Real-time imaging of myosin II regulatory light-chain phosphorylation using a new protein biosensor

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Phosphorylation of the RMLC (regulatory myosin light chain) regulates the activity of myosin II, which is critically involved in the motility of both muscle and non-muscle cells. There are both Ca^{2+} -dependent and -independent pathways for RMLC phosphorylation in smooth-muscle cells, and the latter pathway is often involved in an abnormal contractility in pathological states such as asthma and hypertension. Therefore pharmacological interventions of RMLC phosphorylation may have a therapeutic value. In the present study, we developed a new genetically encoded biosensor, termed CRCit (ECFP-RMLC-Citrine, where ECFP is enhanced cyan fluorescent protein), that detects RMLC phosphorylation using fluorescence resonance energy transfer between two variants of the green fluorescent protein fused to both

the N- and C-termini of RMLC. When expressed in primary cultured vascular smooth-muscle cells, CRCit detected the Ca^{2+} -dependent RMLC phosphorylation with a high spatiotemporal resolution. Furthermore, we could specifically assay the agonist-induced Ca^{2+} -independent phosphorylation of RMLC when Ca^{2+} signalling in cells expressing CRCit was suppressed. Thus CRCit may also be used for the high throughput screening of compounds that inhibit abnormal smooth-muscle contraction.

Key words: fluorescence resonance energy transfer (FRET), green fluorescent protein (GFP), myosin, phosphorylation, protein biosensor, Rho-kinase.

INTRODUCTION

Myosin II plays important roles in regulating various cellular functions, including cytokinesis [1], migration [2], neuronal outgrowth [3,4] and smooth-muscle contraction [5]. The activity of smooth-muscle or non-muscle myosin II is mainly regulated by the phosphorylation of the RMLC (regulatory myosin light chain) in both Ca^{2+} -dependent and -independent manners. In smooth-muscle cells, an increase in the intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) induced by chemical or electrical stimulation causes Ca^{2+} -bound calmodulin to activate the MLCK (myosin light-chain kinase). This serine/threonine kinase phosphorylates RMLC at Ser¹⁹ and to a lesser extent at Thr¹⁸, increasing the actomyosin ATPase activity and initiating smooth-muscle contraction [6,7]. On the other hand, MLCP (smooth-muscle myosin light-chain phosphatase) dephosphorylates RMLC, resulting in smooth-muscle relaxation [8].

In addition to the Ca^{2+} -dependent phosphorylation of RMLC, certain agonists increase the Ca^{2+} sensitivity of smooth-muscle contraction through an inhibitory effect on MLCP or by the direct phosphorylation of RMLC in a Ca^{2+} -independent manner [9]. Rho-kinase, which is activated by the small monomeric G-protein RhoA, is a key molecule that induces the Ca^{2+} sensitization of vascular smooth-muscle cells during responses to vasoconstrictor agonists such as noradrenaline, histamine, angiotensin II, endothelin and thromboxane A₂ [10,11]. It was demonstrated that an up-regulation of Rho-kinase and the resultant phosphorylation of RMLC induces strong vasoconstriction including vasospasm in several hypertensive rat models [12,13]. Not only Rho-kinase but also other molecules, including the C-kinase-potentiated inhibitor of myosin phosphatase (CPI-17) [14], arachidonic acid [15], integrin-linked kinase [16] and ZIP kinase [17], were shown to modulate the phosphorylation of RMLC in a Ca^{2+} -independent manner. These Ca^{2+} -independent phosphorylation mechanisms of RMLC are often implicated in the diphosphorylation (simultan-

eous phosphorylation of Thr¹⁸ and Ser¹⁹) of RMLC and abnormal contractility [18,19]. Therefore a new class of drugs that inhibit the Ca^{2+} -independent RMLC phosphorylation may have a useful therapeutic value for pathophysiological states such as hypertension and asthma [18,19].

In the present study, we constructed a protein indicator of RMLC phosphorylation by fusing two variants of the GFP (green fluorescent protein) to both the N- and C-termini of RMLC. The FRET (fluorescence resonance energy transfer) between two GFP variants is highly sensitive to the distance and relative orientation of the fluorescent proteins [20]. Thus we expected that the conformational change of RMLC on phosphorylation might result in a change in the FRET efficiency. Indeed, the FRET between the two GFP variants of the protein indicator correlated with RMLC phosphorylation in vascular smooth-muscle cells. Using this protein indicator, we succeeded in real-time imaging of the extent of both Ca^{2+} -dependent and -independent (Rho-kinase-mediated) phosphorylations of RMLC in vascular smooth-muscle cells with high spatiotemporal resolutions. This imaging method can be used to construct an effective assay system for identifying new chemicals that inhibit Ca^{2+} -independent RMLC phosphorylation underlying abnormal smooth-muscle contractility.

EXPERIMENTAL

Construction of RMLC phosphorylation biosensor

The expression vector for the biosensor CRCit (ECFP-RMLC-Citrine, where ECFP is enhanced cyan fluorescent protein) was generated by using the retrovirus expression vector pMX (a gift from Dr T. Kitamura, The University of Tokyo). Fragments of DNA that encode ECFP and RMLC were generated by PCR using pECFP-c1 (Clontech, Palo Alto, CA, U.S.A.) and rat lung cDNA library (Clontech) as templates respectively. The primers

Abbreviations used: (E)CFP, (enhanced) cyan fluorescent protein; RMLC, regulatory myosin light chain; CRCit, ECFP-RMLC-Citrine; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; MLCK, myosin light-chain kinase; for brevity, the single-letter system for amino acids has been used, Q69 for example means Gln⁶⁹.

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used were as follows: for ECFP, 5'-TAGCGCGAGCTCGCATA-TGGTGAGCAAGGGCGAGGAGCTGTTTC-3', and 5'-TATTC-CCCGCGGAGCGGATCCCTTGTACAGCTCGTCCATGCCGA-3' and for RMLC, 5'-TATTCGCCGCGGTTCGAGCAAAA-GAGCGAAGACCAAG-3' and 5'-GTAGGGACTAGTGTTCAT-CTTTGTCTTTCGCTCCGTGC-3'. A fragment of DNA that encoded Citrine was generated by PCR using a vector carrying Citrine as a template, and the primers used were 5'-TATAGG-AGTAGTCCCAAGCTTGTGAGCAAGGGCGAGGAGCTGTT-CAC-3' and 5'-GCCGCGCTCGAGCTTGTACAGCTCGTCC-ATGCCGAGA-3'. Citrine is one of the enhanced yellow variants of GFP and identical in sequence with another yellow variant named 10C [21] except for the substitution of Gln⁶⁹ by methionine [22]. Therefore the mutation Q69M (Gln⁶⁹ → Met) was incorporated into the pEYFP-c1 vector (Clontech) containing the four amino acid substitutions, previously published as 10C, to generate the vector carrying Citrine. The Q69M mutation was introduced into pEYFP-c1 by connecting the two fragments generated by two PCRs using the pEYFP-c1 vector as a template. The primers used for the first fragment were 5'-CGGGATCC-TCGAGCAAAAAGAGCGAAGACCAAGACCAC-3' and 5'-GG-GATATCGGGCGAAGCACATCAGGCCGTAGCCGAAGGT-GG-3' and those for the second fragment were 5'-CCCGAT-ATCCCGACCACATGAAGCAGC-3' and 5'-GCCGCGCTCG-AGCTTGTACAGCTCGTCCATGCCGAGA-3'. Then these fragments that encode ECFP, RMLC and Citrine were subcloned into the pBluescript SK(−) vector (Stratagene, La Jolla, CA, U.S.A.) and combined together. After digestion with *Eco*RI and *Sal*I, the fragment that encoded CRCit was cloned into the *Eco*RI and *Sal*I sites in pMX. Protein linker regions were suitably changed by PCR and mutagenesis was accomplished by PCR to generate mutants of CRCit (S19A, T18A and S19A/T18A). Nucleotide sequences of all PCR products were confirmed by sequencing.

Cell culture and protein expression

Vascular smooth-muscle cells were obtained from the aorta of male Wistar rats (7 weeks old) and maintained in D-MEM/F-12 (Invitrogen, Carlsbad, CA, U.S.A.) with 10% (v/v) fetal bovine serum (JRH Biosciences, Lenexa, KS, U.S.A.) and supplemented with 0.1 mM non-essential amino acids (Invitrogen), 50 units/ml penicillin and 50 µg/ml streptomycin. Early-passage (P1) de-differentiated proliferative cells grown on uncoated glass-bottomed dishes (MatTek, Ashland, MA, U.S.A.) were infected with the retrovirus vector. To produce infectious retrovirus particles, a packaging cell line, GP293 (Clontech), was co-transfected with 12 µg of the retrovirus vector carrying CRCit and 1 µg of pVSVG (Clontech) using the LIPOFECTAMINE 2000 reagent (Invitrogen). Viral particles were harvested from the medium 2 days later and used to infect vascular smooth-muscle cells after concentration by centrifugation (9250 g for 16 h). The medium was replaced 48 h after infection with differentiation medium (D-MEM/F-12 without fetal bovine serum and supplemented with 0.1 mM non-essential amino acids, 50 units/ml penicillin and 50 µg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin and 5 ng/ml selenium). The cells were cultured for another 48 h for differentiation.

Fluorescence imaging

The fluorescence images of the cells were captured using an inverted microscope (IX70; Olympus, Tokyo, Japan) equipped with a 40× objective (NA 0.80; Olympus), an Orca-ER charge-coupled-device camera, AquaCosmos Ratio Imaging Module and W-View Optics (Hamamatsu Photonics, Hamamatsu, Japan) at an excitation wavelength of 430 nm. Using this system, pairs of images at two emission wavelengths (480 and 535 nm) were

captured simultaneously. FRET was evaluated on the basis of the ratio of fluorescence intensity at 535 nm to that at 480 nm. All experiments were performed at room temperature (23 ± 1 °C).

Western blotting

Smooth-muscle cells were grown on 12-well culture plates, and infection with retrovirus and differentiation was performed as described above. After differentiation, cells were suspended in SDS sample buffer [8% (w/v) SDS, 20% sucrose, 20% (w/v) 2-mercaptoethanol, 0.008% Bromophenol Blue and 250 mM Tris/HCl, pH 6.8] and boiled for 5 min. Proteins were electrophoresed on an SDS/12% polyacrylamide gel and separated products were transferred on to a PVDF membrane (Bio-Rad, Hercules, CA, U.S.A.). Non-specific binding sites were blocked with 5% (w/v) non-fat dried milk in Tris-buffered saline (100 mM NaCl and 20 mM Tris/HCl, pH 7.8). For detection, the blot was probed with 1:3000 anti-GFP polyclonal antibody (MBL, Nagoya, Japan) and 1:8000 anti-rabbit IgG conjugated with horseradish peroxidase (MBL), then developed using chemiluminescence reagents (Western Lightning™; PerkinElmer LifeSciences, Great Shelford, Cambridge, U.K.).

RESULTS AND DISCUSSION

Generation of RMLC phosphorylation biosensor (CRCit)

We designed a biosensor of the RMLC phosphorylation by fusing two GFP variants to RMLC with the expectation that a phosphorylation-dependent conformational change in RMLC might induce a change in the FRET efficiency between the GFP variants. Among the five constructs we designed and tested, one with ECFP (donor) and Citrine (acceptor) fused at the N- and C-termini respectively of the RMLC, with linkers consisting of 2–5 amino acids, showed the most acceptable properties (CRCit, Figure 1A). CRCit was expressed in cultured vascular smooth-muscle cells using a retrovirus vector. Western blotting with the anti-GFP antibody confirmed the expression of CRCit after infection (Figure 1B), although the level of expression of CRCit was much lower compared with that of native RMLC as indicated by Western blotting using an anti-RMLC antibody (results not shown). CRCit fluorescence displayed fibrous patterns after the differentiation of vascular smooth-muscle cells (Figure 1C), but showed more hazy patterns under the de-differentiated state (results not shown). These results suggest that CRCit is incorporated into myosin filaments partially displacing the native RMLC.

FRET imaging in cells expressing CRCit

At an excitation wavelength of 430 nm, CFP emits fluorescence at 480 nm. Citrine is hardly excited at this excitation wavelength and emits minimal fluorescence at 535 nm (results not shown). However, when CRCit was expressed, we could observe a significant fluorescence intensity at 535 nm. This indicates that FRET between CFP and Citrine within the CRCit molecule results in the fluorescence of Citrine.

To test the capability of CRCit to detect RMLC phosphorylation, we examined fluorescence signals from CRCit before and after stimulation of vascular smooth-muscle cells. In smooth-muscle cells, the extracellular application of high-K⁺ solution causes membrane depolarization and activates voltage-gated Ca²⁺ channels, which then allow an influx of external Ca²⁺, resulting in the activation of Ca²⁺-calmodulin-dependent MLCK. The application of 80 mM K⁺ solution significantly increased fluorescence intensity at 535 nm with a concomitant decrease in fluorescent intensity at 480 nm (Figure 1D, upper panel), suggesting that FRET efficiency between ECFP and Citrine increased on RMLC

Figure 1 Construction and properties of CRCit

(A) Schematic structure of CRCit with the linker sequences shown above. The two phosphorylation sites (Thr¹⁸ and Ser¹⁹) on the RMLC domain of CRCit are indicated. MLC, myosin light chain. (B) Western blots of whole-cell lysate from wild-type smooth-muscle cells or cells expressing 75 kDa CRCit probed with the anti-GFP antibody. (C) Fluorescence image of CRCit expressed in cultured vascular smooth-muscle cells. (D) Upper panel: time courses of fluorescence intensities at 535 nm (green line) and 480 nm (blue line) of a single CRCit-expressing vascular smooth-muscle cell on application of 80 mM K⁺ solution. Application of 80 mM K⁺ solution is indicated by a shaded box. The sampling interval was 1 s. Lower panel: time course of change in FRET response (F_{535}/F_{480}). (E) F_{535}/F_{480} fluorescence intensity ratio images before and after the application of 80 mM K⁺ solution in the same cell as in (D). Each panel shows the average of five consecutive images around the indicated time.

phosphorylation. We used the ratio of fluorescence intensity at 535 nm to that at 480 nm to represent the FRET efficiency of CRCit. The change in the FRET efficiency observed in the present study was completely reversible (Figure 1D, lower panel) and a similar change in FRET efficiency was observed when we challenged the cells with agonists such as noradrenaline, angiotensin II and vasopressin (results not shown). These results are consistent with the notion that RMLC phosphorylation induces a conformational change of CRCit, which in turn changes the FRET efficiency. Furthermore, we could observe the spatial distribution of FRET response within a cell, although there was an almost homogeneous increase in the FRET signal for high-K⁺ stimulation (Figure 1E).

Correlation between RMLC phosphorylation and change in FRET efficiency of CRCit

We further examined the correlation between the change in FRET efficiency of CRCit and RMLC phosphorylation. Since high-K⁺-induced RMLC phosphorylation requires the influx of Ca²⁺ through the voltage-gated Ca²⁺ channel, the removal of external Ca²⁺ is expected to inhibit the high-K⁺-mediated RMLC phos-

phorylation. Indeed, 80 mM K⁺-induced CRCit signal was not observed in the absence of external Ca²⁺ (Figures 2B and 2E), although the high-K⁺-induced FRET change was repetitively observed in the presence of external Ca²⁺ (Figures 2A and 2E).

To exclude the possibility that CRCit responded directly to an increase in [Ca²⁺]_i, we examined the effects of MLCK inhibitors on the high-K⁺-induced changes in FRET efficiency of CRCit. Both ML-7 and wortmannin are MLCK inhibitors and act on the catalytic domain of MLCK [23,24]. It was also reported that wortmannin inhibits RMLC phosphorylation without inhibiting the increase in [Ca²⁺]_i during high-K⁺-induced depolarization in smooth-muscle cells [25]. Both ML-7 and wortmannin significantly attenuated the high-K⁺-induced change in the FRET efficiency of CRCit (Figures 2C–2E). These results indicate that the change in the FRET efficiency of CRCit accurately detects RMLC phosphorylation mediated by MLCK.

CRCit with mutations at phosphorylation sites

It was reported that smooth-muscle RMLC has two phosphorylation sites, Thr¹⁸ and Ser¹⁹ [26]. MLCK predominantly phosphorylates Ser¹⁹ and the phosphorylation at this site results in an

Figure 2 Effects of the MLCK inhibitors and withdrawal of external Ca^{2+} on the high- K^{+} -induced change in FRET efficiency of CRCit

(A) Time course of FRET response of CRCit during repeated applications of high- K^{+} solution. The high- K^{+} -induced response was observed reversibly and repetitively more than five times. Ratios of F_{535}/F_{480} were normalized by the value before the high- K^{+} application. The sampling interval was 5 s. (B) The application of high- K^{+} solution in the absence of external Ca^{2+} resulted in no significant increase in FRET signal. (C, D) Inhibition of high- K^{+} -induced FRET response by 10 μM ML-7 or by 10 μM wortmannin (WM). (E) Composite results of experiments shown in (A–D). White bars indicate the FRET response to the first stimulation, and black bars indicate those to the second stimulation (for B) or the third stimulation (for A, C and D). Means $\pm$ S.E.M., $n = 14, 6, 5$ and 7 for control, Ca^{2+} -free, ML-7 and WM respectively. $^{**}P < 0.01$ (t test).

increase in the activity of actin-activated myosin ATPase [26]. On the other hand, Thr¹⁸ is phosphorylated only slowly by MLCK or by other protein kinases [27]. To examine whether phosphorylation at these residues is responsible for the change in the FRET efficiency of CRCit, we generated three types of mutant CRCit in which amino acids at the phosphorylation sites were substituted

Figure 3 High- K^{+} -induced change in FRET signal of CRCit with amino acid substitution at the phosphorylation site

(A) Time course of FRET efficiency of wild-type (WT) and mutant CRCit when $[\text{Ca}^{2+}]_i$ was increased by application of 80 mM K^{+} or 1 μM ionomycin. The sampling interval was 5 s. (B) Compiled results of effects of mutations on the Ca^{2+} -dependent change in the FRET efficiency. Means $\pm$ S.E.M., $n = 11, 8, 15$ and 15 for wild-type (WT), T18A, S19A and T18A/S19A respectively. $^{**}P < 0.01$ (t test).

by alanine residues. The substitution of Thr¹⁸ by an alanine residue (T18A) had hardly any effect on the FRET efficiency of CRCit during the application of high- K^{+} solution or ionomycin (Figure 3). On the other hand, substitution of Ser¹⁹ with an alanine (S19A) resulted in a partial inhibition of the CRCit response

during high- K^+ stimulation, but had no major effect on FRET efficiency during intense stimulation by ionomycin (Figure 3). Furthermore, the substitution of both Ser¹⁹ and Thr¹⁸ with alanine residues (T18A/S19A) completely abolished the FRET response of CRCit not only to high- K^+ stimulation but to ionomycin treatment (Figure 3). Taken together, these results indicate that the Ca^{2+} -induced increase in the FRET signal of CRCit reflects the MLCK-mediated phosphorylation of CRCit at Ser¹⁹ or at both Ser¹⁹ and Thr¹⁸ of the RMLC domain.

FRET of CRCit on Rho-kinase activation

Finally, we examined whether CRCit also indicates Ca^{2+} -independent RMLC phosphorylation induced by Rho-kinase activation. U46619, a mimetic of thromboxane A₂, activates Rho-kinase in smooth-muscle cells [28]. Thus we examined the effect of U46619 under conditions where that the $[Ca^{2+}]_i$ increase was inhibited. Before agonist application, vascular smooth-muscle cells were pretreated with 1 μ M thapsigargin in the absence of extracellular Ca^{2+} to deplete the intracellular Ca^{2+} store. U46619 was then applied to the cells in the presence of Ca^{2+} -free external solution containing 0.1 mM EGTA to block any influx of Ca^{2+} . The absence of a Ca^{2+} response during U46619 application was confirmed by the measurement of $[Ca^{2+}]_i$ using a fluorescent Ca^{2+} indicator, fura 2. The ratio of fluorescence intensity of fura 2 at 340 nm to that at 380 nm was hardly affected by the application of U46619, although it significantly increased after the application of 2 mM Ca^{2+} solution ($\Delta F_{340}/F_{380} = -0.01 \pm 0.001$ and 0.26 ± 0.04 respectively, $n = 12$). On the other hand, the FRET efficiency of CRCit significantly increased after the application of 30 nM U46619 (Figure 4A, black trace). Notably, this reaction was abolished in the presence of Y-27632, an inhibitor of Rho-kinase [12] (Figures 4A, dotted trace, and 4B). Furthermore, T18A/S19A-mutant CRCit, in which phosphorylation sites of Rho-kinase were substituted by alanine residues, showed hardly any FRET response (Figures 4A and 4B). Both T18A- and S19A-mutant CRCits showed an increase in the FRET response during the application of 30 nM U46619 (results not shown). These results suggest that CRCit also indicates Rho-kinase-dependent RMLC phosphorylation at Thr¹⁸ and Ser¹⁹.

CRCit-based assay systems

We generated a new indicator for real-time monitoring of RMLC phosphorylation with a high spatiotemporal resolution. The analysis can be performed in living cells, since CRCit is genetically coded and various methods can be used to introduce the indicator into cells and tissues. This CRCit-based assay system may be useful for understanding cell biological issues, namely relationship between RMLC phosphorylation and Ca^{2+} dynamics such as Ca^{2+} oscillation or the difference and significance of spatio-temporal patterns of RMLC phosphorylation induced by various agonists. CRCit may also be useful for clarifying the physiological significance of Ca^{2+} -independent RMLC phosphorylation induced by protein kinases other than Rho-kinase.

In addition, CRCit provides an extremely rapid assay system for RMLC phosphorylation. Indeed, the action of Rho-kinase inhibitor Y-27632 on RMLC phosphorylation was demonstrated within only a few minutes (Figure 4). This suggests that a high throughput screening system may be generated to identify compounds that inhibit Ca^{2+} -independent RMLC phosphorylation. Mutants of CRCit (T18A and S19A) may also be useful for screening new chemicals that preferentially block the phosphorylation of RMLC at Thr¹⁸, which is not the main Ca^{2+} -dependent phosphorylation site but is considered to be involved in abnormal contractility

Figure 4 Rho-kinase-dependent change in FRET signal of CRCit

(A) Time course of FRET efficiency of wild-type CRCit after application of U46619 in the absence (black continuous line) and presence (broken line) of Y-27632, a Rho-kinase inhibitor. Time course of FRET efficiency of mutant CRCit (T18A/S19A) is also shown (grey line). The intracellular Ca^{2+} store was depleted by treatment with thapsigargin before the application of the thromboxane A₂ analogue in the absence of extracellular Ca^{2+} . The sampling interval was 5 s. (B) Composite results of effects of Y-27632 and T18A/S19A mutations on the U46619-mediated change in FRET efficiency of CRCit. Means $\pm$ S.E.M., $n = 22$ and 11 for wild-type (WT) CRCit in the absence and presence of Y-27632 respectively and $n = 24$ for T18A/S19A. ** $P < 0.01$ (t test).

[18]. Such compounds may provide a new therapeutic basis for the treatment of abnormal contraction of smooth-muscle cells in pathophysiological states such as hypertension, asthma and urinary incontinence.

In conclusion, our study provided a novel tool for monitoring RMLC phosphorylation in living cells. CRCit may be used to study the dynamics of phosphorylation signals in living cells with high temporal and spatial resolutions. It may also become a powerful tool for the screening of new drugs, which may relax abnormal contractions of smooth-muscle cells.

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REFERENCES

- De Lozanne, A. and Spudich, J. A. (1987) Disruption of the Dictyostelium myosin heavy chain gene by homologous recombination. *Science* **236**, 1086–1091
- Horwitz, A. R. and Parsons, J. T. (1999) Cell migration—movin' on. *Science* **286**, 1102–1103
- Wylie, S. R. and Chantler, P. D. (2001) Separate but linked functions of conventional myosins modulate adhesion and neurite outgrowth. *Nat. Cell Biol.* **3**, 88–92
- Bridgman, P. C., Dave, S., Asnes, C. F., Tullio, A. N. and Adelstein, R. S. (2001) Myosin IIb is required for growth cone motility. *J. Neurosci.* **21**, 6159–6169

- 5 Somlyo, A. P. and Somlyo, A. V. (1994) Signal transduction and regulation in smooth muscle. *Nature (London)* **372**, 231–236
- 6 Ikebe, M. and Hartshorne, D. J. (1985) Phosphorylation of smooth muscle myosin at two distinct sites by myosin light chain kinase. *J. Biol. Chem.* **260**, 10027–10031
- 7 Ikebe, M., Hartshorne, D. J. and Elzinga, M. (1986) Identification, phosphorylation, and dephosphorylation of a second site for myosin light chain kinase on the 20,000-dalton light chain of smooth muscle myosin. *J. Biol. Chem.* **261**, 36–39
- 8 Somlyo, A. P. and Somlyo, A. V. (2003) Ca^{2+} sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. *Physiol. Rev.* **83**, 1325–1358
- 9 Somlyo, A. P. and Somlyo, A. V. (2000) Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J. Physiol. (Cambridge, U.K.)* **522**, 177–185
- 10 Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K. et al. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). *Science* **273**, 245–248
- 11 Kureishi, Y., Kobayashi, S., Amano, M., Kimura, K., Kanaide, H., Nakano, T., Kaibuchi, K. and Ito, M. (1997) Rho-associated kinase directly induces smooth muscle contraction through myosin light chain phosphorylation. *J. Biol. Chem.* **272**, 12257–12260
- 12 Uehata, M., Ishizaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M. et al. (1997) Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature (London)* **389**, 990–994
- 13 Seko, T., Ito, M., Kureishi, Y., Okamoto, R., Moriki, N., Onishi, K., Isaka, N., Hartshorne, D. J. and Nakano, T. (2003) Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. *Circ. Res.* **92**, 411–418
- 14 Kitazawa, T., Eto, M., Woodsome, T. P. and Brautigan, D. L. (2000) Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. *J. Biol. Chem.* **275**, 9897–9900
- 15 Gong, M. C., Fuglsang, A., Alessi, D., Kobayashi, S., Cohen, P., Somlyo, A. V. and Somlyo, A. P. (1992) Arachidonic acid inhibits myosin light chain phosphatase and sensitizes smooth muscle to calcium. *J. Biol. Chem.* **267**, 21492–21498
- 16 Deng, J. T., Van Lierop, J. E., Sutherland, C. and Walsh, M. P. (2001) Ca^{2+} -independent smooth muscle contraction. A novel function for integrin-linked kinase. *J. Biol. Chem.* **276**, 16365–16373
- 17 Niiro, N. and Ikebe, M. (2001) Zipper-interacting protein kinase induces Ca^{2+} -free smooth muscle contraction via myosin light chain phosphorylation. *J. Biol. Chem.* **276**, 29567–29574
- 18 Katsumata, N., Shimokawa, H., Seto, M., Kozai, T., Yamawaki, T., Kuwata, K., Egashira, K., Ikegaki, I., Asano, T., Sasaki, Y. et al. (1997) Enhanced myosin light chain phosphorylations as a central mechanism for coronary artery spasm in a swine model with interleukin-1 β . *Circulation* **96**, 4357–4363
- 19 Shimokawa, H., Seto, M., Katsumata, N., Amano, M., Kozai, T., Yamawaki, T., Kuwata, K., Kandabashi, T., Egashira, K., Ikegaki, I. et al. (1999) Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm. *Cardiovasc. Res.* **43**, 1029–1039
- 20 Miyawaki, A. (2003) Visualization of the spatial and temporal dynamics of intracellular signaling. *Developmental Cell* **4**, 295–305
- 21 Örmö, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y. and Remington, S. J. (1996) Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* **273**, 1392–1395
- 22 Heikal, A. A., Hess, S. T., Baird, G. S., Tsien, R. Y. and Webb, W. W. (2000) Molecular spectroscopy and dynamics of intrinsically fluorescent proteins: coral red (dsRed) and yellow (Citrine). *Proc. Natl. Acad. Sci. U.S.A.* **97**, 11996–12001
- 23 Nakanishi, S., Kakita, S., Takahashi, I., Kawahara, K., Tsukuda, E., Sano, T., Yamada, K., Yoshida, M., Kase, H., Matsuda, Y. et al. (1992) Wortmannin, a microbial product inhibitor of myosin light chain kinase. *J. Biol. Chem.* **267**, 2157–2163
- 24 Saitoh, M., Ishikawa, T., Matsushima, S., Naka, M. and Hidaka, H. (1987) Selective inhibition of catalytic activity of smooth muscle myosin light chain kinase. *J. Biol. Chem.* **262**, 7796–7801
- 25 Longbottom, E. R., Luckas, M. J., Kupittayanant, S., Badrick, E., Shmigol, T. and Wray, S. (2000) The effects of inhibiting myosin light chain kinase on contraction and calcium signalling in human and rat myometrium. *Pflugers Arch.* **440**, 315–321
- 26 Ikebe, M., Koretz, J. and Hartshorne, D. J. (1988) Effects of phosphorylation of light chain residues threonine 18 and serine 19 on the properties and conformation of smooth muscle myosin. *J. Biol. Chem.* **263**, 6432–6437
- 27 Sakurada, K., Seto, M. and Sasaki, Y. (1998) Dynamics of myosin light chain phosphorylation at Ser19 and Thr18/Ser19 in smooth muscle cells in culture. *Am. J. Physiol.* **274**, C1563–C1572
- 28 Fu, X., Gong, M. C., Jia, T., Somlyo, A. V. and Somlyo, A. P. (1998) The effects of the Rho-kinase inhibitor Y-27632 on arachidonic acid-, $\text{GTP}\gamma\text{S}$ -, and phorbol ester-induced Ca^{2+} -sensitization of smooth muscle. *FEBS Lett.* **440**, 183–187

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