0026-895X/06/7001-319–328$20.00
MOLECULAR PHARMACOLOGY
Vol. 70, No. 1
20511/3124430
Mol Pharmacol 70:319–328, 2006
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.

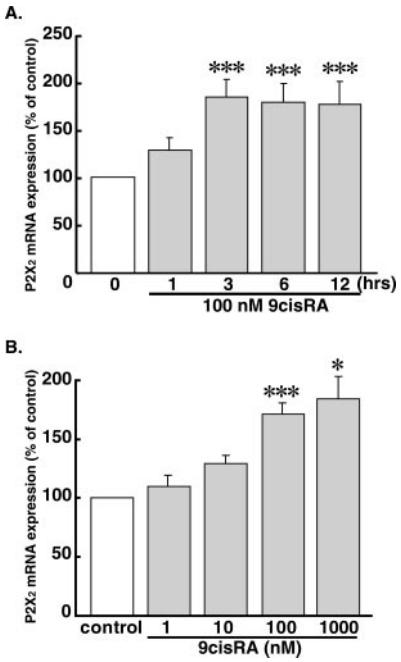

Fig. 2. Increase in the level of $P2X_2$ receptor mRNA by 9-*cis*-retinoic acid. PC-12 cells were treated with 100 nM 9-*cis*-RA for 1, 3, 6, and 12 h (A) or with different concentrations of 9-*cis*-RA (1–1000 nM) (B) followed by real-time RT-PCR analysis of $P2X_2$ and GAPDH mRNAs. $P2X_2$ mRNA levels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

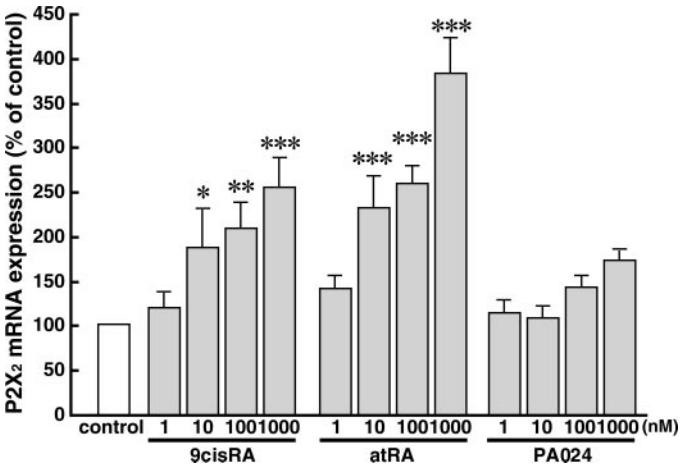

Fig. 3. Effects of selective RAR and RXR agonists on the level of $P2X_2$ receptor mRNA. PC-12 cells were treated with 9-*cis*-RA, atra, or PA024 at different concentrations for 3 h in serum-free condition followed by real-time RT-PCR analysis of $P2X_2$ and GAPDH mRNAs. $P2X_2$ mRNA levels were normalized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

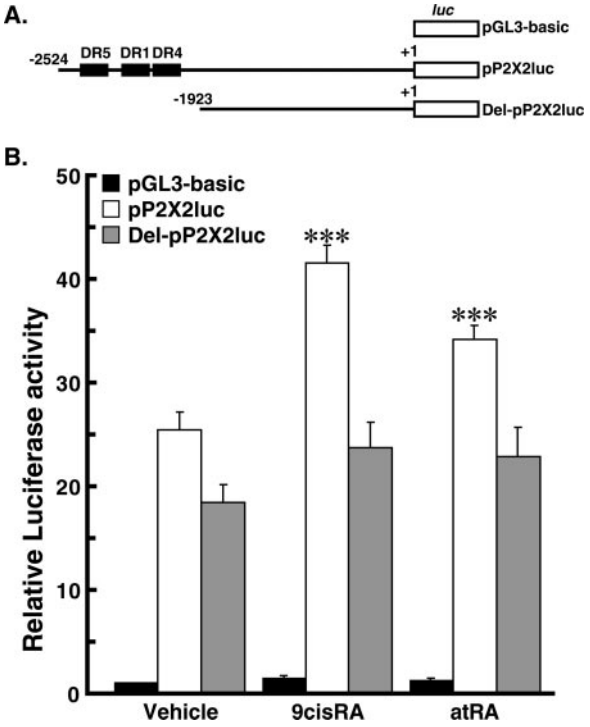

Fig. 4. Transcriptional activity of the 5′-flanking region of *P2rx2* by retinoic acids. The two constructed vectors (pP2X2luc and Del-pP2X2luc) and the empty vector (pGL3-basic) used in the experiment, as described under *Materials and Methods*, are schematically illustrated. Each construct was transfected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

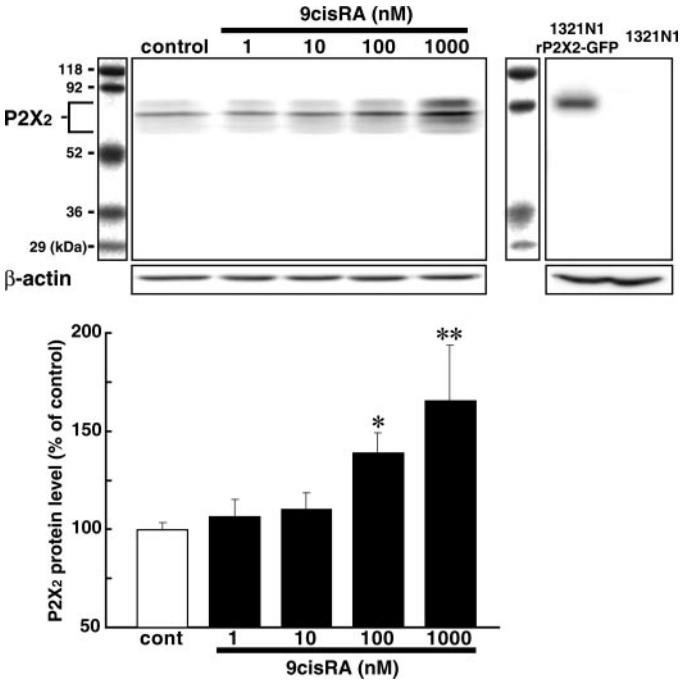

Fig. 5. Increase in $P2X_2$ protein expression by 9-*cis*-RA. Total protein from PC-12 cells treated with or without 9-*cis*-RA (range 1–1000 nM) for 24 h was subjected to Western blot analysis. The proteins of $P2X_2$ receptor and β-actin were detected by their specific antibodies. The intensities of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

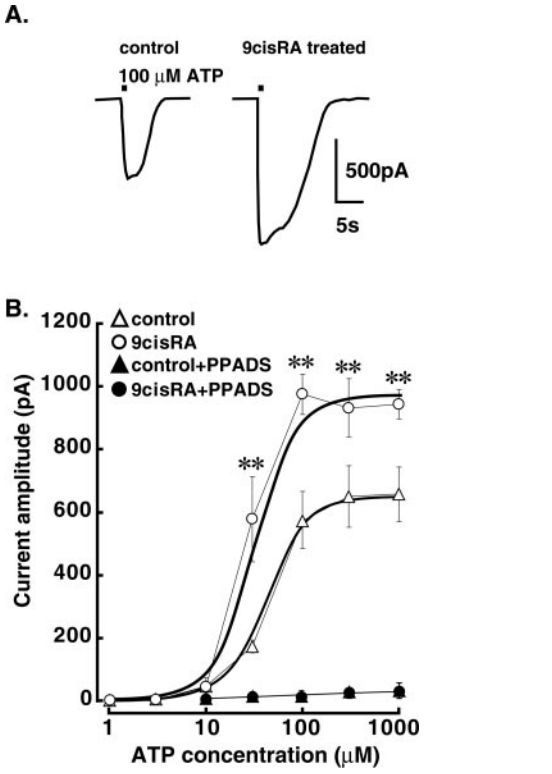

Fig. 6. Effect of 9-*cis*-RA on ATP-induced whole cell current in PC-12 cells. A, representatives traces were the currents evoked by 100 μM ATP in PC-12 cells with or without 100 nM 9-*cis*-RA for 24 h. Cells were voltage-clamped at −60 mV. B, concentration-dependent curves were made by measuring currents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

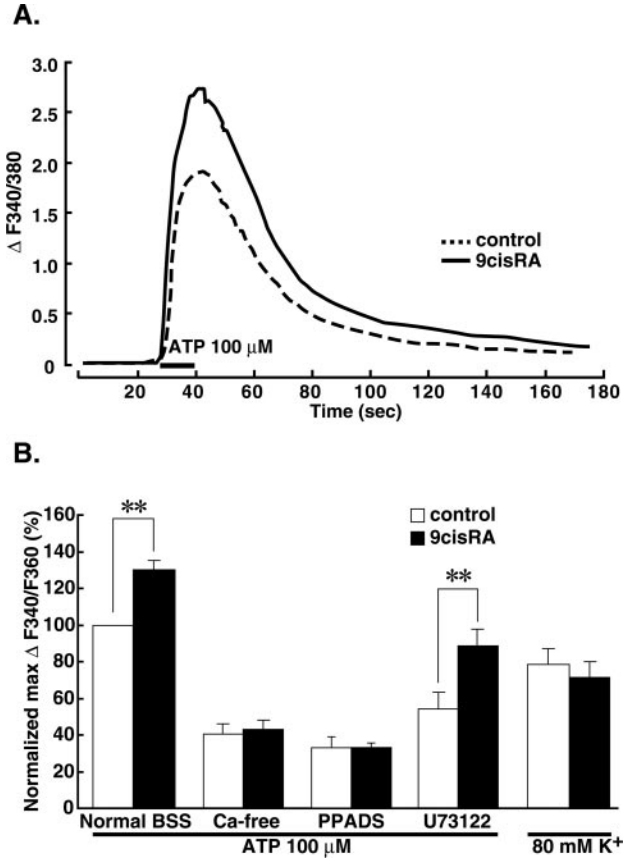

Fig. 7. Effect of 9-*cis*-RA on ATP-induced $[Ca^{2+}]_i$ elevation in PC-12 cells. A, traces showing the records of the fura-2 emission ratios from PC-12 cells onto which 100 μM ATP was applied with or without 100 nM 9-*cis*-RA for 24 h. B, ATP-induced $[Ca^{2+}]_i$ elevations were measured in several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

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).

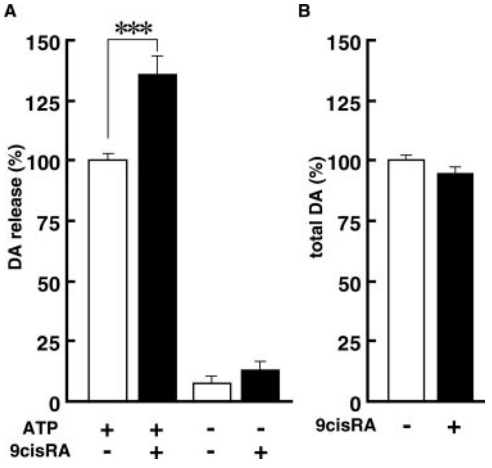

Fig. 8. Enhancement of ATP-evoked dopamine release from PC-12 cells by 9-*cis*-RA. PC-12 cells were incubated with or without 9-*cis*-RA for 24 h. A, the extracellular contents of DA after the application of 30 μM ATP for 1 min were measured with the high-performance liquid chromatography combined with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

Discussion

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

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

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

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

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

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

Acknowledgments

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

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