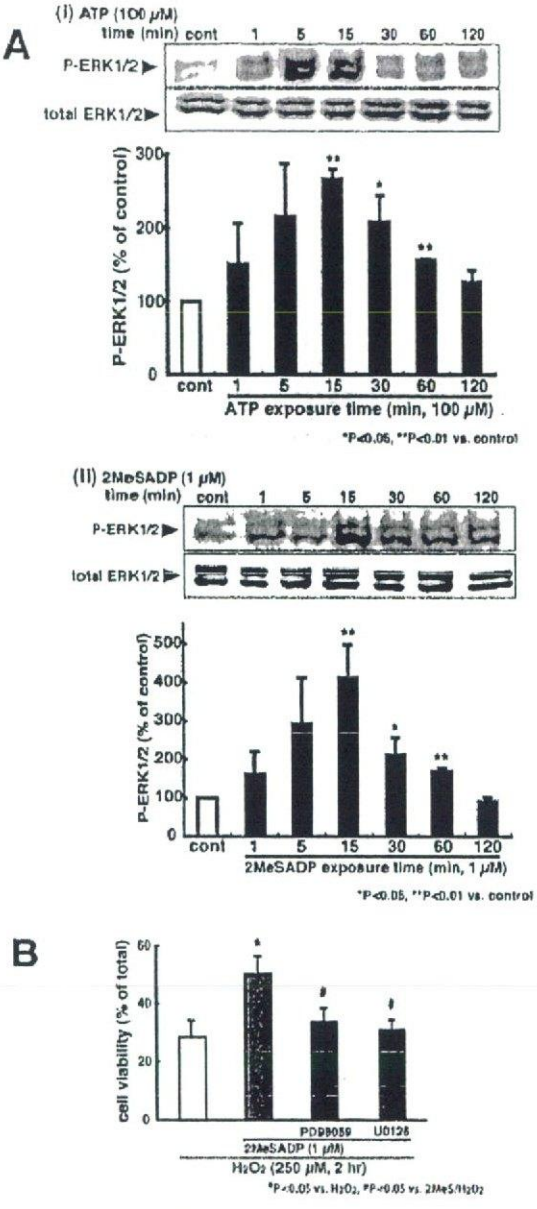

Fig. 6. Analysis of ATP-stimulated ERK1/2 activation in astrocytes. **A**: The time course of ATP-induced ERK1/2 activation. ATP (100 μM) activated ERK1/2 transiently (1–15 min after stimulation) (i). 2MeSADP (1 μM) also activated ERK1/2 transiently (1–15 min after stimulation) (ii). ATP and 2MeSADP did not affect the total amount of ERK1/2. Asterisks show significant difference from control (*$P < 0.05$, **$P < 0.01$ vs. control, Student's *t*-test). **B**: The effect of 2MeSADP-stimulated ERK1/2 on the $P2Y_1$ receptor-mediated cytoprotective effect. The MEK1/2 inhibitors PD98059 (10 μM) and U0126 (20 μM) strongly inhibited the protective effect induced by 2MeSADP (1 μM, 24 h). The MEK1/2 inhibitors were added to the cells 1 h before the 2MeSADP treatment and were washed out before the H_2O_2 treatment. Asterisks show significant difference from the response evoked by H_2O_2 (*$P < 0.05$ vs. 250 μM H_2O_2, Student's *t*-test). Sharps show significant difference from 2MeSADP/H_2O_2 (#$P < 0.05$ vs. 2MeSADP/H_2O_2, Student's *t*-test). Results were expressed as means + SEM of triplicate measurements ($n = 3$).

TABLE 1. List of PTP Genes Upregulated by ATP in Astrocytes

Title	Fold increase (RT-PCR)	Gene ontology[a]
Protein tyrosine phosphatase 4a1	1.7 (5.7)	Protein tyrosine phosphatase activity (GO:0004725) Nucleus (GO:0005634)
Protein tyrosine phosphatase, receptor type, O	1.8 (4.4)	Protein tyrosine phosphatase activity (GO:0004725) Nervous system development (GO:0007399)
	1.6	Protein tyrosine phosphatase activity (GO:0004725) Nervous system development (GO:0007399)

[a]GO ontology defined by Gene Ontology Consortium (www.godatabase.org/cgi-bin/amigo/go.cgi).

(Heffetz et al., 1990; Shisheva and Shechter, 1993) on the ATP- and 2MeSADP-induced cytoprotective action in astrocytes. Na_3VO_4 concentration-dependently reversed the cytoprotective effect by ATP and 2MeSADP against H_2O_2 (Fig. 7B). Na_3VO_4 alone did not affect the cell viability of the astrocytes (light gray column). Additionally, Na_3VO_4 also reversed the inhibition by ATP or 2MeSADP of the H_2O_2-evoked phosphorylation of ERK1/2 in astrocytes (Fig. 7C). Then, we studied the effect of the selective $P2Y_1$ receptor antagonist MRS2179 (10 μM) on the PTP activity. H_2O_2 decreased the PTP activity to about one half. 2MeSADP restored the PTP activity and this effect was reversed by MRS2179 [Fig. 7D(1)]. As previously reported, $P2Y_1$ receptor activation also upregulates oxidoreductases such as TrxR, thereby protecting astrocytes against H_2O_2 (Shinozaki et al., 2005). The TrxR inhibitor auranofin (1 μM) also reversed the 2MeSADP-restored PTP activity. Furthermore, the thiol-containing antioxidant *N*-acetyl cystein (NAC, 10 mM) restored the H_2O_2-decreased PTP activity. In addition, we studied the effect of 2MeSADP, MRS2179 (10 μM), auranofin (1 μM), and NAC (10 mM) on the H_2O_2-evoked ERK1/2 activation [Fig. 7D(2)]. The inhibition of P-ERK1/2 activation by 2MeSADP (1 μM) was reversed by MRS2179 (10 μM) and auranofin (1 μM). In contrast, the H_2O_2-evoked ERK1/2 activation was prevented by NAC (10 mM). MRS2179 was added to the cells 1 h before the 2MeSADP treatment. Auranofin and NAC were added to the cells 1 h before H_2O_2 stimulation.

Involvement of src Tyrosine Kinase Family on H_2O_2-Evoked Cell Death and ERK1/2 Activation

Using Western blotting analysis, we studied whether H_2O_2 induces protein tyrosine phosphorylation. H_2O_2 evoked protein tyrosine phosphorylation, which was inhibited by pretreatment with ATP (100 μM, 24 h) or 2MeSADP (1 μM, 24 h) (Fig. 8A). This ATP- and

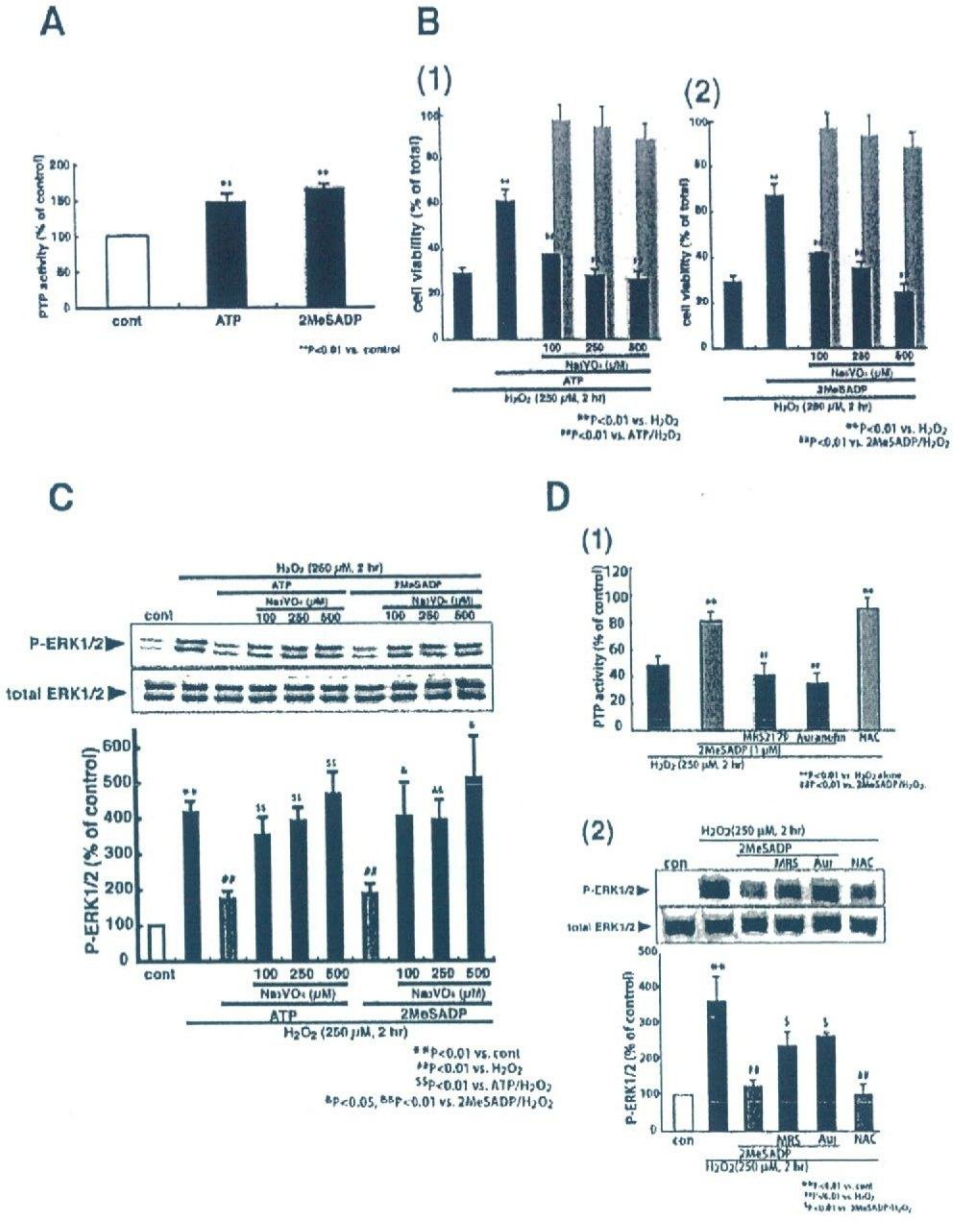

Fig. 7. The effect of ATP-induced PTP upregulation on H_2O_2-evoked cell death and ERK1/2 activation. **A**: ATP- and 2MeSADP-induced an increase of the PTP activity. ATP (100 μM, 24 h) and 2MeSADP (1 μM, 24 h) significantly increased the PTP activity (ATP: 148.3% ± 8.8%; 2MeSADP: 168.8% ± 4.8%, vs. control). Asterisks show significant difference from control ($^{**}P < 0.01$ vs. control, Student's *t*-test). **B**: PTP participates in ATP- and 2MeSADP-induced cytoprotective effect. The PTP inhibitor Na_3VO_4 (100–500 μM, 1 h) reversed the ATP(100 μM, 24 h)- and 2MeSADP(1 μM, 24 h)-induced protective effect concentration-dependently (black column). Na_3VO_4 alone did not affect the cell viability of the astrocytes (light gray column). Asterisks show significant difference from the response evoked by H_2O_2 ($^{**}P < 0.01$ vs. 250 μM H_2O_2, Student's *t*-test). Sharps show significant difference from the response by ATP/H_2O_2 or 2MeSADP/H_2O_2 ($^{\#\#}P < 0.01$ vs. ATP/H_2O_2 or 2MeSADP/H_2O_2, Student's *t*-test.). **C**: PTP participates in ATP- and 2MeSADP-induced inhibition of H_2O_2-evoked ERK1/2 activation. Na_3VO_4 (100–500 μM, 1 h) reversed the ATP(100 μM, 24 h)- and 2MeSADP(1 μM, 24 h)-induced inhibition of H_2O_2-evoked ERK1/2 activation. The cells were treated with ATP and 2MeSADP 24 h before and during H_2O_2 treatment. Na_3VO_4 was added to the cells 1 h before the H_2O_2 (250 μM) treatment. Asterisks show significant difference from control ($^{**}P < 0.01$ vs. control, Student's *t*-test). Sharps show significant difference from H_2O_2 ($^{\#\#}P < 0.01$ vs. H_2O_2, Student's *t*-test). Dollar marks show significant difference from ATP/H_2O_2 or 2MeSADP/H_2O_2 ($^{\$}P < 0.05$, $^{\$\$}P < 0.01$ vs. ATP/H_2O_2 or 2MeSADP/H_2O_2, Student's *t*-test.). **D**: The effect of MRS2179, auranofin, and NAC on PTP and ERK1/2 activity. (1) 2MeSADP (1 μM) restored the H_2O_2 (250 μM, 2 h)-decreased PTP activity and the effect was reversed by MRS2179 (10 μM) and auranofin (1 μM). NAC (10 mM) restored the PTP activity decreased by H_2O_2. Asterisks show significant difference from the response evoked by H_2O_2 ($^{**}P < 0.01$ vs. 250 μM H_2O_2, Student's *t*-test). Sharps show significant difference from the response by 2MeSADP/H_2O_2 ($^{\#\#}P < 0.01$ vs. 2MeSADP/H_2O_2). (2) The effect of MRS2179, auranofin, and NAC on H_2O_2-evoked ERK1/2 activation. MRS2179 (10 μM) and auranofin (1 μM) reversed the ERK1/2 activity inhibited by 2MeSADP. NAC (10 mM) prevented the H_2O_2-evoked ERK1/2 activation. Asterisks show significant difference from control ($^{**}P < 0.01$ vs. control, Student's *t*-test). Sharps show significant difference from H_2O_2 ($^{\#\#}P < 0.01$ vs. H_2O_2, Student's *t*-test). Dollar marks show significant difference from 2MeSADP/H_2O_2 ($^{\$}P < 0.05$ vs. 2MeSADP/H_2O_2, Student's Results were expressed as means *t*-test.). Results were expressed as means ± SEM of triplicate measurements ($n = 3$).

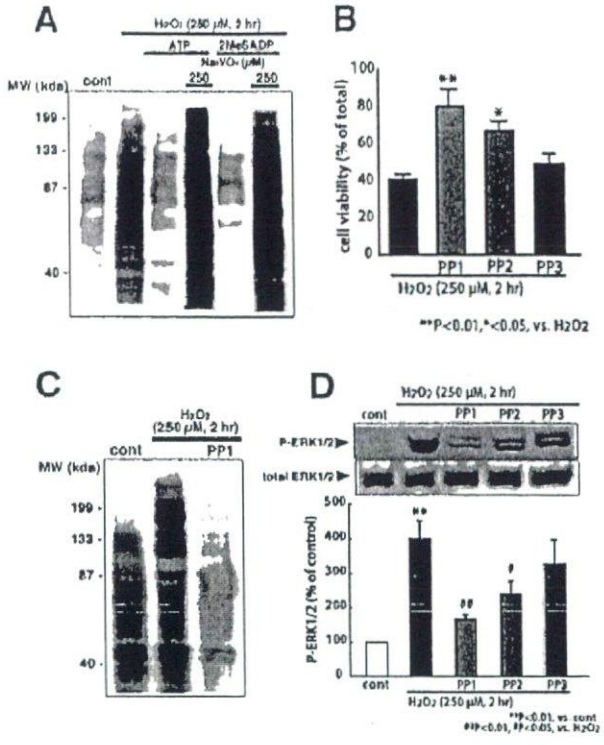

Fig. 8. The effects of the src family on the H_2O_2-evoked cell death and ERK1/2 activation. **A**: ATP and 2MeSADP prevented the H_2O_2-evoked protein tyrosine phosphorylation via PTP upregulation. H_2O_2 (250 μM) evoked the protein tyrosine phosphorylation. Na_3VO_4 (250 μM) markedly inhibited the ATP-(100 μM, 24 h) and 2MeSADP (1 μM, 24 h)-induced prevention of the H_2O_2-evoked protein tyrosine phosphorylation. Na_3VO_4 was applied to the cells 1 h before and during the H_2O_2 treatment. **B**: The effect of selective src family inhibitors on the H_2O_2-evoked cell death. PP1 and PP2 (250 nM) prevented the H_2O_2-evoked cell death but PP3 (250 nM) did not. Asterisks show significant difference from the response evoked by H_2O_2 ($^*P < 0.05$, $^{**}P < 0.01$ vs. 250 μM H_2O_2. Student's *t*-test) . **C**: The effect of PP1 on H_2O_2-evoked protein tyrosine phosphorylation. PP1 (250 nM) strongly inhibited the H_2O_2-evoked protein tyrosine phosphorylation. **D**: The effect of selective src family inhibitors on H_2O_2-evoked ERK1/2 activation. PP1 and PP2 (250 nM) inhibited H_2O_2-evoked ERK1/2 activation but PP3 (250 nM) did not. PP1, PP2, and PP3 were applied to the cells 1 h before and during the H_2O_2 treatment. Asterisks show significant difference from control ($^{**}P < 0.01$ vs. control, Student's *t*-test). Sharps show significant difference from H_2O_2 ($^{\#\#}P < 0.01$ vs. H_2O_2, Student's *t*-test). Results were expressed as means ± SEM of triplicate measurements ($n = 3$).

2MeSADP-induced prevention disappeared when the astrocytes were treated with Na_3VO_4 to inhibit the PTP activity, suggesting that ATP and 2MeSADP would inhibit tyrosine phosphorylation through a pathway(s) mediated by PTP. As it has been reported that H_2O_2 especially activates the src tyrosine kinase family of PTK (Lee and Esselman, 2002; Nishida et al., 2000), we pharmacologically studied whether the src family participates in the H_2O_2-evoked cell death and ERK1/2 activation. When the selective src family inhibitors PP1 (250 nM) and PP2 (250 nM) (Hanke et al., 1996) were added to the cells 1 h before H_2O_2 treatment, the H_2O_2-evoked cell death in astrocytes was abolished (Fig. 8B). The inactive analogue PP3 (250 nM) did not inhibit the H_2O_2-evoked cell death. PP1 (250 nM) also inhibited the H_2O_2-evoked protein tyrosine phosphorylation (Fig. 8C). In addition, the H_2O_2-evoked ERK1/2 activation was inhibited by PP1 and PP2 (250 nM) but not by PP3 (Fig. 8D). PP1, PP2, and PP3 were added to the cells 1 h before H_2O_2 stimulation.

DISCUSSION

In the present study, we demonstrated that ERK1/2 and src family are important molecules that promote the H_2O_2-evoked astrocytic cell death, and that ATP upregulates PTP expression and its activity, thereby preventing the H_2O_2-evoked src family and following ERK1/2 activation, resulting in the protection of astrocytes against H_2O_2-evoked cell death.

We clearly showed that the H_2O_2-evoked activation of ERK1/2 and accumulation of P-ERK in nuclei were critical events that promote cell death in astrocytes. ATP itself, however, which exhibited a protective effect against H_2O_2 in our study, is also known to activate ERK1/2 in astrocytes (Neary et al., 1999, 2003; Panenka et al., 2001). In fact, ATP and 2MeSADP activated ERK1/2 in astrocytes. However, the ERK1/2 activation by ATP, in contrast to that by H_2O_2, was transient (5–15 min after stimulation) and did not affect the astrocyte cell viability. Furthermore, 2MeSADP-activated ERK1/2 seems to function rather as an essential signal that prevents cell death and induces the upregulation of oxidoreductases and PTP gene expression. Thus, such a discrepancy appears to result from spatio- and temporal-behavioral differences of P-ERK1/2. It is known that, after faint brain ischemia, neuronal cells acquire tolerance to a subsequent more serious ischemic injury (Chen and Simon, 1997; Dawson and Dawson, 2000; Schaller and Graf, 2002). The similarity between such preconditioning against ischemia and the preconditioning of ERK1/2 against H_2O_2-evoked cell death is very interesting.

In the present study, the extent of the ERK1/2 activity appeared to be important in the H_2O_2-evoked cell death because of the correspondence between the concentration-dependency of the H_2O_2-evoked ERK1/2 activity and that of the H_2O_2-evoked cell death (Shinozaki et al., 2005). Although recent studies also have reported that H_2O_2-activated ERK1/2 evokes cell death in glioma and osteoblastic cells (Choi et al., 2005; Levinthal and Defranco, 2005), the spatio- and temporal behavior of P-ERK1/2 remained unclear. P-ERK1/2 is known to translocate into the nucleus (Rosenberger et al., 2001) and accumulate there (Brand et al., 2001; Stanciu and DeFranco, 2002), thereby inducing neuronal death. In the immunocytochemical analysis, stimulation by 250 μM but not by 50 μM H_2O_2 for 2 h induced P-ERK1/2 accumulation in the nucleus. Most of the total ERK1/2 existed in the cytoplasm and was not affected by H_2O_2 (50 or 250 μM). Accordingly, it is conceivable that only P-ERK1/2, activated by high concentrations (i.e. 250 μM) of H_2O_2, accumulates in the nucleus and induces cell

death. In a spatiotemporal analysis of P-ERK1/2, although there was a time-dependent activation of ERK1/2 by 50 and 250 μM H_2O_2 at the whole cell level, the fraction of N = E was increased transiently (~15 min after stimulation) by 50 μM H_2O_2 but time-dependently by 250 μM. These results suggest that the long-term accumulation of P-ERK1/2 into the nucleus participates in the H_2O_2-evoked cell death.

In physiological conditions, it is suggested that the P-ERK1/2 translocated into the nucleus is dephosphorylated by MAPK phosphatase (MAPKP), especially by the ERK1/2 selective phosphatase MAPK phosphatase-3 (MKP-3) (Dowd et al., 1998; Groom et al., 1996) and is exported from the nucleus depending on MKP-3 (Karlsson et al., 2004). The interaction between MKP-3 and ERK1/2 requires arginine residues of MKP-3 (Nichols et al., 2000). Because many amino acids, including arginines, in protein are oxidized by H_2O_2 (Amici et al., 1989; Moskovitz et al., 2002; Stadtman and Berlett, 1997; Taborsky, 1973), the interaction of MKP-3 and ERK1/2 and the nuclear export of ERK1/2 may be affected by H_2O_2. Furthermore, the ERK1/2 inactivating enzyme MAPKP is inactivated by H_2O_2 (Foley et al., 2004; Levinthal and Defranco, 2005). In conditions with oxidative stress, it is conceivable that the dephosphorylation and nuclear export of activated-ERK1/2 are attenuated because of the decreased MAPKP function and the association between MAPKP and ERK1/2, which thereby induces the prolonged activation and nuclear accumulation of ERK1/2.

With regard to the upstream molecule that activates ERK1/2 in response to H_2O_2, we found that the src family is important. Furthermore, we demonstrated that activation of $P2Y_1$ receptors inhibits the activation of src family and subsequent signaling cascades by upregulating the PTP expression and activity. We previously reported that ATP upregulates the thiol-containing protein TrxR (Shinozaki et al., 2005). The enzymatic activity of PTP requires reduction of the cystein residue in its active center (Cho et al., 2004; Persson et al., 2004). Accordingly, the redox state of the cystein residue in PTP is considered to crucially affect its phosphatase activity. The decreased activity of PTP in an oxidative state is recovered by adding thiol-containing protein such as glutathione (Salmeen et al., 2003) and thioredoxin (Lee and Esselman, 2002). In fact, the $P2Y_1$ receptor activation-induced restoration of the PTP activity was reversed by auranofin, indicating that TrxR restores the PTP activity. Additionally, the antioxidant NAC restored the PTP activity decreased by H_2O_2 (250 μM). The protective effect of PTP induced by ATP requires either an increase in the amount/activity of PTP or a reduction, in which the upregulated TxrR would have a critical role (Shinozaki et al., 2005). Furthermore, such ATP-induced oxidoreductases may preserve the MKP-3 activity, thereby enhancing the dephosphorylation and inactivation of ERK1/2 and the nuclear export of ERK1/2.

PTP and PTK regulate protein tyrosine phosphorylation in close coordination with each other. ATP prevented the H_2O_2-induced protein tyrosine phosphorylation by increasing the PTP activity. The inhibition of the H_2O_2-evoked cell death by PP1 and PP2 and the tyrosine phosphorylation by PP1 indicates that the src tyrosine kinase family is related to the H_2O_2-evoked cell death. Additionally, PP1 and PP2 inhibited the H_2O_2-evoked ERK1/2 activation, indicating that ERK1/2 is activated following src family activation. As the src family is activated by cystein oxidation independently of tyrosine phosphorylation (Akhand et al., 1999; Pu et al., 1996), under oxidative stress such as by H_2O_2 treatment or in condition of ischemia/reperfusion-injury, the src family could be activated independent of tyrosine phosphorylation. In contrast to src activation, PTP and MAPKP are inactivated by oxidation of the cystein residue under oxidative conditions (Cho et al., 2004; Foley et al., 2004; Meng et al., 2004). Briefly, the "irresponsive to control" signal transduction could be caused under oxidative conditions.

In conclusion, we clearly demonstrated that the src family activation followed by strong ERK1/2 activation and prolonged P-ERK1/2 accumulation into the nucleus participated in the H_2O_2-evoked cell death of astrocytes. $ATP/P2Y_1$ receptor activation inhibits H_2O_2-evoked ERK1/2 activation by preventing the H_2O_2-evoked src activation via upregulation of PTP expression/activity. Our present findings suggest that the gliotransmitter ATP protects astrocytes against oxidative stress by counteracting the intracellular signaling pathway that evokes cell death.

ACKNOWLEDGMENTS

We thank Tomoko Obama for technical assistance.

REFERENCES

Akhand AA, Pu M, Senga T, Kato M, Suzuki H, Miyata T, Hamaguchi M, Nakashima I. 1999. Nitric oxide controls src kinase activity through a sulfhydryl group modification-mediated Tyr-527-independent and Tyr-416-linked mechanism. J Biol Chem 274:25821–25826.

Alessandrini A, Namura S, Moskowitz MA, Bonventre JV. 1999. MEK1 protein kinase inhibition protects against damage resulting from focal cerebral ischemia. Proc Natl Acad Sci USA 96:12866–12869.

Alessi DR, Cuenda A, Cohen P, Dudley DT, Saltiel AR. 1995. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo. J Biol Chem 270:27489–27494.

Amici A, Levine RL, Tsai L, Stadtman ER. 1989. Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. J Biol Chem 264:3341–3346.

Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW. 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci USA 98:13681–13686.

Boschert U, Muda M, Camps M, Dickinson R, Arkinstall S. 1997. Induction of the dual specificity phosphatase PAC1 in rat brain following seizure activity. Neuroreport 8:3077–3080.

Boulton TG, Nye SH, Robbins DJ, Ip NY, Radziejewska E, Morgenbesser SD, DePinho RA, Panayotatos N, Cobb MH, Yancopoulos GD. 1991. ERKs: A family of protein-serine/threonine kinases that are activated and tyrosine phosphorylated in response to insulin and NGF. Cell 65:663–675.

Brand A, Gil S, Seger R, Yavin E. 2001. Lipid constituents in oligodendroglial cells alter susceptibility to H_2O_2-induced apoptotic cell death via ERK activation. J Neurochem 76:910–918.

Brugge JS, Cotton PC, Queral AE, Barrett JN, Nonner D, Keane RW. 1985. Neurones express high levels of a structurally modified, activated form of pp60c-src. Nature 316:554–557.

Chen J, Simon R. 1997. Ischemic tolerance in the brain. Neurology 48:306–311.
Chen RH, Sarnecki C, Blenis J. 1992. Nuclear localization and regulation of erk- and rsk-encoded protein kinases. Mol Cell Biol 12:915–927.
Cho SH, Lee CH, Ahn Y, Kim H, Ahn CY, Yang KS, Lee SR. 2004. Redox regulation of PTEN and protein tyrosine phosphatases in H_2O_2-mediated cell signaling. FEBS Lett 560(1–3):7–13.
Choi JS, Park HJ, Kim HY, Kim SY, Lee JE, Choi YS, Chun MH, Chung JW, Lee MY. 2005. Phosphorylation of PTEN and Akt in astrocytes of the rat hippocampus following transient forebrain ischemia. Cell Tissue Res 319:359–366.
Dawson VL, Dawson TM. 2000. Neuronal ischaemic preconditioning. Trends Pharmacol Sci 21:423–424.
Dowd S, Sneddon AA, Keyse SM. 1998. Isolation of the human genes encoding the pyst1 and Pyst2 phosphatases: Characterisation of Pyst2 as a cytosolic dual-specificity MAP kinase phosphatase and its catalytic activation by both MAP, SAP kinases. J Cell Sci 111(Part 22):3389–3399.
Fam SR, Gallagher CJ, Salter MW. 2000. P2Y(1) purinoceptor-mediated Ca(2+) signaling and Ca(2+) wave propagation in dorsal spinal cord astrocytes. J Neurosci 20:2800–2808.
Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, Feeser WS, Van Dyk DE, Pitts WJ, Earl RA, Hobbs F, Copeland RA, Magolda RL,Scherle PA, Trzaskos JM. 1998. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem 273:18623–18632.
Fialkow L, Chan CK, Rotin D, Grinstein S, Downey GP. 1994. Activation of the mitogen-activated protein kinase signaling pathway in neutrophils. Role of oxidants. J Biol Chem 269:31234–31242.
Fields RD, Stevens-Graham B. 2002. New insights into neuron-glia communication. Science 298:556–562.
Foley TD, Armstrong JJ, Kupchak BR. 2004. Identification and H_2O_2 sensitivity of the major constitutive MAPK phosphatase from rat brain. Biochem Biophys Res Commun 315:568–574.
Gonzalez FA, Seth A, Raden DL, Bowman DS, Fay FS, Davis RJ. 1993. Serum-induced translocation of mitogen-activated protein kinase to the cell surface ruffling membrane and the nucleus. J Cell Biol 122:1089–1101.
Groom LA, Sneddon AA, Alessi DR, Dowd S, Keyse SM. 1996. Differential regulation of the MAP, SAP and RK/p38 kinases by Pyst1, a novel cytosolic dual-specificity phosphatase. EMBO J 15:3621–3632.
Guo J, Meng F, Zhang G, Zhang Q. 2003. Free radicals are involved in continuous activation of nonreceptor tyrosine protein kinase c-Src after ischemia/reperfusion in rat hippocampus. Neurosci Lett 345:101–104.
Guyton KZ, Liu Y, Gorospe M, Xu Q, Holbrook NJ. 1996. Activation of mitogen-activated protein kinase by H_2O_2. Role in cell survival following oxidant injury. J Biol Chem 271:4138–4142.
Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. 1996. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. J Biol Chem 271:695–701.
Hansson E, Ronnback L. 2003. Glial neuronal signaling in the central nervous system. FASEB J 17:341–348.
Heffetz D, Bushkin I, Dror R, Zick Y. 1990. The insulinomimetic agents H_2O_2 and vanadate stimulate protein tyrosine phosphorylation in intact cells. J Biol Chem 265:2896–2902.
Inoue K. 2002. Microglial activation by purines and pyrimidines. Glia 40:156–163.
Karlsson M, Mathers J, Dickinson RJ, Mandl M, Keyse SM. 2004. Both nuclear-cytoplasmic shuttling of the dual specificity phosphatase MKP-3 and its ability to anchor MAP kinase in the cytoplasm are mediated by a conserved nuclear export signal. J Biol Chem 279:41882–41891.
Kimura M, Maeda K, Hayashi S. 1992. Cytosolic calcium increase in coronary endothelial cells after H_2O_2 exposure and the inhibitory effect of U78517F. Br J Pharmacol 107:488–493.
Konishi H, Matsuzaki H, Takaishi H, Yamamoto T, Fukunaga M, Ono Y, Kikkawa U. 1999. Opposing effects of protein kinase C δ and protein kinase B α on H_2O_2-induced apoptosis in CHO cells. Biochem Biophys Res Commun 264:840–846.
Lee K, Esselman WJ. 2002. Inhibition of PTPs by H_2O_2 regulates the activation of distinct MAPK pathways. Free Radic Biol Med 33:1121–1132.
Levinthal DJ, Defranco DB. 2005. Reversible oxidation of ERK-directed protein phosphatases drives oxidative toxicity in neurons. J Biol Chem 280:5875–5883.
Marshall CJ. 1995. Specificity of receptor tyrosine kinase signaling: Transient versus sustained extracellular signal-regulated kinase activation. Cell 80:179–185.
McLaughlin MM, Kumar S, McDonnell PC, Van Horn S, Lee JC, Livi GP, Young PR. 1996. Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase. J Biol Chem 271:8488–8492.
Meng TC, Buckley DA, Galic S, Tiganis T, Tonks NK. 2004. Regulation of insulin signaling through reversible oxidation of the protein-tyrosine phosphatases TC45 and PTP1B. J Biol Chem 279:37716–37725.
Moskovitz J, Yim MB, Chock PB. 2002. Free radicals and disease. Arch Biochem Biophys 397:354–359.
Murray B, Alessandrini A, Cole AJ, Yee AG, Furshpan EJ. 1998. Inhibition of the p44/42 MAP kinase pathway protects hippocampal neurons in a cell-culture model of seizure activity. Proc Natl Acad Sci USA 95:11975–11980.
Namura S, Iihara K, Takami S, Nagata I, Kikuchi H, Matsushita K, Moskowitz MA, Bonventre JV, Alessandrini A. 2001. Intravenous administration of MEK inhibitor U0126 affords brain protection against forebrain ischemia and focal cerebral ischemia. Proc Natl Acad Sci USA 98:11569–11574.
Neary JT, Kang Y, Bu Y, Yu E, Akong K, Peters CM. 1999. Mitogenic signaling by ATP/P2Y purinergic receptors in astrocytes: Involvement of a calcium-independent protein kinase C, extracellular signal-regulated protein kinase pathway distinct from the phosphatidylinositol-specific phospholipase C/calcium pathway. J Neurosci 19:4211–4220.
Neary JT, Kang Y, Willoughby KA, Ellis EF. 2003. Activation of extracellular signal-regulated kinase by stretch-induced injury in astrocytes involves extracellular ATP, P2 purinergic receptors. J Neurosci 23:2348–2356.
Nichols A, Camps M, Gillieron C, Chabert C, Brunet A, Wilsbacher J, Cobb M, Pouyssegur J, Shaw JP, Arkinstall S. 2000. Substrate recognition domains within extracellular signal-regulated kinase mediate binding and catalytic activation of mitogen-activated protein kinase phosphatase-3. J Biol Chem 275:24613–24621.
Nishida M, Maruyama Y, Tanaka R, Kontani K, Nagao T, Kurose H. 2000. G α(i) and G α(o) are target proteins of reactive oxygen species. Nature 408:492–495.
Ohtsuki T, Matsumoto M, Kitagawa K, Mabuchi T, Mandai K, Matsushita K, Kuwabara K, Tagaya M, Ogawa S, Ueda H, Kamada T, Yanagihara T. 1996. Delayed neuronal death in ischemic hippocampus involves stimulation of protein tyrosine phosphorylation. Am J Physiol 271(4, Part 1):C1085–C1097.
Oppenheim RW. 1991. Cell death during development of the nervous system. Annu Rev Neurosci 14:453–501.
Panenka W, Jijon H, Herx LM, Armstrong JN, Feighan D, Wei T, Yong VW, Ransohoff RM, MacVicar BA. 2001. P2X7-like receptor activation in astrocytes increases chemokine monocyte chemoattractant protein-1 expression via mitogen-activated protein kinase. J Neurosci 21:7135–7142.
Persson C, Sjoblom T, Groen A, Kappert K, Engstrom U, Hellman U, Heldin CH, den Hertog J, Ostman A. 2004. Preferential oxidation of the second phosphatase domain of receptor-like PTP-α revealed by an antibody against oxidized protein tyrosine phosphatases. Proc Natl Acad Sci USA 101:1886–1891.
Pu M, Akhand AA, Kato M, Hamaguchi M, Koike T, Iwata H, Sabe H, Suzuki H, Nakashima I. 1996. Evidence of a novel redox-linked activation mechanism for the Src kinase which is independent of tyrosine 527-mediated regulation. Oncogene 13:2615–2622.
Rosenberger J, Petrovics G, Buzas B. 2001. Oxidative stress induces proorphanin FQ, proenkephalin gene expression in astrocytes through p38- and ERK-MAP kinases and NF-κB. J Neurochem 79:35–44.
Salmeen A, Andersen JN, Myers MP, Meng TC, Hinks JA, Tonks NK, Barford D. 2003. Redox regulation of protein tyrosine phosphatase 1B involves a sulphenyl-amide intermediate. Nature 423:769–773.
Schaller B, Graf R. 2002. Cerebral ischemic preconditioning. An experimental phenomenon or a clinical important entity of stroke prevention? J Neurol 249:1503–1511.
Segal RA, Greenberg ME. 1996. Intracellular signaling pathways activated by neurotrophic factors. Annu Rev Neurosci 19:463–489.
Shinozaki Y, Koizumi S, Ishida S, Sawada JI, Ohno Y, Inoue K. 2005. Cytoprotection against oxidative stress-induced damage of astrocytes by extracellular ATP via P2Y(1) receptors. Glia 49:288–300.
Shisheva A, Shechter Y. 1993. Role of cytosolic tyrosine kinase in mediating insulin-like actions of vanadate in rat adipocytes. J Biol Chem 268:6463–6469.
Stadtman ER, Berlett BS. 1997. Reactive oxygen-mediated protein oxidation in aging and disease. Chem Res Toxicol 10:485–494.
Stanciu M, DeFranco DB. 2002. Prolonged nuclear retention of activated extracellular signal-regulated protein kinase promotes cell death generated by oxidative toxicity or proteasome inhibition in a neuronal cell line. J Biol Chem 277:4010–4017.
Subramaniam S, Zirrgiebel U, Von Bohlen Und Halbach O, Strelau J, Laliberte C, Kaplan DR, Unsicker K. 2004. ERK activation promotes neuronal degeneration predominantly through plasma membrane damage and independently of caspase-3. J Cell Biol 165:357–369.

Sugrue MM, Brugge JS, Marshak DR, Greengard P, Gustafson EL. 1990. Immunocytochemical localization of the neuron-specific form of the c-src gene product, pp60c-src(+), in rat brain. J Neurosci 10:2513–2527.

Taborsky G. 1973. Oxidative modification of proteins in the presence of ferrous ion and air. Effect of ionic constituents of the reaction medium on the nature of the oxidation products. Biochemistry 12:1341–1348.

Takahashi M, Berk BC. 1996. Mitogen-activated protein kinase (ERK1/2) activation by shear stress and adhesion in endothelial cells. Essential role for a herbimycin-sensitive kinase. J Clin Invest 98:2623–2631.

Takano S, Fukuyama H, Fukumoto M, Kimura J, Xue JH, Ohashi H, Fujita J. 1996. PRL-1, a protein tyrosine phosphatase, is expressed in neurons and oligodendrocytes in the brain and induced in the cerebral cortex following transient forebrain ischemia. Brain Res Mol Brain Res 40:105–115.

Tan J, Town T, Mori T, Wu Y, Saxe M, Crawford F, Mullan M. 2000. CD45 opposes β-amyloid peptide-induced microglial activation via inhibition of p44/42 mitogen-activated protein kinase. J Neurosci 20:7587–7594.

Ushio-Fukai M, Alexander RW, Akers M, Yin Q, Fujio Y, Walsh K, Griendling KK. 1999. Reactive oxygen species mediate the activation of Akt/protein kinase B by angiotensin II in vascular smooth muscle cells. J Biol Chem 274:22699–22704.

Wang X, Martindale JL, Liu Y, Holbrook NJ. 1998. The cellular response to oxidative stress: Influences of mitogen-activated protein kinase signalling pathways on cell survival. Biochem J 333(Part 2):291–300.

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME. 1995. Opposing effects of ERK, JNK-p38 MAP kinases on apoptosis. Science 270:1326–1331.

Yu XM, Salter MW. 1999. Src, a molecular switch governing gain control of synaptic transmission mediated by *N*-methyl-D-aspartate receptors. Proc Natl Acad Sci USA 96:7697–7704.

0026-895X/06/7001-319-328$20.00
MOLECULAR PHARMACOLOGY

Mol Pharmacol 70:319–328, 2006

Vol. 70, No. 1
20511/3124430
Printed in U.S.A.

Retinoic Acids Increase $P2X_2$ Receptor Expression through the 5′-Flanking Region of *P2rx2* Gene in Rat Phaeochromocytoma PC-12 Cells

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Received November 1, 2005; accepted April 25, 2006

ABSTRACT

The $P2X_2$ receptor is a subtype of ionotropic ATP receptor and plays a significant role in regulating fast synaptic transmission in the nervous system. Because the expression level of the $P2X_2$ receptor is known to determine its channel properties and functional interactions with other neurotransmitter channels, elucidating the mechanisms underlying the regulation of $P2X_2$ receptor expression in neuronal cells is important. Here, we identified three motifs that correspond to the retinoic acid response element in the 5′-flanking region of the rat $P2X_2$ gene. In rat pheochromocytoma PC-12 cells, treatment with 9-*cis*-retinoic acid as well as all-*trans*-retinoic acid significantly increased the mRNA and protein level of $P2X_2$ receptor. In addition, in PC-12 cells transiently transfected with a luciferase reporter gene driven by the promoter region of the rat $P2X_2$ gene, both 9-*cis*-retinoic acid and all-*trans*-retinoic acid increased the luciferase activity, whereas their effects were diminished by truncation of the retinoic acid response elements in the promoter. Furthermore, 9-*cis*-retinoic acid enhanced the ATP-evoked whole cell currents and intracellular Ca^{2+}- and ATP-evoked dopamine release, indicating the up-regulation of functional $P2X_2$ receptors on the plasma membrane. These results provide the molecular mechanism underlying the transcriptional regulation of $P2X_2$ receptors and suggest that retinoid is an important factor in regulating $P2X_2$ receptors in the nervous system.

P2X receptors, of which seven subtypes ($P2X_1$–$P2X_7$) have so far been cloned, are a family of ligand-gated cation channels activated by extracellular ATP and are widely expressed in the peripheral and central nervous system (North, 2002; Illes and Alexandre Ribeiro, 2004). A growing body of evidence indicates that P2X receptors expressed in neurons play important roles in mediating (Galligan and Bertrand, 1994), facilitating presynaptically (Khakh et al., 2003; Shigetomi and Kato, 2004), and modulating postsynaptically fast excitatory and inhibitory synaptic transmission (Wang et al., 2004). It remains unclear which P2X receptor subtypes are the main targets for ATP at synapses, but several lines of evidence have suggested the $P2X_2$ receptor as a candidate. In several regions of the nervous system, neurons express functional $P2X_2$ receptors (North, 2002; Illes and Alexandre Ribeiro, 2004) as well as both the mRNA and protein of $P2X_2$ receptors (Kanjhan et al., 1999). An electron microscopic study has shown that $P2X_2$ receptors are localized at the postsynaptic membrane in the cerebellum and the CA1 region of the hippocampus (Rubio and Soto, 2001). In addition, it has been reported that $P2X_2$ receptors are abundant in the biochemically fractionated presynaptic active zone in the hippocampus (Rodrigues et al., 2005). A recent study has shown that ATP facilitates excitatory glutamate transmission onto stratum radiatum interneurons, a population of the ATP-

This work was partly supported by The National Institute of Biomedical Innovation (Medical Frontier Project; MF-16), The Health Science Foundation in Japan, and a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.105.020511.

ABBREVIATIONS: RARE, retinoic acid response element; RAR, retinoic acid receptor; RXR, retinoid X receptor; DA, dopamine; VDCC, voltage-dependent calcium channel; RA, retinoic acid; RT-PCR, reverse transcriptase polymerase chain reaction; bp, base pair(s); PCR, polymerase chain reaction; TESS, transcription element search system; RACE, rapid amplification of cDNA ends; $P2X_2R$, $P2X_2$ receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; BSS, balanced salt solution; PCA, perchloric acid; AP, adaptor protein; atRA, all-*trans*-retinoic acid; PPADS, pyridoxal phosphate-6-azophenyl-2′4′-disulfonic acid; U-73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; DR, direct repeat; ANOVA, analysis of variance.

responding neurons that is markedly reduced in hippocampus slices from $P2X_2$-deficient mice (Khakh et al., 2003). These results indicate that in several regions $P2X_2$ receptors localized at pre- and/or postsynapses regulate fast synaptic transmission. Furthermore, $P2X_2$ receptors are associated directly with other neurotransmitter channels such as nicotinic acetylcholine receptors, 5-hydroxytryptamine receptors or $GABA_A$ receptors, and activation of both receptors produces nonadditive cross-inhibitory responses (Khakh et al., 2000; Boue-Grabot et al., 2003). It is noteworthy that the functional interaction of $P2X_2$ receptors with other channels is decreased at lower densities of channel expression (Khakh et al., 2000), suggesting that their expression levels affect cellular events resulting from activation of $P2X_2$ receptors at synapses. In addition, the expression level of $P2X_2$ receptors also changes their channel properties (Fujiwara and Kubo, 2004). Moreover, an increase in the expression of $P2X_2$ receptors in neuronal cells has been implicated in the development of several pathological states such as brain ischemia and chronic pain (Xu and Huang, 2002; Cavaliere et al., 2003). Therefore, to understand the physiological and pathological roles of $P2X_2$ receptors in the functioning of the nervous system, it is of particular importance to determine how the expression of $P2X_2$ receptors is regulated in neuronal cells.

In the present study, we cloned the 5′-flanking region of the rat $P2X_2$ gene (*P2rx2*) and identified three sites corresponding to a motif of retinoic acid response element (RARE). RARE is a binding site of nuclear receptors, including retinoic acid receptor (RAR) and retinoid X receptor (RXR), and is required for the gene expression induced by retinoids (Chambon, 1996). We further found that retinoids increase both the mRNA and protein expression of the $P2X_2$ receptor and enhance release of the neurotransmitter dopamine (DA) evoked by ATP through activating $P2X_2$ receptors from rat pheochromocytoma PC-12 cells, a neuronal model (Shafer and Atchison, 1991). Therefore, these results suggest that retinoids are regulators of the expression of $P2X_2$ receptors in neuronal cells in the nervous system.

Materials and Methods

PC-12 Cells. PC-12 cells (passage 55–70) were cultured according to Inoue and Kenimer (1988), and undifferentiated cells were used. Cells were cultured in Dulbecco's modified eagle's medium supplemented with 7.5% fetal bovine serum, 7.5% horse serum, and 4 mM L-glutamine. For reverse transcription-polymerase chain reaction (RT-PCR) and Western blot experiments, cells were plated on 60-mm collagen (Virtogen-100)-coated dishes for 2 days. For whole cell patch-clamp recording and intracellular calcium imaging, cells were plated on collagen-coated coverslips placed on the bottom of 35-mm polystyrene dishes. For the measurement of DA release, cells were plated on collagen-coated 35-mm polystyrene dishes.

Cloning of the $P2X_2$ Upstream Region. Sequences for the 5′-flanking region of *P2rx2* were obtained from National Center for Biotechnology Information Rat Genome Resources. The genomic 2.5-kb upstream sequence of the putative Wistar rat *P2rx2* transcription starting site was targeted as $P2X_2$ mRNA (GenBank accession number NM_053656) upstream sequence. The following primers were designed for amplification of the 5′-flanking region of *P2rx2*: forward primer, GAACCTCGAGTGAGCCACAACCAGAACACT; reverse primer, GACAAGATCTATGGCCCAAGGAGCTCGGT. Genomic DNA extracted from the tail of a female Wistar rat was used for the polymerase chain reaction. Four individual reactions were carried out, and amplicons were inserted in a pGEM-T vector (Promega, Madison, WI) for sequencing. Each insert was sequenced, and the exact sequence was estimated by comparing the four sequences. The relative location of the cloned sequence is confirmed to be just upstream of the first exon of *P2rx2* without any intervening inserts. Using primers specific to the third exon of *P2rx2* and −164 position of the cloned sequence, approximately 750-bp single-band amplification was obtained by PCR. This amplicon included the sequence comprising the 5′ site of $P2X_2$ mRNA (RefSeq sequence NM_053656) exactly as published, and the sequence was determined to be the 5′-flanking region without any additional intervening sequence. The sequence data from the 5′-flanking region of *P2rx2* has been deposited in GenBank with the accession number AY749416. Putative sites for the transcription element were analyzed using Transcription Element Search System (TESS) site (http://www.cbil.upenn.edu/tess).

"Oligo-Capping" 5′ Rapid Amplification of cDNA Ends of $P2X_2$ mRNA. Modified rapid amplification of 5′ cDNA ends (5′ RACE) was performed according to oligo-capping method developed by Maruyama and Sugano (1994). Total RNA (5 μg) extracted from PC-12 cells was treated with 1 unit of bacterial alkaline phosphatase (Takara, Kyoto, Japan) in supplied buffer with 100 units of RNase inhibitor (Toyobo, Osaka, Japan) at 37°C for 30 min to hydrolyze the phosphate of truncated mRNA 5′ ends. After extraction with phenol/chloroform (1:1) twice, chloroform once, and ethanol precipitation, tobacco acid pyrophosphatase (20 units; Wako Pure Chemicals, Osaka, Japan) was reacted (37°C; 15 min) in kit supplied buffer with RNase inhibitor to remove the cap structure of complete mRNAs. After phenol/chloroform extraction and ethanol precipitation, ligation reaction was carried with T4 RNA ligase (Takara) and 0.5 μg of 5′-adapter RNA oligonucleotide to obtain the oligonucleotide composed by mRNAs attached with 5′-adapter RNA oligonucleotide at 5′ ends that originally had the cap structure. After unligated 5′-adapter oligonucleotide was removed by repeating ethanol precipitation with high salt concentration, reverse transcription reaction was performed using ReverTra Ace (Toyobo) with antisense primer of $P2X_2$ mRNA, which was designed from +531 of NM_053656, and PCR was carried out with obtained cDNAs and primers for adapter and $P2X_2$ mRNA sequence, which were designed to cross the border of exons 1 and 2. The reaction mixture was electrophoresed in agarose gel, and all of amplicon was gel extracted and restricted by XhoI, whose restriction site was designed in adapter sequence. The fragments were cloned into pcDNA3 vector which restricted by XhoI and EcoRV and sequenced. The adapter and primers sequences are as follows. The 5′-adapter RNA oligonucleotide was 5′-GUCUGAGCUCUCGAGAUAGA-3′; the primer for reverse transcription, 5′-GTTGTCAGAAGTTCCATCCTCCAC-3′; the primer for 5′-adapter, 5′-GTCTGAGCTCTCGAGATAGA-3′; and the reverse primer for target amplification, 5′-CGATGAAGACGTACCACACGAA-3′.

Real-Time Quantitative RT-PCR (TaqMan RT-PCR). Retinoids were dissolved in ethanol and added to the culture medium so that the ethanol represented 0.1% of the v/v concentration. Total cellular RNA was prepared using the RNeasy method from QIAGEN (Valencia, CA) according to the manufacturer's instructions and included an on-column DNase I digestion to minimize genomic DNA contamination. The TaqMan One-Step RT-PCR Master Mix Reagent kit (Applied Biosystems, Foster City, CA) was used with each custom designed, gene-specific primer/probe set to amplify and quantify each transcript of interest. Reactions (25 μl) contained 50 ng of total RNA, 200 nM forward and reverse primers, 100 nM TaqMan probe, and RNase Inhibitor Mix in the Master Mix solution. RT-PCR amplification and real-time detection were performed using an ABI PRISM 7700 sequence detection system (Applied Biosystems) for 30 min at 48°C (reverse transcription), 10 min at 95°C (AmpliTaq Gold activation), 38 cycles of denaturation (15 s at 95°C), and annealing/extension (60 s at 60°C). Data were analyzed using ABI Prism Sequence Detection Software, version 1.1. The following primers and probes were used. The TaqMan probe for $P2X_2R$ was 5′-5-carboxy-

fluorescein-CACTACTCCCAGGATCAGCCACCCA-5-carboxytetramethylrhodamine-3'; the forward primer for $P2X_2R$, 5'-CATATCCCTCCCCCACCTA-3'; and the reverse primer, 5'-GTTGGTCCTTCACCTGATGGA-3'. Sense and antisense primers and probes for GAPDH were obtained from Rodent GAPDH Control Reagents (Applied Biosystems).

Plasmids. The 5'-flanking region of *P2rx2* (described above) was inserted into multicloning sites of the pGL3-basic vector (termed pP2X2luc; Promega). The sequence between two KpnI sites (one site is in the multicloning site and other site is at the −1923 position) in the vector was restricted by KpnI (Takara) and ligated to construct a deletion mutant which lacks 501 bp of the 5' end in the pP2X2luc insert (Del-pP2X2luc). The P2X2-GFP vector was a kind gift from Dr. Murrell-Lagnado (Department of Pharmacology, Cambridge University, Cambridge, UK).

Transient Transfections and Luciferase Assays. Transient transfection was carried out with Superfect (QIAGEN) according to the manufacturer's protocol. Fifty percent confluent cells seeded on 48-well plates were transfected with reporter plasmid (pP2X2luc, Del-pP2X2luc, P2X2-GFP). The phRL-TK vector (Promega) was cotransfected to monitor the transfection efficiency. After 48 h incubation, the cells were lysed. Firefly and *Renilla reniformis* luciferase activity were measured by 1420 ARVOsx multilabel counter (PerkinElmer Wallac, Turku, Finland) using a dual-luciferase reporter assay system (Promega). The transfection efficiency was corrected by normalizing the firefly luciferase activity to the *R. reniformis* luciferase activity.

Western Blot of $P2X_2$ Receptor Protein. After treatment of the cells with 9-*cis*-retinoic acid (9-*cis*-RA) for 1 day, the cells were washed with phosphate-buffered saline(−) twice and lysed in buffer containing 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 5 mM EGTA, 0.5% mM Nonidet P-40, and 0.5% deoxycholate. The protein concentration was measured by bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL). Proteins (10–30 μg/lane) were mixed with SDS sample buffer, loaded onto a 10% polyacrylamide gel, electrophoresed, and transferred onto a nitrocellulose membrane. The membrane was then blocked in 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20. The membrane was incubated with the anti-rabbit $P2X_2$ polyclonal antibody (1:200; Calbiochem, San Diego, CA) or β-actin (1:5000; Sigma-Aldrich, St. Louis, MO) overnight at 4°C, followed by incubation with the horseradish peroxidase-conjugated anti-rabbit antibody (1:2000; GE Healthcare, Little Chalfont, Buckinghamshire, UK). The blots were probed with an ECL Western blot detection system (GE Healthcare). Quantification of immunoreactive bands was performed by scanned image analysis on a computer.

Whole Cell Patch-Clamp Recording. The cells were placed in a recording chamber and continuously superfused at room temperature (22–24°C) in an extracellular solution containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 11.1 mM D-glucose, and 10 mM HEPES; pH adjusted to 7.4 with NaOH. Patch pipettes were filled with an intracellular solution containing 150 mM CsCl, 10 mM HEPES, and 5 mM EGTA; pH adjusted to 7.3 with CsOH. With this solution, patch electrode resistances ranged between 5 and 8 MΩ. The whole cell patch-clamp was made, and cells were voltage-clamped at −60 mV. ATP was diluted with extracellular solution and applied to the patched cell by gravity from a tube (300-μm inner diameter) attached to an electrically controlled valve. Currents were recorded with an Axopatch 200-B amplifier (Molecular Devices, Sunnyvale, CA) and analyzed using pClamp5 software (Molecular Devices).

Measurement of DA Released from PC-12 Cells. Cells were plated on 35-mm dishes and washed twice with 1 ml of balanced salt solution (BSS) containing 150 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES, and 10 mM D-glucose and then incubated for 1 h with 1 ml of BSS at room temperature. The cells were again washed with BSS and then stimulated by BSS with or without 30 μM ATP for 1 min. BSS was collected in 1.5-ml tubes loaded with 250 μl of 1 N perchloric acid (PCA), and 1 ml of 0.2 N PCA was added to the dishes and incubated for 2 h on ice. Both the collected PCA solutions were centrifuged, and then the supernatants were used for DA measurement. The amount of DA in the solution was measured using high-performance liquid chromatography combined with electrochemical detection.

Intracellular Calcium Imaging. The increase in $[Ca^{2+}]_i$ in single cells was measured by the fura-2 method with minor modifications. Cells were washed with BSS and incubated with 10 μM fura-2 acetoxymethyl ester at 37°C in BSS for 45 min. The coverslips were mounted on an inverted epifluorescence microscope (TMD-300; Nikon, Tokyo, Japan) equipped with a 75-W xenon lamp and band-pass filters of 340-nm wavelength for measurement of the Ca^{2+}-dependent signal (F_{340}) and 360-nm wavelength for measurement of the Ca^{2+}-independent signal (F_{360}).

Results

Homology Search for Transcription Factor Binding Sites in the 5'-Flanking Region of *P2rx2*. The *P2rx2* is located at rat chromosome 12 and has 11 exons between 5'- and 3'-untranslated region (National Center for Biotechnology Information Entrez GeneID 114115). $P2X_2$ mRNA sequence has been first determined by Brake et al. (1994). Of 11 splicing variants registered in GenBank database, only two variants are reported to express functional channel (*P2rx2*, NM_053656; *P2X 2b*, Y10473). The information of the 5'-flanking region of the rat *P2rx2* was obtained from National Center for Biotechnology Information Rat Genome Resources. In the *Rattus norvegicus* (Norway rat) chromosome 12 genomic contig from whole genome shotgun sequence (NW_047378), putative transcription start site of *P2rx2* is predicted by searching the sequence location of rat $P2X_2$ mRNA (NM_053656) using BLAST. Then, a 2524-bp fragment upstream of the Wistar rat *P2rx2* was cloned in the pGL3 vector. Whether the cloned sequence is located in the 5'-flanking region of *P2rx2* is confirmed by sequencing the 743-bp amplicon obtained by genome PCR using specific primers for the third exon of *P2rx2* and our cloned sequence. The homology between database sequence and the cloned sequence was more than 99.8% match. In the cloned sequence, we found three putative RAREs that conformed with a general canonical sequence in which two directly repeated hexanucleotide motifs [consensus (A/G)G(G/T)TCA] are separated by one (DR1: −2309/−2321), four (DR4: −2299/−2314), and five nucleotides (DR5: −2408/−2424) (Fig. 1). We used TESS to verify these sites and confirmed that they were predicted as RAREs. The sequence analysis using TESS also predicted the presence of many consensus sequences for various transcription factors in the cloned fragment such as simian virus 40 protein 1 (Sp-1), AP-1, AP-2, GATA-1, nuclear factor-κB, and cAMP response element-binding protein binding motifs. Sequence data from the 5'-flanking region of the Wistar rat *P2rx2* have been deposited in GenBank with the accession number AY749416. Furthermore using oligo-capping 5' RACE, we could obtain single sequence that encodes 5' region of $P2X_2$ mRNA, suggesting that transcription starting site of *P2rx2* in PC-12 cells is located in 27 bases upstream of RefSeq sequence (NM_053656). Consensus sequences of GC-box (GGGCGG) and initiator (YYANWYY), which are expected to form core promoter region, were found in −67 and −52 bp upstream of transcription starting site determined with oligo-capping 5' RACE.

$P2X_2$ 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

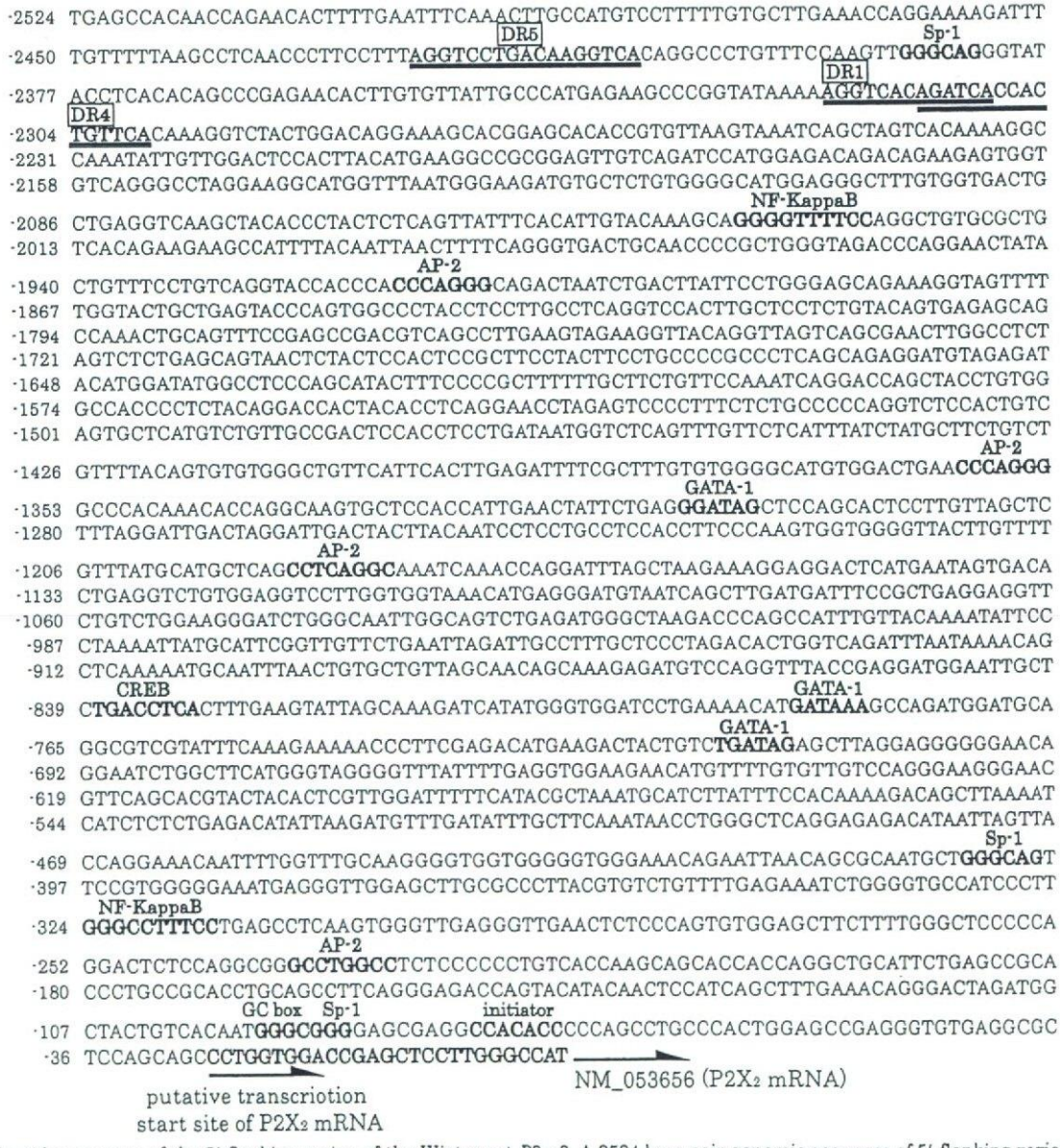

Fig. 1. Nucleotide sequence of the 5′-flanking region of the Wistar rat *P2rx2*. A 2524-base pair genomic sequence of 5′-flanking region of *P2rx2* was cloned and sequenced (GenBank accession no. AY749416) and analyzed to search for consensus motifs interacting with transcription factors using TESS. 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.

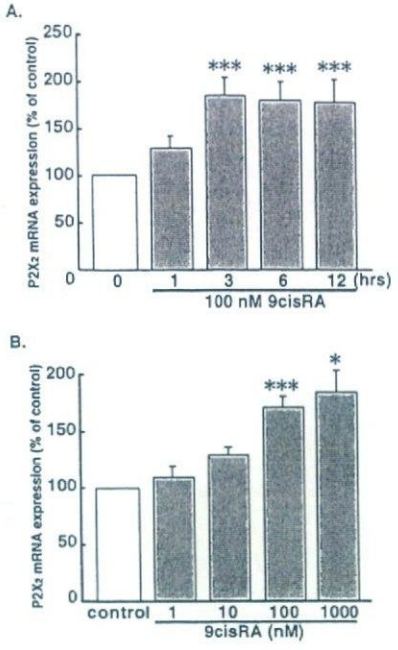

Fig. 2. Increase in the level of $P2X_2$ receptor mRNA by 9-*cis*-retinoic acid. PC-12 cells were treated with 100 nM 9-*cis*-RA for 1, 3, 6, and 12 h (A) or with different concentrations of 9-*cis*-RA (1–1000 nM) (B) followed by real-time RT-PCR analysis of $P2X_2$ and GAPDH mRNAs. $P2X_2$ mRNA levels 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).

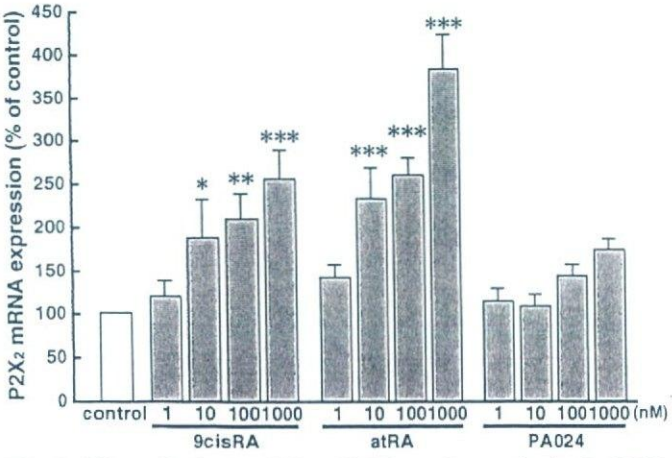

Fig. 3. Effects of selective RAR and RXR agonists on the level of $P2X_2$ receptor mRNA. PC-12 cells were treated with 9-*cis*-RA, atra, or PA024 at different concentrations for 3 h in serum-free condition followed by real-time RT-PCR analysis of $P2X_2$ and GAPDH mRNAs. $P2X_2$ mRNA levels were normalized 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-

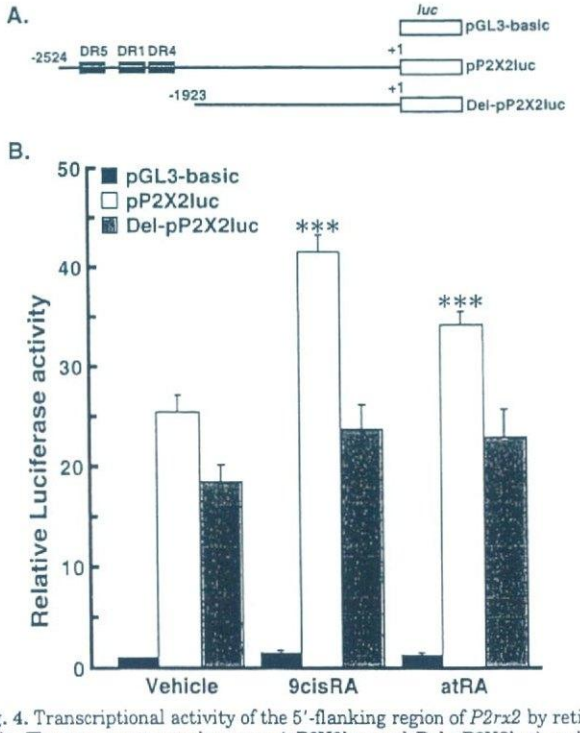

Fig. 4. Transcriptional activity of the 5′-flanking region of *P2rx2* by retinoic acids. The two constructed vectors (pP2X2luc and Del-pP2X2luc) and the empty vector (pGL3-basic) used in the experiment, as described under *Materials and Methods*, are schematically illustrated. Each construct was transfected 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

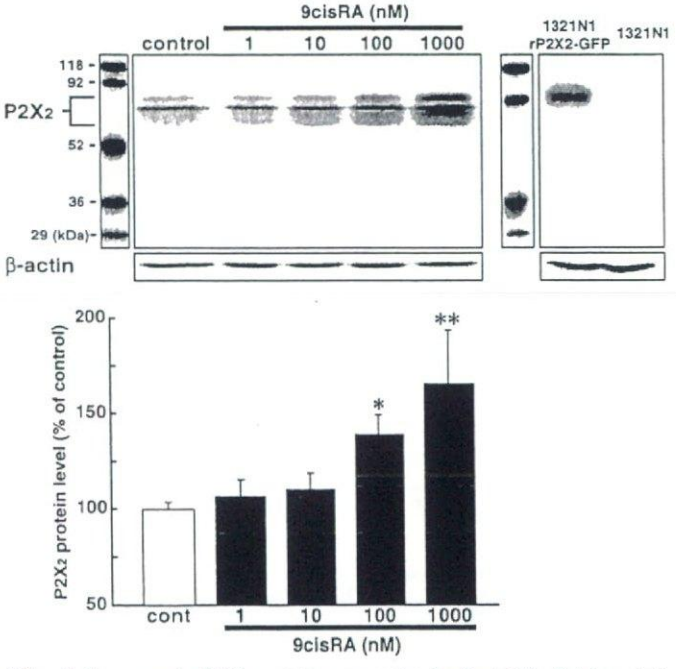

Fig. 5. Increase in $P2X_2$ protein expression by 9-*cis*-RA. Total protein from PC-12 cells treated with or without 9-*cis*-RA (range 1–1000 nM) for 24 h was subjected to Western blot analysis. The proteins of $P2X_2$ receptor and β-actin were detected by their specific antibodies. The intensities of 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).

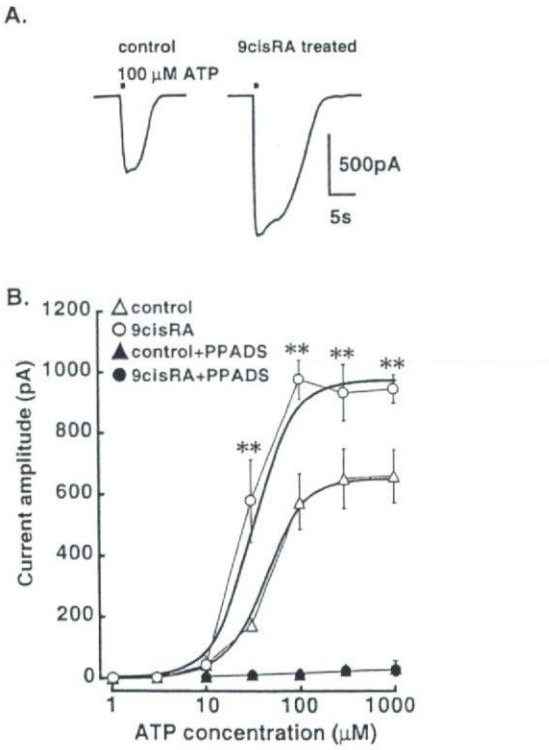

Fig. 6. Effect of 9-*cis*-RA on ATP-induced whole cell current in PC-12 cells. A, representatives traces were the currents evoked by 100 μM ATP in PC-12 cells with or without 100 nM 9-*cis*-RA for 24 h. Cells were voltage-clamped at −60 mV. B, concentration-dependent curves were made by measuring currents 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).

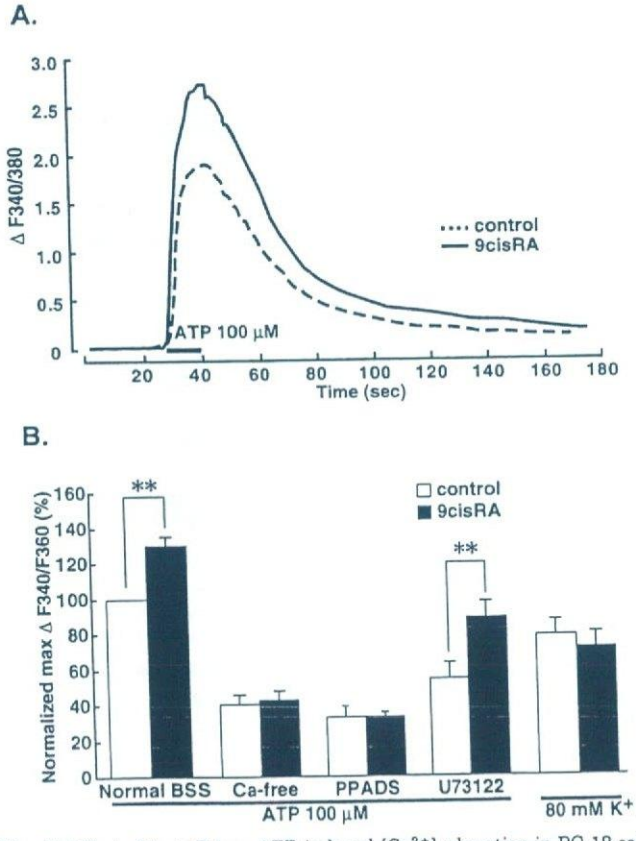

Fig. 7. Effect of 9-*cis*-RA on ATP-induced $[Ca^{2+}]_i$ elevation in PC-12 cells. A, traces showing the records of the fura-2 emission ratios from PC-12 cells onto which 100 μM ATP was applied with or without 100 nM 9-*cis*-RA for 24 h. B, ATP-induced $[Ca^{2+}]_i$ elevations were measured in several 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).

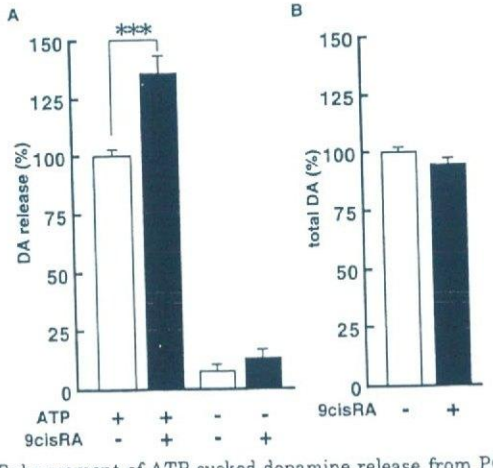

Fig. 8. Enhancement of ATP-evoked dopamine release from PC-12 cells by 9-*cis*-RA. PC-12 cells were incubated with or without 9-*cis*-RA for 24 h. A, the extracellular contents of DA after the application of 30 μM ATP for 1 min were measured with the high-performance liquid chromatography combined with 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 *P2X₂* 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.

References

Aranda A and Pascual A (2001) Nuclear hormone receptors and gene expression. *Physiol Rev* 81:1269–1304.

Batistatou A and Greene LA (1993) Internucleosomal DNA cleavage and neuronal cell survival/death. *J Cell Biol* 122:523–532.

Boniece IR and Wagner JA (1995) NGF protects PC12 cells against ischemia by a mechanism that requires the N-kinase. *J Neurosci Res* 40:1–9.

Boue-Grabot E, Barajas-Lopez C, Chakfe Y, Blais D, Belanger D, Emerit MB, and Seguela P (2003) Intracellular cross talk and physical interaction between two classes of neurotransmitter-gated channels. *J Neurosci* 23:1246–1253.

Brake AJ, Wagenbach MJ, and Julius D (1994) New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature (Lond)* 371:519–523.

Cavaliere F, Florenzano F, Amadio S, Fusco FR, Viscomi MT, D'Ambrosi N, Vacca F, Sancesario G, Bernardi G, Molinari M, et al. (2003) Up-regulation of $P2X_2$, $P2X_4$ receptor and ischemic cell death: prevention by P2 antagonists. *Neuroscience* 120:85–98.

Chambon P (1996) A decade of molecular biology of retinoic acid receptors. *FASEB J* 10:940–954.

Dong D and Noy N (1998) Heterodimer formation by retinoid X receptor: regulation by ligands and by the receptor's self-association properties. *Biochemistry* 37: 10691–10700.

Dong D, Ruuska SE, Levinthal DJ, and Noy N (1999) Distinct roles for cellular retinoic acid-binding proteins I and II in regulating signaling by retinoic acid. *J Biol Chem* 274:23695–23698.

Fasolato C, Pizzo P, and Pozzan T (1990) Receptor-mediated calcium influx in PC12 cells. ATP and bradykinin activate two independent pathways. *J Biol Chem* 265:20351–20355.

Fujiwara Y and Kubo Y (2004) Density-dependent changes of the pore properties of the $P2X_2$ receptor channel. *J Physiol (Lond)* 558:31–43.

Galligan JJ and Bertrand PP (1994) ATP mediates fast synaptic potentials in enteric neurons. *J Neurosci* 14:7563–7571.

Gorodeski GI (2002) Expression, regulation and function of $P2X_4$ purinergic receptor in human cervical epithelial cells. *Am J Physiol* 282:C84–C93.

Illes P and Alexandre Ribeiro J (2004) Molecular physiology of P2 receptors in the central nervous system. *Eur J Pharmacol* 483:5–17.

Inoue K and Kenimer JG (1988) Muscarinic stimulation of calcium influx and norepinephrine release in PC12 cells. *J Biol Chem* 263:8157–8161.

Inoue K, Nakazawa K, Fujimori K, and Takanaka A (1989) Extracellular adenosine 5′-triphosphate-evoked norepinephrine secretion not relating to voltage-gated Ca channels in pheochromocytoma PC12 cells. *Neurosci Lett* 106:294–299.

Inoue K, Nakazawa K, Ohara-Imaizumi M, Obama T, Fujimori K, and Takanaka A (1991a) Antagonism by reactive blue 2 but not by brilliant blue G of extracellular ATP-evoked responses in PC12 phaeochromocytoma cells. *Br J Pharmacol* 102: 851–854.

Inoue K, Nakazawa K, Ohara-Imaizumi M, Obama T, Fujimori K, and Takanaka A (1991b) Selective and competitive antagonism by suramin of ATP-stimulated catecholamine-secretion from PC12 phaeochromocytoma cells. *Br J Pharmacol* 102:581–584.

Ishida S, Shigemoto-Mogami Y, Kagechika H, Shudo K, Ozawa S, Sawada J, Ohno Y, and Inoue K (2003) Clinically potential subclasses of retinoid synergists revealed by gene expression profiling. *Mol Cancer Ther* 2:49–58.

Kanjhan R, Housley GD, Burton LD, Christie DL, Kippenberger A, Thorne PR, Luo L, and Ryan AF (1999) Distribution of the $P2X_2$ receptor subunit of the ATP-gated ion channels in the rat central nervous system. *J Comp Neurol* 407:11–32.

Khakh BS, Gittermann D, Cockayne DA, and Jones A (2003) ATP modulation of excitatory synapses onto interneurons. *J Neurosci* 23:7426–7437.

Khakh BS, Zhou X, Sydes J, Galligan JJ, and Lester HA (2000) State-dependent cross-inhibition between transmitter-gated cation channels. *Nature (Lond)* 406: 405–410.

Kurokawa R, DiRenzo J, Boehm M, Sugarman J, Gloss B, Rosenfeld MG, Heyman RA, and Glass CK (1994) Regulation of retinoid signalling by receptor polarity and allosteric control of ligand binding. *Nature (Lond)* 371:528–531.

MacDonald PN, Bok D, and Ong DE (1990) Localization of cellular retinol-binding protein and retinol-binding protein in cells comprising the blood-brain barrier of rat and human. *Proc Natl Acad Sci USA* 87:4265–4269.

Maruyama K and Sugano S (1994) Oligo-capping: a simple method to replace the cap structure of eukaryotic mRNAs with oligoribonucleotides. *Gene* 138:171–174.

Minucci S, Leid M, Toyama R, Saint-Jeannet JP, Peterson VJ, Horn V, Ishmael JE, Bhattacharyya N, Dey A, Dawid IB, et al. (1997) Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. *Mol Cell Biol* 17:644–655.

Mori T (1978) Study of a growth factor for *Mycobacterium lepraemurium*. I. Minimal medium. *Int J Lepr Other Mycobact Dis* 46:125–132.

Nakazawa K and Inoue K (1992) Roles of Ca^{2+} influx through ATP-activated channels in catecholamine release from pheochromocytoma PC12 cells. *J Neurophysiol* 68:2026–2032.

North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* 82:1013–1067.

Raha S, de Souza LR, and Reed JK (1993) Intracellular signalling by nucleotide receptors in PC12 pheochromocytoma cells. *J Cell Physiol* 154:623–630.

Rodrigues RJ, Almeida T, Richardson PJ, Oliveira CR, and Cunha RA (2005) Dual presynaptic control by ATP of glutamate release via facilitatory $P2X_1$, $P2X_{2/3}$, and $P2X_3$ and inhibitory $P2Y_1$, $P2Y_2$ and/or $P2Y_4$ receptors in the rat hippocampus. *J Neurosci* 25:6286–6295.

Rubio ME and Soto F (2001) Distinct Localization of P2X receptors at excitatory postsynaptic specializations. *J Neurosci* 21:641–653.

Shafer TJ and Atchison WD (1991) Transmitter, ion channel and receptor properties of pheochromocytoma (PC12) cells: a model for neurotoxicological studies. *Neurotoxicology* 12:473–492.

Shigetomi E and Kato F (2004) Action potential-independent release of glutamate by Ca^{2+} entry through presynaptic P2X receptors elicits postsynaptic firing in the brainstem autonomic network. *J Neurosci* 24:3125–3135.

Takahashi B, Ohta K, Kawachi E, Fukasawa H, Hashimoto Y, and Kagechika H (2002) Novel retinoid X receptor antagonists: specific inhibition of retinoid synergism in RXR-RAR heterodimer actions. *J Med Chem* 45:3327–3330.

Virginio C, North RA, and Surprenant A (1998) Calcium permeability and block at homomeric and heteromeric $P2X_2$ and $P2X_3$ receptors and P2X receptors in rat nodose neurones. *J Physiol (Lond)* 510:27–35.
Wang Y, Haughey NJ, Mattson MP, and Furukawa K (2004) Dual effects of ATP on rat hippocampal synaptic plasticity. *Neuroreport* 15:633–636.
Waterman SA (2000) Voltage-gated calcium channels in autonomic neuroeffector transmission. *Prog Neurobiol* 60:181–210.
Werner EA and Deluca HF (2002) Retinoic acid is detected at relatively high levels in the CNS of adult rats. *Am J Physiol* 282:E672–E678.
Xu GY and Huang LY (2002) Peripheral inflammation sensitizes P2X receptor-mediated responses in rat dorsal root ganglion neurons. *J Neurosci* 22:93–102.

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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 P2Y2 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 P2Y2 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 P2Y2 and P2Y4 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 P2Y2 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

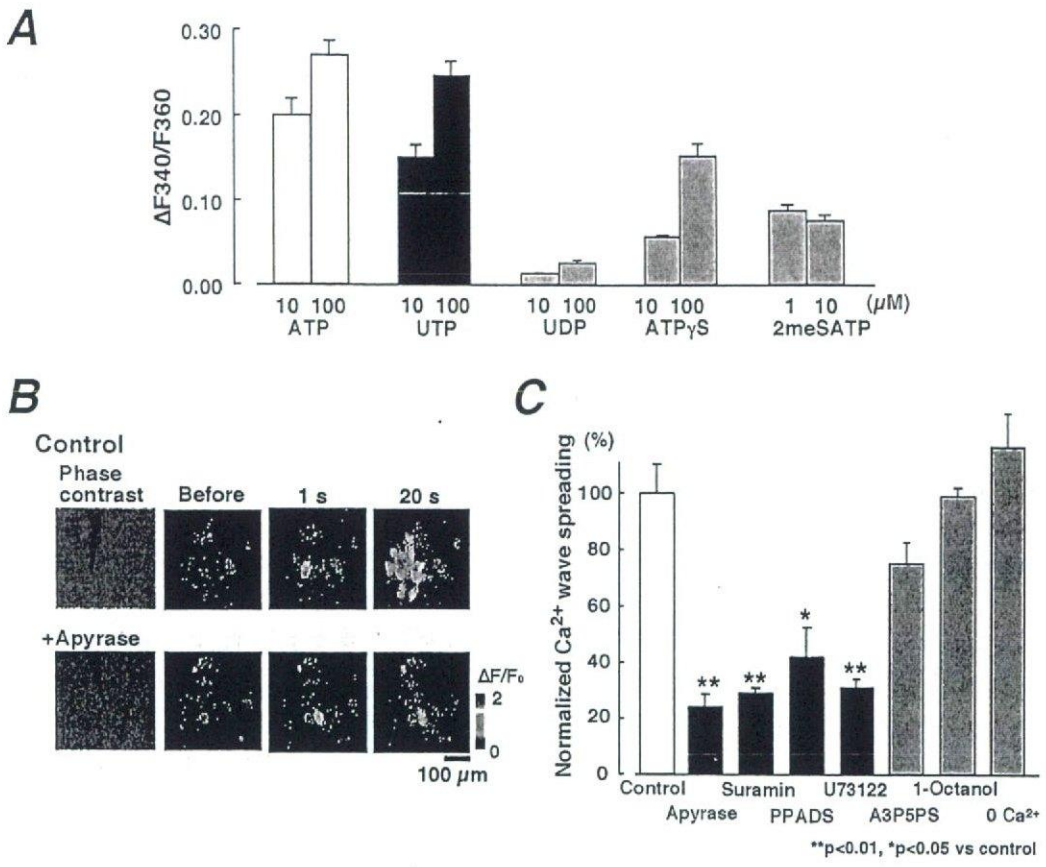

Fig. 1 Pharmacological characterization of increases in $[Ca^{2+}]i$ in NHEKs.
(A) Changes in $[Ca^{2+}]i$ in NHEKs were assessed by a conventional fura-2 fluorescence method. The value show ratiometric fura-2 fluorescence (ΔF340/F360) in responses to various ATP analogues. ATP and UTP were almost equipotent 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 P2Y2 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 P2Y1 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（P2Y2 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 P2Y2 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