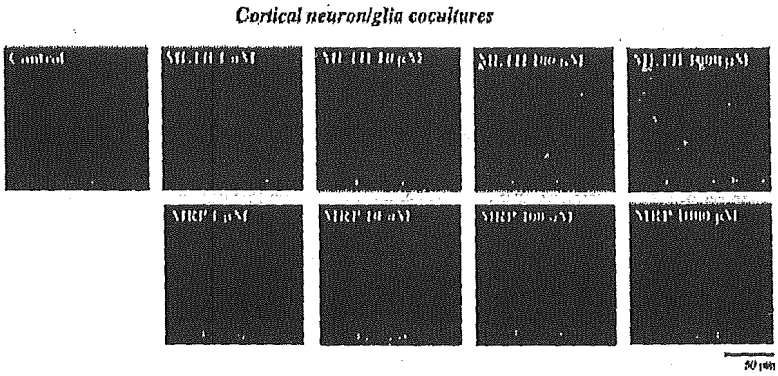
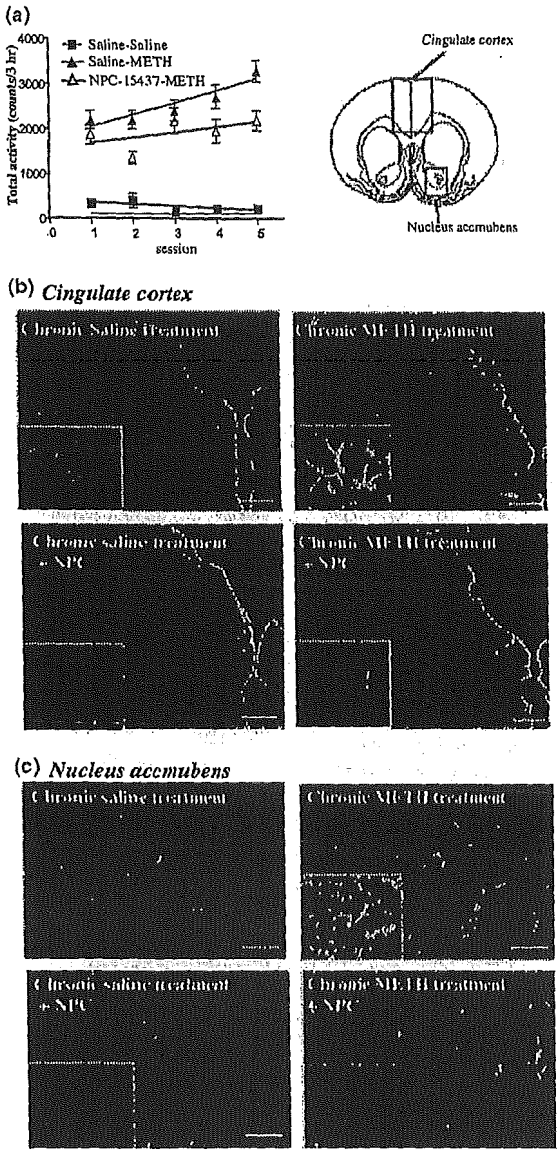


Fig. 7 High concentration of METH, but not MRP, causes a neuronal cell death in cortical neuron/glia co-cultures. (a) Cortical neuron/glia co-cultures were incubated with normal medium or METH (1–1000 μM) for 3 days. (b) Cortical neuron/glia co-cultures were incubated with MRP (1–1000 μM) for 3 days. All cells were stained with a rabbit polyclonal antibody to cleaved caspase-3.



In conclusion, the present data clearly provide direct evidence for the distinct mechanisms between METH and MRP on the astroglial and neuronal responses. Nevertheless opioids, such as MRP, have been used worldwide to control chronic pain, the appearance of opioid addiction following chronic administration of opioids seriously limits their use for the relief of moderate to severe pain. The information of the reversibility of astroglial response and behavioral sensitization with no neuronal cell death induced by MRP could break through the definition of 'opioid addiction' and the misleading of concept that morphine is dangerous. Furthermore, the long-lasting maintenance of behavioral sensitization to METH and neuronal cell death by high concentration of METH observed in this study strongly support the idea for the high risk of the psychostimulant use in humans.

Fig. 8 GFAP-like immunoreactivity (IR) in the cingulate cortex and nucleus accumbens of mice by repeated *in vivo* treatment with METH with or without cotreatment with a specific PKC inhibitor NPC-15437. (a) METH (2 mg/kg, s.c.) or saline was repeatedly given to mice every 96 h, and the total activity was counted for 3 h after each treatment. Repeated injection of METH produced a progressive elevation of the METH-induced locomotor-enhancing effect, indicating the development of sensitization to METH-induced hyper-locomotion ($F_{1,190}$ = 430.20, $p < 0.001$ vs. Saline–Saline). Another group of mice were given METH intermittently in combination with NPC (1 mg/kg, s.c.) every 96 h. NPC or saline was pretreated at 30 min before METH administration (2 mg/kg, s.c.). Intermittent co-administration of NPC-15437 significantly suppressed the development of sensitization to METH-induced hyper-locomotion ($F_{1,90}$ = 20.54, $p < 0.001$ vs. Saline–METH). There were no significant differences between the 1st and 5th administration in NPC-15437-treated mice. The density of GFAP-IR was increased in the cingulate cortex (b) and nucleus accumbens (c) of mice during the development of sensitization to METH. There were no changes in the density of GFAP-IR in the cingulate cortex (b) and nucleus accumbens (c) of mice treated with METH in combination with NPC-15437 as compared to saline treatment. Scale bar (unbroken line): 100 μm; scale bar (broken line): 20 μm (b) or 50 μm (c).

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Upregulation of P2Y2 receptors by retinoids in normal human epidermal keratinocytes

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Abstract

Retinoids, vitamin A derivatives, are important regulators of the growth and differentiation of skin cells. Although retinoids are therapeutically used for several skin ailments, little is known about their effects on P2 receptors, known to be involved in various functions in the skin. DNA array analysis showed that treatment of normal human epidermal keratinocytes (NHEKs) with all-*trans*-retinoic acid (ATRA), an agonist to RAR (retinoic acid receptor), enhanced the expression of mRNA for the P2Y2 receptor, a metabotropic P2 receptor that is known to be involved in the proliferation of the epidermis. The expression of other P2 receptors in NHEKs was not affected by ATRA. ATRA increased the mRNA for the P2Y2 receptor in a concentration-dependent fashion (1 nM to 1 μM). Am80, a synthesized agonist to RAR, showed a similar enhancement, whereas 9-*cis*-retinoic acid (9-*cis*RA), an agonist to RXR (retinoid X receptor), enhanced P2Y2 gene expression to a lesser extent. Ca^{2+} imaging analysis showed that ATRA also increased the function of P2Y2 receptors in NHEKs. Retinoids are known to enhance the turnover of the epidermis by increasing both proliferation and terminal differentiation. The DNA microarray analysis also revealed that ATRA upregulates various genes involved in the differentiation of NHEKs. Our present results suggest that retinoids, at least in part, exert their proliferative effects by upregulating P2Y2 receptors in NHEKs. This effect of retinoids may be closely related to their therapeutic effect against various ailments or aging events in skins such as over-keratinization, pigmentation and re-modeling.

Abbreviations: ATRA – all-trans retinoic acid; $[Ca^{2+}]i$ – intracellular calcium concentration; 9-cisRA – 9-cis retinoic acid; $InsP_3$ – inositol 1,4,5-trisphosphate; 2MeSADP – 2-methyl-thio-ADP; NHEKs – normal human epidermal kratinocytes; RAR – retinoic acid receptor; RXR – retinoid X receptor; UTP – uridine 5′-triphosphate

Introduction

The epidermis, the outermost part of the skin tissue, is a stratified squamous epithelium composed principally of keratinocytes with a highly dynamic structure [1]. Basal keratinocytes are located immediately above the basement membrane that separates the epidermal and dermal compartments. The basal layer is a proliferative layer from which keratinocytes withdraw from the cell cycle and commit to terminal differentiation. Differentiating cells migrate to the skin surface, going through suprabasal layers known as the spinous and granular layers. At the skin surface, dead and terminally-differentiated keratinocytes compose the stratum corneum, the so-called skin barrier. The skin covers most of the body and is exposed to multiple external stimuli such as dryness, chemicals, noxious heat, and UV light.

Endogenous chemical transmitters such as ATP, bradykinin, and histamine modulate skin physiology under normal conditions, after skin injury, and during inflammatory diseases and allergic reactions. Epidermal cells change the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) in response to various environmental stimuli [2–5]. The change in $[Ca^{2+}]i$ is an essential factor for the homeostasis of the skin epidermis and the regulation of the growth of epidermal keratinocytes [6–8]. Because of exposure to such stimuli, the skin causes an aberrant change in $[Ca^{2+}]i$ and often suffers aging damages such as over-keratinization, pigmentation and re-modeling (e.g., formation of wrinkles).

Vitamin A is known as one of the vital nutrients for the body. Retinyl esters and β-carotene from diet are converted

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to retinol and retinal and transported to the tissues by the circulation. They are then metabolized to retinoic acid (RA), a biologically active metabolite of retinoids [9]. Retinoids have been used clinically to prevent aging events or other disorders in the skin. RA works through gene activation as a ligand of the nuclear receptors for retinoic acid (RARα, β and γ), and the retinoid X receptors (RXRα, β and γ) [10, 11]. RARs bind all-*trans*-RA (ATRA), whereas RXRs interact exclusively with 9-*cis*RA stereo-isomers. Retinoid signals are believed to be transduced by RAR–RXR heterodimers [11], but RXRs have also been shown to be heterodimeric partners of a number of other members of the nuclear receptor superfamily (e.g., vitamin D receptors and peroxisome proliferators) [12]. The epidermis expresses RXRα, RXRβ, RARα and RARγ. RXRα and RARγ are dominant in their expression [9]. Although retinoids acting on these nuclear receptors regulate both epidermal proliferation and differentiation [13–15], little is known about the molecular cascades linking the retinoid receptors to cell growth/differentiation.

Adenosine 5′-triphosphate (ATP) is now recognized as an important extracellular molecule that mediates cell-to-cell communication via ATP receptors, P2 receptors. P2 receptors are classified into two subfamilies; the ligand-gated channel P2X receptors (P2X1-7) and the G protein-coupled P2Y receptors (P2Y1,2,4,6,11-14). P2 receptors are distributed in almost all tissues in the body including the skin. Exogenously applied ATP causes an increase in $[Ca^{2+}]i$ in human epidermal keratinocytes [2]. Cultured human keratinocytes can release ATP in response to mechanical stimulation [16] or even spontaneously [3]. Skin cells express P2 receptors, especially P2X5, P2X7, P2Y1 and P2Y2 receptors, each of which is expressed in a spatially distinct zone of the epidermis and has distinct functions in epidermal cell growth and/or differentiation [17]. Recently, we have reported that mechanical stimulation of single normal human epidermal keratinocytes (NHEKs) produces a propagating Ca^{2+} wave that is mediated by extracellular ATP and the activation of P2Y2 receptors [16]. The P2Y2 receptor has been found to be a critical molecule that regulates the proliferation of the basal layer of the epidermis [17]. These findings suggest that intracellular signals mediated by P2Y2 receptors are closely involved in various epidermal functions. We hypothesize that regulation of P2Y2 receptor-mediated signals could lead to therapeutic effects against several ailments or aging events in skin such as over-keratinization, pigmentation and re-modeling.

In the present study, we report that the treatment of cells with retinoids selectively upregulated the mRNA and function of P2Y2 receptors in NHEKs. We also demonstrate that the upregulation is mainly mediated by RAR, presumably RARα. The RARα agonist ATRA mimics an increases in P2Y2 receptors without increasing other P2 receptors in NHEKs. Our present findings suggest that retinoids might at least in part exert their therapeutic effects by controlling ATP/P2Y2 receptor-mediated signals.

Materials and methods

Chemicals

All-*trans* retinoic acid (ATRA), 9-*cis* retinoic acid (9-*cis*RA) and uridine 5′-triphosphate (UTP) were purchased from Sigma Chemical Co. (St. Louis, MO). Am80 was a kind gift from Prof. Kagechika (Tokyo Univ.) [18]. Retinoids and Am80 were dissolved in ethanol and stored at −30 °C.

Cells and cell culture

Normal human epidermal keratinocytes (NHEKs) were obtained as cyropreserved first-passage cells from neonatal foreskins (Kurabo, Osaka, Japan). Cells were cultured in serum-free keratinocyte growth medium, Humedia-KB2 (Kurabo, Osaka, Japan) supplemented with bovine pituitary extract (0.4% v/v), human recombinant epidermal growth factor (0.1 ng/ml), insulin (10 μg/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml) and amphotericin-B (50 ng/ml). The medium was replaced every 2–3 days. In the case of retinoid treatment, the normal culture medium was replaced with the non-supplemented Humedia-KB2 about 24 h before the retinoid treatment in order to remove possible effects from the medium. For Ca^{2+} imaging experiments, cells were plated on collagen-coated coverslips.

Total RNA preparation

NHEKs were prepared in collagen-coated 60 mm dishes (1.5×10^5 cells/dish). After washing the cells twice with PBS, total RNA was prepared with an RNeasy Mini total RNA Preparation Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

Quantitative RT-PCR of P2 receptors

RT-PCR amplifications were performed using Taqman One-step RT-PCR Master Mix Reagents, and 200 nM of each P2 receptor specific primer and 100 nM of Taqman probe. Using Primer Express computer software, (Applied Biosystems Japan Ltd., Tokyo, Japan), clone-specific primers were designed to recognize human P2Y1 (Taqman Probe, 5′-tcagaccccagcaatgtgtgctttca-3′; forward, 5′-gagggcccggctt-gatt-3′; reverse, 5′-atacgtggcataaaccctgtca-3′), P2Y2 (Taqman probe, 5′-aaccttactgcagcatcctcttcctcacc-3′; forward, 5′-tggtgcgcttcctcttctaca-3′; reverse, 5′-accggtgca-cgctgatg-3′), and P2Y11 (Taqman probe, 5′-cgacgacaaactcagtgggttccagg-3′; forward, 5′-ctgccctgccaacttcttg-3′; reverse, 5′-accag-tatgggccacaggaa-3′) receptors mRNA sequences. All primers had similar melting temperatures for running the same cycling program for all samples. RT-PCR was done by 30 min reverse transcription at 48 °C, 10 min AmpliTaq Gold activation at 95 °C, then 15 s denaturation at 95 °C, 1 min annealing and elongation at 60 °C for 40 cycles in a PRISM7700 (Applied Biosystems Japan Ltd). To exclude

the contamination of unspecific PCR products such as primer dimmers, melting curve analysis was applied to all final PCR products after the cycling protocol. Primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Applied Biosystems Japan Ltd) was used for normalization.

DNA micro-array analysis

Converting total RNA to the targets for Affymetrix GeneChip DNA microarray hybridization was done according to the manufacturer's instructions. The targets were hybridized to human genome U95A GeneChip DNA microarray (Affymetrix, Santa Clara, CA, USA) for 16–24 h at 45 °C. After the hybridization, the DNA microarrays were washed and stained on Fluidics Station (Affymetrix) according to the protocol provided by Affymetrix. Then, the DNA microarrays were scanned, and the images obtained were analyzed by Microarray Suite Expression Analysis Software (version 4.0; Affymetrix). The DNA microarray analysis data was obtained from six independent samples ($n = 6$).

Ca^{2+} imaging in single NHEKs

NHEKs were cultured in collagen-coated glass coverslips at density of 1×10^5 cells/ml. Changes in the intracellular calcium concentration ($[Ca^{2+}]i$) in single cells were measured by the fura-2 method as described by Grynkiewicz et al. [19] with minor modifications [20]. In brief, the culture medium of cells grown on a coverslip was replaced with balanced salt solution (BSS) of the following composition (mM): NaCl 150, KCl 5.0, $CaCl_2$ 1.8, $MgCl_2$ 1.2, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) 25, and D-glucose 10 (pH = 7.4). The cells were loaded with 5 μM fura-2 acetoxymethylester (fura-2 AM) (Molecular Probes Inc., Eugene) at room temperature (20–22 °C) in BSS for 45 min, followed by BSS and a further 15 min incubation to allow de-esterification of the loaded dye. The coverslips were mounted on an inverted epifluorescence microscope (TE-2000-U, Nikon, Tokyo, Japan). Fluorescent images were obtained by alternate excitation at 340 (F340) and 380 (F380) nm. The emission signal at 510 nm was collected by a charge-coupled device camera (C-6790, Hamamatsu Photonics, Hamamatsu, Japan) coupled with an image intensifier (GaAsP, C8600-03, Hamamatsu Photonics), and digitized signals were stored and processed using an image processing system (Aquacosmos, Hamamatsu Photonics, Hamamatsu, Japan). Drugs were dissolved in BSS and applied by superfusion.

Detection of ATP release

NHEKs were prepared in collagen-coated 22 × 40 mm chamber glasses at a density of 1×10^5 cells/ml. After superfusion with BSS for 5 min, the cell chamber was filled with a luciferin–luciferase reagent (ATP bioluminescence assay kit CLS, Roche Diagnostics GmbH, Mannheim, Germany). ATP bioluminescence was detected and visualized with a VIM camera (C2400-35, Hamamatsu Photonics, Hamamatsu, Japan) using an integration time of 10 s. The absolute ATP concentration was estimated by using standard ATP solution (Roche Diagnostics GmbH). Data were imaged with Aquacosmos software (Hamamatsu Photonics) and analyzed with NIH-image 1.61 software (Apple computer, Inc., USA). For mechanical stimulation, a single NHEK in the center of the microscopic field was probed with a glass micropipette using a micromanipulator (Narishige, Tokyo, Japan). Under visible light, the tip of the micropipette was positioned approx. 2 μm over the cell to be stimulated. When sampling, the micropipette was rapidly lowered by approx. 2 μm and then rapidly returned to its original position. If the stimulated cell showed any sign of damage (abnormal morphology), the experiment was eliminated.

Statistics

Experimental results are expressed as mean ± SEM Statistical differences between two groups were determined by Student's *t*-test (including the DNA microarray experiments). The multiple linear regression was used to analyze the effect of various concentrations of retinoids on P2Y2 receptor expression. The percentage of the P2Y2 receptor mRNA expression was chosen as outcome variable, and the exposure time of retinoids at each concentration was dummy coded and used as predictor variables.

Results

Retinoids upregulate mRNAs for P2Y2 receptors in NHEKs

The skin expresses multiple P2 receptors. We previously showed that the expression of P2Y1, P2Y2 and P2Y11 is relatively higher in NHEKs [16]. Firstly, we examined the changes in the mRNAs for the P2 receptors induced by retinoids in NHEKs using DNA array analysis. Unfortunately, the DNA microarray we used (U95A GeneChip DNA microarray) does not contain all cloned *P2* receptor genes (for example, P2Y11 receptors) but it contains *P2Y1*, *P2Y2*, *P2Y6*, *P2X1*, *P2X3*, *P2X4*, *P2X5* and *P2X7* receptor genes. Treatment of NHEKs with 0.1 μM all-*trans* retinoic acid (ATRA) for 6 h caused a drastic increase in the mRNA for P2Y2 receptor (304.1 ± 38.1% of control, $n = 3$). Interestingly, ATRA did not affect the expression of any other P2 receptors included in U95A GeneChip (Table 1), suggesting that ATRA selectively upregulates P2Y2 receptors in NHEKs. This result was confirmed quantitatively using real-time RT-PCR (Figure 1). Treatment of NHEKs with ATRA induced a similar increase in the mRNA for P2Y2 receptors (264.4 ± 59.1% of control, $n = 3$) but not for other P2 receptors such as P2Y1 and P2Y11 (P2Y1, 67.5 ± 6.67, $n = 3$; P2Y11, 70.0 ± 11.7% of control, $n = 3$).

t1.1 *Table 1.* ATRA-induced changes in expression pattern of P2 receptors in NHEKs.

t1.2 Gene title	Abbreviations	Percentage of control (%)	Statistics
t1.3 Purinergic receptor P2X, ligand-gated ion channel, 1	P2RX1	94.9 ± 8.5	
t1.4 Purinergic receptor P2X-like 1, orphan receptor	P2RXL1	28.2 ± 40.1	
t1.5 Purinergic receptor P2X, ligand-gated ion channel, 3	P2RX3	138.9 ± 36.7	
t1.6 Purinergic receptor P2X, ligand-gated ion channel, 4	P2RX4	88.9 ± 23.4	
t1.7 Purinergic receptor P2X, ligand-gated ion channel, 5	P2RX5	184.5 ± 41.7	
t1.8 Purinergic receptor P2X, ligand-gated ion channel, 7	P2RX7	78.1 ± 17.4	
t1.9 Purinergic receptor P2Y, G-protein coupled, 1	P2RY1	58.6 ± 18.7	
t1.10 Purinergic receptor P2Y, G-protein coupled, 2	P2RY2	304.1 ± 38.1	**
t1.11 Pyrimidinergic receptor P2Y, G-protein coupled, 6	P2RY6	76.4 ± 9.7	

t1.12 Cells were incubated with 0.1 μM ATRA for 6 h. The expression levels of each gene are shown as the average of triplicate microarray measurements. Asterisks show significant difference from control groups (**$P < 0.01$). Data were normalized by the signals in control (0.5% ethanol).

We next investigated the time-course and concentration dependency of changes in the mRNA expression for P2Y2 receptors induced by ATRA, its stereo-isomer 9-*cis* retinoic acid (9-*cis*RA) and the synthetic RAR agonist Am80. All these retinoids tested caused significant and drastic increases in the mRNA expression in a concentration- and incubation time-dependent manner (Figure 2). After treatment with 1 μM ATRA and Am80 for 24 h, the expression level reached to 768.4 ± 458.1 and 862.2 ± 24.7% of control, respectively (Figure 2A, B). 9-*cis*RA, an agonist to RXRs and possibly to RARs, showed a moderate but significant rise in P2Y2 receptor mRNA in NHEKs (Figure 2C). These results suggest that the upregulation of P2Y2 receptors by retinoids would be mainly mediated by RARs in NHEKs.

Enhancement by ATRA of the UTP-evoked increase in $[Ca^{2+}]i$ in NHEKs

We next investigated whether ATRA increases the function of P2Y receptors in NHEKs. Activation of phospholipase C (PLC)-linked P2Y2 receptors in NHEKs results in an increase in $[Ca^{2+}]i$ via inositol-1,4,5-trisphosphate ($InsP_3$)-mediated Ca^{2+} release from stores [21]. We therefore investigated the effect of ATRA on the increase in $[Ca^{2+}]i$ evoked by UTP, an agonist to P2Y2 receptors, in NHEKs. The cells were stimulated with 0.1 μM ATRA or Am80 for 6 h, and then were incubated with normal culture medium for an additional 18 h. UTP (100 μM) produced an increases in $[Ca^{2+}]i$ in NHEKs that were significantly enhanced by the ATRA- and Am80-treatment (133.2 ± 4.1 and 127.8 ± 6.3% of control, respectively (Figure 3B)). Similar enhancement of Ca^{2+} responses to UTP in retinoids-treated and -untreated cells was observed even in the absence of extracellular Ca^{2+} (Figure 3A). These results suggest that ATRA and Am80 upregulates functional P2Y2 receptor in NHEKs without changing the nature of Ca^{2+} signals.

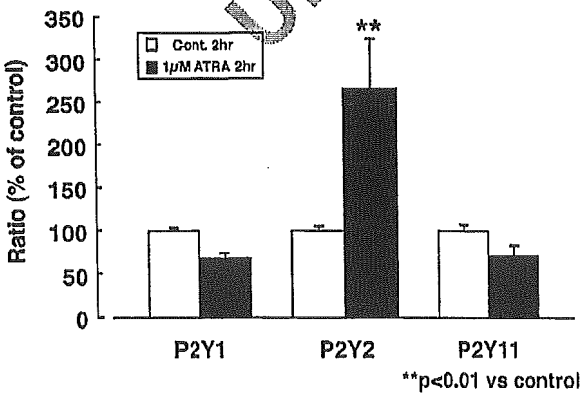

Figure 1. Changes in mRNA expression for P2Y receptors induced by ATRA in NHEKs. Diagram shows the percentage of the quantity after amplification by real-time RT-PCR for P2Y1, P2Y2 and P2Y11 receptor mRNAs extracted from NHEKs treated with 1 μM ATRA for 2 h. Asterisks show significant difference from control groups (*white columns*, **$P < 0.01$). mRNAs of P2Y2 receptors were increased by more than two-fold vs. control. Data were obtained from at least three independent experiments.

ATRA decreases spontaneous ATP release from NHEKs

NHEKs release ATP in response to mechanical stimulation [16] or even spontaneously [3]. Endogenously released ATP and activation of P2Y2 receptors form propagating Ca^{2+} waves in NHEKs [16]. We thus investigated whether ATRA affects the release of ATP from NHEKs. Similarly as in Figure 3, the cells were treated with 0.1 μM ATRA for 6 h, and then incubated for another 18 h with normal culture medium. The cells were bathed in solution containing luciferin–luciferase reagent and photons were counted every 10 s prior to and after mechanical stimulation of the NHEKs in a dark box. The left panels in Figure 4Aa & a′ show phase contrast images of microscopic fields, and the remaining panels (b–e & b′–e′) in Figure 4A show bioluminescence images 10 s before (b & b′) and 10 (c & c′), 20 (d & d′), 30 s (e & e′) after mechanical stimulation in the same field. ATP was released and diffused from the stimulated site in both cultures. We found that the release of ATP peaked around 10 s after mechanical stimulation, and then gradually decreased to the pre-stimulated level in 60 s. The release of ATP at 10 s after mechanical stimulation in ATRA-treated cells is higher than that in control cells (Figure 4A c vs. c′). The photons derived from ATP with and without mechanical stimulation were accumulated for 60 s in both ATRA-treated and -untreated cells and

compared, which was summarized in B. For photon counting, we defined a rectangle (50 μms squares) at the center of the stimulated site, and then measured the averaged photon intensity within the squares (see Figure 4Aa & a' white squares), which was then converted to the absolute ATP concentration using a standard ATP-photon-intensity curve. Mechanical stimulation produced a significant rise in the extracellular ATP concentration in ATRA-treated and control NHEKs (Figure 4B). The extracellular ATP concentrations 60 s after stimulation in ATRA-treated and control NHEKs were 7.30 ± 1.31 (n = 17) and 5.28 ± 0.94 (n = 14) μM, respectively ($P < 0.05$). However, the basal ATP concentration in ATRA-treated NHEKs was significantly lower than that in control cells (Figure 4B, 3.09 ± 0.88 (n = 17) vs. 1.40 ± 0.16 (n = 14) μM, $P < 0.05$).

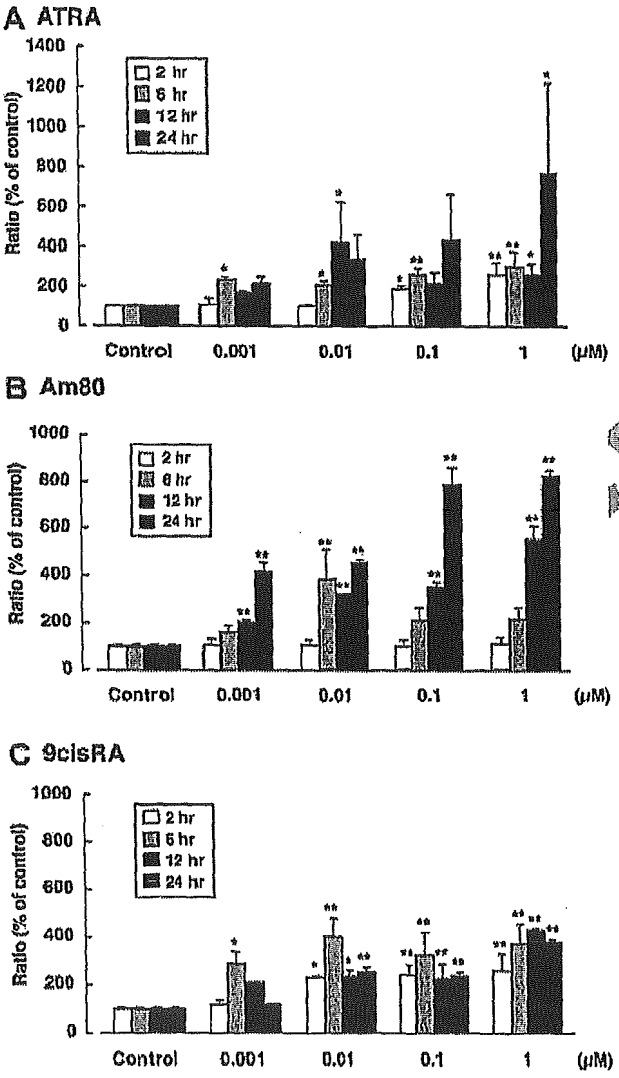

Figure 2. Time- and concentration-dependency of three different retinoids-induced changes in mRNAs in NHEKs. Diagram shows the quantity of P2Y2 mRNAs detected by real-time RT-PCR after treatment with 0.001–1 μM ATRA (A), Am80 (B) and 9-*cis*RA (C) for 2–24 h. The P2Y2 mRNA levels in cells treated with various concentrations of retinoids were normalized by those in retinoids-untreated control cells at each incubation period (2, 6, 12 and 24 h), and expressed as "percentage (%) of control." All these retinoids, and especially Am80, caused a linear increase in P2Y2 mRNAs in a concentration- and time-dependent fashion. *Asterisks* show significant difference in the P2Y2 mRNA levels from control groups (*$P < 0.05$; **$P < 0.01$). Data were obtained from at least three independent experiments.

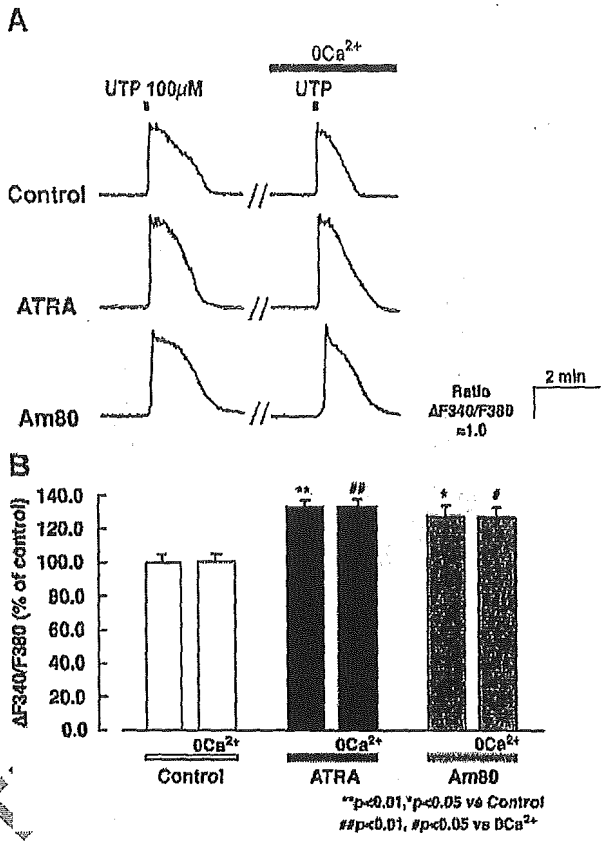

Figure 3. Enhancement by ATRA and Am80 of P2Y2 receptor-mediated increase in $[Ca^{2+}]i$ in NHEKs. A. Typical traces of the UTP-evoked changes in $[Ca^{2+}]i$ in NHEKs. NHEKs were incubated with 0.1 μM ATRA (*middle*) or Am80 (*bottom*) for 6 h, incubated with normal culture medium for another 18 h, and then the fura-2 based $[Ca^{2+}]i$ measurement was performed. UTP (100 μM) was applied to cells for 10 s and the increase in the ΔF340/F380 ratio was calculated (n = 110–125). After the initial UTP-application, the extracellular Ca^{2+} was removed (0 Ca^{2+}), and the second UTP was applied to the cells in the absence of extracellular Ca^{2+}. Effect of ATRA and Am80 on the UTP-evoked elevation in $[Ca^{2+}]i$ in NHEKs in the presence and absence of extracellular Ca^{2+} was summarized in B. *Asterisks* show significant difference from control (without retinoids) (*$P < 0.05$; **$P < 0.01$).

Discussion

In the present study, we demonstrated that ATRA and Am80, a synthesized agonist to RARs, selectively increased the expression of P2Y2 receptors in cultured NHEKs. P2Y2 receptors are relatively localized at the proliferative basal layer of keratinocytes [17], and activation of P2Y2 receptors results in proliferation of the epidermis *in vivo* [17] and *in vitro* [3]. Retinoids are known to induce both epidermal proliferation and differ-

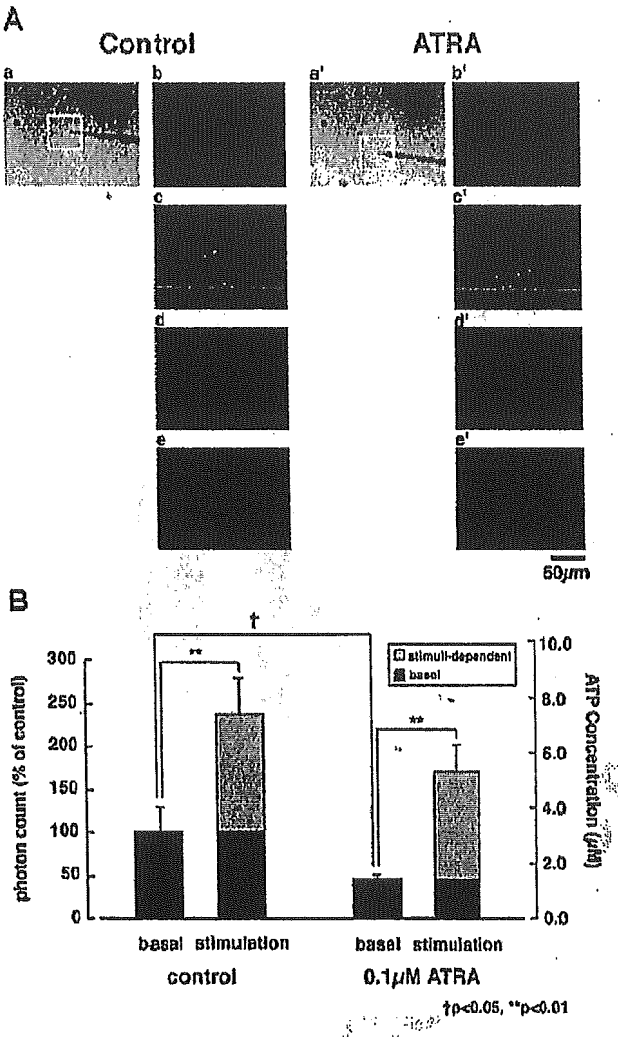

Figure 4. Visualization of release of ATP from NHEKs. The image panels in A show ATP-derived photons (*white dots*) in a field of ATRA-treated (*right*) and -untreated control NHEKs (*left*). NHEKs were incubated with 0.1 μM ATRA for 6 h. Cells were bathed in luciferin–luciferase reagent and the bioluminescence signals were obtained with a VIM camera (see Materials and methods Section) with an exposure time of 10 s. Sequential images show the ATP-derived photon-signals before (−10 s; b & b′) and 10 (c & c′), 20 (d & d′) and 30 s (e & e′) after mechanical stimulation. The positions of the pipettes are shown in phase-contrast images of NHEKs (a & a′). In B, the accumulative photon intensity in 60 s was converted to the absolute extracellular ATP concentration using a standard ATP-photon intensity relationship curve determined with an ATP standard solution (control, n = 17; ATRA-treated, n = 14). Photons within 50 μms squares around the stimulated site (shown as white squares in a & a′ panels in A) were calculated. Asterisks show significant difference from basal groups (**P < 0.01) and dagger shows significant difference from control basal groups (†P < 0.05).

entiation. These findings strongly suggest that upregulation of P2Y2 receptors by retinoids may be the mechanism by which retinoids induce the cell growth of proliferative basal keratinocytes.

Ever since the discovery of retinoic acid, there has been growing interest in retinoid-induced pleiotropic effects [11]. With regard to the skin, retinoids have been used clinically for various skin disorders or problems such as psoriasis [25], wrinkles [26],[23] acne [27][21] and cancer [28–30]. Although retinoids can act on organs other than the epidermis and reveal their therapeutic effects, for example, facilitation of collagen formation in the dermis and inhibition of sebaceous gland activities, they directly act on the epidermis itself and regulate both its proliferation and differentiation [13–15]. The epidermis expresses RARs and RXRs, especially RARγ and RXRα [9]. Activation of RARs causes keratinocytic differentiation and hyperproliferation [31][29] [14]. RXRα are involved in retinoid-induced cell proliferation in adult mouse skin [32],[29] and RARγ/RXRα heterodimers in suprabasal keratinocytes are required for retinoid-induced epidermal hyperplasia [15]. In the present study, we showed that ATRA and Am80 dramatically and selectively increased the expression of P2Y2 receptors. Subcutaneous injection of UTP, an agonist to P2Y2 receptors, generates epidermal hyperplasia that results from hyperproliferation of basal keratinocytes *in vivo* [17]. Incubation of cultured keratinocytes with UTP causes epidermal proliferation *in vitro* [3]. These findings suggest that P2Y2 receptors in basal keratinocytes may be an important target for retinoid-induced proliferation, i.e., retinoids acting mainly on RARs, presumably RARγ, in the epidermis upregulate the expression and function of P2Y2 receptors, thereby leading to the proliferation of basal keratinocytes. Xiao et al. (1999) have already shown that activation of RARγ/RXRα heterodimers in epidermis resulted in the upregulation of heparin-binding epidermal growth factor (HB-EGF), by which retinoid induces the cell growth of basal keratinocytes [14]. However, the production of HB-EGF occurs not in basal layer but in differentiated suprabasal cells which in turn stimulate basal keratinocytes to proliferate in a paracrine manner. We used cultured NHEKs and demonstrated that both ATRA and Am80 selectively upregulated *P2Y2* receptor genes and their functions. Keratinocytes cultured *in vitro* are considered to be similar to basal layer keratinocytes *in vivo* based on their ability to proliferate and express basal cell-specific genes such as keratin-5 or keratin-14 [33].[30] P2Y2 receptors are mainly localized in the proliferative basal layer of keratinocytes *in situ* [17]. Thus, in addition to differentiated suprabasal cells [14], retinoids seem to affect the function of basal keratinocytes directly to cause cell growth. NHEKs release ATP in response to mechanical stimulation, and retinoids had no significant effect on the evoked release of ATP (Figure 4). It is therefore suggested that ATP released from the basal cell layer and acting on P2Y2 receptors could function in an autocrine manner to control the proliferation in basal keratinocytes. Retinoids may cause proliferation by facilitating the ATP/P2Y2 autocrine signals. Although retinoids had no significant effect on the release of ATP in response to mechanical stimulation, they reduced the basal ATP release in NHEKs (Figure 4B). This complexity in the ATP release in NHEKs might explain the mixed and multiple effects of retinoids.

Basal keratinocytes also express P2Y1 receptors *in situ* [17]. However, the effect of the P2Y1 agonists 2methylthio-ADP (2MeSADP) on epidermal proliferation *in vivo* is

much weaker than that of UTP, suggesting that P2Y2 receptors have a more significant role in the proliferation [17]. P2Y1 and P2Y2 receptors share Gq/11-coupled intracellular signal cascades. Activation of both receptor results in inositol 1,4,5-trisphosphate ($InsP_3$) production, leading to Ca^{2+} mobilization from the store [34–36]. In addition, activation of both receptors also induces ERK1/2 MAPkinase cascades in some cells such as astrocytes [37, 38]. However, P2Y2 receptors seem to be much more closely related to proliferation of the epidermis. This discrepancy may be explained by the lower expression level of P2Y1 receptors in basal keratinocytes. In fact, the P2Y1 receptor agonist 2meSADP caused only a slight increase in $[Ca^{2+}]i$ in cultured NHEKs [16], though quantitative analysis of the expression levels and localization of both receptors *in situ* are required to clarify this issue. In any case, retinoids upregulated the P2Y2 receptor but not P2Y1 receptor expression in NHEKs (Table I and Figure 1). The published sequence of the human *P2Y2* receptor gene promoter shows that the P2Y2 receptor has putative RAREs in the upstream promoter region (data not shown), which may also support that *P2Y2* receptors are a likely target gene for retinoids.

In summary, we demonstrated that retinoids upregulate P2Y2 receptors via mainly RAR in NEHKs. Judging from the well-known finding that activation of P2Y2 receptors regulates the proliferation of the epidermis, retinoids may exert their therapeutic effects through the upregulation of P2Y2 receptors in the skin.

Acknowledgements

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Glia in press

"Possible involvement of increase in spinal fibronectin following peripheral nerve injury in upregulation of microglial $P2X_4$, a key molecule for mechanical allodynia"

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Running title: Fibronectin upregulates microglial $P2X_4$

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Key words: ATP, purinergic, glia, extracellular matrix, pain

Abstract

We have recently demonstrated that the $P2X_4$ receptor, an ATP-gated cation channel, in spinal microglia is a key molecule that mediates the mechanical allodynia induced by peripheral nerve injury. Although microglial $P2X_4$ receptor expression is increased after peripheral nerve injury, the molecular mechanism(s) underlying its upregulation remains largely unknown. Fibronectin is a member of the extracellular matrix molecules and is actively produced in response to injury and diseases in the CNS. Here, we describe the influence of fibronectin on $P2X_4$ receptor expression in microglia and the upregulation of fibronectin after peripheral nerve injury. Microglia that were cultured on fibronectin-coated dishes showed a marked increase in $P2X_4$ receptor expression, both at the mRNA and protein levels, as compared to those cultured on control dishes. Fibronectin also enhanced the microglial Ca^{2+} responses mediated by $P2X_4$ receptors. Moreover, Western blot examination of the spinal cord from rat with spinal nerve injury indicated that fibronectin was upregulated on the ipsilateral side. Interestingly, intrathecal injection of ATP-stimulated microglia to the rat lumber spinal cord revealed that microglia cultured on fibronectin-coated dishes was more effective in the induction of allodynia than microglia cultured on control dishes. Taken together, our results suggest that spinal fibronectin is elevated after the peripheral nerve injury and it may be involved in the upregulation of the $P2X_4$ receptor in microglia, which leads to the induction of neuropathic pain.

Introduction

Extracellular nucleotides act as signaling molecules in numerous tissues. Two groups of purinoceptors with distinct signal transduction mechanisms are known to exist. P2X purinoceptors are ligand-gated ion (cation) channels, whereas P2Y purinoceptors are members of the superfamily of G protein-coupled receptors. The P2X family consists of seven different subunits that can form homo- or hetro-oligomeric assemblies, and each subunit has two transmembrane regions with intracellular N- and C-termini. The $P2X_4$ receptor has a broad expression pattern in the periphery and it predominates in the CNS (Le et al. 1998; Soto et al. 1996). With regard to the physiological and pathological importance of $P2X_4$ in the CNS, we have recently showed that $P2X_4$ receptors in the spinal cord are upregulated after peripheral nerve injury, which is responsible for the induction of mechanical allodynia in rats (Tsuda et al. 2003). Interestingly, the $P2X_4$ receptor is upregulated in microglia but not in neurons in the spinal cord. Allodynia is a form of neuropathic pain that is caused by normally innocuous stimuli such as touch, and although the symptom has been recognized for over a century, its cellular mechanisms are largely unknown. Microglial $P2X_4$ receptors in the spinal cord could be a key molecule that induces the mysterious neuropathic pain, allodynia.

Microglia are brain-specific macrophages, and their activation is a general response to pathological processes in the CNS. They are in a quiescent state in the normal brain, but become rapidly activated upon brain injury, inflammation or diseases, transforming from ramified microglia into an amoeboid macrophage-like phenotype. Microglia are known to attach firmly to fibronectin, the upregulation of which is associated with several pathological conditions in the CNS, through β1 integrin and become activated (Milner and Campbell 2002; Milner and Campbell 2003). Fibronectin is one of the extracellular matrix (ECM) molecules and it is a large, multi-domain glycoprotein existing both as a cell surface protein and in plasma. Fibronectin is involved in many cellular processes, including tissue repair, embryogenesis, blood clotting and cell migration/adhesion (Adams and Watt 1993; Hynes 1992; Raghow 1994). The expression of ECM molecules is regionally and developmentally regulated in the brain, and their presence is relatively minor in the normal CNS. Some ECM molecules including fibronectin, however, are upregulated following adult CNS injury (Jones 1996). These data suggest that fibronectin is a key molecule involved in the overexpression of $P2X_4$ in microglia after nerve injury.

In the present study, we demonstrate that (1) culturing primary microglia on fibronectin induces the upregulation of functional $P2X_4$ receptors on the cell surface *in vitro*, (2) increased

expression of fibronectin was observed in the ipsilateral side of the spinal cord taken from allodynia rats and (3) intrathecal administration of ATP-stimulated microglia that had been treated with fibronectin enhanced the allodynic response in rats *in vivo*. All these findings suggest that the increase in spinal fibronectin after a spinal nerve injury is a critical event in the upregulation of microglial $P2X_4$ receptors, which would be important for the onset of mechanical allodynia.

Materials and Methods

Isolation of microglia - The primary cultures of rat microglia were derived from the forebrains of neonatal Wistar rats (Nakajima et al. 1992). In brief, the rat cortices were separated from the meninges, minced, treated with trypsin and with DNase, and then centrifuged to remove dead cells. The pellet was resuspended in DMEM, filtrated and cultured in medium with 10% foetal bovine serum for 12-23 days. Microglia were isolated on day 10 and day 15 by gently shaking the flasks for 2 min.

Quantitative RT-PCR - Microglia were plated on tissue culture dishes that had been coated with fibronectin (Sigma, Missouri, USA) at 10 μg/ml or non-treated, and kept at 37 °C for 3 hr. Then, the cells were washed with warm DMEM twice and the total RNA was extracted using RNeasy mini kit (QIAGEN Japan, Tokyo, Japan). Real time RT-PCR was performed using TaqMan One-Step RT-PCR Master Mix Kit (Applied Biosystems, California, USA), $P2X_4$ primers and TaqMan GAPDH Control Reagents (Applied Biosystems). The forward and reverse primer pairs for $P2X_4$ were :

F : 5'-TGGCGGACTATGTGATTCCA-3'

R: 5'-GGTTCACGGTGACGATCATG-3'

The PCR reaction was carried out by One Step RT-PCR in a total volume of 25 μl using the ABI PRISM 7700 Sequence Detection system (Applied Biosystems). All values were normalized with the GAPDH expression.

Western blotting – Microglia were lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 % NP-40, 1 % SDS, 5 mM EDTA, protease inhibitors cocktail) and mixed with Laemmli sample buffer. For the rat spinal cord homogenates, the L5 corresponding spinal cord was collected from control or allodynia rats of 1-, 3-, 7-day post operation and the area of dorsal horn was excised. Then the tissue was homogenized in homogenization buffer (PBS, 1 % NP-40, 1 % Triton X-100, 5mM EDTA, protease inhibitors cocktail) for 20 sec on ice, centrifuged thoroughly to remove cell debris, and mixed with Laemmli sample buffer. All samples were subject to BCA assay to adjust the loading protein amount. Cell lysates or tissue homogenates were resolved by SDS-PAGE and transferred to nitrocellulose membrane (BioRad, Californina, USA). The membrane was blocked with TBS-Tween 0.05 %, 1 % BSA, 0.02 % NaN_3 and probed with primary antibodies; anti-$P2X_4$ (Alomone, Jerusalem, Israel, 1:200 dilution),

anti-β-actin (Sigma, 1:1000 dilution), anti-ERK2 (Santa Cruz, California, USA, 1:200 dilution) or anti-fibronectin (Dako, Glostrup, Denmark, 1:100 dilution). The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG secondary antibodies (Amersham Biosciences, New Jersey, USA, 1:1000 dilution) and visualized with the ECL system (Amersham Biosciences). Bands were quantified using NIH Image J 1.33u software.

Intracellular calcium concentration ($[Ca^{2+}]i$) measurement - Microglia were cultured for 24 hr at 37 °C on an appropriately coated Flexiperm cover glass. Then the culture medium was replaced with balanced salt solution (BSS at pH 7.4: 150 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgCl_2$, 25 mM HEPES, 10mM D-glucose). Cells were loaded with fura-2 by incubating them with 5 μM fura-2-acetoxymethylester in BSS for 1 hr at room temperature. Changes in $[Ca^{2+}]i$ were assessed by ratiometric images (F340/F380) of fura-2 fluorescence, which were detected with Aquacosmos/HiSca (Hamamatsu Photonics, Hamamatsu, Japan). For TNP-ATP(100 μM), PPADS (10 μM) or 0 Ca^{2+} (removal of extracellular Ca^{2+}) experiments, cells were treated with these antagonists or the 0 Ca^{2+} solution 2 min before and during ATP-applications.

Chung model - All experiments were performed using 8 week old-male Wistar rats. All surgeries were performed under inhalation anesthesia using Forene in 100 % O_2, induced at 5 % and maintained at 2 %. The spinal nerve on the left side was exposed at a proximal location under an aseptic condition. Then the 5^{th} lumber spinal nerve was tightly ligated with a silk suture (5-0) and its peripheral side was completely transected. The muscle and the skin were sutured closed and the animal was allowed to recover before the behavioural testing. To evaluate allodynia, von Frey filaments were applied to the plantar surface of the hindpaw and the withdrawal from mechanical stimulus was monitored as previously reported (Tsuda et al. 2003).

Intrathecal catheterization and injections of microglia - For intrathecal microglia administration, intrathecal catheterization was performed on Wistar rats (12 weeks, male) (Tsuda et al. 2003). Briefly, with the rat under inhalation anesthesia, an incision was made in the atlanto-occipital membrane and the catheter was inserted caudally to the lumber enlargement (close to L4-L5 segments) of the spinal cord. Verification of the catheter placement was made by the observation of hind limb paralysis after intrathecal injection of lidocaine (2 %, 5 μl) 3 days after

catheterization. Animals that failed the verification for the catheter placement were not included in the data analyses. Microglia were cultured on uncoated- or fibronectin-coated dishes for 24 hr at 37 °C, washed twice with PBS and harvested. After adjusting their concentrations, cells were stimulated with ATP at 0, 0.5 and 5 μM and incubated for 1 hr at 37 °C and subsequently microinjected. Animals were subject to the behavioral testing 5 hr after the injection.

Statistical analysis – The von Frey test results were analyzed by Mann-Whitney U-test and values with $p<0.05$ were considered statistically significant. For the other data, the Student's *t* test was performed and values with $p<0.05$ (or $p<0.01$ where appropriate) were considered statistically significant as compared to controls.

Results

Fibronectin increased the expression of $P2X_4$ receptors in microglia both at the mRNA and protein levels.

Microglia were plated onto fibronectin or control plastic and their P2 receptor expression was studied by quantitative RT-PCR (Figure 1). To normalize the results, we used the mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the endogenous control and therefore, the P2 receptor gene expression was given as the ratio P2X(Y)/GAPDH. As shown in the figure, incubation of microglia on fibronectin at 10 μg/ml for 3 hr resulted in the marked upregulation of $P2X_4$ gene expression, whereas the mRNA expressions of $P2X_7$, $P2Y_2$, $P2Y_6$ and $P2Y_{12}$ were all rather diminished (Figure 1), suggesting that the $P2X_4$ receptor is unique among the purinoceptors on microglia.

To confirm this effect of fibronectin on the $P2X_4$ receptor at the protein level, we examined its expression by Western blotting using anti- $P2X_4$ antibody (Figure 2). As seen in Figure 2, microglial $P2X_4$ appeared as a single band at ~75 kDa, and since its predicted molecular weight from its protein sequence is 43 kDa, the molecule seems to be heavily glycosilated (Soto et al. 1996). We previously reported that fibronectin induces profound microglial proliferation through β1 integrin (Nasu-Tada et al. 2005), and thus the protein amount loaded on the gel was carefully adjusted. In addition, β-actin was used as the endogenous control to normalize the Western blot data. Each band was quantified using computing software, and the basal value of β-actin was subtracted from the $P2X_4$ results. The increase in $P2X_4$ expression became evident after 12 hr of fibronectin stimulation (Figure 2) (1.3 fold as compared to 1 hr incubation, $p<0.05$) and the increase continued until it reached an approximately two-fold increase after 24 hr incubation ($p<0.01$).

Microglia cultured on fibronectin showed an increase in $[Ca^{2+}]i$ in response to ATP stimulation.

To confirm that fibronectin upregulates functional $P2X_4$ receptors on microglia, the ATP-evoked increases in $[Ca^{2+}]i$ were subsequently studied. Microglia were cultured for 24 hr on fibronectin or on uncoated Flexiperm cover glass (control), and the changes in $[Ca^{2+}]i$ in response to ATP (50 μM) were detected by conventional fura-2 method., i.e., the ratiometric images of fura-2 fluorescence. The nucleotide receptors that are known to be expressed in microglia include $P2X_4$, $P2X_7$, $P2Y_2$, $P2Y_6$, $P2Y_{12}$ (Inoue 2002; Sasaki et al. 2003; Tsuda et al.

2003), and possibly $P2Y_{13}$ due to its abundant mRNA in the brain and the immune system (Zhang et al. 2002). $P2Y_{12}$ and $P2Y_{13}$ receptors are Gi-coupled P2 receptors, and the activation of these receptors, in general, does not cause an elevation in $[Ca^{2+}]i$ but decreases the intracellular cAMP. In the absence of extracellular Ca^{2+} (Figure 3, 0 Ca^{2+}), neither the control nor microglia cultured with fibronectin showed much response to ATP 50 μM stimulation, suggesting that Gq/11-phospholipase C coupled P2Y receptors, which are dependent on intracellular Ca^{2+} storage, were not relevant to this case. In contrast, in the presence of extracellular Ca^{2+}, microglia on fibronectin showed a significant increase ($p<0.01$) in the Ca^{2+} response to ATP 50 μM (Figure 3, ATP alone), indicating that the expression of the ion-channel type purinoceptors, i.e. P2X receptors, is augmented by fibronectin and that these are likely to be $P2X_4$ receptors, since the $P2X_7$ receptor is activated at a relatively high concentration of ATP (i.e. concentrations greater than 100 μM) (Ralevic and Burnstock 1998). Pretreatment of cells with TNP-ATP (an antagonist of $P2X_{1-4}$ receptors) dramatically reduced the $[Ca^{2+}]i$ response in microglia on both control and fibronectin-coated dishes, indicating that basal response to ATP at 50 μM as well as its augmented response on fibronectin substrate most result from microglial $P2X_4$ receptor. On the other hand, pretreatment with PPDAS (an antagonist of $P2X_{1,2,3,5,7}$) did not fundamentally affect but only slightly reduced the $[Ca^{2+}]i$ response in both populations. PPADS is known to inhibits $P2Y_{1,2}$ receptors as well and therefore, the result indicates that microglial $P2Y_2$ receptor also constitutes the $[Ca^{2+}]i$ response to ATP stimulation. In conclusion, fibronectin upregulated the functional $P2X_4$ receptors on the microglial surface and this lead to an enhancement of the increase in $[Ca^{2+}]i$ evoked by ATP 50 μM via $P2X_4$ receptors.

Fibronectin was upregulated in the allodynia rat spinal cord.

As described earlier, the importance of the microglial $P2X_4$ receptor in the induction of mechanical allodynia after nerve injury has recently become evident (Tsuda et al. 2003). We sought to determine the profile of fibronectin expression in the spinal cord of nerve-injured rats, where the microglial $P2X_4$ receptor expression is increased. L5 spinal cord segments were harvested from rats of control, 3- and 7-day post nerve injury, and the expression of fibronectin was assessed by Western blotting. There has been little evidence for fibronectin in the normal CNS other than in the basement membrane of endothelial, pial and ependymal cells, and our result showed that Naive and Day 1 rats exhibited slight signs of fibronectin (Figure 4). However, spinal fibronectin became evident on the ipsilateral side at 3 and 7 days following the nerve injury (Figure 4, Day3 ipsi; Day7 ipsi). The contralateral side remained unchanged

throughout the experiment.

Upregulation of microglial $P2X_4$ receptors lowered the threshold of pain responses caused by intrathecal transfer of the cells.

In a study by Tsuda et al. (2003), the intrathecal transfer of ATP-treated microglia induced mechanical allodynia in normal rats and microglial $P2X_4$ receptors were mainly responsible for this effect. Therefore, we hypothesized that microglia with more $P2X_4$ receptors expressed on the surface are capable of causing more mechanical allodynia. To examine this hypothesis, microglia were cultured either on fibronectin or on control plastic for 24 hr, stimulated with 0.5, 5 μM of ATP for 1 hr or left untreated as the control, then intrathecally transferred to normal rats, and their pain behavior was monitored 5 hr after the microinjection using von Frey hairs to calculate the 50 % paw withdrawal threshold (Figure 5). Without intrathecal injection of microglia, no rat showed any pain behavior (data not shown). As seen in Figure 5, no pain response was observed at ATP 0 (control) or 0.5 μM. An interesting difference, however, was seen at ATP 5 μM, where a significant increase in the 50 % withdrawal threshold was observed with fibronectin-treated microglia as compared with the non-treated microglia. Additionally, it was clearly demonstrated that intrathecal transfer of microglia which were treated with ATP at 50 μM, in the absence of fibronectin, was capable of inducing allodynia in the recipient rat (Tsuda et al. 2003). Collectively, these results suggest that upregulation of $P2X_4$ receptors by fibronectin lowered the threshold for the response to mechanical allodynia.