Accelerated Ca$_2^+$ entry by membrane hyperpolarization due to Ca$_2^+$-activated K$^+$ channel activation in response to histamine in chondrocytes

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Funabashi K, Ohya S, Yamamura H, Hatano N, Muraki K, Giles W, Imaizumi Y. Accelerated Ca$_2^+$ entry by membrane hyperpolarization due to Ca$_2^+$-activated K$^+$ (K$_{Ca}$) channels in the regulation of intracellular Ca$_2^+$ concentration ([Ca$_2^+$]) in chondrocytes in response to histamine was examined using OUMS-27 cells, as a model of chondrocytes derived from human chondrosarcoma. Application of histamine induced a significant [Ca$_2^+$] rise and also membrane hyperpolarization, and both effects were mediated by the stimulation of H$_1$ receptors. The histamine-induced membrane hyperpolarization was attenuated to ~50% by large-conductance K$_{Ca}$ (BK) channel blockers, and further reduced by intermediate (IK) and small conductance K$_{Ca}$ (SK) channel blockers. The tonic component of histamine-induced [Ca$_2^+$]$_i$ rise strongly depended on the presence of extracellular Ca$_2^+$ ([Ca$_2^+$]$_o$), and was markedly reduced by La$_3^+$ or Gd$^{3+}$ but not by nifedipine. It was significantly attenuated by BK channel blockers, and further blocked by the cocktail of BK, IK, and SK channel blockers. The K$_{Ca}$ blocker cocktail also significantly reduced the store-operated Ca$_2^+$ entry (SOCE), which was induced by Ca$_2^+$ addition after store-depletion by thapsigargin in [Ca$_2^+$]$_o$ free solution. Our results demonstrate that the histamine-induced membrane hyperpolarization in chondrocytes due to K$_{Ca}$ channel activation contributes to sustained Ca$_2^+$ entry mainly through SOCE channels in OUMS-27 cells. Thus, K$_{Ca}$ channels appear to play an important role in the positive feedback mechanism of [Ca$_2^+$]$_i$ regulation in chondrocytes in the presence of articular cartilage inflammation.

store-operated Ca$_2^+$ entry; nonselective cation channels; OUMS-27; articular cartilage

ARTICULAR CHONDROCYTES EMBEDDED in the cartilage of diarthrodial joints play central roles in the physiological function and maintenance of this tissue. These cells synthesize the extracellular matrix by producing, for example, aggrecan and collagens. This synthesis is modulated in response to mechanical stress to the joints, as well as osmotic and ionic changes in the synovial fluid (33, 43). The molecular components and mechanisms that render chondrocytes responsive to a variety of stimuli are not fully understood. However, they include processes that are localized to the plasma membrane, where ion channels are essential for regulation of membrane potential.
**METHODS AND MATERIALS**

**Cell culture.** The cell line, OUMS-27, originally derived from human chondrosarcoma, has been commonly used as a model of chondrocyte functions. These cells were supplied from Japanese Collection of Research Bioresources (JCRB) Cell Bank. OUMS-27 cells were maintained at 37°C, in 5% CO₂ with high glucose Dulbecco’s modified Eagle’s medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 100 U/ml penicillin (Wako, Osaka, Japan), and 0.1 mg/ml streptomycin (Meiji Seika, Tokyo, Japan).

**RNA extraction and real-time PCR.** Total RNA extraction from OUMS-27 was performed by the acid-guanidinium thiocyanate-phenol-chloroform (AGPC) method as reported previously (22). Using the extracted total RNA, reverse transcription (RT) was performed according to GIBCO-BRL’s suggested protocol. One microgram of total RNA and 200 ng of random hexamer (Invitrogen) were heated for 10 min at 70°C and incubated for 10 min at 25°C for annealing. Each sample was incubated for 60 min at 42°C using 150 units of SuperScript II RNase-reverse transcriptase (Invitrogen) in a solution containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 6 mM MgCl₂, 1 mM DTT, and 1 mM individual dNTPs. The primers utilized were as follows: BKα primers, 1222-1331 (GenBank accession no. NM_001014797, human); BKβ₁ primers, 448-548 (NM_0014137, human); BKβ₂ primers, 941-1045 (NM_181361, human); BKβ₃ primers, 617-717 (NM_014407, human); BKβ₄ primers, 995-1098 (NM_014505, human); SK1 primers, 63-754 (NM_002248, human); SK2 primers, 2026-2130 (NM_021614, human); SK3 primers, 2008-2112 (NM_002249, human); SK4 primers, 497-618 (NM_002250, human); and β-actin primers, 411-511 (NM_001101, human). Real-time quantitative PCR was performed by the use of Syber Green chemistry on an ABI 7000 sequence detection system (Applied Biosystems, Foster City, CA). For the amplification described in the present study, the threshold value of the normalized reporter (Rn) fluorescence signal was considered to be 0.2. The PCR cycle at which a statistically significant increase in the Rn is first detected is called the threshold cycle (CT). Target DNA copy number and CT values are calculated, yielding transcriptional quantitation of curves. Unknown quantities relative to the standard curve for a particular genetic analyzer (Applied Biosystems).

**Immunocytochemistry and Western blot analysis.** After fixation and permeabilization, OUMS-27 cells were exposed to anti-BKα, anti-BKβ₁, anti-BKβ₂, anti-BKβ₃, and anti-BKβ₄ (1:100 dilution, Alomone Labs, Jerusalem, Israel), or anti-BKβ₂ (1:100 dilution, Abnova, Taipei, Taiwan) antibodies and then the cells were labeled with Alexa Fluor 488 anti-rabbit IgG or Alexa Fluor 488 anti-mouse IgG (1:100 dilution, Invitrogen). After removal of excess secondary antibody, digital images were analyzed on a laser scanning confocal microscope (A1R, Nikon, Tokyo, Japan). Plasma membrane protein fraction was prepared as previously reported (22), and 10 µg/lane of proteins were subjected to SDS-PAGE (10%). The blots were incubated with anti-BKα or anti-β1–4 subunit antibodies (above) (1:200 dilution) and then incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG (Chemicon International, Temecula, CA). An enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) was used for the detection of the bound antibody. Resulting images were analyzed by a LAS-1000 device (Fujifilm, Tokyo, Japan).

**Electrophysiological measurements.** Collagenase (Amano, Nagoya, Japan) was used to release single chondrocyte from their extracellular matrix. Whole cell patch clamp was applied to single cells using a CEZ-2400 amplifier (Nihon Kohden, Tokyo, Japan) to obtain whole cell current and membrane potential records. The patch pipette resistance ranged from 2 to 5 MΩ when filled with the pipette solution. The seal resistance was ~30 GΩ. Series resistance was partly compensated. Cell capacitance which was very small (6–10 pF) was not neutralized. Current and voltage signals were stored on videotape using a PCM system and were later captured on an IBM computer using DT2801A as an analog-digital converter and an acquisition program for precise analysis as described previously (8). All experiments were carried out at 24 ± 2°C.

**Measurement of intracellular Ca²⁺ concentrations.** OUMS-27 cells were incubated with 10 µM fura-2/AM (Invitrogen) in normal HEPES solution for 30 min at room temperature. Cells loaded with fura-2/AM (Invitrogen) were alternatively illuminated at 340- and 380-nm wavelengths of light from a xenon lamp (Hamamatsu Photonics, Shizuoka, Japan). The fluorescence emissions (>520 nm) were captured with a charge-coupled device camera and analyzed using ARGUS/HisCA software (Hamamatsu Photonics). The frequency of image acquisition was selected to be one image every 2.04 s for those [Ca²⁺], measurements. In some experiments, in which simultaneous measurements of [Ca²⁺], and membrane potential were performed, OUMS-27 was loaded with 50 µM fura-2 (Invitrogen) by diffusion from the recording pipette.

**Chemicals.** Histamine dihydrochloride (Wako) was dissolved in distilled water to 10 mM stock solution. Diphenhydramine (Wako) and ranitidine (Wako) were dissolved in distilled water to 100 mM stock solution. Lanthanum chloride and gadolinium chloride (Wako) were solved with distilled water to 100 mM stock solution. Iberotoxin (BiTx) (Peptide Institute, Osaka, Japan) was solved with distilled water to 100 mM stock solution. Paxilline (Pax, Sigma), TRAM-34 (Sigma), UCL-1684 (Sigma), U73122 (Sigma), and nifedipine (Sigma) were solved with dimethyl sulfoxide (DMSO) to 10 mM stock solution. Thapsigargin (Wako) were dissolved with DMSO to 100 mM stock solution. The final concentration of DMSO was 0.01–0.03%. All of these stock solution of Ca²⁺ were prepared with distilled water.

**Statistical analysis.** Data are expressed as means ± SE. Statistical significance between two or among multiple groups was examined using I Student’s t-test after F-test or 2 Tukey’s test after one-way ANOVA, respectively. P values <0.05 were considered statistically significant.
(340/380) showed a dose-dependent relationship in the range of 0.1 to 10 μM (Fig. 1C).

The sources of the Ca²⁺ responsible for the histamine-induced [Ca²⁺]i rise were examined in OUMS-27 cells. The [Ca²⁺]i increase due to 1 μM histamine was significantly reduced by the pretreatment for 2 min with 1 μM U73122, a phospholipase C (PLC) inhibitor (P < 0.01 vs. control) (Fig. 1D). This result suggests that the [Ca²⁺]i rise by histamine is mainly mediated by formation of inositol-1,4,5 trisphosphate (IP₃) and subsequent Ca²⁺ release from endoplasmic reticulum.

The contribution of Ca²⁺ entry to the histamine-induced [Ca²⁺]i rise in OUMS-27. The removal of Ca²⁺ from the bathing solution did not significantly change the peak [Ca²⁺]i increase (phasic component). In contrast, this maneuver markedly reduced the tonic component (Fig. 2A). Both the phasic and tonic components were significantly reduced in the presence of 10 μM La³⁺, and the reduction in the tonic component (64% reduction) was larger than that in the phasic one (31% reduction) (Fig. 2B). Figure 2C shows that the addition of 10 μM La³⁺ or Gd³⁺ during the prolonged application of 1 μM histamine markedly reduced the tonic phase of [Ca²⁺]i rise (P < 0.01 vs. control). In ~30% of cells examined, substantial oscillatory [Ca²⁺]i changes were superimposed on the tonic component of the histamine-induced [Ca²⁺]i rise (see Fig. 1Ba). The addition of 10 μM La³⁺ abolished the oscillation and also reduced markedly the tonic component (supplemental Fig. 1; the online version of this article contains supplemental data). In contrast, none of [Ca²⁺]i responses induced by 1 μM histamine was significantly affected in the presence of 10 μM nifedipine; relative AUC values of phasic component in the control and in the presence of nifedipine were 0.88 ± 0.03 and 0.82 ± 0.04 (n = 11 and 8; P > 0.05), respectively, and those of tonic components were 0.76 ± 0.08 and 0.72 ± 0.10 (P > 0.05), respectively (see also supplemental Fig. 1). This pattern of result indicates that the tonic phase may be mainly due to sustained Ca²⁺ entry through Ca²⁺-permeable ion channels rather than L-type voltage-gated Ca²⁺ channels.

Histamine-induced membrane hyperpolarization and Ca²⁺ entry. Membrane potential and [Ca²⁺]i were simultaneously measured in OUMS-27 cells loaded with 50 μM fura-2 from recording pipettes under the current clamp in whole cell patch environment (Fig. 3A). The resting membrane potential was −20.2 ± 1.95 mV (n = 5) (see also Fig. 6). Application of 1
μM histamine induced a marked [Ca\(^{2+}\)] increase and concomitant membrane hyperpolarization. In addition, oscillatory [Ca\(^{2+}\)] changes during the tonic phase corresponded to transient membrane hyperpolarizations (Fig. 3Ab). In some preparations, the tonic phase of [Ca\(^{2+}\)] rise was not apparent and only oscillatory changes were observed together with synchronizing transient membrane hyperpolarization (Fig. 3Ac). In the presence of 10 μM La\(^{3+}\), the oscillatory changes in [Ca\(^{2+}\)] and also corresponding membrane hyperpolarization were not observed (n = 2; not shown).

The relationship between membrane potential and [Ca\(^{2+}\)] was determined in OUMS-27 cells under voltage clamp (Fig. 3Ba). In the absence of histamine, a membrane hyperpolarization from −20 to −80 mV resulted in a slowly developing [Ca\(^{2+}\)] rise, and this was reversible when the membrane potential was returned to −20 mV. The [Ca\(^{2+}\)] at −80 mV was significantly higher than that at +20 mV (n = 6) (Fig. 3Bb). This [Ca\(^{2+}\)] change at −80 mV tended to be reduced (but not significantly) by the presence of 10 μM La\(^{3+}\) (open circle; P > 0.05 vs. filled square at −80 mV) (Fig. 3Bb).
These findings suggest that membrane hyperpolarization per se facilitates Ca\textsuperscript{2+} entry in OUMS-27 cells.

Next, the influence of membrane potential on histamine-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise was examined at holding potentials of 0 and -60 mV (Fig. 3 C). The phasic component of histamine-induced [Ca\textsuperscript{2+}]\textsubscript{i} rise at holding potential of -60 mV tended to be larger than 0 mV, but the tendency was not statistically significant. In contrast, the tonic component at -60 mV was significantly larger than that at 0 mV (P < 0.01). These results suggest that 1) histamine-induced Ca\textsuperscript{2+} release is not significantly modulate by membrane potential and that 2) Ca\textsuperscript{2+} entry during the tonic phase in the presence of histamine is, in contrast, strongly dependent upon the membrane potential. Specifically, membrane hyperpolarization facilitates Ca\textsuperscript{2+} entry under these conditions.

Molecular characterization of K\textsubscript{Ca} channels in OUMS-27.

Real-time PCR analyses revealed that mRNA for the BK channel \textalpha-subunit is predominantly expressed among five human K\textsubscript{Ca} channel (BK, SK1, SK2, SK3, and SK4) \textalpha-subunits examined in OUMS-27 cells (Fig. 4 A). Transcripts of SK1, SK3, and SK4 were also detected. In addition, the four types of human BK channel \textbeta-subunit (BK\textbeta\textsubscript{1}-BK\textbeta\textsubscript{4}) transcripts were expressed but not as strongly as that of BK\textalpha-subunit. Western blot analysis of the membrane fraction from OUMS-27 cells indicated the substantial expression of BK\textalpha-subunit, BK\textbeta\textsubscript{2}, and BK\textbeta\textsubscript{3} (Fig. 4 B). The immunoreactive bands suggesting the expression of BK\textbeta\textsubscript{1} and BK\textbeta\textsubscript{4} proteins were not clearly dissolved from nonspecific bands. Immunocytochemical analyses of BK\alpha-subunits (Fig. 4 C) demonstrated the expression of the BK\alpha-subunit protein on plasma membrane. In contrast, none of BK\textbeta\textsubscript{1}-BK\textbeta\textsubscript{4} expression, except BK\textbeta\textsubscript{2}. 

**Fig. 3. Relationship between change in membrane potential and [Ca\textsuperscript{2+}]\textsubscript{i} in OUMS-27 cells.** To obtain the relationship between membrane potential and Ca\textsuperscript{2+} signals, whole cell patch techniques were applied to OUMS-27 cells, which had been loaded with 50 \muM fura-2 from recording pipettes. A: typical simultaneous recording of [Ca\textsuperscript{2+}]\textsubscript{i} signals (top) and membrane potential (bottom) under current-clamp mode (a). Simultaneous recording of oscillatory [Ca\textsuperscript{2+}]\textsubscript{i} signals and membrane potential with no tonic changes in [Ca\textsuperscript{2+}]\textsubscript{i} (b). B: [Ca\textsuperscript{2+}]\textsubscript{i} signals were measured during voltage-clamp protocols in the absence of 1 \muM histamine. The holding potentials are shown at top (a). The averaged ratio values in the absence (■) and presence of 10 \muM La\textsuperscript{3+} (○) are plotted against the holding potentials (b); n = 6. C: the black and gray lines indicate the ratio measured in the presence of 1 \muM histamine at holding potential of -60 and 0 mV, respectively (a). The relative AUC was obtained from results typically shown in a in the same manner as shown in Fig. 2A (b). Numbers of cells examined are shown in parentheses. *, **Statistical significance versus control at P < 0.05 and P < 0.01, respectively.
was detected on plasma membrane of OUMS-27 cells. Detectable fluorescent signals of BKβ1 and BKβ4 were observed but mainly in the nuclear and the intracellular spaces, respectively. Under whole cell voltage clamp, outward currents were elicited upon depolarization from holding potential of \(-60\) mV in OUMS-27 (Fig. 5). When the pipette solution contained small amount of EGTA (50 \(\mu\)M) to buffer weakly intracellular \(\text{Ca}^{2+}\) (pCa \(\approx 7\)), noisy outward currents were detected at potentials positive to \(+30\) mV. These outward currents showed very slow inactivation during the 500-ms depolarization. The corresponding current-voltage (I-V) relationships showed the outward rectification at potentials positive to \(-10\) mV (Fig. 5A). An addition of 100 nM iberiotoxin (IbTX), a selective BK channel blocker, markedly reduced this outward current at potentials positive to 0 mV (\(P < 0.05\) vs. control at \(+60\) mV).

These outward currents were also recorded using the pipette filling solution, in which the pCa was buffered at 6.0 with \(\text{Ca}^{2+}\)-EGTA (see METHODS AND MATERIALS). The average amplitude of peak outward current was significantly larger, when pCa 6.0 solution was used for recording, than when pCa \(-7.0\) was (\(P < 0.05\) at \(+60\) mV) (Fig. 5, Ab and Ba). The IbTX-sensitive outward current component, presumably carried by open BK channels, was noisy and showed no fast inactivation within 500-ms depolarizing step. The outward currents were significantly reduced by the addition of 1 \(\mu\)M Pax, another specific BK channel blocker (Fig. 5, Aa and Bb). In addition to 1 \(\mu\)M Pax, the application of a cocktail of 1 \(\mu\)M UCL-1684 and 1 \(\mu\)M TRAM-34 (SK and IK blockers, respectively), tended to further reduce these outward currents. However, this reduction was not significant with respect to the data obtained in Pax alone (\(P > 0.05\)) (Fig. 5Bb). Some outward current remained in the presence of three KCa blockers. The outward current elicited by depolarization from \(-60\) to \(+40\) mV was substantially enhanced by application of 1 \(\mu\)M histamine (Fig. 5Ca). The pipette filling solution used here was that used in experiments shown in Fig. 5A (50 \(\mu\)M EGTA). The time course of histamine-induced enhancement of the outward current varied widely from cell to cell and lasted during the histamine application for 3 min (Fig. 5Cb) or only for the first \(-1\) min. The current at \(+40\) mV was significantly enhanced by 1 \(\mu\)M histamine (\(n = 4\); \(P < 0.01\)) (Fig. 5Cc). Moreover, effects of 1 \(\mu\)M Pax on the enhancement of outward currents by 1 \(\mu\)M histamine were examined. The application of Pax reduced the peak current at \(+40\) mV from 686 \(\pm\) 36 to 476 \(\pm\) 36 pA (\(n = 3\), \(P < 0.05\)). The addition of 1 \(\mu\)M histamine in the presence of Pax did not induce any increase in the peak current at \(+40\) mV (374 \(\pm\) 29 pA, \(n = 3\), \(P > 0.05\) vs. Pax and \(P < 0.01\) vs. control).

**Effects of KCa channel blockers on histamine-induced membrane hyperpolarization.** Effects of KCa channel blockers on histamine-induced membrane hyperpolarization were examined under current-clamp mode in OUMS-27 cells. These recordings were performed using a pipette filling solution containing 50 \(\mu\)M EGTA in the same manner as in Fig. 5A. The application of 1 \(\mu\)M histamine resulted in a large transient, followed by a sustained, membrane hyperpolarization (Fig. 6Aa). Oscillatory potential changes were often observed during the sustained component (see Fig. 3A). The average amplitude of the initial transient hyperpolarization was 24.1 \(\pm\) 4.3 mV (\(n = 7\)) (Fig. 6, A and D). A application of 1 \(\mu\)M Pax did not change the resting membrane potential (Fig. 6B), but significantly reduced the histamine-induced membrane hyperpolarization (51.4%, Fig. 6D). An addition of 1 \(\mu\)M UCL-1684 and 1 \(\mu\)M TRAM-34 (in the presence of 1 \(\mu\)M Pax) did not change the resting membrane potential (Fig. 6C),
but further reduced histamine-induced membrane hyperpolarization (Fig. 6D). The hyperpolarization in the presence of the cocktail (3.5 ± 0.9 mV, n = 7) was significantly smaller than that in the presence of Pax alone (11.7 ± 2.0 mV, n = 9; P < 0.01). These results indicate that histamine-induced membrane hyperpolarization is mainly due to BK channel activation, with small contribution from SK and IK channel activation.

Effects of KCa channel blockers on [Ca2+]i rise by histamine-induced and store-operated Ca2+ entry. Effects of Pax on [Ca2+]i increase in response to 1 μM histamine and on changes in [Ca2+], induced by store-operated Ca2+ entry (SOCE) (after store depletion) were examined in OUMS-27 cells (Fig. 7, A and B). The tonic component of this [Ca2+]i increase was significantly reduced by 1 μM Pax, but the phasic one was not (Fig. 7A). In cells, where the oscillatory [Ca2+]i changes on the tonic component were prominent, the oscillation was also suppressed by 1 μM Pax (see supplemental Fig. 2). SOCE was induced by the addition of 2.2 mM Ca2+ after pretreatment with 10 μM thapsigargin for 30 min in the absence of extracellular Ca2+ (Fig. 7, B and D). Note that

Fig. 5. Membrane currents and effects of histamine on them in OUMS-27. A: membrane currents were measured under whole cell voltage clamp in OUMS-27 cells. The pipette filling solution contained 50 μM EGTA. Test pulses lasting 500 ms were applied in 10-mV increments from −100 to +60 mV, under control conditions and then in the presence of 100 nM iberiotoxin (IbTX; □). The current-voltage relationships of peak outward or the least inward currents were obtained in the control (■) and in the presence of 100 nM IbTX (●) (b). B: membrane currents were measured using a pipette filling solution in which pCa was stabilized at 6.0 with Ca2+-EGTA buffer. The current-voltage relationships were obtained in the control ([□], in the presence of 1 μM paxilline (Pax) ([●]) and in the presence of a drug cocktail (●); 1 μM Pax, UCL-1648 and TRAM-34, BK, SK, and IK channel blockers, respectively (a). The averaged values of peak currents amplitude at +60 mV under control conditions, in the presence of Pax and cocktail, were compared (b). C: effects of 1 μM histamine on membrane currents were measured using the pipette filling solution containing 50 μM EGTA. Cells were depolarized from −60 to +40 mV once every 10 s. The black and gray lines indicate the currents in the absence (control) or presence of histamine, respectively (a). The time course of changes in the peak amplitude of outward current at +40 mV during the application of histamine (His) is shown (b). The black and gray triangles indicate the times at which the original recordings shown in a were obtained. Summarized data are shown for the peak amplitude of outward current before (control) and during the application of 1 μM histamine (c). Numbers of cells examined are shown in parentheses. **Statistical significance versus control at P < 0.01.
application of 1 μM Pax significantly reduced the \([\text{Ca}^{2+}]_i\) increase due to SOCE (Fig. 7B).

The cocktail of three KCa blockers, 1 μM Pax, UCL-1684, and TRAM-34, significantly reduced both phasic and tonic components of 1 μM histamine-induced \([\text{Ca}^{2+}]_i\) rise (Fig. 7C). The reduction of tonic component by the cocktail was significantly larger than that due to Pax alone (relative AUC; Pax: 0.54 ± 0.07 and cocktail: 0.32 ± 0.04, \(P < 0.01\)). The SOCE-induced \([\text{Ca}^{2+}]_i\) increase was also reduced significantly by the cocktail; however, the differences in the reduction between Pax alone and cocktail were not significant. \((P > 0.05)\) (Fig. 7, B and D).

**DISCUSSION**

Application of histamine to OUMS-27 cells caused dose-dependent increases in \([\text{Ca}^{2+}]_i\) via \(\text{H}_1\) receptor stimulation, as judged by the block with diphenhydramine. This \([\text{Ca}^{2+}]_i\) rise was triggered by the \(\text{Ca}^{2+}\) release from endoplasmic reticulum, which was due to the production of \(\text{IP}_3\) via PLC activation,
since U73122 strongly inhibited the [Ca$^{2+}$]$_i$ rise (1). Ranitidine did not show any effect on histamine-induced [Ca$^{2+}$]$_i$ rise. The contribution of H$_2$ receptor stimulation to the observed [Ca$^{2+}$]$_i$ increase must be very small. However, the possibility that H$_2$ receptor stimulation is involved in increased BK channel activity via phosphorylation by A-kinase (13) cannot be ruled out.

The phasic component of histamine-induced [Ca$^{2+}$]$_i$ rise appears to be mainly due to the IP$_3$-induced Ca$^{2+}$ release. In contrast, the tonic component was strongly dependent on Ca$^{2+}$ entry. This response was not affected by nifedipine, but markedly reduced by La$^{3+}$ and Gd$^{3+}$. When the membrane potential was clamped at −80 mV, [Ca$^{2+}$]$_i$ was slowly and slightly but significantly increased even in the absence of histamine. This increase was strongly inhibited by La$^{3+}$. The Ca$^{2+}$ entry in the presence of histamine was larger at holding potential of −60 than at 0 mV. These results indicate that this Ca$^{2+}$ entry pathway is not traditional voltage-gated Ca$^{2+}$ channels, but instead a voltage-independent nonselective cation pathway. The electromotive force of Ca$^{2+}$ entry through these channels...
depends on the difference between the reversal potential (near 0 mV) and the membrane potential. Accordingly, it is increased significantly by membrane hyperpolarization. Our results show that the application of histamine causes a large membrane hyperpolarization in OUMS-27 cells, and this hyperpolarization facilitated Ca\(^{2+}\) entry. However, it could be possible that the expression of voltage-gated Ca\(^{2+}\) channel in OUMS-27 was substantially reduced in the process to be established as a cell line. In native chondrocytes, its functional expression has been suggested in some studies (29, 45).

The histamine-induced membrane hyperpolarization was mainly attributable to K\(_\text{Ca}\) channel activation following a [Ca\(^{2+}\)]\(_i\) increase, since Pax, a BK channel blocker (10), effectively reduced the hyperpolarization and subsequent addition of TRAM-34 (46) and UCL-1684 (14), IK and SK channel blockers, respectively, also significantly reduced this hyperpolarization. Since the functional expression of voltage-gated K\(^+\) (Kv) channels in chondrocytes has been reported (18, 20, 24, 40, 41), the possibility that Kv channel activation by histamine contributes to the membrane hyperpolarization cannot be excluded completely. This may be, however, unlikely based on the amplitude of remaining hyperpolarization induced by histamine in the presence of BK, IK, and SK channel blockers. It is known that K\(_\text{Ca}\) channels can play an essential role in positive feedback mechanisms of [Ca\(^{2+}\)]\(_i\) regulation in nonexcitable cells (21). In these cells, voltage-independent nonselective cation channels are the main routes for Ca\(^{2+}\) entry, and membrane hyperpolarization due to K\(_\text{Ca}\) activation facilitates [Ca\(^{2+}\)]\(_i\) entry, which results in more activation of K\(_\text{Ca}\) channels (48).

A predominant expression of BK\(\alpha\)-subunit mRNA among BK, SK1, SK2, SK3, and SK4 (IK) \(\alpha\)-subunit transcripts was revealed by our quantitative PCR analyses in OUMS-27 cells. Correspondingly, immunocytochemical analysis clearly demonstrated the expression of BK\(\alpha\) localized to plasma membrane. Electrophysiological results gave further evidence supporting a significant contribution of BK channels to the outward current activated by depolarization, and also to the histamine-induced membrane hyperpolarization. It is, however, noteworthy that membrane hyperpolarization by itself reduces the BK channel activity since gating of BK channels depends on both [Ca\(^{2+}\)]\(_i\) and membrane potential (12, 37).

BK channels are composed of tetrameric sets consisting of a pore-forming \(\alpha\)-subunit and an auxiliary \(\beta\)-subunit (38). The \(\alpha\)-subunit has a characteristic extracellular NH\(_2\)-terminal region coupled with the \(\beta\)-subunit, seven transmembrane segments, and a long intracellular COOH-terminal region that is essential for Ca\(^{2+}\) sensing and tetramerization (26, 28). Only one major type of BK\(\alpha\) (and several of BK\(\alpha\) splice variants; KCNMA1) is expressed in a wide variety of tissues. In contrast, four different \(\beta\)-subunits with tissue-specific distribution have been identified (KCNMB1-4) (2, 9, 36). These \(\beta\)-subunits share a prototypic topology of two transmembrane domains with intracellular NH\(_2\) and COOH terminals. Coexpression of \(\beta\)-subunits with \(\alpha\)-subunits dramatically alters the biophysical and pharmacological properties of the BK channel \(\alpha\)-subunit, such as apparent Ca\(^{2+}\) sensitivity, voltage dependency, gating kinetics, and pharmacological sensitivity. Thus the \(\beta\)-subunit contributes to the diversity in BK channel function (6, 23). In summary, BK\(\beta\)-subunits provide the distinct properties of BK channels, when expressing as auxiliary components to the predominant BK\(\alpha\)-subunit. The \(\beta\)-subunit expression is responsible also for the tissue-specific properties of BK channels.

In the present study, the type of \(\beta\)-subunit of BK channels (\(\beta_1\)-\(\beta_4\)) functionally expressed in OUMS-27 was not clearly identified from results obtained by quantitative PCR. Molecular analyses by Western blotting and immunocytochemical staining suggested the expression of BK\(\beta_2\)- and BK\(\beta_3\)-subunits on the cell membrane. BK\(\beta_1\) and BK\(\beta_3\) expression in OUMS-27 cells may be low, if any. The property of the current due to BK channel in OUMS-27 cells was, however, not consistent with the functional expression of \(\beta_2\)-subunit. It includes an inactivation ball in the NH\(_2\)-terminus and produces rapid inactivation of BK channels (39). The protein expression of BK\(\alpha\) appears to be higher than that of BK\(\beta_2\) and, therefore, the major part of BK channels might consist of BK\(\alpha\) tetramer without \(\beta\)-subunit, as has been suggested in some types of cells (34). Further experiments are required to identify types of BK\(\beta\)-subunits, which are functional in OUMS-27 cells.

In the present study, IK and SK currents susceptible to TRAM-34 and UCL-1648, respectively, could not be detected under voltage-clamp mode. In contrast, under current-clamp mode, histamine-induced membrane hyperpolarization was significantly reduced by a cocktail of these blockers. It is noteworthy that the activities of IK and SK channels are not dependent on membrane potential, whereas that of BK channel is strongly voltage-dependent, i.e., increased by depolarization (37). Since the input resistance of OUMS-27 cells is high (\(\sim 2 \Omega\)\(_{\Omega}\) at resting membrane potential (\(\sim -20 \text{ mV}\)), even small IK/SK currents, which cannot be detected apparently under voltage clamp, may very effectively induce membrane hyperpolarization. Moreover, the Ca\(^{2+}\) sensitivity of IK and SK channels is derived from calmodulin, which is involved in the channel complex as Ca\(^{2+}\)-sensing unit (47) and, therefore, is higher than that of BK channel. Thus, in general, IK and SK channels could be more capable than BK channel as a key unit of the positive feedback mechanism of [Ca\(^{2+}\)]\(_i\) regulation and sustained membrane hyperpolarization in response to stimulation by agonists in nonexcitable cells. However, in OUMS-27 cells, their expression levels may be lower than that of BK channel. Actually, the finding that the tonic component of histamine-induced [Ca\(^{2+}\)]\(_i\) rise was further reduced by addition of TRAM-34 and UCL-1648 to Pax alone suggests some amount of contribution of IK and/or SK channels to the proposed positive feedback mechanism.

Histamine-induced Ca\(^{2+}\) entry in OUMS-27 cells appears to be mediated mainly by receptor-operated and/or store-operated nonselective cation channels. The predominant Ca\(^{2+}\) entry pathway observed during membrane hyperpolarization to \(\sim 80 \text{ mV}\) under voltage clamp in the absence of histamine had similar characteristics to those of the tonic component of histamine-induced membrane hyperpolarization, including La\(^{3+}\) sensitivity and the dependence on membrane potential. The positive feedback mechanism including the activation of K\(_\text{Ca}\) channels and further facilitation of Ca\(^{2+}\) entry by membrane hyperpolarization appeared to play a role in SOCE in OUMS-27 treated with thapsigargin. Expressions of some TRP channels has been reported in chondrocytes (5). However, detailed identification of Ca\(^{2+}\) entry pathway, including the functional expression of Orai and STIM as the SOCE channel (25), remains to be demonstrated in this cell line. Even though it became clear in this study that application of histamine activated Ca\(^{2+}\) entry pathway and, thereby, facilitated
correlation between the membrane hyperpolarization and Ca$^{2+}$/entry, in comparison with much slower [Ca$^{2+}$], rise by hyperpolarization from −20 to −80 mV under voltage-clamp in the absence of histamine.

In conclusion, the present study shows that H$_3$ receptor stimulation induces [Ca$^{2+}$], rise and concomitant membrane hyperpolarization in OUMS-27 cells. This membrane hyperpolarization is due to activation of K$_{Ca}$ channels and facilitates Ca$^{2+}$/ entry through nonselective cation channels, including SOCE channels. Thus, K$_{Ca}$ channels appears to be key molecules in the positive feedback mechanism of [Ca$^{2+}$], regulation in chondrocytes during articular cartilage inflammation. For that reason they may be considered to be a potential target of drug development for arthritis therapy.

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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