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Species Difference in Sensitivity of Human and Mouse P2X7 Receptors to Inhibitory Effects of Divalent Metal Cations

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P2X7 receptor (P2X7R), a purinergic receptor, is involved in pathophysiological events such as inflammation and cell death, and thus is an attractive target for therapeutic approaches. It is reported that divalent metal cations (DMCs) inhibit P2X7R activation and that there are species differences in their inhibitory effects. To extrapolate the findings in experimental animals to humans, these species differences have to be clarified, but species differences in the sensitivity of P2X7R to DMCs between man and mouse have not been demonstrated. Here we performed direct comparison of the inhibitory effects of DMCs on human and mouse P2X7R activation. Cell lines constitutively expressing human and mouse P2X7R were used, and their P2X7R activation was evaluated as means of YO-PRO-1 dye uptake. MgCl₂, NiCl₂, ZnCl₂, CuCl₂ and CaCl₂ dose-dependently decreased agonist-induced YO-PRO-1 uptake *via* both human and mouse P2X7Rs. Apparent differences in the inhibitory profiles for NiCl₂ and CaCl₂ between them were found, and the IC₅₀ values of DMCs were in the order of CaCl₂ > MgCl₂ > NiCl₂ ≈ ZnCl₂ > CuCl₂ for both human and mouse P2X7Rs. In this study, we demonstrate that human P2X7R exhibits different sensitivity to nickel and calcium compared with the case of the mouse one, while there is no species difference in the sensitivity of their P2X7Rs to magnesium, zinc and copper, suggesting that the effects of magnesium, zinc and copper on P2X7R-associated pathophysiological events in humans might be predicted from those in mice.

Key words P2X7 receptor; divalent metal cation; species difference; IC₅₀ value

ATP is an energy currency, and the finding of purinergic receptors (P2Rs) revealed another role of it as a signaling molecule. For instance, ATP acts as a neuro- and gliotransmitter in the neuronal system, and as a regulator of inflammatory response in immune system.^{1–3)} P2Rs are classified into two groups, ionotropic P2XRs and metabotropic P2YRs.^{4,5)} Of P2XRs, P2X7R has unique characteristics, and its affinity for ATP is extremely low and its prolonged activation leads to formation of channels/pores.^{6,7)} In neuronal cells, extensive activation of P2X7Rs causes the death of neurons,^{8,9)} and induces microglial activation¹⁰⁾ and astrocytic engulfing activity.^{11,12)} In immune cells, ATP-induced P2X7R activation triggers Nod-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome activation, followed by generation/release of inflammatory cytokines.^{13–15)} Kurashima *et al.* demonstrated that activation of P2X7R expressed by resident mast cells in the colon induces initiation of inflammation through the release of chemokines and cytokines, and exacerbation of inflammation through the recruitment of neutrophils, and robust release of cytokines in inflammatory bowel disease model mice and Crohn's disease patients.¹⁶⁾ These findings strongly indicate that P2X7Rs are an attractive target for the development of therapeutic approaches for various diseases such as neurodegenerative disorders, inflammatory diseases, *etc.* Thus, the development of P2X7R antagonists has been aggressively and continuously carried out, and some of them are currently under investigation in clinical trials.^{17–19)}

Divalent metal cations (DMCs) have inhibitory effects on P2X7R activation, and their inhibitory effects are reported to

result from allosteric modulation of the receptor by agonist binding²⁰⁾ rather than their chelating ability as to the free acid form of ATP (ATP^{4–}).^{21,22)} It is reported that the rank order for the inhibition potency of DMCs as to P2X7R activation is Cu²⁺ > Cd²⁺ ~ Zn²⁺ > Ni²⁺ > Mg²⁺ ~ Co²⁺ > Mn²⁺ > Ca²⁺ = Ba²⁺ >> Sr²⁺.^{20,23)} and calcium and magnesium at concentrations found *in vivo* inhibited agonist-induced P2X7R activation *in vitro* experiments.^{24,25)} In contrast, the other P2XRs, except for P2X1R, are known to be potentiated or unaffected by DMCs.^{26–32)} Therefore, there is the possibility that DMCs might exhibit specific antagonism as to P2X7R without any effect on other P2XRs.

The inhibitory effects of DMCs on P2X7R activation have been observed beyond species of man and rodents,^{20,33,34)} but there are species difference in the sensitivity of P2X7R to agonists and antagonists between man, mouse and rat,^{35–37)} and in the inhibitory effects of magnesium and zinc on P2X7R activation between man and rat, and between mouse and rat, respectively.^{38,39)} In the development of therapeutic approaches, mouse experimental models play crucial roles, and thus understanding of the species differences between man and mouse is critical. However, to our limited knowledge, there is no available information on species differences in the inhibitory effects of DMCs on P2X7R activation between man and mouse obtained through their direct comparison. Here, we performed detailed investigation of the inhibitory potency of DMCs as to activation of human and mouse P2X7Rs using HEK293T cell lines constitutively expressing them.

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MATERIALS AND METHODS

Chemicals Some chemicals and reagents were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and all other chemicals and reagents were purchased commercially and were of analytical grade requiring no further purification, except where otherwise noted.

Human and Mouse P2X7R-Expressing HEK293T Cells HEK293T cells were cultured in 10% fetal bovine serum (FBS)- and 2 mM glutamine-containing Dulbecco's modified Eagle's medium (DMEM). Mouse P2X7R-expressing HEK293T (clone#1, HEK293T/mP2X7R) cells were cloned and cultured as previously described.⁹⁾ Human P2X7R cDNA (accession No. AB590390.1) was purchased from Kazusa DNA Research Institute (pK1KE1090, Product No. FXC09980M, Chiba, Japan) and was ligated between the *Xho*I-*Sac*I sites in the pF5A CMV-neo Flexi[®] Vector (Promega, WI, U.S.A.) (pF5A/hP2X7R). The plasmid pF5A/hP2X7R was diluted to 1333 ng/mL with OPTI-MEM medium in the presence of Lipofectamine 2000 (Invitrogen, CA, U.S.A.). After standing at room temperature for 20 min, the DNA-liposome complex mixture was added to HEK293T cells, of which the confluency was 70–80%, and they were incubated for 6 h. The transfected amount of DNA of the plasmid for hP2X7R was 1600 ng/well of 24-well plates. After 48 h-culturing in fresh culture medium, the cells were re-plated onto 10-cm culture dishes and then cultured in fresh culture medium containing 2 mg/mL of geneticin (Wako). Some colonies obtained were picked up and then cultured in the culture medium containing 1 mg/mL geneticin. By repeating this selection step, we finally obtained two colonies (HEK293T/hP2X7R #1 and #2), which were maintained in the medium containing 1 mg/mL geneticin. In this study, we used HEK293T/hP2X7R clone#1, which exhibits a greater expression level of hP2X7R. Both the HEK293T/hP2X7R and HEK293T/mP2X7R cells used for experiments were cultured in antibiotic-free culture medium. As control cells, empty vector (pF5A CMV-neo and pcDNA3.1/Zeo(+))-transfected HEK293T cells were also cloned and obtained by culturing with the respective antibiotics, being denoted as hMock and mMock cells, respectively.

Immunocytochemistry As described previously,¹¹⁾ immunostaining was performed for cells grown on glass cover slips and fixed by 20 min or more incubation in ice-cold 4% paraformaldehyde, followed by 3 min sequential dehydration steps in 70, 90 and 100% ethanol. Cells were incubated for 1 h at 4°C in a blocking buffer (0.1 M phosphate-buffered saline (PBS) containing 2% goat serum, 0.2% Triton X-100 and 0.1% bovine serum albumin), and then with rabbit anti-P2X7 antibodies (1:200, Cat. No. APR-004, Alomone Labs, Jerusalem, Israel) diluted in the blocking buffer overnight at 4°C. After six rinses in PBS, the cells were incubated with goat anti-rabbit immunoglobulin G (IgG) antibodies conjugated with Alexa Fluor 488 (1:1000, Cat. No. A11008, Life Technologies, CA, U.S.A.), which were diluted with the blocking buffer for 1 h at room temperature, rinsed, mounted on a glass slide, and then photographed under a laser confocal microscope (LSM510 META, Carl Zeiss, Jena, Germany). The nuclei were counterstained with Hoechst 33258 (2 µg/mL) (Dojindo, Kumamoto, Japan).

Western Blotting Protein homogenates were obtained from cell cultures, subjected to sodium dodecyl sulfate (SDS)-

polyacrylamide gel electrophoresis, and then transferred to polyvinylidene fluoride (PVDF) membranes, as described previously.¹¹⁾ After blocking with a blocking buffer, comprising 5% skim milk and 0.05% sodium azide in PBS-T (0.1% Tween-20 in PBS), each membrane was incubated overnight at 4°C with rabbit anti-P2X7 (1:1000) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:1000, Cat. No. 016-25523, Wako) antibodies diluted with the same blocking buffer. After several washes, each membrane was incubated with horseradish peroxidase (HRP)-linked anti-rabbit (1:10000, Cat. No. PI-1000, Vector Laboratories, CA, U.S.A.) or anti-mouse (1:10000, Cat. No. PI-2000, Vector Laboratories) IgG antibodies diluted in the same dilution buffer as that for the primary antibodies for 1 h at room temperature. The membrane was then washed and the signal was detected with ECL reagent (PerkinElmer, Inc., Boston, MA, U.S.A.).

Dye Uptake Activation of P2X7R was assessed as YO-PRO-1 dye uptake, a marker for channel/pore formation. As previously reported,¹¹⁾ we used a balanced salt solution (BSS) comprising 3.1 mM KCl, 134 mM NaCl, 1.2 mM CaCl₂, 1.2 mM MgSO₄, 0.25 mM KH₂PO₄, 15.7 mM NaHCO₃ and 2 mM glucose, the pH being adjusted to 7.2 under equilibration with 5% CO₂ at 37°C, and a DMC-free BSS as experimental buffers. Experiments were initiated by replacing the culture medium with DMC-free BSS. After 10 min pre-incubation, cells were incubated with an agonist (0.3 mM 2'(3')-O-(4-benzoylbenzoyl)adenosine-5'-triphosphate (BzATP) or 5 mM ATP^{6,7,40)} and 1 µM YO-PRO[®]-1 iodide (YP, Molecular Probes, OR, U.S.A.) in the presence or absence of the designated concentrations of a DMC for 30 min in a 5% CO₂ incubator at 37°C. The incubation was terminated by washing three times with an excess amount of warmed BSS, and then fluorescence photomicrographs were taken (three to five random fields/well) within 10 min using a fluorescence microscope (IX51, Olympus, Tokyo, Japan) equipped with a filter system of 470–490 nm for excitation and 510–550 nm for emission, an LCAch 40X object lens, and a digital camera (coolSNAP, Nippon Roper, Tokyo, Japan).

YP uptake was evaluated in three to five randomly selected fields in 3–4 wells in each experiment. The fluorescence intensity of all nuclei present in each field (more than 20) was measured after background subtraction using the histogram program of the Photoshop[®] CS6 software (Adobe, CA, U.S.A.). The values obtained were averaged to generate a single value for each experiment. Based on YP uptake–DMC concentration curves, the half inhibition concentration (IC₅₀ value) of each DMC was calculated using GraphPad Prism[®] 6 software (GraphPad Prism Software, Inc., CA, U.S.A.).

Statistical Analysis All data are expressed as the mean ± standard deviation (S.D.). Data, of which the population was considered to exhibit a normal distribution,⁴¹⁾ were analyzed with StatView statistical software (ver. 5.0; Abacus Concepts, CA, U.S.A.) using the parametric Student's *t*-test and Turley–Kramer test for comparison between two groups and among three or more groups, respectively. A *p*-value of 0.05 or less was considered to be significant.

RESULTS

Functional Expression of P2X7R Whether the HEK293T/hP2X7R and HEK293T/mP2X7R cells used in this

Fig. 1. Functional Expression of P2X7Rs in HEK293T/hP2X7R and HEK293T/mP2X7R Cells

Representative immunocytochemistry (a) and Western blotting (b) for P2X7R in HEK293T/hP2X7R and HEK293T/mP2X7R cells in three independent experiments are shown. In panel b, transient transfectants of hP2X7R and mP2X7R act as positive controls. Immunoreactivity of P2X7R (a), and a band at approximately 75 kDa corresponding to the mass size of P2X7R (b) were detected for HEK293T/hP2X7R and HEK293T/mP2X7R, but not the respective Mock cells. Scale bar=10 μ m. (c, d) Representative photomicrographs of agonist-induced YP uptake by HEK293T/hP2X7R and HEK293T/mP2X7R cells. After 10 min preincubation, cells were incubated with 1 μ M YP in the presence or absence of 0.3 mM BzATP (c) or 5 mM ATP (d) for 30 min at 37°C. Representative photomicrographs from three to four independent experiments are shown, the quantitative results being given in panels e and f, respectively. Taking the YP uptake by hMock or mMock cells as 100%, YP uptake by HEK293T/hP2X7R and HEK293T/mP2X7R is given. Each column represents the mean $\pm$ S.D. * p <0.05 (vs. YP uptake by the corresponding Mock cells).

study express functional P2X7Rs or not was examined. As shown in Figs. 1a and b, clear immunoreactivity for P2X7Rs was observed on immunocytochemistry and Western blotting, respectively, differing from the cases of the respective Mock cells, in which no immunoreactivity was detected. On functional assessment by means of YP uptake, treatment of HEK293T/hP2X7R and HEK293T/mP2X7R cells with 0.3 mM BzATP (Figs. 1c, e) and 5 mM ATP (Figs. 1d, f) induced significant YP uptake by them, but there was no YP uptake by the Mock cells. These results demonstrate that P2X7Rs were functionally expressed by HEK293T/hP2X7R and HEK293T/mP2X7R cells.

Effects of DMCs on YP Uptake Administration of MgCl_2 , NiCl_2 , ZnCl_2 and CuCl_2 decreased the BzATP-induced YP uptake dose-dependently by both HEK293T/hP2X7R and HEK293T/mP2X7R cells, and YP uptake–DMC concentration curves were similar for both cell types. In preliminary experiments, CaCl_2 at concentrations of 3 or more mM formed an insoluble complex with BzATP (0.9 mM) (data not shown), and we could not use high concentrations of CaCl_2 , which were considered to give a 50% or more decrease in YP uptake, ATP being used as the agonist in this experimental setting. The ATP-evoked YP uptake by HEK293T/hP2X7R and HEK293T/

mP2X7R cells was decreased by CaCl_2 dose-dependently, but the inhibitory effect was greater for the former than for the latter (Fig. 2e).

Based on the data shown in Fig. 2, we calculated the IC_{50} values of DMCs for YP uptake (Table 1). On comparison of the IC_{50} values between hP2X7R and mP2X7R, there were no significant differences for MgCl_2 , ZnCl_2 and CuCl_2 , while that for NiCl_2 was significantly greater for the former than for the latter. For both hP2X7R and mP2X7R, the IC_{50} values of DMCs were greater in the order of $\text{CaCl}_2 > \text{MgCl}_2 > \text{NiCl}_2 \approx \text{ZnCl}_2 > \text{CuCl}_2$.

Next, we directly compared the inhibitory effects on YP uptake among DMCs. In this experimental setting, we used 0.3 mM BzATP as the agonist and 200 μ M of an inhibitor, NiCl_2 , ZnCl_2 , MgCl_2 or CaCl_2 . CuCl_2 at the concentration of 200 μ M formed an insoluble complex with BzATP (0.9 mM) (data not shown), while it gave almost complete inhibition of YP uptake at the lowest concentration *via* both hP2X7R and mP2X7R (Fig. 2e), and thus we used CuCl_2 at 25 μ M. As shown in Fig. 3, the relative inhibitory potency was greater in the order of $\text{CuCl}_2 > \text{NiCl}_2 > \text{ZnCl}_2 \approx \text{MgCl}_2 > \text{CaCl}_2$.

Fig. 2. Effects of DMCs on Agonist-Induced YP Uptake by HEK293T/hP2X7R and HEK293T/mP2X7R Cells

After 10 min-preincubation, cells were incubated with $1 \mu\text{M}$ YP in the presence of 0.3 mM BzATP (a–d) or 5 mM ATP (e) in the presence or absence of 0 – $1430 \mu\text{M}$ MgCl_2 (a), 0 – $500 \mu\text{M}$ NiCl_2 (b), 0 – $360 \mu\text{M}$ ZnCl_2 (c), 0 – $25 \mu\text{M}$ CuCl_2 (d), or 0 – $7000 \mu\text{M}$ CaCl_2 (e) for 30 min at 37°C . Taking the YP uptake by HEK293T/hP2X7R or HEK293T/mP2X7R cells as 100%, YP uptake by HEK293T/hP2X7R (open circles) and HEK293T/mP2X7R (closed circles) cells in the presence of each concentration of a DMC is given. Each point represents the mean $\pm$ S.D. for three to four independent experiments.

Table 1. IC_{50} Values of MgCl_2 , NiCl_2 , ZnCl_2 , CuCl_2 and CaCl_2 for Inhibition of Human and Mouse P2X7Rs

Divalent metal cations	hP2X7R (μM)	mP2X7R (μM)
MgCl_2	409 ± 165	270 ± 31
NiCl_2	153 ± 46	$56.1 \pm 18.0^*$
ZnCl_2	168 ± 64	183 ± 22
CuCl_2	9.18 ± 4.19	12.5 ± 3.3
CaCl_2	1680 ± 440	>7000

IC_{50} values were calculated using the data shown in Fig. 2 with a Graphpad Prism software. Each value represents the mean $\pm$ S.D. of three to four independent experiments. * $p < 0.05$ (vs. hP2X7R).

DISCUSSION

In this study, we examined the inhibitory effects of DMCs on activation of hP2X7R and mP2X7R, the following findings being obtained: (1) the sensitivity of P2X7R to inhibition by magnesium, zinc and copper was comparable between man and mouse, while there was a species difference in the sensitivity to nickel and calcium between hP2X7R and mP2X7R, and (2) the inhibition potency of DMCs as to activation of P2X7R was in the order of $\text{CuCl}_2 > \text{NiCl}_2 > \text{ZnCl}_2 \approx \text{MgCl}_2 > \text{CaCl}_2$. Therefore, we have to pay careful attention to extrapolation of the effects of DMCs on P2X7R-mediated events in mice to those in humans, but as for magnesium, zinc and copper their effects in mice might predict those in humans.

Fig. 3. Comparison of Inhibition Potency of DMCs as to BzATP-Induced YP Uptake by HEK293T/hP2X7R and HEK293T/mP2X7R Cells

After 10 min-preincubation, cells were incubated with $1 \mu\text{M}$ YP in the presence of 0.3 mM BzATP in the presence or absence of $25 \mu\text{M}$ CuCl_2 , or $200 \mu\text{M}$ MgCl_2 , NiCl_2 , ZnCl_2 or CaCl_2 for 30 min at 37°C . Relative inhibition potency of 1 means that the BzATP-induced YP uptake by HEK293T/hP2X7R (open columns) and HEK293T/mP2X7R (closed columns) cells was decreased by a DMC compared to that in the absence of BzATP. Each column represents the mean $\pm$ S.D. for three independent experiments.

Virginio *et al.* reported the inhibitory potency of DMCs on rat P2X7R (rP2X7R) activation, the order being copper $>$ zinc $>$ nickel $>$ magnesium $>$ calcium, there being differences in the potencies of nickel and zinc compared with our findings.²⁰⁾ In their study, furthermore, the IC_{50} value of zinc for rP2X7R

activation was $8\mu\text{M}$, this value being apparently different from those for hP2X7R ($183\mu\text{M}$) and mP2X7R ($168\mu\text{M}$) obtained here. It is reported that the amino acid sequences involved in zinc-mediated inhibition of rP2X7R activation are histidine (His)²¹⁹ and His^{267,42)} and His⁶² and aspartic acid (Asp)^{197,41)}. On comparison of these sequences between hP2X7R (accession No. AB590390.1) and mP2X7R (accession No. NM_011027.3) used in this study, the amino acid positioned at 197 was found to be Asp and His, respectively. As aforementioned, however, we found that there was no difference in the IC_{50} values of zinc between hP2X7R and mP2X7R. Thus, it seems that the lower sensitivity of hP2X7R and mP2X7R to zinc might be due to the difference in other domains rather than the amino acid located at 197.

Parts of the binding sites of ATP and BzATP on P2X7R are reported to be different.³⁷⁾ In the study of Virginio *et al.*, ATP was used as the agonist,²⁰⁾ while we used BzATP to activate P2X7R. Moore and Mackenzie demonstrated that in the presence of zinc, the EC_{50} value of ATP for rP2X7R was increased, but the ATP-induced channel/pore activity of mP2X7R was enhanced.³⁸⁾ Thus, the differences in the agonists used to activate P2X7R might explain the different inhibitory potencies of zinc and nickel as to P2X7R activation found by Virginio *et al.* and us, but detailed investigations are needed to clarify this.

On the other hand, we used BzATP and ATP as the agonists for experiments on MgCl_2 , NiCl_2 , ZnCl_2 and CuCl_2 , and CaCl_2 , respectively, and thus direct comparison of their IC_{50} values was considered to be not appropriate. To overcome this, the inhibitory effects of DMCs on hP2X7R and mP2X7R were evaluated using BzATP as the agonist, the order of their inhibitory potency being comparable with that based on their IC_{50} values, this strongly validating our findings.

It is reported that calcium at concentrations of 10 or more mM cause almost complete inhibition of rP2X7R activation.^{20,43)} In this study, we could not calculate the IC_{50} value of calcium for mP2X7R, but it was reasonably considered to be 7 or more mM. This IC_{50} value for mP2X7R was approximately 5 or more -fold greater than that for hP2X7R (1.7 mM). In addition, the inhibitory effect of calcium on P2X7R activation was greater in man than in mouse. Thus, these differences might reflect the species difference in the inhibitory potency of calcium as to P2X7R activation between man and mouse.

YP is a dead cell marker, but is known to be taken up into live cells under low DMC conditions *via* hemichannels including pannexin-1, which is reported to be a P2X7R-induced YP uptake pathway.^{44–47)} In this study, exposure of HEK293T/hP2X7R and HEK293T/mP2X7R cells to DMCs decreased, but not increased, their YP uptake dose-dependently, denying decrease of their viability under our experimental condition. As for pannexin-1, Bhaskaracharya *et al.* reported that neither carbenoxolone (up to $100\mu\text{M}$) nor ¹⁰Panx peptide ($100\mu\text{M}$), both of which are a pannexin-1 blocker, had any effects on ATP-induced uptake of ethidium, a pannexin-1-permeable dye, by hP2X7R-expressing HEK293 cells,⁴⁸⁾ and Browne *et al.* demonstrated that P2X7R channels allow direct permeation of nanometer-sized dyes including YP and ethidium in rP2X7R-expressing HEK293 cells.⁴⁹⁾ Taken together, we consider that inhibitory effects of DMCs on BzATP/ATP-evoked YP uptake by HEK293T/hP2X7R and HEK293T/mP2X7R cells reflect the inhibition of agonist-evoked P2X7R activation, but not

pannexin-1, in the cells.

CONCLUSION

In this study, we demonstrated that the inhibitory effects of DMCs on both hP2X7R and mP2X7R differed among them, the order of their potency being copper>nickel>zinc≈magnesium>calcium, while there was a species difference in the sensitivity of P2X7R to nickel and calcium, but not copper, zinc or magnesium, between man and mouse. These findings are considered to constitute valuable information on extrapolation of the effects of DMCs on P2X7R-related events in mice to those in humans.

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Conflict of Interest Kazuki Nagasawa received a research Grant from Ako Kasei Co., Ltd.

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