were frozen in liquid nitrogen and stored at −80°C. The quantity of MLCK in cell lysates was determined by immunoblotting including various amounts of purified rabbit smooth muscle MLCK from Sf9 cells as standard and probing with anti-MLCK monoclonal antibody (K36 from Sigma-Aldrich). CaM was measured similarly by Western blot with a monoclonal antibody from Santa Cruz Biotechnology [9].

2.4. MLCK assays

To determine the Ca^{2+}/CaM-dependent activity of wild-type and CaM sensor MLCK, ^{32}P incorporation into purified RLC was measured [9]. Maximal kinase activity was determined in reactions containing 50 mM MOPS at pH 7.0, 10 mM $MgSO_4$, 1 mM DTT, 0.3 mM $CaCl_2$, 1 mM [γ-^{32}P]adenosine triphosphate (ATP) (300–350 cpm/pmol), 1 μM CaM, 26.5 μM RLC, and diluted MLCK. Lysates were freshly diluted in 25 mM MOPS at pH 7.0, 5 mM $MgSO_4$, and 1 mM DTT and added to reaction mixtures. Final concentrations of MLCK showed linear phosphorylation rates with respect to time and enzyme concentration. K_m and V_{max} values were obtained from Lineweaver–Burk plots after measuring the rates of ^{32}P incorporation under varying RLC concentrations. Minimal Ca^{2+}/CaM-independent kinase activity in lysates was observed in the presence of 4 mM EGTA and 1.0 μM CaM.

2.5. FRET measurement

Fluorescence measurements were performed using a Perkin Elmer 650-10S fluorescence spectrophotometer. Reaction volumes (150 μl) were incubated at room temperature using a Starna micro-square cuvette. Excitation and emission slit widths were 5–8 nm. Fluorescence intensity was measured by excitation at 430 nm and emission at 480 and 525 nm in a buffer containing 25 mM MOPS at pH 7.0, 5 mM $MgSO_4$, and 4 mM EGTA. Aliquots of standard Ca^{2+}–methanesulfonate solutions were added to achieve various free [Ca^{2+}] [9].

2.6. Immunoprecipitation of myosin

Cells were homogenized in buffer containing 25 mM Tris–HCl, 100 mM sodium pyrophosphate, 100 mM NaF, 250 mM NaCl, 10 mM EGTA, 5 mM ethylenediamine tetraacetic acid (EDTA), 1% Nonidet P-40, 0.25 mM E64 and 0.25 mM DIFP at pH 8.8. Aliquots of homogenate were incubated with Pansorbin *Staphylococcus aureus* cells, which were bound with IgG from rabbit anti-bovine tracheal myosin antiserum. After overnight incubation, the immune complexes were collected by centrifugation at 3000×*g* for 5 min. The pellet was washed twice with phosphate-buffered saline (PBS) buffer plus 0.1% Triton X-100. The antigen–antibody complexes were eluted by boiling in 8 M urea sample buffer. Eluted proteins were subjected to urea/glycerol–polyacrylamide gel electrophoresis (PAGE) to separate phosphorylated forms of RLC and measured by immunoblotting after transfer to nitrocellulose [9].

2.7. FRET and myosin RLC phosphorylation in cells

HEK-293 stably transfected cells were grown on coverslips to 80–90% confluence, washed twice in Ca^{2+}-free buffer (20 mM piperazine-1,4-bis-2-ethanesulfonic acid (PIPES) pH 6.8, 4 mM EGTA, 5 mM magnesium methanesulfonate, 90 mM potassium methanesulfonate and 4 mM ATP) and incubated at room temperature for 20 min in Ca^{2+}-free buffer containing 15 μg/ml α-toxin (hemolysin). Free Ca^{2+} concentrations were achieved by injecting different amounts of 10 or 100 mM stock Ca^{2+}–methanesulfonate solution in Ca^{2+}-free EGTA-containing buffer, and the extent of RLC phosphorylation measured by urea/glycerol–PAGE and immunoblotting [16]. FRET was also measured in the fluorescence spectrophotometer on permeable cells on coverslips placed in a Teflon frame to hold the coverslip at a 45° angle for the incident light in a temperature controlled cuvette [16].

3. Results and discussion

Fluorescent biosensors based on green fluorescent protein (GFP) and its spectral variants [17] provide powerful tools for studying biochemical processes in cells. Early approaches allowed visualization of protein localizations and translocations in individual cells by fluorescent imaging and advances have led to quantitative improvements [18]. FRET-based biosensors for Ca^{2+} and CaM were among the first to exploit GFP for intracellular detection of specific ligands and proteins [12,19,20]. Recently Chew et al. (2002) reported on a biosensor MLCK for determination of its localization and relative Ca^{2+}/CaM binding state in living cells by linking fluorescent indicator proteins to the C-terminus of MLCK [8]. Upon blue fluorescent protein (BFP) excitation the FRET to GFP decreased when Ca^{2+}/CaM bound to the CaM binding sequence linking the two fluorophores. In individual cells MLCK was activated in lamellae of migrating cells but not the retracting tail and was activated just before cleavage furrow constriction.

We developed a different CaM sensor MLCK capable of monitoring MLCK activation in stably transfected cells to obtain quantitative information on Ca^{2+}/CaM binding to MLCK. We used ECFP and EYFP variants linked by the smooth muscle MLCK CaM binding sequence [11] to monitor FRET as a 480/525 emission ratio with the ECFP donor fluorophore excited at 430 nm, a longer wavelength than that used to excite BFP.

3.1. Ca^{2+}/CaM activation of MLCK is quantitatively linked to FRET changes

CaM sensor MLCK was expressed in stably transfected HEK-293 cells (Fig. 1A). Western blot analysis with an anti-

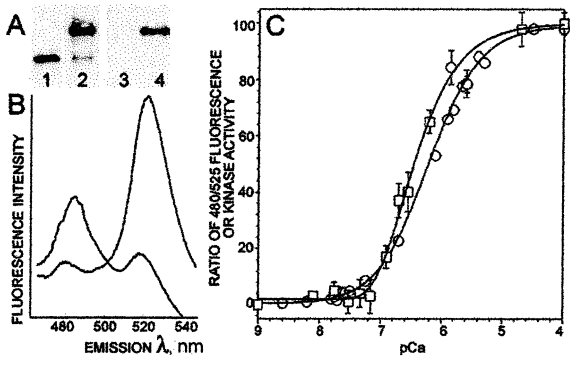

Fig. 1. CaM sensor MLCK expression in HEK-293 cells. A: Western blot analysis with antibodies specific for the N-terminus of MLCK and GFP in untransfected (lanes 1 and 3) and stably transfected HEK-293 cells (lanes 2 and 4). B: Ca^{2+}-dependent changes in the CaM sensor MLCK fluorescence emission in cell lysates. CaM sensor MLCK was incubated with EGTA (curve with longest emission at 525 nm) or Ca^{2+} in the presence of CaM (curve with lower emission at 525 nm). C: Ca^{2+}-dependent changes in the ratio of fluorescence emission at 480 to 525 nm (open circles) and kinase activity (open squares) of CaM sensor MLCK in lysates of stably transfected HEK-293 cells. Results represent means ± standard deviations for five (kinase activity) or three (FRET) experiments.

body specific for the N-terminus of MLCK showed immunoreactivity of endogenous MLCK (140 kDa) in untransfected and stably transfected HEK-293 cells. The stably transfected cells showed immunoreactivity at a high molecular mass predicted for the CaM sensor MLCK (190 kDa), which also reacted with an antibody to GFP (Fig. 1A). The amount of endogenous MLCK appeared to decrease in the stably transfected cells. The reason for the decrease is not clear; however, the CaM sensor MLCK is the dominant form expressed.

In the absence of Ca^{2+}, excitation of ECFP at 430 nm in CaM sensor MLCK resulted in peak fluorescence emission at 525 nm from EYFP due to FRET (Fig. 1B). Upon Ca^{2+}/CaM binding FRET decreased as shown by increased emission at 480 nm and decreased emission at 525 nm. The emission spectrum of the HEK-293 lysate in the absence and presence of Ca^{2+}/CaM was similar to that previously reported for the sensor moiety not linked to MLCK [19]. The ratio of fluorescence emission at 480 to 525 nm increased from 0.45 to 1.55 with Ca^{2+}/CaM binding.

We further characterized the biochemical properties of CaM sensor MLCK relative to wild-type MLCK. The catalytic properties of CaM sensor MLCK were 21 pmol ^{32}P/min/ng kinase and 12 μM K_m^{RLC}. For wild-type MLCK the V_{max} value was 18 pmol ^{32}P/min/ng kinase and K_m^{RLC} was 14 μM. Thus, the addition of the sensor moiety did not significantly change the catalytic properties of the kinase.

HEK-293 cell lysates contain endogenous CaM which is a small fraction of the amount (1 μM) added in reaction mixtures. Thus, we measure Ca^{2+}/CaM activation by increasing [Ca^{2+}] to increase Ca^{2+}/CaM [9]. Increasing [Ca^{2+}] in lysates showed a half-maximum FRET ratio change at *p*Ca 6.2 (Fig. 1C). MLCK activity showed a similar dependence on [Ca^{2+}]. Therefore, Ca^{2+}-dependent change in FRET provides a quantitative measure of Ca^{2+}/CaM binding and MLCK activation.

3.2. Ca^{2+} dependence of RLC phosphorylation and FRET changes are similar in permeable HEK-293 cells

RLC phosphorylation was measured in stably transfected HEK-293 cells. In control experiments different phosphorylated forms of RLC were analyzed by urea/glycerol–PAGE [16]. In cell lysates preincubated in the absence of ATP two major bands were detected by Western blot (Fig. 2, left panel). When cells were incubated in buffer containing 2 mM ATP and 10 μM free Ca^{2+}, two additional bands appeared. Immunoprecipitation experiments with myosin antibody indicated that only the three faster migrating bands were coprecipitated (Fig. 2, right panel). Because the slowest migrating band was not immunoprecipitated, it is not a subunit of myosin. The three bands immunoprecipitated with myosin antibody

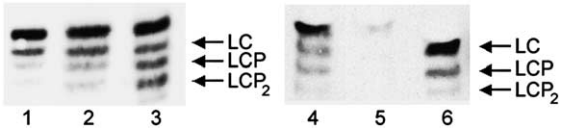

Fig. 2. RLC phosphorylation in cell lysates of stably transfected HEK-293 cells. Left panel: Cell lysates were incubated in Ca^{2+}-free buffer with 0.1 mM EDTA and without ATP (lane 1) or with 1 mM ATP and 5 mM EGTA (lane 2) or 10 μM Ca^{2+} (lane 3). Right panel: Immunoprecipitation of myosin from stably transfected HEK-293 cells. Samples before (lane 1) and after (lanes 2 and 3, supernatant and pellet, respectively) immunoprecipitation. Results are representative of three experiments.

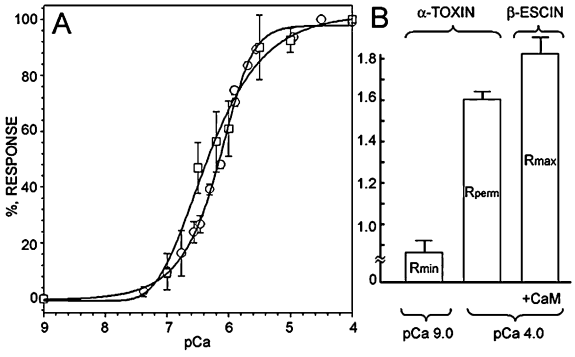

Fig. 3. A: Ca^{2+} dependence of FRET (open circles) and RLC phosphorylation (open squares) in stably transfected, α-toxin permeable HEK-293 cells. B: The effect of Ca^{2+} and CaM on CaM sensor MLCK FRET in stably transfected α-toxin or β-escin permeable HEK-293 cells.

correspond to unphosphorylated, monophosphorylated and diphosphorylated RLC [16].

The total percent phosphorylation of RLC in permeable, stably transfected HEK-293 cells in the absence of Ca^{2+} was 35%. RLC phosphorylation decreased to 15% in the presence of 100 μM indomethacin and in the absence of Ca^{2+}. This result is consistent with recent evidence that other kinases may phosphorylate RLC in a Ca^{2+}-independent manner [15]. Indomethacin is a cyclooxygenase inhibitor which probably decreases arachidonate metabolites that inhibit myosin phosphatase activity [21]. The extent of RLC phosphorylation increased to 72% in the presence of 100 μM Ca^{2+}. Normalizing the minimum and maximum RLC phosphorylation values, the *p*Ca value for the half-maximal response was 6.4 (Fig. 3A). The half-maximal response for FRET was obtained at *p*Ca 6.2, a value similar to the one obtained for RLC phosphorylation. The curves for both RLC phosphorylation and FRET were similar.

We also determined R_{perm} in α-toxin permeable cells which is the maximal FRET response for activated CaM sensor MLCK by endogenous Ca^{2+}/CaM (Fig. 3B). The R_{perm} value depends on the availability of cellular Ca^{2+}/CaM for binding to MLCK and is determined 10 min or later after increasing [Ca^{2+}]$_i$ and obtaining stable FRET values [11]. To determine the emission ratio corresponding to maximal activation of MLCK (R_{max}), cells were permeabilized with β-escin in the presence of 10 μM Ca^{2+} and 1 μM CaM [11]. The R_{max} value was greater than the R_{perm} value, indicating that CaM sensor MLCK is only partially activated in α-toxin permeable cells by endogenous Ca^{2+}/CaM at high [Ca^{2+}]$_i$. The partial activation of CaM sensor MLCK in stably transfected HEK-293 cells raised the possibility that the kinase was overexpressed relative to the amount of CaM. However, quantitative analyses showed that the total intracellular CaM concentration was 10.3 ± 2.0 μM, a value greater than 2.0 ± 0.4 μM MLCK as measured in the stably transfected HEK-293 cells. These data support the concept that even though the total CaM concentration in cells exceeds a specific high-affinity target protein, there may be a limiting pool of available CaM because of its participation in both Ca^{2+}-dependent and Ca^{2+}-independent interactions with many target proteins [3,9]. The maximal biosensor response is consistent with a 50 nM free Ca^{2+}/CaM concentration. Increases in MLCK activation may be coupled

to decreases in Ca^{2+}/CaM binding to other CaM targets through reduction in the size of the limiting pool of CaM [13].

Heller et al. (2003) recently showed that $(Ca^{2+})_2$/CaM has a similar collapsed structure as $(Ca^{2+})_4$/CaM when both are bound to MLCK although they are at different positions relative to the catalytic cleft of the kinase [22]. The $(Ca^{2+})_2$/CaM structure is closer to the catalytic cleft and the kinase is not active. When all four Ca^{2+} binding sites in CaM are saturated, the collapsed CaM structure translocates further away from the cleft resulting in an activated MLCK. Based on these structural analyses, both $(Ca^{2+})_2$/CaM and $(Ca^{2+})_4$/CaM are predicted to decrease FRET similarly. The fact that changes in FRET and kinase activity were coincident in cell lysates as well as in permeable cells argues against $(Ca^{2+})_2$/CaM tethered to MLCK at a low Ca^{2+} concentration (100 nM) typically found in resting cells [14,15].

In summary, there is a tight coupling between Ca^{2+}/CaM binding to MLCK and activation in cells. However, the amount of cellular CaM available for activation appears limiting, even at high $[Ca^{2+}]_i$.

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