

# TRPV3 in keratinocytes transmits temperature information to sensory neurons via ATP

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**Abstract** Transient receptor potential V3 (TRPV3) and TRPV4 are heat-activated cation channels expressed in keratinocytes. It has been proposed that heat-activation of TRPV3 and/or TRPV4 in the skin may release diffusible molecules which would then activate termini of neighbor-

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ing dorsal root ganglion (DRG) neurons. Here we show that adenosine triphosphate (ATP) is such a candidate molecule released from keratinocytes upon heating in the co-culture systems. Using TRPV1-deficient DRG neurons, we found that increase in cytosolic  $Ca^{2+}$ -concentration in DRG neurons upon heating was observed only when neurons were co-cultured with keratinocytes, and this increase was blocked by P2 purinoreceptor antagonists, PPADS and suramin. In a co-culture of keratinocytes with HEK293 cells (transfected with P2X<sub>2</sub> cDNA to serve as a bio-sensor), we observed that heat-activated keratinocytes secretes ATP, and that ATP release is compromised in keratinocytes from TRPV3-deficient mice. This study provides evidence that ATP is a messenger molecule for mainly TRPV3-mediated thermotransduction in skin.

**Keywords** TRPV4 · TRPV3 · TRP channel · Keratinocytes · Thermosensation · ATP

## Introduction

Thermal information detected by the skin layers has to be converted to electrical signals, which then is transmitted to the central nervous system. It is thought that temperature-activated receptors expressed in sensory processes within the skin are directly activated by changes in temperature. For example, capsaicin receptor TRPV1 and menthol receptor TRPM8 expressed in the sensory nerve endings cause depolarization by cation influx through the channels in response to hot and cold stimuli, respectively, followed by action potential generation [5, 27, 32]. These thermo-sensitive transient receptor potential (TRP) channels belong to the TRP ion channel super family, which consists of six related protein families in mammals [10, 34, 38]. In

addition to TRPV1 and TRPM8, seven TRP channels are shown to be involved in thermosensation with distinct temperature thresholds. In contrast to the four heat- or cold-sensitive TRPV1, TRPV2, TRPM8, and TRPA1, two TRPV (TRPV3 and TRPV4) and three TRPM (TRPM2, TRPM4, and TRPM5) channels have been found to be activated by warm stimuli [10, 13, 38, 39]. Interestingly, TRPV3 and TRPV4 are predominantly expressed in skin keratinocytes, suggesting that warm stimuli can be initially detected in skin [7, 10, 13, 33, 38]. Indeed, mice lacking TRPV3 or TRPV4 have been reported to exhibit abnormal thermo-related behaviors [25, 28].

Intercellular signaling mediated by purines is present early in evolution and, therefore, is a widespread route for cell-to-cell communication. The concept of purinergic transmission has been well accepted since the report showing that adenosine triphosphate (ATP) is a transmitter in non-adrenergic, non-cholinergic inhibitory nerves [3]. Now it is recognized that ATP acts as either sole transmitter or a co-transmitter in most nerves in both the peripheral nervous system and central nervous system [4]. The epidermis is extensively innervated by axons arising from peripheral sensory neurons, which convey sensory input to the central nervous system. These sensory afferents are intimately associated with keratinocytes, mast cells, and Langerhans cells, indicating the capacity of peripheral sensory endings to monitor the status of the skin [2]. Signaling processes in keratinocytes have been investigated largely from the aspect of mechanisms such as differentiation and metabolism. However, several lines of evidence indicate a complex system of communication between skin cells and the sensory afferents innervating the skin [10, 14, 26, 38].

The concept of skin keratinocytes initially detecting ambient temperatures indicates that temperature information has to be transmitted to adjacent sensory neurons [13, 24, 31]. Electron microscopic studies have not identified synapse-like structures between keratinocytes and sensory nerve endings [6], raising the possibility that some diffusible molecules can function like neurotransmitters between keratinocytes and sensory nerve endings. However, no such evidence has been provided. In this study, we identified the candidate molecule, ATP, transferring temperature information from keratinocytes to sensory neurons by establishing a co-culture system.

## Materials and methods

### Animals

Neonatal (P2) wild-type (C57BL/6-strain, SLC, Shizuoka, Japan), TRPV1-deficient (from Dr. Julius D), TRPV3-

deficient (from Dr. Patapoutian A), or TRPV4-deficient mice (from Dr. Suzuki M) were housed in a controlled environment. All the procedures were in accordance with our institute (National Institute of physiological Sciences) and the National Institute of Health guides for the care and use of laboratory animals.

### DRG neuron and keratinocyte cultures from P2 mice

Dorsal root ganglion (DRG) neurons were isolated as previously described [29]. Freshly isolated neurons were plated onto cover slips and cultured in keratinocyte medium with added 100 ng/ml 7S nerve growth factor at 33°C with 5% CO<sub>2</sub> for 2 days. Keratinocytes were obtained by modifying earlier described method [7]. Briefly, keratinocytes from P2 mice were cultured in MCDB keratinocyte medium (Sigma, St. Louis, MO) containing (mg/ml) 5 insulin, 0.4 hydrocortisone, 10 transferrin, 14.1 phosphorylethanolamine, 0.01 EGF, 25 gentamicin, 50 streptomycin, and 40 bovine pituitary gland extract (BPE; Kyokuto, Tokyo, Japan) with 50 U/ml penicillin and 1% fetal bovine serum (FBS). Cells,  $5 \times 10^4$ , plated on cover slips were incubated over night in MCDB keratinocyte medium, before replacing with FBS-free MCDB medium containing BPE and CaCl<sub>2</sub> (0.04 mM). Cells were differentiated in FBS-free MCDB medium containing BPE and 1.2 mM CaCl<sub>2</sub> for 24 h at 33°C and 5% CO<sub>2</sub>. For keratinocytes in co-culture with DRG neurons, MCDB was replaced with keratinocyte medium [7].

### Co-culture of HEK293 cells and keratinocytes

Human embryonic kidney-derived 293 (HEK293) cells maintained in DMEM with FBS/penicillin/streptomycin were co-transfected with 1 µg rat P2X<sub>2</sub> or 1 µg mouse 5HT<sub>3A</sub> and 0.1 µg of pGREEN LANTAN 1 cDNAs, using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA) for ATP or serotonin bio-sensor assays, respectively. For a glutamate bio-sensor assay, HEK293 cells were co-transfected with 0.5 µg mouse NR1/0.5 µg mouse NR2B and 0.1 µg of pGREEN LANTAN 1 cDNAs in Basal Medium Eagle (BME; Sigma). Following transfection, HEK293 cells were re-plated to be co-cultured with keratinocytes in MCDB medium containing BPE and 1.2 mM CaCl<sub>2</sub>. Glutamate-induced cytotoxicity to NR1/NR2B expressing HEK293 cells in co-culture with keratinocytes was overcome by adding ketamine (Sigma, 500 µM) and D-AP5 (Sigma, 250 µM) to medium.

### Immunocytochemistry

DRG neuron and keratinocyte co-cultures were triple-immunostained with primary antibodies; mouse monoclonal

anti- $\beta$  tubulin (Sigma) for DRG neurons, rabbit polyclonal anti-MK14 (Covance, Berkeley, CA) for basal keratinocytes, and 4',6-diamidino-2-phenylindole (DAPI; Dojindo, Kumamoto, Japan) for nuclei. Secondary antibodies included Alexa Fluor 546 anti-mouse IgG and Alexa Fluor 488 anti-rabbit IgG (Molecular probes, Eugene, OR, USA).

#### Ca<sup>2+</sup>-imaging using fura-2 or fluo-4

Fura-2 or fluo-4 fluorescence was measured in the bath solution containing (mM) 140 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, 10 HEPES, and 10 glucose (pH 7.4) using a Nikon microscope (Nikon, Tokyo, Japan) connected to a Hamamatsu CCD camera (Hamamatsu Photonics, Shizuoka, Japan) and represented as 340/380 nm ratio for fura-2 or F/Fbackground for fluo-4, respectively. Heating in the experiments with fluo-4 was done by using a microscope stage heater (PH-1 and TC-344B, Warner Instruments, Hamden, CT, USA) to avoid washout of released ATP from keratinocytes. Heating in the experiments with fura-2 was done by using an inline-heater (SH-27B and TC-344B, Warner Instruments). A thermocouple (TA-30, Warner Instruments) was used to measure temperature.

#### Electrophysiology

Whole-cell patch-clamp recordings and sampling from transfected HEK293 cells in co-culture with keratinocytes were carried out as described before [5]. Bath solution was the same as in the Ca<sup>2+</sup>-imaging, and pipette solution contains (mM) 140 KCl, 5 EGTA, 10 HEPES, pH 7.4 (adjusted with KOH). Voltage-ramps (−100 to +100 mV with 5-s intervals) were applied. In a glutamate bio-sensor assay, magnesium-free bath solution and CsCl pipette solution were used. Heating was done by using a microscope stage heater.

#### Measurement of ATP release

The mouse scrotal skin was used. The mouse was anesthetized with pentobarbital sodium (70 mg/kg, i.p.) and hairs of scrotum were removed by depilator cream. The scrotal skin was then removed under surgically clean condition, and put in a small chamber (volume: 1.5 ml) and superfused 1.5 ml/min with sterilized Krebs–Henseleit solution (containing (in mM) 110.9 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25.0 NaHCO<sub>3</sub>, and 20 glucose) continuously bubbled with a gas mixture of 95% O<sub>2</sub> and 5% CO<sub>2</sub>. In half the cases, ruthenium red (RR, 50  $\mu$ M) was applied to the superfusate, and experiments were started after pre-incubation for >20 min. Temperature in the chamber was continuously monitored near the orifice of the sampling tube by a thermocouple (Physitemp,

Clifton, USA) and kept constant (either 24.9 $\pm$ 0.0°C). Before warm stimulation at least 1 h was elapsed. The superfusate was sampled at 0.8 ml/min for 15 s using a peristaltic pump through plastic tubing with a metal tube (2 mm in diameter) at one end. The tip of the metal tube was placed near the orifice of overflow opposite to the inflow. The delay time (15 s) passing through this piping was compensated later for analysis. The superfusate was sampled every 15 s for 2.5 min during the period of steep temperature change and before and after that 1–5-min intervals. Temperature was increased up to 41.9 $\pm$ 0.2°C in about 1 min by changing temperature of the water circulating outside the bath and inlet tubing. The ATP concentration was measured with luciferin–luciferase method. Briefly, 100  $\mu$ l aliquot of the bath sample was added to 100  $\mu$ l of ATP assay mix (Enliten ATP assay system, Promega, Madison, WI, USA) and the luminescence was measured with a luminometer (Lumat LB 9507, Berthold Technologies, Germany) for 10 s. Net ATP release in the sample was calculated by subtracting the basal concentration measured before stimulation from that induced by the thermal stimulation. The surface area of the skin was measured afterward, and the ATP release was normalized to that of 100 mm<sup>2</sup>.

#### Cell death assay

Same amounts of wild-type keratinocytes co-cultured with TRPV1-deficient DRG neurons were treated without or with heat. Then, propidium iodide (PI) was added to detect the dead cells in the dishes. Numbers of PI-positive dead cells were counted in glass cover slips (12 cover slips each).

#### Data analysis

Values are shown as mean  $\pm$  SEM. *p* values <0.05 were considered significant.

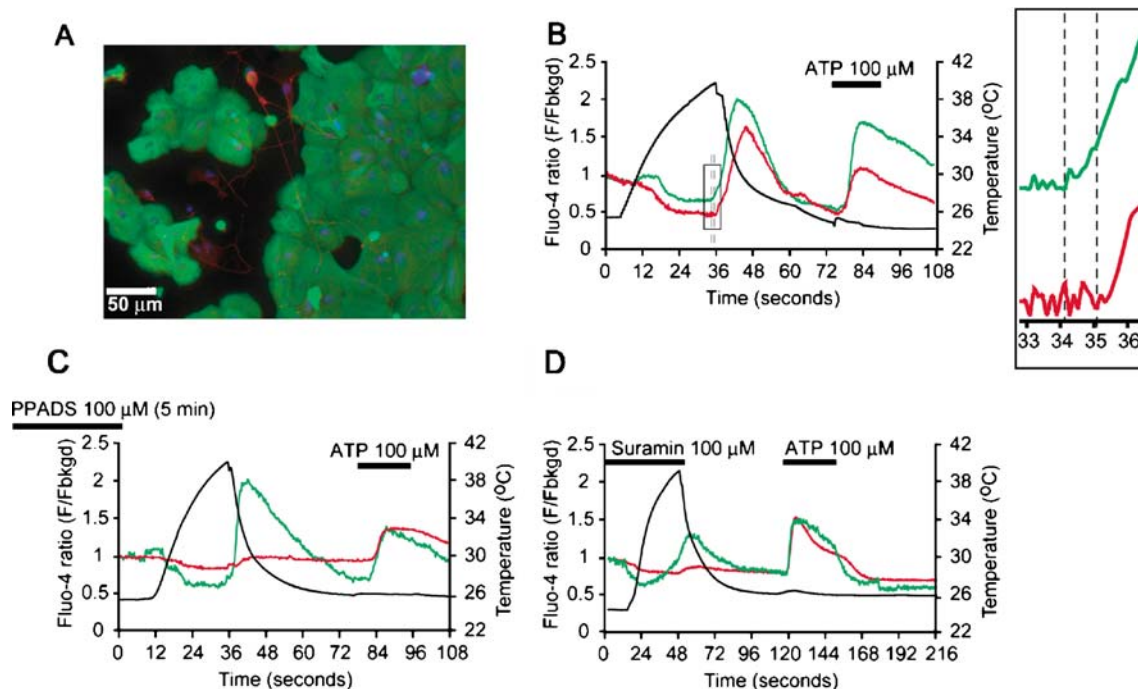
## Results

#### Intracellular Ca<sup>2+</sup> increase in sensory neurons secondary to the one in keratinocytes involves ATP signals

We first examined changes in intracellular Ca<sup>2+</sup> levels ([Ca<sup>2+</sup>]<sub>i</sub>) of DRG neurons from wild-type (WT) and TRPV1-deficient (TRPV1KO) mice. We found that heat application (~45°C) evoked increases in [Ca<sup>2+</sup>]<sub>i</sub> in all capsaicin-sensitive neurons (0.74 $\pm$ 0.03, *n*=190; Supplemental Fig. 1a, c). However, no such response was observed in DRG neurons from TRPV1KO mice (0.08 $\pm$ 0.01, *n*=220; Supplemental Fig. 1b, c) while DRG neurons from both WT and TRPV1KO mice responded to ATP similarly, indicating

an unaltered purinergic system in the TRPV1-deficient DRG neurons (Supplemental Fig. 1a, b). This result prompted us to establish a co-culture of keratinocytes and DRG neurons from TRPV1KO mice to avoid the cell-autonomous TRPV1-mediated heat responses in DRG neurons. Immunostaining of the co-culture with antibodies showed close proximity of neuronal terminals and keratinocytes (Fig. 1a). To obtain a sensitive readout of the signaling between such keratinocytes and DRG neurons in close proximity, the single wavelength-excitation  $\text{Ca}^{2+}$ -indicating dye, fluo-4, was used to capture responses less than a second time frame. To avoid the washout of candidate molecules released from keratinocytes upon heating, we used a stage heater without solution flow. Nonspecific reduction of fluo-4 ratio was observed upon heating (Fig. 1b, c, d) because of temperature-dependent change of the signals. We found that heat application ( $\sim 40^\circ\text{C}$ ) to DRG neuron-keratinocyte co-culture from TRPV1KO mice evoked a  $[\text{Ca}^{2+}]_i$  increase in both keratinocytes and DRG neurons as shown in Fig. 1a ( $n=244$ , Fig. 1b), while neurons with their terminals not in close proximity of

keratinocytes did not show such heat-evoked  $[\text{Ca}^{2+}]_i$  increase (data not shown). The heat-induced  $[\text{Ca}^{2+}]_i$  increase in a DRG neuron cell body was delayed ( $1.21 \pm 0.17$  s,  $n=8$ ) compared to that in a keratinocyte, lying in close proximity to the axon terminal originating from the neuron cell body (Fig. 1b), suggesting that the DRG neuron response was secondary to the keratinocyte response. Both a DRG neuron and a keratinocyte responded to ATP ( $100 \mu\text{M}$ ) with a similar time course (Fig. 1b). Pretreatment of the co-culture with global P2 receptor antagonists, pyridoxal-phosphate-6-azophenyl-2',4'-disulfonate (PPADS;  $100 \mu\text{M}$ ) or suramin ( $100 \mu\text{M}$ ), completely inhibited the heat-induced  $[\text{Ca}^{2+}]_i$  increase in the DRG neuron cell bodies, but not in the keratinocytes ( $n=270$  with PPADS in Fig. 1c, and  $n=43$  with suramin in Fig. 1d). On the other hand, ATP application ( $100 \mu\text{M}$ ) showed subdued purinergic responses in both cell types in the experiments with PPADS, possibly due to incomplete washout of the compound (Fig. 1c). Similar results were obtained in the co-culture of DRG neurons and keratinocytes from wild-type mice (data not shown). We



**Fig. 1** Cytosolic  $\text{Ca}^{2+}$  increase in DRG neurons upon heating was secondary to that in adjacent keratinocytes via ATP. **a** A representative co-culture showing the proximity between DRG neurons and keratinocytes from TRPV1-deficient mice, immunostained with antibodies; anti- $\beta$ -tubulin III for DRG (red), anti-MK14 for keratinocytes (green) and DAPI for nuclei (blue;  $n=8$ ). **b** A representative trace showing heat application (a black trace), evoked a rise in intracellular  $\text{Ca}^{2+}$  levels in a DRG neuron cell body (a red trace) secondary to that in a keratinocyte (a green trace). Dotted black lines represent the time difference ( $1.21 \pm 0.17$  s) in commencement of response between a keratinocyte and a DRG neuron cell body. Further application of ATP ( $100 \mu\text{M}$ ) confirmed purinergic responses in both cell types. Traces

are representative of typical experiments ( $N=33$  experiments). The neurons with nerve terminals in close proximity of keratinocytes responded to heat stimulus ( $n=244$ ). An inset is expanded from the square box. Flow was stopped during heating to avoid washout of the candidate molecules released from keratinocytes. **c, d** A representative trace showing that a 5-min pretreatment with PPADS ( $100 \mu\text{M}$ , **c**) or treatment with suramin ( $100 \mu\text{M}$ , **d**) blocked heat-induced response in a DRG neuron (a red trace) but not in a keratinocyte (green trace) in close proximity. Further application of ATP ( $100 \mu\text{M}$ ) confirmed purinergic responses in both cell types. Traces are representative of typical experiments ( $n=27$  with PPADS and  $n=43$  with suramin)

confirmed that heat stimulus did not cause cell death in these experiments (Supplemental Fig. 2). These results suggest that ATP is responsible for the transmission of the heat stimulus from keratinocytes to sensory neurons in culture.

Evidence of ATP release from keratinocytes via predominant TRPV3 activation upon heating in the bio-sensor system

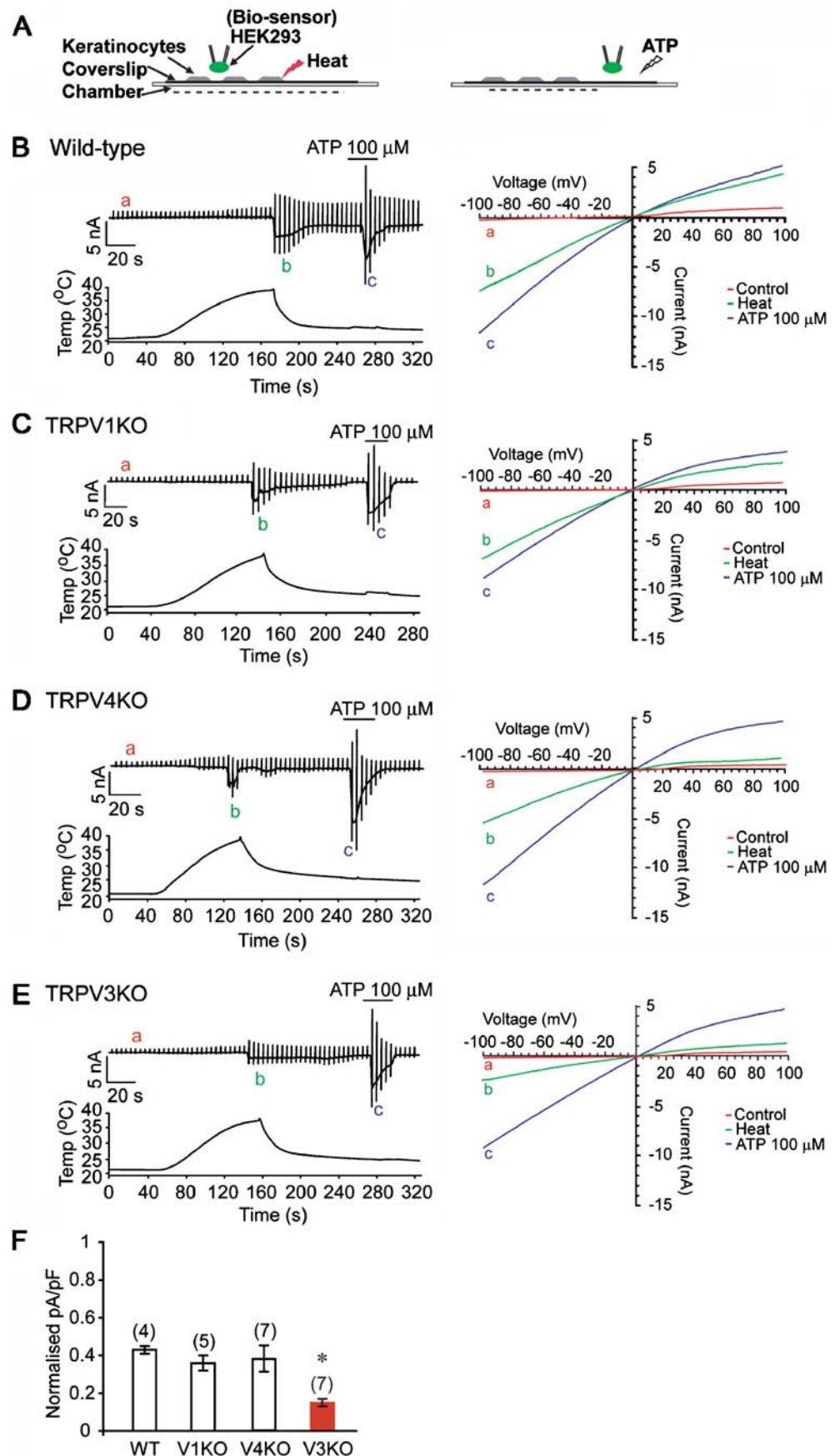
We then tested directly whether keratinocytes secrete ATP upon heating. In order to detect the local ATP released within the microenvironment of warmed keratinocytes, we established a bio-sensor system [17]. In this system, ionotropic receptors expressed in HEK293 cells placed closely to keratinocytes detect the molecules released from keratinocytes upon stimulus (Fig. 2a). During heat stimulus ( $\sim 40^\circ\text{C}$ ) to the co-culture of wild-type keratinocytes with HEK293 cells expressing P2X<sub>2</sub> receptors, P2X<sub>2</sub>-like inwardly rectifying currents were recorded from the whole-cell patch-clamped HEK293 cells only when placed in close proximity to the keratinocytes (less than 10  $\mu\text{m}$  in the distance; Fig. 2b,  $n=4$ ), suggesting ATP release from the keratinocytes upon heating. Functional expression of P2X<sub>2</sub> receptors in the HEK293 cell was confirmed by ATP (100  $\mu\text{M}$ ) application in the condition with two cell types apart to avoid possible effect of ATP-induced ATP release from keratinocytes (Fig. 2b). Similar P2X<sub>2</sub> receptor-mediated currents in the HEK293 cells were observed in the co-culture with keratinocytes from TRPV1KO mice (Fig. 2c,  $n=5$ ), confirming the results shown in Fig. 1. Because TRPV3 and TRPV4 are possible candidates for heat detectors in keratinocytes, heat-evoked P2X<sub>2</sub> receptor-mediated currents were examined in the co-culture with keratinocytes from either TRPV4-deficient (TRPV4KO, Fig. 2d) or TRPV3-deficient (TRPV3KO, Fig. 2e) mice. Heat-evoked P2X<sub>2</sub> receptor-mediated currents in the co-culture with keratinocytes from TRPV3KO mice were significantly smaller ( $0.15 \pm 0.02$  pA/pF,  $n=7$ ) than those in the co-culture with keratinocytes from WT ( $0.43 \pm 0.02$  pA/pF,  $n=4$ ), TRPV1KO ( $0.36 \pm 0.04$  pA/pF,  $n=5$ ), or TRPV4KO ( $0.39 \pm 0.07$  pA/pF,  $n=7$ ) mice (Fig. 2f,  $p < 0.05$ ). These results suggest that TRPV3 is predominantly required for heat-induced ATP release. A semi-quantitative estimate for the amount of heat-induced ATP release from keratinocytes was obtained by equating the heat-evoked P2X<sub>2</sub> receptor-mediated currents in the co-culture with ATP-evoked P2X<sub>2</sub> receptor-mediated currents not in co-culture (Supplemental Fig. 3a). This comparison resulted in an estimated release of about 1  $\mu\text{M}$  ATP from the TRPV3KO keratinocytes and about 10  $\mu\text{M}$  ATP from other keratinocytes. In addition, the Ca<sup>2+</sup>-imaging experiments showed that the percentage of keratinocytes from

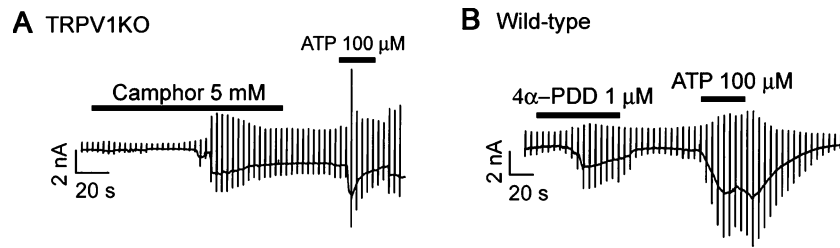
TRPV3KO mice responding to heat ( $26.6 \pm 4.4\%$ , 179/640 cells) was significantly lower ( $p < 0.005$ ) than those from WT ( $76.4 \pm 2.7\%$ , 538/708 cells), TRPV1KO ( $79.1 \pm 2.9\%$ , 507/648 cells) or TRPV4KO ( $77.3 \pm 5.4\%$ , 567/611 cells) mice (Supplemental Fig. 3b), consistent with the data shown in Fig. 2f. We examined the involvement of TRPV3 or TRPV4 in the ATP release from keratinocytes in the co-culture system by applying the ligand, camphor (5 mM), or 4 $\alpha$ -PDD (1  $\mu\text{M}$ ), respectively. Because TRPV1 has been reported to be activated by camphor [42] and to be expressed in keratinocytes [12], we used TRPV1-deficient keratinocytes in the co-culture for the experiment with camphor. To avoid the washout of molecules released from keratinocytes upon stimulus, we applied the agonists for 20 s and stopped the flow. Both camphor ( $n=5$ ) and 4 $\alpha$ -PDD ( $n=5$ ) could cause activation of P2X<sub>2</sub> channels expressed in HEK293 cells in the system (Fig. 3a, b) although time to activation of P2X<sub>2</sub>-mediated currents varied because of the stopped flow and the fact that it requires 1–2 min for 4 $\alpha$ -PDD to activate the TRPV4 in heterologous expression system [40]. These results suggest that activation of both TRPV3 and TRPV4 causes ATP release from keratinocytes, a little different from the results in Fig. 2.

Serotonin or glutamate was not released from keratinocytes upon heating

We also tested if other candidate molecules are secreted from keratinocytes upon heating. We used additional bio-sensor systems to detect serotonin or glutamate release from keratinocytes upon heating ( $\sim 40^\circ\text{C}$ ). We looked for serotonin, since this transmitter has been reported to be involved in taste signal transmission from taste cells to afferent neurons [35], although ATP has been recently shown to transmit taste information between taste cells and gustatory nerves [15]. We also looked for glutamate since glutamate release from Merkel cells in skin is known to be associated with mechanotransduction [16]. To detect heat-induced release of serotonin or glutamate from keratinocytes, HEK293 cells expressing the serotonin receptor, 5HT<sub>3A</sub>, or the glutamate receptor subunits, NR1/NR2B, were co-cultured with keratinocytes from wild-type mice. No serotonin- ( $n=10$ ) or glutamate- ( $n=10$ ) evoked currents were observed in HEK293 cells in close proximity to keratinocytes exposed to heat stimulus (Fig. 4a, b). Functional expression of 5HT<sub>3A</sub> or NR1/NR2B receptors in the patch-clamped HEK293 cells was confirmed by applying serotonin (100  $\mu\text{M}$ ; Fig. 4a) or a combination of glutamate (50  $\mu\text{M}$ ) with glycine (10  $\mu\text{M}$ ; Fig. 4b), respectively, in a condition where the two cell types were apart to avoid possible effect of serotonin- or glutamate-induced signaling from keratinocytes.

**Fig. 2** P2X<sub>2</sub>-mediated currents were observed upon heating in HEK293 cells expressing P2X<sub>2</sub> co-cultured with mouse keratinocytes. **a** Cartoons showing a bio-sensor system. HEK293 cells expressing bio-sensors were placed very close to the heated keratinocytes, then the ability of the bio-sensors to detect the molecules were confirmed by applying their agonists away from the keratinocytes. **b–e** Representative traces showing that heat application evoked whole-cell current responses (the *left panels*) with inward rectification (green traces in the *right panels*) in the HEK293 cells transfected with P2X<sub>2</sub> cDNA in co-culture with keratinocytes from wild-type (WT, **b**), TRPV1-deficient (TRPV1KO, **c**), TRPV4-deficient (TRPV4KO, **d**) or TRPV3-deficient (TRPV3KO, **e**) mice. Further application of ATP (100 μM) was used to confirm P2X<sub>2</sub>-mediated responses (the *left panels*) with inward rectification (blue traces in the *right panels*). A ramp-pulse (from -100 mV to +100 mV for 500 ms) was applied with 5-s intervals. Holding potential was -60 mV. Traces are representative of typical experiments (N=4–7). **f** Heat-induced P2X<sub>2</sub>-mediated currents were normalized to the responses evoked by 100 μM ATP from four to seven typical experiments (**b–e**). *Parenthesis* indicate the numbers of cells tested. \**p*<0.05, student *t* test





**Fig. 3** Camphor and 4 $\alpha$ -PDD response in keratinocytes. **a, b** Representative whole-cell current traces in P2X<sub>2</sub>-expressing HEK293 cells co-cultured with TRPV1-deficient (TRPV1KO; **a**) or

wild-type (**b**) keratinocytes upon application of camphor (5 mM, **a**) or 4 $\alpha$ -PDD (1  $\mu$ M, **b**). Similar results were observed in other five cells tested

ATP-evoked current activation upon heating in the co-culture of keratinocytes and sensory neurons

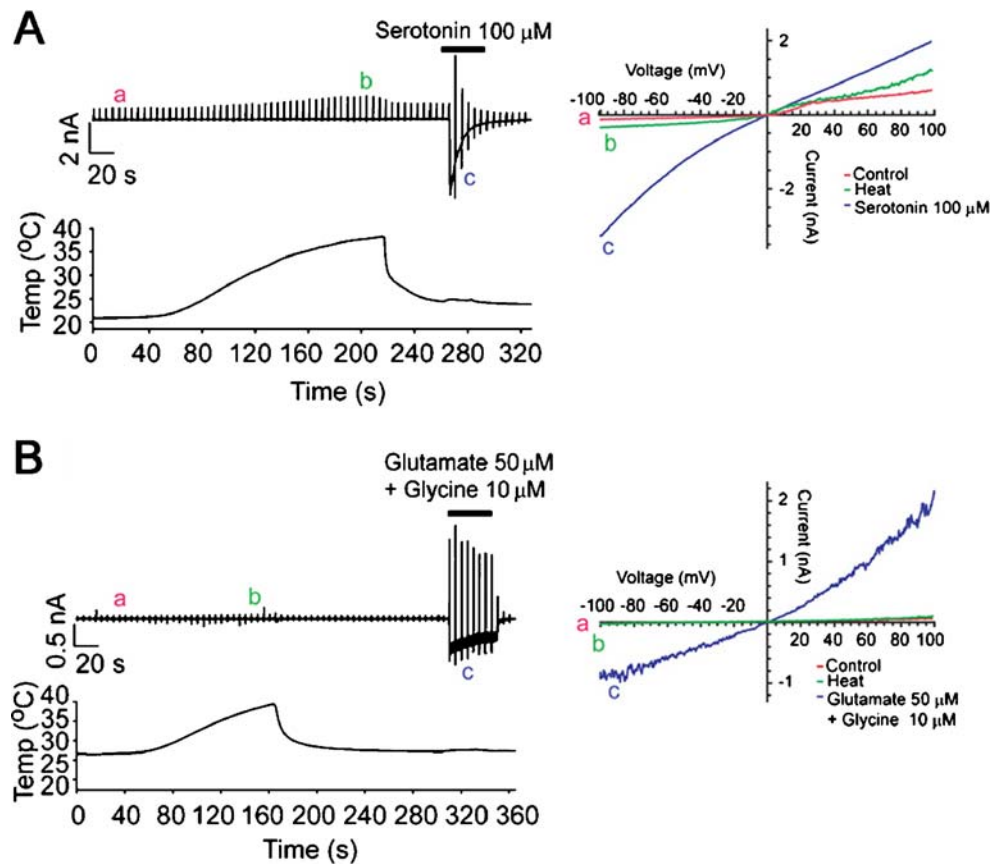
In order to confirm ATP-mediated signal transmission to sensory neurons from the keratinocytes upon heating, we performed patch-clamp experiments using DRG neurons and keratinocytes from TRPV1KO mice. We added acutely dissociated DRG neurons to the keratinocyte culture because whole-cell patch-clamped DRG neurons had to be lifted up and moved close to the keratinocytes. We observed transient inward currents ( $0.33 \pm 0.11$  nA,  $n=9$ ) in the small DRG neurons (cell capacitance  $7.5 \pm 0.5$  pF) in close proximity to the heated keratinocytes (Fig. 5a). ATP

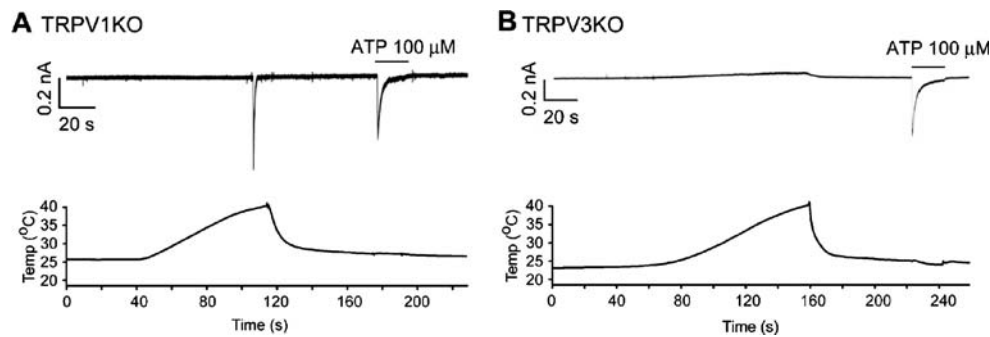
(100  $\mu$ M) also activated transient inward currents, indicative of the opening of P2X<sub>3</sub> channels. We also heated TRPV3KO keratinocytes co-cultured with TRPV1KO DRG neurons ( $n=18$ ). However, no significant ATP-activated current responses were obtained (Fig. 5b), supporting the predominant involvement of TRPV3 in heat-evoked ATP release shown in Fig. 2.

Measurement ATP release upon heating from skin in the skin-nerve preparation

Finally, we assessed whether heat-evoked ATP release is observed in tissue level. The excised mouse scrotal skin,

**Fig. 4** 5HT<sub>3A</sub>- nor NMDA-receptor-mediated responses were observed in HEK293 cells upon heating. **a, b** There was no 5HT<sub>3A</sub>-mediated (**a**) or NMDA-receptor-mediated (**b**) responses upon heat application to the co-culture while control inward currents with typical rectifications were observed in response to serotonin (100  $\mu$ M; **a**) or a combination of glutamate (50  $\mu$ M)+glycine (10  $\mu$ M; **b**). Traces are representative of typical experiments ( $n=10$ )





**Fig. 5** Heat-evoked ATP-mediated currents in DRG neurons with TRPV1-deficient keratinocytes, but not with TRPV3-deficient keratinocytes. Representative traces showing that heat application evoke a

current response in a DRG neuron with keratinocytes from TRPV1-deficient (TRPV1KO) mice (**a**,  $n=9$ ), but not from TRPV3-deficient (TRPV3KO) mice (**b**,  $n=18$ )

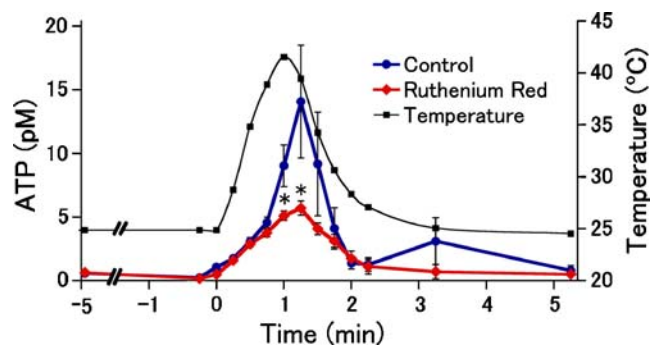
where excellent warm-sensitivity and warm-sensitive fibers have been reported [18, 21], was superfused in a small bath, and ATP concentration in the bath solution was measured by luciferin–luciferase assay. Upon heat application ( $\sim 42^\circ\text{C}$ ), temperature-dependent increase of ATP concentration was observed (Fig. 6, blue). This heat-induced ATP release was significantly reduced by ruthenium red (RR,  $50\ \mu\text{M}$ ), a broad TRP channel inhibitor (Fig. 6, red) at higher temperature range: in the presence of ruthenium red, the RR-insensitive ATP release activated at lower temperature range ( $>25^\circ\text{C}$ ) was still observed. However, RR-sensitive fraction of ATP release was apparent first at  $\sim 40^\circ\text{C}$  (Fig. 6, at 1 min; temperature range of the fraction,  $40.3\text{--}41.9^\circ\text{C}$ ), showing a good agreement with the temperature needed for ATP release from keratinocytes and activation of TRPV3- or TRPV4-expressing HEK293 cells. These results support that TRPV3 and TRPV4 would be involved in heat-evoked ATP release in the skin.

## Discussion

We showed heat-induced ATP release from keratinocytes and that purinoreceptor antagonists blocked the heat-activated communication between the skin and nerve in the  $\text{Ca}^{2+}$ -imaging. Furthermore, small amount of local ATP, but not serotonin or glutamate released from keratinocytes upon heating could be detected using a patch-clamp-based bio-sensor system with ionotropic ATP receptors expressed in HEK293 cells. This bio-sensor system could be used for detecting many other molecules locally released from not only keratinocytes but also other cell types including neurons and glia. The involvement of ATP in physiological signal transmission in thermosensation clarified in this study is consistent with the well-documented concept of sensory afferent signaling via purinergic nerves innervating various tissues [9, 22, 23], the report that  $\text{P2X}_3$ -deficient mice exhibited warm-coding deficits [36], and that warm

temperature sensation has been proposed to involve fast communication between the skin and CNS via the DRG [10, 13, 24, 31, 38].

Our data suggests that TRPV3 mainly mediates the release of ATP from keratinocytes upon heating, being consistent with the recent report showing that keratinocytes participate in thermal pain transduction through TRPV3 in keratinocytes although  $\text{PGE}_2$  is an intercellular messenger [19]. Our results are also agreement with that *DS-Nh* mice having TRPV3 gain-of-function mutation exhibited higher  $\text{Ca}^{2+}$  influx upon thermal stimulation in the epidermal sheet preparation [20, 30]. However, we cannot rule out contributions by TRPV4. Since  $4\alpha\text{-PDD}$  caused activation of  $\text{P2X}_2$  channels to the extent comparable to that evoked by  $100\ \mu\text{M}$  of ATP (Fig. 3b), and since heat-evoked  $\text{P2X}_2$ -mediated current responses were not completely abolished in our bio-sensor system using TRPV3KO keratinocytes (Fig. 2e). These data suggest the existence of other thermosensors in vivo. The results that none of 18 TRPV1KO DRG neurons showed  $\text{P2X}$ -like current responses upon



**Fig. 6** Heat-evoked ATP release from mouse scrotal skin in the skin-nerve preparation. Heat application (*a* black line,  $n=18$ ) caused ATP release from the isolated mouse scrotal skin in the normal bath solution (control, blue circles,  $n=9$ ). The response was decreased in the presence of ruthenium red ( $50\ \mu\text{M}$  started at  $-5$  min, red diamonds,  $n=9$ ). The ATP level was plotted at the middle of each sampling period (15 s).  $*p<0.05$ , vs. control by Mann–Whitney test



heating of keratinocytes (Fig. 5) contradict the residual response observed in the co-culture with TRPV3KO keratinocytes (Fig. 2e). This is partially because the mechanisms connecting TRP channel activation and ATP release are largely unknown. In neuronal cells, depolarizing stimulation resulted in exocytotic release of ATP in hippocampal slices [41]. However, in non-excitabile cells including HEK293 cells, the mechanism of ATP release is still a matter of debate. In astrocytes, there have been several reports that ATP can be released via chloride channels [11], gap junction hemi-channels [37], ATP-binding cassette [1], and exocytosis [8] some of which require  $[Ca^{2+}]_i$  increase. Keratinocytes and astrocytes might share the same mechanism for the ATP release. In any case, ATP release from keratinocytes could potentially signal to a variety of P2X- or P2Y-expressing sensory terminals within the epidermis to transmit innocuous temperature information; however, a role in noxious heat transmission cannot be ruled out.

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