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ATP stimulates interleukin-6 production via P2Y receptors in human HaCaT keratinocytes

Hirohide Yoshida [a,1], Daisaku Kobayashi [a,1], Satoko Ohkubo [a,b,*], Norimichi Nakahata [a,b]

[a] *Department of Cellular Signaling, Tohoku University, Aoba 6-3, Aramaki, Aoba-ku, Sendai 980-8578, Japan*
[b] *21st century COE program "CRESCENDO", Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba 6-3, Aramaki, Aoba-ku, Sendai 980-8578, Japan*

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Abstract

We evaluated the role of ATP in functions of human HaCaT keratinocytes. ATP was released from HaCaT cells by changing the culture medium. Reverse transcription-polymerase chain reaction analysis revealed that HaCaT cells expressed multiple P2 purinergic receptor mRNAs. UTP was the most potent agonist to increase the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). UTP and ATP caused the accumulation of [^{3}H]inositol phosphates, suggesting that UTP binds to the $G_{q/11}$-coupled P2Y receptor. UTP increased IL-6 mRNA and protein levels, and the increases were inhibited by a P2 purinergic receptor antagonist (suramin, 300 μM). While a protein kinase C inhibitor (GF109203X, 10 μM) was without effect, an intracellular free Ca^{2+} chelator (BAPTA-AM, 50 μM) suppressed UTP-mediated IL-6 induction. These results suggest that 1) ATP is released from HaCaT cells upon physical stimulation and may act as an autocrine molecule, and 2) the stimulation of P2Y receptors causes IL-6 production via mRNA expression through $[Ca^{2+}]_i$ elevation.

Keywords: ATP; UTP; P2Y receptor; HaCaT cell; Keratinocyte; Interleukin-6

1. Introduction

Skin consists of the dermis and the epidermis. The epidermis is a laminar squamous epithelium composed of keratinocytes. Proliferating keratinocytes exist in the basal layer, and they gradually differentiate, migrating to the skin surface to form the stratum corneum, which acts as the main cutaneous barrier.

Epidermal keratinocytes produce various cytokines, including transforming growth factor (TGF)-α (Elder et al., 1989), tumor necrosis factor (TNF)-α (Kock et al., 1990), interleukin (IL)-6 (Neuner et al., 1991) and IL-8 (Kondo et al., 1993). IL-6 is known to be a pleiotropic cytokine, causing the growth and differentiation of numerous cell types of dermal and epidermal origin (Naka et al., 2002). In addition, IL-6 has been also suggested to be involved in a number of skin diseases, such as psoriasis (Grossmann et al., 1989) and lichen planus (Yamamoto and Osaki, 1995). Furthermore, the IL-6 level is increased following cutaneous wounds (Gallucci et al., 2000), ultraviolet irradiation to skin (Urbanski et al., 1990) and thermal damage of skin (Kawakami et al., 1997), suggesting the involvement of this cytokine in pathological conditions. Numerous recent studies have indicated that IL-6 has a role in skin repair by stimulating keratinocyte migration and cell proliferation (Gallucci et al., 2004).

Skin is continuously exposed to a variety of stimuli including UV irradiation, thermal changes, harmful chemicals and physical contacts. It has been shown that ATP is released from cells via an unknown mechanism in response to physical stimuli such as stretch and shear forces, and that the released ATP acts as an autocrine and/or paracrine molecule to regulate cell function via interaction with P2 purinergic receptors (Harden et al., 1997; Newman, 2001). Because skin is an elastic tissue that can expand and contract or receive mechanical stimuli, it is possible that ATP is released from the dermal and

* Corresponding author. Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University Aoba 6-3, Aramaki, Aoba-ku, Sendai 980-8578, Japan. Tel.: +81 22 795 5924; fax: +81 22 795 3847.
E-mail address: s-ohkubo@mail.pharm.tohoku.ac.jp (S. Ohkubo).
[1] Both authors contributed equally to this work.

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epidermal cells in response to such physical stimuli. Indeed, it has been shown that dermal cells (fibroblasts) and epidermal cells (keratinocytes) release ATP in response to mechanical stress (Boudreault and Grygorczyk, 2004; Koizumi et al., 2004). These phenomena suggest that ATP released from keratinocytes might act as a regulator of keratinocytes themselves and of neighboring cells which are components of cutaneous tissues. Furthermore, when cutaneous tissues are damaged, a large amount of ATP is released from the breakdown of plasma membranes, which also might activate surrounding cells.

P2 purinergic receptors are divided into ionotropic P2X ($P2X_{1, 2, 3, 4, 5, 6, 7}$) and G protein-coupled P2Y ($P2Y_{1, 2, 4, 6, 11, 12, 13, 14}$) receptors based on the molecular structure, signal transduction and pharmacological properties (Illes and Ribeiro, 2004; Ralevic and Burnstock, 1998). P2X receptors form nonselective cation channels to increase intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$). Among P2Y receptors, $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors are linked to $G_{q/11}$, which stimulates phospholipase C, resulting in the production of two second messengers. Inositol 1,4,5-trisphosphate (IP_3) releases Ca^{2+} from the endoplasmic reticulum, whereas diacylglycerol activates protein kinase C in the presence of Ca^{2+} and phosphatidylserine. Several reports suggest that keratinocytes express P2 receptors (Burrell et al., 2003; Dixon et al., 1999; Koizumi et al., 2004). For example, ATP and UTP stimulate keratinocyte proliferation through $P2Y_2$ receptors (Dixon et al., 1999; Greig et al., 2003). Greig et al. (2003) also reported that multiple P2 purinergic receptors, such as $P2X_5$, $P2X_7$, $P2Y_1$ and $P2Y_2$ receptors, are expressed in spatially distinct zones of the developing epidermis, suggesting that each receptor has a role in keratinocyte proliferation, differentiation and apoptosis in the different zones of the epidermis. However, the physiological and/or pathophysiological roles of P2 purinergic receptors in the epidermis remain to be elucidated. In the present study, we show that human HaCaT keratinocytes release ATP in response to mechanical stimuli and that the released ATP stimulates IL-6 production via an increase in $[Ca^{2+}]_i$ through activation of P2Y receptors in HaCaT cells.

2. Materials and methods

2.1. Cell culture

Human HaCaT keratinocytes, which were provided by Dr. Fusenig N.E. (German Cancer Research Center, Heidelberg, Germany), were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (50 units/ml) and streptomycin (50 μg/ml) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. The cells were used within 10 passages after starting the culture from stored frozen cells.

2.2. Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured by monitoring the intensity of Fura 2 fluorescence, using suspended HaCaT cells as described previously (Ohkubo et al., 1998). Trypsinized HaCaT cells were loaded with 1 μM 1-[6-amino-2-(5-carboxy-2-oxazolyl)-5-benzofuranyloxy]-2-(2-amino-5-methylphenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethylester (Fura 2-AM) for 15 min at 37 °C. Cells were washed twice, and finally suspended in modified Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.18 mM $CaCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.4). $[Ca^{2+}]_i$ was measured in 1.5 ml of the cell suspension in quartz cells with constant stirring at 37 °C, using a fluorescence spectrophotometer (Hitachi, F-2000). Fura 2 fluorescence at 510 nm was monitored every 1 s with excitation at 340 and 380 nm. $[Ca^{2+}]_i$ was calculated by using the K_d value of Fura 2 for Ca^{2+} (224 nM).

2.3. Measurement of phosphoinositide hydrolysis

Cells were seeded in a 12-well plate at the density of 5×10^4 cells/well and grown for 2 days. Cells were labeled with DMEM containing [^{3}H]inositol (1.2 μCi/well) for 18–24 h before the experiment. Cells were washed twice and incubated with 10 mM LiCl in modified Tyrode solution for 10 min at 37 °C. After incubation, cells were stimulated with agonists for 10 min at 37 °C. The reaction was terminated by addition of 5% trichloroacetic acid (TCA) after aspiration of the incubation medium. Total [^{3}H]inositol phosphates in the ether-washed TCA extracts were separated on an anion exchange column (AG 1X-8, formate form, Bio-Rad Laboratories, Hercules, CA, USA). Samples were loaded onto the anion exchange column, and glycerophosphates were washed out with 50 mM ammonium formate (6 ml). Total [^{3}H]inositol phosphates were then eluted with 1 M ammonium formate/0.1 M formic acid (4 ml). For determining [^{3}H]phosphoinositides, TCA precipitate was dissolved in 0.5 ml of 1 N NaOH and [^{3}H]radioactivity was measured after neutralization.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from agonist-stimulated or non-stimulated cells was extracted with the ISOGEN reagent (Nippon gene Co. Ltd, Tokyo, Japan), according to the manufacturer's protocol. A first-strand cDNA primed by Oligo(dT) primer (Promega Co., Madison, WI, USA) was prepared from total RNA (1 μg) by ReverTraAce (Toyobo Co Ltd., Tokyo, Japan) and was diluted 5 times with water to use as a template for the PCR analysis. Primers for human IL-6 and human P2 purinergic receptor families were designed based on the published cDNA sequences as summarized in Table 1. β−actin mRNA was determined as a positive control. PCR was carried out in 10 μl of solution containing 10 × PCR buffer (1 μl), RT template (1 μl), 2.5 mM dNTP mixture (0.5 μl), water (6.45 μl) and Taq polymerase (0.05 μl). The PCR conditions were: 94 °C for 2 min, followed by 27 cycles (for IL-6), 30 cycles (for P2 purinergic receptors) or 17 cycles (for β−actin) of 30 s at 94 °C, 30 s at 56 °C, and 2 min at 72 °C, with final extension at 72 °C for 10 min. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. The intensity of the

Table 1
Primers for RT-PCR used in the present study

	Accession no.	Primer sequences	Target	Expected size (bp)
$P2X_1$	NM_002558	GGCTCTGGAATTGGCATCTT	1179-1198 (S)	520
		CGGGTTGGATAATGAGAGTC	698-1679 (AS)	
$P2X_2$	NM_016318	CAGCATCCACTACCCCAAAT	531-550 (S)	490
		TAGCAAGATCCAGTCGCACA	1020-1001 (AS)	
$P2X_3$	NM_002559	GAGGAGAAATACCGCTGTGT	496-515 (S)	256
		TCAGGTTGGGAAGGAGGTTT	751-732 (AS)	
$P2X_4$	NM_031594	GATCCCTTCTGCCCCATATT	770-789 (S)	504
		TAGGCGTCACTGGTTCATCT	1273-1254 (AS)	
$P2X_5$	NM_002561	AAGAACCACTACTGCCCCAT	702-728 (S)	310
		GTTCACCATCACGTCAAAGC	1011-992 (AS)	
$P2X_6$	AF065385	CTGCCGCTATGAACCACAAT	695-714 (S)	398
		GCAGTAGCAGGTCACAGAAA	1092-1073 (AS)	
$P2X_7$	NM_002562	CCATCACTGCCATCCCAAAT	876-895 (S)	303
		ATATGGGAGCGACAGCAGTT	1178-1159 (AS)	
$P2Y_1$	NM_002563	GGTCTAGCAAGTCTCAACAG	1068-1087 (S)	359
		AAGCTAAGTGTGGATGTGGG	1426-1407 (AS)	
$P2Y_2$	NM_002564	TTGCCGTCATCCTTGTCTGT	877-896 (S)	433
		CTGCCCAACACATCTCCTAT	1309-1290 (AS)	
$P2Y_4$	NM_002565	TGTCCTTTTCCTCACCTGCA	366-385 (S)	440
		AGTAAATGGTGCGGGTGATG	805-786 (AS)	
$P2Y_6$	NM_004154	CACATCACCAAGACAGCCTA	1074-1093 (S)	340
		TCTTAACTCCATGCCCAGCT	1413-1394 (AS)	
$P2Y_{11}$	NM_002566	TCCACCCTCTACTCTACATG	947-966 (S)	443
		TGTAGAGTAGAGGGTGGACA	1389-1370 (AS)	
$P2Y_{12}$	NM_022788	GTGTCAAGTTACCTCCGTCA	532-551 (S)	274
		ATGCCAGACTAGACCGAACT	805-786 (AS)	
IL-6	XM_004777	AGAGTAGTGAGGAACAAGCC	463-482 (S)	238
		TACATTTGCCGAAGAGCCCT	700-681 (AS)	
β-actin	NM031144	AΓΓΓAAATXΓTΓXΓTΓAXAT	616-635 (S)	467
		TXXTΓXTTΓXTΓATXXAXAT	1082-1063 (AS)	

bands corresponding to IL-6 and β−actin was analyzed with NIH-image (version 1.55, Macintosh). The value of IL-6 mRNA was divided by the corresponding value of β−actin.

2.5. Measurement of IL-6 by enzyme-linked immunosorbent assay (ELISA)

HaCaT cells were seeded at 2.5×10^4 cells/well in a 24-well plate and grown for 2 days. HaCaT cells were stimulated with various concentrations of UTP for various times, and the incubation medium (250 μl) was collected after centrifugation to remove floating cells. Human IL-6 was measured by a sandwich-ELISA method using a capture antibody for human IL-6, biotin-conjugated anti-human IL-6 antibody and avidin-horseradish peroxidase (all antibodies from eBioscience Inc., San Diego, CA. USA), according to the manufacturer's protocol. Absorbance at 450 nm was measured with a plate-reader (SUNRISE; TECAN Group Ltd, Maennedorf, Switzerland), after incubation with the substrate (*o*-phenylenediamine dihydrochloride, OPD, from Sigma-Aldrich Co., St. Louis, MO, USA) for horseradish peroxidase. The amount of human IL-6 in a well was calculated as pg/well.

2.6. Measurement of ATP

Extracellular ATP level was measured with a commercially available kit (ENLITEN® ATP Assay System; Promega Co., Madison, WI, USA). Cells in a 24-well plate were washed twice and incubated in modified Tyrode solution (0.5 ml) for the indicated times. Incubation buffer (0.25 ml) was collected, and ATP level in the buffer was measured with a luminometer (GENE LIGHT 55; Microtec Co. Ltd., Chiba, Japan).

2.7. Materials

DMEM was purchased from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). Fetal bovine serum, ATP, ADP, UTP, UDP, α,β−methylene ATP (αβMeATP) and benzoyl benzoic ATP (BzATP) were purchased from Sigma-Aldrich Co. 3-[1-[3-(Dimethylamino)propyl]-1*H*-indol-3-yl]-4-(1*H*-indol-3-yl)-1*H*-pyrrole-2, 5-dione (GF109203X) was purchased from Wako Pure Chemicals Co. Ltd. (Osaka, Japan). Fura 2-AM and *O*,*O*′-bis(2-aminophenyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM) were obtained from Dojindo Laboratories (Kumamoto, Japan). Suramin was purchased from Merck Ltd. (Tokyo, Japan). [1,2-^{3}H]Myo-inositol was from American Radiolabeled Chemicals (St. Louis, MO, USA). All other chemicals used were of reagent grade or the highest quality available.

2.8. Data analysis

Data are expressed as means±S.E.M. Statistical analyses were performed by the paired Student's *t*-test for two data

comparison and one-way analysis of variance with the Dunnett two-tailed test for multiple data comparison.

3. Results

3.1. Mechanical stress-induced ATP release from HaCaT cells

Changing the medium of cultured adherent cells is recognized as causing mechanical stress to the cells, resulting in ATP release from cells via an unknown mechanism (Lazarowski et al., 2000). We therefore measured the extracellular ATP level after changing the medium of HaCaT cells (Fig. 1). The basal extracellular ATP level was about 8×10^{-11} M (0.04 pmol/well), but this increased by 62-fold immediately after changing the medium, and gradually decreased in a time-dependent manner. These results suggest that keratinocytes release ATP in response to mechanical stimuli.

3.2. Expression of P2 purinergic receptors in HaCaT cells

To evaluate the role of ATP in keratinocytes, we next analyzed the expression of mRNA for P2 purinergic receptor subtypes in HaCaT cells (Fig. 2A). HaCaT cells expressed $P2X_5$ and $P2X_6$ receptor mRNAs as P2X receptor subtypes. $P2X_7$ receptor mRNA was also detected to a small extent. P2Y receptor mRNAs were abundantly expressed in HaCaT cells, such as $P2Y_1$, $P2Y_2$, $P2Y_6$, $P2Y_{11}$ and $P2Y_{12}$ receptors. Slight expression of $P2Y_4$ receptor was also detected by more than 33 cycles of PCR reaction (data not shown). It has been shown that $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$ receptors stimulate the $G_{q/11}$-phospholipase C–Ca^{2+} pathway. Indeed, several P2 receptor agonists caused an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner in HaCaT cells (Fig. 2B). It is known that ATP is a broad P2 purinergic receptor agonist; that ADP is an agonist for $P2Y_1$ receptor among the $G_{q/11}$-coupled P2Y receptors; that UTP is an agonist for $P2Y_2$ and $P2Y_4$ receptors; that UDP is a $P2Y_6$ receptor agonist; that αβMeATP is an agonist for $P2X_1$ and $P2X_3$ receptors; and that BzATP is a $P2X_7$ receptor agonist. As shown in Fig. 2B, the potency in causing $[Ca^{2+}]_i$ mobilization was UTP > ATP > UDP > ADP ≥ BzATP >> αβMeATP in HaCaT cells. UTP is known to stimulate both $P2Y_2$ and $P2Y_4$ receptors (Ralevic and Burnstock, 1998). When the amount of $P2Y_2$ and $P2Y_4$ receptor mRNA was detected by the real-time RT-PCR, the expression of $P2Y_2$ receptor was dominant in HaCaT cells (the relative amount of $P2Y_2$ and $P2Y_4$ receptor mRNA, was 100 and 2.3, respectively; data not shown). Therefore, UTP-induced $[Ca^{2+}]_i$ mobilization might be mediated via mainly $P2Y_2$ receptors in HaCaT cells.

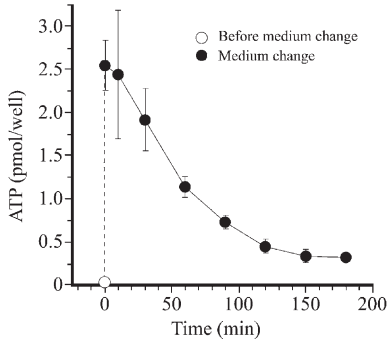

Fig. 1. ATP release from HaCaT cells by medium change. HaCaT cells were seeded at 2.5×10^4 cells/well in a 24-well plate and grown for 2 days. Cells were washed twice with modified Tyrode solution (0.5 ml), and 0.25 ml was collected as a sample after the indicated times. Released ATP in the buffer was determined by the luciferin–luciferase assay as described in Materials and methods. Data are expressed as the mean±S.E.M. of three determinations, and the results are representative of three independent experiments.

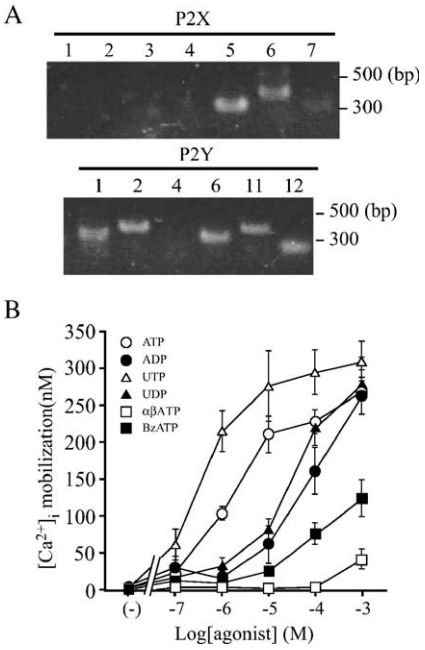

Fig. 2. Expression of P2 receptor subtypes in HaCaT cells. (A) Total RNA was isolated from HaCaT cells, and the expression of P2 purinergic receptor subtypes was analyzed by RT-PCR using the specific primers for each P2 purinergic receptor subtype as mentioned in Table 1. (B) HaCaT cells were loaded with Fura 2-AM (1 μM), and $[Ca^{2+}]_i$ mobilization was analyzed by measuring the Fura 2 fluorescences at 510 nm with excitation at 340 and 380 nm. Agonist-induced increase in $[Ca^{2+}]_i$ was averaged and expressed as the mean±S.E.M. of 4–12 determinations. The basal $[Ca^{2+}]_i$ level was 132±15 nM (n=5).

We next analyzed the hydrolysis of phosphoinositides in [^{3}H]inositol-prelabeled HaCaT cells (Fig. 3). UTP and ATP, potent agonists in increasing $[Ca^{2+}]_i$ (Fig. 2), led to the accumulation of [^{3}H]inositol phosphates in a concentration-dependent manner, suggesting that these agonists bind to the $G_{q/11}$-coupled P2Y receptor to stimulate phospholipase C following $[Ca^{2+}]_i$ mobilization.

3.3. UTP-induced increases in IL-6 mRNA and protein levels in HaCaT cells

To clarify the physiological role of P2Y receptors expressed in keratinocytes, we determined IL-6 production in response to P2 purinergic receptor agonists in HaCaT cells. As shown in Fig. 4A and B, UTP increased IL-6 mRNA and IL-6 protein levels in a time-dependent manner, with a peak at 1 and 3 h after stimulation, respectively. UTP also increased IL-6 mRNA and

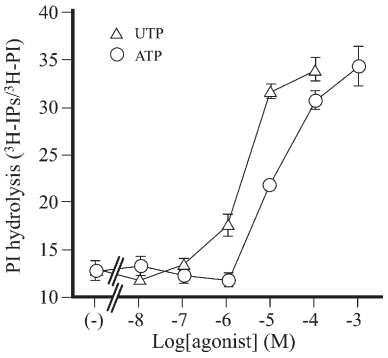

Fig. 3. Agonist-dependent phosphoinositide hydrolysis in HaCaT cells. [^{3}H] Inositol-labeled HaCaT cells in a 24-well plate were stimulated with increasing concentrations of UTP or ATP in the presence of 10 mM LiCl for 10 min at 37 °C. Total [^{3}H]inositol phosphates were separated as described in Materials and methods. PI hydrolysis was calculated as the percentage of total [^{3}H]inositol phosphates (^{3}H-IPs, dpm)/[^{3}H]phosphoinositides (^{3}H-PI, dpm). The basal levels of total [^{3}H]inositol phosphates and [^{3}H]phosphoinositides (dpm) were 3371±279 (n=3) and 26201±6876 (n=3), respectively. Data are expressed as the mean±S.E.M. of three determinations, and the results are representative of three independent experiments.

IL-6 protein levels in a concentration-dependent manner (Fig. 4C and D). In contrast, a broad P2 purinergic receptor antagonist suramin suppressed the UTP-induced increase in IL-6 mRNA expression and protein production (Fig. 5A and B).

3.4. UTP-induced transcription of IL-6 mRNA in HaCaT cells

It has been shown that mRNA levels are regulated by two mechanisms, by the activation of transcription and by the inhibition of mRNA degradation. We therefore analyzed the effect of actinomycin D, an inhibitor of mRNA transcription, on IL-6 mRNA level. The UTP-induced increase in IL-6 mRNA was completely suppressed by pretreatment with actinomycin D (Fig. 6A), suggesting that UTP might stimulate the transcription of IL-6 mRNA in HaCaT cells. Corresponding to the result shown in Fig. 6A, actinomycin D completely suppressed UTP-induced IL-6 protein production (Fig. 6B). Interestingly, actinomycin D also decreased the basal levels of IL-6 mRNA and protein (Fig. 6A and B), suggesting that the production/degradation cycle of IL-6 mRNA is rapid.

3.5. Involvement of $[Ca^{2+}]_i$ elevation in P2Y receptor-mediated IL-6 mRNA transcription

As shown in Fig. 3, UTP stimulated phospholipase C, which resulted in activation of protein kinase C and an increase in $[Ca^{2+}]_i$. To examine the mechanism of UTP-induced IL-6 mRNA expression and protein production, we used GF109203X, a protein kinase C inhibitor, and BAPTA-AM, an intracellular Ca^{2+} chelator. GF109203X at 10 μM had little effect on the UTP-induced change in IL-6 mRNA or protein level (Fig. 7A and C). In contrast, pretreatment of cells with BAPTA-AM (50 μM) resulted in complete inhibition of UTP-induced IL-6 mRNA expression and its protein production (Fig. 7B and C), suggesting that an increase in $[Ca^{2+}]_i$ is involved in IL-6 mRNA transcription.

4. Discussion

In the present study, we demonstrated that keratinocytes released ATP in response to mechanical stimuli and that one possible physiological role of the released ATP is the induction

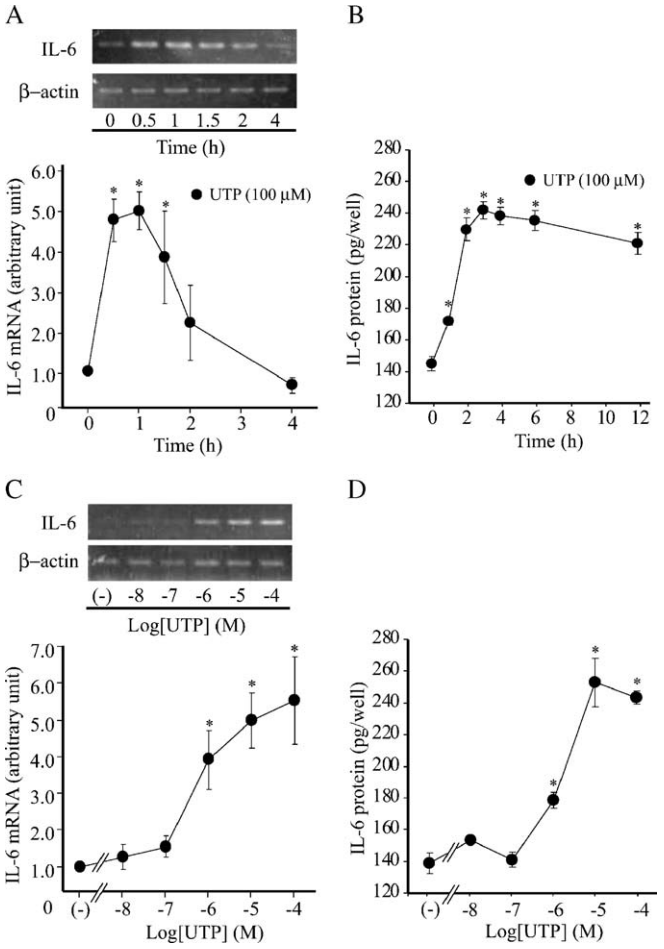

Fig. 4. UTP-induced increase in IL-6 mRNA and protein levels in HaCaT cells. (A) Time-dependent increase in IL-6 mRNA expression induced by UTP. HaCaT cells in a 6-well plate were stimulated with UTP (100 μM) for the indicated times, and IL-6 mRNA levels were analyzed by RT-PCR. β−actin mRNA was determined to analyze the variation in template cDNA. The band intensity corresponding to IL-6 mRNA was divided by that of β−actin mRNA. The results are shown as the fold increase above basal. Data are expressed as the mean±S.E.M. of three determinations. Significant difference from the value at time 0 is shown as *P<0.05. (B) Time-dependent IL-6 production induced by UTP. HaCaT cells in a 24-well plate were incubated with UTP (100 μM) for the indicated times, and IL-6 protein in the incubation medium was analyzed by a sandwich ELISA as described in Materials and methods. Data are expressed as the mean±S.E.M. of three determinations. Significant difference from the value at time 0 is shown as *P<0.05. The basal IL-6 level (pg/well) was 141.1±2.57 nM (n=9). (C) Concentration-dependent increases in IL-6 mRNA expression induced by UTP. HaCaT cells were incubated with increasing concentrations of UTP for 1 h. IL-6 mRNA levels were analyzed by RT-PCR, and normalized by β-actin mRNA levels. Results are shown as the fold increase above basal. The data are expressed as the mean±S.E.M. of three experiments. Significant difference from the value without UTP is shown as *P<0.05. (D) Concentration-dependent IL-6 production induced by UTP. HaCaT cells were incubated with increasing concentrations of UTP for 4 h, and the IL-6 level in the incubation medium was analyzed by a sandwich ELISA. The data are expressed as the mean±S.E.M. of three determinations. Significant difference from the value without UTP is shown as *P<0.05.

of IL-6 via P2Y receptors, possibly $P2Y_2$ receptors. The P2Y receptor-mediated increase in $[Ca^{2+}]_i$ may be involved in the activation of IL-6 mRNA transcription. This is the first observation that P2Y receptors in keratinocytes have a role in cytokine production.

Multiple P2 purinergic receptor mRNAs were identified in HaCaT cells, as determined with RT-PCR (Fig. 2A). UTP and ATP increased $[Ca^{2+}]_i$ more effectively than did other P2 purinergic receptor agonists (Fig. 2B), and both agonists stimulated phosphoinositide hydrolysis (Fig. 3), suggesting that these agonists stimulate $G_{q/11}$-coupled P2Y receptors.

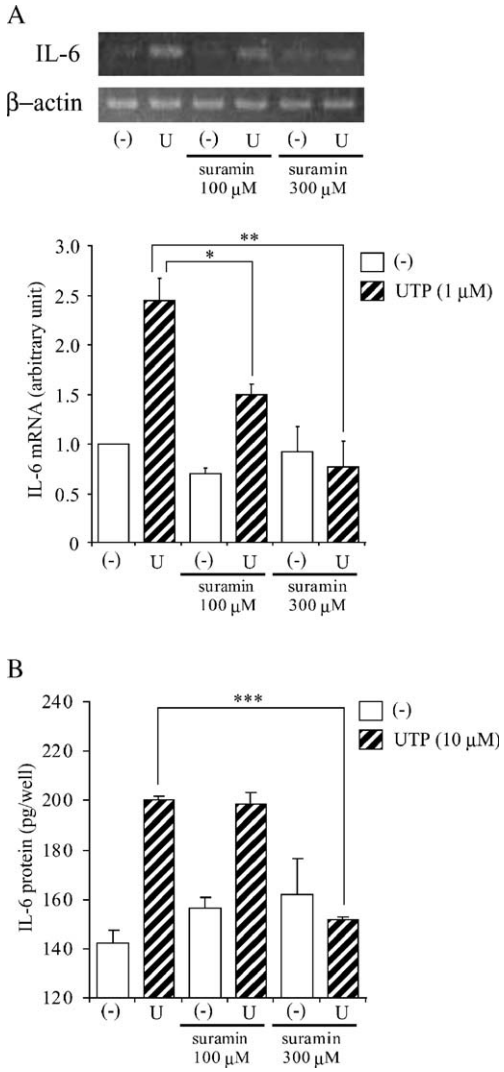

Fig. 5. Effect of suramin on UTP-induced IL-6 mRNA expression and protein production. (A) Inhibition of the UTP-induced increase in IL-6 mRNA level by suramin. Suramin (100 or 300 μM), a P2 receptor antagonist, was preincubated for 10 min before addition of UTP (1 μM). IL-6 mRNA levels were normalized by β−actin mRNA levels. Data are expressed as the mean±S.E.M. of three determinations. Significant differences from UTP alone are shown as $^{*}P<0.05$ and $^{**}P<0.01$. (B) Inhibition of UTP-induced IL-6 protein production by suramin. HaCaT cells were preincubated with suramin for 30 min before addition of UTP (10 μM) for 4 h. IL-6 level in the incubation medium was analyzed by a sandwich ELISA as described in Materials and methods. Data are expressed as the mean±S.E.M. of three determinations. Significant difference from UTP alone is shown as $^{***}P<0.001$.

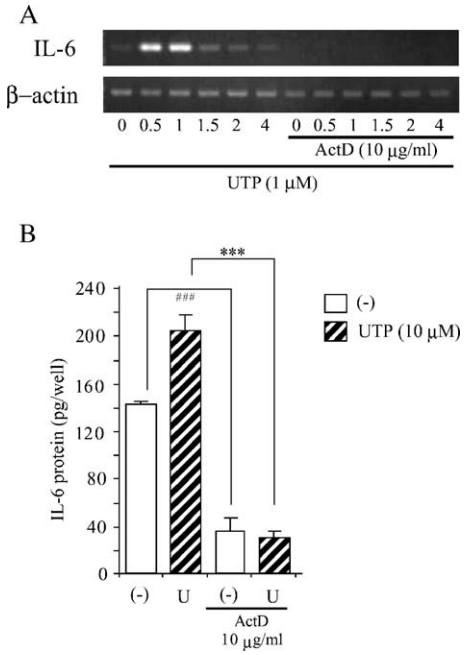

Fig. 6. Effect of actinomycin D on UTP-induced increase in IL-6 mRNA and protein levels. (A) Effect of actinomycin D on IL-6 mRNA levels. HaCaT cells were pretreated with or without actinomycin D (10 μg/ml) for 2 h and then incubated with UTP (10 μM) for the indicated times. IL-6 mRNA levels were analyzed by RT-PCR. The representative result is shown in three independent examinations. (B) Effect of actinomycin D on IL-6 protein production. HaCaT cells were preincubated with actinomycin D (10 μg/ml) for 30 min before addition of UTP (10 μM) for 4 h. IL-6 level in the incubation medium was analyzed by a sandwich ELISA as described in Materials and methods. Data are expressed as the mean±S.E.M. of three determinations. Significant difference between UTP alone and UTP in the presence of actinomycin D is shown as $^{***}P<0.001$. Significant difference from control without any drug is shown as $^{\#\#\#}P<0.001$.

Although UTP stimulates $P2Y_2$ and $P2Y_4$ receptors as an agonist, it remains unclear which subtype mediates UTP-induced responses in HaCaT cells. However, the $P2Y_2$ receptor may be a major receptor in mediating UTP-induced responses in HaCaT cells, because the mRNA expression of $P2Y_2$ receptor was about 40 times higher than that of the $P2Y_4$ receptor. Furthermore, the effectiveness of suramin on UTP-induced IL-6 mRNA and protein production (Fig. 5) also supports the suggestion that $P2Y_2$ receptors might be a major receptor, because suramin has been shown to antagonize $P2Y_2$ receptors but not $P2Y_4$ receptors (Charlton et al., 1996; Communi et al., 1996).

Other P2 purinergic receptor agonists, UDP and ADP, also increased $[Ca^{2+}]_i$ (Fig. 2B), mediated by possibly $P2Y_6$ and $P2Y_1$ receptors, respectively. These P2 purinergic receptor mRNAs were also identified in HaCaT cells (Fig. 2A). It is, however, unknown whether $P2Y_6$ or $P2Y_1$ receptor activation causes IL-6 production in HaCaT cells. Furthermore, we confirmed that ATP is potent in increasing the IL-6 level in HaCaT cells (Kobayashi D., unpublished observation). Thus, it is necessary to clarify the physiological and/or pathophysiological roles of other P2 purinergic receptors expressed in keratinocytes in the future.

UTP dramatically increased IL-6 mRNA expression, with a peak around 1 h after stimulation of HaCaT cells. The IL-6

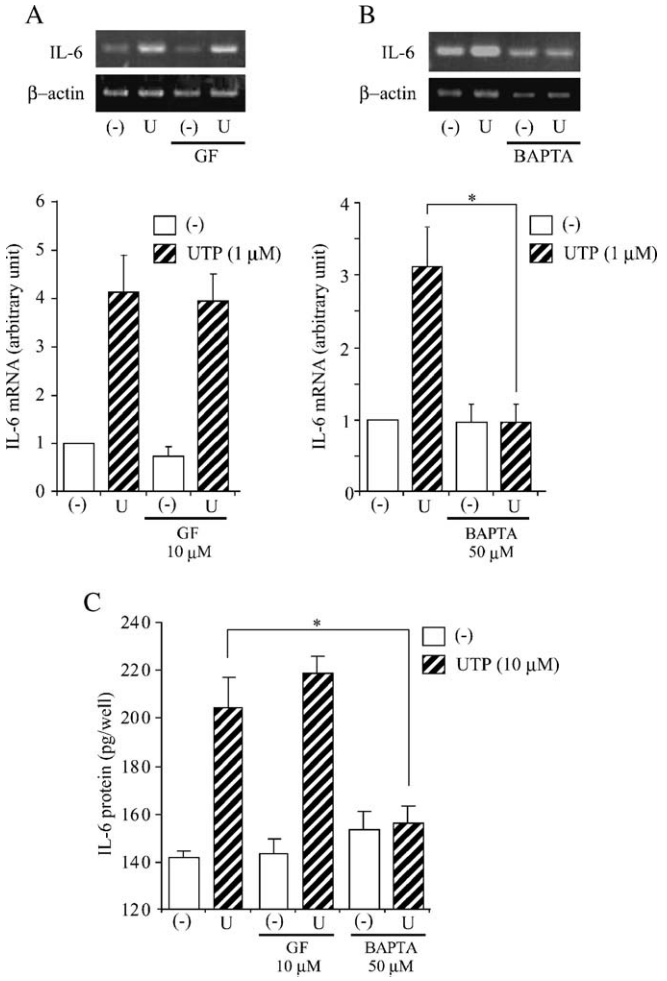

Fig. 7. Effects of GF109203X and BAPTA-AM on UTP-induced increase in IL-6 mRNA and protein levels. (A) Effect of GF109203X on IL-6 mRNA levels. HaCaT cells were pretreated with GF109203X (10 μM) for 10 min and then incubated with UTP (1 μM) for 1 h. IL-6 mRNA levels were normalized by β−actin mRNA levels. Data are expressed as the mean±S.E.M. of three determinations. (B) Effect of BAPTA-AM on IL-6 mRNA levels. HaCaT cells were pretreated with BAPTA-AM (50 μM) for 30 min and then incubated with UTP (1 μM) for 1 h. IL-6 mRNA levels were normalized by β−actin mRNA levels. Data are expressed as the mean±S.E.M. of three determinations. Significant difference from UTP alone is shown as $*P<0.05$. (C) Effects of GF109203X and BAPTA-AM on IL-6 protein level. HaCaT cells were preincubated with GF109203X (10 μM) or BAPTA-AM (50 μM) for 30 min and then incubated with UTP (10 μM) for 4 h. IL-6 level in the incubation medium was analyzed by a sandwich ELISA as described in Materials and methods. Data are expressed as the mean±S.E.M. of three determinations. Significant difference from UTP alone is shown as $*P<0.05$.

protein level also reached a maximum at 3 h. The EC_{50} values of UTP for causing $[Ca^{2+}]_i$ elevation, phosphoinositide hydrolysis, increase in IL-6 mRNA and protein levels were quite similar, i.e., approx. 0.5–2 μM, suggesting that the series of events could be associated with each other and possibly caused by $P2Y_2$ receptor activation. Indeed, an intracellular Ca^{2+} chelator BAPTA-AM inhibited the increase in IL-6 mRNA, indicating that the $G_{q/11}$-phospholipase C–Ca^{2+} pathway might be relevant to the IL-6 production stimulated by UTP in HaCaT cells.

Actinomycin D, which suppresses mRNA transcription, completely inhibited the UTP-induced increase in IL-6 mRNA expression (Fig. 6), suggesting that UTP could stimulate the transcription of IL-6 mRNA. Recent lines of evidence indicate that cytokine mRNA is regulated by both transcriptional and post-transcriptional mechanisms. In the present study, we showed that IL-6 mRNA transcription could be the principle mechanism for the UTP-induced increase in IL-6 mRNA level, but we could not rule out the possibility that UTP also affects the stability of IL-6 mRNA. Recently, it has become evident that the 3′-untranslated region of mRNA contributes to the regulation of gene expression by affecting the subcellular localization of mRNA, its translation or degradation (Conne et al., 2000). IL-6 mRNA has been reported to have AU-rich elements in the 3′-untranslated region that is involved in mRNA stability and translation through the binding of AU-rich element binding proteins (Bevilacqua et al., 2003; Winzen et al., 1999). Actinomycin D suppressed not only UTP-stimulated IL-6 mRNA expression but also the basal IL-6 mRNA level (Fig. 6), suggesting that IL-6 mRNA might be rapidly produced and degraded by transcriptional and post-transcriptional mechanisms.

While a protein kinase C inhibitor GF109203X was without effect, BAPTA-AM completely suppressed the UTP-induced increase in IL-6 mRNA expression (Fig. 7), demonstrating that an increase in $[Ca^{2+}]_i$ could be involved in the transcription of IL-6 mRNA. Characterization of the human IL-6 promotor has revealed the presence of a NF-κB-binding element between positions −73 and −63 and multiple response elements, consisting of CRE and a binding site for the CCATT enhancer-binding protein (C/EBPβ or NF-IL6), between −173 and −145 and an activator protein-1 site located between −283 and −277 (Vanden Berghe et al., 1998, 1999). The binding sites of NF-κB and NF-IL6 have been reported to serve as obligatory elements for IL-6 induction, especially in epithelial cells (Aragane et al., 1996). Matsubara et al. (2005) reported that histamine-induced IL-6 production is inhibited by BAPTA pretreatment in normal human epidermal keratinocytes, and our results are consistent with this. They indicate that the inhibitory κB (IκB) kinase (IKK)/IκB-α/NF-κB cascade is necessary for IL-6 induction, while Ca^{2+}-dependent protein kinase C could be also involved upstream of the activation of NF-κB. Our results show that protein kinase C might not be involved in UTP-induced mRNA expression, while an increase in $[Ca^{2+}]_i$ is enough to induce the response. Jeong et al. (2002) reported that BAPTA-AM completely inhibited IgE-stimulated IL-6 mRNA induction as well as IKKβ activation and IκB-α phosphorylation in RBL-2H3 cells. With regard to P2Y receptor-mediated IL-6 production, Ihara et al. (2005) reported that ATP-stimulated IL-6 synthesis through P2Y receptors is mediated by $[Ca^{2+}]_i$ mobilization through phospholipase C activation in human osteoblasts, and our results are consistent with this. The participation of protein kinase C in IL-6 production should be investigated in detail using other methods instead of a pharmacological inhibitor in the future.

We demonstrated that ATP was released from HaCaT cells by changing the medium. Because skin receives physical stimuli including stretch, press, sharp force injury and exposure

to harmful chemicals, ATP might be released from keratinocytes in response to such stimuli. Furthermore, wounds or thermal damage accompany the release of a large amount of ATP from dead cells, which in turn might activate $P2Y_2$ receptors and the rapid IL-6 release from keratinocytes. In fact, it has been shown that IL-6 mRNA is expressed at high levels in epidermal keratinocytes around the leading edge of a wound (Gallucci et al., 2000). Furthermore, IL-6 is released within a short time (15–20 min) after an injury caused by a sharp force in human skin (Grellner, 2002), suggesting a possible role of ATP as a trigger of IL-6 expression.

Recent studies have shown that IL-6 has a physiological role in the repair process of wounds (Gallucci et al., 2000) beyond its participation in inflammatory or immune reactions. Furthermore, IL-6 belongs to the neurotrophic cytokine superfamily and may play a key role in neuronal survival and maintenance of neuronal functions (Gruol and Nelson, 1997). For instance, sensory impairments as well as impairments of regeneration of sensory functions in the sciatic nerve were observed in IL-6-deficient mice, suggesting an essential role of IL-6 in the modulation of sensory functions in vivo (Zhong et al., 1999). It has recently been reported that keratinocytes and sensory dorsal root ganglion neurons may communicate, i.e., ATP released from keratinocytes by mechanical stimuli might transmit the signal to dorsal root ganglion neurons (Koizumi et al., 2004). Furthermore, IL-6 and its soluble receptor have been shown to support the survival of sensory dorsal root ganglion neurons (Thier et al., 1999), reminding us that ATP released from keratinocytes stimulates keratinocyte $P2Y_2$ receptors to release IL-6, which in turn maintains the survival and function of sensory neurons.

In the present study, we show that ATP stimulates IL-6 production, mediated via mainly $P2Y_2$ receptors in human HaCaT keratinocytes as autocrine cells. Further study will clarify the role of ATP in physiological and pathophysiological conditions of cutaneous tissue.

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References

Aragane, Y., Yamada, H., Schwarz, A., Pöppelmann, B., Luger, T.A., Tezuka, T., Schwarz, T., 1996. Transforming growth factor-α induces interleukin-6 in the human keratinocyte cell line HaCaT mainly by transcriptional activation. J. Invest. Dermatol. 106, 1192–1197.

Bevilacqua, A., Ceriani, M.C., Capaccioli, S., Nicolin, A., 2003. Post-transcriptional regulation of gene expression by degradation of messenger RNAs. J. Cell. Physiol. 195, 356–372.

Boudreault, F., Grygorczyk, R., 2004. Cell swelling-induced ATP release is tightly dependent on intracellular calcium elevations. J. Physiol. 561, 499–513.

Burrell, H.E., Bowler, W.B., Gallagher, J.A., Sharpe, G.R., 2003. Human keratinocytes express multiple P2Y-receptors: evidence for functional $P2Y_1$, $P2Y_2$, and $P2Y_4$ receptors. J. Invest. Dermatol. 120, 440–447.

Charlton, S.J., Brown, C.A., Weisman, G.A., Turner, J.T., Erb, T., Boarder, M.R., 1996. Clooned and transfected $P2Y_4$ receptors: characterization of a suramin and PPADS-insensitive response to UTP. Br. J. Pharmacol. 119, 1301–1303.

Communi, D., Motte, S., Boeynaems, J.-M., Pirotton, S., 1996. Pharmacological characterization of the human $P2Y_4$ receptor. Eur. J. Pharmacol. 317, 383–389.

Conne, B., Stutz, A., Vassalli, J.-D., 2000. The 3′-untranslated region of messenger RNA: a molecular 'hotspot' for pathology? Nat. Med. 6, 637–641.

Dixon, C.J., Bowler, W.B., Littlewood-Evans, A., Dillon, J.P., Bilbe, G., Sharpe, G.R., Gallagher, J.A., 1999. Regulation of epidermal homeostasis through $P2Y_2$ receptors. Br. J. Pharmacol. 127, 1680–1686.

Elder, J.T., Fischer, G.J., Lindquist, P.B., Bennett, G., Pittelkow, M., Coffey, E., Ellingsworth, L., Derynck, R., Voorhees, J., 1989. Overexpression of transforming growth factor alpha in psoriatic epidermis. Science 243, 811–814.

Gallucci, R.M., Simeonova, P.P., Matheson, J.M., Kommineni, C., Guriel, J.L., Sugawara, T., Luster, M.I., 2000. Impaired cutaneous wound healing in interleukin-6-deficient and immunosuppressed mice. FASEB J. 14, 2525–2531.

Gallucci, R.M., Sloan, D.K., Heck, J.M., Murray, A.R., O'Dell, S.J., 2004. Interleukin 6 indirectly induces keratinocyte migration. J Invest. Dermatol. 122, 764–772.

Greig, A.V.H., Linge, C., Cambrey, A., Burnstock, G., 2003. Purinergic receptors are part of a signaling system for keratinocyte proliferation, differentiation, and apoptosis in human fetal epidermis. J. Invest. Dermatol. 121, 1145–1149.

Grellner, W., 2002. Time-dependent immunohistochemical detection of proinflammatory cytokines (IL-1β, IL-6, TNF-α) in human skin wounds. Forensic Sci. Int. 130, 90–96.

Grossmann, R.M., Krueger, J., Yourish, D., Granelli-Piperno, A., Murphy, D.P., May, L.T., Kupper, T.S., Sehgal, P.B., Gottlieb, A.B., 1989. Interleukin 6 is expressed in high levels in psoriatic skin and stimulates proliferation of cultured human keratinocytes. Proc. Natl. Acad. Sci. U. S. A. 86, 6367–6371.

Gruol, D.L., Nelson, T.E., 1997. Physiological and pathological roles of interleukin-6 in the central nervous system. Mol. Neurobiol. 15, 307–339.

Harden, T.K., Lazarowski, E.R., Boucher, R.C., 1997. Release, metabolism and interconversion of adenine and uridine nucleotides: implications for G protein-coupled P2 receptor agonist activity. Trends Pharmacol. Sci. 18, 43–46.

Ihara, H., Hirukawa, K., Goto, S., Togari, A., 2005. ATP-stimulated interleukin-6 synthesis through P2Y receptors on human osteoblasts. Biochem. Biophys. Res. Commun. 326, 329–334.

Illes, P., Ribeiro, J.A., 2004. Molecular physiology of P2 receptors in the central nervous system. Eur. J. Pharmacol. 483, 5–17.

Jeong, H.-J., Hong, S.-H., Lee, D.-J., Park, J.-H., Kim, K.-S., Kim, H.-M., 2002. Role of Ca^{2+} on TNF-α and IL-6 secretion from RBL-2H3 mast cells. Cell. Signal. 14, 633–639.

Kawakami, M., Kaneko, N., Anada, H., Terai, C., Okada, Y., 1997. Measurement of interleukin-6, interleukin-10, and tumor necrosis factor-a levels in tissues and plasma after thermal injury in mice. Surgery 121, 440–448.

Kock, A., Schwarz, T., Kirnbauer, R., Urbanski, A., Perry, P., Ansel, J.C., Luger, T.A., 1990. Human keratinocytes are a source for tumor necrosis factor α: evidence for synthesis and release upon stimulation with endotoxin or ultraviolet light. J. Exp. Med. 172, 1609–1614.

Koizumi, S., Fujishita, K., Inoue, K., Shigemoto-Mogami, Y., Tsuda, M., Inoue, K., 2004. Ca^{2+} waves in keratinocytes are transmitted to sensory neurons: the involvement of extracellular ATP and $P2Y_2$ receptor activation. Biochem. J. 380, 329–338.

Kondo, S., Kono, T., Sauder, D.N., McKenzie, R.C., 1993. IL-8 gene expression and production in human keratinocytes and their modulation by UVB. J. Invest. Dermatol. 101, 690–694.

Lazarowski, E.R., Boucher, R.C., Harden, T.K., 2000. Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. J. Biol. Chem. 275, 31061–31068.

Matsubara, M., Tamura, T., Ohmori, K., Hasegawa, K., 2005. Histamine H1 receptor antagonist blocks histamine-induced proinflammatory cytokine

production through inhibition of Ca^{2+}-dependent protein kinase C Raf/MEK/ERK and IKK/IκB/NF-κB signal cascades. Biochem. Pharmacol. 69, 433–449.

Naka, T., Nishimoto, N., Kishimoto, T., 2002. The paradigm of IL-6: from basic science to medicine. Arthritis Res. 4 (Suppl. 3), S233–S242.

Neuner, P., Urbanski, A., Trautinger, F., Moller, A., Kirnbauer, R., Kapp, A., Schopf, E., Schwarz, T., Luger, T.A., 1991. Increased IL-6 production by monocytes and keratinocytes in patients with psoriasis. J. Invest. Dermatol. 97, 27–33.

Newman, E.A., 2001. Propagation of intercellular calcium waves in retinal astrocytes and Muller cells. J. Neurosci. 21, 2215–2223.

Ohkubo, S., Matsuoka, I., Kimura, J., Nakanishi, H., 1998. Inhibition of ATP-induced cAMP formation by 5′-p-fluorosulfonylbenzoyladenosine in NG108-15 cells. Naunyn Schmiedeberg's Arch. Pharmacol. 358, 153–159.

Ralevic, V., Burnstock, G., 1998. Receptors for purines and pyrimidines. Pharmacol. Rev. 50, 413–492.

Thier, M., März, P., Otten, U., Weis, J., Rose-John, S., 1999. Interleukin-6 (IL-6) and its soluble receptor support survival of sensory neurons. J. Neurosci. Res. 55, 411–422.

Urbanski, A., Schwarz, T., Neuner, P., Krutmann, J., Kirnbauer, R., Kock, A., Luger, T.A., 1990. Ultraviolet light induces increased circulating interleukin-6 in humans. J. Invest. Dermatol. 94, 808–811.

Vanden Berghe, W., Plaisance, S., Boone, E., Bosscher, K.D., Schmitz, M.L., Fiers, W., Haegeman, G., 1998. p38 and extracellular signal-regulated kinase mitogen-activated protein kinase pathway are required for nuclear factor-κB p65 transactivation mediated by tumor necrosis factor. J. Biol. Chem. 273, 3285–3290.

Vanden Berghe, W., Bosscher, K.D., Boone, E., Plaisance, S., Haegeman, G., 1999. The nuclear factor-κB engages CBP/p300 and histone acetyltransferase activity for transcriptional activation of the interleukin-6 gene promoter. J. Biol. Chem. 274, 32091–32098.

Winzen, R., Kracht, M., Ritter, B., Wilhelm, A., Chen, C.-Y.A., Shyu, A.-B., Müller, M., Gaestel, M., Resch, K., Holtmann, H., 1999. The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism. EMBO J. 18, 4969–4980.

Yamamoto, T., Osaki, T., 1995. Characteristic cytokines generated by keratinocytes and mononuclear infiltrates in oral lichen planus. J. Invest. Dermatol. 104, 784–788.

Zhong, J., Dietzel, I.D., Wahle, P., Kopf, M., Heumann, R., 1999. Sensory impairments and delayed regeneration of sensory axons in interleukin-6-deficient mice. J. Neurosci. 19, 4305–4313.