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RESEARCH ARTICLE

Inhibition of TRPM8 function by prostacyclin receptor agonists requires coupling to Gq/11 proteins

Cosmin Trif[1] | Alexandra-Maria Banica[1] | Alexandra Manolache[2] | Sorina Andreea Anghel[1] | Debora-Elena Huţanu[2] | Teodora Stratulat[1,2] | Rodica Badea[1] | George Oprita[2] | Tudor Selescu[2] | Stefana M. Petrescu[1] | Marco Sisignano[3] | Stefan Offermanns[4] | Alexandru Babes[2] | Sorin Tunaru[1,5]

[1]Cell Signalling Research Group, Institute of Biochemistry of the Romanian Academy, Bucharest, Romania

[2]Department of Anatomy, Physiology, and Biophysics, Faculty of Biology, University of Bucharest, Bucharest, Romania

[3]Institute of Clinical Pharmacology, Pharmazentrum Frankfurt/ZAFES, University Hospital, Goethe-University, Frankfurt am Main, Germany

[4]Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany

[5]Prothanor Biotech S.R.L., Bucharest, Romania

Correspondence
Alexandru Babes, Department of Anatomy, Physiology, and Biophysics, Faculty of Biology, University of Bucharest, Splaiul Independenței 91-95, 050095 Bucharest, Romania.
Email: alexandru.babes@bio.unibuc.ro

Sorin Tunaru, Cell Signalling Research Group, Institute of Biochemistry of the Romanian Academy, Splaiul Independentei 296, 060031 Bucharest, Romania.
Email: sorin.tunaru@gmail.com

Abstract

Background and Purpose: The TRPM8 ion channel is involved in innocuous cold sensing and has a potent anti-inflammatory action. Its activation by lower temperature or chemical agonists such as menthol and icilin induces analgesic effects, reversing hypersensitivity and reducing chronic pain. On the other hand, prostacyclin (PGI_2) enhances pain and inflammation by activating the IP receptors. Due to the critical roles of TRPM8 and IP receptors in the regulation of inflammatory pain, and considering their overlapping expression pattern, we analysed the functional interaction between human TRPM8 and IP receptors.

Experimental Approach: We transiently expressed human TRPM8 channels and IP receptors in HEK293T cells and carried out intracellular calcium and cAMP measurements. Additionally, we cultured neurons from the dorsal root ganglia (DRGs) of mice and determined the increase in intracellular calcium triggered by the TRPM8 agonist, icilin, in the presence of the IP receptor agonist cicaprost, the IP receptor antagonist Cay10441, and the Gq/11 inhibitor YM254890.

Key Results: Activation of IP receptors by selective agonists (cicaprost, beraprost, and iloprost) inhibited TRPM8 channel function, independently of the Gs-cAMP pathway. The potent inhibition of TRPM8 channels by IP receptor agonists involved Gq/11 coupling. These effects were also observed in neurons isolated from murine DRGs.

Conclusions and Implications: Our results demonstrate an unusual signalling pathway of IP receptors by coupling to Gq/11 proteins to inhibit TRPM8 channel function. This pathway may contribute to a better understanding of the role of TRPM8 channels and IP receptors in regulating pain and inflammation.

KEYWORDS
inflammatory pain, prostacyclin, prostacyclin receptor, TRPM8

Abbreviations: FBS, fetal bovine serum; HBSS, Hank's buffered salt solution; PKA, protein kinase A; PBS, phosphate buffered saline; PL(A_2,C,D), phospholipase (A_2,C,D).

Cosmin Trif and Alexandra-Maria Banica should be considered joint first author.

Alexandru Babes and Sorin Tunaru should be considered joint senior author.

1 | INTRODUCTION

Pain perception and heat production are hallmarks of the inflammatory response that require the expression and modulation of an intricate and elaborate network of chemical mediators, membrane proteins, and cellular signalling pathways (Ronchetti et al., 2017). Among the most important membrane proteins involved in the regulation of inflammation and pain perception are ion channels and GPCRs (Che, 2021; Veldhuis et al., 2015).

The **transient receptor potential melastatin subtype 8 (TRPM8)** is an ion channel activated by a mild temperature decrease, as well as by supercooling agents such as **icilin** and **menthol** (Bautista et al., 2007; Brauchi et al., 2004). Its activation in unmyelinated type-C afferent fibres by low temperatures and supercooling agents induces transient increases in the cytosolic calcium and sodium ions, leading to depolarization and initiation of action potentials (McCoy et al., 2011). Furthermore, TRPM8 is a negative regulator of inflammation and neuropathic pain, mediating analgesic effects (B. Liu et al., 2013; Proudfoot et al., 2006; Ramachandran et al., 2013). TRPM8 is also expressed in macrophages and pulmonary epithelial cells. In macrophages, the activation of TRPM8 channels induces anti-inflammatory effects, by increasing **IL-10** and decreasing the expression of **TNF-α** (Khalil et al., 2016; Silverman et al., 2020), whereas its activation in pulmonary epithelial cells induces the expression of pro-inflammatory **IL-1** (Sabnis et al., 2008). Moreover, in hypothalamic neurons, TRPM8 activation induced by cold stress leads to TRPM8 overexpression, reduced nuclear localization of the transcription factor NF-kB, and a decrease in transcription of NF-kB-dependent genes, leading to a down-regulation of TNF expression and a diminished inflammatory response (Wang et al., 2017).

The prostacyclin (PGI_2) receptor (IP receptor) belongs to the family of prostanoid receptors within the large superfamily of GPCRs. Extensive research has demonstrated that PGI_2 and its synthetic mimetics, acting through this cognate IP receptor, play critical roles in vascular homeostasis, inhibiting platelet aggregation and promoting arterial smooth muscle relaxation (Moncada & Vane, 1979; Ricciotti & FitzGerald, 2011). These effects are mediated by the interaction of IP receptors with intracellular heterotrimeric Gs proteins, triggering a positive effect on **adenylyl cyclase**, with subsequent increases in intracellular **cAMP** and **PKA**-dependent activation of several intracellular signalling pathways (Woodward et al., 2011). Studies performed on mice genetically modified to lack the *Ptgir* gene that encodes the IP receptor also demonstrated its involvement in pain and inflammation, as inflammatory and pain responses were significantly reduced in IP receptor-deficient mice (Murata et al., 1997).

Apart from temperature or exogenous chemical agonists, TRPM8 channels are modulated by several other factors such as lysophospholipids and polyunsaturated fatty acids, (Andersson et al., 2007). **Phosphatidylinositol 4,5-bisphosphate (PIP2)** is an important intracellular modulator of TRPM8 channels (Rohacs, 2014), as PIP2 depletion shifted its voltage dependence towards more positive potentials, thus reducing cold- and menthol-induced currents (Daniels et al., 2009). The modulation of TRPM8 channels by GPCRs remains a matter of continuing exploration, as it appears to be dependent on the interacting heterotrimeric G proteins of the receptor. On this note, TRPM8 channels are was shown to be inhibited following the activation of **α_{2A}-adrenoceptors**, in a Gi-dependent fashion, involving a decrease in PKA-dependent phosphorylation of the channel (Bavencoffe et al., 2010), while another study found no influence of the PKA pathway on TRPM8 activity in dorsal root ganglion (DRG) neurons (Sarria & Gu, 2010). In contrast, earlier studies had found that the intracellular cAMP elevation inhibits TRPM8 channels in a PKA-dependent manner (De Petrocellis et al., 2007; Linte et al., 2007). Taken together, these contradictory results point to a complex regulation of TRPM8 channel function by G proteins and GPCRs, possibly depending on the cellular context the experiments were performed in, and indicate that further studies are needed to draw a clear picture of TRPM8 modulation by GPCRs. To add a new layer of complexity to the regulation of TRPM8 channels by GPCRs, other studies showed that Gq-coupled GPCRs inhibited these ion channels, an effect mediated by the direct binding of the Gαq subunit to TRPM8, thus challenging the idea of the mechanism of inhibition through PIP2 depletion as a result of **PLCβ** activation by Gq-coupled GPCRs (B. Liu & Qin, 2005; X. Zhang, 2019; X. Zhang et al., 2012).

The aim of our present study was to determine whether there is a functional regulation of TRPM8 channel activity by IP receptors, given the opposing effects they have on pain and inflammatory responses. By employing a combination of cellular pharmacology experiments carried out on recombinant TRPM8 expressed in heterologous expression systems and on TRPM8 channels natively expressed in neurons isolated from mouse DRGs, we demonstrated that the activation of IP receptors by synthetic agonists inhibited icilin and cooling-induced

What is already known

- IP receptors and TRPM8 channels exert opposing effects on pain and inflammation.
- IP receptors and TRPM8 channels are co-expressed in cell types relevant to inflammatory pain.

What does this study add

- IP receptor agonists inhibited TRPM8 channels in HEK293T cells and neurons isolated from mouse DRGs.
- Inhibition of TRPM8 channels by IP receptor agonists involved an unusual G protein coupling.

What is the clinical significance

- IP receptors are targets of FDA-approved drugs that can also affect TRPM8 channel activity.

activation of TRPM8 channels in a Gq/11-dependent manner. Moreover, the typical signalling pathway of IP receptors, involving Gs proteins, activation of adenylyl cyclase and cAMP increase did not play a role in the modulation of TRPM8 activity following the activation of IP receptors. The functional interaction between TRPM8 channels and IP receptors was also shown in mouse DRG neurons, as the pre-activation of IP receptors with **cicaprost** resulted in a significant reduction of icilin-induced calcium transients, an effect that was reversed by the IP receptor antagonist, **Cay10441**, and the Gq/11-protein inhibitor **YM254890**. Our data support the idea that the Gs-cAMP-PKA pathway does not play any role in the modulation of TRPM8 activity and strongly indicates that Gq proteins have an inhibitory effect on TRPM8 channels. Moreover, our results point to a novel role of IP receptors in the modulation of an important regulator of pain and inflammation by an unusual signalling pathway involving Gq/11 proteins.

2 | METHODS

2.1 | Cell cultures and determination of intracellular cAMP levels

HEK293T cells were from ATCC (Cat. # CRL-3216 RRID:CVCL_0063; LGC Standards Sp. KielpinLomianki, Poland), grown in DMEM containing 10% FBS, were seeded in clear bottom, white-walled 96-well plates. The next day, cells were transfected with plasmids containing cDNA for the indicated membrane proteins and the cytosolic cAMP-sensitive bioluminescent p22F probe (Cat. # E2301, Promega, Madison, WI, USA) by using Lipofectamine 2000 reagent (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations. After 48 h, cells were removed from the incubator and the growth medium was replaced with HBSS containing 2% (v/v) endotoxin-free, luciferin (Cat. # E1290, Promega, Madison, WI, USA), 2-mM $CaCl_2$, and 10 mM glucose. Following 2 h of incubation in the dark, at room temperature, intracellular cAMP was determined by recording the light generated after stimulation of cells with indicated ligands for 10 min at room temperature. The light was recorded by using a 96-well plate reader (Mithras LB940, Berthold, Bad Wildbad, Baden-Württember, Germany) with an integration time of 500 ms and expressed as relative luminescence units (RLU).

2.2 | Determination of cAMP response element (CRE) protein activity

HEK293T cells, grown in DMEM containing 10% FBS, were seeded on clear bottom, white-walled 96-well plates. The next day, cells were transfected with plasmids containing cDNA for the indicated membrane proteins and the cytosolic pGL4.29[luc2P/CRE/Hygro] probe (Promega, Madison, WI, USA) by using Lipofectamine 2000 reagent (ThermoFisher Scientific, Waltham, MA, USA) following the manufacturer's recommendations. After 48 h, cells were removed from the incubator and the growth medium was replaced with DMEM without FBS containing indicated concentrations of ligands. Following 5 h of incubation in a 5% CO_2 incubator at 37°C, media with ligands was aspirated and the cells were lysed using Universal lysis buffer (NanoLight Technology, Pinetop, AZ, USA) and then a solution of D-luciferin (NanoLight Technology, Pinetop, AZ, USA) was added in each well. CRE activity was determined by measuring the light generated using a 96-well plate reader (Mithras LB940, Berthold, Bad Wildbad, Baden-Württember, Germany) with an integration time of 500 ms and expressed as relative luminescence units (RLU).

2.3 | Intracellular calcium determination

HEK293T cells, seeded on clear bottom white-walled 96-well plates, were transfected with plasmids encoding the indicated membrane proteins and the cytosolic calcium-sensitive fusion protein between aequorin and GFP (termed G5A) (Baubet et al., 2000) by using Lipofectamine 2000 reagent (ThermoFischer Scientific, Waltham, MA, USA) following manufacturer's recommendations. After 48 h, the growth medium was replaced with HBSS containing 5 μM coelenterazine h (Invitrogen, Waltham MA, USA), 2 mM $CaCl_2$ and 10 mM glucose. Following 2 h of incubation at 37°C, in the absence of CO_2, intracellular calcium was determined by continuously recording the light generated for 100 seconds after stimulation with the indicated ligands. Measurements were performed by using a luminometric plate reader (FlexStation 3, Molecular Devices, San Jose, CA, USA), and the area under each calcium transient was calculated and expressed as AUC by using SoftMaxPro 6 software.

2.4 | Heterologous expression of human TRPM8 channels in HEK293T cells

HEK293T cells were transiently co-transfected with either hTRPM8 or an hTRPM8 triple mutant R364Q, R368Q, R470Q and prostacyclin receptor IP (DNA ratio 1:10) using the jetPEI transfection reagent (Polyplus transfection, Illkirch, France). The pcDNA3.1 vector containing human TRPM8 was generously provided by Dr. Gordon Reid, University College Cork, while the triple TRPM8 mutant was a kind gift from Dr. Xuming Zhang, University of Warwick, UK. After transfection, cells were plated onto 24 mm borosilicate glass coverslips (0.17 mm thick), which had been treated with poly-D-lysine (Sigma-Aldrich, 0.1 $mg{\cdot}ml^{-1}$ for 30 min). All experiments were performed within 48 h from the transfection.

2.5 | Immunoprecipitation and western blot analysis

The immunoblot procedures complied with the recommendations made by the British Journal of Pharmacology (Alexander et al., 2018). To assess the effects of Gq/11 protein on expression of human

TRPM8, HEK293T cells grown in 6-well plates were transiently transfected with pcDNA3.1-GQ209L plasmid encoding for the constitutive active mutant form of Gq/11 (kindly provided by Dr. Xuming Zhang) together with pcDNA3.1-c-Myc-TRPM8 plasmid, encoding for N-terminally c-Myc-tagged TRPM8 (generated in-house by subcloning untagged human TRPM8 in an N-terminally c-Myc tagged pcDNA 3.1 vector), at plasmid DNAs ratio 1:1, using PEI reagent (Sigma-Aldrich, St. Louis, USA). Forty-eight hours after transfection, cells were lysed in a lysis buffer containing 50 mM Tris-HCl, 150 mM NaCl, 10 mM $MgCl_2$, 1% NP-40, pH 7.4, supplemented with 0.25% N-dodecyl-beta-maltoside detergent (D310S, Anatrace, Maumee, USA), protease inhibitors cocktail (ThermoFischer Scientific, Waltham, USA), 1 mM PMSF (AppliChem, GmbH, Germany), 1 mM ortho-vanadate, 5 mM iodoacetic acid, 5 mM NaF (HGNT lysis buffer) for 45 min at 4°C. After that, the lysates were clarified by a centrifugation step at 25.000 g, 4°C for 45 min. The protein concentration of each sample was quantified by using BCA protein assay kit (ThermoFischer Scientific, Waltham, USA). Samples were diluted in 5× SDS sample buffer and incubated for 10 min at 70°C. Equal amounts of the indicated samples were separated by SDS-PAGE electrophoresis and transferred to PVDF membranes (Merck-Millipore, Burlington, USA) followed by blocking with 5% milk in TBST (0.1% Tween-20) for 1 h at room temperature. Next, membranes were incubated over-night at 4°C, with rotation, with antibodies anti-c-Myc (9E10)-HRP (Cat. # sc-40, Santa Cruz Biotechnology, Dallas, USA, RRID:AB_627268, mouse monoclonal IgG1 raised against amino acids 408–439 of human c-Myc), anti-Gα q/11/14 Antibody (G-7) (Cat. # sc-365906, Santa Cruz Biotechnology, Dallas, USA, RRID:AB_10842057, mouse monoclonal IgG1 raised against amino acids 60–359 of mouse and human Gα11) and anti-GAPDH-HRP (FL-335) (Cat. # sc-25778, Santa Cruz Biotechnology, Dallas, USA, RRID:AB_10167668, rabbit polyclonal IgG raised against amino acids 1–335 of human GAPDH). The immunoblotting signals were detected with Immobilon Crescendo Western HRP Substrate (Merck-Millipore, Burlington, USA). Specific bands were visualized using the ChemiDoc MP Imaging System (Bio-Rad Laboratories Inc., Hercules, USA) and processed with ImageLab 4.1 software (Bio-Rad Laboratories Inc., Hercules, USA). To study the interaction between c-Myc-tagged human TRPM8 and human N-terminally tagged HA prostacyclin receptor (HA-IP-R), HEK293T cells grown in 75 cm^2 flasks were transiently transfected with pcDNA3.1-c-Myc-TRPM8 and pcDNA3.1-HA-IP-R plasmids, at plasmid ratio 1:1, using PEI reagent. After 48 h, cells were washed twice with ice-cold PBS and stored at −80°C until processing. Cells were lysed in HGNT-lysis buffer as described above. For immunoprecipitation, 500 µg total protein of each sample was incubated both with 1 µg anti-HA.11 Epitope Tag Antibody (Cat. # 901523, BioLegend, San Diego, USA, RRID:AB_2734657, mouse monoclonal antibody, IgG1k isotype) and with 1 µg of anti-c-Myc overnight at 4°C, with rotation. On the second day, 10 µl of protein G-Sepharose 4 FastFlow (Cytiva, Marlborough, USA) was added to the samples, and incubated for 3 h at 4°C with rotation. After washing three times with ice-cold HGNT-lysis buffer, proteins bound to the beads were eluted in 2×SDS sample buffer and incubated at 75°C for 10 min. Equal amounts of indicated samples of lysate and immunoprecipitates were separated by SDS-PAGE and transferred to PVDF membranes as described above and immunoblotted with antibodies anti-HA.11 Epitope Tag Antibody and anti-c-Myc. For loading control of cell lysates, an anti-GAPDH-HRP (FL-335) antibody was used. Visualization and processing of the immunoblotting signals were performed as described above.

2.6 | Cultures of neurons from mouse DRGs

All animal care and experimental procedures were approved by the institutional ethics committee of the Faculty of Biology, University of Bucharest (Decision no. 105/23.06.2016 of the Committee for Ethics in Scientific Research of the University of Bucharest). Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020).

Mice were provided by the animal facility of the "Cantacuzino" National Institute for Medical-Military Research and Development (INCDMM "Cantacuzino", Bucharest, Romania). Mice were kept in a conventional (non-SPF) facility and were housed separated by sexes, up to four in a standard individually ventilated cage, kept on a 12 h light-dark cycle with ad libitum access to food and water. The bedding material consisted of large wood shavings. The temperature was maintained in the range of 21 to 22 °C, and the relative humidity between 45% and 55%. In the animal facility of the user (University of Bucharest), animals were kept for up to 24 h, until the end of the experiment and were provided with the same conditions as in the husbandry facility, except for the individually ventilated cages.

DRG neurons were obtained from adult male C57Bl6 mice, (humanely killed by inhalation of CO_2 followed by decapitation), as previously described (Babes et al., 2010). DRGs from all spinal level were dissected out and incubated at 37°C for 60 min, in the IncMix solution (NaCl, 155 mM; K_2HPO_4, 1.5 mM; HEPES, 5.6 mM; HEPES, 4.8 mM; glucose, 5 mM; and gentamicin 50 $mg{\cdot}ml^{-1}$) containing 3 $mg{\cdot}ml^{-1}$ dispase (Gibco, Grand Island, NY) and 1.5 $mg{\cdot}ml^{-1}$ collagenase (type XI, Sigma-Aldrich, St. Louis, MO, USA).

Following incubation, individual neurons were obtained by triturating DRGs using a fire-polished glass Pasteur pipette coated with silicone. The cells were then plated onto glass coverslips (24 mm diameter; 0.17 mm thickness), which had been treated for 30 min with poly-D-lysine (0.1 $mg{\cdot}ml^{-1}$). The coverslips were placed in a 5% CO_2 incubator at 37°C and kept in a 1:1 mixture of DMEM and Ham's F12 medium (Sigma-Aldrich, St. Louis, MO, USA), containing 50 $\mu g{\cdot}ml^{-1}$ gentamicin, 7.4 mM glucose, and 10% horse serum. The neurons were used for calcium microfluorimetry recordings between 12 and 24 h after plating.

2.7 | Intracellular non-ratiometric calcium microfluorimetry on mouse DRG neurons and HEK293T cells

For intracellular non-ratiometric calcium microfluorimetry, a standard extracellular solution (ECS) was used, which contained (in mM) NaCl,

140; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; HEPES, 10; NaOH, 4.54; and glucose, 5 (pH 7.4 at 25°C). The ECS also contained the vehicle used for dissolving the drugs. The cells plated on coverslips were incubated in ECS containing 0.02% Pluronic F-127 and 2-μM Calcium Green-1 AM (both from Life Technologies GmbH, Darmstadt, Germany), for 30 min at 37°C. After a recovery period of another 30 min, in standard ECS, coverslips were mounted in a Teflon chamber (MSC TD, Digitimer, Welwyn Garden City, UK), which was then positioned on the stage of an Olympus IX70 inverted microscope. A CCD camera (Cohu 4910, Pieper GmbH, Schwerte, Germany) was used for recording the changes in fluorescence. The excitation light at 470 nm was emitted by a Dual OptoLED light source (Cairn Research, Faversham, UK), controlled by the Axon Imaging Workbench 2.2 software (Axon Instruments, Union City, CA, USA). The same software was also used for image acquisition and analysis.

Temperature stimuli were applied through local superfusion using a Peltier-based system described elsewhere (Reid et al., 2001). To measure the temperature experienced by the cells during the experiment, a miniature thermocouple (1T-1E, Physitemp, Clifton, NJ, USA) was placed very close to the imaged cells and the temperature was measured in real time.

Custom software was used for the analysis of the change in calcium indicator emission, and the amplitude of the response was calculated as ratio of maximal fluorescence change during stimulation to the initial fluorescence ($\Delta F/F_0$). Cells that responded with a $\Delta F/F_0$ > 0.1 during the application of a chemical compound were considered responsive to that stimulus. Unless otherwise stated, the experiments were performed at 25°C.

2.8 | Data and statistical analysis

The data and statistical analysis in this study comply with the recommendations of *British Journal of Pharmacology* on experimental design and analysis (Curtis et al., 2022). In this study, the experiments were designed to generate groups of equal size, using randomization and blinded analysis where possible. In case of experiments performed on single cells (as presented in Figures 1c-e, 4a-e, S1 and S2), the groups could not be designed to be of equal size due to the isolation and preparation of the cells. Statistical analysis was undertaken only for experiments where each group size was at least n = 5 independent values, using GraphPad Prism 6 (RRID:SCR_002798) software. Concentration-response curves are presented as mean ± SEM plotted on a semi-logarithmic scale. Log (agonist) vs response curves were generated using non-linear regression curve fit.

Normalization of the response amplitude (ΔF/F0) to the magnitude of the first response to a series of identical thermal or chemical stimuli was performed for each individual cell, to reduce the inter-cell variability of TRPM8 expression. When using normalized data, comparisons with the first responses (normalized to themselves and thus lacking SEM) were avoided, and therefore parametric tests were still possible. We only compared the degree of desensitization between different populations of treated and control cells, two sets of data which were normally distributed. Data normalization presented in Figures 1g and 3e-f was required because of the different assays employed to determine the cellular effects in the presence of the indicated inhibitors (Figure 1g and 3e or when comparing TRPM8 wild-type with TRPM8 mutant (Figure 3f). Statistical analyses of differences between the two groups were performed by non-parametric, unpaired, two-tailed Mann–Whitney tests. *P* values less than 0.05 were considered to show significant differences between means.

2.9 | Materials

Cicaprost, **beraprost**, **iloprost**, **forskolin**, **treprostinil**, YM254890, and Cay10441 were from Cayman Chemicals (Ann Arbor, MI, USA). Gallein, polyethyleneimine (PEI, 40872-7), cholera toxin (CTX), SQ22,536, **PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyramidine)**, **U-73122** were from Sigma-Aldrich (St. Louis, MO, USA), Lipofectamine™ 2000 Transfection Reagent (11668019) was from ThermoFisher Scientific (Waltham, MA, USA). Icilin and **WS-12** were from Tocris (Bristol, UK).

DMEM supplemented with 4.5 $g{\cdot}L^{-1}$ glucose, L-glutamine, sodium pyruvate, 1.5 $g{\cdot}L^{-1}$ $NaHCO_3$, Cat. #P04-03596, was from Pan Biotech (Aidenbach, Germany), FBS (Cat. # 16000044), 100× MEM Non-Essential Amino Acids (NEAA) (Cat. #11140), 100-mM sodium pyruvate (Cat. #11360070), and 1× PBS (Cat. #18912-014) used in cell culture were from Gibco (ThermoFisher Scientific, Waltham, MA, USA).

2.10 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2021/22 (Alexander, Christopoulos, et al., 2021; Alexander, Fabbro et al., 2021; Alexander, Mathie, et al., 2021).

3 | RESULTS

3.1 | Agonists of IP receptors inhibit icilin- and cold-induced activation of TRPM8 channels

To test whether there is a functional relationship between activation of IP receptors and TRPM8 activity, we transiently co-expressed human TRPM8 and IP receptors in HEK293T cells and determined the effect of icilin (1 μM) on intracellular calcium in cells pretreated for 10 min with increasing concentrations of synthetic agonists of IP receptors, such as iloprost, beraprost, treprostinil, and cicaprost. As shown in Figure 1a, all IP receptor agonists potently inhibited icilin-induced TRPM8 activation in a concentration-dependent manner, demonstrating that there is a strong functional effect of IP receptors on TRPM8 activity. Because there was no difference in maximal

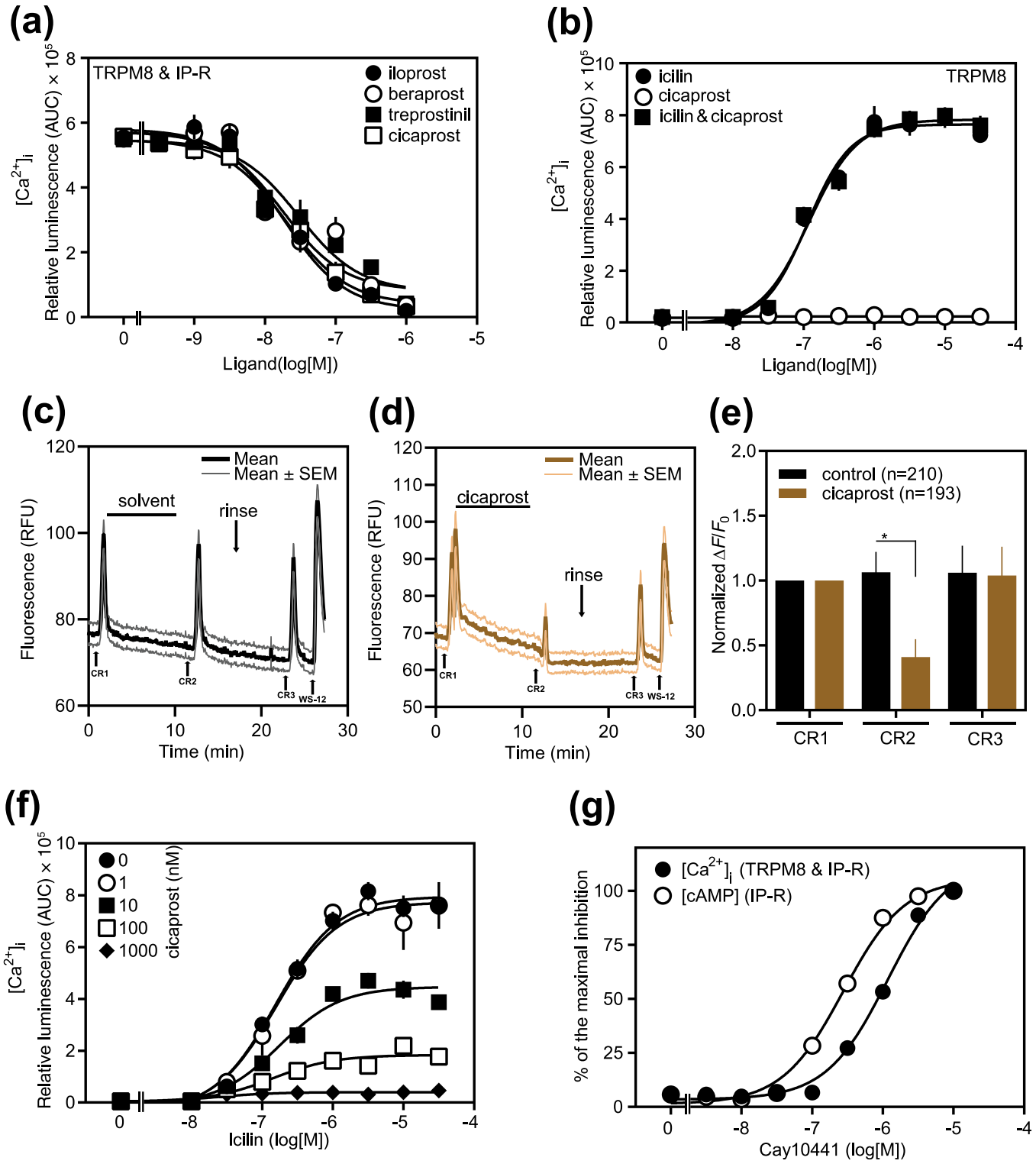

FIGURE 1 Legend on next page.

FIGURE 1 Prostacyclin receptor inhibits the activation of TRPM8 channels by icilin and cold temperature. (a) Effect of preincubation for 10 min with increasing concentrations of the indicated IP receptor agonists on $[Ca^{2+}]_i$ evoked by 1-μM stimulation with icilin of HEK293T cells expressing TRPM8 channels and IP receptors, together with the calcium-sensitive cytosolic-localized probe (G5A). (b) Effect of increasing concentrations of icilin, cicaprost and icilin plus with 1 μM cicaprost on $[Ca^{2+}]_i$ in cells expressing TRPM8 channels together with G5A. (c–e) Calcium microfluorimetry in HEK293T cells expressing TRPM8 channels together with IP receptors and stimulated with three cold-ramps (CR1–3) in the absence (c, n = 104) or presence of 1 μM cicaprost (d, n = 72). The selective TRPM8 channel agonist WS-12 (5 μM) was applied at the end of each experiment, to confirm functional expression of TRPM8 channels. Data shown are means (continuous traces) ± SEM (dotted traces). (e) Mean responses to cooling ramps CR2 and CR3 quantified as $\Delta F/F_0$ normalized to the response evoked by the first cooling ramp (CR1). Data shown are means ± SEM. * $P \leq 0.05$, significantly different as indicated; Student's two-tailed *t* test. (f) Effect of increasing concentrations of icilin on $[Ca^{2+}]_i$ in the presence of 10 min prestimulation with indicated concentrations of cicaprost in HEK293T expressing TRPM8 channels and IP receptors together with G5A. (g) Normalized quantification of the effect of increasing concentrations of the IP receptor antagonist Cay10441 on cicaprost-induced cAMP accumulation in HEK293T cells expressing only IP receptors, together with a cytosolic cAMP-sensitive probe (p22F-Glo) (white dots, [cAMP](IP-R)) or on icilin (1 μM)-induced increases in $[Ca^{2+}]_i$ in cells expressing TRPM8 channels and IP receptors together with G5A, and prestimulated with 1 μM cicaprost, for 10 min (black dots, $[Ca^{2+}]_i$ (TRPM8-IP-R)). (a,b,f,g). Data shown are means ± SEM; n = 5.

inhibition and potency among the tested IP receptor agonists, we continued our study by using cicaprost, a chemically stable and a widely used IP receptor agonist to examine various functions of the IP receptor. To exclude the possibility that the observed effects are due to the interference of cicaprost with TRPM8 activity, either as an agonist (promoting TRPM8 desensitization) or an antagonist, we tested its effects on TRPM8 heterologously expressed in HEK293T, in the absence of IP receptors. As shown in Figure 1b, whereas icilin induced a robust concentration-dependent activation of TRPM8 channels, stimulation of cells expressing TRPM8 with increasing concentrations of cicaprost did not produce any significant calcium transients, ruling out the possibility that cicaprost might act as an agonist of TRPM8 channels. On the other hand, when icilin was tested at increasing concentrations together with a maximal concentration of cicaprost (1 μM), no effect on the potency or efficacy of icilin-induced TRPM8 activation was observed (Figure 1b), clearly demonstrating that cicaprost does not act as an antagonist of icilin-dependent activation of TRPM8 channels. Collectively, these results prove that the inhibition of TRPM8 channels by cicaprost is specifically mediated through IP receptors. The physiological role of TRPM8 channels is to elicit cellular responses triggered by cooling. Therefore, we sought to examine whether activation of IP receptors by cicaprost also inhibits cooling-induced TRPM8 activation. As shown in Figure 1c–e, cicaprost pretreatment of HEK293T cells expressing TRPM8 together with IP receptors significantly reduced the activation of TRPM8 in response to cooling ramps (from ~32°C to ~22°C, 60s duration). It is important to note that in Figure 1d, the addition of cicaprost induced a calcium transient in mouse DRG neurons, resulting in a second fluorescence peak. As shown in Figure S1, the calcium peak induced by cicaprost originates from the internal stores, as the removal of the extracellular calcium does not affect cicaprost-induced calcium transient. In HEK293T cells only expressing TRPM8 and an empty backbone vector, cicaprost had no effect on the cold-induced calcium transients mediated by TRPM8 (Figure S2). All the data presented so far point to a specific inhibition of TRPM8 channel function by IP receptor agonists. In the next series of experiments, we sought to determine the type of inhibition exerted by IP receptor agonists on TRPM8 channels by performing a pharmacological profile of icilin on cells co-expressing TRPM8 and IP receptors, in the presence of increasing concentrations of cicaprost. As shown in Figure 1f, raising the concentration of cicaprost from 1 nM to 1 μM resulted in a marked decrease of the efficacy of icilin, whereas its potency remained unchanged. These results suggest that the effect of cicaprost/IP receptor on TRPM8 functionality resembles a non-competitive type of inhibition, which would be compatible with the idea of a negative regulation of TRPM8 function by IP receptor agonists through a cellular mediator that acts on a different binding site from that of icilin at TRPM8. Because the main physiological intracellular mediators of IP receptors are Gs proteins that activate adenylyl cyclase with subsequent generation of cAMP, we measured the concentration dependence of the inhibitory effect of a competitive antagonist of cicaprost at IP receptors, Cay10441, on the cAMP accumulation induced by 1 μM of cicaprost in IP receptor-expressing HEK293T cells and also on the inhibition of TRPM8 function produced by the same concentration of cicaprost in HEK293T cells co-expressing IP receptors and TRPM8 channels. As shown in Figure 1g, the effect of cicaprost, to inhibit TRPM8 channels, was right-shifted compared with the effect on cAMP accumulation, suggesting that the inhibitory action of cicaprost-activated IP receptors on TRPM8 might include other intracellular components besides the physiological Gs-cAMP pathway. In conclusion, these data strongly indicate that the inhibition of TRPM8 function by IP receptor agonists occurs in a specific manner, most likely involving an intracellular mediator.

3.2 | Inhibition of TRPM8 channels by IP receptors occurs in a cAMP-independent fashion

Our data, presented so far, demonstrate a potent inhibition of cold- and icilin-induced TRPM8 activity by PGI_2 analogues such as cicaprost and related compounds. Because the main physiological actions of IP receptor agonists are mediated by the IP receptors coupling to intracellular Gs proteins to increase intracellular cAMP, we wanted to determine whether the contribution of either the increase in intracellular cAMP levels or the activation of Gs proteins, in a receptor-independent manner, affect TRPM8 function measured by its responses to increasing concentrations of icilin. As shown in Figure 2a,b, raising intracellular cAMP levels by forskolin, which

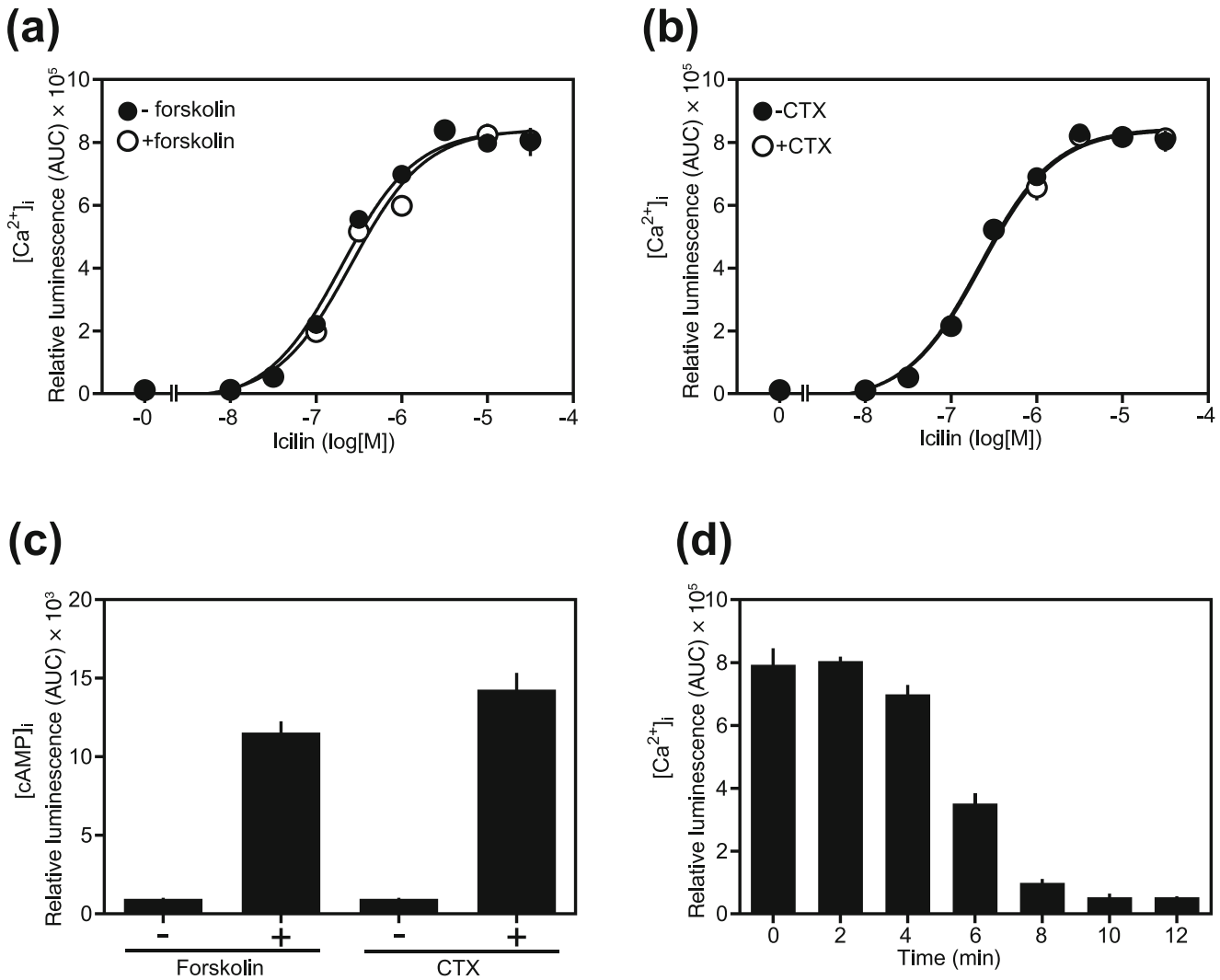

FIGURE 2 TRPM8 channel activity is not affected by Gs proteins or by cAMP-dependent pathway. (a,b) Effect of 10 μM forskolin (a) or 100 ng·ml^{-1} cholera toxin (CTX) (b) on the potency and efficacy of icilin-induced calcium transients in HEK293T cells expressing TRPM8 channels together with G5A. (c) Effect of 10 μM forskolin or 100 ng·ml^{-1} CTX on intracellular cAMP accumulation in HEK293T expressing TRPM8. (d) Time dependence of TRPM8 inhibition by 1 μM cicaprost, in HEK293T cells expressing TRPM8 channels and IP receptors together with G5A followed by stimulated with 1 μM icilin. Data shown are means ± SEM; n = 5.

activates adenylyl cyclase in a GPCR/G protein-independent manner, or by activating Gs proteins with cholera toxin (CTX), did not affect the potency or the efficacy of icilin-induced TRPM8 activation in HEK293T cells expressing TRPM8 and the cytosolic calcium-sensitive probe G5A. Despite the lack of inhibitory effects on TRPM8 channels, both forskolin and CTX strongly increased intracellular cAMP levels (Figure 2c). In addition, the inhibition of adenylyl cyclase with a specific inhibitor, SQ22536, did not affect cicaprost-induced TRPM8 inhibition through IP receptors (Figure S3d), although the inhibitor strongly reduced cicaprost-induced cAMP elevation in HEK293T cells expressing IP receptors (Figure S3c). Moreover, SQ22536 had no effect on the icilin-induced TRPM8 activation in HEK293T and also did not impair cicaprost-induced Gq/11 signalling of IP receptors that led to the intracellular calcium elevation (Figure S3b). These data demonstrate that the Gs-cAMP pathway does not play any role in the modulation of TRPM8 activity in general and, most likely, in the inhibitory effect induced by the activation of IP receptors by cicaprost. Interestingly, the inhibition of TRPM8 by cicaprost through IP receptors was time-dependent, with a half-time of around 5 min of preincubation of HEK293T cells expressing TRPM8 and IP receptors with 1-μM cicaprost (Figure 2d). Taken together, these results demonstrate that the inhibition of TRPM8 channels function by IP receptor agonists occurs in a cAMP-independent manner.

3.3 | Gq/11 proteins mediate the inhibition of TRPM8 channels by IP receptor agonists

Considering the data presented so far, which clearly demonstrated the insensitivity of TRPM8 function to activated Gs proteins or

elevated intracellular cAMP levels, we examined the effect of pharmacological inhibitors of specific intracellular pathways on the negative regulation of TRPM8 channels, by IP receptors, pre-stimulated for 10 min with 1 μM cicaprost. Pretreatment of HEK293T cells expressing TRPM8 and IP receptors with gallein, an inhibitor of G protein βγ-subunits, did not abolish the cicaprost-induced inhibition of TRPM8 function by IP receptor agonists. Similarly, the inhibition of PLCβ with U-73122 and of protein kinases with PP2 did not block the inhibition of TRPM8 function by IP receptor agonists. However, the Gq/11 blocker YM254890 (C. Zhang et al., 2020) could fully restore TRPM8 functionality in response to icilin, in HEK293T cells expressing TRPM8 and IP receptors, incubated with cicaprost (1 μM) for 10 min (Figure 3a). Moreover, in the same cellular system and assay conditions, the effect of YM254890 on restoring TRPM8 response to icilin was dose-dependent, with an EC_{50} of 100 nM (Figure 2b). The results obtained using YM254890 indicate that Gq/11 proteins mediate the inhibitory effects of IP receptor agonists on TRPM8 channels.

Should this be the case, we would expect IP receptors to couple not only to Gs, but also to Gq/11, upon agonist stimulation. When IP receptors were heterologously expressed in HEK293T cells, cicaprost and related agonists increased intracellular cAMP levels in a concentration-dependent manner with expected potencies, which were similar among the different compounds (Figure 3c). Interestingly, the same ligands could promote intracellular calcium increases in

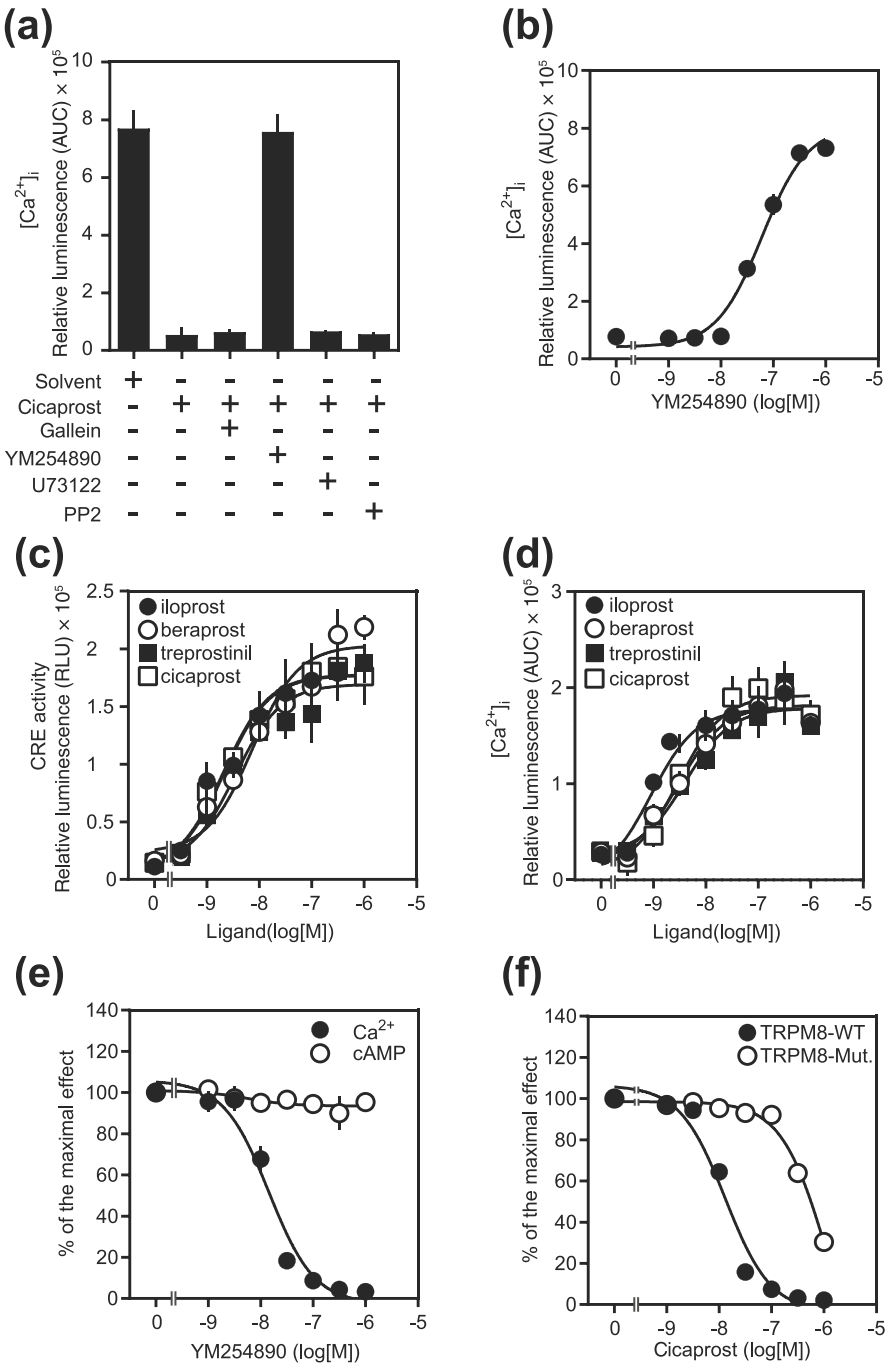

FIGURE 3 Inhibition of TRPM8 function by IP receptor activation occurs in a Gq/11-dependent fashion. (a) Effect of the indicated pharmacological inhibitors on the inhibition of TRPM8 channels by IP receptor agonists. HEK293T cells co-expressing TRPM8 channels with IP receptors and G5A were treated with indicated inhibitors for 30 min before incubation with cicaprost (1 μM) for 10 min. After that, cells were stimulated with icilin (1 μM) and $[Ca^{2+}]_i$ was recorded. Gallein = 30 μM, YM254890 = 1 μM, U73122 = 30 μM, PP2 = 300 μM. (b) Effect of incubation with increasing concentration of YM254890 on icilin (1 μM)-induced $[Ca^{2+}]_i$ increases in HEK293T cells co-expressing TRPM8 channels and IP receptors together with G5A, after 10 min of preincubation with cicaprost (1 μM). (c,d) Effect of increasing concentrations of the indicated ligands on intracellular cAMP accumulation, as determined by CRE-reporter activity (c) or $[Ca^{2+}]_i$ transients in HEK293T cells expressing IP receptors. (e) Normalized representation of the effects of increasing concentrations of YM254890 on cicaprost (1 μM)-induced cAMP accumulation (white dots) or $[Ca^{2+}]_i$ increases in HEK293T expressing IP receptors together with the intracellular probes for cAMP (p22F-Glo) and calcium (G5A), (black dots), respectively. (f) Normalized representation of the effect of increasing concentrations of cicaprost on icilin-induced $[Ca^{2+}]_i$ transients in HEK293T expressing wild-type TRPM8 (TRPM8-WT) and Gq/11-binding deficient TRPM8 mutant (TRPM8-Mut.) together with G5A. Data shown are means ± SEM; n = 5.

these IP receptor expressing HEK293T cells, with a potency similar to that of cAMP accumulation, demonstrating that IP receptors have indeed a dual Gs- and Gq/11-coupling (Figure 3d), a feature that has been previously described (Woodward et al., 2011). Although YM254890 was considered a specific inhibitor of Gq/11-proteins, a recent report evaluated its specificity among other types of G proteins revealing that it may also inhibit, to some extent, Gs proteins (Peng et al., 2021). For this reason, we tested the effect of increasing concentrations of YM254890 on the ability of IP receptors, heterologously expressed in HEK293T cells, to elicit calcium and cAMP responses, after stimulation with 1 μM cicaprost. As shown in Figure 3e, whereas YM25490 potently abolished cicaprost-induced calcium transients, cAMP responses to the IP receptor agonist remained unaltered. These data confirm that, at least in the case of heterologous expression of IP receptors, YM254890 is a specific Gq/11 inhibitor, thus strengthening the idea of a Gq/11-mediated inhibition of TRPM8 by IP receptor agonists. In order to find out how Gq/11 proteins alter TRPM8 activity in the context of IP receptor activation by cicaprost, we analysed the responses of a TRPM8 mutant (TRPM8-TM), defective in binding Gq proteins (X. Zhang, 2019), to 1 μM icilin in HEK293T cells co-expressing IP receptors and subjected to prestimulation with increasing concentrations of cicaprost, and compared them to responses elicited by equivalent concentration of icilin acting on wild-type TRPM8 channels.

As shown in Figure 3f, whereas cicaprost could strongly inhibit wild-type TRPM8 channels, co-expressed with IP receptors, it was much less effective in inhibiting the mutant deficient in binding Gq proteins. Interestingly, although the inhibition is severely reduced, at higher cicaprost concentrations, the activation of TRPM8-TM by icilin is still significantly inhibited. This could be the result of a possible response of TRPM8-TM to G11 proteins, or as a result of a yet unidentified mediator.

Taken together, the presented data clearly support the notion that Gq/11 proteins mediate the inhibitory effect of IP receptor agonists on TRPM8 channel activity.

3.4 | IP receptors mediate, in a Gq/11-dependent manner, inhibition of TRPM8 channels in DRG-derived neurons,

To determine whether the IP receptors can modulate the function of TRPM8 channels in a more physiological context, we decided to explore the possible functional interaction of the two proteins in DRG neurons from mice, which are relevant for pain and inflammatory responses. We identified TRPM8-expressing neurons by their sensitivity to the selective TRPM8 agonist WS-12 (Sherkheli et al., 2008). When these neurons were exposed to icilin, a strong intracellular calcium response was generated (Figure 4a,e). However, preincubation of neurons with cicaprost (1 μM) for 8 min significantly reduced icilin-induced calcium transients (Figure 4b,e). Importantly, this effect was reversed by a maximal concentration of the IP receptor antagonist Cay10441 indicating that the inhibitory effect of cicaprost was mediated by IP receptors co-expressed with TRPM8 channels in mouse DRG-derived neurons (Figure 4c,e). Consistent with the results obtained in the heterologous expression system, pretreatment of DRG neurons with the Gq/11 inhibitor YM254890 (1 μM) fully restored the response of TRPM8 channels to icilin in neurons prestimulated with 1 μM cicaprost for 10 min (Figure 4d,e). The results presented above support the notion that the Gq/11-dependent inhibition of TRPM8 channels by IP receptor agonists can also be observed in DRG neurons.

4 | DISCUSSION

Ion channels and GPCRs expressed in the membranes of nociceptive nerve endings are key players in inflammatory pain states. The TRPM8 channel has been acknowledged as an important cold sensor and also as a contributor to the regulation of inflammation. TRPM8 channels are responsible for detecting temperatures in the innocuous cooling range (i.e., between 32°C and ca. 15°C) and, while they contribute to noxious cold sensing (Bautista et al., 2007), activation of TRPM8 channels also produces analgesia in both neuropathic and inflammatory pain states (Liu et al., 2013; Proudfoot et al., 2006). In contrast, the IP receptor is involved in the initiation and maintenance of inflammatory states, leading to pain hypersensitivity (Murata et al., 1997). Given the co-expression of TRPM8 and IP receptors in the vascular system as well as in a subpopulation of primary afferent neurons (Dhaka et al., 2008; Oida et al., 1995) and their opposing roles in inflammation and pain, we sought to clarify whether there is a functional crosstalk between these proteins. It has been previously shown that TRPM8 activity can be modulated by intracellular heterotrimeric G proteins, especially from the Gi and Gq/11 classes. Gi-mediated inhibition of TRPM8 channels is thought to occur indirectly, involving a decrease in PKA-induced phosphorylation of the channel. Signal transduction pathways downstream of Gq-coupled receptors have been reported to suppress TRPM8 function via several mechanisms: PKC-mediated dephosphorylation of the channel (Premkumar et al., 2005), PIP2 depletion (B. Liu & Qin, 2005) and direct binding of Gαq subunit to TRPM8 (X. Zhang et al., 2012). Of note, the latter mechanism was shown to be solely responsible for the inhibition of TRPM8 channels, which followed activation of **bradykinin B_2 receptors** in primary afferent neurons. The direct modulation of Gαq proteins on TRPM8 activity occurs by electrostatic interactions between the N-terminus of TRPM8 and the Switch III domain in Gαq, while the C-terminus of TRPM8 interferes with the PLCβ-PIP2 pathway by direct binding to the α3 helix of Gαq (X. Zhang, 2019).

In our experiments, the PLCβ inhibitor U73122 had no effect on the cicaprost-induced inhibition of TRPM8 function, indicating that PIP2 hydrolysis does not play a significant role in this effect. Zhang (2019) demonstrated that binding of the B_2 receptor to TRPM8 renders the channel insensitive to PIP2 depletion (X. Zhang, 2019). A similar mechanism may be responsible for the apparent TRPM8 insensitivity to PIP2 depletion following the activation of IP receptors. To explore that possibility, we examined whether TRPM8 channels

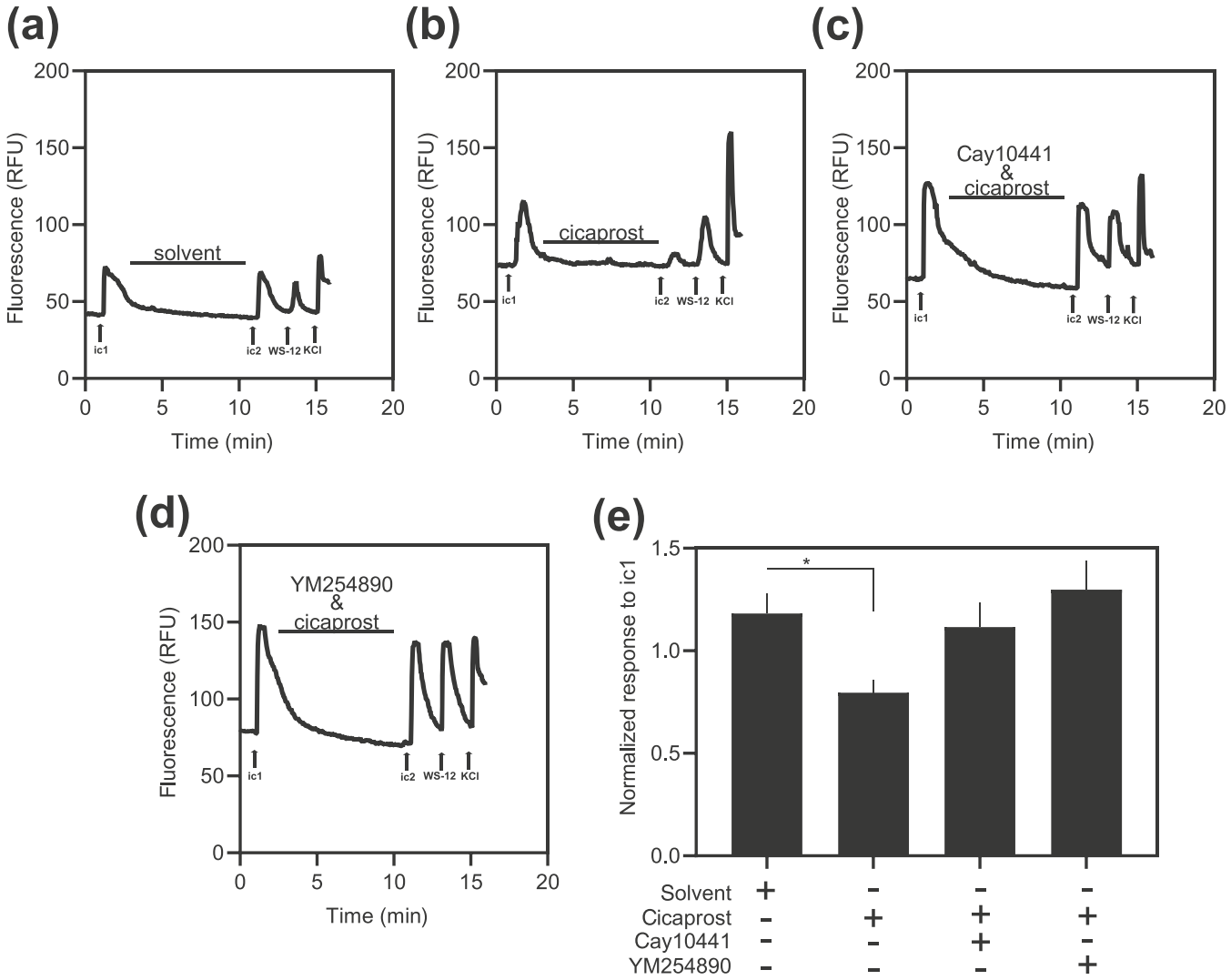

FIGURE 4 Inhibition of TRPM8 channels by IP receptor agonists in DRG neurons is mediated by Gq/11 proteins. (a–d) Representative recordings of calcium transients in DRG-derived neurons subjected to two icilin stimulations (1 μM, ic1 and ic2) followed by stimulation with a selective TRPM8 channel agonist, WS-12 (5 μM), in the absence (a) or in the presence of 1 μM cicaprost (b–d) and 30 μM Cay10441 (c) or 1 μM YM254890 (d). A high potassium-containing solution (KCl, 50 mM) was applied at the end of each experiment to depolarize neurons and select viable ones (i.e., neurons that responded with calcium transients to KCl). (e) Quantification of the response ($\Delta F/F_0$) evoked by the second icilin stimulation (ic2, normalized to the response evoked by the first icilin stimulation, ic1) in DRG-derived neurons in the absence or in the presence of cicaprost, together with 30-μM Cay10441 and 1-μM YM254890. Data shown are means ± SEM, from n = 26, solvent; n = 34, cicaprost; n = 29, Cay10441; n = 27, YM254890. * $P \leq 0.05$, significantly different as indicated; Student's two-tailed *t* test.

and IP receptors can physically interact with each other. As shown in Figure S4, when heterologously expressed in HEK293T, human c-Myc-N-terminally tagged TRPM8 channels interacted with human N-terminally HA-tagged IP receptors in an immunoprecipitation assay. However, this interaction is unlikely to explain the effect of IP receptors on TRPM8 channels, as the inhibition of TRPM8 channels by IP receptor agonists, was fully reversed by a small molecule inhibitor of Gq/11 proteins (Figure 3a,b). Our results demonstrate that while Gs proteins and cAMP do not alter TRPM8 function, the selective Gq/11 inhibitor YM254890 fully restored the sensitivity of TRPM8 to icilin in the presence of cicaprost supporting the hypothesis of a direct effect of Gq/11 proteins on TRPM8. Consistent with a direct effect of Gq/11 proteins on TRPM8, co-expression of a constitutively active mutant of Gαq, GQ209L (F. Liu et al., 2005), completely blocked icilin-induced TRPM8 activation (Figure S5). This conclusion is also strengthened by the fact that the ability of IP receptor agonists to inhibit TRPM8 function was substantially diminished in a TRPM8 mutant, which lacks the ability to bind Gαq (Figure 3f). Previous studies of IP receptors have shown that besides the physiological Gs coupling, Gi and Gq/11 coupling might also occur in a cell-dependent manner and appear to be species-specific. When heterologously expressed, hIP receptors coupled to Gq/11 and Gs proteins in HEK293T and COS cells (Smyth et al., 1998). However, studies on endogenously expressed IP receptors in platelets (Nishimura et al., 1995) and vascular smooth muscle cells (Cottafava et al., 1978) confirmed the coupling to Gs but not to Gq/11. Early work

demonstrated that the coupling of mouse IP receptors to Gq/11 proteins depends on initial coupling to Gs proteins ultimately leading to the activation of PKA and subsequent phosphorylation of Ser356. In human IP receptors, that critical serine appears at position 328 (Ser328) forming a PKC phosphorylation site, and this modification did not appear to affect the coupling of hIP receptors to Gq/11 proteins (Miggin & Kinsella, 2002). However, that paper was retracted in 2018 (Miggin & Kinsella, 2018). IUPHAR acknowledges that dual Gs and Gq/11 coupling of IP receptors might occur but also mentions that it is still unclear whether the Gq/11 coupling happens widely in vivo. Our results indicate that Gq/11 coupling of IP receptors occurs in mouse DRG neurons, resulting in the inhibition of endogenously expressed TRPM8 (Figure 4a–e). While it is known that icilin also acts as an agonist of **TRPA1**, a polymodal receptor-channel also expressed in mouse DRG neurons, in our experiments we used a very low concentration of icilin (1 μM), which is insufficient to activate mouse TRPA1 channels, as we demonstrate in Figure S6. Moreover, of the total 116 icilin-sensitive neurons identified, 108 (93.1%) also responded to WS-12, indicating a very high correlation of icilin sensitivity with functional expression of TRPM8. We are therefore confident that the icilin-evoked calcium transients we recorded in cultured mouse DRG neurons were solely mediated by TRPM8 channels.

The reported co-expression of TRPM8 and IP receptors is relatively limited in murine primary afferent neurons (Jung et al., 2023), a feature that may explain that in DRG neurons the inhibition of TRPM8 by IP receptors is only around 30%, compared with almost 100% in a heterologous expression system. However, given the robust inhibition of TRPM8 channels following activation of IP receptors, it may be inferred that this inhibitory action, which removes the anti-inflammatory effects of the cold and menthol receptor TRPM8, underlies at least to some extent the pro-inflammatory peripheral action of prostacyclin. Further studies will be needed to determine the role of this interaction in the regulation of inflammatory responses and pain.

AUTHOR CONTRIBUTIONS

Cosmin Trif: Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); software (equal); validation (equal); writing—review and editing (equal). **Alexandra Maria Banica:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); software (equal); validation (equal); visualization (equal); writing—original draft (equal). **Alexandra Manolache:** Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (equal); software (supporting); validation (supporting); writing—original draft (supporting); writing—review and editing (supporting). **Andreea Sorina Anghel:** Data curation (supporting); formal analysis (supporting); funding acquisition (supporting); investigation (supporting); methodology (supporting); software (supporting); validation (supporting); visualization (supporting); writing—original draft (supporting); writing—review and editing (supporting). **Debora Hutanu:** Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); validation (supporting); visualization (supporting); writing—original draft (supporting); writing—review and editing (supporting). **Teodora Stratulat:** Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); software (supporting); validation (supporting); visualization (supporting); writing—original draft (supporting); writing—review and editing (supporting). **Rodica Aura Badea:** Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); writing—review and editing (supporting). **George Oprita:** Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); writing—review and editing (supporting). **Tudor Selescu:** Data curation (supporting); investigation (lead); visualization (supporting). **Stefana Maria Petrescu:** Conceptualization (equal); data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); project administration (equal); resources (equal); supervision (equal); validation (equal); writing—original draft (equal); writing—review and editing (equal). **Marco Sisignano:** Data curation (equal); formal analysis (equal); investigation (equal); methodology (equal); supervision (equal); validation (equal); writing—original draft (equal); writing—review and editing (equal). **Stefan Offermanns:** Conceptualization (equal); funding acquisition (equal); investigation (equal); methodology (equal); supervision (equal); validation (equal); visualization (equal); writing—original draft (equal); writing—review and editing (equal). **Alexandru Babes:** Conceptualization (lead); data curation (lead); formal analysis (lead); investigation (lead); methodology (lead); project administration (lead); resources (equal); software (lead); supervision (lead); validation (lead); visualization (lead); writing—original draft (lead); writing—review and editing (lead). **Sorin Tunaru:** Conceptualization (lead); data curation (lead); formal analysis (lead); funding acquisition (lead); investigation (lead); methodology (lead); project administration (lead); resources (lead); software (lead); supervision (lead); validation (lead); visualization (lead); writing—original draft (lead); writing—review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

None.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation, and as recommended by funding agencies, publishers, and other organizations engaged with supporting research.

ORCID

Marco Sisignano https://orcid.org/0000-0002-7581-0951

Sorin Tunaru https://orcid.org/0000-0002-6005-1737

REFERENCES

Alexander, S. P., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Southan, C., Davies, J. A., Abbracchio, M. P., Alexander, W., al-Hosaini, K., Bäck, M., Barnes, N. M., Bathgate, R., … Ye, R. D. (2021). The Concise Guide to PHARMACOLOGY 2021/22: G protein-coupled receptors. *British Journal of Pharmacology*, *178*-(Suppl 1), S27–S156. https://doi.org/10.1111/bph.15538

Alexander, S. P., Fabbro, D., Kelly, E., Mathie, A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Southan, C., Davies, J. A., Boison, D., Burns, K. E., Dessauer, C., Gertsch, J., Helsby, N. A., Izzo, A. A., Koesling, D., … Wong, S. S. (2021). THE CONCISE GUIDE TO PHARMACOLOGY 2021/22: Enzymes. *British Journal of Pharmacology*, *178*(S1), S313–S411. https://doi.org/10.1111/bph.15542

Alexander, S. P., Mathie, A., Peters, J. A., Veale, E. L., Striessnig, J., Kelly, E., Armstrong, J. F., Faccenda, E., Harding, S. D., Pawson, A. J., Southan, C., Davies, J. A., Aldrich, R. W., Attali, B., Baggetta, A. M., Becirovic, E., Biel, M., Bill, R. M., Catterall, W. A., … Zhu, M. (2021). The Concise Guide to PHARMACOLOGY 2021/22: Ion channels. *British Journal of Pharmacology*, *178*(Suppl 1), S157–S245. https://doi.org/10.1111/bph.15539

Alexander, S. P. H., Roberts, R. E., Broughton, B. R. S., Sobey, C. G., George, C. H., Stanford, S. C., Cirino, G., Docherty, J. R., Giembycz, M. A., Hoyer, D., Insel, P. A., Izzo, A. A., Ji, Y., MacEwan, D. J., Mangum, J., Wonnacott, S., & Ahluwalia, A. (2018). Goals and practicalities of immunoblotting and immunohistochemistry: A guide for submission to the British Journal of Pharmacology. *British Journal of Pharmacology*, *175*(3), 407–411. https://doi.org/10.1111/bph.14112

Andersson, D. A., Nash, M., & Bevan, S. (2007). Modulation of the cold-activated channel TRPM8 by lysophospholipids and polyunsaturated fatty acids. *The Journal of Neuroscience*, *27*(12), 3347–3355. https://doi.org/10.1523/JNEUROSCI.4846-06.2007

Babes, A., Fischer, M. J., Reid, G., Sauer, S. K., Zimmermann, K., & Reeh, P. W. (2010). Electrophysiological and neurochemical techniques to investigate sensory neurons in analgesia research. *Methods in Molecular Biology*, *617*, 237–259. https://doi.org/10.1007/978-1-60327-323-7_19

Baubet, V., Le Mouellic, H., Campbell, A. K., Lucas-Meunier, E., Fossier, P., & Brulet, P. (2000). Chimeric green fluorescent protein-aequorin as bioluminescent Ca^{2+} reporters at the single-cell level. *Proceedings of the National Academy of Sciences of the United States of America*, *97*(13), 7260–7265. https://doi.org/10.1073/pnas.97.13.7260

Bautista, D. M., Siemens, J., Glazer, J. M., Tsuruda, P. R., Basbaum, A. I., Stucky, C. L., Jordt, S. E., & Julius, D. (2007). The menthol receptor TRPM8 is the principal detector of environmental cold. *Nature*, *448*(7150), 204–208. https://doi.org/10.1038/nature05910

Bavencoffe, A., Gkika, D., Kondratskyi, A., Beck, B., Borowiec, A. S., Bidaux, G., Busserolles, J., Eschalier, A., Shuba, Y., Skryma, R., & Prevarskaya, N. (2010). The transient receptor potential channel TRPM8 is inhibited via the alpha 2A adrenoreceptor signaling pathway. *The Journal of Biological Chemistry*, *285*(13), 9410–9419. https://doi.org/10.1074/jbc.M109.069377

Brauchi, S., Orio, P., & Latorre, R. (2004). Clues to understanding cold sensation: Thermodynamics and electrophysiological analysis of the cold receptor TRPM8. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(43), 15494–15499. https://doi.org/10.1073/pnas.0406773101

Che, T. (2021). Advances in the treatment of chronic pain by targeting GPCRs. *Biochemistry*, *60*(18), 1401–1412. https://doi.org/10.1021/acs.biochem.0c00644

Cottafava, F., Bertolotto, M., Brida di Prio, S., Moni, L., & Grossi-Bianchi, M. L. (1978). Clinical and immunological study of a case of mixed connective tissue disease (MCTD) with onset in childhood. *Minerva Pediatrica*, *30*(24), 1927–1932.

Curtis, M. J., Alexander, S. P., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Patel, H. H., Sobey, C. G., Stanford, S. C., Stanley, P., Stefanska, B., Stephens, G. J., Teixeira, M. M., Vergnolle, N., & Ahluwalia, A. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, *179*, 3907–3913. https://doi.org/10.1111/bph.15868

Daniels, R. L., Takashima, Y., & McKemy, D. D. (2009). Activity of the neuronal cold sensor TRPM8 is regulated by phospholipase C via the phospholipid phosphoinositol 4,5-bisphosphate. *The Journal of Biological Chemistry*, *284*(3), 1570–1582. https://doi.org/10.1074/jbc.M807270200

De Petrocellis, L., Starowicz, K., Moriello, A. S., Vivese, M., Orlando, P., & Di Marzo, V. (2007). Regulation of transient receptor potential channels of melastatin type 8 (TRPM8): Effect of cAMP, cannabinoid CB_1 receptors and endovanilloids. *Experimental Cell Research*, *313*(9), 1911–1920. https://doi.org/10.1016/j.yexcr.2007.01.008

Dhaka, A., Earley, T. J., Watson, J., & Patapoutian, A. (2008). Visualizing cold spots: TRPM8-expressing sensory neurons and their projections. *The Journal of Neuroscience*, *28*(3), 566–575. https://doi.org/10.1523/JNEUROSCI.3976-07.2008

Jung, M., Dourado, M., Maksymetz, J., Jacobson, A., Laufer, B. I., Baca, M., Foreman, O., Hackos, D. H., Riol-Blanco, L., & Kaminker, J. S. (2023). Cross-species transcriptomic atlas of dorsal root ganglia reveals species-specific programs for sensory function. *Nature Communications*, *14*(1), 366. https://doi.org/10.1038/s41467-023-36014-0

Khalil, M., Babes, A., Lakra, R., Försch, S., Reeh, P. W., Wirtz, S., Becker, C., Neurath, M. F., & Engel, M. A. (2016). Transient receptor potential melastatin 8 ion channel in macrophages modulates colitis through a balance-shift in TNF-alpha and interleukin-10 production. *Mucosal Immunology*, *9*(6), 1500–1513. https://doi.org/10.1038/mi.2016.16

Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P. H., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M. M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the British Journal of Pharmacology: Updated guidance for 2020. *British Journal of Pharmacology*, *177*, 3611–3616. https://doi.org/10.1111/bph.15178

Linte, R. M., Ciobanu, C., Reid, G., & Babes, A. (2007). Desensitization of cold- and menthol-sensitive rat dorsal root ganglion neurones by inflammatory mediators. *Experimental Brain Research*, *178*(1), 89–98. https://doi.org/10.1007/s00221-006-0712-3

Liu, B., Fan, L., Balakrishna, S., Sui, A., Morris, J. B., & Jordt, S. E. (2013). TRPM8 is the principal mediator of menthol-induced analgesia of acute and inflammatory pain. *Pain*, *154*(10), 2169–2177. https://doi.org/10.1016/j.pain.2013.06.043

Liu, B., & Qin, F. (2005). Functional control of cold- and menthol-sensitive TRPM8 ion channels by phosphatidylinositol 4,5-bisphosphate. *The Journal of Neuroscience*, *25*(7), 1674–1681. https://doi.org/10.1523/JNEUROSCI.3632-04.2005

Liu, F., Ruiz, M. S., Austin, D. A., & Webster, N. J. (2005). Constitutively active Gq impairs gonadotropin-releasing hormone-induced intracellular signaling and luteinizing hormone secretion in LβT2 cells. *Molecular Endocrinology*, *19*(8), 2074–2085. https://doi.org/10.1210/me.2004-0145

McCoy, D. D., Knowlton, W. M., & McKemy, D. D. (2011). Scraping through the ice: Uncovering the role of TRPM8 in cold transduction. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology*, *300*(6), R1278–R1287. https://doi.org/10.1152/ajpregu.00631.2010

Miggin, S. M., & Kinsella, B. T. (2002). Investigation of the mechanisms of G protein: Effector coupling by the human and mouse prostacyclin

receptors. Identification of critical species-dependent differences. *The Journal of Biological Chemistry*, *277*(30), 27053–27064. https://doi.org/10.1074/jbc.M203353200

Miggin, S. M., & Kinsella, B. T. (2018). Investigation of the mechanisms of G protein: Effector coupling by the human and mouse prostacyclin receptors. Identification of critical species-dependent differences. *The Journal of Biological Chemistry*, *293*(31), 12285. https://doi.org/10.1074/jbc.W118.004804

Moncada, S., & Vane, J. R. (1979). The role of prostacyclin in vascular tissue. *Federation Proceedings*, *38*(1), 66–71.

Murata, T., Ushikubi, F., Matsuoka, T., Hirata, M., Yamasaki, A., Sugimoto, Y., Ichikawa, A., Aze, Y., Tanaka, T., Yoshida, N., Ueno, A., Oh-ishi, S., & Narumiya, S. (1997). Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature*, *388*(6643), 678–682. https://doi.org/10.1038/41780

Nishimura, T., Yamamoto, T., Komuro, Y., & Hara, Y. (1995). Antiplatelet functions of a stable prostacyclin analog, SM-10906 are exerted by its inhibitory effect on inositol 1,4,5-trisphosphate production and cytosolic Ca^{++} increase in rat platelets stimulated by thrombin. *Thrombosis Research*, *79*(3), 307–317. https://doi.org/10.1016/0049-3848(95)00117-a

Oida, H., Namba, T., Sugimoto, Y., Ushikubi, F., Ohishi, H., Ichikawa, A., & Narumiya, S. (1995). In situ hybridization studies of prostacyclin receptor mRNA expression in various mouse organs. *British Journal of Pharmacology*, *116*(7), 2828–2837. https://doi.org/10.1111/j.1476-5381.1995.tb15933.x

Peng, Q., Alqahtani, S., Nasrullah, M. Z. A., & Shen, J. (2021). Functional evidence for biased inhibition of G protein signaling by YM-254890 in human coronary artery endothelial cells. *European Journal of Pharmacology*, *891*, 173706. https://doi.org/10.1016/j.ejphar.2020.173706

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Würbel, H. (2020). The ARRIVE guidelines 2.0: updated guidelines for reporting animal research. *PLOS Biology*, *18*, e3000410. https://doi.org/10.1371/journal.pbio.3000410

Premkumar, L. S., Raisinghani, M., Pingle, S. C., Long, C., & Pimentel, F. (2005). Downregulation of transient receptor potential melastatin 8 by protein kinase C-mediated dephosphorylation. *The Journal of Neuroscience*, *25*(49), 11322–11329. https://doi.org/10.1523/JNEUROSCI.3006-05.2005

Proudfoot, C. J., Garry, E. M., Cottrell, D. F., Rosie, R., Anderson, H., Robertson, D. C., Fleetwood-Walker, S. M., & Mitchell, R. (2006). Analgesia mediated by the TRPM8 cold receptor in chronic neuropathic pain. *Current Biology*, *16*(16), 1591–1605. https://doi.org/10.1016/j.cub.2006.07.061

Ramachandran, R., Hyun, E., Zhao, L., Lapointe, T. K., Chapman, K., Hirota, C. L., Ghosh, S., McKemy, D. D., Vergnolle, N., Beck, P. L., Altier, C., & Hollenberg, M. D. (2013). TRPM8 activation attenuates inflammatory responses in mouse models of colitis. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(18), 7476–7481. https://doi.org/10.1073/pnas.1217431110

Reid, G., Amuzescu, B., Zech, E., & Flonta, M. L. (2001). A system for applying rapid warming or cooling stimuli to cells during patch clamp recording or ion imaging. *Journal of Neuroscience Methods*, *111*(1), 1–8. https://doi.org/10.1016/s0165-0270(01)00416-2

Ricciotti, E., & FitzGerald, G. A. (2011). Prostaglandins and inflammation. *Arteriosclerosis, Thrombosis, and Vascular Biology*, *31*(5), 986–1000. https://doi.org/10.1161/ATVBAHA.110.207449

Rohacs, T. (2014). Phosphoinositide regulation of TRP channels. *Handbook of Experimental Pharmacology*, *223*, 1143–1176. https://doi.org/10.1007/978-3-319-05161-1_18

Ronchetti, S., Migliorati, G., & Delfino, D. V. (2017). Association of inflammatory mediators with pain perception. *Biomedicine & Pharmacotherapy*, *96*, 1445–1452. https://doi.org/10.1016/j.biopha.2017.12.001

Sabnis, A. S., Reilly, C. A., Veranth, J. M., & Yost, G. S. (2008). Increased transcription of cytokine genes in human lung epithelial cells through activation of a TRPM8 variant by cold temperatures. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *295*(1), L194–L200. https://doi.org/10.1152/ajplung.00072.2008

Sarria, I., & Gu, J. (2010). Menthol response and adaptation in nociceptive-like and nonnociceptive-like neurons: Role of protein kinases. *Molecular Pain*, *6*, 47. https://doi.org/10.1186/1744-8069-6-47

Sherkheli, M. A., Gisselmann, G., Vogt-Eisele, A. K., Doerner, J. F., & Hatt, H. (2008). Menthol derivative WS-12 selectively activates transient receptor potential melastatin-8 (TRPM8) ion channels. *Pakistan Journal of Pharmaceutical Sciences*, *21*(4), 370–378.

Silverman, H. A., Chen, A., Kravatz, N. L., Chavan, S. S., & Chang, E. H. (2020). Involvement of neural transient receptor potential channels in peripheral inflammation. *Frontiers in Immunology*, *11*, 590261. https://doi.org/10.3389/fimmu.2020.590261

Smyth, E. M., Li, W. H., & FitzGerald, G. A. (1998). Phosphorylation of the prostacyclin receptor during homologous desensitization. A critical role for protein kinase c. *The Journal of Biological Chemistry*, *273*(36), 23258–23266. https://doi.org/10.1074/jbc.273.36.23258

Veldhuis, N. A., Poole, D. P., Grace, M., McIntyre, P., & Bunnett, N. W. (2015). The G protein-coupled receptor-transient receptor potential channel axis: Molecular insights for targeting disorders of sensation and inflammation. *Pharmacological Reviews*, *67*(1), 36–73. https://doi.org/10.1124/pr.114.009555

Wang, X. P., Yu, X., Yan, X. J., Lei, F., Chai, Y. S., Jiang, J. F., Yuan, Z. Y., Xing, D. M., & du, L. J. (2017). TRPM8 in the negative regulation of TNFalpha expression during cold stress. *Scientific Reports*, *7*, 45155. https://doi.org/10.1038/srep45155

Woodward, D. F., Jones, R. L., & Narumiya, S. (2011). International Union of Basic and Clinical Pharmacology. LXXXIII: Classification of prostanoid receptors, updating 15 years of progress. *Pharmacological Reviews*, *63*(3), 471–538. https://doi.org/10.1124/pr.110.003517

Zhang, C., Qin, J., Zhang, S., Zhang, N., Tan, B., Siwko, S., Zhang, Y., Wang, Q., Chen, J., Qian, M., Liu, M., & du, B. (2020). ADP/$P2Y_1$ aggravates inflammatory bowel disease through ERK5-mediated NLRP3 inflammasome activation. *Mucosal Immunology*, *13*(6), 931–945. https://doi.org/10.1038/s41385-020-0307-5

Zhang, X. (2019). Direct $G\alpha_q$ gating is the sole mechanism for TRPM8 inhibition caused by bradykinin receptor activation. *Cell Reports*, *27*(12), 3672–3683 e3674. https://doi.org/10.1016/j.celrep.2019.05.080

Zhang, X., Mak, S., Li, L., Parra, A., Denlinger, B., Belmonte, C., & McNaughton, P. A. (2012). Direct inhibition of the cold-activated TRPM8 ion channel by $G\alpha_q$. *Nature Cell Biology*, *14*(8), 851–858. https://doi.org/10.1038/ncb2529

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