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$P2Y_1$ Receptor Activation of the TRPV4 Ion Channel Enhances Purinergic Signaling in Satellite Glial Cells*

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Background: The function of TRP channels in satellite glial cells is unknown.
Results: The proinflammatory, mechanosensitive TRPV4 channel is expressed by satellite glial cells. $P2Y_1$ receptors cause protein kinase C-dependent activation of TRPV4.
Conclusion: TRPV4 enhances purinergic signaling in non-neuronal cells of sensory ganglia.
Significance: TRPV4-mediated signaling in satellite glial cells may contribute to inflammatory pain.

Transient receptor potential (TRP) ion channels of peripheral sensory pathways are important mediators of pain, itch, and neurogenic inflammation. They are expressed by primary sensory neurons and by glial cells in the central nervous system, but their expression and function in satellite glial cells (SGCs) of sensory ganglia have not been explored. SGCs tightly ensheath neurons of sensory ganglia and can regulate neuronal excitability in pain and inflammatory states. Using a modified dissociation protocol, we isolated neurons with attached SGCs from dorsal root ganglia of mice. SGCs, which were identified by expression of immunoreactive Kir4.1 and glutamine synthetase, were closely associated with neurons, identified using the pan-neuronal marker NeuN. A subpopulation of SGCs expressed immunoreactive TRP vanilloid 4 (TRPV4) and responded to the TRPV4-selective agonist GSK1016790A by an influx of Ca^{2+} ions. SGCs did not express functional TRPV1, TRPV3, or TRP ankyrin 1 channels. Responses to GSK1016790A were abolished by the TRPV4 antagonist HC067047 and were absent in SGCs from *Trpv4*$^{-/-}$ mice. The $P2Y_1$-selective agonist 2-methylthio-ADP increased $[Ca^{2+}]_i$ in SGCs, and responses were prevented by the P2Y1-selective antagonist MRS2500. $P2Y_1$ receptor-mediated responses were enhanced in TRPV4-expressing SGCs and HEK293 cells, suggesting that $P2Y_1$ couples to and activates TRPV4. PKC inhibitors prevented $P2Y_1$ receptor activation of TRPV4. Our results provide the first evidence for expression of TRPV4 in SGCs and demonstrate that TRPV4 is a purinergic receptor-operated channel in SGCs of sensory ganglia.

Transient receptor potential (TRP)[3] ion channels are important sensory proteins in the pathways to pain, itch, and neurogenic inflammation. They are prominently expressed by unmyelinated small diameter or lightly myelinated medium diameter primary sensory neurons and participate in thermal, mechanical, and chemical sensation (1). In addition to these direct mechanisms of TRP activation, TRP channels also function as receptor-operated proteins that can be indirectly regulated by receptor tyrosine kinases (2) and G protein-coupled receptors (GPCRs) (3). TRP channels are non-selective cation channels that, once activated, enhance neuronal excitability and stimulation of voltage-operated Na^+ channels, which generate action potentials that underlie central transmission.

TRP channels are also expressed by non-neuronal cells (*e.g.* keratinocytes) of peripheral sensory pathways, where activation is important for the initiation of nociception, itch, and inflammation (4–6). In addition, TRP channels are expressed by astrocytes and microglial cells of the central nervous system. These immune-like cells support neuronal health and activity, where TRP channel ion flux in glia has been associated with proliferation, osmo-sensation, cytokine production, and maintenance of the blood-brain barrier (7–9). To our knowledge, TRP channel expression and function have not been closely examined in satellite glial cells (SGCs) of peripheral sensory pathways.

SGCs of primary sensory ganglia, including dorsal root ganglia (DRG) and trigeminal ganglia, tightly ensheath the soma of sensory neurons. This intimate association of SGCs and neurons facilitates bidirectional regulation of SGC function and neuronal excitability. During injury and inflammation, neuronal hyperexcitability can lead to SGC activation. Activation can induce glial cell proliferation, increased formation of gap junctions for rapid trans-cellular exchange of small molecules,

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[3] The abbreviations used are: TRP, transient receptor potential; SGC, satellite glial cell; TRPV, transient receptor potential vanilloid; TRPA, transient receptor potential ankyrin; GPCR, G protein-coupled receptor; DRG, dorsal root ganglia; IR, immunoreactive; GSK101, GSK1016790A; 2-MeSADP, 2-methylthio-ADP; PKC, protein kinase C.

increased protein expression (*e.g.* glial fibrillary acid protein), and release of inflammatory mediators. Collectively, these changes may contribute to inflammatory and neuropathic pain (10–12). SGCs also express Kir4.1 and display a high K^+ conductance that is hypothesized to control $[K^+]$ of the perineuronal environment and regulate neuronal excitability. Reduction in Kir4.1 expression and mediated currents has been demonstrated during inflammation, and Kir4.1 silencing in sensory peripheral ganglia enhances pain (13–17). In inflammatory pain and disease states, SGCs can release ATP and cytokines that may increase neuronal excitability (18–21). Thus, SGCs actively contribute to the induction and maintenance of pain.

We sought to determine whether TRP channels are expressed and serve an important function in non-neuronal cells of sensory ganglia. To do so, we established a neuron-SGC culture system wherein SGCs remained closely attached to DRG neurons. This approach is advantageous for maintaining physiologically relevant neuro-glial interactions and glial morphology and minimizing phenotypic changes associated with long term culturing of primary cells (22). Using single cell Ca^{2+} imaging, functional responses were exclusively measured in SGCs that remained attached to neurons. This approach, combined with pharmacological, genetic, and immunochemical studies, revealed that a subpopulation of SGCs express functional TRPV4. TRPV4 is a mechanosensitive and receptor-operated cation channel that contributes to mechanical hyperalgesia, neurogenic inflammation, and edema formation (23–25). The mechanism of activation and function of TRPV4 in SGCs are unknown.

Purines contribute to nociceptive pathways by activating primary sensory neurons and SGCs (19, 20, 26, 27). We investigated whether purinergic signaling is a mechanism of receptor-operated TRPV4 activation in SGCs. By examining purinergic signaling in SGCs from wild type and *Trpv4*$^{-/-}$ mice, we found that TRPV4 contributes to $P2Y_1$ receptor purinergic signaling in a subpopulation of SGCs. Studies using kinase inhibitors revealed that protein kinase C (PKC) activity mediates $P2Y_1$ receptor-stimulated activation of TRPV4. Together, these data provide the first evidence for expression and function of the TRPV4 ion channel in SGCs and reveal that TRPV4 is a purinergic receptor-operated channel in SGCs of sensory ganglia. TRPV4 activation within SGCs of sensory ganglia may contribute to inflammatory and neuropathic pain controlled by non-neuronal cells.

Experimental Procedures

Reagents—2-Methylthio-ADP (2-MeSADP), MRS2500, Go6976, and Go6983 were from Tocris Bioscience (Bristol, UK). HC067047 was from Santa Cruz Biotechnology, Inc. Other reagents were from Sigma-Aldrich.

Animals—The Animal Ethics Committee of Monash University approved all experiments with mice. C57BL/6, *Trpv4*$^{-/-}$, and *Trpv4*$^{+/+}$ (littermates) mice were studied (28). Mice (6–8 weeks, male and female, 20–25 g) were from the Monash Animal Research Platform (Clayton, Victoria, Australia) and were maintained under temperature-controlled (22 ± 4 °C) and light-controlled (12-h light/dark cycle) conditions with free access to food and water.

Dissociation and Culture of DRG Cells—Mice were killed by cervical dislocation. Whole DRGs were collected from all spinal levels, taking care to remove the spinal roots, which minimized aggregation during dissociation. Ganglia were pooled and incubated in Ca^{2+}-, Mg^{2+}-free Hanks' balanced salt solution containing 0.6 mg/ml of both collagenase type 2 and type 4, 0.3 mg/ml dispase II, and 0.1 mg/ml DNase I for 25 min at 37 °C. DRG neurons and SGCs were partially dissociated by gentle mechanical trituration with fire-polished Pasteur pipettes. The cell suspension was washed by repeated centrifugation (2 × 500 × *g*, 5 min) and resuspension in DMEM supplemented with antibiotic-antimitotic (Life Technologies, Mulgrave, Australia). Dissociated cells were plated onto coverslips coated with poly-L-lysine and 100 μg/ml laminin (lysine/laminin). Cells were maintained in DMEM containing antibiotic-antimitotic, 10% FBS, and N-1 supplement in a humidified incubator at 37 °C (95% O_2, 5% CO_2) for 24 h before experiments (29, 30).

Measurement of $[Ca^{2+}]_i$ in DRG Cultures—Dissociated DRG were loaded with Fura-2/AM ester (5 μM, 45 min, 37 °C) in calcium assay buffer (10 mM HEPES, 0.5% BSA, 10 mM D-glucose, 2.2 mM $CaCl_2{\cdot}H_2O$, $MgCl_2{\cdot}6H_2O$, 2.6 mM KCl, 150 mM NaCl) containing 4 mM probenecid and 0.05% pluronic F127. Cells were washed and incubated in calcium assay buffer for 30 min before imaging. Cells were observed using a Leica DMI-6000B microscope with an HC PLAN APO 0.4 numerical aperture ×10 objective maintained at 37 °C. Images were collected at 1-s intervals (excitation, 340 nm/380 nm; emission, 530 nm). Cells were challenged with 2-MeSADP (10 μM; $P2Y_1$ agonist), GSK1017690A (GSK101) (10 μM to 10 nM; TRPV4 agonist), UTP (5 μM; purinergic agonist), or capsaicin (0.5 μM; TRPV1 agonist). In some experiments, cells were pretreated with HC067047 (10 μM; TRPV4 antagonist), MRS2500 (1 μM; $P2Y_1$ antagonist), Go6976 (1 μM; PKC inhibitor (31)), or DMSO (vehicle control) 30 min prior to the addition of agonists. Ionomycin (1 μM) was applied at the end of each experiment to obtain maximal $[Ca^{2+}]_i$.

Cell Lines—HEK293 cell lines stably expressing human TRPV4 were generated as described (29). Cells were maintained in DMEM containing 10% tetracycline-free FBS, 150 μg/ml hygromycin, and 5 μg/ml blasticidin. To induce TRP channel expression, 0.1 μg/ml tetracycline was added to the medium 16 h before use (25). Parental untransfected cells HEK293 were used as control.

Measurement of $[Ca^{2+}]_i$ in HEK293 Cells—$[Ca^{2+}]_i$ was measured as described (25). Briefly, HEK293 cells were seeded onto 96-well plates (25,000 cells/well) coated with poly-D-lysine (100 μg/ml) and cultured for 48 h. Cells were loaded with Fura-2/AM ester (2 μM, 45 min, 37 °C) in calcium buffer. Fluorescence was measured at 4-s intervals (excitation, 340 nm/380 nm; emission, 520 nm) using a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA). Cells were challenged with 2-MeSADP (31.6 nM, $P2Y_1$ agonist) and GSK1017690A (3.16 nM, TRPV4 agonist). In some experiments, cells were pretreated for 30 min with antagonists as described under "Results" (32). Ionomycin (1 μM) was applied at the end of each experiment to obtain maximal $[Ca^{2+}]_i$.

Analysis of Ca^{2+} Signals—Results are expressed as the 340/380 nm fluorescence emission ratio, which is proportional to

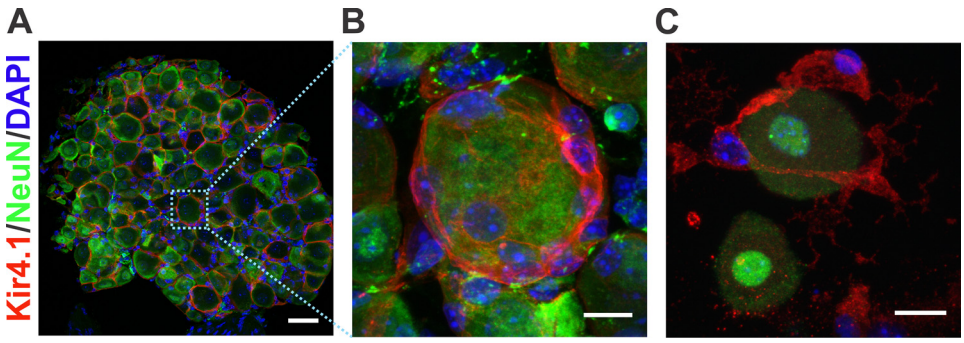

FIGURE 1. **Localization of SGCs in DRG.** *A* and *B*, section of intact DRG showing localization of Kir4.1-IR (*red*; SGC marker) and NeuN-IR (*green*; neuronal marker). *B*, enlargement of *boxed area*. *C*, localization of Kir4.1-IR and NeuN-IR in cultured DRG (24 h). *Scale bars*, 40 μm (*A*) and 10 μm (*B* and *C*).

changes in $[Ca^{2+}]_i$. Data are presented as F/F_{max} relative to baseline, where F is the measured fluorescence intensity and F_{max} is the maximal fluorescence intensity in saturating calcium (upon ionomycin addition), unless mentioned otherwise. For SGCs, a positive response was counted if the F/F_{max} measurement was ≥0.2 ratiometric units above baseline. For cells isolated from *Trpv4*$^{-/-}$ mice or treated with antagonists, the whole population was included for analysis. For DRG neuron-glia co-cultures, only glial cells attached to the neurons were selected as a region of interest. All non-neuronal cells in the co-culture were omitted from analysis because SGCs detached from the neuron could not be reliably distinguished from other cell types. Images were processed using ImageJ software, version 1.49m (National Institutes of Health) (25). The Ratio Plus plugin for ImageJ was used to aid in identification of SGCs and generation of ratiometric images.

Immunofluorescence—Intact DRGs were removed and fixed in 4% paraformaldehyde for 3 h at 4 °C. DRGs were washed, incubated in 30% sucrose in PBS overnight at 4 °C, and embedded in OCT. Sections of DRG (14–16 μm) were cut and mounted on poly-L-lysine-coated slides. Dissociated DRG cultures and HEK293 cells were plated onto coverslips coated with lysine/laminin. Cells were washed with PBS (pH 7.4) and fixed (4% paraformaldehyde in PBS, pH 7.4, 15 min on ice). Cells and tissue sections were incubated in blocking buffer (5% normal horse serum in PBS with 0.1% Triton X-100, 1 h at room temperature) and then incubated with primary antibodies at 4 °C overnight. Primary antibodies used were mouse anti NeuN (1:300; MAB 377, Millipore, Billerica, MA), rabbit anti-Kir4.1 (1:2500; APC-035, Alomone, Jerusalem, Israel), guinea pig anti-Kir4.1 (1:1000; AGP-012, Alomone), mouse anti-glutamine synthetase (1:1000; MAB302 Millipore), and mouse anti-TRPV4 (1:1000; ab39260 Abcam, Cambridge, UK). Cells or tissue sections were washed with PBS (3 times) and incubated with donkey anti-mouse 647, donkey anti-rabbit 594, donkey anti-rabbit 488, donkey anti-guinea pig 594, or donkey anti-mouse 488 (1:500, 1 h, room temperature; Jackson ImmunoResearch). Coverslips or slides were mounted with ProLong Gold Antifade (Life Technologies, Inc.). Images were obtained with a Leica TCS SP8 laser-scanning confocal microscope using an HC PLAN APO 1.3 numerical aperture ×40 and 1.4 numerical aperture ×63 oil immersion objective.

Statistical Analysis—Results are expressed as the mean ± S.E. and were compared by Student's t test (two-tailed) or repeated measures analysis of variance and Dunnett's post hoc test using GraphPad Prism, version 6.05. Differences were considered significant when p was <0.05.

Results

Maintaining Neuron-SGC Interactions in Dissociated and Cultured DRG—The expression and function of TRP ion channels has been reported in neurons and glial cell types of the central and peripheral nervous systems (6, 33, 34). However, TRP channel activity in SGCs has not been investigated in detail. We therefore sought to determine whether TRP channels are expressed and functional in SGCs of the peripheral sensory pathway. To do so, we developed a protocol to dissociate and culture cells of mouse DRG, using a protease digestion protocol that ensured neuron-glia interactions would remain intact.

In order to identify neurons and SGCs in DRG, we localized the pan-neuronal marker NeuN and the inwardly rectifying potassium channel Kir4.1. Kir4.1 is confined to SGCs of sensory ganglia, where it is proposed to control K^+ concentrations in the perineuronal environment and thereby regulate neuronal excitability (13–17). In intact DRG, we found that Kir4.1-immunoreactive (IR) SGCs completely ensheathed NeuN-IR neurons (Fig. 1, *A* and *B*). In DRG cultures, we also observed that most NeuN-IR neurons were ensheathed by Kir4.1-IR SGCs (Fig. 1*C*). Thus, although proteolytic digestion and trituration could separate SGCs and neurons, mild digestion conditions preserve the close association of these cells in culture.

Neurons and Attached SGCs Differentially Express Functional TRP Channels—To investigate functional expression of TRP ion channels in SGCs, we measured $[Ca^{2+}]_i$ of individual cells in DRG cultures. Previous studies have shown that $[Ca^{2+}]_i$ can be measured in individual SGCs adhered to the neurons independent of the neuronal responses (35–37). We observed that SGCs preferentially take up Fura-2/AM compared with adherent neurons (Fig. 2, *A* and *B*). Whether the preferential uptake is because SGCs ensheath neurons and physically impede neuronal loading remains to be determined. However, prolonged incubation (150 min) can enhance neuronal loading with Fura-2/AM (37).

By carefully defining regions of interest, we were able to selectively measure changes in $[Ca^{2+}]_i$ that were specific to neurons and adherent SGCs. We sequentially stimulated cells with the purinergic agonist ATP (10 μM), a known stimulator of

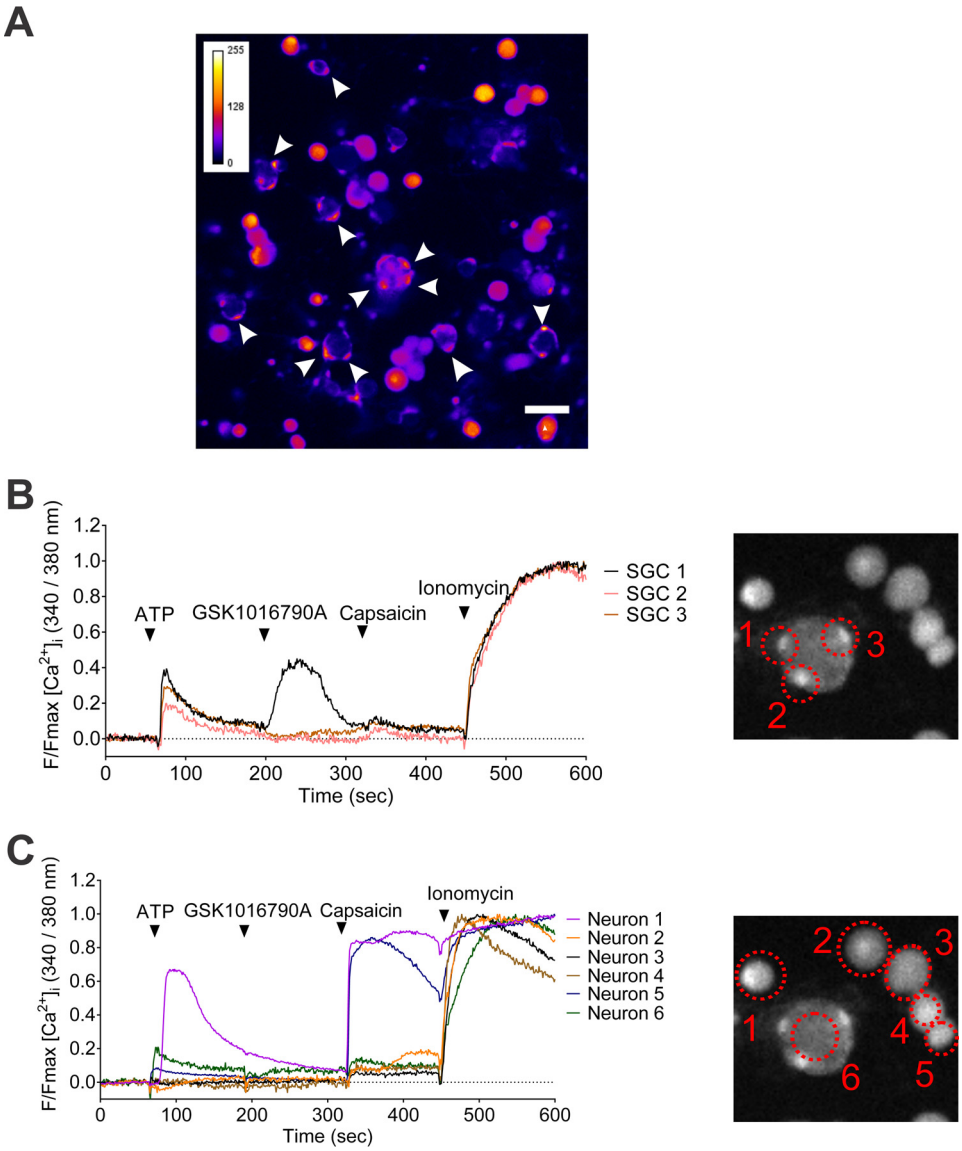

FIGURE 2. **Measurement of $[Ca^{2+}]_i$ in neurons and SGCs of cultured DRG.** *A*, DRG culture loaded with Fura-2/AM. SGCs (*white arrows*) more efficiently take up the dye than neurons. *Scale bar*, 40 μm. *B* and *C*, measurement of $[Ca^{2+}]_i$ in SGCs (*B*) and neurons (*C*) sequentially challenged with ATP (10 μM), GSK101 (1 μM), capsaicin (0.5 μM), and ionomycin (1 μM). *Insets*, imaged cells (*red circles*).

neurons and glia (18, 19), the TRPV4 agonist GSK101 (1 μM), and TRPV1 agonist capsaicin (500 nM) to identify small and medium diameter neurons, followed by ionomycin (1 μM). All SGCs responded to ATP, and unexpectedly, a subpopulation of SGCs also responded to GSK101 but not to capsaicin (Fig. 2*B*). Representative data demonstrated that small diameter neurons (18–19 μm) responded to ATP and capsaicin (Fig. 2*C*). Neuronal responses to GSK101 were also observed (data not shown), which is consistent with our previous findings (25, 29). These results show that we can record differential and physiologically relevant Ca^{2+} responses from neurons and associated SGCs.

Detection of Functional TRPV4 in a Subpopulation of SGCs—In order to assess the expression of functional TRP channels by SGCs, we challenged cultured DRG with agonists of the key nociceptive TRP channels, including TRPA1 (100 μM allyl isothiocyanate), TRPV3 (50 μM carvacrol), TRPV1 (500 nM capsaicin), and TRPV4 (1 μM GSK101). GSK101 stimulated a robust increase in $[Ca^{2+}]_i$ in individual SGCs, whereas none of the other TRP agonists elicited a response (Fig. 3). Thus, SGCs express functional TRPV4 but not functional TRPA1, TRPV1, or TRPV3. GSK101 (10 nM to 10 μM) caused a concentration-dependent increase in $[Ca^{2+}]_i$ in SGCs with an EC_{50} of 199.9 ± 66 nM and a maximal response at 10 μM (Fig. 4*A*). There was also a concentration-dependent increase in the proportion of SGCs that responded to GSK101 (Fig. 4*B*). The maximal concentration of GSK101 (1 μM) stimulated a detectable increase in $[Ca^{2+}]_i$ in 26 ± 3% of all neuron adherent SGCs (205 cells, $n \geq 4$ experiments). Preincubation of cultures with the TRPV4 antagonist HC067047 (10 μM) abolished GSK101-evoked Ca^{2+} responses of SGCs (Fig. 4*C*). GSK101 did not stimulate Ca^{2+} responses in SGCs in DRG cultures from *Trpv4*$^{-/-}$ mice (Fig. 4*D*). Thus, a subpopulation of neuronally adherent SGCs express functional TRPV4 in cultures of mouse DRG.

To confirm the expression of TRPV4 in SGCs within whole ganglia, we colocalized TRPV4-IR and glutamine synthetase-IR, a SGC marker, in sections of intact mouse DRG. We

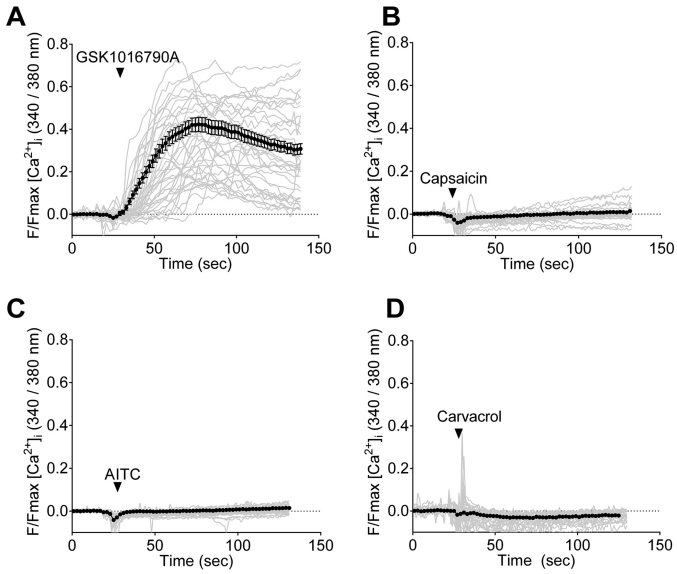

FIGURE 3. **Functional characterization of TRP ion channel expression in SGCs.** $[Ca^{2+}]_i$ was measured in SGCs. *A*, TRPV4 agonist GSK101 (1 μM; $n = 120$ cells). *B*, TRPV1 agonist capsaicin (0.5 μM; $n = 121$ cells). *C*, TRPA1 agonist allyl isothiocyanate (*AITC*) (100 μM, $n = 267$ cells). *D*, TRPV3 agonist carvacrol (50 μM; $n = 175$ cells). Results are from $n = 3$ separate experiments. *Gray lines*, individual traces from 30 random cells; *black lines*, mean responses.

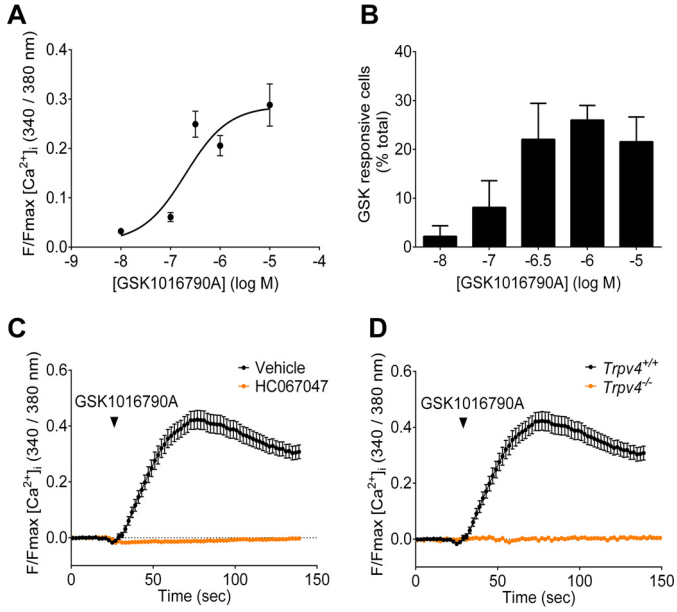

FIGURE 4. **Functional characterization of TRPV4 ion channel in SGCs.** $[Ca^{2+}]_i$ was measured in SGCs. *A* and *B*, concentration response curves to GSK101 showing the maximal response (*A*) and the proportion of GSK-responsive cells (*B*). The maximally effective GSK101 concentration (1 μM) increased $[Ca^{2+}]_i$ in 26 ± 3% of the whole SGC population ($n = 205$ cells), indicating that a subset of SGCs express functional TRPV4. *C* and *D*, GSK101 (1 μM) evoked $[Ca^{2+}]_i$ responses were abolished by the TRPV4 antagonist HC067047 (*C*) and not detected in cells from *Trpv4*$^{-/-}$ mice. $n > 4$ experiments. *Error bars*, S.E.

detected TRPV4-IR in glutamine synthetase-IR SGCs (Fig. 5*A*, *insets i* and *ii*). TRPV4-IR was detected at the plasma membrane and within the cytosol of SGCs. TRPV4-IR was detected in a subpopulation of glutamine synthetase-IR SGCs, consistent with functional data. TRPV4-IR was also observed in a subpopulation of neurons, consistent with previous findings (Fig. 5*A*, *inset iii*) (38–40). Preadsorption of the TRPV4 antibody with the TRPV4 fragment that was used for immunization abolished staining, suggesting specificity (Fig. 5*B*). In acutely dissociated cultures (24 h), TRPV4-IR was detected in Kir4.1-positive SGCs still adhered to the neuron (Fig. 5*C*). HEK293 cells stably expressing TRPV4 and untransfected HEK293 cells were used as positive and negative controls for TRPV4-selective IR, respectively (Fig. 5*D*). These findings support the presence of TRPV4 in a subset of SGCs of mouse DRG.

Purinergic P2Y$_1$ Receptors Activate TRPV4 in SGCs—Purinergic signaling has been implicated in the glial modulation of neurons during pathological pain (41–43). Purinergic receptors can also regulate TRP channels (44). To determine whether purinergic receptors can regulate TRPV4 in SGCs, we measured $[Ca^{2+}]_i$ in SGCs in response to ATP (10 μM), UTP (5 μM), and the selective TRPV4 agonist GSK101 (1 μM). ATP and UTP both evoked rapid and transient increases in $[Ca^{2+}]_i$ in 78 ± 4.5% (521 cells, $n = 6$ experiments) and 85.5 ± 8.8% (458 cells, $n = 4$ experiments) of SGCs, respectively (Fig. 6, *A* and *B*). To determine whether the presence of TRPV4 could alter purinergic responsiveness, we segregated SGCs based on the ability of cells to respond to GSK101. The maximal Ca^{2+} responses to ATP and UTP were similar in GSK101-responsive and GSK101-unresponsive SGCs (Fig. 6, *C* and *D*). However, both the ATP- and UTP-evoked Ca^{2+} responses were significantly larger at 90 s post-stimulation in GSK101-unresponsive SGCs (Fig. 6, *A–D*). The more sustained Ca^{2+} response in SGCs lacking TRPV4 may be related to interplay between ionotropic purinergic receptors and TRPV4, which was not further investigated.

TRPV4 activity can be regulated by receptor-operated mechanisms involving GPCRs (3, 25). To assess the functional expression of P2Y receptors in SGCs from mouse DRG, we examined the effect of 2-MeSADP (10 μM), an agonist of $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors, but not P2X ion channel family members (45). 2-MeSADP caused a transient increase in $[Ca^{2+}]_i$ in SGCs, consistent with stimulation of a $G\alpha_q$-coupled receptor (Fig. 7*A*). To further identify the receptor responding to 2-MeSADP in SGCs, cells were preincubated with MRS2500 (1 μM), a $P2Y_1$ receptor-selective antagonist (46), which significantly inhibited 2-MeSADP-stimulated responses (Fig. 7, *A–B*). This confirmed functional expression of $P2Y_1$ receptor in SGCs, as reported previously for sensory ganglia (19, 27, 47, 48). Removal of extracellular Ca^{2+} significantly attenuated the Ca^{2+} response to 2-MeSADP relative to vehicle (Fig. 7, *C* and *D*). These results suggest that SGCs express functional $P2Y_1$ receptors, and 2-MeSADP responses in SGCs are highly Ca^{2+}-dependent.

To examine the contribution of TRPV4 to $P2Y_1$ receptor-evoked Ca^{2+} signaling in SGCs, we stimulated cells sequentially with 2-MeSADP (10 μM) and GSK101 (1 μM). Populations of SGCs were categorized into GSK101-responsive and GSK101-unresponsive SGCs based on Ca^{2+} response to the agonist GSK101. The magnitude of 2-MeSADP response was larger in GSK101 positive than in GSK101 negative cells, as reflected by significantly increased maximal and integrated (area under curve) Ca^{2+} (Fig. 8, *A–C*). To confirm the involvement of TRPV4 in 2-MeSADP Ca^{2+} responses, we compared responses in SGCs from *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice. The maximal and

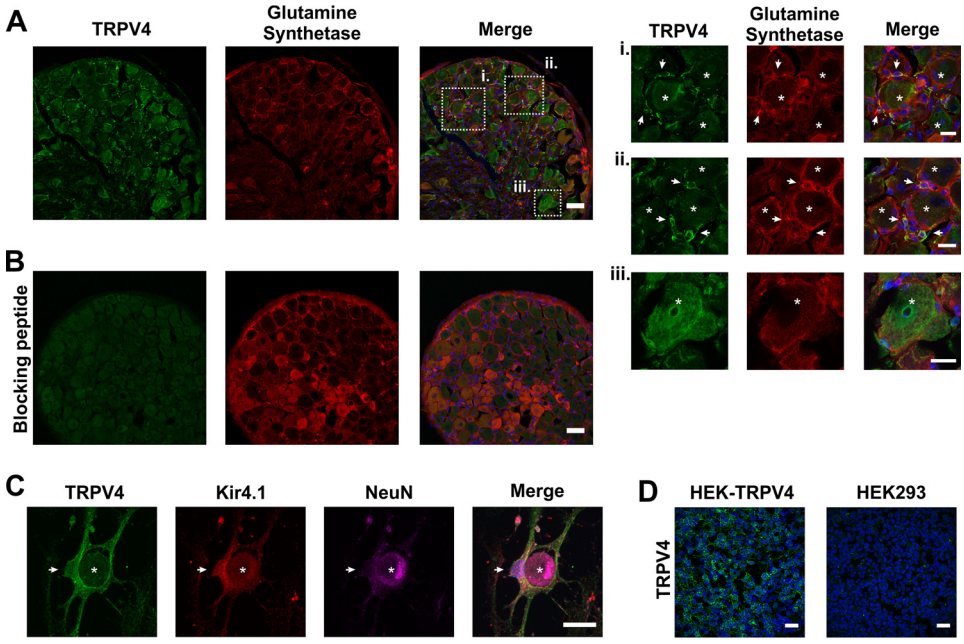

FIGURE 5. **Localization of TRPV4 in SGCs.** *A*, detection of TRPV4-IR and glutamine synthetase-IR in a subset of SGCs of intact DRG. *Scale bar*, 40 μm. *Insets i* and *ii* demonstrate heterogeneity in TRPV4-IR even in SGCs surrounding the same neuron. *Inset iii* demonstrates TRPV4 staining in neurons. *White arrows*, TRPV4-positive SGCs; *asterisks*, TRPV4-positive neurons. *Scale bar*, 15 μm. *B*, preadsorption of the TRPV4 antibody with the peptide used for immunization abolished the signal. *Scale bar*, 40 μm. *C*, detection of TRPV4-IR and Kir4.1-IR SGC surrounding a neuron in an acutely dissociated DRG culture. *Scale bar*, 15 μm. *D*, TRPV4-IR in HEK-TRPV4 cells but not untransfected HEK293 cells, DAPI counterstain. *Scale bar*, 40 μm.

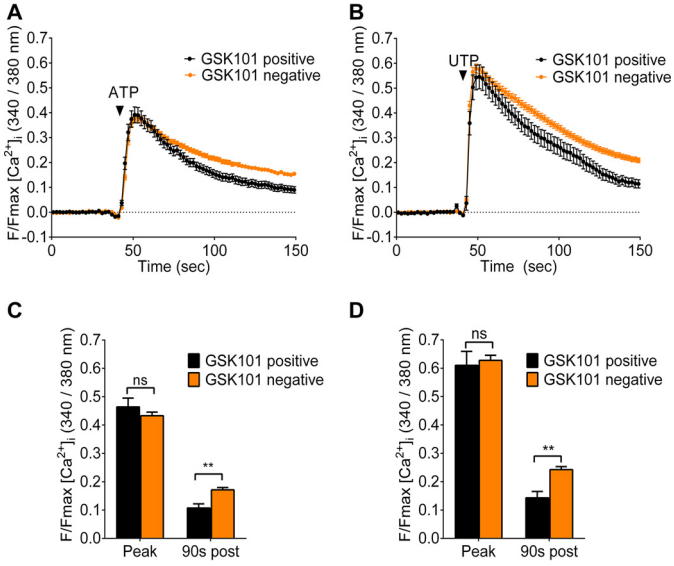

FIGURE 6. **Purinergic signaling in SGCs.** *A* and *B*, ATP (10 μM; ($n = 521$ cells) (*A*) and UTP (5 μM; $n = 428$ cells) (*B*) Ca^{2+} response in SGCs. *C* and *D*, magnitude of Ca^{2+} responses to ATP (*C*) and UTP (*D*) at peak and at 90 s after stimulation. Responses are segregated between GSK101-responsive (GSK101-positive) and GSK101-unresponsive (GSK101-negative) cells. n = 4–6 experiments; **, $p < 0.01$ relative to vehicle, Student's t test. *ns*, not significant. *Error bars*, S.E.

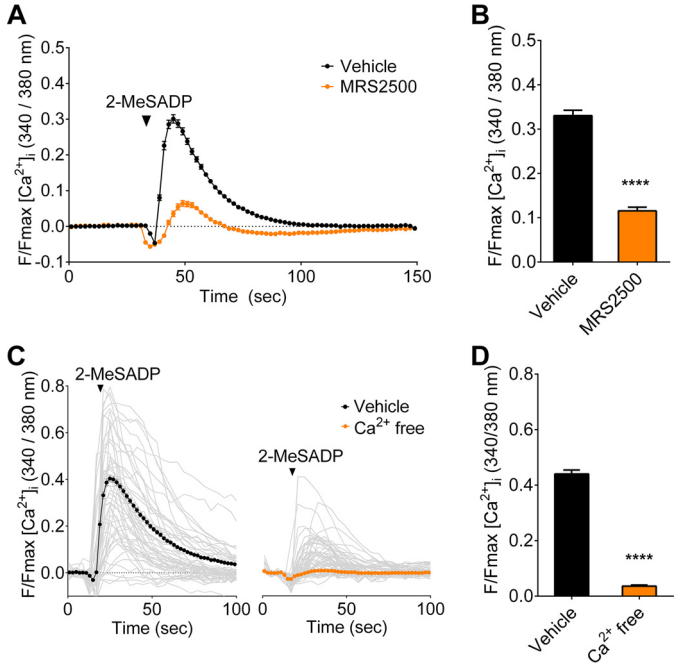

FIGURE 7. **Functional $P2Y_1$ receptors of SGCs.** Time course (*A*) and peak Ca^{2+} responses (*B*) of SGCs to 2-MeSADP (10 μM) in the presence of vehicle or MRS2500 (1 μM) (vehicle, $n = 543$ cells; MRS2500, $n = 603$ cells; $n = 3$ experiments). Shown are the time course (*C*) and peak Ca^{2+} responses (*D*) of SGCs to 2-MeSADP (10 μM) in the presence of vehicle or in the absence of extracellular Ca^{2+} (vehicle, $n = 365$ cells; $n = 4$ experiments; Ca^{2+}-free, $n = 225$ cells). *Gray lines*, individual traces from 30 random cells; *solid lines*, mean responses. $n = 3$ experiments. ****, $p < 0.001$; Student's t test. *Error bars*, S.E.

integrated responses to 2-MeSADP were significantly larger in SGCs from *Trpv4*$^{+/+}$ mice compared with SGCs from *Trpv4*$^{-/-}$ mice, and differences were more apparent when only GSK101-responsive cells from Trpv4$^{+/+}$ mice were compared (Fig. 8, *D–F*).

We categorized SGCs based on the functional expression of $P2Y_1$ receptors (2-MeSADP-responsive) and TRPV4 (GSK101-responsive). This analysis showed that 17.2 ± 5.5% of the SGC population expressed both functional $P2Y_1$ receptors and TRPV4 (Table 1). These results suggest that $P2Y_1$ receptors can activate TRPV4 in SGCs, which mediates an influx of extracellular Ca^{2+} ions contributing to the magnitude of the $P2Y_1$ response.

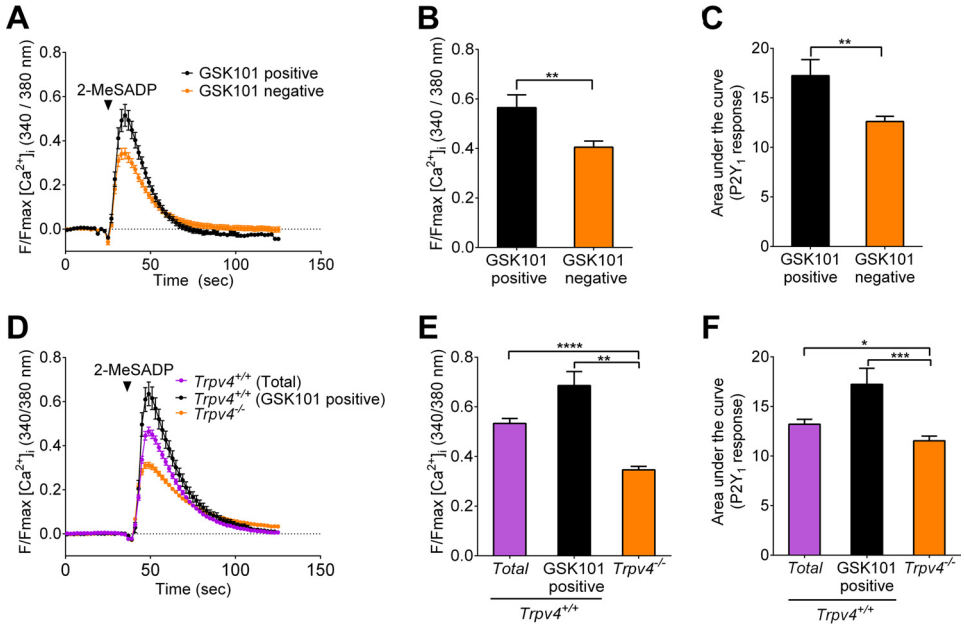

FIGURE 8. **$P2Y_1$ receptor-TRPV4 coupling in SGCs.** *A–C*. 2-MeSADP (10 μM) Ca^{2+} responses in SGCs from wild type mice. Responses are segregated between GSK101-responsive (GSK101-positive) and GSK101-unresponsive (GSK101-negative) cells. The time course (*A*), peak response (*B*), and integrated response (*C*) are shown. $n = 387$ cells from 4 experiments. *D* and *E*, 2-MeSADP (10 μM) Ca^{2+} responses in SGCs from $Trpv4^{+/+}$ and $Trpv4^{-/-}$ mice. Responses of the $Trpv4^{+/+}$ mice are segregated between GSK101-responsive (GSK101-positive) and all (total) cells. The time course (*D*), peak response (*E*), and integrated response (*F*) are shown. $n = 629$ cells from 4 experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ relative to vehicle, Student's *t* test or one-way analysis of variance with Dunnett's post hoc test. *Error bars*, S.E.

Purinergic $P2Y_1$ Receptors Sensitize TRPV4 in SGCs—$P2Y_1$ receptors can enhance responses to TRP agonists and thus sensitize TRP channels (49). To examine whether $P2Y_1$ receptors could sensitize TRPV4 in SGCs, we pretreated SGCs with 2-MeSADP (10 μM) or vehicle (control) and 120 s later challenged cells with a submaximal concentration of GSK101 (200 nM) (Fig. 4*A*). Activation of $P2Y_1$ receptors changed the temporal profile of GSK101-evoked Ca^{2+} responses in SGCs compared with vehicle (Fig. 9*A*). The magnitude of Ca^{2+} responses to GSK101 was significantly increased in SGCs treated with 2-MeSADP compared with vehicle (Fig. 9*B*). These results indicate that $P2Y_1$ stimulation can sensitize TRPV4, resulting in an increase in influx of extracellular Ca^{2+}.

PKC Mediates $P2Y_1$ Receptor-evoked Activation of TRPV4 in SGCs—We have previously examined receptor-operated activation of TRPV4 in HEK293 cells with stable TRPV4 expression under the control of a tetracycline-inducible promoter (25). These cells endogenously express purinergic receptors, including $P2Y_1$ receptors (50), and are thus a convenient system to study $P2Y_1$ receptor-mediated activation of TRPV4. In HEK293 cells, 2-MeSADP stimulated a concentration-dependent increase in $[Ca^{2+}]_i$ (Fig. 10*A*). The $P2Y_1$ receptor antagonist MRS2500 (1 μM) abolished 2-MeSADP-evoked Ca^{2+} responses (Fig. 10*B*). This confirms that $P2Y_1$ receptor-selective Ca^{2+} signaling can be measured, and the contribution of TRPV4 to $P2Y_1$ receptor responses can be further examined. 2-MeSADP-evoked Ca^{2+} responses were compared in untransfected HEK293 cells (control) and HEK-TRPV4 cells. The maximal and the integrated (area under curve) responses to 2-MeSADP (31.6 nM) were significantly larger in HEK-TRPV4 cells compared with untransfected HEK293 cells (Fig. 10, *C*, *E*, and *F*). Pretreatment of HEK-TRPV4 cells with the TRPV4-selective antagonist HC067047 (10 μM) reduced basal $[Ca^{2+}]_i$ and the maximal Ca^{2+} response and abolished the sustained phase of $P2Y_1$ response to $[Ca^{2+}]_i$ levels lower than HEK293 control, thus indicating that TRPV4 enhances $P2Y_1$-mediated Ca^{2+}-dependent processes (Fig. 10, *C*, *E*, and *F*).

TABLE 1

Proportion of SGCs responding to $P2Y_1$ and TRPV4 agonists ($n = 377$ cells from 6 experiments)

	$TRPV4^+$	$TRPV4^-$	Total
	%	%	%
$P2Y1^+$	17.2 ± 5.5	36.9 ± 5.43	55 ± 8.5
$P2Y1^-$	8.3 ± 2.1	36 ± 7.1	44.3 ± 8.4
Total	25 ± 5.1	73 ± 5.6	

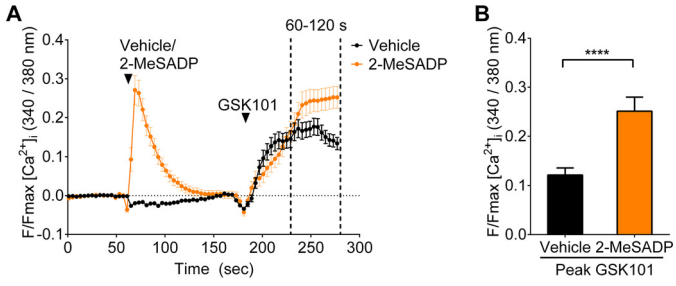

FIGURE 9. **$P2Y_1$-dependent sensitization of TRPV4 in SGCs.** *A*, time course of 2-MeSADP or vehicle stimulation followed by GSK101 Ca^{2+} responses in SGCs from $Trpv4^{+/+}$ mice. *B*, peak GSK101-mediated Ca^{2+} responses from SGCs pretreated with 2-MeSADP relative to vehicle. $n = 117$ cells from 3 experiments. **, $p < 0.01$; ***, $p < 0.001$ relative to vehicle; Student's *t* test. *Error bars*, S.E.

The phosphorylation status of TRP channels strongly influences gating. GPCRs have been reported to regulate TRP channels by modulating protein kinase activity (29, 49, 51–53). To determine whether PKC mediates $P2Y_1$ sensitization or activation of TRPV4, we preincubated HEK-TRPV4 cells with

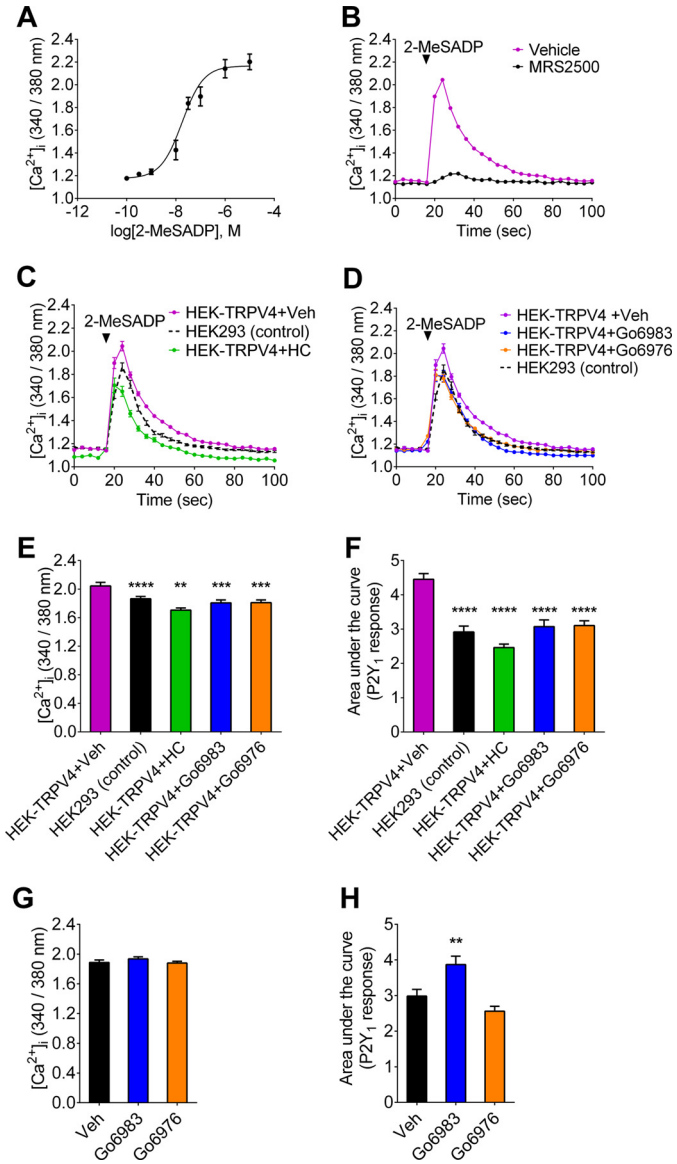

FIGURE 10. **$P2Y_1$-dependent activation of TRPV4 in HEK-TRPV4 cells.** *A*, concentration response curve for 2-MeSADP-induced $[Ca^{2+}]_i$ in HEK293 cells. *B*, time course of 2-MeSADP (31.6 nM) Ca^{2+} response in the presence of vehicle or MRS2500 (1 μM). Shown are time course (*C* and *D*), peak Ca^{2+} responses (*E*), and area under the curve (*F*) for 2-MeSADP (31.6 nM) Ca^{2+} responses in HEK-TRPV4 cells in the presence of vehicle, TRPV4 antagonist HC067047 (10 μM), or the PKC inhibitors Go6983 (1 μM) and Go6976 (1 μM) and in HEK293 (control) cells. Shown are peak Ca^{2+} responses (*G*) and area under the curve (*H*) for 2-MeSADP (31.6 nM) Ca^{2+} responses in HEK293 control cells in the presence of vehicle or the PKC inhibitors Go6983 (1 μM) and Go6976 (1 μM) to determine the effect of PKC inhibitors in the absence of TRPV4. $n = 4$ experiments. **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ relative to vehicle; one-way analysis of variance with Dunnett's post hoc test. *Error bars*, S.E.

Go6983 (1 μM; a broad spectrum PKC inhibitor (32)), Go6976 (1 μM; a selective inhibitor of PKCα and PKCβ1 isoforms (31)), or vehicle (control). Both inhibitors suppressed the maximal and integrated Ca^{2+} responses to 2-MeSADP (Fig. 10, *E* and *F*). In contrast, PKC inhibitors did not suppress Ca^{2+} responses to 2-MeSADP in HEK293 control cells when compared with vehicle-treated cells (Fig. 10, *G* and *H*). This result is consistent with previous reports of PKC-mediated inhibition of $P2Y_1$ desensitization (54). These results suggest that PKCα and PKCβ1 isoforms diminish $P2Y_1$ receptor-mediated activation of TRPV4 in HEK293 cells.

To examine whether PKCα and β1 contribute to the $P2Y_1$ receptor-mediated activation of TRPV4 in SGCs, we preincubated SGCs with Go6976 (1 μM) and challenged them with 2-MeSADP (10 μM) followed by GSK101 (1 μM). Go6976 pretreatment induced a modest increase in maximal Ca^{2+} response upon 2-MeSADP stimulation, indicating the potential for PKC-mediated inhibition of $P2Y_1$ desensitization. In contrast, at 60 s after 2-MeSADP addition, the magnitude of Ca^{2+} response was diminished relative to that of vehicle pretreatment (Fig. 11, *A* and *B*). The maximal Ca^{2+} response to a subsequent stimulation with GSK101 was also reduced (Fig. 11*C*). Together, these results suggest that PKCα or PKCβ1 isoforms contribute to $P2Y_1$ receptor-mediated activation or sensitization of TRPV4 in SGCs.

Discussion

We report the discovery that TRPV4 and $P2Y_1$ receptor are co-expressed in a subset of SGCs of the mouse DRG, and these proteins functionally interact. We detected immunoreactive TRPV4 in a subset of SGCs in intact sensory ganglia and observed that a TRPV4-selective agonist activated TRPV4 in SGCs in short term culture. The agonist response was prevented by TRPV4 antagonism and genetic deletion and is thus mediated by this channel. By comparing $P2Y_1$ receptor signals in SGCs from wild-type and *Trpv4*$^{-/-}$ mice and in untransfected or TRPV4-expressing HEK293 cells, we obtained evidence that the $P2Y_1$ receptor functionally couples to TRPV4. This functional coupling was prevented by inhibition of PKC, in particular PKCα and PKCβ1 isoforms. To our knowledge, this is the first report of TRPV4 expression and of $P2Y_1$ receptor-TRPV4 coupling in SGCs of sensory ganglia. SGCs ensheath neurons within sensory ganglia and can regulate neuronal hyperexcitability and pain transmission. We propose that the functional coupling of $P2Y_1$ receptor and TRPV4 in SGCs may contribute to the regulation of neuronal excitability during inflammatory and neuropathic pain.

TRPV4 Is Expressed by SGCs of Sensory Ganglia—Peripheral sensory ganglia are composed of primary afferent neurons, SGCs, fibroblasts, immune cells, and vasculature. The expression and function of numerous TRP channels have been reported in sensory neurons, yet the functional importance of nociceptive TRP channels in glial cells that regulate sensory neuronal activity has not been characterized. To examine functional expression of TRP channels in SGCs, we utilized a mild enzymatic digestion DRG dissociation protocol to maintain intercellular associations between neurons and SGCs (37). The intimate relationship between SGCs and neurons was confirmed by immunostaining for Kir4.1-IR, glutamine synthetase-IR, and NeuN-IR, which demonstrated that SGCs ensheath neurons in whole ganglia and dispersed ganglia cultures. In these neuron-glia co-cultures, we were able to selectively record distinct Ca^{2+} signals in SGCs and neurons. We discovered that the TRPV4 agonist GSK101 increased $[Ca^{2+}]_i$ in ~25% of the neuron-associated SGC population. This response was abolished by a TRPV4-selective antagonist and was not observed in SGCs from *Trpv4*$^{-/-}$ mice and is thus attributable to TRPV4 activity. In contrast, SGCs did not express functional TRPV1, TRPV3, or TRPA1. We detected TRPV4-IR in a subset of SGCs in neuron-glia co-cultures and in fixed whole DRG sections. Furthermore, TRPV4-IR co-distributed with a sub-

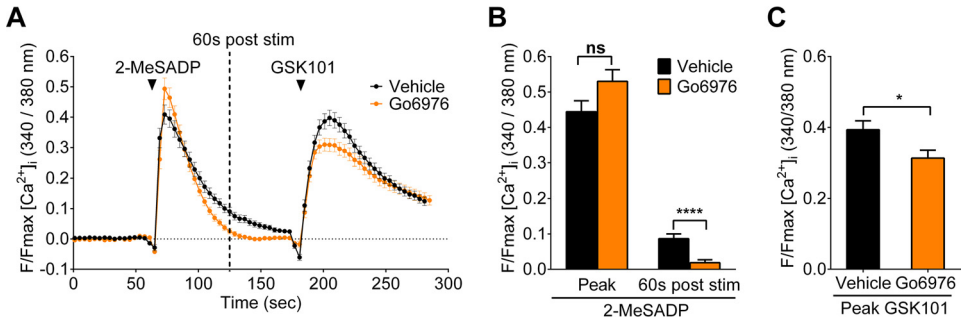

FIGURE 11. **$P2Y_1$-dependent activation of TRPV4 in SGCs.** *A*, time course of 2-MeSADP (10 μM)-induced and GSK101 (1 μM)-induced Ca^{2+} response in GSK101-positive SGCs pretreated with vehicle or PKC inhibitor Go6976 (1 μM). *B*, peak Ca^{2+} responses and $[Ca^{2+}]_i$ measured at 60 s after the addition of 2-MeSADP. *C*, peak GSK101-mediated Ca^{2+} responses from SGCs pretreated with Go6976 relative to vehicle (vehicle, $n = 80$ GSK-positive cells (from a total of 365 SGCs); Go6976-pretreated, $n = 109$ GSK-positive cells (from a total of 347 SGCs)). $n = 4$ experiments. *, $p < 0.05$ relative to vehicle; Student's *t* test. *ns*, not significant. *Error bars*, S.E.

population of glutamine synthetase-IR SGCs. TRPV4-positive staining was also observed in a subpopulation of primary afferents, as reported previously (29, 40, 55, 56). Consistent with these results, studies on glial cells of the central nervous system have demonstrated that TRPV4 is expressed by a subpopulation of astrocytes and microglia, suggesting that TRPV4 regulates Ca^{2+}-dependent processes in glia throughout central and peripheral nervous systems (33, 57, 58).

In the periphery, TRPV4 is expressed on Aδ- and C-fibers, colonic epithelium, visceral afferents, and keratinocytes, where activation induces neurogenic inflammation, contributes to thermal and mechanical hyperalgesia, and mediates skin barrier formation (24, 28, 56, 59–61). TRPV4 is sensitive to cell swelling, mechanical stress, and warm temperatures (>27 °C) and can be stimulated by arachidonic acid and its metabolites, such as eicosanoic acids generated by inflammatory processes or receptor-mediated signaling (40, 59, 62–65). Purinergic signaling is a well established mediator of neuron-glia interactions and is important for the initiation and development of chronic pain (66). This study explored purinergic receptor-operated TRPV4 channel activity in SGCs to determine how neuron-dependent activation of TRPV4 may occur.

SGCs Express Functional $P2Y_1$ Receptors—Metabotropic purinergic receptors perform multiple roles in glial cells to regulate nociceptive processes. $P2Y_1$, $P2Y_{12}$, and ionotropic P2X4 receptors mediate ATP-dependent chemotaxis of microglia (67, 68). ATP stimulates $P2Y_1$ receptor-dependent glutamate release from astrocytes, thereby increasing neuronal excitability (69). Activated microglia can also release ATP, triggering astrocyte-mediated modulation of excitatory neurotransmission and neuroprotective effects via $P2Y_1$ (70, 71). Within sensory ganglia, $P2X_7$, $P2Y_1$, $P2Y_2$, and $P2Y_{12}$ receptors have been identified in SGCs (19, 26, 27). Furthermore, P2Y receptor signaling in SGCs of rat trigeminal ganglia can be potentiated by nociceptive or pro-algogenic mediators from neurons, including calcitonin gene-related peptide and prostaglandin E_2 (20). Antagonism of $P2Y_2$ but not $P2Y_1$ receptors inhibits facial allodynia and SGC activation in an inflammatory model of pain (27). Thus, activation of purinoceptors on SGCs can modulate neuronal sensory transmission, yet the mechanisms underlying SGC purinergic signaling are not well characterized.

We observed that ATP and UTP, which can activate ionotropic and metabotropic purinergic receptors, increased $[Ca^{2+}]_i$ in most SGCs. Due to the complexity of ATP and UTP signaling and previous evidence of GPCR-operated TRPV4 activity, $G\alpha_q$-coupled purinergic receptor signaling was further assessed using 2-MeSADP, a selective agonist for $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptors (45). MeSADP increased $[Ca^{2+}]_i$ in ~55% of SGCs. This response was markedly inhibited by the $P2Y_1$ receptor-selective antagonist MRS2500, supporting functional $P2Y_1$ expression in these cells. To our knowledge, the $P2Y_1$ receptor has not previously been identified in SGCs from mouse DRGs.

TRPV4 Couples to $P2Y_1$ Receptors in SGCs—TRP channels are major downstream targets of many GPCRs that mediate pain, itch, and neurogenic inflammation (3). We report that the $P2Y_1$ receptor couples to TRPV4 in SGCs and in HEK-TRPV4 cells. We observed that a $P2Y_1$ receptor, 2-MeSADP, increased $[Ca^{2+}]_i$ in SGCs from wild type mice and that both the magnitude and integrated response were significantly attenuated by TRPV4 antagonism or deletion. Similarly, the amplitude of $P2Y_1$ receptor Ca^{2+} response of HEK-TRPV4 cells was elevated when compared with untransfected HEK293 cells or attenuated in the presence of a TRPV4 antagonist. These results suggest that TRPV4 functions as a receptor-operated channel in SGCs and HEK293 cells, whereby activation of the $P2Y_1$ receptor leads to *trans*-activation of TRPV4 and the elevation of Ca^{2+} dependent processes. We also observed that prestimulation of SGCs with the $P2Y_1$ receptor agonist 2-MeSADP amplified the response to the TRPV4 agonist GSK101, indicating $P2Y_1$-mediated TRPV4 sensitization. Thus, TRPV4 is a downstream target of purinergic GPCR signaling in sensory pathways. Other GPCRs that sensitize TRPV4 include the bradykinin B_2, protease-activated receptor 2, 5-hydroxytryptamine 3, histamine H_1 receptor, and muscarinic M1 receptors (25, 29, 39, 72, 73).

Second messenger kinases can phosphorylate TRP channels and alter channel gating and are thus important mediators of GPCR-TRP coupling. We observed that Go6983 (broad spectrum PKC inhibitor) and Go6976 (selective inhibitor of PKCα and PKCβ1 isoforms) (31, 32) diminished $P2Y_1$ receptor-mediated activation of TRPV4 Ca^{2+} flux in HEK-TRPV4 cells. Inhibition of PKCα and PKCβ1 also prevented $P2Y_1$ receptor-mediated sustained Ca^{2+} flux and sensitization of TRPV4 in GSK101-responsive SGCs. PKC performs essential roles in $P2Y_1$ signaling and receptor trafficking, where inhibition of PKCα can attenuate $P2Y_1$ desensitization to promote an increase in agonist-induced $P2Y_1$ signaling (54, 74). This may

explain the elevated $[Ca^{2+}]_i$ peak in Go6976-pretreated SGCs and HEK293 control cells. Our results suggest that PKC is an integrator of the $P2Y_1$ receptor-TRPV4 coupling pathway. In sensory or inflammatory pathways, PKC is similarly known to function as a second messenger for other GPCR-TRPV4 coupling pathways, including those stimulated by protease-activated receptor 2, bradykinin B_2, 5-hydroxytryptamine 3, and muscarinic acetylcholine receptors (25, 29, 39, 52, 72, 73). Other mechanisms of GPCR-TRPV4 coupling include PKA-dependent channel phosphorylation (29) and the generation of endogenous lipid mediators such as anandamide and arachidonic acid metabolites, 5′,6′- and 8′,9′-epoxyeicosatrienoic acid (64, 75). Whether these mechanisms also regulate TRPV4 in SGCs remains to be studied.

In contrast to the observed TRPV4-mediated responses to 2-MeSADP, responses to ATP and UTP were more sustained in GSK101-negative cells. To our knowledge, no evidence of TRPV4-dependent down-regulation of purinergic signaling has been reported in the literature. Although this may involve complex metabotropic-ionotropic signaling events, the elevated Ca^{2+} response is sustained and appears to be mediated by ion channels, indicating functional interplay between TRPV4 and ionotropic purine receptors on SGCs. Further studies are required to investigate and confirm TRPV4-mediated down-regulation of purinergic responses.

Contributions of TRPV4 and $P2Y_1$ Receptors of SGCs to Nociception—Our finding that the pro-nociceptive and pro-inflammatory TRPV4 is expressed by SGCs of sensory ganglia and functions as an effector of purinergic signaling has implications for the mechanism and treatment of pain. Hyperalgesic priming is a preclinical model of chronic pain whereby a prior acute inflammatory or neuropathic stimuli can induce neuroplastic changes in nociceptors that contribute to the transition from acute to chronic pain (76, 77). Proinflammatory mediators that cause priming in peripheral terminals fail to induce priming when directly administered to sensory ganglia, suggesting the existence of distinct mechanisms in the neuronal soma and its peripheral terminals that underlie the transition to chronic pain (78). It is possible that SGCs contribute to the mechanisms of sustained pain within sensory ganglia. Thus, TRPV4 may become activated in SGCs of sensory ganglia as a result of neuronal activity and consequent activation of $P2Y_1$ receptors or other GPCRs. This mechanism may, in turn, regulate neuronal hyperexcitability. Further experiments are required to examine this possibility and to determine the contribution of SGCs to conditions of TRPV4-mediated chronic pain or inflammation (79, 80).

Author Contributions—P. R. designed and performed all experiments and wrote the paper. D. P. P., N. A. V., and N. W. B. conceived, designed, and coordinated the study, provided technical assistance, and contributed to the preparation of the manuscript and figures. W. L. provided $Trpv4^{-/-}$ mice for the study. All authors analyzed the results and approved the final version of the manuscript.

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