Involvement of calmodulin and myosin light chain kinase in activation of mTRPC5 expressed in HEK cells

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MATERIALS AND METHODS

Cell culture and transient transfection. Human embryonic kidney (HEK-293) cells (ATCC, Manassas, VA) were maintained according to the supplier’s recommendations. For transient transfection, cells were seeded in 12-well plates. The following day, 1.5–2 μg/well of pcDNA vector containing the cDNA for TRPC5 or pTracer-cytomegalovirus vector containing the cDNA for CaM1234 mutant were mixed with 50–100 ng/well of pEGFP-C1 (Clontech), and transfected into the cells using the transfection reagent FuGENE 6 (Roche Molecular Biochemicals) according to the manufacturer’s protocol.

Whole cell patch-clamp experiment. Isolated cells were transfected to a small chamber on the stage of an inverted microscope (model TE2000, Nikon) and were constantly perfused with physiological salt solution (PSS) at a rate of 2–3 ml/min. A glass microelectrode with a resistance of 2–5 MΩ was used to make a gigahm seal. The conventional whole cell patch-clamp technique was adopted to hold the membrane potential at −60 mV using an Axopatch 1-D patch-clamp amplifier (Axon Instruments). For data acquisition and the application of command pulses, pCLAMP software version 6.0 (Axon Instruments) was used. Data were filtered at 5 kHz and displayed on a digital oscilloscope (PM 3350, Philips), a pen recorder (model 220, Gould), and a computer monitor. Data were analyzed with the use of pCLAMP and Microcal Origin software, version 6.0.

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RNA preparation and RT-PCR. Total RNA was extracted using RNeasy Mini Kit (Qiagen) and reverse transcription of total RNA was performed using a random hexamer primer and Superscript II-RT (Life Technologies), following the manufacturer’s instruction. PCR primers are shown as follows: the first PCR amplification with upstream primers (MLCK1-OF, 5′-GGCAATGCCAAGCGGTGA-3′ for endothelial cell MLCK; MLCK2-OF, 5′-GACCAGACT-GCCTTCAGCAT-3′ for smooth muscle MLCK; MLCK3-OF, 5′-CGGTGTGGCCAGGTCCAG-3′ for cardiac muscle MLCK; MLCK4-OF, 5′-GTCTTGGATGTAATCAGG-3′ for skeletal muscle MLCK), and downstream primer (MLCK1-OR, 5′-CAGGTGGCTTCTCCAAGACT-3′), MLCK2-OR, 5′-TCCAAATGAGGTCGTGTT-3′; MLCK3-OR, 5′-CATGTAAGTGAGTGACTCCCA-3′; MLCK4-OR, 5′-CAGGATGGTGAGCT-3′) were performed for 40 cycles under the following conditions: denaturing at 94°C for 2 min; annealing at 60°C for 30 s; and polymerization at 72°C for 1 min. Nested PCR amplifications with primers (MLCK1-IF, 5′-GGAGGCCAAGAACTCTCCA-3′; MLCK2-IF, 5′-CAGGTGGCTTCTCCAAGACT-3′; MLCK3-IF, 5′-TGCTCTTGCCAGAATCCTCCA-3′; MLCK4-IF, 5′-AAGTCTTTGGCCTCGTCTGA-3′; MLCK1-IR, 5′-TTC-GTCTGTGGGGAATGAGACA-3′; MLCK2-IR, 5′-CAGGATGGTGAGCT-3′; MLCK3-IR, 5′-GTCTTGGATGTAATCAGG-3′; MLCK4-IR, 5′-CAGGATGGTGAGCT-3′) were performed for 20 cycles under the following conditions: denaturing at 94°C for 2 min; annealing at 60°C for 30 s; and polymerization at 72°C for 1 min. The PCR product, predicted as 699 bp (endothelial cell MLCK, U48959), 500 bp (smooth muscle MLCK, AB037663), 490 bp (cardiac muscle MLCK, NM_182493), and 450 bp (skeletal muscle MLCK, NM_033118) in size, were separated on 1.0% agarose gel by electrophoresis. The identification of the PCR product was confirmed by DNA sequencing.

RNA interference. One day before transfection, 3 × 10⁵ HEK-293 cells were plated in 2 ml of growth medium per well without antibiotics. Cells were 90% confluent at the time of transfection. For each transfection sample, Stealth RNAi-Lipofectamine 2000 complexes were prepared by following the manufacturer’s directions (Invitrogen). The 510 nl of Stealth RNAi-Lipofectamine 2000 complexes were added to each well containing cells and medium. The complexes were prepared by following the manufacturer’s directions (Invitrogen). The 510 nl of Stealth RNAi-Lipofectamine 2000 complexes were added to each well containing cells and medium. The cells were incubated at 37°C in a humidified CO₂ incubator.

Solutions and drugs. PSS contained (in mM) 135 NaCl, 5 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 glucose, and 10 HEPES, and pH was adjusted to 7.4 with the use of NaOH. Cs⁺-rich external solution was made by replacing NaCl and KCl with equimolar CsCl. CaCl₂ was simply omitted for Ca²⁺-free PSS. The pipette solution contained (in mM) 140 CsCl, 10 HEPES, 0.5 Tris-GTP, 0.5 EGTA, and 3 Mg-ATP, and its pH was adjusted to 7.3 with CsOH.

Calmidazolium (CMZ), W-7, GTPyS, CaM, CaMKII inhibitor [CAMK-IP(381-309)], ML-7, and protein kinase C inhibitory peptide [PKC-IP(19–36)]. Arg-Phe-Ala-Arg-Lys-Ala-Leu-Arg-Glu-Lys-Asn-Val-His-Val-Arg-Asn were purchased from Calbiochem (La Jolla, CA), and CCh, HEPES, and Y27632 were from Sigma (St. Louis, MO).

Statistics. All data are expressed as means ± SE. Statistical significance was determined using the Student’s paired or unpaired t-tests. P values < 0.05 were considered statistically significant, and n refers to the number of cell recordings.
The subtraction to get the relationship (Fig. 1C), obtained by subtracting current in the absence of CCh (Fig. 1A.b) from that in the presence of CCh (Fig. 1A.c), showed a typical doubly rectifying shape (Fig. 1B). We used only results obtained from cells showing the typical I-V relationship of TRPC5. mTRPC5 currents were not activated by CCh in mock transfected cells (Fig. 1B). Among GFP-positive cells, ~60% responded to CCh and 90% to intracellular GTPγS. Responding cells showed mTRPC5 currents with amplitude of >400 pA and typical I-V relationship.

The TRPC5 current activated by stimulation of muscarinic receptors decayed spontaneously to the basal level even during the first application (Fig. 1A). This phenomenon is called desensitization. The degree of desensitization was variable among cells. When we applied 50 μM CCh 5 min after the first application, the current was activated much less than the first application (Fig. 2A). When we applied 50 μM CCh 5 min after the second application, the current was activated much less than the second application (Fig. 2A). During the second application, the inward component of CCh-induced current decreased more than the outward component. During the third application of CCh, both inward and outward currents were decreased.

Intracellularly applied CaM (150 μg/ml) increased the amplitude of mTRPC5 current activated by muscarinic stimulation (Fig. 3). The amplitudes were $-234.1 \pm 47.8$ pA/pF ($n = 7$) and $-94.5 \pm 16.3$ pA/pF ($n = 7$) at −100 mV with and without intracellularly applied CaM, respectively (Fig. 3C). CaM increased the inward component of CCh-induced current more than the outward component (Fig. 3B). However, CaM did not show any effect on the desensitization.

**RESULTS**

Effect of CaM on TRPC5 expressed in HEK cells. Whole cell currents were recorded using patch-clamp techniques. In the beginning, whole cell currents were recorded under the condition of normal Tyrode solution (140 mM [Na$^+$]o) and [Cs$^+$]. To determine the current-voltage (I-V) relationship, we applied a ramp pulse from +100 mV to −100 mV for 1 s. After the external solution was exchanged from normal Tyrode to 140 mM [Cs$^+$]o solution, basal currents slightly increased due to a constitutive activity of TRPC5. Sometimes the currents activated up to 1 nA without any stimulation of TRPC5 with CCh. Thus we usually waited for at least 2 min before the application of CCh. Whole cell current was also recorded under the condition of 140 mM [Cs$^+$]o and [Cs$^+$], as a control current for the subtraction to get the I-V relationship of mTRPC5 activated by CCh. When 50 μM CCh was applied at a holding potential of −60 mV, an inward current was activated (Fig. 1A). The I-V relationship (Fig. 1C.c-b), obtained by subtracting current in the absence of CCh (Fig. 1A.b) from that in the presence of CCh (Fig. 1A.c), showed a typical doubly rectifying shape (Fig. 1B). We used only results obtained from cells showing the typical I-V relationship of TRPC5. mTRPC5 currents were not activated by CCh in mock transfected cells (Fig. 1B). Among GFP-positive cells, ~60% responded to CCh and 90% to intracellular GTPγS. Responding cells showed mTRPC5 currents with amplitude of >400 pA and typical I-V relationship.

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Activation of mTRPC5 current. Pretreatment of 5 μM PKC inhibitory peptide added to internal pipette solution. Slow ramp depolarizations from +100 to −100 mV were applied from a holding potential of −60 mV. I-V relationships showed a typical doubly rectifying shape. A: whole cell currents were recorded under the condition of 140 mM [Cs⁺]. To attenuate the desensitization, a PKC inhibitory peptide was added to internal pipette solution. Slow ramp depolarizations from +100 to −100 mV were applied from a holding potential of −60 mV. I-V relationships showed a typical doubly rectifying shape. B: different concentrations of W-7 were applied during the activation of CCh-induced current. W-7 inhibited mTRPC5 current dose dependently. C: I-V relationships showed a typical doubly rectifying shape.

Effect of MLCK on TRPC5 expressed in HEK cells. Inhibitors of MLCK inhibited the activation of mTRPC5 currents (Fig. 7). Pretreatment of 3 μM ML-7 inhibited the activation of mTRPC5 current by CCh (Fig. 7A). After washout of ML-7, CCh activated an inward current, which showed a typical doubly rectifying shape (Fig. 7B). Inclusion of 10 μM CaMKII inhibitor within the pipette, however, did not inhibit the activation of mTRPC5 current by CCh (Fig. 7C). In the presence of 10 μM CaMKII inhibitor within the pipette, CCh activated an inward current, which showed a typical doubly rectifying shape (Fig. 7C, right panel). The effect of ML-7 and 10 μM CaMKII inhibitor within the pipette is summarized in Fig. 8. The current density is shown in Fig. 6 (12.0 ± 1.7 s, n = 8) (Fig. 6C). The averaged mTRPC5 current density is shown in Fig. 6B. The current in control decreased from 65.9 ± 9.8 pA/pF (n = 7) to 3.7 ± 0.7 pA/pF (n = 4) and 3.5 ± 0.7 pA/pF (n = 5) in the presence of CMZ and W-7, respectively. When W-7 was applied intracellularly, CCh did not induce an inward current at a holding potential of −60 mV (n = 10; Fig. 6A). When dominant negative mutant of CaM (CaM1234 mutant) was coexpressed with mTRPC5, CCh did not activate an inward current at a holding potential of −60 mV (n = 12).

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MLCK mRNA was also knocked down by siRNA for MLCK inhibitory effect on the activation of mTRPC5 (Fig. 9). Only one GACTTCCATCGATTCTAT to investigate which MLCK is and one siRNA for CaMKII (NM_171825, 1318 GGGCCTGGAGGAAACACCTCCA for siH1; GGATCTCCATCCAATAATGACCAAA) and one siRNA for CaMKII (NM_171825, 1318 GGGCCTGGAGGAAACACCTCCA) to investigate which MLCK is responsible for the activation of mTRPC5 (Fig. 9). Only one siRNA for cardiac muscle type MLCK (NM_182483) had an inhibitory effect on the activation of mTRPC5 (Fig. 9B). MLCK mRNA was also knocked down by siRNA for MLCK (NM_182483) (Fig. 9D). Other types of MLCK mRNAs were not knocked down by siRNA for cardiac muscle type MLCK (NM_182483) (Fig. 9E). To confirm the involvement of siRNA for cardiac muscle type MLCK, we made three kinds of siRNA

for cardiac muscle type MLCK (NM_182483, CACAGTGGTGAGGAAACACCTCCA for siH1; GGATCTCCATCCAATAATGACCAAA for siH2; and AGAACCTCAGGACTG-GCTTG GAATT for siH3). Similar inhibitory effects were obtained (Fig. 10). SiRNAs did not inhibit GTPγS-induced current (siH1, 110 ± 20 pA/pF; siH2, 130 ± 20 pA/pF; siH3, 100 ± 20 pA/pF) but inhibited CCh-activated TRPC5 currents (siH1, 20 ± 5 pA/pF; siH2, 20 ± 5 pA/pF; siH3, CCh, 25 ± 5 pA/pF).

Intracellular EGTA concentration was increased from 0.5 mM to 2 or 5 mM. With 2 mM [EGTA], ML-7 could still inhibit mTRPC5 current. With 5 mM [EGTA], however, CCh rarely activated an inward mTRPC5 current. The current amplitudes were 42.2 ± 5.3 pA/pF (n = 7) and 10.3 ± 7.4 pA/pF (n = 7) for 2 mM [EGTA], and 5 mM [EGTA], respectively.

**Effect of CaM and MLCK on GTPγS-induced current of TRPC5 expressed in HEK cells.** Inhibitors of CaM or MLCK did not show any effect on GTPγS-induced currents (Fig. 11). Intracellular GTPγS (0.2 mM) induced an inward current at a holding potential of −60 mV. I-V relationships showed a typical doubly rectifying shape (Fig. 11A). mTRPC5 currents were not activated by intracellular GTPγS in mock transfected cells (Fig. 11B). Pretreatment of 5 μM CMZ (Fig. 11C) or 100 μM W-7 (Fig. 12A) did not inhibit the activation of mTRPC5 current by 0.2 mM GTPγS. I-V relationships showed a typical doubly rectifying shape (Fig. 11, A and C). The current in control was 100.7 ± 20.9 pA/pF (n = 5) (Fig. 12C). The current in the presence of W-7 (69.0 ± 10.7 pA/pF, n = 6) and CMZ (97.5 ± 16.6 pA/pF, n = 6) was similar to that in control.
Pretreatment of 50 µM ML-7 did not inhibit the activation of mTRPC5 current by 0.2 mM GTP S.

The effect of small interfering RNA (siRNA) for MLCK and CaMKII on mTRPC5 current using the whole cell patch-clamp technique. Whole cell currents were recorded under the condition of 140 mM [Cs⁺]. Slow ramp depolarizations from +100 to −100 mV were applied from a holding potential of −60 mV. CCh (50 µM) induced an inward current when siRNA for CaMKII was coexpressed with mTRPC5 (A). However, CCh did not activate mTRPC5 current when siRNA for MLCK (NM_182483) was coexpressed (B). C: I-V relationships showed a typical doubly rectifying shape. D: RT-PCR detected all 4 kinds of MLCK. E: endothelial cell MLCK (U48959); Sm, smooth muscle MLCK (AB037663); H, cardiac muscle MLCK (NM_182483); Sk, skeletal muscle MLCK (NM_033118). MLCK mRNA was knocked down by siRNA for cardiac muscle MLCK. M, marker; C, control; S, siRNA. E: a: MLCK mRNA was knocked down by only siRNA for cardiac muscle MLCK. b: summary of effect of RNAi on MLCK (n = 5). Intensity: (MLCKsiRNA/β-actinRNA)/(MLCK in HEK cells/β-actin in HEK cells). F: a: Knockdown of MLCK mRNA by 3 other siRNAs for cardiac muscle MLCK. Three kinds of RNAi (sh1, 2 and 3) were used. They suppressed endogenous MLCK mRNA. b: Summary of effect of sh1, 2 and 3 on MLCK (n = 5). Intensity: (MLCKsiRNA/β-actinRNA)/(MLCK in HEK cells/β-actin in HEK cells). * P < 0.05.

**DISCUSSION**

The following results obtained in this study suggest that CaM and MLCK are involved in the activation of mTRPC5 as well as NSCC in guinea pig antral myocytes or portal vein myocytes. First, CaM increased the current amplitude of mTRPC5. Second, inhibitors of CaM inhibited the activation of mTRPC5. Third, inhibitor of MLCK inhibited the activation of mTRPC5 but inhibitor of CaMKII did not. Fourth, RNAi for MLCK inhibited the activation of mTRPC5. Fifth, CaM inhibitors or MLCK inhibitors did not inhibit the activation of GTP S-induced current in TRPC5-expressing cells. Finally, application of both Rho kinase inhibitor and MLCK kinase inhibitor decreased GTP S-induced currents.

We previously showed that TRPC4/5 is a candidate for NSCC activated by muscarinic stimulation in gastric smooth muscle cells (9, 23). In guinea pig gastric antral myocytes, intracellularly applied CaM inhibited desensitization and rundown phenomenon of CCh-activated NSCC (7). MLCK was responsible for the action of CaM on CCh-activated NSCC, whereas CaMKII was not involved (8). Even in portal vein myocytes, MLCK is important for ß2-adrenergic receptor-activated NSCC (1). Our results give more evidence that mTRPC4/5 is
a molecular candidate for NSCC activated by muscarinic stimulation in gastric smooth muscle cells.

In TRPV1, the association of the COOH terminus with PIP2 inhibits the channel activity (4, 11) and its interaction with CaM promotes channel desensitization (10). Although PIP2 hydrolysis activates both TRPV1 and TRPC5, the desensitization process is different between them. In TRPC5, PKC phosphorylation is involved in desensitization (17), whereas CaM is involved in desensitization of TRPV1 (10). In this experiment, there was no effect of CaM on desensitization (Figs. 2 and 3).

Interestingly, constitutive activity of mTRPC5 could be clearly seen in the presence of internal CaM, and constitutive activity of mTRPC5 was not desensitized during the application of CCh (Fig. 3). The activation mechanism of constitutive activity of mTRPC5 seems different from the activation mechanism of mTRPC5 by CCh.

MLCK seems to act upstream of the activation of G proteins by muscarinic stimulation because inhibitors of MLCK did not inhibit GTPyS-activated currents in mTRPC5-expressing cells (Figs. 11 and 12). Because there was no predicted phosphorylation site in mTRPC5 channel itself or G protein α-subunits, mTRPC5 channel itself or G protein α-subunits seem not to be a target for MLCK. The response to muscarinic receptor stimulation is terminated by muscarinic receptor kinase (15, 16). Even in muscarinic receptor kinase, there was no predicted phosphorylation site by MLCK. Recently, Chou et al. (3) showed AVP-induced MLC phosphorylation was associated with a rearrangement of actin filaments in primary cultures of

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**Fig. 10.** Sequence-specific RNAi efficiently suppresses CCh-activated mTRPC5 currents in HEK cells. Three kinds of sequence-specific RNAi [siH 1 (A), 2 (B), and 3 (C)] were used. They did not inhibit GTPyS-activated currents but inhibited CCh-activated currents. (a) representative I-V relationships of GTPyS-activated TRPC5 currents; (b) representative I-V relationships of CCh-activated TRPC5 currents; (c) summary of the current density at −100 mV.
inner medullary collecting duct cells. They suggested that MLC phosphorylation by MLCK represents a downstream effect of AVP-activated Ca\(^{2+}\)/CaM signaling and points to a role of nonmuscle myosin II in regulation of water permeability by vasopressin. Like aquaporin insertion by AVP, mTRPC5 might be inserted into plasma membrane by muscarinic stimulation (see Ref. 2). MLCK phosphorylates MLC and modulates the insertion of vesicles containing mTRPC5. Thus G proteins may activate mTRPC5 independently of the activation of PLC/IP3/Ca/CaM/MLCK pathway because actomyosin-based cytoskeleton action depends on not only the MLCK-mediated pathway but also the Rho kinase-mediated pathway (21). Consequently, GTP\(\gamma\)S-induced currents in mTRPC5-expressing cells were inhibited by simultaneous application of MLCK inhibitor and Rho kinase inhibitor (Fig. 13). These results suggest that the activation of mTRPC5 depends on actomyosin-based cytoskeleton rearrangement as well as specific muscarinic receptor/G proteins/PLC pathway. Hypotonic cell swelling could increase nonselective cation current activated by muscarinic stimulation in the guinea pig (20).

The role of Ca\(^{2+}\) itself in the activation of TRPC channels is not certain yet. We could not record TRPC5 current under the condition of 10 mM [EGTA] or [BAPTA]. This result suggests that transient Ca\(^{2+}\) release from the Ca\(^{2+}\) stores is very important for the activation of mTRPC5 current. However, there is some controversy as to whether only intracellular Ca\(^{2+}\) can activate mTRPC5. We could record current once under the condition of pCa 6 without stimulation of CCh (n = 6). The time course, however, is slower and the current amplitude is smaller compared with normal stimulation by acetylcholine or CCh. Schaefer et al. (12) also could record mTRPC4 and mTRPC5 currents when they used 1 or 10 \(\mu\)M Ca\(^{2+}\) internal solution. Infusion of solutions with 1 or 10 \(\mu\)M Ca\(^{2+}\) produced a small, slow, transient stimulation of mTRPC4/5 immediately after break-in in many cells (12). In cells that later responded to CCh, the responses to CCh were larger than those responses to Ca\(^{2+}\). The activation of mTRPC5 with CCh seems to need a concerted action of PLC, Ca \(^{2+}\), IP3, and diacylglycerol, among others, on muscarinic receptor stimulation.

In conclusion, mTRPC5 is activated by PLC/IP3/Ca\(^{2+}\)/CaM/MLCK pathway and actomyosin-based cytoskeleton action through the Rho kinase-mediated pathway.

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whole cell currents were recorded under the condition of 140 mM [Cs\(^+\)/H\(_2\)O] washout of both ML-7 and Y27632. Slow ramp depolarizations from 
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Fig. 13. The effect of Rho kinase inhibitor on CCh- or GTP\(_\gamma\)S-activated inward current using the whole cell patch-clamp technique. A: whole cell currents were recorded under the condition of 140 mM [Cs\(^+\)]. CCh (50 \(\mu\)M) induced an inward current in the presence of Y27632. Slow ramp depolarizations from +100 to −100 mV were applied from a holding potential of −60 mV. I-V relationships showed a typical doubly rectifying shape (right). B: whole cell currents were recorded under the condition of 140 mM [Cs\(^+\)], and internal 0.2 mM GTP\(_\gamma\)S. When external solution was changed to 140 mM [Cs\(^+\)], solution, an inward current was slightly activated in the presence of both ML-7 and Y27632. However, CCh activated a larger current after washout of both ML-7 and Y27632. Slow ramp depolarizations from +100 to −100 mV were applied from a holding potential of −60 mV. C: I-V relationships showed a typical doubly rectifying shape.

Fig. 14. The effect of Rho kinase inhibitor on GTP\(_\gamma\)S-activated inward current. Amplitudes of inward currents at −100 mV are summarized. **\(P<0.01\).

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