TRPC5 as a candidate for the nonselective cation channel activated by muscarinic stimulation in murine stomach

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TRPC5 as a candidate for the nonselective cation channel activated by muscarinic stimulation in murine stomach. Am J Physiol Gastrointest Liver Physiol 284: G604–G616, 2003; 10.1152/ajpgi.00069.2002.—We investigated which transient receptor potential (TRP) channel is responsible for the nonselective cation channel (NSCC) activated by carbachol (CCh) in murine stomach with RT-PCR and the electrophysiological method. All seven types of TRP mRNA were detected in murine stomach with RT-PCR. When each TRP channel was expressed, the current-voltage relationship of mTRP5 was most similar to that recorded in murine gastric myocytes. mTRP5 showed a conductance or-

dependent activation indicated a half-maximal activation potential (V1/2) of −50 mV and steepness factor (k) of −15 mV (14, 18, 42). However, the V1/2 value depended on the concentrations of agonists and the type of extracellular cations used for current recordings (19). Second, the channel has the similar permeability to Na+, K+, Cs+, and Li+(15, 23, 45). It is also permeable to Ca2+(21). Third, its activation depends on G protein activity (13, 26). We have also showed that Gα type among GTP-binding proteins is responsible for activating the channel (22). Fourth, the unitary conductance was −25–30 pS (3, 13, 18, 42). The open probability is modulated by extracellular monovalent cation (18). Fifth, it is regulated by intracellular Ca2+ concentration ([Ca2+]i) and calmodulin (22). There is also a desensitization phenomenon depending on [Ca2+]i and protein kinase C (1, 23).

In many tissues, mammalian homologs of the Drosophila transient receptor potential (TRP) channel family (TRPC1–7) have been implicated as molecular candidates for the receptor-operated Ca2+ entry channels (ROCC) and store-operated Ca2+ channels (SOCC). ROCCs are activated by G protein-coupled receptor-PLC (GPCR-PLC) pathway and independently of store depletion by various messengers of the signal transduction. SOCCs are activated by depletion of intracellular Ca2+ stores (8, 32). In contrast to the abundance of reports on the TRP channels in heterologous expression systems, relatively little information is available on their role in native tissues. In most studies on the role of TRPCs in native tissues, these channels have been implicated as a component of SOCC (2, 5, 6, 30, 33, 38, 46). However, a role for TRPCs in store-independent ROCCs has also been proposed (16, 17, 28). It is suggested that second messengers such as G proteins, inositol 1,4,5-trisphosphate (IP3), diacylglycerol (DAG), arachidonic acid, and Ca2+ directly activate TRP channels.

Although smooth muscle cells are known to express voltage-activated Ca2+ channels, the NSCCs stimulated by GPCR (α1-adrenoceptor in vascular smooth muscle or muscarinic receptor in visceral smooth muscle)-PLC activation form an additional important role in control of smooth muscle tone.

IT IS WELL KNOWN THAT ACh and carbachol (CCh) induce depolarization and, consequently, cause the contraction of mammalian gastrointestinal smooth muscle. In mammalian gastrointestinal smooth muscle cells, muscarinic agonists bind to M2 and M3 muscarinic receptors (27, 39) and then activate a nonselective, voltage-sensitive inward current (3). On the other hand, muscarinic agonists inhibit the outward current in the toad stomach (42). Since the first report by Benham et al. (3), the characteristics of nonselective cation channel (NSCC) activated by ACh (NSCCACh) or CCh (NSCCCCh) have been reported. First, the channel is voltage dependent. Initial reports of the voltage-dependent activation indicated a half-maximal activation potential (V1/2) of −50 mV and steepness factor (k) of −15 mV (14, 18, 42). However, the V1/2 value depended on the concentrations of agonists and the type of extracellular cations used for current recordings (19). Second, the channel has the similar permeability to Na+, K+, Cs+, and Li+(15, 23, 45). It is also permeable to Ca2+(21). Third, its activation depends on G protein activity (13, 26). We have also showed that Gα type among GTP-binding proteins is responsible for activating the channel (22). Fourth, the unitary conductance was −25–30 pS (3, 13, 18, 42). The open probability is modulated by extracellular monovalent cation (18). Fifth, it is regulated by intracellular Ca2+ concentration ([Ca2+]i) and calmodulin (22). There is also a desensitization phenomenon depending on [Ca2+]i and protein kinase C (1, 23).

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Ca\(^{2+}\)-entry pathway, i.e., ROCCs in smooth muscle cells. The TRP6 was shown to be the molecular identity for \(\alpha_1\)-adrenoceptor-activated NSCC permeable to Ca\(^{2+}\) (16). Likewise, TRPC6 was proposed to be a molecular component of ROCCs in A7r5 smooth muscle cells (17). For the NSCC activated by AC, Schaefer et al. (40) suggested that mouse (m) TRP4 and 5 might be candidates. We tried to identify the molecular candidate for the NSCC activated by CCh. To pursue the goal, we planned to record the NSC current in the NSCC activated by CCh. To pursue the candidates. We tried to identify the molecular candidates.

METHODS AND MATERIALS

Molecular biological methods. Ion-channel genes were transiently expressed in Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells using the pFx-8 cationic lipid transfection reagent (Invitrogen) according to the manufacturer’s instructions. As a marker of transient transfection in CHO or HEK cells, plasmid DNA (pEGFP-N1) containing the cDNA for Green Fluorescent Protein (Clontech) was cotransfected with the TRP cDNAs.

Single-cell dissociation of mouse stomach. Gastric myocytes were isolated enzymatically from the antral region of the Institute for Cancer Research (ICR) mouse. Mice of either sex weighing 20–30 g were anaesthetized with carbon dioxide and killed by cervical dislocation. Small intestines, from 1 cm of the antral part of the stomach with those of mTRP5.

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Preparation of ICC cells and tissues. ICR mice (0–15 days old) of either sex were anesthetized with carbon dioxide and killed by cervical dislocation. Small intestines, from 1 cm of the antral part of the stomach with those of mTRP5.

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Fig. 1. Tissue distribution of transient receptor potential (TRP) channels. Detection of trp mRNA with RT-PCR in brain (A), stomach of mouse (B), interstitial cells of Cajal (ICC) (C), and digestion (D). Specificity of each primer was tested in the brain. Each primer for each trp mRNA detected all trp mRNA in the brain. D: confirmation of each PCR product with restriction enzyme.
bridge reference electrode was used, and corrections were made for liquid junction potentials. For application of drugs, the experimental chamber was superfused by gravity at a rate of \( \frac{H}{11011} {\text{2}} \text{–} \frac{H}{102} \text{3 ml/min}. \) All drugs were purchased from Sigma. Anti-Gq/11 (SA-232, Biomol) and Go (SA-130, Biomol) antibody were applied intracellularly with dilution (1:1,000). Experiments were carried out at room temperature (20–23°C). Averaged results throughout this paper are given as means ± SE. Student’s unpaired \( t \)-test was performed, and \( P \) values <0.05 were regarded as significant.

**RNA Preparation and RT-PCR.** Total RNAs were extracted from antral smooth muscle tissues (without mucosa layer) of murine stomach, ICC (1 \( \times \) \( 1 \) \( 10^6 \) cells), and brain using a SNAP Total RNA Isolation kit (Invitrogen, Carlsbad, CA) following the procedures of the manufacturer as previously described (41). First-strand cDNA was synthesized from the RNA preparations with a Superscript II RNase Transcriptase kit (GIBCO-BRL, Gaithersburg, MD); RNA (1 pg) was reverse transcribed by using random hexamers (50 \( \mu \)g/\( \mu \)l). To perform nested PCR, the following sets of primers were used: mtrp1 forward (nucleotides 1583–1600, 1601–1608) and reverse (nucleotides 2283–2300, 2301–2318, gene accession no. NM_011643); mtrp2 forward (nucleotides 2783–2800, 2801–2818) and reverse (nucleotides 3483–3500, 3501–3518, AF111107); mtrp3 forward (nucleotides 1030–1047, 1048–1065) and reverse (nucleotides 1749–1966, 1749–1966, AF190645); mtrp4 forward (nucleotides 1483–1500, 1501–1518) and reverse (nucleotides 2183–2200, 2201–2218, AF190646); mtrp5 forward (nucleotides 1749–1766, 1767–1784) and reverse (nucleotides 2449–2466, 2467–2484, AF060107); mtrp6 forward (nucleotides 603–620, 621–638) and reverse (nucleotides 1303–1320, 1321–1338, AF057748);
and mtrp7 forward (nucleotides 2065–2082, 2083–2100) and reverse (nucleotides 2765–2782, 2783–2800, NM_012035). Complementary DNA (20% of the first-strand reaction) was combined with first sense and antisense primers (20/μH9262M), 1 mM deoxynucleotide triphosphates, 60 mM Tris-HCl (pH 8.5), 15 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 2.5 U of Taq (Bioneer), and RNase-free water to a final volume of 50/μH9262l. The reaction occurred in a PerkinElmer thermal cycler under the following conditions: an initial denaturation at 94°C for 4 min, followed by 40 cycles at 94°C for 30 s, 42°C for 30 s, 72°C for 1 min, with a final extension step at 72°C for 7 min. Five microliters of the first-round PCR product were then added to a new reaction mixture containing all of the components listed above except for second sense and antisense primers (20 μM), and 40 additional cycles of PCR were then performed. PCR products were separated by 2% agarose gel electrophoresis. The sets of primers for mtrp1, mtrp2, mtrp3, mtrp4, mtrp5, mtrp6, and mtrp7 were predicted to yield 700-, 700-, 700-, 718-, 700-, 700-, and 700-bp products, respectively. Two sets of negative control experiments were performed by including primers without cDNA or by including primers with RNA that had not been reverse transcribed (no RT added). To confirm murine TRP channels, PCR products of mtrp1–8 were digested with restriction enzymes. PCR product of mtrp1 was digested into 395 and 305 bp by EcoRI. PCR product of mtrp2 was digested into 378 and 322 bp by MluI. PCR product of mtrp3 was digested into 391 and 319 bp by SmaI. PCR product of mtrp4 was digested into 400 and 318 bp by MluI. PCR product of mtrp5 was digested into 400 and 300 bp by BamHI. PCR product of mtrp6 was digested
into 400 and 300 bp by SmaI. And PCR product of mtrp7 was digested into 400 and 300 bp by EcoRI as expected from the nucleotide sequences of murine TRP channels. Primers were designed with the aid of the designer program Primer3 at http://www-genome.wi.mit.edu/cgi-bin/primer/primer3.cgi using the nucleotide sequences of murine TRP channels. Primers were checked for hairpin loops and palindromes using the corresponding murine mRNA sequences. The specificity of the primers for the target gene was checked against the databases using Fasta3 at http://www2.ebi.ac.uk/fasta3/, and primers were checked for hairpin loops and palindromes using the Cybergene utility at http://www.cybergene.se/primer.html. The oligonucleotides were synthesized by Bionics (Seoul, South Korea).

RESULTS

RT-PCR and expression of TRP channels. We used RT-PCR to see which types of TRP mRNA exist in the murine stomach (Fig. 1). To test whether each primer can detect each TRP mRNA, the murine brain was used. All seven types of TRP mRNA were detected in the brain (Fig. 1A). These primers were used to detect TRP mRNA in murine ICC and gastric myocytes. In the stomach, all TRP mRNAs were detected (Fig. 1B), whereas TRP mRNAs except mtrp5 were detected in ICC (Fig. 1C). To confirm the nucleotide sequences of the PCR products, we digested the PCR products with restriction enzymes based on the nucleotide sequence. We found that the PCR product of each TRP subtype was digested into two fragments of the expected size from the nucleotide sequences (Fig. 1D). Next, we expressed all types of TRP genes except trp2, because TRPC2 is a pseudogene in humans (47) and a potential pseudogene coding for an NH2-terminal truncated protein in the bovine system (48). First, we obtained the ratios to compare each type of TRP channel with a pipette containing intracellular 10 mM BAPTA or intracellular 0.2 mM GTPγS and 140 mM Cs⁺ under the condition of intracellular 140 mM Cs⁺ in control cells transfected with the empty vector (−115 ± 49 pA at −100 mV, mean ± SE, n = 6). We recorded a current from each TRP channel with a pipette containing intracellular 10 mM BAPTA or intracellular 0.2 mM GTPγS under the condition of intracellular and extracellular 140 mM Cs⁺ (Figs. 2 and 3). The currents in human (h) TRP1 (n = 6), hTRP3 (n = 5), mTRP4 (n = 8), mTRP5 (n = 5), mTRP6 (n = 3), and mTRP7 (n = 3) were recorded under the condition of intracellular 10 mM BAPTA and 140 mM Cs⁺ and extracellular 140 mM Cs⁺ by applying ramp pulses from 100 to −100 mV for 2 s from a holding potential of −60 mV (Fig. 2). In Fig. 2G, we obtained the ratios to compare current-voltage (I-V) curves quantitatively: the current (under the condition of intracellular 10 mM BAPTA and 140 mM Cs⁺ and extracellular 140 mM Cs⁺) at 100 mV to the current at 25 mV and the current at −100 mV to the current at 25 mV. When intracellular 0.2 mM GTPγS was used in hTRP1 (n = 3), hTRP3 (n = 3), mTRP4 (n = 5), mTRP5 (n = 3), mTRP6 (n = 4), and mTRP7 (n = 4)-expressing cells, I-V relationships were obtained, respectively (Fig. 3). In Fig. 3G, we obtained the ratios to compare I-V curves quantitatively: the current (under the condition of intracellular 0.2 mM GTPγS and 140 mM Cs⁺ and extracellular 140 mM Cs⁺) at 100 mV to the current at 25 mV and the current at −100 mV to the current at 25 mV.
Cs\(^{+}\)) at 100 mV to the current at 25 mV and the current at \(-100\) mV to the current at 25 mV. In mouse stomach and mTRP5, current ratios of 100/25 and \(-100/25\) mV are similar: 11.8 \(\pm\) 1.1 and \(-7.1 \pm 1.7\) in mouse and 10.8 \(\pm\) 0.8 and \(-9.8 \pm 2.6\) in mTRP5, respectively (\(P > 0.05\)). However, there are differences between the rectifying ratios (100/25 and \(-100/25\) mV) of other TRPs and those of murine stomach (\(P < 0.05\)). TRP6 was suggested as the molecular identity for \(\alpha_1\) adrenoceptor-activated NSCC (16). In our study, the I-V shape of TRP6 has unique voltage dependence. At 0–30 mV, there is a range in which little current flows in the outward direction, whereas at more positive potential (30 mV), a prominent outward rectification is seen. At negative potential (\(-40\) mV), there is a marked voltage-dependent inhibition (Fig. 3E) as shown by Inoue et al. (16) and Jung et al. (17). However, mTRP5 showed a slightly doubly rectifying appearance. In addition, the TRP6 maintained their activity when intracellular GTP\(\gamma\)S was used for recording the currents in our results (data was not shown) as well as other reports (16), whereas the NSCC activated by intracellular GTP\(\gamma\)S in the murine stomach did not [see Activation in murine stomach (see Fig. 11)]. Thus we focused on TRP5 and compared the electrophysiological properties with those recorded in the murine stomach.

**Electrophysiological properties of NSCC\(_{CCh}\) in murine stomach.** We isolated single cells from the murine stomach and used the same protocol as that in the guinea pig to record the NSCC\(_{ACh}\) (18–20, 22). CCh, similar substance to ACh, induced NSCC in isolated smooth muscle cells from the murine stomach (Fig. 4, A and B). The current trace and I-V relationship recorded in murine stomach were similar to those in mTRP5 expressed in HEK cells (Fig. 4, C and D). The currents displayed a reversal potential of 0 mV. In both, the I-V relationship showed slightly voltage-dependent inhibition at negative potential. This voltage-dependent inhibition was also observed for mTRP5 in other studies (35, 40). When the extracellular chloride ion was replaced with aspartate, the current was activated by CCh. The reversal potentials in the presence of aspartate ion (0.8 \(\pm\) 1.3 mV, \(n = 5\)) were not different from those in the presence of Cl\(^{-}\) (0.5 \(\pm\) 1.2 mV, \(n = 6\)).

The channel in murine stomach has similar permeability to monovalent cations such as those in mTRP5 (Fig. 5). Relative permeability was calculated using the biionic equation modified from the Goldman-Hodgkin-Katz equation.

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**Fig. 5.** The I-V relationships of nonselective cation channels (NSCC) activated by CCh in murine stomach and mTRP5 recorded under various ionic conditions. Ramp pulses were applied before and during treatment with CCh. Traces were recorded from different cells and in different ionic conditions, as shown above each I-V relationship. Differences in currents were obtained by digital subtraction and plotted against membrane potential. A-C: murine stomach. D-F: mTRP5.
Katz equation. The reversal potentials for Na\(^+\) and K\(^+\) in the murine stomach were \(-4.0 \pm 1.1\) and \(4.0 \pm 1.0\) mV (\(n = 4\)), respectively. Relative permeability of Cs\(^+\) to Na\(^+\) to K\(^+\) was 1:0.80:1.19. The relative permeability of Cs\(^+\) to Na\(^+\) to K\(^+\) in mTRP5 was 1:0.98:1.1. On the other hand, the order of conductance was Cs\(^+\) > K\(^+\) > Na\(^+\). The current amplitude at \(-60\) mV for Na\(^+\), K\(^+\), and Cs\(^+\) solution in murine stomach was 143 ± 23 (\(n = 3\)), 210 ± 38 (\(n = 3\)), and 700 ± 24 pA (\(n = 10\)), respectively. The current amplitude at \(-60\) mV for Na\(^+\), K\(^+\), and Cs\(^+\) solution in mTRP5 was 137 ± 101 (\(n = 4\)), 690 ± 122 (\(n = 4\)), and 1,400 ± 388 pA (\(n = 7\)), respectively. The relative ratio of the current amplitude (Cs\(^+\) > K\(^+\) > Na\(^+\)) and relative permeability ratio (K\(^+\) > Cs\(^+\) > Na\(^+\)) in murine stomach are similar to those of the NSCC\(_{CCb}\) in mTRP5.

Another property of NSCC\(_{CCb}\) is the dependence upon extracellular Ca\(^{2+}\) concentration (12, 18). The NSCC was also modulated by extracellular calcium in murine stomach (Fig. 6). After the activation of the currents, the external Ca\(^{2+}\) was changed to nominally free solution and then 10 mM Ca\(^{2+}\). The current under the Ca\(^{2+}\)-free condition decreased, and then the current under the 10 mM Ca\(^{2+}\) condition increased in both NSCC\(_{CCb}\) in the murine stomach and mTRP5. In our study, the mTRP5 was shown to be increased when [Ca\(^{2+}\)]\(_o\) was raised above physiological levels as in the other studies of mTRP4/5 (35, 40).

We have used flufenamate to see whether it increases or decreases the current, because Inoue et al. (16) recommended flufenamate could be used to distinguish TRP6 from other TRP channels. This compound has been shown to reversibly enhance the currents mediated by mTRP6, whereas the currents mediated by mTRP3 and mTRP7 were inhibited by the drug (16). Flufenamate (100 \(\mu\)M) inhibited NSCC\(_{CCb}\) by 87 ± 3% in the murine stomach (\(n = 5\)) and 92 ± 3% in mTRP5 (\(n = 5\); Fig. 7). To further confirm the similarity between mTRP5- and carbachol-activated nonselective cation channels in the murine stomach, we investigated the effects of a nonspecific but frequently used cation channel blocker La\(^{3+}\). Interestingly, in this study, La\(^{3+}\) also inhibited the NSCC\(_{CCb}\) in murine stomach. In Fig. 7E, the concentration-inhibition curves for La\(^{3+}\) block of NSCC\(_{CCb}\) in mTRP5 and murine stomach gave similar IC_{50} values (88 and 109 \(\mu\)M, \(n = 4–10\), respectively) and similar Hill coefficients (0.48 and 0.50, respectively).

**Activation mechanism in murine stomach.** The main discrepancy between TRP and NSCC in visceral smooth muscle is the G protein that activates channels. TRPCs are activated downstream of G protein-coupled receptors, which induce PLC-mediated phosphoinositide breakdown. In the TRPCs shown to be activated by store-independent pathways, Gq/11 is the mediator for the activation of channels. On the other hand, in visceral smooth muscle, pertussis toxin-sensitive G protein is responsible for the activation of the channels. When ACh binds to the muscarinic receptor, information is transferred to the channel protein through a pertussis toxin-sensitive GTP binding protein (13, 19). We performed experiments to see whether Gq/11 is involved in the activation of NSCC by muscarinic stimulation in the murine stomach. Anti-Gq/11 antibody blocked the activation of NSCC in the murine stomach (Fig. 8). Anti-Gq/11 antibody decreased the current amplitude from 500 ± 50 (\(n = 5\)) and 600 ± 40 (\(n = 8\)) to 5.0 ± 1.5 (\(n = 4\)) and 15 ± 1.3 pA (\(n = 4\)) in murine stomach and mTRP5-expressing cells, respectively. Anti-Gq/11 antibody did not inhibit the currents in murine stomach and mTRP5; the peak amplitude was 475 ± 140 (\(n = 4\)) and 575 ± 60 pA, respectively (\(n = 4\)). We have tested inactivated anti-Gq/11 antibody for inhibition of currents in murine stomach and mTRP5. Inactivated anti-Gq/11 antibody did not inhibit the currents (\(n = 5\)). PLC inhibitor (500 \(\mu\)M neomycin sulphate and 1 \(\mu\)M U-73122, \(n = 4\)) blocked the activation of NSCC in the murine stomach. U-73122 (1 \(\mu\)M) inhibited NSCC\(_{CCb}\) by 88 ± 2% in the murine stomach. The inhibitors of IP3-induced Ca\(^{2+}\) store-independent pathways, Gq/11 is the mediator for activation of channels. On the other hand, in visceral smooth muscle, pertussis toxin-sensitive G protein is responsible for the activation of the channels. When ACh binds to the muscarinic receptor, information is transferred to the channel protein through a pertussis toxin-sensitive GTP binding protein (13, 19). We performed experiments to see whether Gq/11 is involved in the activation of NSCC by muscarinic stimulation in the murine stomach. Anti-Gq/11 antibody blocked the activation of NSCC in the murine stomach (Fig. 8). Anti-Gq/11 antibody decreased the current amplitude from 500 ± 50 (\(n = 5\)) and 600 ± 40 (\(n = 8\)) to 5.0 ± 1.5 (\(n = 4\)) and 15 ± 1.3 pA (\(n = 4\)) in murine stomach and mTRP5-expressing cells, respectively. Anti-Gq/11 antibody did not inhibit the currents in murine stomach and mTRP5; the peak amplitude was 475 ± 140 (\(n = 4\)) and 575 ± 60 pA, respectively (\(n = 4\)). We have tested inactivated anti-Gq/11 antibody for inhibition of currents in murine stomach and mTRP5. Inactivated anti-Gq/11 antibody did not inhibit the currents (\(n = 5\)). PLC inhibitor (500 \(\mu\)M neomycin sulphate and 1 \(\mu\)M U-73122, \(n = 4\)) blocked the activation of NSCC in the murine stomach. U-73122 (1 \(\mu\)M) inhibited NSCC\(_{CCb}\) by 88 ± 2% in the murine stomach. The inhibitors of IP3-induced Ca\(^{2+}\) release [100 \(\mu\)M 2-aminoethoxydiphenylborate (2-APB) and 1 \(\mu\)M xestospongin C, \(n = 4\)] also blocked the activation of NSCC in the murine stomach. 2-APB (100 \(\mu\)M) inhibited NSCC\(_{CCb}\) by 87 ± 7% in the murine stomach.

![Fig. 6. The effect of external calcium on NSCC activated by CCh in murine stomach and mTRP5. As the external calcium increased, the current increased. The current was activated under the condition of 1.5 mM Ca\(^{2+}\). During the activation of the current, the external Ca\(^{2+}\) was changed first to nominally Ca\(^{2+}\)-free and then 10 mM Ca\(^{2+}\) and finally back to 1.5 mM Ca\(^{2+}\). After a sudden jump of extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_o\)) from 0 to 10 mM, the amplitude of both CCh current (I_CCh) in murine gastric myocytes (A) and mTRP5 (B) increased immediately. The potentiating action of [Ca\(^{2+}\)]\(_o\) on mTRP5 is essentially the same as that in the murine gastric myocytes.](image-url)
stomach. 2-APB (100 μM; n = 5) and U-73122 (1 μM; n = 5) also blocked NSCChCCh by 91 ± 5 and 92 ± 3% in mTRP5-expressing cells, respectively (Fig. 9). U-73343 (1 μM; inactive analog of U-73122), however, also inhibited the activation of NSCC in murine stomach and mTRP5 (n = 3). For DAG as activator, we used 1-oleoyl-2-acetyl-sn-glycerol (OAG). OAG itself activated NSCC a little in the murine stomach (40 ± 5 pA, mean ± SE, n = 3; Fig. 10). Similarly, OAG can activate a little inward current in mTRP5-expressing cells (147 ± 82 pA, n = 4; Fig. 10). OAG activated inward current in murine stomach and mTRP5-expressing cells that has similar I-V relationship to NSCChCCh. On the other hand, OAG did not activate inward currents in control cells transfected with the empty vector (n = 3). However, the current activation by DAG analogs was reported as a characteristic feature of the TRPC3/6/7 subfamily of TRP channels (10, 34), not the TRPC4/5 subfamily (40). In addition, when we coexpressed the muscarinic receptor (M3) and mTRP5 in CHO cells, which have endogenous P2Y purinoceptor but lack endogenous muscarinic receptors, CCh activated currents. When we expressed mTRP5 only or coexpressed mTRP5 and M2 muscarinic receptors in CHO cells, CCh did not activate currents, whereas ATP did (data not shown). It seems that in murine stomach, NSCC is activated by a similar mechanism in the murine portal vein, that is, the muscarinic receptor-Gq/11-PLC pathway.

NSCC in the murine stomach and mTRP5 was activated transiently by intracellular 0.2 mM GTPγS (Fig. 11). The activation was not maintained in the murine stomach and decayed to the level before the activation (desensitization). The facilitation induced by depolarizing the ramp pulse was observed. We tested whether the desensitization process depends on [Ca2+]i or not. The desensitization process was compared under the different concentrations of EGTA. The degree of desensitization was estimated by calculating the relative values of the current 5 min after peak to the current at peak (I_{5min}/I_{peak}). The values for 0.5, 2, 2.5, 3, and 3.5 mM EGTA were 12.0 ± 2.2 (n = 25), 10.0 ± 1.1 (n = 4), 11.0 ± 1.7 (n = 3), 12.0 ± 3.5 (n = 3), and 10.0 ± 3.0% (n = 3), respectively. The nonselective cation current was not activated under the condition of 5 mM EGTA. The desensitization process was not dependant on

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**Fig. 7.** The effect of La³⁺ and flufenamate on NSCC activated by CCh in the murine stomach and mTRP5. A and B: murine stomach. C and D: mTRP5. Cation blockers 1 mM La³⁺ and 100 μM flufenamate inhibited the currents. The effect was reversible. Sharp current deflections are ramp pulses from 100 to −100 mV for 2 s from a holding potential of −60 mV. E: the concentration-inhibition curves for mTRP5 and murine stomach by La³⁺ (n = 4–10). Curves are best nonlinear fits to the Hill equation: 1/(1+([La³⁺]/K_i)^n), where [La³⁺], K_i, and n denote the concentration of La³⁺ applied, dissociation constant, and Hill coefficient, respectively.
[Ca$$^{2+}$$], but PLC$$\beta$$1 seems to be involved in the desensitization process, because the desensitization remarkably slowed in PLC$$\beta$$1 knockout mice (data not shown; $$n$$ = 3).

One unique property of mTRP5 is the constitutive activity (40). We investigated whether NSCC in the murine stomach has such an activity (Fig. 12). In control HEK cells transfected with the empty vector, there was no constitutive activity in normal tyrode and 140 Cs$$^+$$ ($$n$$ = 4). The I-V relationship recorded under the condition of 140 mM Cs$$^+$$ in control HEK cells was not similar to that of NSCC$$\text{CCh}$$ and that activated by mTRP5. When we superfused external Cs$$^+$$ solution without the muscarinic stimulation, there was an increase in the conductance, suggesting that there is a constitutive activity in NSCC$$\text{CCh}$$ in the murine stomach.

**DISCUSSION**

In this study, we investigated the molecular identity of the NSCC activated by muscarinic stimulation (ACh or CCh) in the murine stomach. Similarity between mTRP5 and NSCC$$\text{CCh}$$ in murine gastric myocytes, i.e., unique voltage dependence, permeability ratio ($$K^+ > Cs^+ > Na^+$$) and conductance order ($$Cs^+ > K^+ > Na^+$$), dependence on [Ca$$^{2+}$$]o, block by flufenamic acid and La$$^{3+}$$, activation pathway (muscarinic-G$$\text{q/11}$$.PLC pathway), transient activation by intracellular GTP$$\gamma$$S, activation by OAG, and the constitutive activity, suggests that the mTRP5 protein is a molecular component of NSCC$$\text{CCh}$$ in murine gastric myocytes.

Our results support this conclusion. First, CCh-induced nonselective cation currents in murine gastric myocytes were found to display a characteristic doubly rectifying I-V relationship that resembles the I-V relationship for hTRPC3/6 (10, 24), mTRP5 (16) and mTRP6 (17). On the other hand, mTRP5 showed a slightly doubly rectifying I-V relationship with a peak value of 600 pA at 1 min. In control HEK cells transfected with the empty vector, there was no constitutive activity in normal tyrode and 140 Cs$$^+$$ ($$n$$ = 4). The I-V relationship recorded under the condition of 140 mM Cs$$^+$$ in control HEK cells was not similar to that of NSCC$$\text{CCh}$$ and that activated by mTRP5. When we superfused external Cs$$^+$$ solution without the muscarinic stimulation, there was an increase in the conductance, suggesting that there is a constitutive activity in NSCC$$\text{CCh}$$ in the murine stomach.
rectifying appearance (Figs. 2 and 3). Second, the channel in both the murine stomach and mTRP5-expressing cells has a similar permeability to monovalent cation (K⁺ > Cs⁺ > Na⁺) and conductance order (Cs⁺ > K⁺ > Na⁺; Fig. 4). Third, NSCC was modulated by extracellular calcium, as in murine stomach and mTRP5-expressing cells (Fig. 5). The mTRP6 exhibits a dual dependence on [Ca²⁺]o. The current is partially inhibited by [Ca²⁺]o in the physiological range, and the amplitude increases when [Ca²⁺]o is decreased. Nevertheless, the complete removal of external Ca²⁺ did not further potentiate the currents but rather led to a decrease in the amplitude of inward currents (16, 17).

A similar complex dependence of agonist-evoked cation currents on [Ca²⁺]o has been reported for norepinephrine-evoked currents in rabbit portal vein smooth muscle cells (9, 16). A potentiating effect of decreasing [Ca²⁺]o has been described for hTRPC1 (29, 43), hTRPC3 (29), and mTRPSC7 (34). In our study, however, the mTRP5 was shown to increase when [Ca²⁺]o was raised above physiological levels as in the other study of mTRPC4/5 (35, 40). Fourth, the pharmacological properties of the murine stomach are similar to mTRP5 in our study (Fig. 6). Flufenamate increased mTRPC6 currents but inhibited currents mediated by the TRPC3/7 subfamily (16). Flufenamate decreased NSCC_CCh in the murine stomach. La³⁺ also inhibited the NSCC_CCh in murine stomach. The IC₅₀ value of murine stomach is similar to that of mTRP5. Fifth, both mTRP5 and NSCC activated by CCh in the murine stomach were activated through the M3-Gq/11-PLC pathway (Fig. 7). TRPCs are activated downstream of G protein-coupled receptors, which induce PLC-mediated phosphoinositide breakdown. However, the downstream signaling pathways that finally activate TRPCs remain highly controversial. For nearly all of the functionally expressed TRPCs, there is at least one report proposing a store-operated mechanism of activation (4, 10).

Fig. 10. The activation of current by 1-oleoyl-2-acetyl-sn-glycerol (OAG) in murine stomach and mTRP5-expressing cells. The holding potential was −60 mV. A and B: bath-applied membrane-permeable analogs of diacylglycerol (DAG), OAG (20 μM)-activated NSCC in murine stomach, which have similar I-V relationships to NSCC activated by CCh, although the amplitude was very small compared with that of NSCC activated by CCh and mTRP5. B and C: OAG-activated inward current in mTRP5-expressing cells, which has a similar I-V relationship to NSCC activated by CCh.

Fig. 11. The transient activation of current by intracellularly applied GTPγS. The arrow indicates the rupture of the cell membrane. After the rupture, the current was activated as the 0.2 mM GTPγS diffused into the cell. The activation was not maintained in murine stomach but decayed to the control level (A). During the decay, the application of depolarizing pulses increased the current amplitude transiently, so-called facilitation. B: mTRP5.
TRP5 AND NSCC ACTIVATED BY ACh

Fig. 12. The basal activity of NSCC activated by CCh in murine stomach and mTRP5. A: murine stomach. B: mTRP5. Without the stimulation by CCh, the change of external monovalent cation from Na\(^+\) to Cs\(^+\) increased the current amplitude. The I-V relationship recorded under the condition of 140 mM Cs\(^+\) and normal Tyrode in murine stomach was similar to that of mTRP5-expressing cells. In addition, the I-V relationships recorded under the condition of 140 mM Cs\(^+\) were similar to those of NSCC activated by CCh and mTRP5. C: in control HEK cells transfected with the empty vector, the I-V relationship recorded under the condition of 140 mM Cs\(^+\) was not similar to that of NSCC activated by CCh (n = 4).

25, 31, 36, 37). On the other hand, there is growing evidence for the involvement of store-independent pathways in the regulation of TRPC3 (10, 11, 49), TRPC5 (35), TRPC6 (4, 10), and TRPC7 (34). In the TRPCs shown to be activated by store-independent pathways, G\(_{q/11}\) is the mediator for the activation of channels. In the murine stomach, the NSCC is activated by similar mechanism in the murine portal vein, that is, muscarinic receptor-G\(_{q/11}\)-PLC pathway. Finally, both mTRP5 and NSCC activated by CCh in the murine stomach were activated transiently by intracellular GTP\(_{Y}\)S (Fig. 10). The mTRP6 maintained their activity when intracellular GTP\(_{Y}\)S was used for recording the currents (16), whereas NSCC activated by intracellular GTP\(_{Y}\)S in the murine stomach did not.

When mTRP5 was expressed in HEK in our laboratory, two properties were different from a previous report by Schaefer et al. (40). First, OAG could activate the nonselective cation current in our experiment, although the current amplitude is small compared with that activated by CCh or GTP\(_{Y}\)S. Application of OAG stimulated the current independently of protein kinase C, a characteristic property of the TRPC3/6/7 subfamily (10, 34) not shared with the TRPC4/5 subfamily (40). Schaefer et al. (40) recorded the intracellular calcium change and found that OAG did not increase the [Ca\(^{2+}\)]\(_i\). From our results, we assume that the calcium influx through the activation by OAG is not enough to record the change in the [Ca\(^{2+}\)]\(_i\). Second, La\(^{3+}\) blocked the mTRP5 in our results, whereas Schaefer et al. (40) showed that La\(^{3+}\) increased the [Ca\(^{2+}\)]\(_i\) using the Mn\(^{2+}\) quenching experiment. One characteristic biophysical feature of I\(_{CRAC}\) is a specific block by low micromolar concentrations of La\(^{3+}\). The TRPC3/6/7 subfamily (7, 34) was also blocked by La\(^{3+}\). La\(^{3+}\) (100 \(\mu\)M) did not inhibit, but, similar to 10 mM Ca\(^{2+}\), even potentiated GTP\(_{Y}\)S-induced currents through mTRPC4/5 without changing the reversal potential. The TRPC4/5 subfamily was potentiated by La\(^{3+}\) in two studies (40, 44) but not in another (35). Okada et al. (35) showed that La\(^{3+}\) decreased the [Ca\(^{2+}\)]\(_i\) when mTRP5 was expressed. Inoue et al. (16) also showed that La\(^{3+}\) blocked the current by phenylephrine when mTRP6 was expressed in HEK cells.

In conclusion, we suggest that mTRP5 is a candidate for NSCCACh in the murine stomach.

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