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Functional Expression of TRPV4 Cation Channels in Human Mast Cell Line (HMC-1)

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Mast cells are activated by specific allergens and also by various nonspecific stimuli, which might induce physical urticaria. This study investigated the functional expression of temperature sensitive transient receptor potential vanilloid (TRPV) subfamily in the human mast cell line (HMC-1) using whole-cell patch clamp techniques. The temperature of perfusate was raised from room temperature (RT, 23~25°C) to a moderately high temperature (MHT, 37~39°C) to activate TRPV3/4, a high temperature (HT, 44~46°C) to activate TRPV1, or a very high temperature (VHT, 53~55°C) to activate TRPV2. The membrane conductance of HMC-1 was increased by MHT and HT in about 50% (21 of 40) of the tested cells, and the I/V curves showed weak outward rectification. VHT-induced current was 10-fold larger than those induced by MHT and HT. The application of the TRPV4 activator 4α-phorbol 12,13-didecanoate (4α PDD, 1μM) induced weakly outward rectifying currents similar to those induced by MHT. However, the TRPV3 agonist camphor or TRPV1 agonist capsaicin had no effect. RT-PCR analysis of HMC-1 demonstrated the expression of TRPV4 as well as potent expression of TRPV2. The $[Ca^{2+}]_c$ of HMC-1 cells was also increased by MHT or by 4α PDD. In summary, our present study indicates that HMC-1 cells express Ca^{2+}-permeable TRPV4 channels in addition to the previously reported expression of TRPV2 with a higher threshold of activating temperature.

Key Words: Mast cell, TRPV cation channels, TRPV4 protein, Temperature, Non-selective cation channel, Human

INTRODUCTION

Mammalian mast cells play important roles in the pathogenesis of allergy, asthma, pulmonary fibrosis, and rheumatoid arthritis. In addition, they also contribute to the inflammatory defense and homeostasis of various human tissues [1-3]. As is well-known, the activated mast cells release histamine and other mediators of immune responses, causing allergic inflammatory responses. Mast cells are activated not only by the specific allergens but also by various physical stimuli such as high temperature and mechanical stimuli [4]. The subsequent release of histamine, serotonin and other mediators induce skin rash and wheals, so-called physical urticaria [5-8].

Sustained Ca^{2+} influx and subsequent increase of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) is a critical intracellular signal for the activation of mast cells, especially for the release of pre-formed mediators and newly formed autacoids [9,10]. The transient receptor potential (TRP) family of non-selective cation channels and highly Ca^{2+}-selective CRAC (Ca^{2+}-release activated Ca^{2+}) channel have been implicated in the Ca^{2+} influx pathways of mast cells [11,12].

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of human mast cells from skin, lung, and cord blood commonly detects TRP vanilloid 2 (TRPV2) mRNA [13]. Among the six members of TRPV subfamily, four types (TRPV1-4) are known to be activated by various physicochemical stimuli. The permeability ratio of TRPV1-4 to Ca^{2+} over Na^+ (P_{Ca}/P_{Na}) is relatively moderate, ranging between 1 and 10 [14,15], whereas the other two types (TRPV5 and 6) are highly selective for Ca^{2+} ($P_{Ca}/P_{Na} > 100$) and are less affected by physical stimuli [16-18]. TRPV1-4 channels are commonly activated by the increase of temperature [19-21]. Different levels of temperature have been suggested as threshold stimuli for activating TRPV1 (> 43°C), TRPV2 (>53°C), TRPV3 (>31~35°C), and TRPV4 (>24~33°C) [22-25]. While the range of activating temper-

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ABBREVIATIONS: TRPV, transient receptor potential vanilloid family; HMC-1, human mast cell line-1; 4α PDD, 4α-phorbol 12,13-didecanoate; NSC, nonselective cation channel.

atures overlap for TRPV3 and TPRV4, they can be clearly distinguished by the use of repetitive temperature stimulation ('run-down'), which sensitizes TRPV3 and desensitizes TRPV4 [22,26-32].

TRPV2 is prominently expressed in mast cells, and the thermal activation of TRPV2 may induce serotonin secretion from RBL2H3 mast cells [19]. However, the level of heat (>50°C) required to activate TRPV2 is less likely to be experienced in the cellular environment. In this respect, the role of TRPV channels, which have a lower threshold for temperature, have not been reported in mast cells. The present study investigated the functional expression of TRPV channels in a human mast cell line (HMC-1) using whole-cell patch clamp technique and fura-2 spectrofluorimetry for the measurement of $[Ca^{2+}]_c$.

METHODS

Cell culture

HMC-1 cells were kindly donated by Professor Jae Seung Kang (Department of Anatomy, Seoul National University College of Medicine) and were cultured in IMDM media (GIBCO, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (GIBCO) and 1% penicillin/streptomycin (GIBCO) at 37°C in an atmosphere of 20% O_2/5% CO_2.

RT-PCR and electrophoresis

Total RNA was isolated from HMC-1 cells by use of TRizol (Invitrogen, Carlsbad, CA). Human mRNAs of TRPV1, TRPV2, TRPV3, TRPV4 and GAPDH were analyzed using an established RT-PCR method. Briefly, 1 μg of total RNA was reverse transcribed at 48°C for 20 min, and the produced cDNA was amplified over 30 PCR cycles (55~62°C for 1 min, 72°C for 1 min, and 95°C for 1 min). The nucleotide sequences of the primers used for amplification were TRPV1, 5'-GTGCCAGGCTGCTGTCCCAG-3' and 5'-CGCGCGATCTCCAGGAGCAG-3'; TRPV2, 5'-GAGAACCCACACCAGCCCGC-3' and 5'-GGCGGCCTGCTTCTTCAGGG-3'; TRPV3, 5'-AAGGGCAGACGGCGCTGAAC-3' and 5'-TGCGGGATGGCCTCCTCCTC-3'; TRPV4, 5'-TGGCCGCAACGACACCATCC-3' and 5'-AGCAATGGCCACCAGCGCAT-3'. The PCR products (5 μl) were electrophoresed on a 2% agarose gel at 100 V in a 1× Tris-acetate-EDTA buffer and visualized using ethidium bromide.

Electrophysiological measurements

Electrophysiological recordings were performed in the conventional whole-cell recording mode as well as in the perforated patch recording mode with nystatin (200 μg/ml; ICN Biomedicals, Aurora, OH). Membrane currents were measured using an Axopatch-200B patch clamp amplifier (Axon Instruments, Foster City, CA). pCLAMP software v.10.2 and Digidata-1322A (Axon Instruments) were used to acquire data and apply command pulses, respectively. Cells were transferred into a bath (approximately 0.1 ml) mounted on the stage of an inverted microscope (IX50, Olympus, Osaka, Japan) and perfused with HEPES buffered normal Tyrode (NT) solution at 5 ml/min. Patch pipettes with a free-tip resistance of about 2.5~3.5 MΩ were used.

Fluorimetry

$[Ca^{2+}]_c$ was measured using the fluorescent Ca^{2+} indicator Fura-2 acetoxymethyl ester (Fura-2 AM). HMC-1 cells were loaded with Fura-2 AM (2 μM, 20 min, 25°C) and washed twice with fresh NT solution. Fura-2 AM loaded cells were transferred into a microscope stage bath (approximately 0.1 ml) mounted on the stage of an inverted microscope (IX 70, Olympus) and perfused with HEPES buffered NT solution at 5 ml/min. Fluorescence was monitored using a Polychrome IV monochromator (TILL Photonics, Martinsried, Germany), Cascade 650 CCD camera (Roper Scientific, Sarasota, FL) and Metafluor software (Universal Imaging, Downingtown, PA) at excitation wavelengths of 340 and 380 nm, and an emission wavelength of 510 nm. At the end of each experiment, 5 mM EGTA and Ca^{2+}-free solution were applied to produced a minimum fluorescence ratio (R_{min}; 340/380 nm). Then, 2 μM ionomycin and 10 mM $CaCl_2$ were applied to confirm a maximum ratio of fluorescence (R_{max}). The percent values of $[Ca^{2+}]_c$ were calculated as: $[Ca^{2+}]\% = [(R - R_{min}) \div (R_{max} - R_{min})] \times 100$.

Experimental solution and chemicals

Normal bath solution used for patch clamps and Fura-2 fluorimetry was composed of 3.6 KCl, 145 NaCl, 1 $MgCl_2$, 5 glucose and 10 HEPES (in mM) and was of pH 7.2 adjusted with NaOH. The pipette solutions were contained (in mM) 145 Cs-glutamate, 8 NaCl, 1 $MgCl_2$, 10 HEPES, 1 EGTA and 2 MgATP for the whole-cell patch clamps and 120 Cs-aspartate, 20 CsCl, 1 $MgCl_2$, 10 HEPES, 5 EGTA and 3 MgATP for the perforated patch clamps; the pH of both solutions was 7.2, titrated with CsOH. The fluorescent Ca^{2+} indicator Fura-2 AM was purchased from Molecular Probes (Eugene, OR). 4α-Phorbol 12,13-didecanoate (4α PDD), capsaicin, camphor were purchased from Sigma-Aldrich (St. Louis, MO).

Heat application

The patch clamp and Fura-2 fluorimetry experiments were performed at room temperature (23~26°C). In some experiments for the change of temperature, the bath solution was warmed up using a heating circulator and a home-made water jacket device. The experimental temperatures indicated in the text and figures were directly measured from the bath perfusate using a thermostat.

Data analysis and statistics

Data was managed and analyzed using Origin v.7.0 software (Microcal Software, Piscataway, NJ). Statistical results are presented as the mean±standard error of the mean (SEM). Paired and unpaired Student's *t*-tests were used as appropriate to evaluate for significance, which was accepted for a p value of <0.05.

RESULTS

Under the whole-cell configuration, the current-voltage relationship (I/V curve) was obtained in each HMC-1 cell by applying ramp-like pulses from −100 to 80 mV (0.36 V/s). The temperature of the perfusate measured in the ex-

perimental bath was raised from room temperature (RT, 23~24°C) to moderately high temperature (MHT, 37~39°C), high temperature (HT, 44~46°C), or very high temperature (VHT, 53~55°C) conditions. The membrane conductance was significantly increased by MHT in about 50% (21 of 40) of the tested HMC-1 cells. The I/V curves of MHT-induced current were weakly outward rectifying, and crossed the control I/V curve at −4.1±1.1 mV (Fig. 1A). The other HMC-1 cells tested for MHT showed only a slight increase of membrane conductance, which crossed the control I/V curve at −19.7±3.4 mV (Fig. 1B).

The HT condition induced more prominent increase of membrane conductance than the responses to MHT. The I/V curve of HT-induced current also showed weak outward rectification (Figs. 1C and 1E). VHT induced remarkable increase of membrane conductance of HMC-1 cells, with weakly outward rectifying property. The amplitudes of inward currents at −60 mV were normalized to the membrane area (i.e. electrical capacitance), and the averaged results were compared (Fig. 1E). The data from the relatively weak response group in the MHT-treated experiment (Fig. 1B) were not included in the statistics. The averaged values were −2.2±0.5 (n=33), −5.6±1.2 (n=21), −18.3±5.1 (n=9), −181.4±61.8 (n=3) pA/pF for the control, MHT, HT, and VHT, respectively.

The foregoing results implied that TRPV3 or TRPV4 channels might be expressed in HMC-1 cells. To explore this further, the responses to repetitive applications of MHT were compared. The amplitudes of MHT-induced currents became progressively smaller upon the three applications of thermal stimuli ($1^{st} > 2^{nd} > 3^{rd}$, Figs. 2A and 2B). Since the desensitizing tendency of MHT-induced current was compatible with TRPV4, we also tested the effect of 1 μM 4α PDD, a chemical activator of TRPV4 [22,29-31]. The application of 4α PDD consistently activated weakly outward rectifying current in a reversible manner (Fig. 2C).

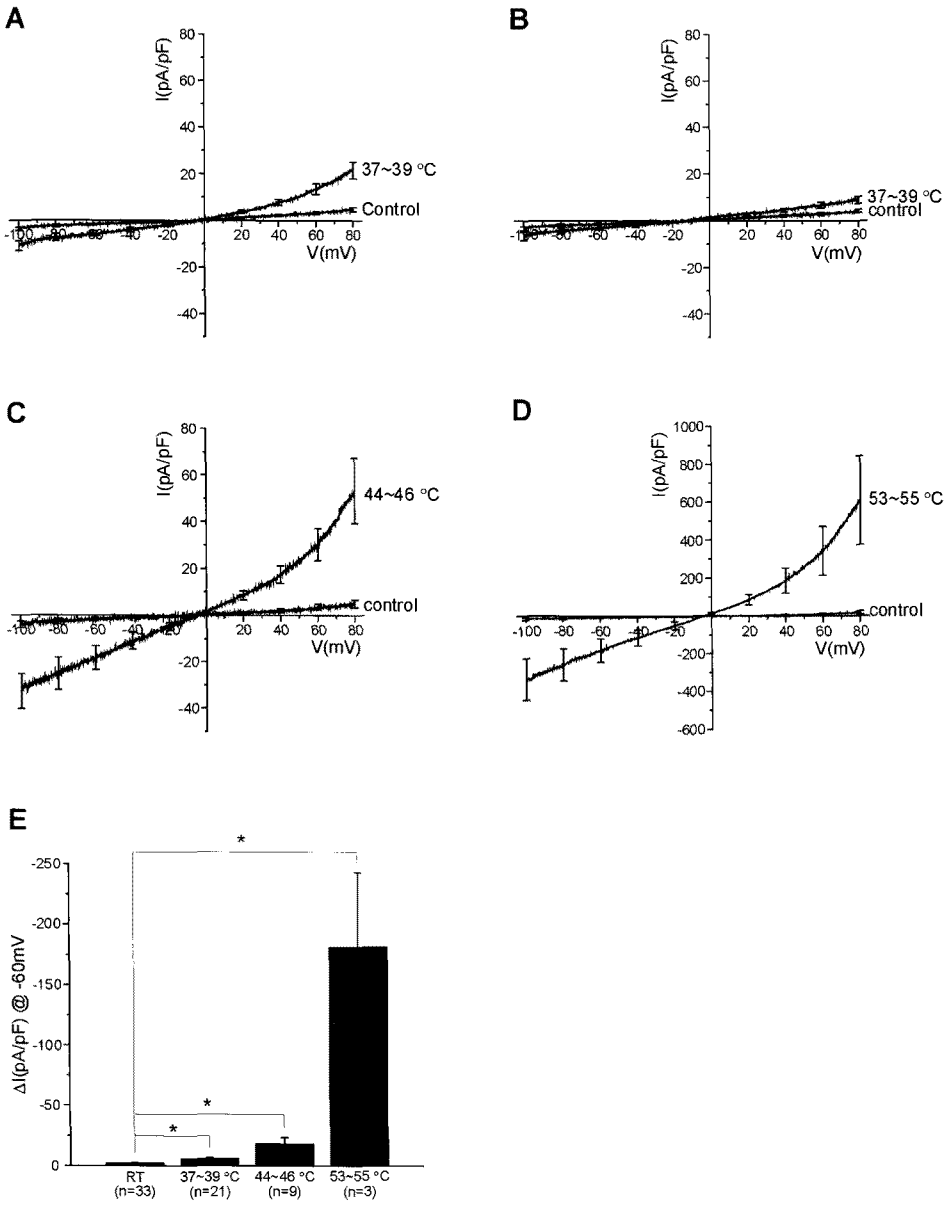

Fig. 1. Heat-evoked currents observed at various ranges of temperature in HMC-1 cells. Current-voltage relations (I-V curves) obtained using the ramp-like pulses from −100 to 80 mV at room temperature (23~25°C) and at different levels of increased temperature. The average values of normalized currents (pA/pF) are plotted against voltage. The responses to (MHT, 37~39°C) are divided into two groups; relatively large increase weakly outward rectifying I/V curves (n=21, A) and small increase with linear I/V curves (n=19, B). (C) Summary of the responses to high temperature stimuli (HT, 44~46°C, n=9) showing weakly outward rectifying I/V curves. (D) Remarkable increase of membrane conductance by very high temperature (VHT, 53~55°C, n=3). (E) Summaries of the average densities of heat-activated current for the control, MHT, HT and VHT at −60. The data acquired from (B) were excluded in this statistics. *$p < 0.05$; control versus MHT, HT and VHT, respectively.

Interestingly, however, the I/V curve of the 4 α PDD-activated current crossed the control I/V curve at 3.5±0.7 mV, which was more positive than the results of the MHT-induced current (Fig. 2D).

Next, the expression of transcripts for the TRPV subfamily was investigated. RT-PCR demonstrated the strong expression of the mRNA for TRPV2. In addition, a signal corresponding to TRPV4 was also observed (Fig. 3A). A faint signal for TRPV1 was also found (data not shown). Consistent with the RT-PCR data, no effect on the membrane currents of HMC-1 cells was evident using the TRPV1 agonist capsaicin (Fig. 3B, n=4) or the TRPV3 agonist camphor (Fig. 3C, n=3).

Finally, we tested whether the activation of TRPV4 increased the $[Ca^{2+}]_c$ of HMC-1 cells using Fura-2 fluorimetry. At RT, the application of 2 μM 4 α PDD increased the fluo-

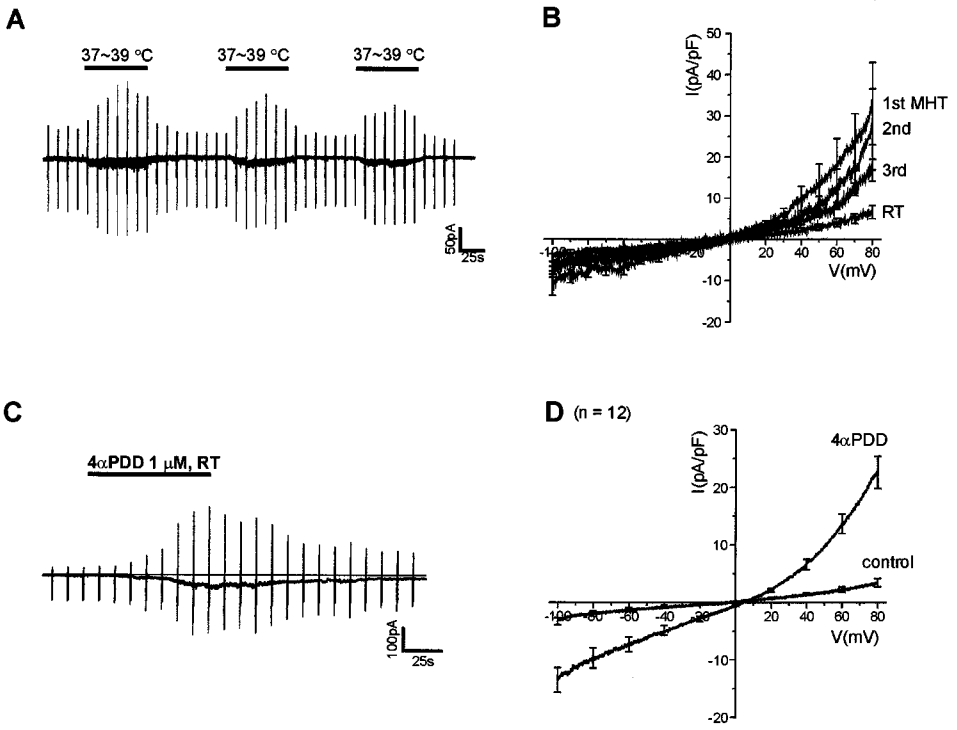

Fig. 2. Desensitization of MHT-induced current and activation by 4aPDD in HMC-1 cells. (A) An exemplary current trace showing of repetitive application of MHT. Vertical lines reflect current responses to the repetitive ramp-like pulses from −100 to 80 mV. (B) Summary of I/V curves obtained by the ramp pulses, showing desensitization by repeated MHT stimuli (1st, 2nd, and 3rd, n=6) from room temperature (RT). The whole-cell current responded to repeated application of heat stimuli was measured using the perforated-patch recording mode. (C) Representative current trace showing the response to 1 μM 4 α PDD. (D) Summary of I-V curves obtained using the ramp-like pulse from −100 to 80 mV at room temperature, showing a weakly outward rectifying current induced by 1 μM 4 α PDD (n=12).

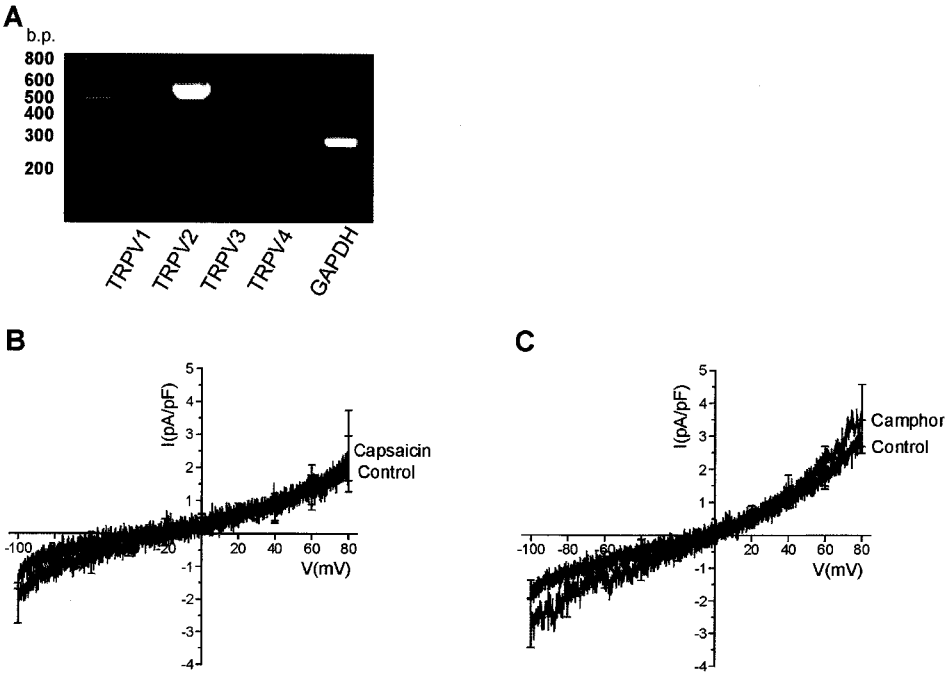

Fig. 3. Expression of TRPV2 and TRPV4 in HMC-1 cells. (A) RT-PCR analysis for TRPV1-4 in HMC-1 cells. Positive signals corresponding to the expected sizes of TRPV2 (573 b.p.) and TRPV4 (244 b.p.) were detected while not for TRPV1 (268 b.p.) and TRPV3 (772 b.p.). GAPDH was used as the control (rightmost lane). (B) and (C) Summary of the I-V curves obtained during the applications of 2 μM capsaicin (n=4) or 2 mM camphor (n=3), respectively.

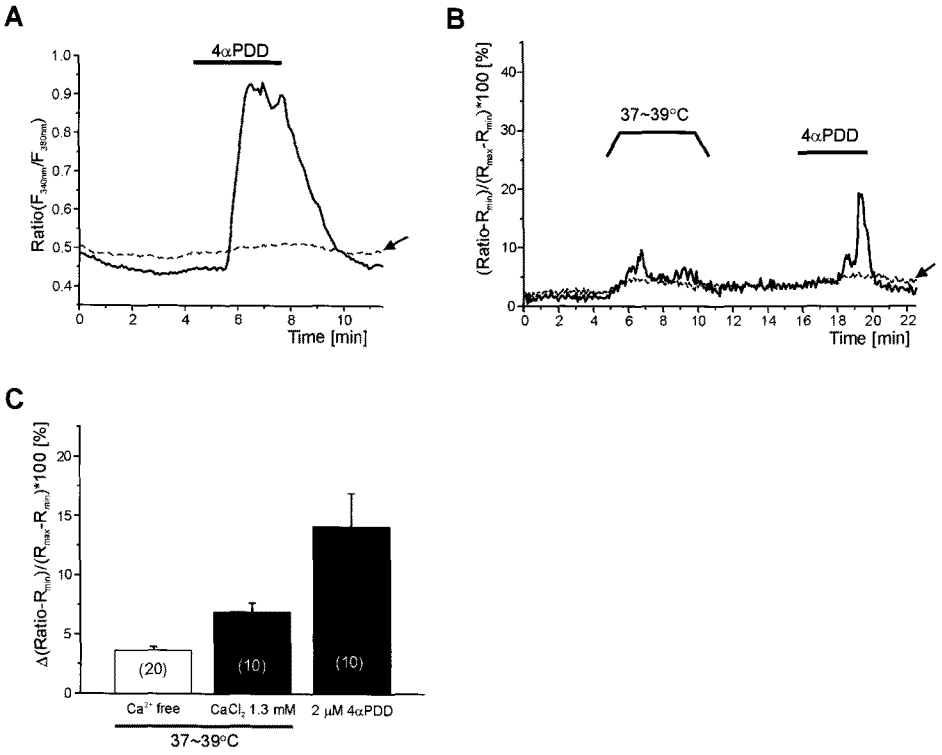

Fig. 4. Increase of intracellular Ca^{2+} concentration by heat and 4 α PDD in HMC-1 cells. (A) Representative traces of F_{340}/F_{380} showing the effect of 2 μM 4 α PDD at room temperature. The gray trace indicated by arrow is a representative negative response to 4 α PDD. (B) Representative traces of normalized F_{340}/F_{380} (see Methods), showing the sequential increases of $[Ca^{2+}]_c$ by MHT and 2 μM 4 α PDD. The gray trace indicated by arrow is a representative negative response to 4 α PDD with slight increase by MHT. (C) Summary of $[Ca^{2+}]_c$ increases caused by 2 μM 4 α PDD and MHT in presence (left bar) and absence (middle bar) of 1.3 mM $CaCl_2$. Number of tested cells are indicated in each bar.

rescence ratio (F_{340}/F_{380}) in 17 of 69 tested cells (Fig. 4A). In comparison, MHT more consistently increased the fluorescence ratio ($\Delta F_{340/380}$); a slight increase was observed when 4 α PDD had no effect (Fig. 4B, gray trace). In the cases showing $[Ca^{2+}]_c$ increase, the response to 4 α PDD was usually larger than MHT (Fig. 4B, black trace). The nonspecific increase in $F_{340}/F380$ by MHT was also observed in the absence of extracellular Ca^{2+} (Ca^{2+}-free Tyrode's solution, Fig. 4C), indicating that there might be an artifact of fluorimetry by the changed temperature.

DISCUSSION

Our present study indicates that HMC-1 cells express TRPV4 channels, albeit non-homogenously, and that the activation of these channels mediates significant $\Delta[Ca^{2+}]_c$. The experimental evidence supporting this conclusion are: 1) MHT for the stimulation, 2) desensitization by repetitive stimuli, 3) sensitivity to the pharmacological agonist 4 α PDD, and 4) presence of mRNA for TRPV4. The presence of TRPV4 mRNA is consistent with the previous reports in RBL2H3 cell, rat basophilic leukemic cell line [19]. Unfortunately, owing to the technical difficulty of cell isolation, we could not yet confirm the above conclusion in the human primary mast cells.

Since the amplitudes of HT-induced current appeared higher than the MHT-induced amplitudes (Fig. 1C), it was initially proposed that TRPV1 might be also expressed in HMC-1 cells. However, the negative responses of membrane conductance to capsaicin and the negligible signal of TRPV1 in the RT-PCR analysis (Figs. 3A and 3B) negated the possibility of the functional expression of TRPV1 in HMC-1 cells. Therefore, the current responses to HT may reflect the further activation of TRPV4 at well above its threshold temperature. Another possibility is a partial activation of the highly expressed TRPV2. Although the tested temperature (HT) was lower than the usually known threshold temperature for TRPV2 (>53°C), only a partial activation of the highly expressed TRPV2 (Fig. 3A) might contribute to a significant increase in the membrane conductance.

Mast cells are central to the pathophysiology of allergic disease through their immunomediator secretory activity in response to IgE-dependent activation. As there are important differences between rodent models and human mast cells with respect to mediator content as well as secretory and pharmacological responsiveness, studies must be ultimately performed on human cells. The human mast cell line, HMC-1, originated from a patient with mast cell leukemia; the cells express several features of mature human mast cells, making it a valuable model for the studies of human mast cell biology [20].

Conductance for Cl^-, K^+, Na^+, and Ca^{2+} have been described in mast cells at rest and after activation [7-13,19]. In general, mast cells are composed of heterogenous cell types, and this might be reflected in the different types of functional currents as shown in the prvious studies. For example, the rodent basophilic leukemic cell line and bone marrow-derived mast cells, which are considered to represent a mucosal mast cell phenotype, express a strong inwardly rectifying K^+ current, which sets a stable resting membrane potential to the hyperpolarized level (i.e. K^+ reversal potential) at about −85 mV. In contrast, rat peritoneal mast cells are electrically silent at rest or express an outwardly rectifying Cl^- conductance [15]. However, the above reports mostly deal with the membrane conductance of resting condition, i.e. without physiologically relevant stimuli. Given this background, our present study of thermosensitive nonselective cation channels (NSCs) might provide insight into the potential mechanisms for the electrical

regulation of mast cell function. Although not investigated in this study, Cl^- channels are also sensitive to nonspecific physicochemical conditions such as acidic pH and temperature [33].

Expression of TRPV2 has been identified in HMC-1 cells as well as RBL cells [19,34]. TRPV2 is expressed in the plasma membrane, leading to a rise in cytosolic free Ca^{2+} and degranulation at the high temperature (>51°C), which is dependent on the presence of extracellular Ca^{2+} [19]. While quantitatively inaccurate, the present RT-PCR analysis indicated that expression of TRPV4 was significantly less than that of TRPV2. However, compared to the non-physiological high temperature threshold for TRPV2 activation, the functional expression of TRPV4 in mast cells might imply a background influx of Na^+ and Ca^{2+} at the physiological temperature *in vivo*. In this respect, TRPV4 might provide a physiological mechanism for the Ca^{2+}-dependent regulation of mast cell functions. However, because heat-activation of TRPV4 produced desensitization, together with the relatively low expression level, the role of TRPV4 at resting conditions might be insignificant. On the other hand, once mast cells undergo cold temperature and subsequently to moderately high temperature, then the recovery of TRPV4 activity might trigger a Ca^{2+}-dependent activation of mast cells, which might contribute to the pathophysiology of heat or cold-induced urticaria.

Apart from the heat stimuli, anandamide and arachidonic acid-derived epoxyeicosatrienoic acids (5',6'-EET) have been suggested as the endogenous ligand for TRPV4 [35], and this process can be potentiated by IP3 receptor stimulation [36]. As anandamide, an agonist for endocannabinoid receptor, stimulates histamine release from mast cells in a receptor-independent manner [37], the activation of TRPV4 by 5',6'-EET might be a putative mechanism.

In conclusion, the present results demonstrate the expression of TRPV4 in HMC-1 cells in addition to the previously known expression of TRPV2. The distinguishing ranges of temperature that activate TRPV2 and TRPV4 imply different physiological roles of these thermosensitive nonselective cation channels.

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