Characteristics of ATP-Induced Current Through P2X$_7$ Receptor in NG108-15 Cells: Unique Antagonist Sensitivity and Lack of Pore Formation

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ABSTRACT—ATP activates the mouse P2X$_7$ receptor and induces a nonselective-cation current in NG108-15 cells. We investigated the effects of five receptor antagonists on the ATP-induced nonselective-cation current through P2X$_7$ receptor ($I_{\text{NS}\cdot\text{P2X7}}$) in NG108-15 cells. Nonselective P2 receptor antagonists, RB-2, PPADS and suramin inhibited the $I_{\text{NS}\cdot\text{P2X7}}$ with $IC_{50}$ values of 4.3, 53 and 40 nM, respectively. However, KN-04, which is a potent antagonist of human P2X$_7$ receptors but is not that of rat P2X$_7$ receptors, had only a weak blocking effect. Furthermore, oxidized-ATP (300 nM), an antagonist of the P2X$_7$ receptor-mediated pore-formation, did not affect the $I_{\text{NS}\cdot\text{P2X7}}$. Prolonged ATP application did not increase the membrane permeability to large molecules, N-methyl-D-glucamine or Yo-Pro-1, indicating that pore-formation was not promoted by the P2X$_7$ receptor activation in NG108-15 cells. These results suggest that antagonist sensitivities and pore-forming properties of the P2X$_7$ receptors in NG108-15 cells are different from those of other cell types.

Keywords: ATP, P2X$_7$ receptor, Antagonist, Pore formation, NG108-15 cell

Extracellular ATP exerts its diverse physiological effects through activation of P2 receptors. P2X receptors are ligand-gated nonselective cation channels and currently seven subtypes (P2X$_1$–7) are known (1, 2). P2X$_7$ receptors are unique in several respects compared to other P2X receptors (3, 4). First, while other P2X (P2X$_1$–6) receptors are activated by submicromolar concentrations of ATP, the P2X$_7$ receptor requires much higher concentrations (>0.3 mM) of ATP for activation (5). Second, the effective form of ATP that activates the P2X$_7$ receptor is the free base of ATP$^4$ (6) and thus the ATP-induced nonselective-cation current through P2X$_7$ receptor ($I_{\text{NS}\cdot\text{P2X7}}$) is potentiated by reducing external Mg$^{2+}$ (7). Third, benzoylbenzoic ATP (BzATP) is a more potent agonist than ATP (7 – 9). Fourth, brief stimulation of the P2X$_7$ receptor opens nonselective cation channels, while sustained or repetitive activation of the P2X$_7$ receptor results in the formation of large pores (9, 10), which might be the mechanism of the involvement of the P2X$_7$ receptor in ATP-induced cell death of monocytes and macrophages (11, 12).

NG108-15 cells are hybrids of mouse neuroblastoma N18TG-2 and rat glioma C6Bu-1 cells (13). We previously found that NG108-15 cells express functional P2X$_7$ receptors, which are originated from mouse N18TG-2 cells (7, 14). In NG108-15 cells, the P2X$_7$-receptor agonists BzATP and high concentrations of ATP (>500 µM) open the P2X$_7$ receptor-nonselective cation channels, which lead to a Ca$^{2+}$ influx from the extracellular space and sustained increase in intracellular Ca$^{2+}$ concentration (7, 15, 16).

The P2X$_7$ receptor is inhibited by several compounds, including RB-2, PPADS, suramin (10, 17 – 19), KN-04 (20, 21) and oxidized-ATP (22, 23). The antagonistic effects of these compounds are different among species and they also vary among cell types even within the same species. For example, KN-04 is a potent antagonist of human P2X$_7$ receptor (20, 21), but does not antagonize rat P2X$_7$ receptors (21). In the present study, we investigated the effects of P2 antagonists on the mouse P2X$_7$ receptor current in NG108-15 cells, using the whole cell voltage clamp. We also investigated whether prolonged application of ATP induces pore formation in NG108-15 cells.

MATERIALS AND METHODS

Cell culture

NG108-15 cells were grown in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 7% fetal
bovine serum, 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine at 37°C in a humidified atmosphere of 10% CO2 and 90% air (24). For electrophysiological study, cells were seeded in 12-well tissue culture plates at a density of 1 x 10^4 cell/well and cultured for 2–3 days until becoming confluent. Before experiments, cells were harvested from each well and dispersed in an experimental chamber.

Electrophysiological study

Electrophysiological experiments were carried out using the whole-cell configuration of the patch-clamp technique (25) as described previously (7, 14, 26). Patch pipettes had a tip resistance of about 2 MΩ when filled with an intracellular solution containing 110 mM CsOH, 30 mM CsCl, 50 mM Dl-aspartic acid, 5 mM MgATP, 3 mM MgCl2, 5 mM potassium creatine phosphate, 10 mM EGTA and 20 mM HEPES (adjusted to pH 7.2 with Dl-aspartic acid). The cells were dispersed in an experimental chamber perfused with Tyrode solution containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 0.33 mM NaH2PO4, 5.5 mM glucose and 5 mM HEPES (adjusted to pH 7.4 with NaOH). The temperature of the external solution was maintained at approximately 37°C by a silicon tube water jacket.

Membrane potentials were controlled by a model CEZ-2300 patch-clamp amplifier (Nihon Kohden, Tokyo). Membrane currents were acquired on-line and subsequently analyzed with a computer (DimensionV333C; Dell, Round Rock, TX, USA) using pCLAMP7 software (Axon, Union City, CA, USA). The current-voltage (I-V) relation was obtained by ramp pulses from a holding potential of –10 mV, initially depolarized to 60 mV, then hyperpolarized to –120 mV, and depolarized back to the holding potential with a speed of 1 V/s. The ramp pulse was applied every 3 s.

All the test agents were added to the superfusate. ATP (1 mM) was applied for 10–20 s repetitively with 1-min intervals to induce the I_{NS-P2X}. Although I_{NS-P2X} did not run-down significantly, we induced the I_{NS-P2X} at least twice to confirm that the current magnitudes were similar, and then an antagonist (RB-2, PPADS, suramin or KN-04) was applied. After perfusing the antagonist for 30 s, ATP was added. The effect of an antagonist was expressed as a percent inhibition, which was calculated by dividing the I_{NS-P2X} magnitude in the presence of an antagonist by that just before introducing the antagonist. Since the inhibition by oxidized-ATP required an incubation period of 120 min (27), the current densities were compared between the cells treated with and those without oxidized-ATP. The current density was obtained by dividing the current by the membrane capacitance (Cm). Cm was calculated using the test protocol in pCLAMP7 software with the following equation:

\[ C_m = i_c dt/dv \]

where i_c is the capacitive current. Data are expressed as means ± S.E.M. IC_{50} values are determined by probit analysis using EXCEL software and expressed as geometric means with 95% confidence.

To determine whether pore formation of P2X7 receptors occurs, the reversal potential of the I_{NS-P2X} activated by 3 mM ATP was first measured in the Tyrode solution containing 140 mM Na⁺ and then the external solution was changed to one containing 140 mM N-methyl-D-glucamine (NMDG) instead of Na⁺. The divalent cation concentrations were the same in both solutions.

Yo-Pro-1 uptake

P2X7 receptor-mediated pore formation was also evaluated by uptake of Yo-Pro-1, a fluorescence DNA chelating dye (28). Cells resuspended at 1–2 x 10^6 cells/ml in the HEPES buffer containing 10 μM Yo-Pro-1 (Molecular Probes, Eugene, OR, USA) were transferred into a 10 x 10 mm quartz cuvette placed in the thermostat-regulated sample chamber of a dual-excitation beam spectrofluorometer (F-2000; Hitachi, Tokyo). Cells was continuously stirred with a circular stir and stimulated with 1 mM ATP at 37°C. The fluorescence change in Yo-Pro-1-containing cell suspension was continuously monitored with an excitation wavelength of 490 nm, and emission of 509 nm. At the end of the experiments, digitonin (50 μg/ml) was added to the cell suspension to obtain maximal fluorescence.

Drugs

ATP (adenosine 5‘-triphosphate 2Na; Wako, Osaka) and oxidized-ATP (Sigma, St. Louis, MO, USA) were dissolved in water and diluted with Tyrode solution. The pH was adjusted to 7.4 with NaOH. RB-2 (reactive blue 2) (RBI, Natick, MA, USA), PPADS (pyridoxalphosphate-6-azophenyl-2‘,4‘-disulfonic acid) (Tocris, Bristol, UK), suramin (RBI), KN-04 ([N-1-[N-methyl-p-(5-isoquinolinesulfonyl)benzyl]-2-(4-phenylpiperazine)ethyl]-5-isoquinolinesulfonamide) (Seikagaku Corporation, Tokyo), KN-62 ([1-[N,O-bis (5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine]) (Seikagaku Corporation) were dissolved in dimethylsulfoxide (DMSO) and diluted with Tyrode solution. The final concentration of DMSO was less than 0.1%.

RESULTS

Effects of RB-2, PPADS and suramin on I_{NS-P2X}

We first investigated the effects of the non-selective P2 receptor antagonists, RB-2, PPADS and suramin. RB-2 (3 μM) did not affect the basal current, but it inhibited the I_{NS-P2X} induced by 1 mM ATP (Fig. 1A). The inhibition by
RB-2 was almost fully reversed after washing out the drug for 2 min (Fig. 1A). Figure 1B shows the I-V curves of the net $I_{NS·P2X7}$ obtained by subtracting the control current from the current in the presence of ATP before (left), in the presence of (middle) and after washing out (right) RB-2. The I-V curves of the $I_{NS·P2X7}$ showed a slight inward rectification at the negative potentials.

PPADS (100 μM) and suramin (100 μM) were tested using the same protocol as that for RB-2 and the results are shown in Figs. 2 and 3, respectively. These two antagonists also inhibited the $I_{NS·P2X7}$ and the inhibition was also completely reversed after washing out the drugs for 1 min.

Concentration-inhibition curves for the three antagonists were plotted by measuring the net $I_{NS·P2X7}$ at −100 mV (Fig. 4). The inhibitory effects of RB-2, PPADS and suramin were concentration-dependent with IC$_{50}$ (geometric means with 95% confidence intervals) values of 4.3 (3.0 – 5.9), 53 (25 – 81) and 40 (26 – 60) μM, respectively. The data with RB-2, PPADS and suramin could be fit with Hill coefficients of 0.76, 1.8 and 2.4, respectively.

Effect of KN-04 on $I_{NS·P2X7}$

A Ca$^{2+}$-calmodulin-dependent protein kinase II inhibitor, KN-62, and its inactive analogue, KN-04, in nanomolar concentrations inhibit human P2X$_7$ receptor-mediated responses (20, 21). In NG108-15 cells, the $I_{NS·P2X7}$ was little affected by 1 μM KN-04, which totally inhibits the human P2X$_7$ receptor-mediated effect (Fig. 5). Even at 10 μM, KN-04 only decreased $I_{NS·P2X7}$ to 61.3 ± 9.5% (n = 4) of the control value. The inhibition was reversible, as the current almost fully recovered after washing out KN-04 for 1 min.

Effect of oxidized-ATP on $I_{NS·P2X7}$

Oxidized-ATP is an irreversible inhibitor of the P2Z receptor in human lymphocytes (22) and of the cloned expressed human P2X$_7$ receptor (9). We incubated NG108-15 cells with 300 μM oxidized-ATP for 120 min, and compared the $I_{NS·P2X7}$ densities between oxidized-ATP-treated and non-treated cells. When $I_{NS·P2X7}$ was induced by ATP concentrations between 0.5 and 5 mM, no difference was found between the current in the oxidized-ATP-treated cells and that in non-treated cells (Fig. 6). This indicates that oxidized-ATP does not inhibit the P2X$_7$ receptor in NG108-15 cells.

Effect of prolonged ATP application on P2X$_7$ receptors

The inhibitory effects of oxidized-ATP on the P2X$_7$ receptor have been demonstrated with ATP-induced cell lysis and pore formation (23, 27) but not with the membrane current. The failure of oxidized-ATP to inhibit the
ATP-induced current in NG108-15 cells suggested that the pore-forming ability of P2X7 receptor in these cells are different from that in other cells. Therefore, using the whole cell clamp, we investigated whether P2X7 receptor activation induces pore formation in NG108-15 cells. Figure 7A shows the current activated by 3 mM ATP...
applied for 5 min. During this continuous superfusion of ATP, the current did not increase and its reversal potential did not change in Tyrode solution containing 140 mM Na⁺ (Fig. 7: B and C). Figure 7D shows a similar experiment in which the external solution was switched from one containing 140 mM Na⁺ to one containing 140 mM NMDG about 30 s after starting the application of ATP. Upon substituting Na⁺ with NMDG, the current magnitude suddenly decreased and the reversal potential shifted from 5 mV to −55 mV (Fig. 7E). The decreased current remained almost the same during the following 6 min of ATP superfusion in the NMDG solution. When the external solution was changed from one containing NMDG back to one containing Na⁺ in the presence of ATP, the current magnitude and the reversal potential were restored nearly to their initial value. These results indicate that prolonged stimulation with ATP does not promote the P2X₇ receptors to form pores large enough for the permeation of NMDG and Na⁺ equally into NG108-15 cells.

We examined whether Yo-Pro-1 uptake occurs during P2X₇ receptor stimulation in NG108-15 cells. ATP (1 mM) did not increase Yo-Pro-1 fluorescence during a 4 min-exposure (Fig. 8). In contrast, membrane disruption by digitonin (50 µg/ml) markedly increased Yo-Pro-1 fluorescence (Fig. 8). Yo-Pro-1 or ethidium bromide uptake through the P2X₇ receptors is reported to be facilitated at low concentrations of external divalent cations (9, 29). However, in our experiment, even in a low divalent cation-medium (Mg²⁺-free and 0.18 mM CaCl₂), ATP was unable to stimulate the Yo-Pro-1 uptake in NG108-15 cells (data not shown). This result also indicates that large pores were not formed in NG108-15 cells by prolonged application of ATP.

**DISCUSSION**

P2X₇ receptor stimulation has been reported to lead to two types of molecular responses, namely the opening of intrinsic nonselective cation channels and the formation of large pores, resulting in diverse cellular reactions such as Ca²⁺ influx, uptake of large molecules, cytokine release, activation of phospholipase D, shedding L-selectin from the cell surface, and cell lysis (23, 27, 30, 31). Our results demonstrated that the ATP-induced current through the P2X₇ receptor activation in NG108-15 cells had unique antagonist sensitivity compared with those in other cell types, and that prolonged stimulation of the P2X₇ receptor did not induce pore formation in NG108-15 cells.
Antagonists of P2X<sub>7</sub> Receptor

In NG108-15 cells, RB-2 was more potent than PPADS and suramin. The IC<sub>50</sub> values of RB-2, PPADS and suramin were 4.3, 53 and 40 μM, respectively. This order of potency is different from that with human P2X<sub>7</sub> receptors because PPADS is more potent (IC<sub>50</sub> = 1 μM) than suramin (IC<sub>50</sub> = 70 μM) in inhibiting the human P2X<sub>7</sub> receptor current in HEK-293 cells (18). The I<sub>NSS-P2X<sub>7</sub></i> from NTW8 microglial cells is more sensitive to RB-2 than PPADS (29), which is similar to our results with NG108-15 cells. Thus, the difference noted above may be due to the species differences because the P2X<sub>7</sub> receptors in both NG108-15 and NTW8 cells are from mouse origin (26). The Hill coefficient of RB-2, is close to 1, while those of PPADS and suramin are greater than 1, indicating that one molecule of RB-2 and more than one molecule of PPADS and suramin may be involved in the inhibition of one functional P2X<sub>7</sub> receptor.

Chessell et al. (29) observed no effect of 100 μM suramin on the rising phase of the current in mouse NTW8 cells when they applied ATP for only 0.5 s with their quick solution change method. In contrast, we observed that suramin considerably diminished the initial rising state of the I<sub>NSS-P2X<sub>7</sub></i> and greatly inhibited the maximal current 10–20 s after ATP application. It is likely that the P2X<sub>7</sub> receptors differ in different cell lines even though the cell lines originated from the same species. However, the possibility that the discrepancy is due to the different methods of ATP application cannot be excluded.

Unlike other P2X receptor subtypes, several selective antagonists have been found for the P2X<sub>7</sub> receptors. The calmodulin kinase II inhibitor KN-62 and its ineffective analogue KN-04 inhibit the human lymphocyte P2X<sub>7</sub>-mediated Ba<sup>2+</sup> influx and ethidium uptake at nanomolar concentrations (20), but did not affect the responses mediated through the rat P2X<sub>7</sub> receptors (30). Human and rat P2X<sub>7</sub> receptors have 80% homology in amino acid sequences (21). Introduction of the first 335 amino acids of the human P2X<sub>7</sub> receptor to the rat P2X<sub>7</sub> receptor conferred KN-62 sensitivity (21), suggesting that the drug interacts with residues in the amino-terminal half of the human P2X<sub>7</sub> receptor which contains the large extracellular loop. In NG108-15 cells, KN-04 at 1 μM had no effect and it only partly inhibited the I<sub>NSS-P2X<sub>7</sub></i> at 10 μM. Similar weak inhibition was found for KN-62. These results can not be explained by species differences, because KN-62 inhibits both native and recombinant mouse P2X<sub>7</sub> receptors from...
the NTW8 microglial cell line with IC₅₀ of 1.17 μM and 180 nM, respectively (31). A significant difference was also obtained with oxidized-ATP, another selective blocker of human, rat and mouse P2X₇ receptors (22, 23, 27, 32). Oxidized-ATP had no effect on the INS-P2X₇ in NG108-15 cells. This also cannot be explained by species differences. However, it should be noted that the effects of KN-62 (33) and oxidized-ATP (27) on mouse P2X₇ receptors were demonstrated with ATP-induced pore formation, but not with the activation by ATP of non-selective cation channels. Several lines of evidence suggest that the induction of non-selective cationic current does not always correlate with pore formation. In recombinant human P2X₇ receptors, PPADS inhibits ATP-induced pore formation with an IC₅₀ of 15 nM, while inhibition of the current requires a 100-fold higher concentration of PPADS (18). Calmidazolium inhibits the rat P2X₇ receptor current without affecting pore formation (34). In addition, the rat P2X₇ receptor expressed in Xenopus oocytes does not form a pore (35). Therefore, channel activation and pore formation appear to be separate events that are affected differently by KN-compounds and oxidized-ATP.

With respect to the P2X₇ receptor pore formation, our results suggest that prolonged stimulation of the P2X₇ receptor did not induce pore formation in NG108-15 cells. The P2X₇ receptor in NG108-15 cells is less permeable to NMDG than to Na⁺ (7). The rat P2X₇ receptor in macrophages, which has a marked pore forming activity, is equally permeable to NMDG and Na⁺ (35). In addition, Khakh et al. (36) and Virginio et al. (37) showed that prolonged activation of P2X₂ and P2X₆ receptors progressively increased their permeability to NMDG and proposed a pore dilatation property for these P2X receptor subtypes. We did not observe such a change in the permeability to NMDG in NG108-15 cells during the application of 3 mM ATP for 6 min. Pore formation was facilitated at low concentrations of divalent cations at 32°C in mouse NTW8 microglial cells (29). Yo-Pro-1 uptake was observed at 1 mM CaCl₂ at 37°C in the mouse P2X₇ receptor in HEK-293 cells and in the native NTW8 cells (33). In this study, we showed that prolonged stimulation of the P2X₇ receptor in NG108-15 cells did not increase Yo-Pro-1 uptake even in a low divalent cations-medium. Thus, we conclude that the mouse P2X₇ receptors in NG108-15 cells do not form large pores. These results might be related to the lack of effects of KN-04 and oxidized-ATP in NG108-15 cells.

An alternative explanation for the different sensitivities to antagonists and lack of pore formation in NG108-15 cells would be that some other cellular protein is either deficient or present in these cells that alters the properties of the P2X₇ channels. Another possibility is that the P2X₇ receptor may be heteromeric. Heteromeric P2X receptors have been demonstrated as combinations of P2X₂ and P2X₃ (38, 39), P2X₆ and P2X₇ (40), and P2X₄ and P2X₆ receptor subtypes (41). In NG108-15 cells, considerable levels of mRNAs for P2X₆ and P2X₇ receptors were detected (I. Matsuoka, unpublished data). However, we could not measure any membrane current at lower concentrations of ATP, which is an indication that homomeric P2X3 or P2X4 receptors are not expressed in these cells. The interaction between these P2X receptor subtypes and the P2X₇ receptor would be an important subject for future study.

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REFERENCES
1 Valera S, Hussy N, Evans RJ, Adami N, North RA, Surprenant A and Buell G: A new class of ligand-gated ion channel defined by P2X receptor for extracellular ATP. Nature 371, 516 – 519 (1994)
12 Di Virgilio F: The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death. Immunol Today 16, 524 – 528
(1995)


30 Humphreys BD and Dubyak GR: Induction of the P2z/P2X1 nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN-gamma in the human THP-1 monocytic cell line. J Immunol 157, 5627 – 5637 (1996)


36 Khakh BS, Bao XR, Labarca C and Lester HA: Neuronal P2X transmitter-gated cation channels change their ion selectivity in seconds. Nat Neurosci 2, 322 – 330 (1999)


39 Virgilio C, North RA and Surprenant A: Calcium permeability and block at homomeric and heteromeric P2X2 and P2X3 receptors, and P2X2 receptors in rat nodose neurones. J Physiol (Lond) 510, 27 – 35 (1998)


41 Le KT, Babinski K and Seguela P: Central P2X5 and P2X6 channel subunits coassemble into a novel heteromeric ATP receptor. J Neurosci 18, 7152 – 7159 (1998)