ABSTRACT—We investigated the effects of anions on different P2 receptors by measuring ATP-induced increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) in fura-2-loaded NG108-15 and PC12 cells. In NG108-15 cells, ATP at 100 \(\mu\)M and 1 mM induced a transient and a sustained [Ca\(^{2+}\)], increase, respectively. The former, but not the latter, was inhibited by U-73122, indicating that the former was via the P2Y\(_7\) receptor and the latter via the P2X\(_7\) receptor. When external Cl\(^{-}\) was replaced by other anions, the [Ca\(^{2+}\)], increase mediated by the P2Y\(_7\) receptor was not changed, but that mediated by the P2X\(_7\) receptor varied in the order of aspartate\(^{-}\) > methanesulfonate\(^{-}\) > Cl\(^{-}\) > Br\(^{-}\) > I\(^{-}\). In PC12 cells, transient [Ca\(^{2+}\)], increases mediated by the P2Y\(_7\) and P2X\(_7\) receptors were not affected by various anions. These results suggest that modulation by anions is unique to the P2X\(_7\) receptor and does not occur in P2Y\(_7\) and P2X\(_7\) receptors. This may be because the mechanism of ATP binding to the P2X\(_7\) receptor may be different than that to other P2 receptors.

Keywords: ATP, P2X\(_7\) receptor, Anion, NG108-15 cell, PC12 cell

NG108-15 cells are hybrids of mouse neuroblastoma N18TG-2 and rat glioma C6Bu-1 cells. NG108-15 cells possess two types of functional P2 receptors, P2X\(_7\) and P2Y\(_7\) (1–7). P2X\(_7\) receptors are nonselective cation channels, which cause influx of extracellular Ca\(^{2+}\) (2–4, 8, 9). P2Y\(_7\) receptors are G-protein coupled receptors that are involved in the production of inositol-1,4,5-trisphosphate (IP\(_3\)) via activation of phospholipase C (PLC) and induce Ca\(^{2+}\) release from IP\(_3\)-sensitive intracellular Ca\(^{2+}\) stores (1).

Using the whole-cell voltage clamp, Kaiho et al. (3) found that the current through the P2X\(_7\) receptor was enhanced in NG108-15 cells when extracellular Cl\(^{-}\) was replaced by aspartate\(^{-}\) (Asp\(^{-}\)) or methanesulfonate\(^{-}\) (MS\(^{-}\)), while it was reduced when Cl\(^{-}\) was replaced by Br\(^{-}\) or I\(^{-}\). Extracellular ATP elevates intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]) via P2X\(_7\) and P2Y\(_7\) receptors (1–7) in NG108-15 cells. Ca\(^{2+}\) signals by these two receptors can be distinguished from each other because the P2Y\(_7\) receptor was activated by a low concentration of ATP (100 \(\mu\)M), while the P2X\(_7\) receptor requires a higher concentration of ATP (>0.3 mM) for activation. In the present study, using fura-2-loaded cells, we investigated whether anions modulate Ca\(^{2+}\) permeability through the P2X\(_7\) receptors by monitoring ATP-induced [Ca\(^{2+}\)] elevation. We also investigated whether the effects of anions were unique to the P2X\(_7\) receptor by comparing them with the effects on P2Y\(_7\) and P2X\(_7\) receptors in NG108-15 and PC12 cells.

MATERIALS AND METHODS

Cell culture

NG108-15 cells were grown in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 7% (v/v) fetal bovine serum, 100 \(\mu\)M hypoxanthine, 0.4 \(\mu\)M aminopterin and 16 \(\mu\)M thymidine (7). Cells were maintained in 100-mm tissue culture dishes and incubated at 37\(^\circ\)C in a humidified atmosphere of 10% CO\(_2\) and 90% air.

PC12 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) fetal bovine serum, 5% (v/v) heat-inactivated horse serum, and 2 mM L-glutamine (10). Cells were maintained in 100-mm tissue culture dishes and incubated at 37\(^\circ\)C in a humidified atmosphere of 5% CO\(_2\) and 95% air.

Solutions and drugs

The standard Cl\(^{-}\) external solution contained 130 mM NaCl, 1.8 mM CaCl\(_2\), 4.7 mM KCl, 4 mM NaHCO\(_3\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 11.5 mM glucose, 10 mM HEPES, and 0.1% (w/v) bovine serum albumin (BSA) (pH 7.4). To replace Cl\(^{-}\) with Asp\(^{-}\) and MS\(^{-}\), 130 mM of each anion was added and the pH was adjusted to 7.4 with NaOH. In replacing Cl\(^{-}\) with Br\(^{-}\) and I\(^{-}\), 130 mM of the Na\(^{+}\) salt of each anion was used instead of NaCl. ATP

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(Wako, Osaka) and UTP (Sigma, St. Louis, MO, USA) were dissolved in water, diluted with the external solution, and the pH was adjusted to 7.4 with NaOH. 1-[6-[(17b-3-methoxyestra-1,3,5(10)-tri-en-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione (U73122) (Sigma) was dissolved in DMSO and diluted with the external solution. The final DMSO concentration was less than 0.1%.

**Measurements of \([\text{Ca}^{2+}]_i\)**

NG108-15 or PC12 cells were washed twice with standard external solution, pelleted by centrifugation at 200 × g for 2 min and resuspended. The cell suspension was incubated at 37°C for 20 min with 3 μM fura-2-acetoxymethyl ester (fura-2-AM). The cells were then washed twice and finally resuspended at 1–2 × 10^6 cells/ml in the standard external solution. The cells were kept at room temperature during the experiments. Aliquots of cell suspensions (1 ml) were centrifuged at 200 × g for 1 min in a microcentrifuge, and the cells were washed and resuspended in 2 ml of fresh standard external solution or the anion solution that had been pre-warmed to 37°C. The cell suspension was transferred into a 10- × 10-mm quartz cuvette placed in the thermostat-regulated sample chamber of a dual excitation beam spectrophotometer (F-4500; Hitachi, Tokyo). The cell suspension was stirred continuously with a stirring bar. The excitation wavelengths were 340 and 380 nm, and fura-2 fluorescence emission was measured at 510 nm. At the end of the measurements, triton X-100 was added to the cell suspension to obtain maximal fluorescence and then excess EGTA was added to obtain minimal fluorescence. \([\text{Ca}^{2+}]_i\) was calculated from the ratio of the fluorescence at the two excitation wavelengths, with a K_d value of 224 nM for the fura-2-Ca^{2+} equilibrium (11).

**Statistical analyses**

Statistical analyses were performed by the Dunnett multiple comparisons test, with P<0.05 considered significant.

**RESULTS**

**Separation of P2Y_2 and P2X_7 receptor mediated \([\text{Ca}^{2+}]_i\) signals in NG108-15 cells**

NG108-15 cells have P2Y_2 and P2X_7 receptors (1–7). We first examined whether a low concentration of ATP (100 μM) and a high concentration of ATP (1 mM) can distinguish between P2Y_2 and P2X_7 receptor-mediated \([\text{Ca}^{2+}]_i\) signals using a specific PLC inhibitor, U73122 (12, 13). Figure 1 shows that 100 μM ATP induced a transient \([\text{Ca}^{2+}]_i\) increase, while 1 mM ATP induced a sustained \([\text{Ca}^{2+}]_i\) increase. In the presence of U73122, the transient \([\text{Ca}^{2+}]_i\) increase induced by 100 μM ATP was suppressed, but not that induced by 1 mM ATP. This indicates that the \([\text{Ca}^{2+}]_i\) increase induced by 100 μM ATP was due to PLC activation and therefore was mediated by P2Y_2 receptors, while the sustained \([\text{Ca}^{2+}]_i\) increase induced by 1 mM ATP was due to activation of P2X_7 receptors. This is consistent with results from this laboratory reported previously by Kaiho et al. (2).

![Fig. 1](image-url)
Effects of anions on ATP-induced \([Ca^{2+}]\) increase in NG108-15 cells

Figure 2A shows the effects of various anions on the \([Ca^{2+}]\) increase in NG108-15 cells induced by 100 \(\mu\)M and 1 mM ATP. The transient \([Ca^{2+}]\) increases induced by 100 \(\mu\)M ATP were not significantly different in the different anionic solutions. However, the responses to 1 mM ATP were larger in Asp\(^{-}\)/G2d and MS\(^{-}\)/G2d than in Cl\(^{-}\)/G2d, but smaller in Br\(^{-}\)/G2d or I\(^{-}\)/G2d than in Cl\(^{-}\)/G2d (Fig. 2A). The peak values of \([Ca^{2+}]\) increases induced by 100 \(\mu\)M and 1 mM ATP in solutions of various anions are summarized in Fig. 2B. In six experiments, the average values of the \([Ca^{2+}]\) increase induced by 100 \(\mu\)M ATP were similar with the various anions. However, the average \([Ca^{2+}]\), peaks induced by 1 mM ATP varied in the order of Asp\(^{-}\) > MS\(^{-}\) > Cl\(^{-}\) > Br\(^{-}\) > I\(^{-}\). This indicates that the P2Y\(_2\) receptor-mediated transient \([Ca^{2+}]\), increase was not affected by anions, but the P2X\(_7\) receptor-mediated \([Ca^{2+}]\), increase was modulated by external anions.

Effects of anions on ATP-induced \([Ca^{2+}]\) increase in PC12 cells

We next examined whether anions affect other P2X and P2Y receptors using PC12 cells, which have P2X\(_2\) and P2Y\(_2\) receptors (14, 15). We applied UTP (100 \(\mu\)M) to activate the P2Y\(_2\) receptor and subsequently ATP (100 \(\mu\)M) in the presence of UTP to activate the P2X\(_2\) receptor, because continuous application of UTP should desensitize the P2Y\(_2\) receptor but not the P2X\(_2\) receptor. As shown in Fig. 3A, 100 \(\mu\)M UTP and subsequent application of 100 \(\mu\)M ATP elevated \([Ca^{2+}]\), transiently in the presence of 5 different anions. The UTP-induced transient \([Ca^{2+}]\), increase appeared to be slightly larger in Asp\(^{-}\), MS\(^{-}\) and Cl\(^{-}\) than in Br\(^{-}\) and I\(^{-}\). On the other hand, the ATP-induced transient \([Ca^{2+}]\), increases were similar in the presence of all the anions examined.

The average maximum \([Ca^{2+}]\), values induced by UTP and ATP from four experiments with each anion are summarized in Fig. 3B. The peak \([Ca^{2+}]\), values were not significantly different among the different anions with UTP and ATP.

Effects of NMDG and mannitol on ATP-induced \([Ca^{2+}]\) increase in NG108-15 and PC12 cells

We examined the effects of cations on \([Ca^{2+}]\), increase in NG108-15 cells induced by two different concentrations of ATP. As shown in Fig. 4, replacing external Na\(^{+}\) with N-methyl-D-glucamine (NMDG) did not change the \([Ca^{2+}]\), response to 100 \(\mu\)M ATP, but dramatically increased the

Fig. 2. Effects of various anions on ATP-induced \([Ca^{2+}]\), increases in NG108-15 cells. A: Typical chart recordings of \([Ca^{2+}]\), increase induced by 100 \(\mu\)M (left) and 1 mM ATP (right) in aspartic acid (Asp\(^{-}\)), methansulfonic acid (MS\(^{-}\)), Cl\(^{-}\), Br\(^{-}\) and I\(^{-}\). B: Summarized data of peak \([Ca^{2+}]\), in response to 100 \(\mu\)M and 1 mM ATP in the presence of indicated anions. Each column indicates the mean ± S.E.M. from 6 cells. *P<0.05, **P<0.01, compared with in Cl\(^{-}\).
Fig. 3. Effects of various anions on UTP- and ATP-induced [Ca$^{2+}$], increase in PC12 cells. A: Typical traces of [Ca$^{2+}$], increase induced by 100 μM ATP and 100 μM UTP in the presence of aspartic acid (Asp$^-$), methansulfonic acid (MS$^-$), Cl$^-$, Br$^-$, and I$^-$.
B: Summarized data of peak [Ca$^{2+}$] in response to 100 μM ATP and 100 μM UTP obtained in panel A. Each column indicates the mean ± S.E.M. from 4 cells.

Fig. 4. Effects of NMDG and mannitol on ATP-induced [Ca$^{2+}$], increase in NG108-15 cells. A: Typical [Ca$^{2+}$] responses to 100 μM and 1 mM ATP in NaCl (top panel), NMDG-Cl (middle panel) and mannitol (lower panel) solutions. B: Summarized data of peak [Ca$^{2+}$] in response to 100 μM and 1 mM ATP in panel A. Each column indicates the mean ± S.E.M. from 6 to 11 cells. **P<0.01, compared with in NaCl.
sustained \([Ca^{2+}]\), increase induced by 1 mM ATP. This may indicate that Na\(^+\) competes with Ca\(^{2+}\) for passage through the P2X\(_7\) channels. If Na\(^+\) inhibits Ca\(^{2+}\) permeation, and if Cl\(^-\)/G2d inhibits ATP binding, then elimination of both Na\(^+\) and Cl\(^-\)/G2d should additively enhance the Ca\(^{2+}\) signal mediated by the P2X\(_7\) receptor. This hypothesis was tested by replacing NaCl with an equiosmotic concentration of uncharged mannitol. In mannitol solution, the \([Ca^{2+}]\) increase induced by 1 mM ATP became significantly larger than that in NMDG-Cl, but that induced by 100 \(\mu\)M UTP was not changed.

We performed the same experiment with PC12 cells. As shown in Fig. 5, both UTP- and ATP-induced transient \([Ca^{2+}]\), increases were the same in NaCl and NMDG-Cl solutions. However, in mannitol, the transient \([Ca^{2+}]\) increase induced by 100 \(\mu\)M UTP was slightly increased, but that induced by 100 \(\mu\)M ATP was significantly larger than that in NMDG-Cl or NaCl.

DISCUSSION

In the present study, a transient \([Ca^{2+}]\), increase was induced in NG108-15 cells by a low concentration (100 \(\mu\)M) of ATP. This was inhibited by U73122, a PLC inhibitor (12, 13), indicating that 100 \(\mu\)M ATP stimulated the P2Y receptor. In NG108-15 cells, a functional P2Y receptor subtype, P2Y\(_2\), has been identified (1–7). On the other hand, a high concentration (1 mM) of ATP induced a sustained \([Ca^{2+}]\), increase, which was insensitive to U73122 (Fig. 1), indicating that the P2X\(_7\) receptor, the only functional P2X receptor subtype in NG108-15 cells (1–7), was stimulated.

Kaiho et al. (3) reported that replacement of external Cl\(^-\) with various anions modulated the magnitude of the nonselective cation current through the P2X\(_7\) receptor, depending on the anionic species and concentrations. The magnitude of the current through the P2X\(_7\) receptor varied in the order of Asp\(^-\)/G2d > Ms\(^-\)/G3e > Cl\(^-\)/G2d > Br\(^-\)/Gb3 > I\(^-\)/G2d in NG108-15 cells. Replacing external Cl\(^-\) with glutamate also enhanced the ATP-induced current through human, rat, and mouse recombinant P2X\(_7\) receptors expressed in HEK cells (16, 17).

In fura-2-loaded NG108-15 cells, the magnitude of \([Ca^{2+}]\) elevation induced by 1 mM ATP varied in the order of anions identical to that for the current modulation. Thus the effects of anions on \([Ca^{2+}]\), are consistent with the P2X\(_7\) receptor-mediated current results, suggesting that anions do not modulate the permeability ratio between Ca\(^{2+}\) and monovalent cations through the nonselective cation channels of the P2X\(_7\) receptor, but possibly modulate ATP binding to the receptor as hypothesized by Kaiho et al. (3) from the observation of the shift by these anions of the concentration-response curve of ATP.

The transient \([Ca^{2+}]\), increase induced by 100 \(\mu\)M ATP
was not affected by varying external anions, indicating that the P2Y<sub>2</sub> receptor is not affected by external anions. Thus, anions affect the P2X<sub>7</sub>- but not the P2Y<sub>2</sub>-receptor in NG108-15 cells. We performed similar experiments with PC12 cells, which possess P2X<sub>2</sub> and P2Y<sub>2</sub> receptors but not the P2X<sub>7</sub>-receptor. In PC12 cells, 100 μM UTP induces [Ca<sup>2+</sup>]<sub>i</sub> elevation via the P2Y<sub>2</sub>-receptor (18, 19) and subsequent application of 100 μM ATP in the presence of UTP induces [Ca<sup>2+</sup>]<sub>i</sub>; elevation via the P2X<sub>7</sub>-receptor (14, 20). Replacing external Cl<sup>-</sup> with Asp<sup>4</sup>, MS<sup>+</sup>, Br<sup>-</sup>, or I<sup>-</sup> did not affect [Ca<sup>2+</sup>]<sub>i</sub> elevation induced by UTP and ATP in PC12 cells. These results suggest that the effects of anions are unique to P2X<sub>7</sub>-receptors, and not seen with P2Y<sub>2</sub> and P2X<sub>2</sub> receptors.

Kaiho et al. (3) reported that anions inhibit ATP-binding to the receptor and that the order of inhibition correlates with the hydration energy of each anion. ATP<sup>4-</sup> has been reported to be the effective agonist form of ATP for the P2X<sub>7</sub>-receptor (21). If ATP<sup>4-</sup> is a unique agonist for the P2X<sub>7</sub>-receptor but not for other P2X or P2Y receptor subtypes, then it can be hypothesized that the mechanism of ATP binding to the P2X<sub>7</sub>-receptor is unique and different than that for other P2 receptors.

We also tested the effect of external monovalent cations by replacing Na<sup>+</sup> with NMDG, which is less permeable than Na<sup>+</sup> through the P2X<sub>7</sub>-receptor channels (2, 22). The transient [Ca<sup>2+</sup>]<sub>i</sub>, increases in PC12 and NG108-15 cells induced by 100 μM UTP and 100 μM ATP were not affected by NMDG. However, the P2X<sub>7</sub>-receptor-mediated [Ca<sup>2+</sup>]<sub>i</sub> increase in NG108-15 cells was significantly enhanced by NMDG. Wiley et al. (23) also reported that replacement of Na<sup>+</sup> with NMDG, choline, and K<sup>+</sup> enhanced 1 mM ATP-induced [Ca<sup>2+</sup>]<sub>i</sub>, increase in human lymphocytes. Thus it is likely that Na<sup>+</sup> ions inhibit Ca<sup>2+</sup>-permeability through the P2X<sub>7</sub>-receptor but not through the P2X<sub>2</sub>-receptor.

If the external anion interferes with ATP binding to the receptor, and if external cations inhibit Ca<sup>2+</sup>-permeability through the channel, then eliminating both ions should enhance the ATP-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>, additively. This was demonstrated when mannitol was substituted equiosmotically for NaCl. [Ca<sup>2+</sup>]<sub>i</sub>, elevations induced by 1 mM ATP via P2X<sub>7</sub>-receptors in NG108-15 cells and by 100 μM ATP via P2X<sub>7</sub>-receptors in PC12 cells were dramatically enhanced compared to those in NMDG solution. However, mannitol did not affect P2Y<sub>2</sub>-receptor-mediated [Ca<sup>2+</sup>]<sub>i</sub>; in both NG108-15 and PC-12 cells. P2X receptors are non-selective cation channels, which allow passage of not only Na<sup>+</sup>, but also Ca<sup>2+</sup>. When Na<sup>+</sup>, the main permeating ion, is absent, permeation of Ca<sup>2+</sup> may increase. An opposite but similar phenomenon has been demonstrated with the L-type Ca<sup>2+</sup> channels, whose permeability to Ca<sup>2+</sup> is 1000-fold higher than that to Na<sup>+</sup> under physiological ionic conditions, but in Ca<sup>2+</sup>-free solution, Na<sup>+</sup> permeates through the channel (24 – 26).

In PC12 cells, although P2X<sub>7</sub>-receptor-mediated [Ca<sup>2+</sup>]<sub>i</sub>, elevation was similar in NaCl and NMDG solutions, and was not modulated by anions, it became significantly larger in mannitol. This is a puzzling result. One possibility is that permeability to Ca<sup>2+</sup> may have increased dramatically, when all the other permeating ions were absent. The relative permeability ratio of the P2X<sub>7</sub>-channel to Ca<sup>2+</sup> and Na<sup>+</sup> in NG108-15 cells is 0.22 (2) and that of the P2X<sub>7</sub>-channel in PC12 cells is 2.2 (22). Permeability to Ca<sup>2+</sup> is larger than to Na<sup>+</sup> in PC12 cells but smaller in NG108-15 cells and this difference may be reflected in the different sensitivities to NMDG.

We conclude that anions modulate [Ca<sup>2+</sup>]<sub>i</sub>, through the P2X<sub>7</sub>-receptor in a manner similar to that for the P2X<sub>2</sub>-current. This effect of anions is unique to P2X<sub>7</sub>-receptors and does not occur with P2Y<sub>2</sub> and P2X<sub>2</sub> receptors. This difference may be due to ATP<sup>4-</sup>-binding to the P2X<sub>7</sub>-receptor, unlike other P2 receptors, which may require forms of ATP other than ATP<sup>4-</sup>.

Acknowledgments
We thank Ms S. Sato and Dr. T. Ono for their technical assistance. This work was supported by the Japan Health Science Foundation (21279), the Smoking Research Foundation, and Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (10670092, 11770047). K. Ogawa was a 4th year medical student at FMU at the time of the study.

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