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DELETION OF THE MURINE ATP/UTP RECEPTOR P2Y₂ ALTERS MECHANICAL AND THERMAL RESPONSE PROPERTIES IN POLYMODAL CUTANEOUS AFFERENTS

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Abstract

P2Y₂ is a member of the P2Y family of G protein-coupled nucleotide receptors that is widely co-expressed with TRPV1 in peripheral sensory neurons of the dorsal root ganglia. To characterize P2Y₂ function in cutaneous afferents, intracellular recordings from mouse sensory neurons were made using an *ex vivo* preparation in which hindlimb skin, saphenous nerve, dorsal root ganglia and spinal cord are dissected intact. The peripheral response properties of individual cutaneous C-fibers were analyzed using digitally controlled mechanical and thermal stimuli in male P2Y₂^{+/+} and P2Y₂^{-/-} mice. Selected sensory neurons were labeled with Neurobiotin and further characterized by immunohistochemistry. In wildtype preparations, C-fibers responding to both mechanical and thermal stimuli (CMH or CMHC) preferentially bound the lectin marker IB4 and were always immunonegative for TRPV1. Conversely, cells that fired robustly to noxious heat, but were insensitive to mechanical stimuli, were TRPV1-positive and IB4-negative. P2Y₂ gene deletion resulted in reduced firing by TRPV1-negative CMH fibers to a range of heat stimuli. However, we also identified an atypical population of IB4-negative, TRPV1-positive CMH fibers. Compared to wildtype CMH fibers, these TRPV1-positive neurons exhibited lower firing rates in response to mechanical stimulation, but had increased firing to noxious heat (43–51 °C). Collectively, these results demonstrate that P2Y₂ contributes to response properties of cutaneous afferents, as P2Y₂ deletion reduces responsiveness of conventional unmyelinated polymodal afferents to heat and appears to result in the acquisition of mechanical responsiveness in a subset of TRPV1-expressing afferents.

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INTRODUCTION

A growing body of evidence implicates signaling by extracellular nucleotides in sensory neurotransmission, particularly in the transduction of noxious stimuli. P2Y₂ is a member of the P2Y family of G protein-coupled nucleotide receptors that is highly expressed in sensory neurons of the dorsal root ganglion (DRG) (Molliver et al., 2002; Kobayashi et al., 2006; Malin et al., 2008). In previous studies, P2Y₂ has been implicated in the excitation of nociceptive sensory neurons, and has been shown to enhance TRPV1 signaling, resulting in thermal hypersensitivity (Moriyama et al., 2003; Malin et al., 2008).

Unlike P2X channels, which are selectively activated by ATP, P2Y₂ can be activated by either ATP or UTP. ATP and UTP evoke prolonged depolarization and action potential firing in a subset of sensory neurons, including a large proportion of neurons expressing TRPV1 (Molliver et al., 2002; Stucky et al., 2004). Mice lacking P2Y₂ have reduced behavioral responses to noxious heat and capsaicin and fail to develop heat hypersensitivity in response to inflammatory injury (Malin et al., 2008). However, it has not been resolved whether these differences are a direct consequence of changes in the functional properties of cutaneous sensory neurons. In order to directly examine the contribution of P2Y₂ to the physiological response properties of primary sensory neurons, cutaneous afferents in P2Y₂^{+/+} and P2Y₂^{-/-} mice were analyzed using intracellular recording in an *ex vivo* preparation in which the hairy skin of the hindpaw, the DRG and spinal cord remain connected (McIlwrath et al., 2007). In previous studies with this technique, we demonstrated that, in naïve mice, immunoreactivity for TRPV1 in cutaneous afferents is restricted to a population of mechanically insensitive C-fibers that are highly responsive to noxious heat (CHs) (Lawson et al., 2008). In contrast, cutaneous afferents responsive to both thermal and mechanical stimuli are negative for TRPV1 staining and bind the lectin marker IB4, a traditional marker for non-peptidergic nociceptors.

In the current study we found that null mutation of the P2Y₂ gene resulted in a decrease in responsiveness to noxious heat of the conventional CMH/CMHC population, and a reduction in the proportion of mechanically-sensitive C-fibers responding to both heat and cold.

Intriguingly, immunohistochemical analysis of recorded cells in P2Y₂^{-/-} mice revealed that a substantial proportion of CMH fibers expressed TRPV1 and were negative for IB4. These atypical TRPV1-containing P2Y₂^{-/-} CMHs exhibited reduced firing in response to mechanical stimuli, increased responsiveness to noxious thermal stimulation (43–51 °C), and decreased responses to non-noxious thermal stimulation (37–41 °C) compared to wildtype CMH neurons. Based on these results, we conclude that nucleotide signaling through P2Y₂ is required for the establishment of the normal profile of neuronal response properties in cutaneous unmyelinated afferents.

EXPERIMENTAL PROCEDURES

Animals

Experiments were performed using adult (4–6 weeks) male $P2Y_2^{+/+}$ and $P2Y_2^{-/-}$ C57/Bl6 mice obtained from Jackson Laboratories. A total of 90 cutaneous primary afferent neurons from 10 $P2Y_2^{-/-}$ mice and 150 primary afferent neurons from 20 $P2Y_2^{+/+}$ mice were physiologically characterized by intracellular recording. We focused our analyses on C-fibers, resulting in a total of 67 $P2Y_2^{-/-}$ and 118 $P2Y_2^{+/+}$ characterized C-fibers. All animals were group housed and maintained on a 12-h light–dark cycle with *ad libitum* access to food and water. All procedures were carried out in accordance with protocols approved by the Institutional Care and Use Committee at the University of Pittsburgh.

Ex vivo preparation

The *ex vivo* skin/nerve/DRG/spinal cord preparation utilized in these experiments is described elsewhere (McIlwrath et al., 2007). Briefly, mice were anesthetized with a 90/10 mg/kg mixture of ketamine and xylazine (i.m.) and were transcardially perfused with oxygenated (95% O_2 –5% CO_2) artificial cerebrospinal fluid (aCSF; in mM: 1.9 KCl, 1.2 KH_2PO_4 , 1.3 $MgSO_4$, 2.4 $CaCl_2$, 26.0 $NaHCO_3$, and 10.0 D-glucose) containing 253.9 mN sucrose at 12–15 °C. The spinal cord and the right hindlimb was then dissected and placed in a bath of cold aCSF, and the hairy skin, saphenous nerve, DRG, and spinal column were dissected in continuity. The preparation was transferred to a separate chamber containing chilled oxygenated aCSF in which the sucrose was replaced with 127.0 mN NaCl. The skin was placed on an elevated grid, such that the epidermis was exposed to air and the dermis was continually perfused. Once the preparation was stabilized the temperature of the aCSF was warmed to 31 °C prior to recording.

Electrophysiological recording and stimulation

Sensory neuron somata were impaled with quartz microelectrodes containing 5% Neurobiotin (Vector Laboratories, Burlingame, CA, USA) in 1 M potassium acetate. Orthograde electrical search stimuli were administered through a suction electrode placed on the saphenous nerve to locate sensory neuron somata innervating the skin. Receptive fields (RF) were localized with a paintbrush, blunt glass stylus, and von Frey filaments. If an electrically driven cell with no mechanical RF was located a thermal search was performed by applying hot (~51 °C) and/or cold (~0 °C) 0.9% saline to the skin. Previous work has shown that repeated brief applications of hot saline do not result in nociceptor sensitization (Lawson and Waddell, 1991; Jankowski et al., 2009).

Response characteristics of DRG neurons were determined by applying digitally controlled mechanical and thermal stimuli. The mechanical stimulator consisted of a tension/length controller (Aurora Scientific, Aurora, ON, Canada) attached to a 1-mm diameter plastic disk. Computer controlled 5-s square waves of 1, 5, 10, 25, 50, and 100 mN were applied to the cells RF. After mechanical stimulation, a controlled thermal stimulus was applied using a 3-mm² contact area Peltier element (Yale University Machine Shop, New Haven, CT, USA). The temperature stimulus consisted of a 12-s heat ramp from 31 to 52 °C, followed by a 5-s

holding phase. The temperature ramped back down to 31 °C in 12 s. A 30-s resting period was inserted between stimulus presentations. In some instances, fibers that were unable to be characterized by computer-controlled mechanical and/or thermal stimulation, but were phenotyped by von Frey and/or saline stimuli were not included in threshold determination. All elicited responses were recorded digitally for offline analysis (Spike 2 software, Cambridge Electronic Design, Cambridge, UK). After physiological characterization, select cells were labeled by iontophoretic injection of Neurobiotin. Peripheral conduction velocity was then calculated from spike latency and the distance between stimulation and recording electrodes (measured directly along the saphenous nerve). Thermal thresholds were determined to be the temperature change for fibers that did not exhibit ongoing activity prior to thermal stimulation. For those fibers that did have some degree of ongoing activity, threshold was determined as the temperature at the second spike of 2, where the instantaneous frequency exceeds that present in a 30-s window prior to thermal stimulation.

Classification of cutaneous C-fiber sensory neurons

For the purposes of the present experiments we have focused our recording and analyses specifically on C-fibers, identified as sensory neurons with a conduction velocity of <1.2 m/s (Lawson and Waddell, 1991; Lawson et al., 1993). C-fibers were classified as follows: (1) those that responded to mechanical and heat stimuli (CMH); (2) cells that responded to mechanical, heat and cold stimulation (CMHC); (3) those that responded only to mechanical stimulation of the skin (CM); (4) those that responded to mechanical and cooling stimuli (but not heating) (CMC); (5) those that were cold and mechanically insensitive, but heat sensitive (CH); and (6) those that were heat and mechanically insensitive but responded to cooling of the skin (CC).

Tissue processing and analysis of recorded cells

Once a sensory neuron was characterized and intracellularly filled with Neurobiotin, the DRG containing the injected cell was removed and immersion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min at 4 °C. Ganglia were then embedded in 10% gelatin, postfixed in 4% paraformaldehyde, and cryoprotected in 20% sucrose. Frozen sections (60 µm) were collected in phosphate buffer and reacted with fluorescently tagged (fluorescein isothiocyanate) avidin to label Neurobiotin-filled cells (Vector Laboratories). Next, each section was labeled with *Griffonia simplicifolia* isolectin IB4 conjugated to AlexaFluor 647 (1:100, Molecular Probes, Eugene, OR, USA) and rabbit anti-TRPV1 (1:500, CalBiochem, Merck KGaA, Darmstadt, Germany) immunohistochemistry. After incubation in primary antiserum, tissue was washed and incubated in Cy3- or Cy5-conjugated donkey anti-rabbit secondary antisera (1:200; Jackson ImmunoResearch, West Grove PA, USA). Distribution of fluorescent staining was determined using an Olympus FluoView™ 500 laser-scanning confocal microscope (Olympus America Inc, Center Valley, PA, USA).

Data analysis

In all analyses we tested for distributions of variances using Levene's Test of Equality of Error Variances or Mauchly's Test of Sphericity, where appropriate. We also analyzed skewness and kurtosis to determine normality of distributions. All samples were normally

distributed and had equal variances. One-way and mixed-design Analyses of Variance (ANOVAs) were used to analyze parametric data including firing rates and frequencies, as well as mechanical and thermal thresholds. *Post hoc* analysis was conducted using Tukey's test. Neurons were sorted by functional type to construct relative distributions. Comparisons between distributions (non-parametric) were analyzed using χ^2 . Responses to heat ramp were normalized by multiplying the average spikes per degree by the percentage of cells responding at that temperature. Statistical significance was maintained at $\alpha=.05$.

RESULTS

Functionally identified primary afferent populations in $P2Y_2^{+/+}$ and $P2Y_2^{-/-}$ mice

A total of 90 cutaneous primary afferent neurons from 10 $P2Y_2^{-/-}$ mice and 150 primary afferent neurons from 20 $P2Y_2^{+/+}$ mice were physiologically characterized by intracellular recording. We focused our analyses on C-fibers, resulting in a total of 67 $P2Y_2^{-/-}$ and 118 $P2Y_2^{+/+}$ characterized C-fibers. The distribution of $P2Y_2^{+/+}$ afferents is as follows: 1% CC, 8% CM, 13% CH, 12% CMC, and 41% CMH, and 25% CMHC (Fig. 1). The distribution of characterized $P2Y_2^{-/-}$ afferents is as follows: 1% CC, 12% CM, 7% CH, 13% CMC, 50% CMH, and 17% CMHC. Comparison of the distributions of cells between genotypes revealed a significant increase in the number of CMH, and a significant decrease in the number of CMHC neurons in $P2Y_2^{-/-}$ mice. In addition, there was a trend toward a reduction in the proportion of CHs in $P2Y_2^{-/-}$ mice that did not reach significance.

Deletion of $P2Y_2$ results in mechanical responsiveness in a subset of TRPV1-positive afferents

Previous studies have suggested that $P2Y_2$ plays a role in the transduction of mechanical stimuli and the development of thermal hyperalgesia (Moriyama et al., 2003; Malin et al., 2008). The current experiments examined how deletion of $P2Y_2$ affected primary afferent function, and whether any observed alterations in primary afferent firing properties could contribute to the previously observed behavioral phenotype. To determine if knockout of $P2Y_2$ affected mechanical sensitivity, we examined mechanical thresholds and firing rates in 67 $P2Y_2^{+/+}$ and 42 $P2Y_2^{-/-}$ C-polymodal (CPM, including CMH and CMHC) neurons. Mean mechanical thresholds were 14.84 ± 2.50 mN and 20.07 ± 4.40 mN for $P2Y_2^{+/+}$ and $P2Y_2^{-/-}$ CPMs, respectively. When comparing mean thresholds across all afferents examined, we did not observe any significant differences between genotypes (Fig. 2A). Similarly, no differences were observed when comparing mean peak instantaneous firing frequencies across forces of mechanical stimulation (1–100 mN) for all afferents (Fig. 2B). However, we did observe non-significant trends toward an increase in threshold and a reduction in the average number of spikes generated by $P2Y_2^{-/-}$ neurons in response to mechanical stimulation at nociceptive intensities. We hypothesized that this could be due to the conversion of some TRPV1-containing CHs to relatively insensitive CMH fibers, as TRPV1 is normally only expressed in mechanically insensitive CHs (Lawson et al., 2008) in

naïve mice. To determine whether TRPV1 is expressed in $P2Y_2^{-/-}$ CMHs, a cohort of physiologically characterized afferents was labeled with Neurobiotin and stained for IB4 and TRPV1. In $P2Y_2^{+/+}$ mice, 18/23 CMHs were positive for IB4, while 0/20 were positive for TRPV1. In contrast, of 15 CMH afferents characterized from $P2Y_2^{-/-}$ mice, only 8/15 were positive for IB4 and 5/15 were positive for TRPV1. No afferents were positive for both markers (Fig. 3). CMHs for which complete physiological and histochemical data were available were then separated into two groups, 5 TRPV1-positive afferents and 5 TRPV1-negative afferents, and mechanical threshold data were re-analyzed. Although there were no significant differences in mean mechanical thresholds (Fig. 4A; TRPV1-positive = 11.0 ± 3.29 mN; TRPV1-negative = 15.0 ± 7.87 mN), TRPV1 +CMH neurons exhibited lower mean firing rates to the lower intensities of mechanical stimulation than did TRPV1- CMH neurons (Fig. 4B). These results suggest that in the absence of $P2Y_2$ a subset of TRPV1-positive neurons acquire mechanical sensitivity, but do not respond as robustly as conventional wildtype IB4-positive CMH afferents.

Altered heat responsiveness in CPM afferents from $P2Y_2^{-/-}$ mice

We next examined changes in thermal thresholds and firing properties of CPM afferents, including both CMH and CMHC afferents. We analyzed heat thresholds in 67 $P2Y_2^{+/+}$ and 44 $P2Y_2^{-/-}$ CPMs. No significant differences in heat threshold were detected when comparing $P2Y_2^{+/+}$ (40.76 ± 0.60 °C) and $P2Y_2^{-/-}$ (40.67 ± 0.92 °C) afferents across the entire population (Fig. 5A). We also analyzed cold thresholds in $P2Y_2^{+/+}$ (16.14 ± 1.03 °C) and $P2Y_2^{-/-}$ (16.54 ± 1.60 °C) mice and found no statistically significant difference (Fig. 5B). Similarly, no differences were observed between the 2 genotypes in the average number of spikes generated per second in response to heat stimulation (Fig. 5C).

Although this initial analysis revealed no significant difference in heat thresholds overall, we did observe a trend toward an increase in the average number of spikes observed in $P2Y_2^{-/-}$ CMHs in response to 46–50 °C stimulation (see Fig. 5C). We hypothesized that this trend could represent a diluted effect of the presence of TRPV1 in a subset of the $P2Y_2^{-/-}$ CMH fibers, given that this temperature range represents the optimal stimulus for gating of TRPV1 (Tominaga et al., 1998). To test this possibility, we analyzed the average heat thresholds and the number of spikes generated in response to heat in the same 5 TRPV1-positive and 5 TRPV1-negative $P2Y_2^{-/-}$ CMH neurons analyzed in Fig 4. Although there was no difference in mean heat thresholds between TRPV1-positive and TRPV1-negative CMH neurons (Fig. 6A), the firing frequency was significantly higher in TRPV1-positive CMHs from 43 to 51 °C (Fig. 6B). We also compared heat responses between wildtype and $P2Y_2^{-/-}$ CPMs (CMHs plus CMHCs), excluding the TRPV1-positive afferents (Fig. 6C): $P2Y_2^{-/-}$ CPMs displayed reduced firing across the range of temperatures employed. These results indicate that the TRPV1-positive CMHs observed in $P2Y_2^{-/-}$ mice show increased firing to noxious heat in the temperature range that activates TRPV1, whereas TRPV1-

negative CMH fibers in $P2Y_2^{-/-}$ mice show decreased responsiveness to heat across a broad range of temperatures.

DISCUSSION

The current experiments examine the impact of $P2Y_2$ receptor expression on cutaneous nociceptor function using an *ex vivo* skin/nerve/DRG/spinal cord preparation. Our previous work using this preparation has shown that TRPV1 expression in cutaneous nociceptors is limited to mechanically insensitive CH neurons that *do not* bind IB4. In contrast, CMH neurons, which are sensitive to both mechanical and heat stimuli, are TRPV1-negative and *do* bind IB4 (Lawson et al., 2008; Jankowski et al., 2010; Koerber et al., 2010; Jankowski et al., 2014). In mice lacking $P2Y_2$, CMH fibers showed a deficit in responsiveness to noxious heat across a range of temperatures. In addition, deletion of $P2Y_2$ led to the emergence of an atypical population of CMH neurons that lacked IB4 binding and expressed TRPV1. These atypical CMH fibers were less responsive to mechanical stimuli than conventional CMH fibers (they fired fewer action potentials in response to mechanical stimulation). Consistent with functional expression of TRPV1, these neurons exhibited enhanced firing rates to noxious heat compared to TRPV1-negative CPMs. $P2Y_2^{-/-}$ mice showed a corresponding increase in the proportion of all cutaneous C-fibers responsive to both heat and mechanical stimulation; the percentage of CMHC fibers was also reduced, although the cold thresholds of remaining cold-sensitive afferents were unchanged. We conclude that in the absence of $P2Y_2$, a subset of TRPV1-positive C-fibers, which are normally responsive to heat but not mechanical stimuli, become responsive to mechanical stimuli.

Intriguingly, a similar change in phenotype is seen in response to peripheral inflammation, with TRPV1-positive C-fibers gaining mechanical sensitivity (Jankowski et al., 2009; Koerber et al., 2010; Jankowski et al., 2012). Inflammatory injury also causes an increase in the heat sensitivity of IB4-positive CMH fibers. Analogous findings were reported in human microneurography studies in which C-fiber afferents responsive to noxious heat but not mechanical stimuli acquired mechanical responsiveness after sensitization by inflammatory mediators (Schmidt et al., 1995; Orstavik et al., 2003).

The reduction in firing frequency in response to noxious heat by $P2Y_2^{-/-}$ cutaneous CMH afferents suggests that ATP/UTP signaling through $P2Y_2$ modulates responsiveness to noxious heat in these fibers. The change in phenotype of TRPV1-expressing cutaneous afferents likely results from a compensatory response to the loss of $P2Y_2$ signaling. The similarity of the phenotype to the inflamed state points to the possibility of a role for nucleotide signaling in the inflammation-induced acquisition of mechanical responsiveness in TRPV1 neurons, although it was unexpected that the loss of $P2Y_2$ would lead to such a phenotypic switch. In our previous study, $P2Y_2^{-/-}$ mice did not develop thermal hyperalgesia in response to hindpaw injection of CFA (Malin et al., 2008). It is possible that the emergence of TRPV1-positive CMH fibers in naïve $P2Y_2^{-/-}$ mice, combined with the diminished responses to heat in the mutant TRPV1-negative CMH afferents, occludes the hyperalgesia normally seen in response to inflammation.

Previous work supports a role for P2Y₂ in thermal nociception. P2Y₂^{-/-} mice had higher baseline behavioral thresholds (they were less sensitive) to noxious heat when compared to P2Y₂^{+/+} controls, and deletion of P2Y₂ prevented ATP-evoked as well as the inflammation-evoked heat hyperalgesia normally observed in WT mice (Malin et al., 2008). Several laboratories have reported that P2Y₂ is extensively colocalized with TRPV1 and sensitizes TRPV1 to heat and capsaicin, suggesting that wildtype CH responsiveness is regulated by P2Y₂ (Moriyama et al., 2003). Sensitization of TRPV1 by P2Y₂ is reportedly mediated by activation of protein kinase C epsilon to directly phosphorylate TRPV1 (Moriyama et al., 2003). The attenuation of capsaicin-evoked TRPV1 function reported in P2Y₂^{-/-} mice (Malin et al., 2008) suggests that normal TRPV1 function requires a basal level of phosphorylation, a possibility also suggested elsewhere (Hu et al., 2006; Fioravanti et al., 2008; Srinivasan et al., 2008). Given the previous focus on interactions between P2Y₂ and TRPV1, we were surprised to find no significant decrement in the heat sensitivity of cutaneous TRPV1-positive afferents from P2Y₂^{-/-} mice using the *ex vivo* preparation. However, our previous work in rat sensory neurons suggested that a substantial proportion of IB4-binding neurons also express P2Y₂, leading us to focus on CMH afferents in the current study (Molliver et al., 2002). As a result, we identified a deficit in the heat responsiveness of the IB4-positive CMH population, as well as the appearance of mechanically-sensitive TRPV1-positive afferents. It is worth noting that in the present study we examined heat responses in *ex vivo* afferents with intact peripheral terminals in the skin, whereas the original P2Y₂^{-/-} report used capsaicin to activate TRPV1 in dissociated DRG neurons. However, that study also found that P2Y₂^{-/-} mice had increased behavioral thresholds to noxious heat.

P2Y₂ and the G_{q/11}-coupled ADP receptor P2Y₁ are the principle excitatory P2Y receptors expressed in DRG neurons (Malin et al., 2008). P2Y₁^{-/-} mice also showed deficits in thermosensation using the *ex vivo* preparation; both heat and cold sensitivity were decreased in cutaneous afferents (Molliver et al., 2011). Furthermore, knockdown of P2Y₁ in DRG with *in vivo* siRNA increased the proportion of mechanically sensitive TRPV1-positive neurons, similar to our results here in P2Y₂^{-/-} mice (Jankowski et al., 2012). These results indicate that both P2Y₁ and P2Y₂ have significant impacts on neuronal response properties of thermally responsive afferents.

Although inflammatory sensitization of CMHs was not examined here, P2Y₂^{-/-} mice failed to develop behavioral heat hyperalgesia in response to CFA injection in the previous study (Malin et al., 2008). Collectively, our results suggest that reducing G_q-coupled P2Y signaling alters nociceptor response properties and results in the emergence of TRPV1-positive/IB4-negative CMH neurons. How P2Y receptors influence nociceptive transmission is likely to be multi-factorial, as seen in the apparent disconnect between the afferent physiology detailed here and the behavioral phenotype published previously (Malin et al., 2008). The reason for this discrepancy is not obvious; a perennial challenge in correlating single-cell physiology with behavioral analysis is that behavior is an emergent property of

the entire nervous system, and alterations in the function of individual classes of neurons may have unexpected consequences at other levels of the pain neuraxis. The physiological analysis presented here provides information specific to the impact of P2Y₂ gene deletion on the function of the primary cutaneous afferents.

While our results demonstrate changes in primary afferent responses to physical stimulation of the skin, we cannot rule the possibility that the phenotype may be impacted by signal transduction changes in the skin itself, although histological changes in skin morphology were not detected (unpublished results). Most cells in the body, including keratinocytes, release ATP and/or UTP in response to tissue-appropriate stimulation as well as explicit damage, and P2Y receptors expressed by keratinocytes, including P2Y₂, contribute to proliferation and wound repair (Braun et al., 2006). In addition, keratinocytes likely participate in sensory stimulus transduction and communicate information to sensory afferents, in part through purinergic signaling (Lumpkin and Caterina, 2007; Dussor et al., 2009; Mandadi et al., 2009). For example, mechanical or thermal stimulation of keratinocytes *in vitro* and injury or inflammation of the skin evoke nucleotide release (Cook and McCleskey, 2002; Inoue et al., 2005; Dussor et al., 2009; Mandadi et al., 2009; Barr et al., 2013). As a result of this complexity, dissecting out cell type-specific mechanisms for purinergic signaling remains a major technical challenge. In ongoing efforts to use genetic tools to target specific cell types, we recently demonstrated that direct optical activation of keratinocytes using channelrhodopsin evokes action potential firing in nociceptive neurons (Baumbauer et al., 2015), and we are currently examining whether this phenomenon is ATP-dependent. Our current results presented here demonstrate dysregulation of nociceptor response properties in the absence of P2Y₂, and provide a new piece of the purinergic puzzle.

Acknowledgments

DCM conceived of the experiments and provided the P2Y₂^{-/-} and P2Y₂^{+/+} mice. KKR, DJS, MPJ and HRK contributed to the experimental design and data acquisition. All authors contributed to the interpretation of results and writing of the manuscript and authorized submission of the final version. This work was supported by R01 NS56122 (DCM) and R01 NS23725, R01 NS052848 (HRK).

Abbreviations

aCSF	artificial cerebrospinal fluid
CPM	C-polymodal
DRG	dorsal root ganglion
RF	receptive fields
CMH	C-mechano-heat
CMHC	C-mechano-heat/cold

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Fig. 1.

Changes in functionally identified primary afferent populations. Phenotypes of C-fibers characterized from P2Y₂^{+/+} (A) and P2Y₂^{-/-} (B) mice. CMH and CMHC fibers constitute the largest percentage of C-fibers in both strains of mice. However, electrophysiological characterization of C-fibers from P2Y₂^{-/-} mice revealed an increase in the percentage of CMHs. This is similar to what is observed following cutaneous inflammation or injury. Asterisks indicate statistically significant relationships, $p < .05$. ($n=118$ C-fibers from 20 P2Y₂^{+/+} mice, 67 C-fibers from 10 P2Y₂^{-/-} mice).

Fig. 2.

No change in global C-polymodal mechanical responsiveness in P2Y₂^{-/-} versus P2Y₂^{+/+} mice. Analysis of mean mechanical thresholds (A) and mean spikes/s at stimulation forces ranging from 5–100 mN (B) of P2Y₂^{+/+} and P2Y₂^{-/-} mice. Mechanical stimuli were administered using a calibrated, computer controlled mechanical stimulator. No differences in firing properties were observed between genotypes, $p > .05$. ($n = 67$ P2Y₂^{+/+} CPMs from 16 mice, 42 P2Y₂^{-/-} CPMs from 12 mice).

Fig. 3.

Histochemical classification of recorded neurons. (A) Representative traces of mechanical and thermal responses of the two histochemically distinct types of CMH fibers seen in $P2Y_2^{-/-}$ mice: “conventional” IB4-positive/TRPV1-negative fibers, and IB4-negative/TRPV1-positive CMH fibers, which were never seen in wildtype mice. Stimulus magnitudes for mechanical (in mN) and thermal (in °C) stimuli are shown below the traces. (B–J) Cells were iontophoretically labeled with neurobiotin during *ex vivo* electrophysiological recording and analyzed by immunohistochemistry. CMHs characterized from $P2Y_2^{+/+}$ mice show IB4 binding (C), but not TRPV1 staining (D), while some CMHs characterized from $P2Y_2^{-/-}$ mice do not bind IB4 (F) and stain positively for TRPV1 (G). This pattern of staining is normally observed in wildtype C-heat fibers (H–J).

Fig. 4.

Diminished mechanical responsiveness in P2Y₂^{-/-} (TRPV1-positive) CMH afferents. Average mechanical thresholds (A) and mean number of spikes/s (B) for 5 TRPV1-positive and 5 TRPV1-negative CMHs characterized from 4 P2Y₂^{-/-} mice. No significant differences in average mechanical threshold were observed between the two populations of neurons, $p > .05$. However, TRPV1-positive CMHs exhibited decreased responsiveness (a lower mean firing rate) to the lowest-intensity (5 and 10 mN) mechanical stimuli. Asterisks indicate statistically significant relationships, $p < .05$.

Fig. 5.

No change in global C-polymodal thermal responsiveness in P2Y₂^{-/-} versus P2Y₂^{+/+} mice. Average response threshold to heat (A) and cold stimulation (B) in CMHs characterized from P2Y₂^{+/+} and P2Y₂^{-/-} mice. No differences in response thresholds were detected, $p > 0.05$. The average number of spikes/s generated in response to heat stimulation was also examined (C). The apparent increase in responsiveness of P2Y₂^{-/-} afferents to stimuli from 46° to 50°C did not reach significance, but led us to perform the more refined analysis in Fig. 6. ($n=67$ P2Y₂^{+/+} CPMs from 16 mice, 42 P2Y₂^{-/-} CPMs from 12 mice).

Fig. 6.

Altered responsiveness to noxious heat in TRPV1-positive and TRPV1-negative c-polymodal fibers from P2Y₂^{-/-} mice. Average heat thresholds (A) and average number of spikes/s (B) for the TRPV1-positive and TRPV1-negative CMHs analyzed in Fig. 4 and for (C) CPMs (CMHs plus CMHCs) with TRPV1-positive fibers excluded. A: No differences in heat threshold were observed when comparing the two populations of neurons, $p > .05$. B: However, TRPV1-positive CMHs exhibited an increase in the mean number of spikes generated from 43° to 51°C. C: When TRPV1-positive CPMs were excluded from the analysis, P2Y₂^{-/-} afferents showed reduced firing rates to heat compared to wildtype, indicating that “conventional” CPM fibers are less responsive to heat in the absence of P2Y₂. For C, $n=37$ P2Y₂^{-/-} CPMs from 12 mice. Asterisks indicate statistically significant relationships, $p < .05$.