$P2X_2$ mRNA Level Is Increased by Retinoids Treatment in PC-12 Cells. The presence of putative RAREs in the 5′-flanking region of the *P2rx2* indicated the possibility that retinoids may change the expression of $P2X_2$ receptors. We examined the level of the $P2X_2$ mRNA expression in PC-12 cells that had been treated with or without 9-*cis*-RA, an active form of an endogenous vitamin A derivative, using real-time quantitative RT-PCR analysis. We found that the $P2X_2$ mRNA in 9-*cis*-RA (100 nM)-treated PC-12 cells was markedly increased and the highest level was observed as early as 3 h later ($n = 4$; ***, $p < 0.001$), and the increase persisted for at least 12 h after the treatment with 9-*cis*-RA ($n = 4$; ***, $p < 0.001$) (Fig. 2A). The increase in the level of $P2X_2$ mRNA by 9-*cis*-RA was dose-dependent, and a significant increase was seen at 100 and 1000 nM 9-*cis*-RA (Fig. 2B).

9-*cis*-RA is known to be an activator of the nuclear receptors RXR and RAR (Aranda and Pascual, 2001). RXR can form as homodimers and as heterodimers with a number of other nuclear receptors such as RAR (Aranda and Pascual, 2001). To clarify the nuclear receptors involved in the increase in the level of $P2X_2$ mRNA, we used two ligands, all-*trans*-retinoic acid (atRA) (Aranda and Pascual, 2001) and PA024 (Takahashi et al., 2002), agonists preferentially of RAR and RXR, respectively. In this experiment, PC-12 cells were cultured in serum-free medium to detect only the effects of RAR and RXR agonists because serum contains large amounts of retinoids and binding protein (Mori, 1978). In this condition, a dose-dependent increase in the level of $P2X_2$ mRNA was also observed in cells treated with 9-*cis*-RA (Fig. 3) as in cells grown in medium with serum (Fig. 2). We treated PC-12 cells with atRA and found that the level of

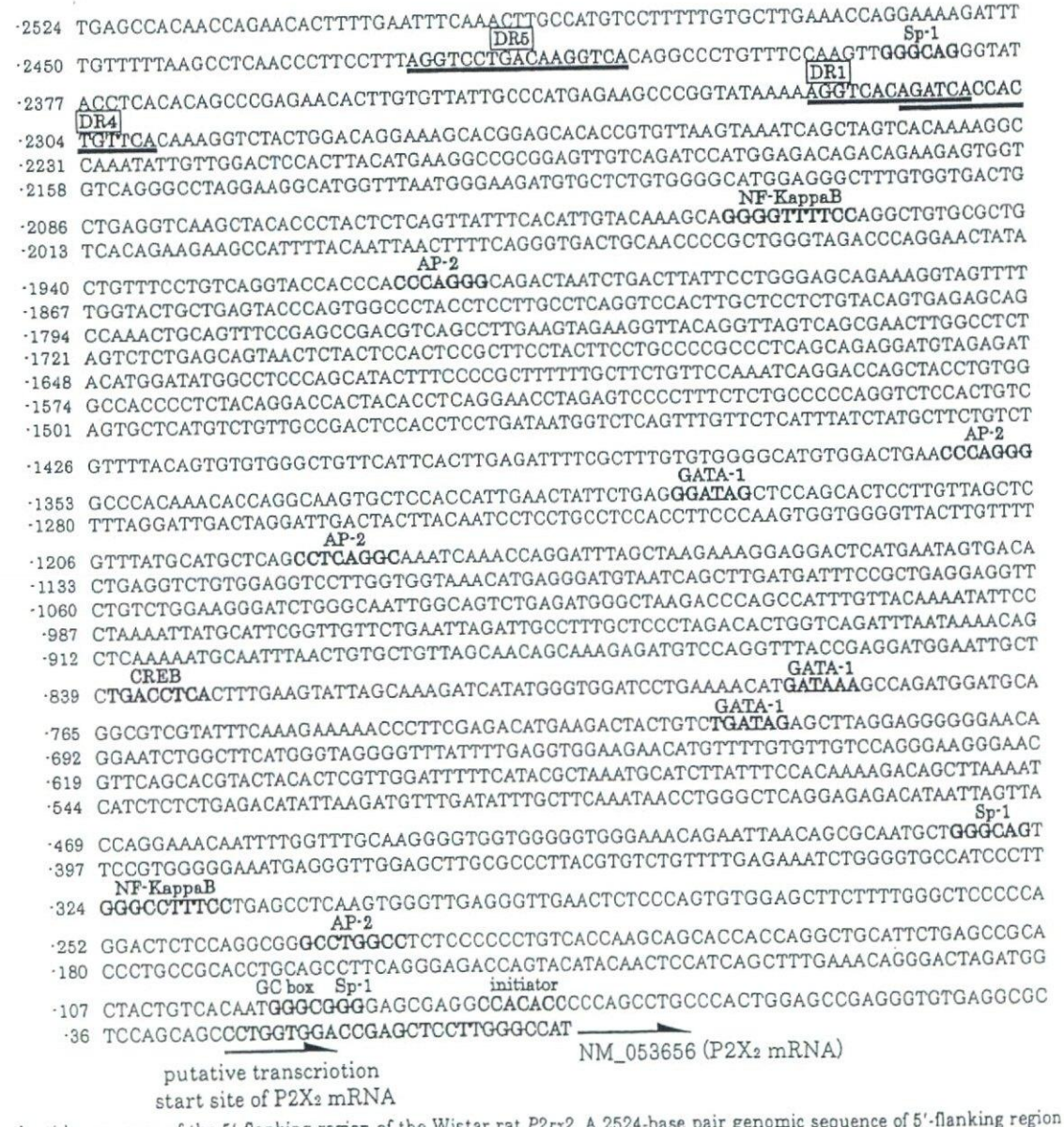

Fig. 1. Nucleotide sequence of the 5′-flanking region of the Wistar rat *P2rx2*. A 2524-base pair genomic sequence of 5′-flanking region of *P2rx2* was cloned and sequenced (GenBank accession no. AY749416) and analyzed to search for consensus motifs interacting with transcription factors using TESS. Predicted RAREs, sequences are underlined and indicated in bold. Other potential transcription binding sites predicted by TESS are indicated in bold. Arrows represent the location of $P2X_2$ mRNA sequences indicated by RefSeq sequence and 5′ RACE analysis.

$P2X_2$ mRNA was markedly increased. The increase was in a dose-dependent manner, and a significant increase was seen at the range of 10 to 1000 nM atRA (Fig. 3). By contrast, the preferential agonist of RXR, PA024 (1–100 nM), did not increase the level of $P2X_2$ mRNA. Because PC-12 cells undergo apoptotic cell death by serum deprivation (Batistatou and Greene, 1993), we maintained cells in serum-containing medium for other experiments.

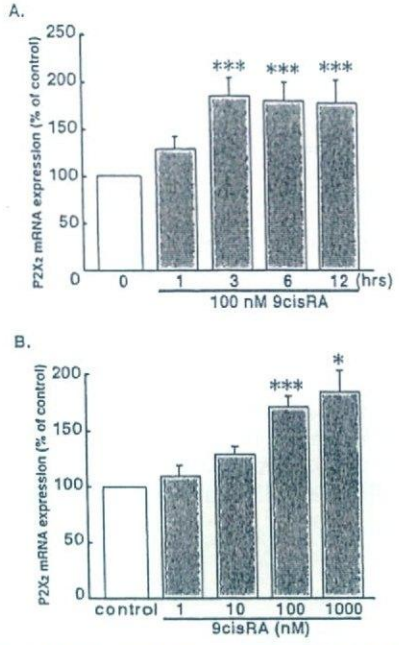

Fig. 2. Increase in the level of $P2X_2$ receptor mRNA by 9-*cis*-retinoic acid. PC-12 cells were treated with 100 nM 9-*cis*-RA for 1, 3, 6, and 12 h (A) or with different concentrations of 9-*cis*-RA (1–1000 nM) (B) followed by real-time RT-PCR analysis of $P2X_2$ and GAPDH mRNAs. $P2X_2$ mRNA levels were normalized by GAPDH mRNA levels, and each set of data represents the means ± S.E.M. of percentages of control from four individual experiments (***, $p < 0.001$; *, $p < 0.05$, multiple comparisons versus control group using Bonferroni *t* test after one-way ANOVA).

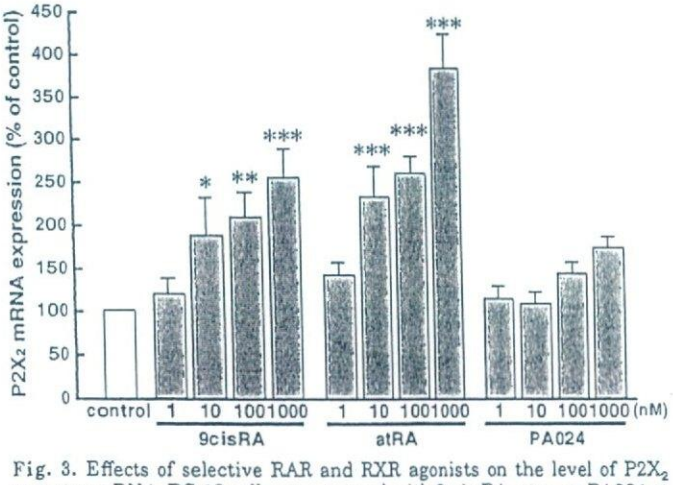

Fig. 3. Effects of selective RAR and RXR agonists on the level of $P2X_2$ receptor mRNA. PC-12 cells were treated with 9-*cis*-RA, atra, or PA024 at different concentrations for 3 h in serum-free condition followed by real-time RT-PCR analysis of $P2X_2$ and GAPDH mRNAs. $P2X_2$ mRNA levels were normalized by the GAPDH mRNA levels, and each set of data represents the means ± S.E.M. of the percentage over the value of the control group from four individual experiments (***, $p < 0.001$; **, $p < 0.01$; *, $p < 0.05$, multiple comparisons versus control group using Bonferroni *t* test after one-way ANOVA).

Retinoids Stimulate the Promoter Activity Driven by the 5′-Flanking Region of *P2rx2*. To determine whether 9-*cis*-RA increases $P2X_2$ mRNA at the transcriptional level, we examined the transcriptional activity of the 5′-flanking region of *P2rx2* (Fig. 4) using a dual-luciferase reporter assay method. The 5′-flanking region of *P2rx2* (a 2524-bp fragment upstream of the putative transcription start site) was inserted into the multicloning site of the pGL3-basic firefly luciferase assay vector (termed pP2X2luc) (Fig. 4A), which was transiently transfected into PC-12 cells. The cloned sequence increased basal luciferase activity by 25-fold. This confirmed that the sequence can promote downstream transcription. When stimulated with 1 μM 9-*cis*-RA, pP2X2luc exhibited higher luciferase activity (from 25.7 ± 2.1 to 42 ± 2.0, 65% increase; $n = 8$; ***, $p < 0.001$) (Fig. 4B). A similar increase in the luciferase activity was also observed with atRA (from 25.7 ± 2.1 to 34.8 ± 2.9., 35% increase; $n = 8$; ***, $p < 0.001$). These results indicate that 9-*cis*-RA and atRA increase the promoter activity of the cloned 5′-flanking region of *P2rx2*. Furthermore, the increases in luciferase activity by 9-*cis*-RA and atRA were lost in cells transfected with a vector lacking the fragment from −2524 to −1924 (Del-pP2X2luc) where three putative RAREs are located (Fig. 4A). In addition, the pGL3-basic vector without the 5′-flanking region of *P2rx2* showed no transcriptional activity, the RAR agonists caused no change, and basal activity of Del-

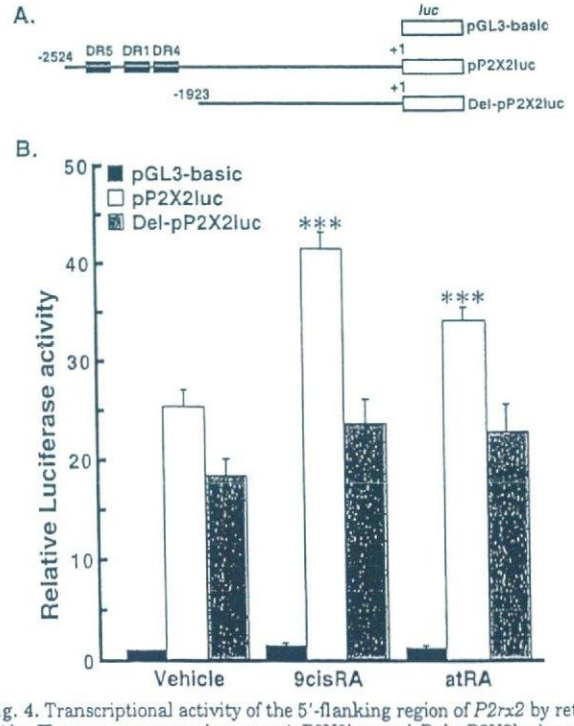

Fig. 4. Transcriptional activity of the 5′-flanking region of *P2rx2* by retinoic acids. The two constructed vectors (pP2X2luc and Del-pP2X2luc) and the empty vector (pGL3-basic) used in the experiment, as described under *Materials and Methods*, are schematically illustrated. Each construct was transfected into PC-12 cells, and the firefly luciferase activity, normalized to the *R. reniformis* luciferase activity driven by the cotransfected phRL-TK, was determined 24 h after the transfection in the presence or absence of 1 μM 9-*cis*-RA or 1 μM atRA (pGL3-basic, open columns; pP2X2luc, closed columns; and Del-pP2X2luc, gray columns). Each value represents the mean ± S.E.M. of the relative light activities to the control treated pGL3-basic vector activity ($n = 8$; ***, $p < 0.001$ by Student-Newman-Keuls method after two-way ANOVA, compared with the value of control group).

pP2X2luc was decreased to 19-fold greater than pGL3, compared with 25-fold grater than pGL3 for pP2X2luc. These results indicate that the RAREs mediate the transcriptional activity of the 5′-flanking region of the *P2rx2* by retinoids.

The Protein Level of $P2X_2$ in PC-12 Cells Is Increased by 9-*cis*-RA Treatment. To investigate whether 9-*cis*-RA increases the level of $P2X_2$ protein as a consequence of an increase in the mRNA level, we performed Western blot analyses to detect $P2X_2$ protein by using a specific antibody for the $P2X_2$ receptor. The specificity of antibody was confirmed by comparing protein blots of 1321N1 cells transfected or untransfected with rP2X2-GFP. In cells transfected with rP2X2-GFP, a single band is detected at approximately 90 kDa, consistent with the molecular mass sum of $P2X_2$ and GFP, whereas no band was detected in untransfected cells. In PC-12 cells, the antibody detected an intense band at approximately 70 kDa with a weak smear ranging from 60 to 80 kDa that was postulated to be glycosylated $P2X_2$ protein. In PC-12 cells that had been treated with 9-*cis*-RA (1–1000 nM) for 24 h, the $P2X_2$ protein was significantly increased in a concentration-dependent manner up to approximately 65% (n = 4–14; *, $p < 0.05$, **, $p < 0.01$) (Fig. 5) in comparison with the level expressed in control. The increase in the $P2X_2$ receptor protein by 9-*cis*-RA was consistent with that in $P2X_2$ mRNA.

9-*cis*-RA Increased the Amplitude of ATP-Evoked Whole-Cell Current in PC-12 Cells. $P2X_2$ receptors form nonselective cation channels, and ATP evokes an inward current (North, 2002). Thus, to investigate whether 9-*cis*-RA increases the level of $P2X_2$ receptors in PC-12 cells as functional channels, we performed whole-cell patch-clamp recordings to examine the ATP-activated inward current. Treatment of cells with 100 nM 9-*cis*-RA for 24 h significantly increased the amplitude of the ATP-evoked inward current (**, $p < 0.01$; Fig. 6, A and B). The concentration-response curves for the ATP-activated currents in control and 9-*cis*-RA-treated cells showed that 9-*cis*-RA did not change the Hill coefficient (control cells, 1.9; 9-*cis*-RA-treated cells, 2.1) and EC_{50} value (control cells, 33; 9-*cis*-RA-treated cells, 30) but enhanced the maximal response (Fig. 6B). Furthermore, 20 μM PPADS almost completely blocked ATP-induced current, which means PPADS-insensitive $P2X_4$ expression is too low to evoke the whole cell current, even though mRNA expression is detectable by RT-PCR. The membrane capacitance, reversal potential, inward rectification property (data not shown), and activation kinetics estimated from the current trace were not significantly changed in the 9-*cis*-RA-treated cells, compared with untreated controls. These results indicate that the expression of functional $P2X_2$ receptors is increased on the plasma membrane of 9-*cis*-RA-treated PC-12 cells.

9-*cis*-RA Facilitates P2X-Mediated $[Ca^{2+}]_i$ Elevation. $P2X_2$ receptors are reported to be highly permeable to Ca^{2+} (Virginio et al., 1998). We monitored the level of $[Ca^{2+}]_i$ in individual PC-12 cells using the Ca^{2+}-sensitive fluorescent

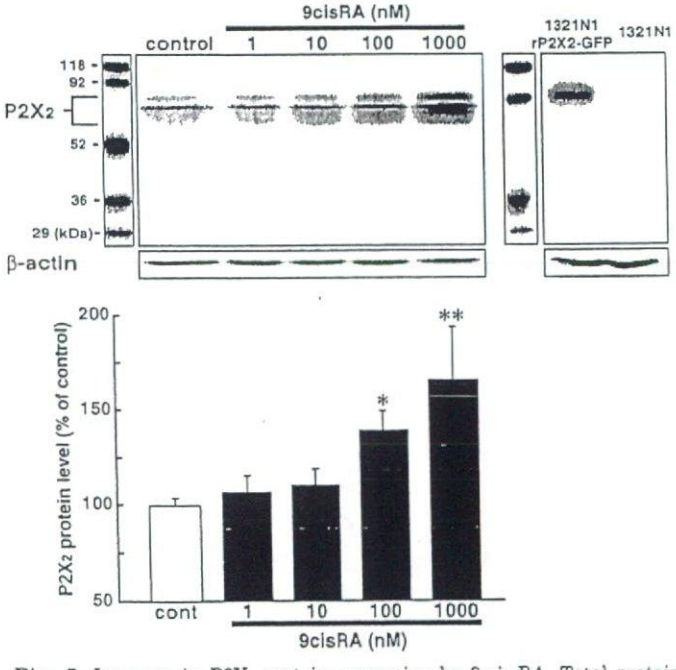

Fig. 5. Increase in $P2X_2$ protein expression by 9-*cis*-RA. Total protein from PC-12 cells treated with or without 9-*cis*-RA (range 1–1000 nM) for 24 h was subjected to Western blot analysis. The proteins of $P2X_2$ receptor and β-actin were detected by their specific antibodies. The intensities of the bands were quantified, and the relative values of $P2X_2$ protein were normalized by the values of the β-actin protein levels for the loading control. The anti-$P2X_2$ antibody was tested on the lysate of 1321N1 cells with or without transfection of P2X2-GFP expression vector. Each set of data represents the mean ± S.E.M. of the percentage over the control (n = 4–14; *, $p < 0.05$; **, $p < 0.01$ by multiple comparisons versus control group using Bonferroni t test after one-way ANOVA).

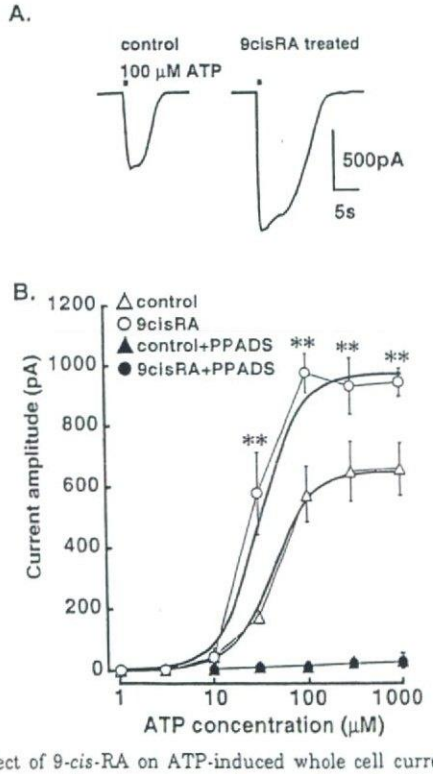

Fig. 6. Effect of 9-*cis*-RA on ATP-induced whole cell current in PC-12 cells. A, representatives traces were the currents evoked by 100 μM ATP in PC-12 cells with or without 100 nM 9-*cis*-RA for 24 h. Cells were voltage-clamped at −60 mV. B, concentration-dependent curves were made by measuring currents elicited by a series of ATP concentrations with or without 20 μM PPADS. Each point represents the mean values ± S.E.M. of the maximum amplitude of the ATP-evoked currents (n = 10–13; **, $p < 0.01$ by t test, compared with the value of the corresponding control group) and was fitted to a sigmoidal curve to calculate Hill coefficient and the EC_{50} values.

dye fura-2 and examined the effects of 9-*cis*-RA on the ATP-evoked $[Ca^{2+}]_i$ elevation. Applying 100 μM ATP produced an increase in the 340/360 emission ratio for fura-2 (n = 21 cells), indicating that ATP caused an increase in $[Ca^{2+}]_i$ in the PC-12 cells (Fig. 7A), as shown previously (Fasolato et al., 1990). Treatment of the cells with 100 nM 9-*cis*-RA for 24 h significantly enhanced the ATP-evoked increase in $[Ca^{2+}]_i$ by approximately 30% (**, $p < 0.01$) (Fig. 7, A and B). PC-12 cells express not only $P2X_2$ but also P2Y (presumably $P2Y_2$) receptors (Raha et al., 1993), both of which increase $[Ca^{2+}]_i$ after their activation. It has been shown that the P2X and P2Y receptor-mediated $[Ca^{2+}]_i$ elevations can be distinguished by using an extracellular recording solution (BSS) without Ca^{2+} to remove P2X component and by treating cells with the phospholipase Cβ inhibitor U-73122 to remove the P2Y component. When Ca^{2+} was not added to the extracellular solution, the increase in $[Ca^{2+}]_i$ evoked by ATP was markedly reduced by ~55% (n = 24 cells) (Fig. 7B). On the other hand, U-73122 (5 μM) reduced the ATP-evoked increase in $[Ca^{2+}]_i$ by approximately 40%. PC-12 cells that had been treated with 9-*cis*-RA did not show any enhancement of the ATP-evoked $[Ca^{2+}]_i$ elevation in the extracellular recording solution without Ca^{2+} but did after treatment with U-73122 (Fig. 7B). Furthermore, inhibition of $P2X_2$ but not $P2X_4$ by 20 μM PPADS reduced ATP-evoked $[Ca^{2+}]_i$ elevation to the level in Ca^{2+}-free BSS both in 9-*cis*-RA-treated or untreated PC-12 cells (Fig. 7B). This result suggests ATP-evoked Ca^{2+} influx through P2X receptors does not include a $P2X_4$ response. Application of 80 mM K^+ evoked the release of DA presumably via activating voltage-dependent Ca^{2+} channels (VDCCs) (Waterman, 2000), but the $[Ca^{2+}]_i$ elevation evoked by 80 mM K^+ was not altered by the treatment with 9-*cis*-RA (Fig. 7B). Together, these results indicate that 9-*cis*-RA up-regulates the expression of $P2X_2$ receptors in PC-12 cells, and activating them by ATP increases Ca^{2+} influx, which contributes to enhancing the neurotransmitter release.

ATP-Induced DA Release from PC-12 Cells Is Enhanced by 9-*cis*-RA Treatment. PC-12 cells are known as a model of neuronal cells (Shafer and Atchison, 1991) and are able to release neurotransmitters such as catecholamines by various extracellular stimuli, including ATP (Nakazawa and Inoue, 1992). The ATP-evoked DA release requires Ca^{2+} influx into cells mediated through opening $P2X_2$ receptor channels but not VDCCs (Inoue et al., 1989). Thus, we investigated whether the ATP-evoked release of DA from PC-12 cells is modulated by 9-*cis*-RA. Stimulation of PC-12 cells with 30 μM ATP for 1 min caused the release of DA as shown previously (Nakazawa and Inoue, 1992). By contrast, in PC-12 cells treated with 100 nM 9-*cis*-RA for 24 h, the ATP-evoked DA release was significantly enhanced by 35.7 ± 7.3% (n = 9; ***, $p < 0.001$; Fig. 8A) without significant change in the total DA content in the cells (94.4 ± 2.4%; p = 0.07; Fig. 8B). 9-*cis*-RA did not affect the spontaneous release of DA from PC-12 cells (control cells, 7.7 ± 2.5%; 9-*cis*-RA-treated cells, 12.8 ± 3.1%; p = 0.23; Fig. 8A).

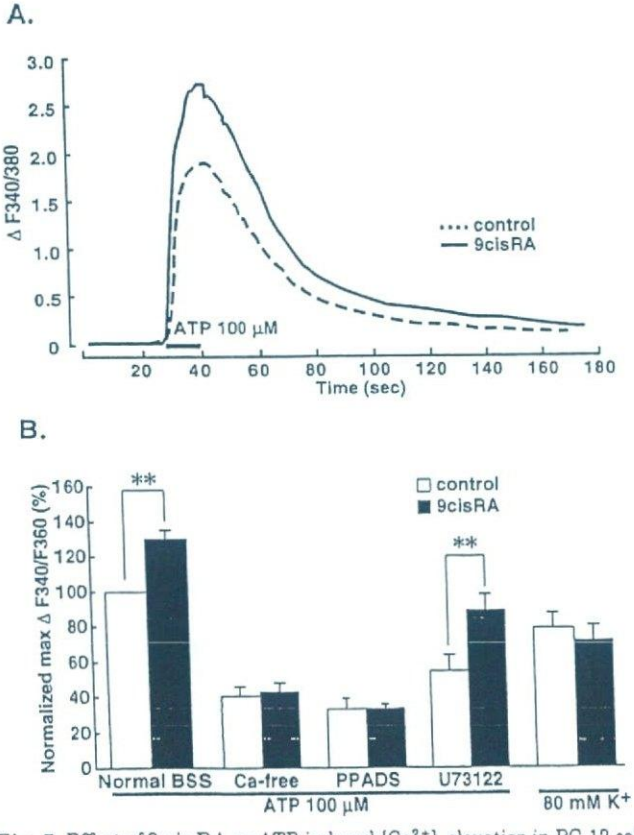

Fig. 7. Effect of 9-*cis*-RA on ATP-induced $[Ca^{2+}]_i$ elevation in PC-12 cells. A, traces showing the records of the fura-2 emission ratios from PC-12 cells onto which 100 μM ATP was applied with or without 100 nM 9-*cis*-RA for 24 h. B, ATP-induced $[Ca^{2+}]_i$ elevations were measured in several different conditions (from left: normal BSS, n = 9; Ca^{2+}-free BSS, n = 5; 20 μM PPADS, n = 3; and 5 μM U-73122, n = 6). To measure the $[Ca^{2+}]_i$ elevation by the depolarizing stimulation, BSS containing a high concentration of potassium (80 mM; n = 5) was applied. Each set of data represents the mean ± S.E.M. of the maximum responses of the ratiometric fura-2 fluorescence ($\Delta F_{340}/\Delta F_{360}$), which were normalized by the value obtained from control PC-12 cells (**, $p < 0.01$ by Student-Newman-Keuls method after two-way ANOVA, compared with the value of control group).

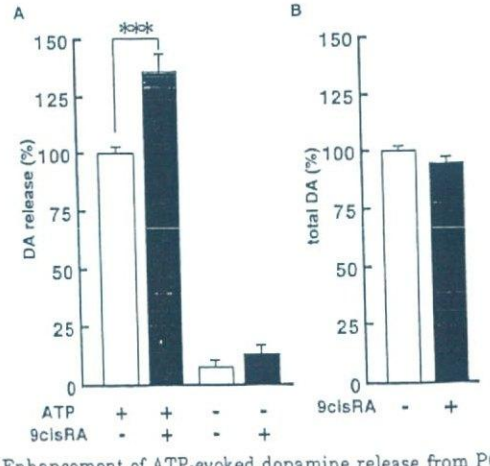

Fig. 8. Enhancement of ATP-evoked dopamine release from PC-12 cells by 9-*cis*-RA. PC-12 cells were incubated with or without 9-*cis*-RA for 24 h. A, the extracellular contents of DA after the application of 30 μM ATP for 1 min were measured with the high-performance liquid chromatography combined with electrochemical detection system. B, measured amount of extracellular and intracellular DA was compared as percentage of 9-*cis*-RA untreated cells. Amount of DA released by ATP was calculated by dividing supernatant values by the sum of supernatant and pellet values and shown as the mean ± S.E.M. of the percentage of the ATP-evoked DA release in 9-*cis*-RA-untreated control cells (n = 9; ***, $p < 0.001$ by t test).

Discussion

In the present study, we first identified three motifs that are canonical consensus sequences of RAREs in the cloned 5′-flanking region of the Wistar rat *P2rx2* and found that 9-*cis*-RA, an endogenous vitamin A derivative, increases the expression of the $P2X_2$ receptor at the transcriptional level in the neuronal model PC-12 cells. The transcriptional effects of 9-*cis*-RA are primarily mediated by activating two families of nuclear receptors, RARs and RXRs (Chambon, 1996). RXRs can form as homodimers and as heterodimers with a number of other nuclear receptors such as thyroid hormone receptor, vitamin D receptor, and RAR (Aranda and Pascual, 2001). Among them, the RXR/RAR heterodimer is known to respond specifically to the RAR activator atRA (Kurokawa et al., 1994). The present study did not show direct binding of RAR and RXR with 5′-flanking region of *P2rx2* but did demonstrate that atRA-treated PC-12 cells also show an increase in the level of $P2X_2$ mRNA expression, suggesting the involvement of RAR in regulating the $P2X_2$ receptor expression in PC-12 cells. PA024 did not increase the level of $P2X_2$ mRNA. A slight, but not significant, increase in $P2X_2$ mRNA was seen. This finding corresponds with the fact that a low activity of PA024 alone was observed in an experiment of retinoid-induced HL-60 differentiation (Ishida et al., 2003). That PA024 scarcely increased the $P2X_2$ mRNA expression is consistent with the findings of previous studies showing that a single application of RXR-selective agonists does not induce gene transcription (Minucci et al., 1997; Ishida et al., 2003) and is supported by the notion that the RXR ligand induces homodimerization of RXR and inhibits heterodimerization without dimerization partner ligands; moreover, a partner ligand is sufficient for heterodimerization (Dong and Noy, 1998). The RXR/RAR heterodimer generally binds to the DR5 RARE (Kurokawa et al., 1994) and also binds to DR1 (Kurokawa et al., 1994). We determined the $P2X_2$ mRNA transcription start site by 5′ RACE, which is located near the site supposed by RefSeq entry (NM_053656). We also had the predictions for some transcription factor binding sites in the 5′-flanking region of the rat *P2rx2* cloned in the current study, which include the DR5 and DR1 sequences located at −2381/−2397 and −2292/−2294 from the transcription start site. It includes other factors such as simian virus 40 protein 1, activator protein-2, nuclear factor-κB, GATA-1, cAMP response element binding protein, GC-box, and initiator sequence as well. Consensus sequences for GC-box and initiator found in our cloned sequence imply that core promoter region would exist near the 5′ end of our cloned sequence. Although the factors we showed here were just the candidates estimated by the electrical search system, we confirmed that the cloned fragment has sensitivity to retinoid treatment and deletion of a fragment containing DR elements lead to abolishing the 9-*cis*-RA- and atRA-mediated and parts of basal transcriptional activities. On the other hand, the deleted fragment also contains DR4, but this is known as a binding site of RXR heterodimerized with nuclear receptors other than RARs (Aranda and Pascual, 2001). Because RAR/RAR homodimerization has not been reported, our series of results could suggest that retinoic acids activate RAR/RXR heterodimers that bind to RAREs (DR5 and/or to DR1-responsive elements) located at the distant place from transcription start site in the promoter region of the *P2rx2*, which in turn work as activators of basal transcription machinery and lead to an increase in the transcription of $P2X_2$ receptors in PC-12 cells.

The biochemical analysis in the present study indicated that the increase in $P2X_2$ transcription resulted in an increase in the level of $P2X_2$ protein. Furthermore, we found that the maximal responses of ATP-evoked currents were enhanced in 9-*cis*-RA-treated PC-12 cells. The inward currents evoked by ATP in PC-12 cells have been demonstrated to be inhibited by suramin, PPADS, and reactive blue 2 (Inoue et al., 1991a,b), a pharmacological profile that fits rat $P2X_2$ receptors, thus suggesting an increase in the level of functional $P2X_2$ protein. This view is strongly supported by the finding that the Ca^{2+} response evoked by ATP in 9-*cis*-RA-treated PC-12 cells was enhanced in the presence of a phospholipase Cβ inhibitor, which abolishes P2Y-mediated Ca^{2+} responses. It could be possible that ATP produces an inward current via activating another P2X subtype. Indeed, in addition to $P2X_2$ receptors $P2X_4$ transcript was also detected in PC-12 cells by our RT-PCR analysis (our unpublished observation). However, 20 μM PPADS almost completely blocked ATP-induced inward currents and $[Ca^{2+}]_i$ elevation, and 9-*cis*-RA did not alter the EC_{50} and Hill coefficient value of the ATP-evoked currents in the PC-12 cells. It is suggested that functional $P2X_4$ receptor is not expressed on the cell membrane. In addition, the mRNA level of the $P2X_4$ receptor in the PC-12 cells was not changed by treatment with 9-*cis*-RA (our unpublished observation). In human cervical epithelial cells, however, the expression of $P2X_4$ mRNA has been reported to be increased by atRA (Gorodeski, 2002). This discrepancy may be due to differences in the species, the basal expression levels of $P2X_4$ receptors, and the expression of RAR and RXR isoforms or the large numbers of coregulators.

In the nervous system, a key function of $P2X_2$ receptors is to increase release of neurotransmitters (Khakh et al., 2003). PC-12 cells are frequently used in studies investigating stimulus-induced vesicular transmitter release (Shafer and Atchison, 1991). We have observed that retinoid significantly enhanced the ATP-evoked release of DA from PC-12 cells. Because retinoid treatment might lead to the changes in many gene transcriptions involved in $[Ca^{2+}]_i$ elevation, exocytotic machinery, or packaging in vesicles, the enhancement of DA release seen in the present study might include multiple interpretations. However, we found that enhancement by 9-*cis*-RA of the $P2X_2$ receptor protein expression level and ATP-activated Ca^{2+} entry was almost identical to that of the ATP-evoked DA release. In addition, 9-*cis*-RA did not affect basal release or the total content of DA in PC-12 cells, suggesting the 9-*cis*-RA affects neither DA biosynthesis nor exocytotic machinery itself. Calcium is one of the most important factors to regulate exocytosis, and we previously showed that the ATP-evoked DA release from PC-12 cells is induced by Ca^{2+} influx directly via $P2X_2$ channels but not via VDCCs (Nakazawa and Inoue, 1992). Together with this, the most probable interpretation of the results could be that 9-*cis*-RA up-regulates $P2X_2$ receptor mRNAs and proteins, thereby leading to enhancement of $P2X_2$ receptor-mediated Ca^{2+} entry and DA release in PC-12 cells.

In native neurons, activating P2X receptors on the presynapses facilitates the release of neurotransmitters by directing Ca^{2+} influx through P2X receptors (Shigetomi and Kato,

2004). This raises the possibility that retinoids may increase the synaptic effects of ATP in modulating neurotransmitter release in native neurons by up-regulating $P2X_2$ receptors. In the adult brain, relatively high levels of retinoic acid are detected (Werner and Deluca, 2002). In particular, in the hippocampal region it has been shown that molecules required for retinoid signaling pathways are expressed (MacDonald et al., 1990; Werner and Deluca, 2002). These include cellular retinol binding proteins that facilitate retinol uptake into cells; retinal dehydrogenases, which are enzymes for the synthesis of retinoids; and cellular retinoic acid binding proteins, which are thought to deliver atRA to RAR in cell nuclei, as well as RARs and RXRs (Dong et al., 1999). The hippocampus is one of the areas where the roles of $P2X_2$ receptors in facilitating neurotransmitter release have been investigated (Khakh et al., 2003; Shigetomi and Kato, 2004). One can question that retinoid effect on the PC-12 cells is the consequence of the differentiation of PC-12 into neurons. However, morphological differentiation of PC-12 cells by retinoic acid requires a period of greater than 3 weeks, and retinoic acid treatment increased differentiation of nerve growth factor-stimulated PC-12 cells (Boniece and Wagner, 1995). Thus, retinoic acid-induced differentiation of PC-12 cells was suggested to be the consequence of complicated molecular modulations. In fact, we observed up-regulation of $P2X_2$ mRNA within 3 h after retinoids treatment. Hence, the effect of retinoids on $P2X_2$ expression could be a notable factor for the differentiation, but it might be distinguished from differentiation of PC-12 cells. The up-regulation of $P2X_2$ receptors by retinoids may be involved in some of the biological effects of retinoids in neuronal function and synaptic plasticity in the nervous system (Wang et al., 2004).

In the present study, we found that the $P2X_2$ receptor is up-regulated by retinoids as a result of increased transcription most likely mediated by the retinoid-activated RAR heterodimerized with RXR acting on RAREs (presumably DR5- and DR1-responsive elements) in the promoter region of *P2rx2* in neuronal cells. An increase in the expression of $P2X_2$ receptors in neuronal cells has recently been implicated in the development of several pathological states, such as brain ischemia (Cavaliere et al., 2003) and chronic pain (Xu and Huang, 2002), and $P2X_2$ receptor might thus be a target for their treatment. It is noteworthy that in an analysis of the human genomic sequence using TESS, we also found a putative DR5-responsive element in the 5′-flanking region of the human *P2X*$_2$ gene. Together, the present results provide the molecular mechanism underlying the expression of $P2X_2$ receptors and may help in understanding the roles of $P2X_2$ receptors in the regulation of neuronal function, synaptic plasticity, and pathophysiology in the nervous system.

Acknowledgments

We thank Dr. Satoko Ohkubo for helpful discussion, Tomoko Obama for assistance with the cell cultures, Yukari Sigemoto-Mogami for technical suggestions, and Dr. Murrell-Lagnado for providing the P2X2-GFP vector.

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PAIN RESEARCH

The Official Journal of the Japanese Association for the Study of Pain

August 2006
Volume 21, Number 3

別　刷

日本疼痛学会誌

第21巻 第3号
〔平成18年8月20日発行〕

PAIN RESEARCH 21 (2006) 133–139

The extracellular ATP-mediated epidermal keratinocyte-to-sensory neuron communication; an involvement of keratinocytic ATP in induction of pain

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[Received 6 January 2006, Accepted 21 July 2006]

Abstract

ATP acts as an intercellular messenger in a variety of cells. Here we characterized the Ca^{2+} wave propagation mediated by extracellular ATP in cultured normal human epidermal keratinocytes (NHEKs) also co-cultured with mouse dorsal root ganglion (DRG) neurons. We also asked about physiological consequence of the ATP-mediated communication in relation to pain by behavioral analysis. Pharmacological characterization showed that NHEKs express functional metabotropic $P2Y_2$ receptors. When a cell was gently stimulated with a glass pipette, an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) was observed, followed by propagating Ca^{2+} waves in neighboring cells in an extracellular ATP-dependent fashion. Using an ATP-imaging technique, the release and diffusion of ATP among NHEKs were confirmed. DRG neurons are known to innervate the epidermis that is mainly composed of keratinocytes. In the co-culture of NHEKs and DRG neurons, mechanical stimulation-evoked Ca^{2+} waves in NHEKs evoked the $[Ca^{2+}]i$ elevation in adjacent DRG neurons, which was also dependent on extracellualr ATP and the activation of $P2Y_2$ receptors. Extracellular ATP is a dominant messenger that forms intercellular Ca^{2+} waves in NHEKs. In addition, Ca^{2+} waves in NHEKs could produce a $[Ca^{2+}]i$ elevation in DRG neurons, suggesting dynamic cross talk between skin and sensory neurons mediated by extracellular ATP. Next we investigated a physiological consequence of the ATP-mediated communications. Injection of the $P2Y_2$ and $P2Y_4$ receptor agonist uridine 5'-triphosphate (UTP) into plantar surface in rats produced the mechanical allodynia in a concentration-dependent manner. The UTP-induced mechanical allodynia was inhibited by the P2 receptor antagonist PPADS (pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate) or antisense oligonucleotide for $P2Y_2$ receptors. Taken together, ATP is a key molecule that mediates pain signaling from skin to sensory neurons.

Key words: ATP; P2Y receptor; Skin-sensory interaction

細胞外ATPを介した表皮ケラチノサイト－知覚神経間コミュニケーション；痛み伝達への関与

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背　景

エネルギーの通貨であるATPは細胞外に放出されて，情報伝達物質としても機能する．ATPを受容する特異的受容体P2受容体は，イオン型P2X受容体とG蛋白共役型P2Y受容体に大別され，それぞれが7および8種類のサブタイプに分類されている．ATPは各種P2受容体に作用して，様々な応答を呈するが，その生理的意義に関する研究は緒についたばかりである．最近ATPが種々の知覚情報シグナルと強くリンクしているとの報告が相次ぎ，特に痛み情報の伝達との関連性で注目を集めている．ATP受容体のなかでも特に，P2X受容体は，痛覚情報の伝達と強く関連していることが指摘されており，実際，一次求心性神経$P2X_3$受容体[1,11]および$P2X_{2/3}$受容体[13]さらに脊髄ミクログリアの$P2X_4$受容体[14]の活性化は，痛覚を伝達する．ところが，一次求心性神経に各種P2Y受容体が存在し[8]，機能していること[6,10]またこれらが痛みとリンクしている可能性が報告されている等，P2Y受容体も痛み情報の制御と関連していること[7,12]が示唆されてきた．一方，知覚神経の末梢端は表皮ケラチノサイトにその終末を伸ばし，皮膚から種々のシグナルを受容していると考えられている．最近表皮ケラチノサイトにP2受容体が存在していること[2~4]，皮膚にのみ存在する感覚受容器がその情報を伝える際にATPを使う可能性が示唆される等[9]，皮膚と感覚神経間のコミュニケーションとATPの関連性に注目が集まっている．

本報告では，表皮ケラチノサイトがATPを用いて細胞間情報連絡を担っていること，またこのケラチノサイト由来ATPは，知覚神経自由終末に作用し，痛覚伝達に関与していることを示す．

実験方法

1. 細胞培養法

ヒト表皮ケラチノサイト(normal human epidermal keratinocytes; NHEKs)は，クラボウ（大阪）から入手し情報に従って培養した．マウス後根神経節細胞（DRG）とNHEKsの共培養は既報に従った[5]．つまり，3T3-J2細胞をfeeder層としてNHEKsを播種し，2日後にNHEKsが完全に接着した後にDRGを播種した．共培養2～3日後に，細胞を実験に供した．

2. 細胞内カルシウム濃度（$[Ca^{2+}]i$）測定

$[Ca^{2+}]i$は既報に従い，fura-2法およびfluo-4を用いた共焦点レーザー法により行った．

3. 免疫組織学的検討

NHEKsおよびDRGsはanti-cytokeratin14 (Cymbus Biotechnology)およびanti-peripherin抗体(Chemicon)を用いて二重染色を行った．

4. ATP放出量の測定

既報[5]に従い，ルシフェリン－ルシフェラーゼ存在下で，機械刺激により惹起される発光をVIMカメラに蓄積し，画像化した．

5. 行動薬理学的検討

痛み行動は既報[13]に従い，von Frey filamentを用いてメカニカルアロディニアを判定した．$P2Y_2$受容体作用薬であるUTPをラット左足底部に投与し（100 μl），15, 30, 60, 120, 180および240分後の痛み行動変化を観察した．アンチセンス（$P2Y_2$, $P2Y_4$および$P2Y_6$）は髄腔より3日間連投した後，痛み実験を行った．カプサイシン神経欠損動物の作成は，新生ラットにカプサイシンを投与することにより行った．

結果および考察

1. NHEKsのP2受容体

ATPおよびそのアナログでNHEKsを刺激すると，ほとんどの細胞で$[Ca^{2+}]i$上昇が認められた．**Fig. 1A**に示すように，ATPおよびUTPは最も強く$[Ca^{2+}]i$を上昇させ，その強さはほぼ同程度であった．ATPγSは次に強く，2MeSATPおよびUDPは非常に弱い作用を有していた．UTPは$P2Y_2$および$P2Y_4$受容体に作用すること，その代謝物UDPは$P2Y_6$受容体には作用するが$P2Y_2$受容体には作用しないこと，さらに$P2Y_2$受容体はUTPおよびATPにより同程度活性化されること等，P2受容体の薬理学的な性質を考えると，本Ca^{2+}応答の主たる責任受容体は$P2Y_2$受容体であると考えられる．本Ca^{2+}応答が確かにP2受容体を介していることを明らかにするために，拮抗薬の作用を検討した．現在$P2Y_2$受容体の選択的拮抗薬は存在しないので，サブクラス非選択的P2受容体拮抗薬suraminおよびPPADS (pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate)の作用を検討し，両拮抗薬がUTPに

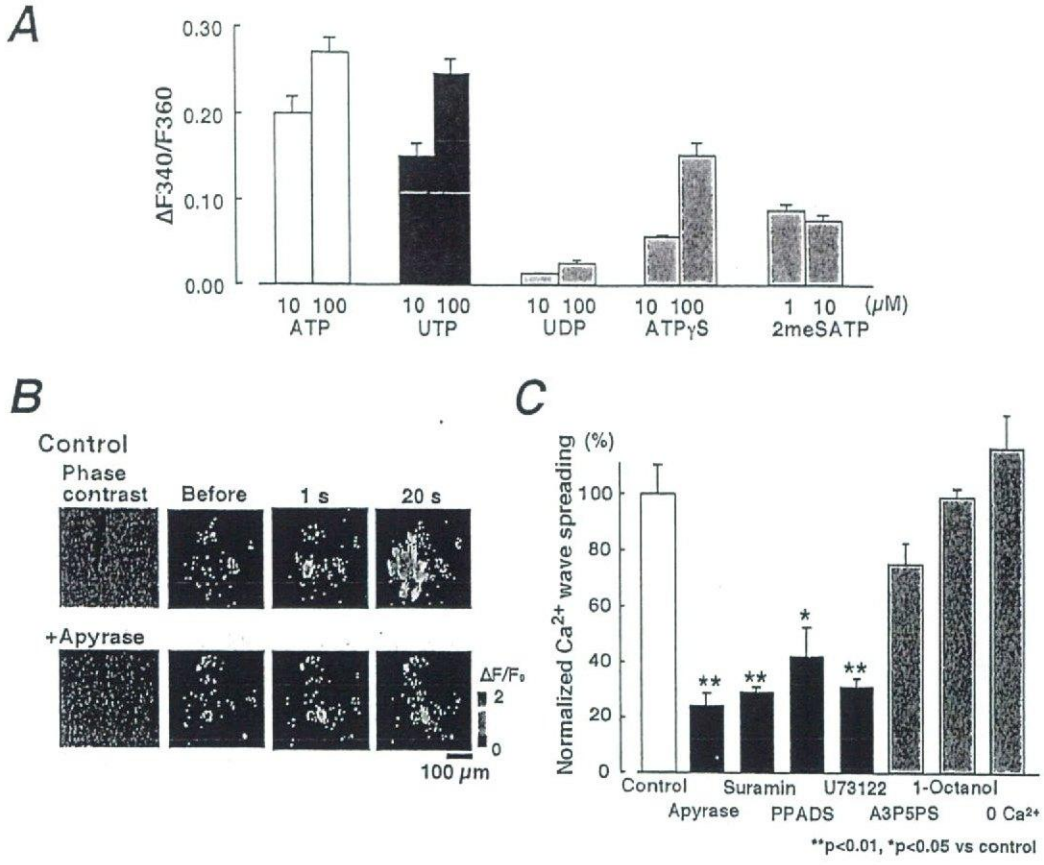

Fig. 1 Pharmacological characterization of increases in $[Ca^{2+}]i$ in NHEKs.
(A) Changes in $[Ca^{2+}]i$ in NHEKs were assessed by a conventional fura-2 fluorescence method. The value show ratiometric fura-2 fluorescence (ΔF340/F360) in responses to various ATP analogues. ATP and UTP were almost equipotent to produce $[Ca^{2+}]i$ in NHEKs. (B) Phase-contrast (left) and pseudo-colored $[Ca^{2+}]i$ images of a field of cultured NHEKs in the absence (upper panels) and presence (lower panels) of apyrase (80 units/ml). Increase in $[Ca^{2+}]i$ was estimated by self-ratio of fluo-4 fluorescence (ΔF/F0), which was obtained by a laser confocal microscopy. A single NHEK was mechanically stimulated. (C) The diameter of the spreading distance of Ca^{2+} wave was calculated in the absence and presence of various chemicals. The average diameter of the Ca^{2+} wave in the control condition was 93.4 ± 9.7 μm (n=12). Suramin (300 μM), PPADS (100 μM) and U73122 (5 μM) also abolished the Ca^{2+} wave propagation, but A3P5PS (100 μM), 1-octanol (500 μM) or removal of extracellular Ca^{2+} (0 Ca^{2+}) failed to inhibit the mechanical stimulation-evoked Ca^{2+} wave in NHEKs (n = 8–12).

よる $P2Y_2$ 受容体刺激を介した $[Ca^{2+}]i$ の上昇を抑制することを確認した（データは示さず）．次に内在性の ATP により同様の応答が観察されるか否かを検討した．共焦点レーザー顕微鏡による fluo4-Ca^{2+} イメージング法を用いた検討により，あるNHEK に機械刺激を加えると，その細胞で $[Ca^{2+}]i$ 上昇が観察され，これはタイムラグを経て周囲のNHEKs へ Ca^{2+} wave となって伝播した（**Fig. 1B**）．この Ca^{2+} wave 伝播は P2 受容体拮抗薬 suramin および PPADS，また ATP 分解酵素 apyrase によりほぼ消失した（**Fig. 1C**）．また $P2Y_1$ 受容体を選択的に抑制する A3P5PS およびギャップ結合を阻害する 1-octanol はこの Ca^{2+} wave 伝播に影響しなかった．したがって，機械刺激による Ca^{2+} wave は ATP および P2 受容体（$P2Y_2$ 受容体）の活性化が重要であること，つまり機械刺激に応答して ATP が放出され，拡散し，周囲の NHEKs の $P2Y_2$ 受容体が活性化されることにより，Ca^{2+} wave が伝播されることが示唆された．そこで次に実際，機械刺激に応じてNHEKs から放出される ATP 量を測定し，可視化した．**Fig. 2** で示すように，ルシフェリン－ルシフェラーゼ存在下で NHEK をガラスピペットで刺激して，30 秒間 ATP に起因するフォトンを蓄積して画像化すると，刺激部位にフォトンが密集した画像が得られた．フォトンの密度と既知の ATP 量の間には高い相関関係が認められることを考慮すると

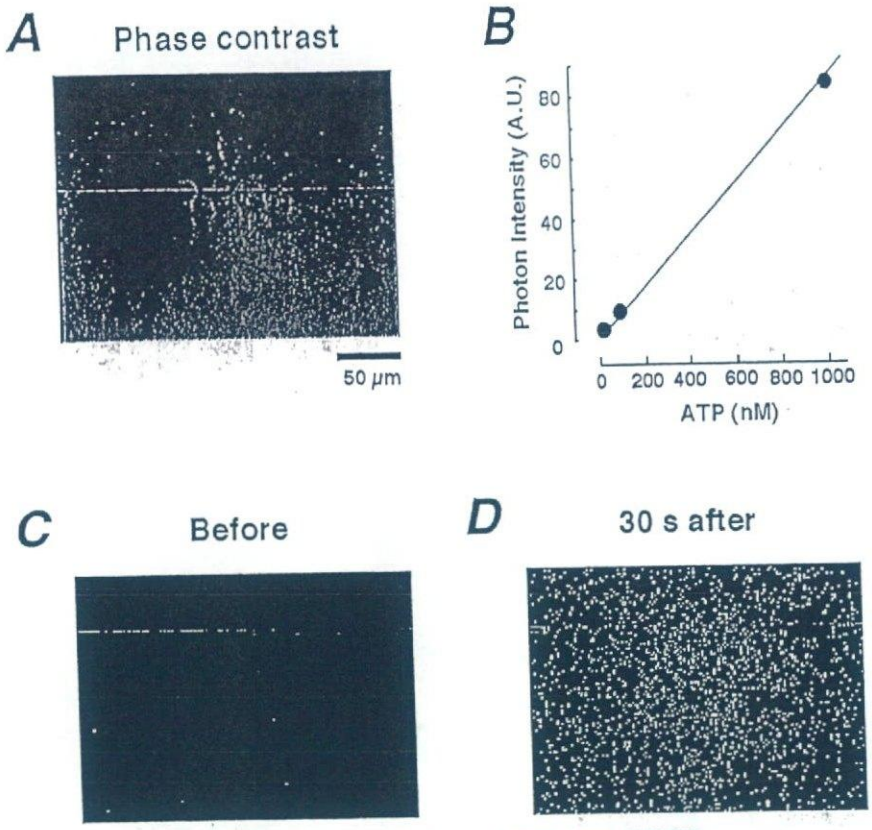

Fig. 2 Visualization of release of ATP from NHEKs.
The images show photon counts (white dots) in a field of NHEKs bathed in luciferin-luciferase reagent before (C) and 30 s after (D) mechanical stimulation. Position of pipette is shown in a phase-contrast image of NHEKs (A). (B) A typical bioluminescence intensity-ATP concentration relationship in this condition. Various concentrations of ATP standard solution were injected in the presence of the luciferin-lucierase reagent, and then photons were accumulated for 30 s.

(**Fig. 2B**)，機械刺激により NHEK は ATP を放出し，これが周辺に拡散していくものと考えられる．

一次求心性神経は P2 受容体を発現しており，またその末梢端は表皮ケラチノサイトにまで達していることが知られている．そこで，DRG ニューロンと NHEKs の共培養系を構築し，NHEKs と DRG ニューロンのコミュニケーションの有無を検討した．Anti-peripherin および anti-cytokerain14 抗体を用いた二重染色法により，小型の C 線維および NHEKs が存在し，これらは互いに物理的に接触していることが明らかとなった（**Fig. 3A**）．このとき，共培養細胞に KCl で脱分極刺激を与えると，ほとんどの peripherin 陽性 DRG ニューロンで $[Ca^{2+}]i$ 上昇が観察されたが，cytokeratin14 陽性 NHEKs では応答が観察されなかった（**Fig. 3B**）．$P2Y_2$ 作用薬 UTP で刺激を加えると，DRG ニューロンでも，NHEKs でも $[Ca^{2+}]i$ 上昇が観察され，これは P2Y 受容体の拮抗薬 suramin で抑制された．つまり小型 DRG ニューロンおよび NHEKs 両者共に機能的 $P2Y_2$ 受容体を発現していることが明らかとなった．

機械刺激を受容した NHEKs は ATP を放出し，細胞間に Ca^{2+} wave を伝播させる．したがって，共培養系を用いた系において，NHEKs の機械受容が，ATP を介して DRG ニューロンに情報を伝えるか否かの検討を行った．共焦点レーザー顕微鏡による Ca^{2+} イメージング法により，単一 NHEK の機械刺激により（**Fig. 3C** および **Fig. 3D** の K1），Ca^{2+} wave の伝播が近傍の NHEKs（**Fig. 3D** の K2 および K3）および DRG ニューロン（**Fig. 3D** の N4）に伝播すること，これが apyrase（**Fig. 3D** の右）および suramin（7.4 ± 1.1% of control, n=7, $p<0.01$）により抑制されたことから，NHEKs と DRG ニューロン間のコミュニケーションが ATP/$P2Y_2$ 受容体依存的に起きていることが明らかとなった（**Fig. 3**）．

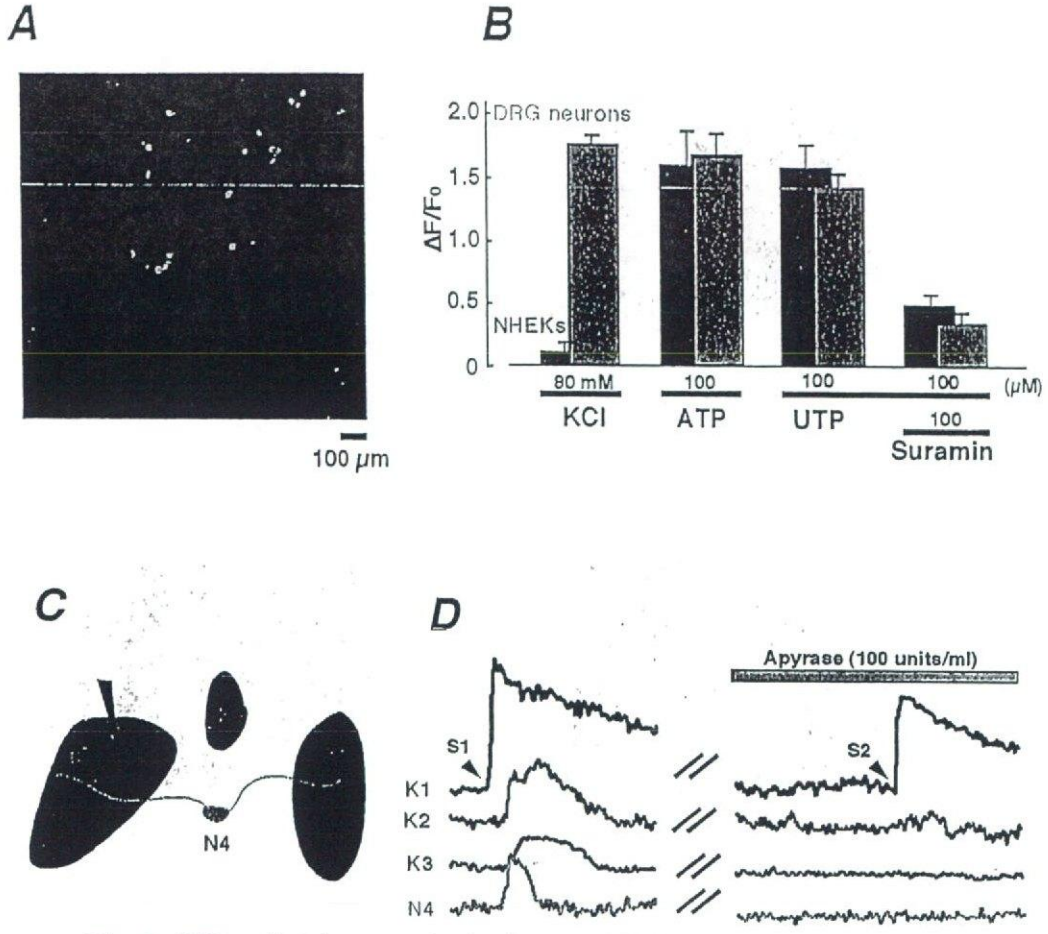

Fig. 3 ATP-mediated communication between DRG neurons and NHEKs.
(A) After some Ca^{2+} imaging experiments, cells were fixed and stained with anti-cytokeratin14 (red) and anti-peripherin (green) antibodies for confirming NHEKs and small-sized DRG neurons, respectively. **(B)** Summary of Ca^{2+} responses obtained from self ratios of fluo-4 fluorescence in anti-peripherin positive DRG neuron (green) and anti-cytokeratin14 positive NHEK (red). Firstly, cells were stimulated with 80 mM KCl for 3 s. Then, they were stimulated with 100 μM ATP and UTP for 10 s separated by 5 min. Finally, UTP (100 μM) was applied to the cells in the presence of 100 μM suramin. Both ATP and UTP produced elevations in $[Ca^{2+}]i$ in about 71% of the DRG neurons (37 out of 52 cells tested) and 73% of the NHEKs (58 out of 79 cells tested) in the co-cultured cells. **(C)** Schematic images of NHEKs (K1–K3) and DRG neuron (N4) were shown. Black arrow shows the position of the micropipette for stimulation. **(D)** The graph shows individual traces of self ratios of fluo-4 fluorescence in keratinocytes (K1–K3) and DRG neuron (N4) shown in C. Keratinocyte 1 was mechanically stimulated twice (arrowheads S1 and S2) separated by 5 min. The first and second mechanical stimulation was performed in the absence and presence of 100 units/ml apyrase (gray horizontal bar).

2. P2Y 受容体と痛覚

皮膚から放出された ATP は，一次求心性神経に到達し，$P2Y_2$ 受容体を活性化し得る．それでは，一次求心性神経における $P2Y_2$ 受容体活性化はどのような生理機能とリンクしているのだろうか？前述したように，一次求心性神経に発現している $P2X_3$ および $P2X_{2/3}$ 受容体が，痛覚伝達と密接にリンクしていることは良く知られている[1,11,13,14]．一方，初代培養 DRG ニューロンには $P2Y_2$ 受容体も存在していた．つまり，$P2Y_2$ 受容体作用薬 UTP の刺激により初代培養神経細胞で $[Ca^{2+}]i$ 上昇が惹起され，これは P2 受容体拮抗薬 suramin および PPADS で抑制された．興味深いことに，UTP による Ca^{2+} 応答は，一次感覚神経の中でも小型のカプサイシン感受性神経にほぼ限局していた．これらは UTP が小型の DRG ニューロンで $[Ca^{2+}]i$ を上昇させ[10]，cyclic AMP response element binding protein を活性化する[6]との先行実験データとよく一致している．

次に $P2Y_2$ 受容体活性化と痛覚伝達の関連性について検討した．ラットの後肢足底部に UTP を投与

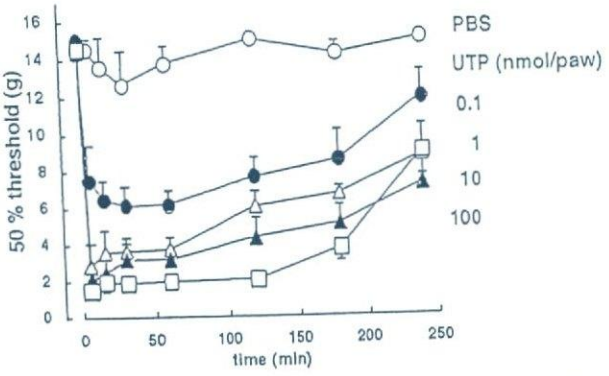

Fig. 4 Mechanical allodynia induced by an intraplantar injection of UTP in rats.
An injection of UTP into the plantar surface of the hindpaw produced mechanical allodynia in a concentration dependent fashion over a concentration range from 0.1 to 100 nmol/paw. Time course of the mean ± s.e.m. paw withdrawal threshold (g) for von Frey filaments after the UTP injection is shown.

すると，濃度依存的にメカニカルアロディニアが観察された（**Fig. 4**）．このUTPによるアロディニアは，$P2X_{2/3}$受容体を介するアロディニアと同程度に強く，しかもより持続的であった[13]．UTP投与直後から240分後までのアロディニアの総和を定量化して（$AUC_{0\text{-}240}$: 0～240分までのアロディニア値をAUCで示した），P2受容体拮抗薬PPADSの効果を検討したところ，PPADSの前処置は，このアロディニアを約8割抑制していた（$AUC_{0\text{-}240}$: 22.8 ± 5.9% of UTP alone, n=8, $p<0.01$）．またカプサイシン感受性神経破壊ラットでは，UTPによるアロディニアが有意に抑制されたことから（$AUC_{0\text{-}240}$: 49.7 ± 9.1% of control rats, n=7, $p<0.05$），小型のカプサイシン感受性一次求心性神経（C線維）がUTPにより興奮し，アロディニアを誘発していると考えられる．責任受容体として，UTPが作用する$P2Y_2$および$P2Y_4$，またUTPの分解産物UDPが作用する$P2Y_6$受容体が挙げられるが，$P2Y_2$アンチセンスオリゴはUTPによるアロディニアを強く抑制するが（$AUC_{0\text{-}240}$: 27.6 ± 6.6% of UTP alone, n=8, $p<0.01$），$P2Y_4$アンチセンスオリゴは影響しないこと（$AUC_{0\text{-}240}$: 94.8 ± 12.8% of UTP alone, n=7, $p>0.05$），$P2Y_6$作用薬UDPはアロディニアを誘発しないことから（$AUC_{0\text{-}240}$: 6.8 ± 4.8% of UTP alone, n=8, $p>0.05$），$P2Y_2$受容体がアロディニア形成の責任受容体と考えられる．

$P2Y_2$活性化が如何にして痛みを伝えるかに関するメカニズムは不明である．最近，P2Y受容体（$P2Y_1$あるいは$P2Y_2$）活性化がPKCを介してカプサイシン受容体TRPV1の反応を増強し，熱刺激に対する痛覚過敏を引き起こすことが報告された[7,12]．しかしUTPによるアロディニアはPKC阻害薬で全く影響されず（$AUC_{0\text{-}240}$: 94.8 ± 13.6% of UTP alone, n=8, $p>0.05$），またTRPV1拮抗薬による影響を受けない（$AUC_{0\text{-}240}$: 89.8 ± 14.6% of UTP alone, n=6, $p>0.05$）．したがって，メカニカルアロディニア発現には必ずしもTRPV1の感受性亢進が関与していない可能性が考えられる．また大型DRGニューロンには$P2Y_1$受容体が発現しており，この$P2Y_1$受容体が触刺激の応答亢進とリンクしている可能性がアフリカツメガエル卵母細胞や，カエル下肢皮膚－感覚神経系で明らかにされている[8]．したがって，同様のメカニズムが小型DRGニューロンの$P2Y_2$受容体を介するシグナル経路に存在している可能性も考えられる．

また，実際の報告はないものの，P2Y受容体と痛覚伝達系を考えたときに，アストロサイトが標的となり得る．ATPは脊髄神経細胞の約1/3で反応性を示すのに対して，脊髄後角アストロサイトでは殆ど全細胞でATPでCa^{2+}応答が認められる．アストロサイトでのATP反応には，$P2Y_1$および$P2Y_2$受容体が介在し，G蛋白質/PLC/$InsP_3$系の活性化が関与している[5]．さらにアストロサイトはATPを放出し，$P2Y_1$および$P2Y_2$受容体を介して隣接するアスロトサイトおよび神経細胞にCa^{2+} waveを伝播させる．アストロサイトは，神経因性疼痛モデルや最近報告された癌性疼痛モデル動物の脊髄内で著明な活性化が認められること，$P2Y_1$受容体が神経因性疼痛モデルの脊髄後角で発現増加分子の1つとしてcDNAマイクロアレイで検出されたこと等を考慮すると，アストロサイトのATP/P2Y受容体シグナル系も痛覚伝達と強くリンクしている可能性が考えられる．

以上，表皮ケラチノサイトが機械刺激に応答してATPを放出し$P2Y_2$受容体を刺激することにより細胞間で情報を交換していること，さらにこのシグナルが皮膚に入力する小型一次求心性神経の$P2Y_2$受容体を活性化し，メカニカルアロディニアを引き起こす可能性を示した．皮膚は，通常の機械刺激だけ

でなく，傷害，紫外線照射等により，容易にATPを放出する．ATPによるシグナルが，皮膚の機能だけでなく，皮膚－知覚神経細胞連関に強く影響すること，またこれまで痛みとの関連性ではイオンチャネル型P2X受容体が注目されていたが，P2Y受容体も痛覚伝達と密接にリンクしている可能性が示唆された．

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特集 痛みの研究最前線

G蛋白質共役型ATP受容体と痛み

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ペインクリニック
Vol.27 No.5（2006.5）別刷

真興交易㈱医書出版部

G蛋白質共役型ATP受容体と痛み

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要　旨
エネルギーの通貨であるATPは，細胞外に放出されて情報伝達物質としても機能する．このATPとその受容体であるP2受容体を介する情報伝達は，知覚情報伝達と強くリンクしており，特にイオンチャネル型P2X受容体は，末梢および中枢の痛覚情報伝達との強い関連性から，特に注目されている．一次求心性神経には，G蛋白質共役型ATP受容体であるP2Y受容体も存在しているが，P2Y受容体と痛覚伝達に関しては情報が少ない．本稿では痛覚伝達とATPに関し，特にP2Y受容体に注目して最近の知見を報告する．
（ペインクリニック 27：560-568，2006）
キーワード：ATP，P2Y受容体，皮膚-知覚神経連関

はじめに

ATP（アデノシン三リン酸）はあらゆる細胞に存在するエネルギーの通貨であるが，1993年に最初のATP受容体（P2受容体）cDNAがクローニングされて以来，その細胞間情報伝達物質として役割が注目されている．P2受容体は，イオンチャネル型P2X受容体とG蛋白質共役型P2Y受容体に大別され，それぞれが7種類と8種類のサブタイプに分類されている（図1）．これらは中枢・末梢を問わず，生体のあらゆる部分に多様に発現しており，そこでの種々の生理反応と密接にリンクしていると考えられている．ATP/P2受容体が担う生理的役割に関してはまだまだ不明な点が多いが，現在，最も解明が進んでいる分野の一つが，痛覚情報伝達との関連性である．1995年に，イオンチャネル型P2X受容体サブクラスの$P2X_3$受容体が末梢一次求心性感覚神経に局在していることが明らかとされたのに端を発し[1,2]，$P2X_3$受容体[1-3]，さらには$P2X_{2/3}$受容体[4]と，特に急性期の疼痛に関しての多くの知見が集積された．また，神経因性疼痛などの慢性疼痛の基礎的研究では，末梢一次求心性感覚神経である坐骨神経や脊髄神経などを人為的に損傷させる動物モデルがよく用いられるが，このような病態モデル動物で誘発される異痛症（アロディニア）の発生および維持には，脊髄後角ミクログリアの$P2X_4$受容体の発現亢進が中心的な役割を果たしていることが明らかとなった[5,6]．このようにP2X受容体系は，末梢の痛み発生・調節に大きく影響を与えるだけでなく，脊髄における知覚情報の伝達の制御と深く関連し，慢性疼痛発生・維持と強

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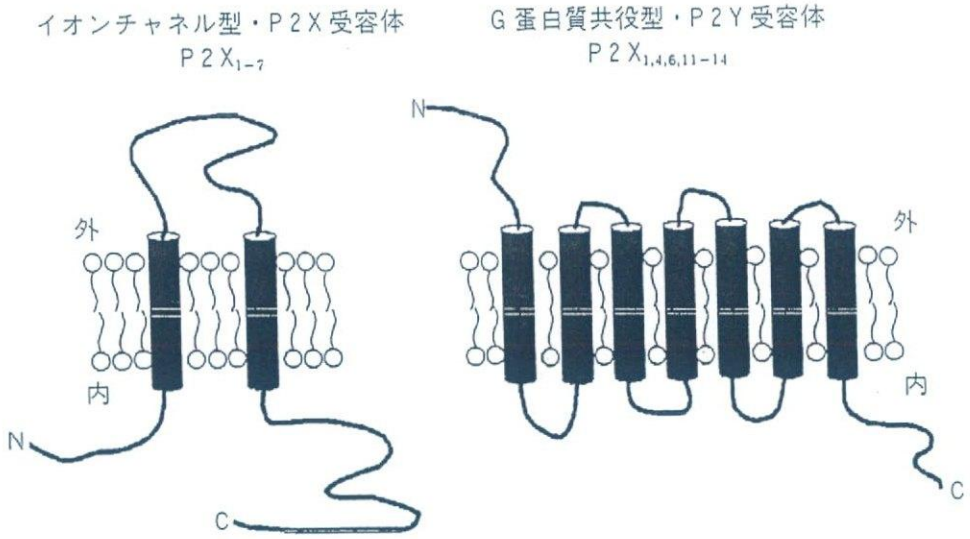

図1　ATP受容体の模式図
イオンチャネル型P2X受容体は$P2X_{1-7}$の7種類，G蛋白質共役型P2Y受容体は$P2Y_{1,2,4,6,11-14}$の8種類が知られている

く関わっている重要な分子である．一方，一次求心性神経およびその周辺細胞にはG蛋白質共役型P2Y受容体も存在していることが明らかとなったが，P2Y受容体と痛覚伝達に関する報告は少ない．本稿では，一次求心性神経の痛覚伝達とATPとの関連性について，特にP2Y受容体に注目して最近の知見を報告する．

1．DRGのP2Y受容体

一次求心性神経にはP2Y受容体も発現している．P2Y受容体と知覚情報の伝達に関する最初の知見は，NakamuraとStrittmatter[7]の報告である．彼らは$P2Y_1$受容体mRNAが，大型後根神経節細胞（dorsal root ganglion：DRG）神経細胞のマーカーであるRT-97に陽性の細胞に発現していることを見出した．さらに，P2Y1受容体を強制発現させたアフリカツメガエル卵母細胞では，触刺激により惹起される内向電流を，低濃度ATP（1 μM未満）が強く増強する．また，カエル下肢皮膚の触刺激による感覚神経の興奮もATPで顕著に増大し，ATP受容体拮抗薬（suramin：pyridoxal-phosphate-6-azophenyl-2',4'-disulfonic acid（PPADS））およびATP分解酵素apyraseにより消失する．また，マウスの急性単離DRG細胞においても，比較的大型のDRG神経細胞で，ATP刺激により細胞内カルシウム濃度上昇が認められ，$InsP_3$受容体拮抗薬および小胞体Ca^{2+}-ATPase阻害薬により消失する．当研究室においても同様の結果を得ており，これらの知見は，$P2Y_1$受容体がAβ線維由来の非侵害性刺激（触刺激）の伝達制御に深く関与している可能性を示唆している．

前述したように，$P2X_3$受容体は小型DRG神経細胞[1,2]に，$P2X_2$および$P2X_3$受容体は中型のDRG神経細胞[4]に強く発現している．痛覚を伝えるこれらの小型DRG神経細胞に，P2Y受容体は発現しているのだろうか？ATPで小型および中型DRG神経細胞を刺激して，細胞内Ca^{2+}濃度（$[Ca^{2+}]i$）変化を観察すると，80％程度のDRG神経細胞で$[Ca^{2+}]i$上昇が観察され

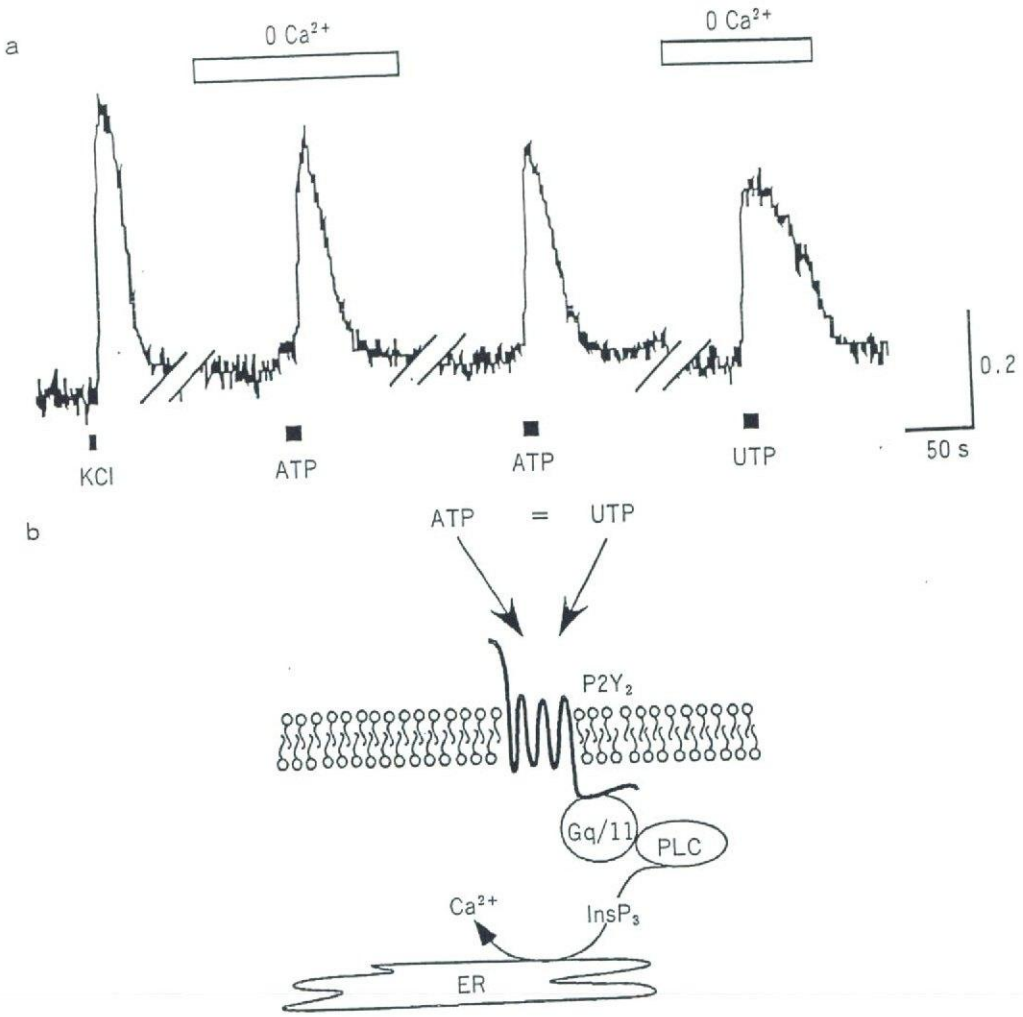

図2　ATP および UTP により惹起される小型 DRG 神経細胞の $[Ca^{2+}]i$ 上昇

a：Fura 2 法により測定した小型 DRG 神経細胞 $[Ca^{2+}]i$ 変動の代表例．ATP (100 μM) により惹起される $[Ca^{2+}]i$ 上昇は，細胞外 Ca^{2+}除去 (0 Ca^{2+}) により影響を受けず，同様の応答は UTP 刺激を行った場合にも観察される．

b：この $[Ca^{2+}]i$ 上昇に関与する責任受容体 $P2Y_2$受容体の模式図．$P2Y_2$受容体は，Gq/11－phospholipase C (PLC) とリンクしており，その刺激により inositol 1, 4, 5-trisphosphate ($InsP_3$) が産生され，小胞体 (ER) $InsP_3$受容体から Ca^{2+}が放出される．ATP および UTP は同程度の強さでこの受容体を活性化する

る．興味深いことに，これらの $[Ca^{2+}]i$ 応答は，細胞外 Ca^{2+}を除いても観察され，また，phospholipase C (PLC) 阻害薬 U73122，小胞体 Ca^{2+}- ATPase 阻害剤 cyclopiazonic acid (CPA) および P2 受容体拮抗薬 suramin で抑制された．さらに，uridine 5'-diphosphate (UTP) によってもほぼ同様の $[Ca^{2+}]i$ 上昇が認められた (図2)．したがって，小型 DRG 神経細胞には，PLC/$InsP_3$系と共役した UTP 感受性の P2Y 受容体，つまり $P2Y_2$ ($P2Y_4$) 受容体が共発現している (図2b)．$P2Y_2$受容体は，ATP および UTP によりほぼ同程度の強さで

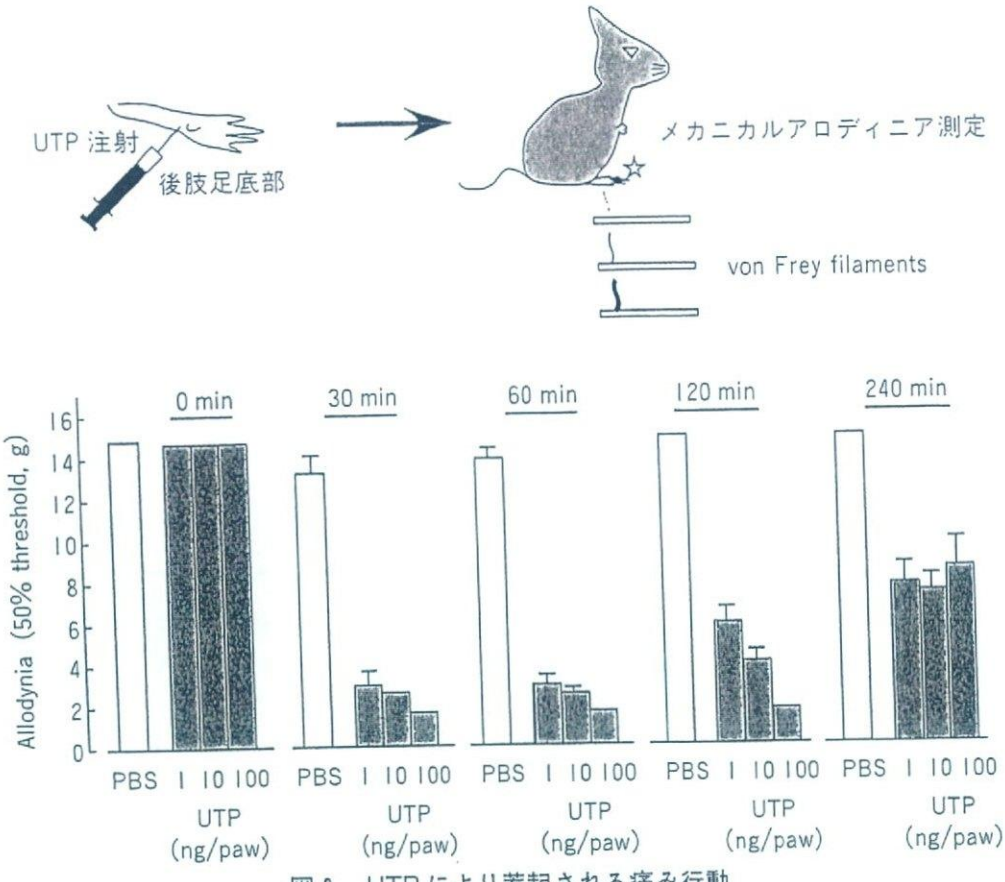

図3　UTPにより惹起される痛み行動

a：実験の模式図．ラット後肢足底部に，各種濃度のUTP（1～100 ng/paw）を注入した後，一定時間ごとに太さの異なるvon Frey filamentsによる触刺激を行い，それに対する足上げ行動を異痛症（allodynia：アロディニア）として観察した．

b：UTP投与後30，60，120および240分後のアロディニア．値は50％閾値（g）を示す

活性化される．これは，小型のDRG神経細胞でUTPが細胞内Ca^{2+}動員を惹起するという報告によっても支持される[8,9]．興味深いことには，UTP応答DRG神経細胞の多くが，TRPV1受容体作用薬カプサイシンにも応答したことである．こられの知見は，P2X受容体系だけでなく，P2Y受容体，特に$P2Y_2$受容体が痛覚伝達の制御と関係していることを強く示唆するものである．

2．痛みとP2Y受容体

DRG神経細胞の$P2Y_1$受容体を刺激すると，N型電位依存性Ca^{2+}チャネル[10]および$P2X_3$受容体[11]が抑制され，知覚情報の伝達が阻害される．それでは，UTP刺激によって$P2Y_2$受容体を活性化すると，知覚情報はどのように制御されるのだろうか？痛みは出るのであろうか．最近，われわれは，ラットの後肢足底部にUTPを投与し，太さの異なるvon Frey filamentで一次求心性神経を刺激することで，触刺激に対する痛み応答，アロディニアが発現することを見出した（図3）．同様のアロディニアは，中型DRG神経細胞に存在する$P2X_{2/3}$受容体を刺激した場合にも惹起されるが[4]，UTPにより惹起されるアロディニアは同程度かそれ以上の強さ