*Short Communication***Contribution of Chloride Channel Conductance to the Regulation of Resting Membrane Potential in Chondrocytes**Kenji Funabashi¹, Masato Fujii¹, Hisao Yamamura¹, Susumu Ohya¹, and Yuji Imaizumi^{1,*}¹Department of Molecular and Cellular Pharmacology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya 467-8603, Japan

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Abstract. The contribution of Cl[−] conductance relative to that of K⁺ in the regulation of membrane potential was examined using OUMS-27 cells, a model cell-line of human chondrocytes. Application of 100 μM niflumic acid (NFA) and other anion-channel blockers induced significant membrane hyperpolarization. The NFA-sensitive membrane current under voltage-clamp was predominantly Cl[−] current. Application of NFA induced small but significant increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i) and markedly enhanced the late component of [Ca²⁺]_i rise induced by 1 μM histamine. In conclusion, Cl[−] conductance substantially contributes to the regulation of resting membrane potential and [Ca²⁺]_i in OUMS-27 cells.

Keywords: chondrocyte, chloride channel, niflumic acid

In articular chondrocytes, in spite of the inactive electrical properties of the cell membrane (1), the control of membrane potential by ion channel activities is suggested to be functionally significant in the regulation of extracellular matrix formation in response to mechanical stress to the joints, as well as osmotic and ionic changes in the synovial fluid (2, 3). 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 and presumably the regulation of membrane potential (4). The contributions of ion channels to chondrocyte functions are mediated by the increase in intracellular Ca²⁺ concentration ([Ca²⁺]_i), and in most cases, they have been suggested based on pharmacological evidence (5).

It has been suggested that the membrane potential is mainly determined by Cl[−] conductance in cultured rabbit articular chondrocytes (6). However, our previous study has shown that K⁺ channels also contribute substantially to the regulation of membrane potential changes by histamine in OUMS-27 cells (7). The OUMS-27 cell-line is originally derived from human chondrosarcoma, presents

all major features of chondrocytes, and is widely used as a chondrocyte model (8, 9). The present study was undertaken to elucidate major determinants of membrane potential in OUMS-27 cells. Results suggest the strong dependency of membrane potential to Cl[−] conductance and also the contribution of Cl[−] conductance to histamine-induced intracellular Ca²⁺ dynamics in chondrocytes.

The cell line, OUMS-27 was supplied from Japanese Collection of Research Bioresources (JCRB) Cell Bank (Osaka) and cultured as mentioned previously (7). For electrophysiological and [Ca²⁺]_i measurements, collagenase was used to release single chondrocytes from their extracellular matrix. Whole-cell patch-clamp was performed in the same manner as described previously (10). The fura-2 fluorescence was measured by the ARGUS/HiSCA system (7) (Hamamatsu Photonics, Hamamatsu). Membrane potentials were also monitored with voltage-sensitive fluorescent dye, DiBAC₄(3). The physiological superfusion solution (HEPES-buffered solution) for electrophysiological experiments and [Ca²⁺]_i measurement contained 137 mM NaCl, 5.9 mM KCl, 2.2 mM CaCl₂, 1.2 mM MgCl₂, 10 mM glucose, and 10 mM HEPES. The pH was adjusted to 7.4 with NaOH. Data are expressed as the mean ± S.E.M. Statistical significance between two or among multiple groups was examined using Student's *t*-test after the *F*-test or Tukey's test after one way ANOVA, respectively. In the figures, sig-

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nificance is expressed by $^{\#}P < 0.05$ or $^{**}P < 0.01$.

First, the membrane potential was measured from OUMS-27 cells under current clamp mode. Application of 100 μ M niflumic acid (NFA) induced marked hyperpolarization, when Cl^- concentration in the pipette solution ($[\text{Cl}^-]_{\text{pip}}$) was 140 mM (Fig. 1A: a). The averaged resting membrane potential was -19.9 ± 2.9 mV and hyperpolarized by 100 μ M NFA to -42.6 ± 6.1 mV ($n = 5$, $P < 0.01$) (Fig. 1A: b). Under the conditions of 30 mM $[\text{Cl}^-]_{\text{pip}}$, the resting membrane potential was -38.8 ± 1.2 mV ($n = 10$) ($P < 0.05$ vs. 140 mM $[\text{Cl}^-]_{\text{pip}}$). Application of 100 μ M NFA induced significant membrane hyperpolarization in a similar manner but to a lower extent than that with 140 mM $[\text{Cl}^-]_{\text{pip}}$. Significant hyperpolarization was also observed when other anion channel blockers, 100 μ M 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), 1 mM 4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid (SITS), and 1 mM anthracene-9-carboxylic acid (9-AC), were applied ($n = 4, 6$, and 5, respectively) (Fig. 1B).

The NFA-induced membrane hyperpolarization may be due to block of Cl^- conductance and subsequent increase in the dependency of resting membrane potential to K^+ conductance. To determine the K^+ channels responsible for the background conductance, effects of several K^+ -channel blockers were examined using 30 mM $[\text{Cl}^-]_{\text{pip}}$. In these experiments, the following K^+ -channel blockers were used: 100 nM iberiotoxin [selective large conductance Ca^{2+} -activated K^+ (BK)-channel blocker; IbTX], 1 mM BaCl_2 (inward rectifier K^+ -channel blocker), and the combination of 1 mM tetraethylammonium (TEA) plus 1 mM 4-aminopyridine (4-AP) (blockers of BK and voltage-gated K^+ channels). However, the pretreatment with these blockers did not affect the hyperpolarization by 100 μ M NFA (Fig. 1C). In contrast, it was significantly reduced when $[\text{Cs}^+]_{\text{pip}}$ was 140 mM ($P < 0.01$ vs. NFA alone) (Fig. 1C). NFA-induced membrane hyperpolarization was also measured using DiBAC₄(3), a voltage-sensitive fluorescent dye in OUMS-27 cells (Fig. 1D: a). The application of 100 μ M NFA significantly reduced the fluorescent intensity ($P < 0.01$ vs. the control, which was measured just before the application in each cell) (Fig. 1D: b).

The membrane currents were measured under the conditions where Cl^- concentrations were 140 mM in both sides of the cell membrane and K^+ currents were blocked by replacing K^+ with Cs^+ in the pipette solution. The $[\text{Ca}^{2+}]_{\text{pip}}$ was pCa 6.0 in Fig. 2A. Both outward and inward currents were reduced by application of 100 μ M NFA (Fig. 2A). When $[\text{Ca}^{2+}]_{\text{pip}}$ was pCa 7.0, the membrane currents were smaller and the NFA application did not significantly reduce the currents (Fig. 2B). The NFA-sensitive components of the membrane currents were il-

lustrated as current-voltage relationships (Fig. 2C). The reversal potentials (E_{rev}) measured from the NFA-sensitive components were very close to 0 mV regardless of the pCa in the pipette solution. It is notable that the currents were apparently larger when $[\text{Ca}^{2+}]_{\text{pip}}$ was pCa 6.0 than when pCa 7.0. Moreover, when $[\text{Cl}^-]_{\text{pip}}$ was reduced from 140 to 30 mM, the E_{rev} obtained from the current-voltage relationships of the NFA-sensitive component at pCa 6.0 shifted from 2.6 ± 2.1 mV ($n = 6$) to -21.6 ± 3.2 mV ($n = 6$) ($P < 0.05$) (Fig. 2D). Since the calculated E_{rev} s were 0 and -30 mV under these conditions, the 24-mV shift of the measured E_{rev} suggests that the current is mostly carried by Cl^- .

Effects of NFA on $[\text{Ca}^{2+}]_i$ change were examined in OUMS-27 cells using fura-2. Application of 100 μ M NFA induced a small but significant rise of $[\text{Ca}^{2+}]_i$, which was removed quickly by washout of NFA (Fig. 3A). The application of 1 μ M histamine induced a $[\text{Ca}^{2+}]_i$ rise, which consisted of a large initial (early: E) and a following tonic/oscillatory (late: L) phases (7). When 1 μ M histamine was applied in the presence of 100 μ M NFA, the E-phase was not affected substantially but the L-phase was markedly enhanced (Fig. 3B: b, average of ratios). The results were calculated as areas under the curves (AUC). In the presence of NFA, the relative AUC was 3 times larger than that in the absence ($P < 0.01$) (Fig. 3B: c).

The present study clearly demonstrated that anion-channel blockers induced significant membrane hyperpolarization, which was presumably due to the block of resting Cl^- conductance and consequently the increased dependence of resting membrane potential to K^+ conductance in OUMS-27 cells. The potency of anion-channel blockers to induce membrane hyperpolarization widely varied between them: NFA > DIDS > SITS > 9-AC. Of importance is that the effect is not specific to NFA but all of them share it. The information about features of Cl^- channels responsible for resting membrane conductance are very limited, while huge conductance of swelling-activated Cl^- channels, which are apparently not active in isotonic conditions, has been reported recently (11). The much smaller resting Cl^- conductance in normal osmotic environments may be attributable to a large conductance and voltage-gated Cl^- channel in rabbit articular chondrocytes (6), in which the addition of 1 mM SITS induced membrane depolarization from resting membrane potential of -42 mV. In contrast, in the present study, NFA induced hyperpolarization of 15 mV from the resting membrane potential of -39 mV in OUMS-27 cells under the same experimental conditions. This NFA-induced hyperpolarization was also detected using DiBAC₄(3). The reason of this apparent discrepancy between the previous report and ours is not clear at present.

Fig. 1. Effects of K^+ channel blockers on NFA-induced hyperpolarization of the membrane potential in OUMS-27 cells. Membrane potentials were measured under current clamp mode in whole cell patch configuration in OUMS-27 cells. All experiments were carried out at $23 \pm 2^\circ\text{C}$. The pipette solution for electrical recordings contained 140 mM KCl, 4 mM MgCl_2 , 0.05 mM EGTA, 10 mM HEPES, and 2 mM ATPNa_2 . The pH was adjusted to 7.2 with KOH. The pipette solution containing 30 mM Cl^- contained 112 mM K-aspartate, 28 mM KCl, 1 mM MgCl_2 , 0.05 mM EGTA, 10 mM HEPES, and 2 mM ATPNa_2 . **A:** Typical recording of membrane potential change induced by $100 \mu\text{M}$ NFA (a) together with summarized data (b). $**P < 0.01$: statistical significance vs. control. The pipette solution contained 140 mM Cl^- . **B:** Effects of anion channel blockers on membrane potential were measured with 30 mM $[\text{Cl}^-]_{\text{pip}}$. Blockers were $100 \mu\text{M}$ NFA, $100 \mu\text{M}$ DIDS, 1 mM SITS, and 1 mM 9-AC. $**P < 0.01$: statistical significance vs. control. **C:** Effects of K^+ -channel blockers on $100 \mu\text{M}$ NFA-induced membrane hyperpolarization. The following K^+ -channel blockers were used: combination of 1 mM TEA + 1 mM 4-AP, 100 nM IbTX, 1 mM BaCl_2 , and 140 mM Cs^+ in the pipette solution. Number of cells used for experiments is shown in parentheses. $^{\#}P < 0.05$: statistical significance vs. NFA + IbTX. $**P < 0.01$: statistical significance vs. NFA alone, NFA + TEA + 4-AP, and NFA + Ba^{2+} . **D:** Effects of $100 \mu\text{M}$ NFA on membrane potential were monitored by DiBAC₄(3), a voltage-sensitive fluorescent dye. The measurement of membrane potential changes by DiBAC₄(3), which is a bis-barbituric acid oxonol dye with excitation maximum at approximately 490 nm, was performed as follows: Before the fluorescence measurements, cells were incubated with 100 nM DiBAC₄(3) in HEPES-buffered solution for 30 min at room temperature. Experiments were carried out in the constant presence of DiBAC₄(3). Hyperpolarization results in the extrusion of the dye from cells and a subsequent decrease in fluorescence intensity. Data collection and analyses were performed by the ARGUS/HiSCA system. The sampling interval of DiBAC₄(3) fluorescence measurements was 5 s. At the end of the experiments, cells were exposed to the bathing solution, in which 140 mM Na^+ was substituted with K^+ , to measure the fluorescent intensity at 0 mV ($F_{140\text{K}^+}$). The relative fluorescent intensity (ratio of $F_x/F_{140\text{K}^+}$) was measured by taking $F_{140\text{K}^+}$ as 1.0. The application of $100 \mu\text{M}$ NFA reduced the ratio (a) and the reduction was significant ($**P < 0.01$) (b). The control indicates the ratio just before the application of NFA. Number of cells used for experiments is shown in parentheses.

Fig. 2. Effects of NFA on membrane currents in OUMS-27 cells. Membrane currents in OUMS-27 cells were measured under whole cell voltage clamp mode. The pipette solution contained 133 mM CsCl, 4 mM $MgCl_2$, 4.25 or 1.82 mM $CaCl_2$, 5 mM EGTA, 10 mM HEPES, and 2 mM $ATPNa_2$. The pH was adjusted to 7.2 with CsOH. The pCa of the solution was calculated to be 6.0 or 7.0 (<http://www.stanford.edu/~cpatton/webmaxc/webmaxcS.htm>). Membrane currents were measured at a potential range of -100 and $+60$ mV by 10 -mV steps from the holding potential of -60 mV once every 10 s. **A:** The pCa of the pipette solution was 6.0 . Application of 100 μ M NFA reduced both inward and outward currents (a). Current-voltage relationships were obtained in the absence and presence of NFA (b). **B:** The pCa of the pipette solution was 7.0 . Application of 100 μ M NFA did not significantly change currents. **C:** The NFA-sensitive components at pCa 6.0 and 7.0 in the pipette solutions were plotted against test potentials. Note that the currents were apparently larger when $[Ca^{2+}]_{pip}$ was pCa 6.0 than when pCa 7.0 . The NFA-sensitive current amplitude at $+40$ mV was 129.0 ± 42.5 ($n = 6$) and 27.9 ± 7.1 pA ($n = 3$) ($P < 0.01$) and that at -40 mV was -114.9 ± 22.2 and -24.2 ± 6.5 pA ($P < 0.01$), when pCa was 6.0 and 7.0 , respectively. **D:** The current-voltage relationships of NFA-sensitive membrane currents when $[Cl^-]_{pip}$ was 140 and 30 mM.

In both studies, the real $[Cl^-]_i$ under the resting conditions and, thereby, the real resting membrane potential, which strongly depends upon $[Cl^-]_{pip}$, could not be determined. However, the finding that the application of NFA induced small but significant $[Ca^{2+}]_i$ rise strongly suggests the membrane hyperpolarization by NFA, since membrane hyperpolarization results in $[Ca^{2+}]_i$ rise via the facilitated Ca^{2+} influx through the non-voltage gated Ca^{2+} -entry pathway in OUMS-27 cells as well as in some other non-excitable cells (7, 12). Moreover, the late component of histamine-induced $[Ca^{2+}]_i$ rise was markedly enhanced by NFA. The membrane hyperpolarization by NFA may remove the oscillatory potential changes, keep the membrane stable at a more negative potential, and facilitate Ca^{2+} influx through the store-operated Ca^{2+}

entry pathway in the presence of histamine.

The K^+ conductance presumably responsible for the membrane hyperpolarization following the block of Cl^- channel was not sensitive to IbTX, TEA, 4-AP, and Ba^{2+} , but blocked by Cs^+ in the pipette solution. These results suggest that the K^+ channels contributing to the resting membrane conductance are not BK, $Kv1.2$, $Kv1.5$, and $Kir2.X$ (13, 14), but remained to be determined.

In conclusion, we showed here the stronger dependence of resting membrane conductance to Cl^- than to K^+ , but there is still a balance between them in OUMS-27 cells. This finding is apparently not consistent with the previous report that the resting membrane potential in chondrocytes is mainly determined by Cl^- but not K^+ conductance in rabbit chondrocytes (6). It is notable that

Fig. 3. Effects of NFA on $[Ca^{2+}]_i$ in OUMS-27 cells. To measure $[Ca^{2+}]_i$, OUMS-27 cells were treated with collagenase and then loaded with 10 μM fura-2/AM (Invitrogen, Carlsbad, CA, USA) in standard HEPES buffered solution for 30 min at room temperature. The ratio of fluorescence intensity elicited by excitation wave lengths of 340 and 380 nm, ratio (340/380), was measured as an indication of $[Ca^{2+}]_i$ changes by ARGUS/HiSCA system. A: Cells were challenged with 100 μM NFA for 5 min (a). The resting ratio (340/380) was shown as the control (b). The peak ratio (340/380) in the presence of 100 μM NFA was significantly higher than the control (** $P < 0.01$). B: Cells were challenged with 1 μM histamine (His) for 5 min twice at an interval of 10 min. The second histamine application was performed in the absence or presence of 100 μM NFA (a). The time course of averaged ratio (340/380) is shown in panel b. The value of AUC of the ratio (340/380) from the resting level for 1.5 min as the early phase (E) and for the following 5 min as the late phase (L) during the application of histamine was obtained by the integration on the computer. The AUC value in the second application was normalized by that of the first challenge (relative AUC). Histamine-induced responses were observed in the absence and presence of NFA, and the relative AUC was shown as AUC in the presence of NFA/that in the absence (c). ** $P < 0.01$: statistical significance vs. control.

the activity of the Cl^- channel depended on both voltage and $[Ca^{2+}]_i$, while the possible contribution of Ca^{2+} -activated Cl^- channels remains to be determined. The Cl^- -channel activity may possibly protect chondrocytes from excess Ca^{2+} influx during stimulation by histamine under pathophysiological conditions as the counterbalance to the activity of Ca^{2+} -activated K^+ channels.

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References

- 1 Sugimoto T, Yoshino M, Nagao M, Ishii S, Yabu H. Voltage-gated ionic channels in cultured rabbit articular chondrocytes. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol.* 1996;115: 223–232.
- 2 Woolley DE, Tetlow LC. Observations on the microenvironmental nature of cartilage degradation in rheumatoid arthritis. *Ann Rheum Dis.* 1997;56:151–161.
- 3 Tetlow LC, Woolley DE. Mast cells, cytokines, and metalloproteinases at the rheumatoid lesion: dual immunolocalisation studies. *Ann Rheum Dis.* 1995;54:896–903.
- 4 Mobasher A, Carter SD, Martin-Vasallo P, Shakibaei M. Integrins and stretch activated ion channels; putative components of functional cell surface mechanoreceptors in articular chondrocytes. *Cell Biol Int.* 2002;26:1–18.
- 5 Wu QQ, Chen Q. Mechanoregulation of chondrocyte proliferation, maturation, and hypertrophy: ion-channel dependent transduction of matrix deformation signals. *Exp Cell Res.* 2000;256: 383–391.
- 6 Tsuga K, Tohse N, Yoshino M, Sugimoto T, Yamashita T, Ishii S, et al. Chloride conductance determining membrane potential of rabbit articular chondrocytes. *J Membr Biol.* 2002;185:75–81.
- 7 Funabashi K, Ohya S, Yamamura H, Hatano N, Muraki K, Giles WR, et al. Accelerated Ca^{2+} entry by membrane hyperpolarization due to Ca^{2+} -activated K^+ channel activation in response to histamine in chondrocytes. *Am J Physiol Cell Physiol.* 2010;298:

- C786–C797.
- 8 Demircan K, Hirohata S, Nishida K, Hatipoglu OF, Oohashi T, Yonezawa T, et al. ADAMTS-9 is synergistically induced by interleukin-1 β and tumor necrosis factor α in OUMS-27 chondrosarcoma cells and in human chondrocytes. *Arthritis Rheum*. 2005;52:1451–1460.
 - 9 Kunisada T, Miyazaki M, Mihara K, Gao C, Kawai A, Inoue H, et al. A new human chondrosarcoma cell line (OUMS-27) that maintains chondrocytic differentiation. *Int J Cancer*. 1998;77:854–859.
 - 10 Hotta S, Morimura K, Ohya S, Muraki K, Takeshima H, Imaizumi Y. Ryanodine receptor type 2 deficiency changes excitation-contraction coupling and membrane potential in urinary bladder smooth muscle. *J Physiol*. 2007;582:489–506.
 - 11 Isoya E, Toyoda F, Imai S, Okumura N, Kumagai K, Omatsu-Kanbe M, et al. Swelling-activated Cl⁻ current in isolated rabbit articular chondrocytes: inhibition by arachidonic acid. *J Pharmacol Sci*. 2009;109:293–304.
 - 12 Yamazaki D, Aoyama M, Ohya S, Muraki K, Asai K, Imaizumi Y. Novel functions of small conductance Ca²⁺-activated K⁺ channel in enhanced cell proliferation by ATP in brain endothelial cells. *J Biol Chem*. 2006;281:38430–38439.
 - 13 Grissmer S, Nguyen AN, Aiyar J, Hanson DC, Mather RJ, Gutman GA, et al. Pharmacological characterization of five cloned voltage-gated K⁺ channels, types Kv1.1, 1.2, 1.3, 1.5, and 3.1, stably expressed in mammalian cell lines. *Mol Pharmacol*. 1994;45:1227–1234.
 - 14 Coghlan MJ, Carroll WA, Gopalakrishnan M. Recent developments in the biology and medicinal chemistry of potassium channel modulators: update from a decade of progress. *J Med Chem*. 2001;44:1627–1653.