

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 μ M). 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. Ca²⁺ 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; [Ca²⁺]_i – intracellular calcium concentration; 9-*cis*RA – 9-*cis* retinoic acid; InsP₃ – inositol 1,4,5-trisphosphate; 2MeSADP – 2-methyl-thio-ADP; NHEKs – normal human epidermal keratinocytes; 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 Ca²⁺ concentration ([Ca²⁺]_i) in response to various environmental stimuli [2–5]. The change in [Ca²⁺]_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 [Ca²⁺]_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 (RAR α , β and γ), and the retinoid X receptors (RXR α , 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 RXR α , RXR β , RAR α and RAR γ . RXR α and RAR γ 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 [Ca²⁺]_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 Ca²⁺ 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 RAR α . The RAR α agonist ATRA mimics an increase 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 cryopreserved 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 μ g/ml), hydrocortisone (0.5 μ g/ml), gentamicin (50 μ g/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 Ca²⁺ imaging experiments, cells were plated on collagen-coated coverslips.

Total RNA preparation

NHEKs were prepared in collagen-coated 60 mm dishes (1.5 \times 10⁵ 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'-tcagaccaccgaatgtgtgcttca-3'; forward, 5'-gagggcccggtgatt-3'; reverse, 5'-atagctggcataaacctgtca-3'), P2Y2 (Taqman probe, 5'-aaccttactgcagcatcctcttctacc-3'; forward, 5'-tggtgcgcttctctctaca-3'; reverse, 5'-accggtgca-cgctgatg-3'), and P2Y11 (Taqman probe, 5'-cgacgacaaactcagtggttccagg-3'; forward, 5'-ctgccctgccaaactcttg-3'; reverse, 5'-accagtatggccacaggaa-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 ($n = 6$).

Ca²⁺ 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, CaCl₂ 1.8, MgCl₂ 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, Mann-

heim, 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 ($304.1 \pm 38.1\%$ 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 ($264.4 \pm 59.1\%$ of control, $n = 3$) but not for other P2 receptors such as P2Y1 and P2Y11 (P2Y1, 67.5 ± 6.67 , $n = 3$; P2Y11, $70.0 \pm 11.7\%$ of control, $n = 3$).

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

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

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 $[\text{Ca}^{2+}]_i$ 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 $[\text{Ca}^{2+}]_i$ via inositol-1,4,5-trisphosphate (InsP₃)-mediated Ca^{2+} release from stores [21]. We therefore investigated the effect of ATRA on the increase in $[\text{Ca}^{2+}]_i$ 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 increase in $[\text{Ca}^{2+}]_i$ 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.

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

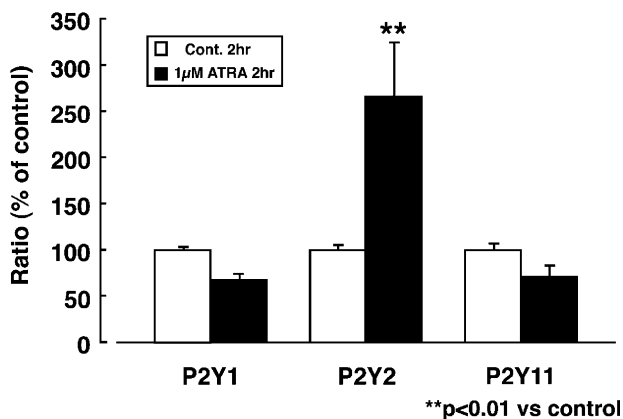


Figure 1. Changes in mRNA expression for P2Y receptors induced by ATRA in NHEKs. Diagram shows the percentage of the quantity after amplification by real-time RT-PCR for P2Y1, P2Y2 and P2Y11 receptor mRNAs extracted from NHEKs treated with 1 μM ATRA for 2 h. Asterisks show significant difference from 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.

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

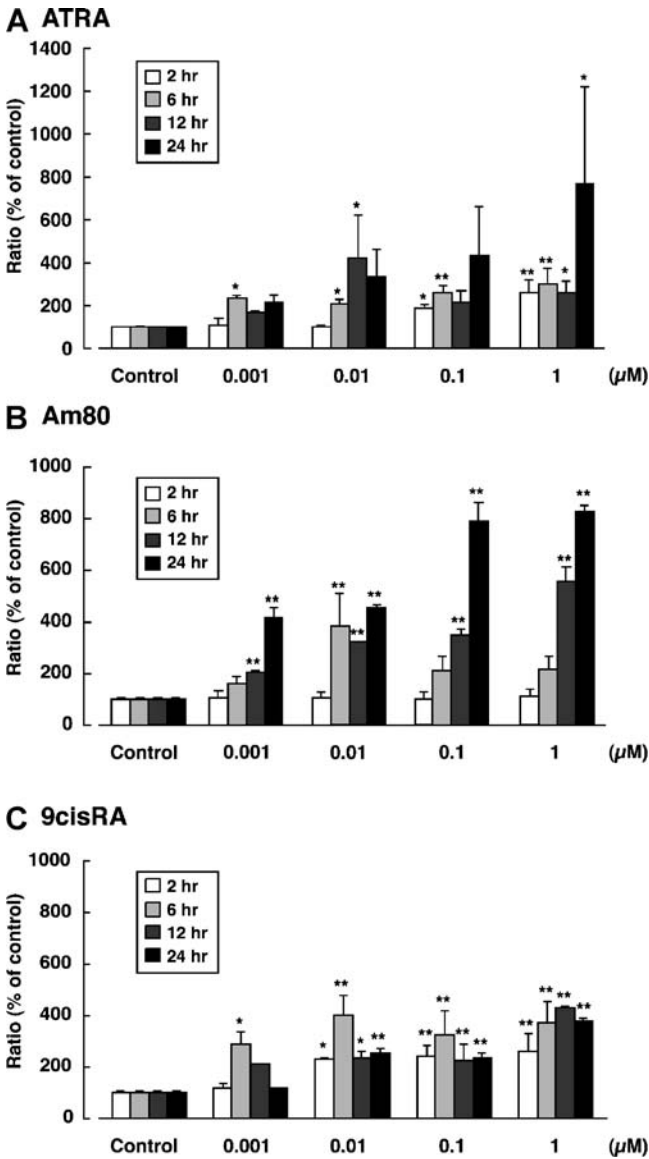


Figure 2. Time- and concentration-dependency of three different retinoids-induced changes in mRNAs in NHEKs. Diagram shows the quantity of P2Y2 mRNAs detected by real-time RT-PCR after treatment with 0.001–1 μM ATRA (A), Am80 (B) and 9-*cis*RA (C) for 2–24 h. The P2Y2 mRNA levels in cells treated with 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.

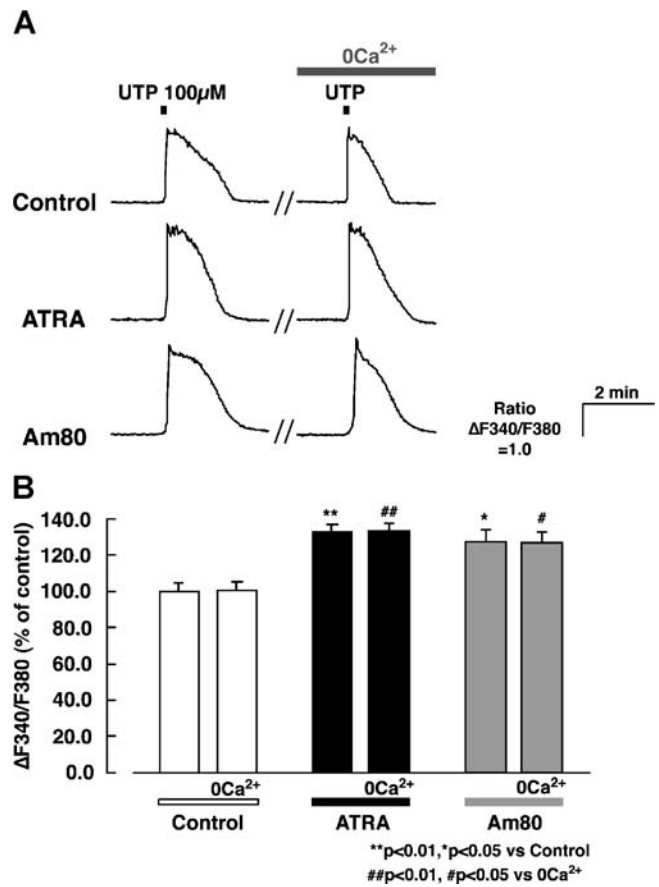


Figure 3. Enhancement by ATRA and Am80 of P2Y2 receptor-mediated increase in $[\text{Ca}^{2+}]_i$ in NHEKs. A. Typical traces of the UTP-evoked changes in $[\text{Ca}^{2+}]_i$ in NHEKs. NHEKs were incubated with 0.1 μM ATRA (middle) or Am80 (bottom) for 6 h, incubated with normal culture medium for another 18 h, and then the fura-2 based $[\text{Ca}^{2+}]_i$ measurement was performed. UTP (100 μM) was applied to cells for 10 s and the increase in the $\Delta\text{F340}/\text{F380}$ 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 $[\text{Ca}^{2+}]_i$ in NHEKs in the presence and absence of extracellular Ca^{2+} was summarized in B. Asterisks show significant difference from control (without retinoids) (* $P < 0.05$; ** $P < 0.01$).

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 , $P < 0.05$).

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-

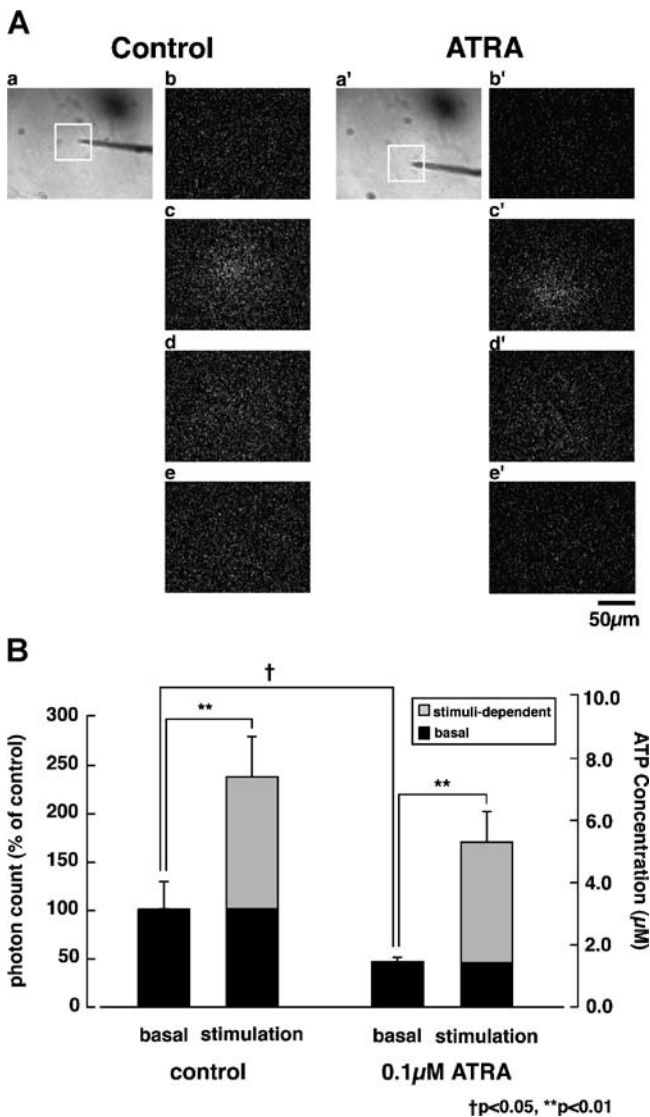


Figure 4. Visualization of release of ATP from NHEKs. The image panels in A show ATP-derived photons (white dots) in a field of ATRA-treated (right) and -untreated control NHEKs (left). NHEKs were incubated with 0.1 µM ATRA for 6 h. Cells were bathed in luciferin-luciferase reagent and the bioluminescence 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 µm 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 [22], wrinkles [23], acne [24] and cancer [25–27]. 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 RAR γ and RXR α [9]. Activation of RARs causes keratinocytic differentiation and hyperproliferation [28] [14]. RXR α are involved in retinoid-induced cell proliferation in adult mouse skin [29], and RAR γ /RXR α 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 RAR γ , 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 RAR γ /RXR α heterodimers in epidermis resulted in the up-regulation 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 [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 (InsP₃) production, leading to Ca²⁺ mobilization from the store [31–33]. In addition, activation of both receptors also induces ERK1/2 MAPkinase cascades in some cells such as astrocytes [34, 35]. 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 [Ca²⁺]_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 1 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 NHEKs. 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.

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