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Institute of Biochemistry

PhD THESIS SUMMARY

Validation of a multiplex platform for the functional analysis of G Protein-Coupled Receptors (GPCRs) and ion channels

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Study aim

G-protein-coupled receptors (GPCRs) and transient receptor potential (TRP) channels are among the most significant membrane proteins in cellular signaling. GPCRs constitute the largest family of receptors, representing the target for approximately 40% of marketed drugs. They mediate numerous physiological processes by transducing extracellular signals into intracellular responses by interacting with G-proteins. On the other hand, TRP channels are a diverse group of ion channels that respond to various stimuli, including temperature, pH, and mechanical stress. Both GPCRs and TRP channels are crucial in the context of pain, inflammation, and sensory perception, making them prime targets in drug discovery. Their ability to regulate complex signaling pathways positions them as potential therapeutic targets for various conditions, including chronic pain, cardiovascular diseases, and neurological disorders.

The overarching aim of this thesis is to validate a multiplex platform initially designed for the HTS screening of GPCR-targeting compounds and use it for the functional analysis of G-protein-coupled receptors and ion channels, with a particular focus on the interaction between prostacyclin receptors (IP-R) and transient receptor potential melastatin 8 (TRPM8) channels.

In the first part of the thesis, we aimed to optimise assays based on the BRET technology to accurately measure cyclic adenosine monophosphate and intracellular calcium levels. These two molecules are crucial second messengers in cellular signaling pathways mediated by GPCRs and ion channels. By refining these assays, we wanted to establish a reliable and sensitive method for evaluating the functional states of GPCRs and ion channels. This optimisation included adjusting assay conditions, validating measurement techniques, and ensuring reproducibility across different experimental setups.

The second part of the thesis focuses on using the optimised multiplex platform to investigate the functional interaction between IP-R and TRPM8 channels. TRPM8 is a well-known ion channel involved in sensing cold temperatures and mediating anti-inflammatory and analgesic effects. Conversely, prostacyclin and its receptor IP-R are associated with promoting inflammation and pain by activating specific signaling pathways.

Given the critical roles of TRPM8 and IP-R in regulating pain and inflammatory responses and their overlapping expression patterns in various tissues, this study explores the hypothesis that IP-R activation can modulate TRPM8 function. Specifically, we examine how

IP-R agonists affect TRPM8 channel activity in heterologous expression systems in HEK293T cells and in DRG neurons from mice.

Furthermore, understanding the specific pathways through which IP-R modulates TRPM8 activity can reveal novel insights into the molecular mechanisms underlying inflammatory pain. This knowledge could potentially inform the development of new therapeutic strategies targeting these pathways to treat chronic pain and inflammatory conditions. By achieving these objectives, this thesis aims to contribute to the broader understanding of GPCR-TRP channel interactions, specifically how prostacyclin receptor signaling influences TRPM8 function.

Chapter 1. INTRODUCTION

1.1. G protein-coupled receptors

G protein-coupled receptors constitute the largest and the most diverse family of membrane proteins, with over 800 members identified in the human genome. They play pivotal roles in virtually every physiological process, including sensory perception, neurotransmission, immune response, and hormone signaling (M. Zhang et al., 2024).

About half of the GPCRs identified in man have sensory functions. They mediate olfaction, taste, light perception, and odour signaling. The rest of the non-sensory GPCRs mediate signaling through various ligands, from small molecules to peptides or large proteins (Alexander et al., 2021). Central to their function is the ability to interact with heterotrimeric G proteins, which serve as molecular switches that transduce signals from the extracellular compartments to intracellular effector proteins (Hamm, 1998).

G protein-coupled receptors share a typical architecture, representing a structurally conserved framework essential for their diverse roles. Each GPCR typically comprises a single polypeptide characterised by an extracellular N-terminus, facilitating ligand binding, and an intracellular C-terminus, which interacts with intracellular signaling molecules. The core of the GPCR consists of seven hydrophobic transmembrane domains (TM1-TM7) arranged in a helical bundle that traverses the lipid bilayer of the cell membrane. These transmembrane helices serve as a scaffold for ligand recognition and signal transduction, with specific amino acids lining the interior of the transmembrane bundle, forming the ligand-binding pocket. Connecting the transmembrane helices are three extracellular loops (ECL1-ECL3) and three intracellular loops (ICL1-ICL3), which provide additional structural support and contribute to

the ligand-binding interface and receptor activation (Katritch et al., 2013). Ligand binding (Figure 1) induces conformational changes in the receptor, activating downstream signaling pathways.

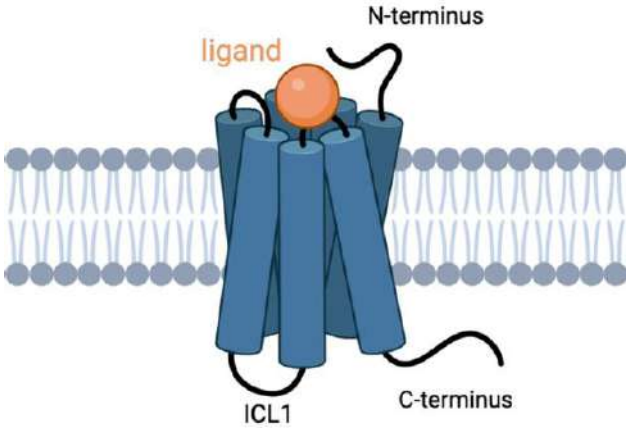

Figure 1. Schematic representation of the three-dimensional structure of GPCR. The transmembrane helices serve as a scaffold for ligand recognition and signal transduction, and specific amino acids within the helices form the ligand-binding pocket. Additionally, extracellular and intracellular loops contribute to receptor activation (Created with BioRender).

GPCRs transduce extracellular signals into intracellular responses by coupling to heterotrimeric G proteins, which serve as molecular switches. These G proteins are composed of three subunits: α, β, and γ. Upon ligand binding to the GPCR, a conformational change occurs, allowing the receptor to interact with the α subunit, leading to its activation and subsequent dissociation from the dimeric βγ subunit.

The G protein cycles between an inactive GDP-bound state and an active GTP-bound state. In its active state, the α subunit is bound to GDP and associated with the βγ subunits. Upon receptor activation, GDP is exchanged for GTP, leading to the dissociation of the α subunit from the βγ dimer. Both the α subunit and βγ dimer can then modulate downstream effector proteins, leading to various cellular responses. Notably, the α subunit possesses intrinsic GTPase activity, allowing it to hydrolyse GTP to GDP, thereby terminating signaling and returning to its inactive state (Wettschureck & Offermanns, 2005).

The functional versatility of the G protein-mediated signaling system arises from its modular design and the presence of multiple G protein subtypes. These subtypes are categorised into four families based on their α subunit, namely $G_{\alpha s}$, $G_{\alpha i}/G_{\alpha o}$, $G_{\alpha q}/G_{\alpha 11}$, $G_{\alpha 12}/G_{\alpha 13}$. Characteristic coupling patterns between receptors and G proteins have been

identified for most receptors, leading to an alternative classification of GPCRs into four main categories based on their association with G_α subtypes.

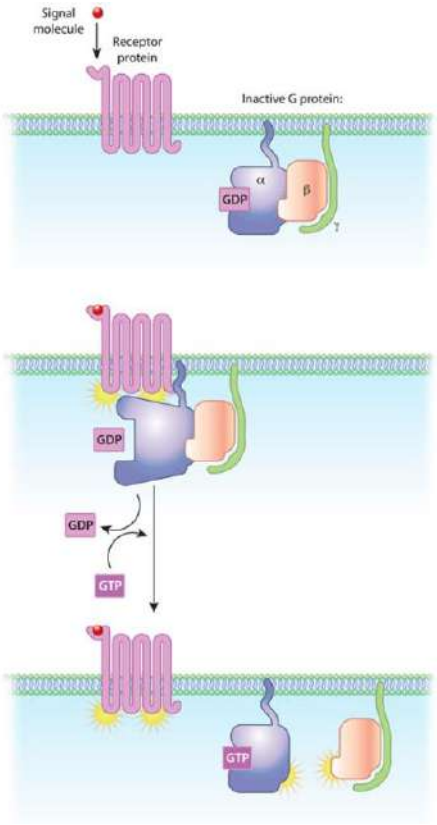

Figure 2. G proteins during receptor activation across the plasma membrane. The α, β, and γ are depicted with different associations to the plasma membrane. Upon GDP-GTP exchange on the α subunit, both α subunit and the βγ the complex can interact with other molecules to promote signaling cascades. Notably, both α subunit and the βγ complex remains anchored to the plasma membrane during their activation. These activated subunits can further interact with ion channels, other membrane proteins and secondary messengers dispersed throughout the cell (Nature Education, 2010).

The $G_{\alpha s}$ family of G proteins, ubiquitously expressed throughout the body, plays a pivotal role in activating the cAMP-dependent pathway. This family comprises the $G_{\alpha s}$ protein encoded by the GNAS gene and $G_{\alpha olf}$, primarily expressed within the olfactory system and encoded by the GNAL gene. While $G_{\alpha s}$ serve as fundamental mediators of intracellular signaling, $G_{\alpha olf}$ plays a more specialised role, primarily involved in olfactory signal transduction.

The cAMP-dependent pathway begins with the activation of GPCRs by extracellular ligands. Upon receptor activation, $G_{\alpha s}$ proteins are stimulated, leading to the dissociation from

the βγ complex. The liberated $G_{\alpha s}$ protein then binds to and activates adenylyl cyclase (AC), catalysing the conversion of ATP to cyclic AMP (cAMP). Increased cAMP levels subsequently activate cAMP-dependent protein kinase, known as PKA, by releasing its regulatory subunits, allowing the catalytic subunits to phosphorylate target proteins. These phosphorylation events modulate the activity of downstream effectors, transcription factors, and ion channels, mediating a wide range of cellular responses.

Another major signaling pathway GPCRs activate is mediated by the $G_{\alpha q/11}$ family of G proteins. This family is also called $G_{q/11/14/15}$ to include closely related family members, although $G_{\alpha q}$ and $G_{\alpha 11}$ appear to be expressed ubiquitously. Like the other signaling pathways, $G_{\alpha q/11}$ are activated through ligand binding to the Gq-coupled GPCRs. This activation causes the $G_{\alpha q}$ subunit to dissociate from the βγ dimer, which then modulates the activity of downstream effectors. The primary effector of this signaling pathway is phospholipase C-β (PLC-β), which hydrolyses phosphatidylinositol 4,5-biphosphate (PIP2) into two essential secondary messengers: inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 diffuses through the cytoplasm to bind to IP3 receptors on the endoplasmic reticulum, triggering the release of calcium ions into the cytosol. The increased intracellular calcium concentration leads to various cellular responses, including muscle contraction, secretion, and metabolism. Concurrently, DAG remains anchored to the plasma membrane due to its hydrophobic nature and activates protein kinase C (PKC), which phosphorylates target proteins to propagate the signal further (Wettschureck & Offermanns, 2005).

1.1.1. IP receptor

The prostacyclin receptor, also known as prostaglandin I_2 Receptor, or IP-R, is a GPCR activated by prostacyclin (PGI_2) and a member of the prostanoid family of lipid mediators. The gene encoding the human IP is PTGIR, located on chromosome 19q13.32. PTGIR expression is widespread but is particularly prominent in the endothelium of blood vessels, platelets, smooth muscle cells, kidneys, and specific immune cells such as macrophages and dendritic cells.

The endogenous ligand of the prostacyclin receptor is PGI_2, which is highly unstable, making it difficult to determine its exact affinity. This instability led to synthesising stable analogues for research and clinical usage. Among these compounds are cicaprost, iloprost, and carbocyclic, with cicaprost exhibiting the highest selectivity and potency than the endogenous

ligand. Several synthetic compounds have been used as antagonists, namely CAY10441, TG6-126, and RO3244794, the latter appearing to be the most specific (Clapp et al., 2023).

The IP receptor primarily couples to Gs proteins, resulting in elevated cAMP levels and mediating prostacyclin's effects. However, depending on the cell type, the IP receptor may also signal through other signaling pathways, including Gq- and Gi-dependent signaling cascades.

Prostacyclin plays a crucial role in maintaining cardiovascular homeostasis. Activation of the IP-R induces vasodilation, lowering blood pressure and improving blood flow. This effect primarily occurs through the cAMP-dependent pathway, which relaxes smooth muscle cells. Additionally, prostacyclin inhibits platelet aggregation, preventing thrombosis occurrence and exerting cardioprotective effects, a process also associated with increased cAMP concentration in platelets. Within the lungs, IP-R promotes pulmonary blood pressure and decreases bronchial hyper-responsiveness, while within the kidneys, it regulates renal blood flow and glomerular filtration rate.

IP-R plays a significant role as a mediator of inflammation. It reduces leukocyte adhesion and migration, downregulates the expression of pro-inflammatory cytokines, and decreases oxidative stress. It also modulates the activity of various immune cells, including macrophages and T cells, thereby influencing the immune response and inflammation. In contrast, within rheumatological conditions, IP-R exhibits pro-inflammatory responses (Stitham et al., 2011).

Furthermore, the prostacyclin receptor exhibits a complex role in pain perception. In sensory neurons, activation of the IP-R can lead to hyperalgesia an increased sensitivity to pain, particularly in inflammation. This hyperalgesic effect is mediated by the elevation of cAMP levels, which enhance the excitability of nociceptive neurons. However, IP-R can also exert analgesic effects under certain conditions by modulating inflammatory processes and reducing the release of pro-inflammatory cytokines. Thus, the IP receptor's involvement in pain perception is context-dependent, with effects varying based on specific pathological conditions.

1.2. Transient Receptor Potential (TRP) channels

Transient receptor potential (TRP) channels detect various cellular and environmental signals. These ion channels are present in numerous tissues and cell types and allow the passage of multiple cations, including Ca^{2+}, Mg^{2+}, Na^{+}, and K^{+}. TRP channels are involved in sensory responses such as heat, cold, pain, stress, vision, and taste, and numerous stimuli can trigger them. Their predominant location on the cell surface, interaction with various physiological

signaling pathways, and unique crystal structure make TRP channels promising targets for drug development and relevant for treating multiple diseases.

TRP channels were first identified over 50 years ago when a visual mutant in Drosophila was discovered to have a transient response to bright light. Due to its electrophysiological properties, this mutant was named "transient receptor potential" (trp). Later, the trp was cloned and recognised as a transmembrane protein. Researchers have since delved into understanding TRP channels, establishing their link to calcium transport and identifying them as light-activated, Ca^{2+} permeable channels. They also observed that TRP-like proteins resemble voltage-gated channels and confirmed the presence of ankyrin repeats in their amino acid sequences (M. Zhang et al., 2023).

At the beginning of this century, efforts were made to standardise TRP nomenclature, resulting in the classification of 28 channel subunit genes into seven subfamilies: TRPA (ankyrin), TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), TRPML (mucolipin), TRPP (polycystin), and TRPN (NO-mechano-potential) (Venkatachalam & Montell, 2007).

1.2.1. Transient Receptor Potential cation channel subfamily M member 8 (TRPM8)

TRPM8, or transient receptor potential melastatin 8, is a gene located on chromosome 2q37.1, encoding a protein composed of 1,104 amino acids. This gene is instrumental in encoding a cold and menthol receptor, pivotal for detecting cold temperatures and the sensation of coolness. TRPM8 is a polymodal ion channel, meaning it responds to various stimuli, including temperature changes, voltage, pressure, cooling agents like menthol and icilin (Pertusa et al., 2023a), and changes in osmolality. These diverse activation modes underline the complexity and versatility of TRPM8 in physiological processes.

The TRPM8 channel is a non-selective cation channel permeable to monovalent and divalent cations. This permeability is crucial in sensory perception, particularly in detecting cold. Activation of TRPM8 involves several signaling pathways, most notably the phosphatidylinositol 4,5-bisphosphate (PIP2) signaling pathway, which is essential for the channel's proper function and regulation (Izquierdo et al., 2021).

The molecular structure of TRPM8 has been elucidated through advanced techniques such as cryo-electron microscopy. This ion channel is a tetramer composed of four subunits, each containing six transmembrane domains (TM1-TM6). The region between TM5 and TM6 forms the channel, which is pore, critical for ion conduction. The N-terminal and C-terminal regions of TRPM8 are intracellular and play significant roles in channel regulation and interaction with other proteins (Palchevskyi et al., 2023).

TRPM8 is predominantly expressed in a subset of sensory neurons within the dorsal root ganglia (DRG) and trigeminal ganglia, responsible for sensing environmental cold and cool stimuli. It is also present in other tissues, such as the skin, cornea, and bladder, contributing to various physiological responses. TRPM8 mediates the cold sensation in these tissues and is involved in cold thermotransduction. When activated, TRPM8 channels allow the influx of cations like Ca^{2+}, leading to neuronal depolarisation and the subsequent transmission of cold signals to the central nervous system.

In addition to its role in sensory perception, TRPM8 modulates cellular responses to inflammation and pain. It interacts with signaling molecules and pathways such as phospholipase C (PLC) and protein kinase C (PKC). For example, TRPM8 activation can modulate inflammatory responses by affecting cytokine production in immune cells. This modulation underscores the importance of TRPM8 in maintaining homeostasis and responding to environmental stimuli (Voets, 2007).

TRPM8 plays a significant role in pain modulation, particularly in cold-induced pain. It is deeply involved in the sensation and modulation of pain, contributing significantly to conditions like cold allodynia, where non-painful cold stimuli become painful following nerve injury or inflammation. TRPM8's activation by cooling agents such as menthol is well-documented for providing analgesic effects. These effects are not only due to the direct activation of TRPM8 but also complex interactions with other pain pathways, including those mediated by TRPV1 channels.

In inflammatory contexts, TRPM8 activation can influence immune cell function. For instance, in macrophages, TRPM8 affects phagocytosis and cytokine production. In T-cells, inhibition of TRPM8 impacts the production of critical cytokines like IL-2 and IL-6, suggesting a regulatory role in immune responses. This dual role in both nociceptive and anti-nociceptive pathways highlights TRPM8's complex nature, where its effects on pain can be highly context-dependent, influenced by the overall state of sensory neurons and the presence of other stimuli (Dussor & Cao, 2016).

TRPM8 is a multifaceted ion channel with significant roles in sensory perception, immune regulation, cancer progression, and several clinical conditions. Its diverse functions and regulatory mechanisms make it a promising target for therapeutic intervention across various health issues. The ongoing research into TRPM8's functions and mechanisms will likely continue to uncover new opportunities for clinical applications and therapeutic innovations.

1.3. GPCRs and ion channel interactions

The interplay between G protein-coupled receptors and transient receptor potential channels represents a significant area of interest in cellular signaling. While GPCRs and TRP channels have been extensively studied individually for their roles in transducing extracellular signals and mediating ionic flows, their interactions introduce additional regulatory complexity and functional diversity layers.

GPCR-TRP channel interactions are pivotal in numerous physiological processes. Upon activation by various ligands, GPCRs initiate signaling cascades that can modulate TRP channel activity directly through G proteins or indirectly via secondary messengers such as diacylglycerol (DAG) and inositol triphosphate (IP3). These interactions influence critical functions, including sensory perception, cardiovascular regulation, and immune responses (Veldhuis et al., 2015).

Understanding the mechanisms behind GPCR-TRP channel interactions provides insights into how cells integrate multiple signals to maintain homeostasis and respond to environmental changes. Dysregulation of these pathways can lead to various pathological conditions, including chronic pain, inflammation, and cardiovascular diseases, highlighting the therapeutic potential of targeting these interactions.

The interactions between G protein-coupled receptors (GPCRs) and transient receptor potential (TRP) channels involve complex signaling mechanisms that enable cells to integrate and respond to various external stimuli. These interactions occur through several pathways, each adding layers of regulatory complexity and functional diversity.

One primary mechanism is direct G protein coupling. Upon activation by their respective ligands, GPCRs undergo conformational changes that facilitate the activation of heterotrimeric G proteins. The activated Gα and Gβγ subunits can directly interact with TRP channels, modulating their activity (Zhao & MacKinnon, 2023). For instance, the Gαi subunit has been shown to activate TRPC4 and TRPC5 channels directly, significantly regulating vascular tone and cardiac function. This direct coupling allows for rapid and precise modulation of TRP channel activity in response to extracellular signals (Kang et al., 2024).

Another significant pathway involves the activation of phospholipase C (PLC). Upon GPCR activation, the Gαq subunit activates PLC, leading to the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol trisphosphate (IP3). DAG can directly activate specific TRP channels, such as TRPC3, TRPC6, and TRPC7, which are implicated in processes like smooth muscle contraction and cell proliferation (Veldhuis et al., 2015). IP3 facilitates calcium release from intracellular stores,

which can indirectly modulate TRP channel activity by altering the intracellular calcium concentration. This PLC pathway exemplifies how GPCRs can indirectly influence TRP channel function through secondary messengers, linking extracellular signals to intracellular calcium dynamics (Clapham, 2003).

GPCR activation can generate secondary messengers such as cyclic AMP (cAMP) and activate protein kinases like PKA and PKC. These secondary messengers and kinases can phosphorylate TRP channels, modulating their activity. For instance, TRPV1 channels, critical in pain perception, can be sensitised by PKA and PKC following GPCR activation by inflammatory mediators like bradykinin (Montell, 2005). This sensitisation enhances the channel's responsiveness to noxious stimuli, illustrating the role of GPCR-TRP interactions in amplifying sensory signals under inflammatory conditions (Ramsey et al., 2006). Conversely, activation of Gαi-coupled receptors decreases adenylyl cyclase activity, reducing cAMP levels and subsequent PKA activation. This pathway is involved in the peripheral analgesic effects of opioids and cannabinoids through their action on Gαi-coupled receptors, which reduces TRPV1 receptor currents (Salzer et al., 2019).

An example of regulation mediated by GPCRs is the modulation of TRPM8, one of this thesis's central points. The regulation of TRPM8 by GPCRs, encompasses a variety of intricate mechanisms that highlight the channel's role in sensory and non-sensory functions. One prominent pathway involves the thyroid hormone derivative 3-iodothyronine (3-T1AM), which can modulate TRPM8 directly or through β-adrenergic receptor-mediated signaling. This interaction involves the βγ subunits of Gi/o proteins, resulting in elevated intracellular calcium levels and the inhibition of TRPV1 activity, thereby showcasing a potential anti-inflammatory effect. The complex interplay between TRPM8 and TRPV1 suggests a nuanced regulatory network influenced by 3-T1AM, impacting both thermoregulation and inflammatory responses (Khajavi et al., 2017).

GPCR-mediated regulation of TRPM8 also involves the PIP2 pathway. Activation of TRPM8 channels leads to calcium influx, which activates PLC. This activation results in the hydrolysis of PIP2, a critical lipid for TRPM8 function, thereby reducing channel activity. Specific residues within TRPM8, such as R688, R850, and R997, are essential for PIP2 binding, highlighting the significance of this interaction (Pertusa et al., 2023b). Additionally, pathways involving the α2A adrenoreceptor (α2A-AR) and Gi proteins lead to decreased cAMP levels and reduced PKA activity, culminating in the inhibition of TRPM8. This pathway underscores the intricate balance of intracellular signaling events that finely tune TRPM8 activity in response to various stimuli (Bavencoffe et al., 2010).

Furthermore, the direct inhibition of TRPM8 by the Gαq subunit represents a unique regulatory mechanism. Inflammatory mediators like bradykinin utilise this pathway by activating bradykinin receptors, where Gαq binds directly to TRPM8, causing a conformational change that inhibits channel activity. This interaction bypasses conventional signaling pathways, emphasising the specificity of Gαq in modulating TRPM8 (X. Zhang et al., 2012). Additionally, endogenous ligands such as anandamide and N-arachidonoyl-dopamine (NADA), which act through cannabinoid receptors like CB1, function as antagonists of TRPM8, further highlighting the diverse regulatory landscape (De Petrocellis et al., 2007). The modulation of TRPM8 by various GPCR pathways illustrates its pivotal role in sensory perception, particularly in cold sensation and pain. It opens avenues for potential therapeutic interventions targeting inflammatory hyperalgesia and cold allodynia.

The initial step in investigating the interactions between GPCRs and TRP channels involves the establishment of robust and precise experimental assays. This process requires meticulous selection and optimisation of assay conditions to accurately reflect the physiological environment and ensure the results' reproducibility and reliability.

The commonly used techniques to study GPCR-TRP channel interactions are co-immunoprecipitation (Co-IP) and pull-down assays. These methods detect and confirm protein-protein interactions in biological samples. For instance, Zhang (2019) utilised pull-down assays to demonstrate the interaction between the Gαq subunit and the TRPM8 channel, shedding light on the signaling mechanisms regulating TRP channels by Gq-coupled GPCRs (X. Zhang, 2019).

However, not all GPCR-TRP channel interactions involve direct contact; many are functional, necessitating techniques like electrophysiology and calcium imaging to study these functional interactions comprehensively. These methods are widely used due to their high resolution in measuring ion channel activity and intracellular calcium dynamics. They are often combined with pharmacological studies using agonists and antagonists and *in vivo* behavioural studies to understand these interactions comprehensively (Quallo et al., 2017).

The interactions between GPCRs and TRP channels hold significant promise in drug discovery, offering numerous opportunities for novel therapeutic interventions. Understanding the intricate crosstalk between these receptor families can lead to identifying new drug targets and designing more effective and specific pharmacological agents.

Targeting the interfaces or modulatory mechanisms of GPCR-TRP channel interactions offers opportunities for developing drugs with enhanced specificity and efficacy. This approach can fine-tune specific signaling pathways and minimise side effects. Studying interactions

where GPCRs influence TRP channel activity indirectly through second messengers or directly via protein-protein interactions, provides new avenues for creating precise therapeutic interventions. These modulatory mechanisms can offer more selective targeting options, potentially leading to fewer side effects and improved patient outcomes. This strategy is particularly promising for conditions like chronic pain, where traditional therapies might fail to deliver effective relief without adverse effects.

Advancements in high-throughput screening technologies and assays have facilitated the identification of novel compounds that modulate GPCR-TRP channel interactions, accelerating the drug discovery process. These technologies enable the efficient screening of large compound libraries, identifying potential drug candidates that target these interactions. The potential for pharmacological synergy, where drugs target both GPCRs and TRP channels, can lead to greater therapeutic effects, particularly in treating complex diseases involving multiple signaling pathways, such as cancer and neurodegenerative disorders.

Understanding GPCR-TRP channel interactions contributes to personalised medicine by identifying patient-specific signaling abnormalities. This knowledge allows for developing tailored therapies that address individual needs more effectively, improving therapeutic outcomes and reducing the likelihood of adverse effects.

In conclusion, studying GPCR-TRP channel interactions is pivotal in modern pharmacology and therapeutic development. These interactions offer numerous opportunities for developing effective treatments for various diseases. Targeting these interactions, from pain management to cardiovascular health, can lead to significant medical advancements.

Chapter 2. MATERIALS AND METHODS

In this study, intracellular cAMP levels were measured using the GloSensor™ technology from Promega, specifically employing the pGlo22F variant (Figure 3). This biosensor is engineered by fusing cAMP binding domains to a mutant form of luciferase derived from the firefly *Photinus pyralis*. Upon binding of cAMP, the biosensor undergoes conformational changes that significantly increase luminescence, offering a highly sensitive and quantifiable measure of intracellular cAMP.

HEK293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and seeded into 96-well plates. The following day, cells were transfected with plasmids encoding the p22F cAMP-sensitive bioluminescent probe

and the membrane proteins of interest using Lipofectamine 2000. After a 48-hour incubation period, the medium was replaced with Hank's Balanced Salt Solution (HBSS) supplemented with endotoxin-free luciferin, calcium chloride, and glucose. The cells were then incubated for an additional 2 hours at room temperature in the dark to minimise potential light interference. Following ligand stimulation, cAMP levels were quantified by measuring the bioluminescent signal using a 96-well plate reader, with results expressed in relative luminescence units (RLU).

For intracellular calcium determination, we employed a genetically encoded calcium indicator, G5A (Figure 5), as described in a previous study by (Baubet et al., 2000). This biosensor is a fusion between green fluorescent protein (GFP) and aequorin, a calcium-sensitive bioluminescent protein derived from jellyfish. The G5A biosensor functions based on the principle of bioluminescence resonance energy transfer (BRET), where the binding of calcium to aequorin induces a conformational change that results in blue light emission. This energy is subsequently transferred to GFP, leading to green light emission. HEK293T cells were transfected with plasmids encoding the G5A biosensor and specific membrane proteins. After 48 hours, the growth medium was replaced with HBSS supplemented with coelenterazine h, calcium chloride, and glucose, and the cells were incubated at 37°C for 2 hours to maintain the stability and functionality of the G5A fusion protein. Following ligand stimulation, the intracellular calcium levels were recorded by capturing the bioluminescent signal using a FlexStation 3 plate reader. The resulting data were analysed to calculate the area under the calcium transient curve (AUC), providing a comprehensive quantification of calcium signalling dynamics within the cells.

Chapter 3. RESULTS

3.1. Assay optimisation

The first step in developing a luminescence-based assay for cAMP determination is target selection and assay optimisation. As our target, we chose the prostacyclin receptor (IP-R) for intracellular cAMP determination due to its well-documented role as a Gs-coupled GPCR. This experiment allowed us to verify the assay's reliability. We used HEK293T cells, transiently transfected with the human IP-R, and assessed the effect of 1 µM of the synthetic specific compound cicaprost. As shown in Figure 3, the presence of cicaprost led to a significant elevation of cAMP levels, indicating minimal constitutive activity of IP-R.

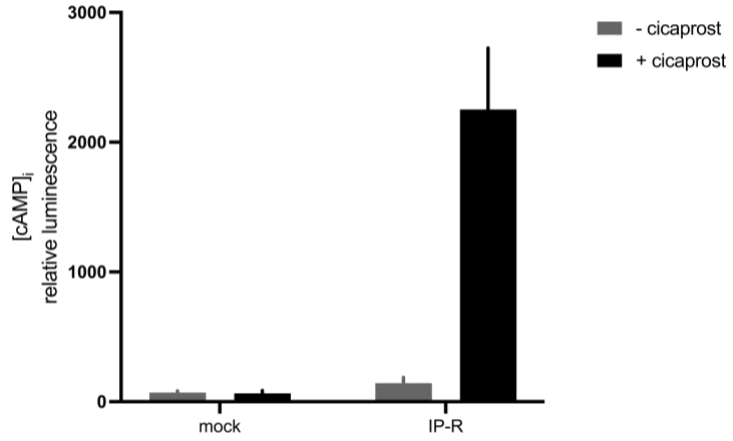

Figure 3. The effect of pre-incubation with 1 μM cicaprost for 10 minutes on cAMP accumulation in HEK293T cells expressing IP-R together with pGlo22F. Expressed as means ± SEM, n=32

Next, we chose another GPCR, GPR101, known for its constitutive activity. To investigate this, we analysed the activity of GPR101 in CHO cells transiently transfected with GPR101 and pGlo22F. As expected, in the absence of ligand stimulation, GPR101-expressing cells exhibit elevated levels of cAMP, as shown in Figure 4 (a). This constitutive activity provided a valuable model for evaluating our assay's performance, distinguishing between constitutive activity and ligand-stimulated effects.

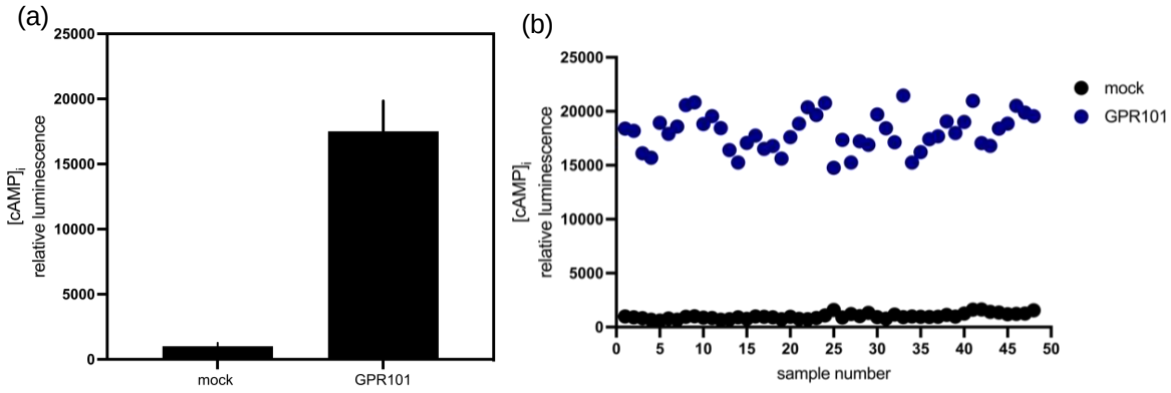

Figure 4. (a) Constitutive activity of GPR101-expressing CHO cells. Data are shown as means ± SEM, n=48; (b) Data distribution for GPR101-expressing CHO cells in the absence of ligand stimulation. Data is shown as relative luminescence, n=48

To further validate the assay's reliability for potential high-throughput screening (HTS) campaigns, we calculated essential parameters, including standard deviation, coefficient of variation, and Z' factor. As illustrated in Figure 4 (b), the data obtained were consistent, indicating the assay's precision. Table 1 presents the calculated parameters for this experiment,

with the Z' factor measured at 0.59. This value confirms the assay's robustness and suitability for HTS applications, ensuring reliable and reproducible detection of cAMP levels.

	mock	**GPR101**
Average (RLU)	981,5	17906
Median (RLU)	955	17945
Standard deviation	205	2105
CV %	0,21	0,12
Maximum	1410	22960
Minimum	610	12450
S: N		82,39
S: B		18,24
Signal window		4,748
Z' score		**0,59**

Table 1. HTS metrics characterisation for GPR101-expressing CHO cells in the absence of ligand stimulation. RLU – relative luminescence units, CV % - coefficient of variation percentage, S:N – signal-to-noise, S:B – signal-to-background

For the intracellular calcium determination assay, we used another well-described Gq-coupled GPCR, namely the human M3 receptor (hM3). HEK293T cells expressing the hM3 receptor and the calcium probe G5A were stimulated with 30 μM of the selective agonist carbachol to measure its activity. As shown in Figure 5 (a), carbachol significantly increased intracellular calcium transients in hM3-expressing cells with G5A. This robust response demonstrated the assay's capability to detect calcium flux accurately.

The data obtained from these experiments were further utilised to measure HTS metrics, which are essential for evaluating the assay's reliability in large-scale applications (Figure 5 (b)). The calculated Z score for this experiment was 0.62, as presented in Table 2. This Z' score indicates a reliable outcome, confirming that the assay is precise and suitable for HTS applications.

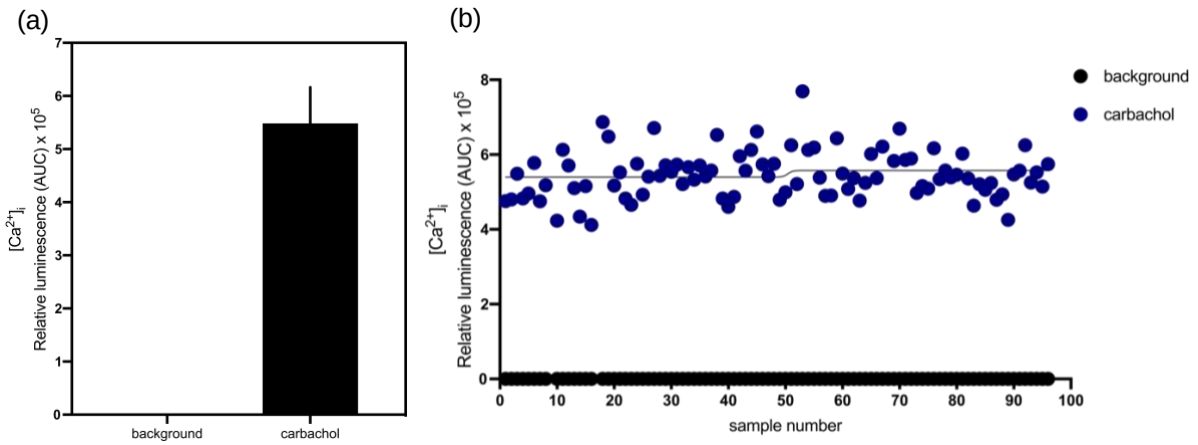

Figure 5. (a) Effect of 30 μM carbachol on HEK293T cells expressing the human M3 receptor. Data are shown as means ± SEM, n=96; (b) Data distribution for HEK293T cells expressing the hM3 receptor. Data expressed relative luminescence, n=96

	background	**carbachol**
Average (AUC)	26,826851	548302,4407
Median (AUC)	0	541454,2928
Standard deviation	40	68265,78991
CV %	1,49	0,12
Maximum	217,39	806746,8634
Minimum	0	411809,8174
S: N		13750,92849
S: B		20438,56878
Signal window		5,029732005
Z' score		**0,62**

Table 2. HTS metrics characterisation for hM3-expressing HEK293T cells in the presence of 30 μM carbachol stimulation. RLU – relative luminescence units, CV % - coefficient of variation percentage, S:N – signal-to-noise, S:B – signal-to-background

To further confirm the assay's reliability, we applied the same experimental approach to measure the activity of GPR40 (FFAR1), a GPCR that predominantly couples to Gq/11 proteins, leading to increased intracellular calcium levels. We stimulated CHO cells expressing the GPR40 receptor and G5A using 100 μM three endogenous ligands: palmitoleic acid, sodium palmitate, and linoleic acid. As shown in Figure 6 (a), this stimulation increased intracellular calcium levels, with linoleic acid inducing a distinct effect compared to the others.

Subsequently, we assessed the effect of a highly potent specific synthetic compound, TAK-895, at a concentration of 0.3 μM. As demonstrated in Figure 6 (b), TAK-895 elicited a

significantly greater response than linoleic acid, with approximately a tenfold increase. These compounds were used as controls in screening campaigns for a project aimed at repurposing FDA-approved drugs for treating type 2 diabetes.

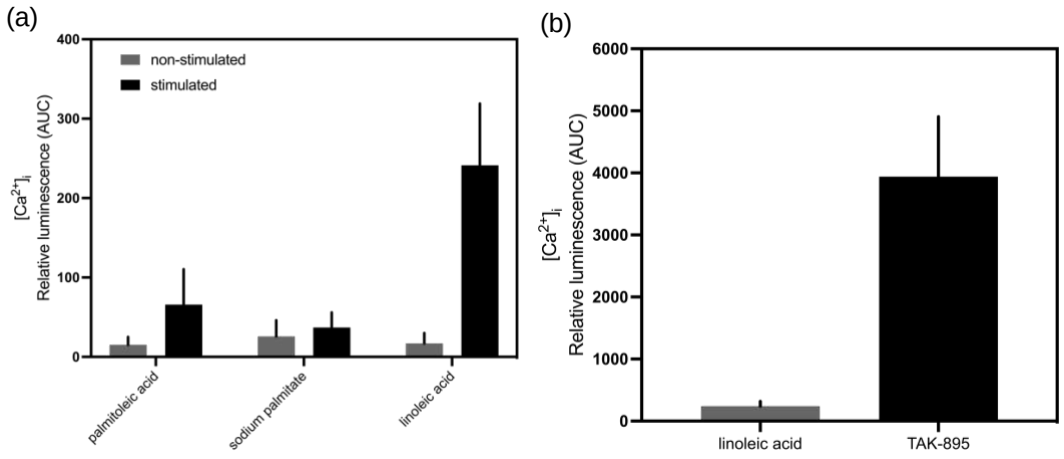

Figure 6. (a) Effect of endogenous ligands on GPR40-expressing CHO cells. Data expressed as means ± SEM, n=16; (b) Comparison between the effect of 100 μM of linoleic acid and 0,3 μM TAK-895. Data are shown as means ± SEM, n=96

We further investigated the impact of oleic acid, another endogenous ligand of GPR40, on CHO cells to evaluate its activity. As shown in Figure 7, when CHO cells expressing both GPR40 and a calcium-sensitive probe were stimulated with increasing concentrations of oleic acid, there was a marked elevation in activity compared to cells expressing only the calcium-sensitive probe. This experiment was conducted as part of a broader study aimed at understanding the role of GPR40 in the regulation of TRPV1 induced by oleic acid.

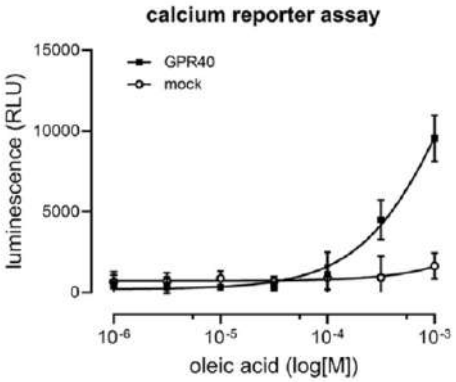

Figure 7. Effect of increasing concentrations of oleic acid on CHO cells co-expressing GPR40 and the calcium-sensitive probe, G5A. Data represented as means ± SEM of n ≥ 3 independent transfections and measurements. From Sendetski et al., 2024

The assay for intracellular calcium determination was initially designed to assess the activity of GPCRs. To explore its applicability for non-GPCR targets, we evaluated its performance with TRPM8, an ion channel that increases intracellular calcium levels. We applied the same experimental approach using HEK293T cells expressing human TRPM8 and G5A. The synthetic compound icilin served as the agonist (Figure 8 a,b).

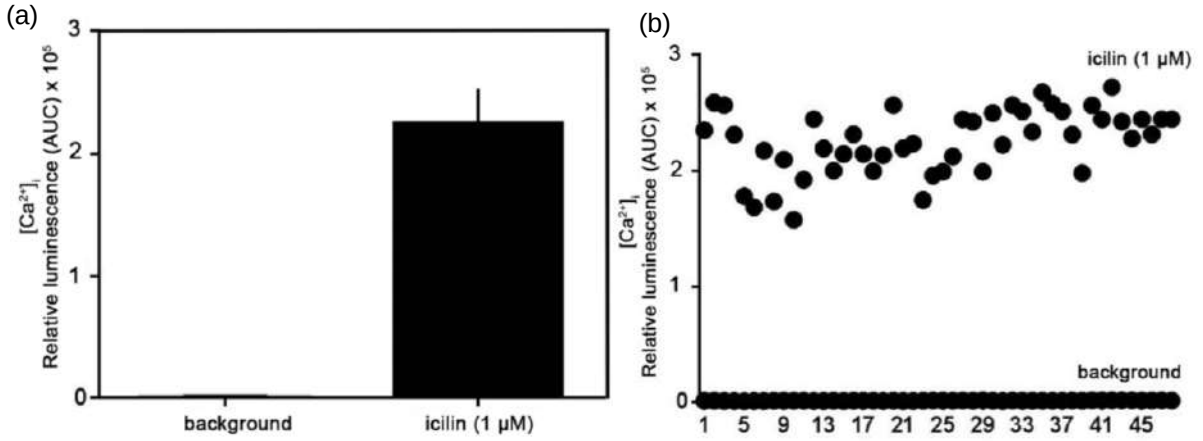

Figure 8. (a) Effect of icilin on HEK293T cells expressing the human TRPM8 and G5A. Data expressed as means ± SEM, n=48; (b) Data distribution for icilin-induced response in HEK293T cells expressing the human TRPM8 and G5A. Data expressed as relative luminescence, n=48

	background	**TRPM8**
Average (RLU)	982,7	224653
Median (RLU)	960	231244
Standard deviation	205	30875,1
CV %	0,21	0,14
Maximum	1410	278839
Minimum	610	144579
S: N		1090,2
S: B		228,605
Signal window		4,2244
Z' score		**0,58**

Table 3. HTS metrics characterisation for icilin-induced response in HEK293T cells expressing the human TRPM8 and G5A. RLU – relative luminescence units, CV % - coefficient of variation percentage, S:N – signal-to-noise, S:B – signal-to-background

The optimisation steps were essential in determining the experimental conditions required for reliable and reproducible results. These optimised conditions were then further

applied to subsequent target validation and characterisation studies, ensuring consistency and accuracy across all experiments.

3.2. Functional interaction between IP-R and TRPM8

The second part of this thesis explores the potential functional interaction between the prostacyclin receptor (IP-R) and the transient receptor potential melastatin 8 (TRPM8) ion channel. This investigation is based on their overlapping expression in specific cell types and their opposing roles in mediating inflammatory pain. IP-R activation generally promotes inflammation, whereas TRPM8 activation can reduce pain sensation.

We heterologously expressed TRPM8 and IP-R to study this interaction in HEK293T cells. The cells were then incubated with increasing concentrations of cicaprost to activate IP-R for 10 minutes. Following this incubation, we measured the impact of IP-R activation on TRPM8 activity by assessing intracellular calcium levels after stimulating TRPM8 with 1 µM icilin. As illustrated in Figure 9, the results showed that increasing concentrations of cicaprost led to a complete inhibition of TRPM8 activity. The response was dose-dependent, confirming the validity of these findings.

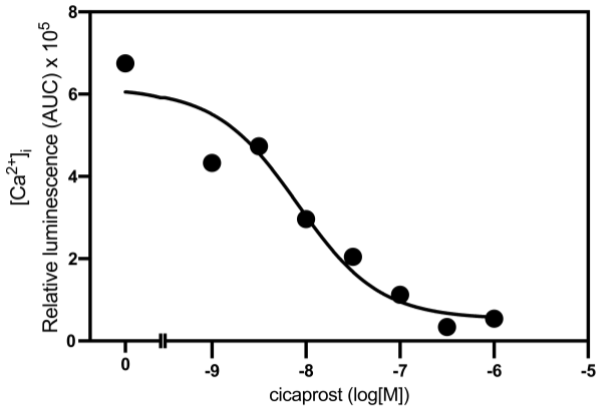

Figure 9. Effect of pre-incubation with increasing concentrations of cicaprost on the intracellular calcium evoked by 1 µM icilin-induced TRPM8 activation in HEK293T cells expressing TRPM8 and IP-R, together with G5A. Data shown as means ± SEM, n=6

To exclude the possibility that the observed effects were due to cicaprost interfering with TRPM8 as either an agonist or an antagonist, we conducted control experiments using HEK293T cells expressing only TRPM8 without IP-R. In one experiment, we directly measured the effect of cicaprost on TRPM8 activity. As shown in Figure 10 (a), increasing

concentrations of cicaprost had no impact on TRPM8 activity, indicating that cicaprost does not act as an agonist for TRPM8.

Next, we tested whether cicaprost could act as an antagonist using the same experimental setup where inhibition of TRPM8 was observed. We pre-incubated TRPM8-expressing cells with increasing concentrations of cicaprost and then stimulated them with 1 µM icilin. As illustrated in Figure 10 (b), calcium transients remained high despite the pre-incubation with cicaprost, demonstrating that cicaprost does not function as an antagonist.

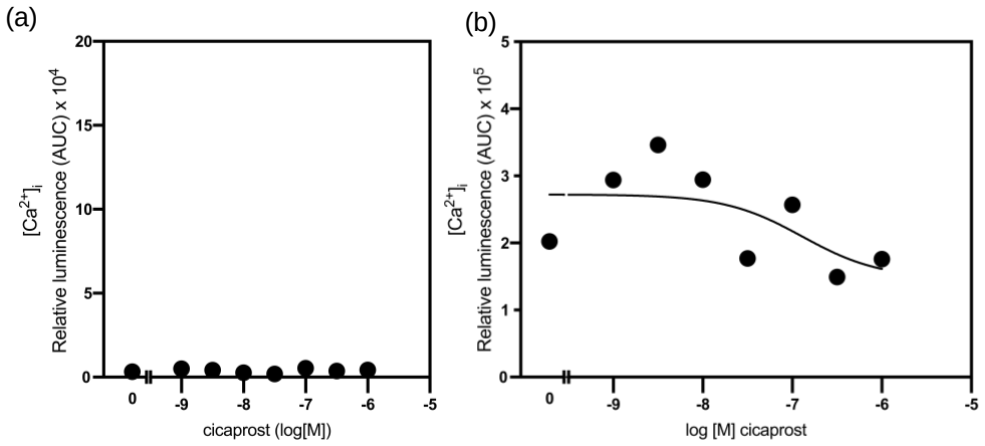

Figure 10. (a) Effect of pre-incubation with increasing concentrations of cicaprost on the TRPM8 activation in HEK293T cells expressing TRPM8 together with G5A. Data shown as means ± SEM, n=3; (b) Effect of pre-incubation with increasing concentrations of cicaprost on the intracellular calcium evoked by 0,3 µM icilin-induced TRPM8 activation in HEK293T cells expressing TRPM8 together with G5A. Data shown as means ± SEM, n=3

All the data presented so far indicate a specific inhibition of TRPM8 channel function by IP receptor agonists. We conducted a pharmacological profiling experiment to elucidate further the type of inhibition exerted by IP receptor agonists on TRPM8 channels. HEK293T cells co-expressing TRPM8 and IP receptors, along with G5A, were pre-incubated with increasing concentrations of cicaprost for 10 minutes. Subsequently, TRPM8 was stimulated with increasing concentrations of icilin.

The resulting data, depicted in Figure 11, demonstrate that cicaprost pre-incubation leads to a dose-dependent inhibition of TRPM8 activity, even at higher concentrations of icilin. Raising the concentration of cicaprost from 1 nM to 100 nM resulted in a marked decrease in the efficacy of icilin, while its potency remained unchanged. This dose-dependent inhibition suggests that the inhibition occurs in a non-competitive fashion. These findings support the hypothesis that the negative regulation of TRPM8 activity by IP-R occurs through a cellular

mediator or a signaling mechanism that does not involve direct competition at the icilin-binding site of TRPM8.

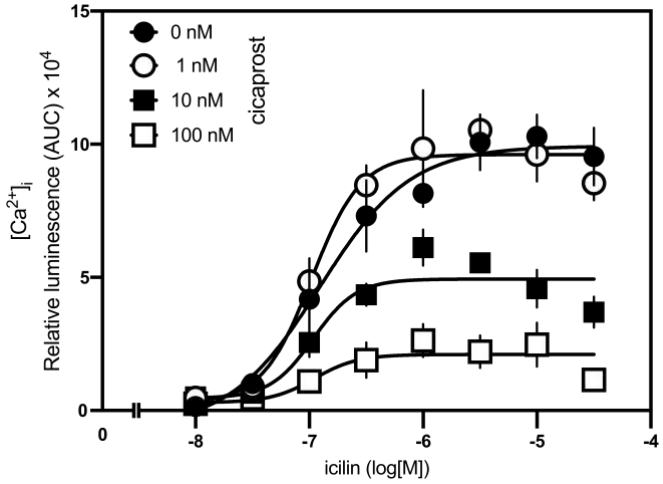

Figure 11. Effect of increasing concentrations of icilin on the intracellular calcium transients in the presence of 10 pre-incubation with increasing concentrations of cicaprost in HEK293T cells heterologously expressing TRPM8, IP-R and G5A. Data shown as means ± SEM, n=3

To further confirm the involvement of IP-R in the inhibition of TRPM8 activity, we tested the effect of a selective competitive antagonist of IP-R, Cay10444. HEK293T cells co-expressing TRPM8 and IP-R were pre-incubated with 3 µM of Cay10444 for 20 minutes before a subsequent 10-minute pre-incubation with 1 µM cicaprost. Following this treatment, we measured the calcium transients after TRPM8 activation by 1 µM icilin.

As shown in Figure 12 (a), the pre-incubation with Cay10444 effectively restored TRPM8 activity, indicating that the inhibition was mediated specifically through IP-R. This result was further corroborated by an additional experiment in which we used increasing concentrations of Cay10444. The data exhibited a dose-dependent response, as illustrated in Figure 12 (b), providing evidence that Cay10444 can competitively antagonise the inhibition of TRPM8 activity by cicaprost.

Additionally, as illustrated in Figure 12 (c), a collaborative experiment revealed that the inhibitory impact of cicaprost on TRPM8 channels exhibited a rightward shift relative to its influence on cAMP accumulation. This observation implies inhibiting TRPM8 by cicaprost-activated IP receptors may engage other intracellular mechanisms beyond the Gs-cAMP signaling pathway. These findings offer compelling evidence that cicaprost-mediated inhibition of TRPM8 is driven explicitly by IP-R activation, potentially involving alternative intracellular mediators.

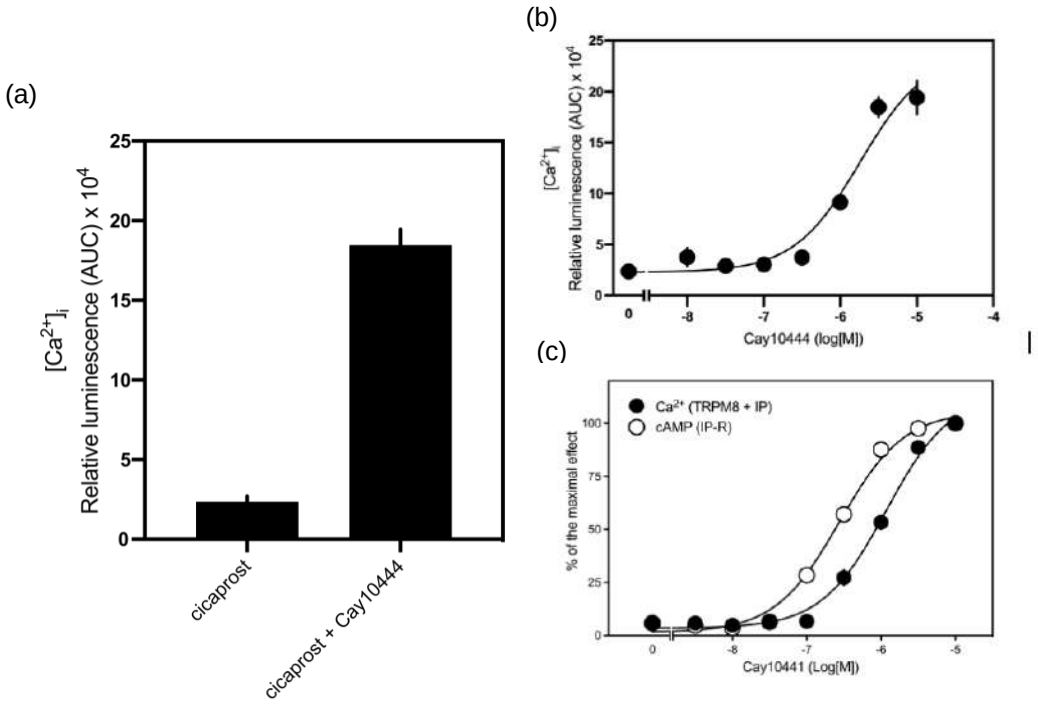

Figure 12. (a) Effect of 3 µM Cay10444 on 1 µM cicaprost-mediated inhibition of TRPM8. Data shown as means ± SEM, n=3; (b) Effect of increasing concentrations of Cay10444 on cicaprost-mediated inhibition of TRPM8. Data expressed as means ± SEM, n=3; (c) Normalised measurement of the effect of increasing concentrations of Cay10441 on cicaprost-induced cAMP accumulation in HEK293T cells heterologously expressing IP receptors and pGlo-22F compared to icilin-induced increases in intracellular calcium in cells co-expressing TRPM8 channels and IP receptors, along with G5A, and pre-treated with 1 µM cicaprost for 10 minutes. Data expressed as means ± SEM, n=5. Adapted from Trif et al., 2024

All experiments were conducted with a 10-minute pre-incubation period with cicaprost, based on the time required for cAMP accumulation. To further investigate the time dependence of IP-R involvement in the inhibition of TRPM8, we measured the effect of cicaprost pre-incubation at various time points. As illustrated in Figure 13, the inhibition of TRPM8 by cicaprost via IP receptors was indeed time-dependent. The data revealed a half-time of approximately 5 minutes for the pre-incubation of HEK293T cells expressing TRPM8 and IP receptors with 1 µM cicaprost. This finding confirms that the inhibitory effect is linked to the duration of IP-R activation, further supporting the role of IP-R in modulating TRPM8 activity.

We also investigated the possibility of functional selectivity of cicaprost by evaluating synthetic IP receptor agonists and prostaglandin analogues, including iloprost, beraprost, and treprostinil. As shown in Figure 14 a, all tested IP receptor agonists inhibited icilin-induced TRPM8 activation. Furthermore, in experiments done in collaboration under the same experimental conditions, these agonists demonstrated a dose-dependent inhibition of icilin-induced TRPM8 activation (Figure 14 b), corroborating previous findings. Given the absence

of significant differences in the maximal response and potency among the tested compounds, we selected cicaprost for subsequent experiments due to its widespread use and chemical stability as an IP agonist.

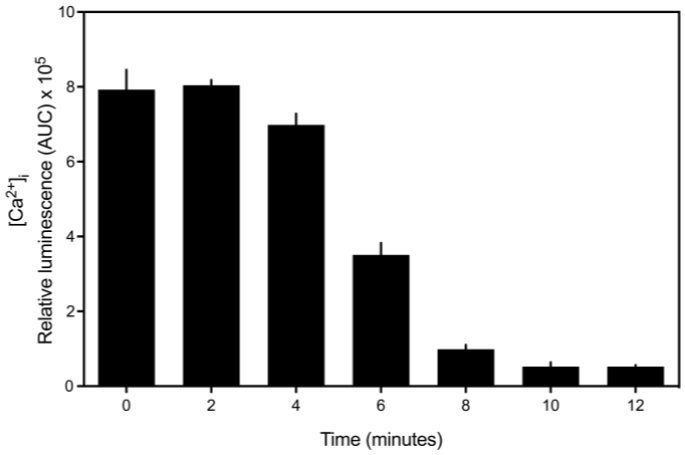

Figure 13. Time dependence of TRPM8 inhibition by 1 µM cicaprost in HEK293T cells co-expressing TRPM8, IP, and G5A, followed by stimulation with 1 µM icilin. Data are presented as means ± SEM; n = 5. From Trif et al., 2024

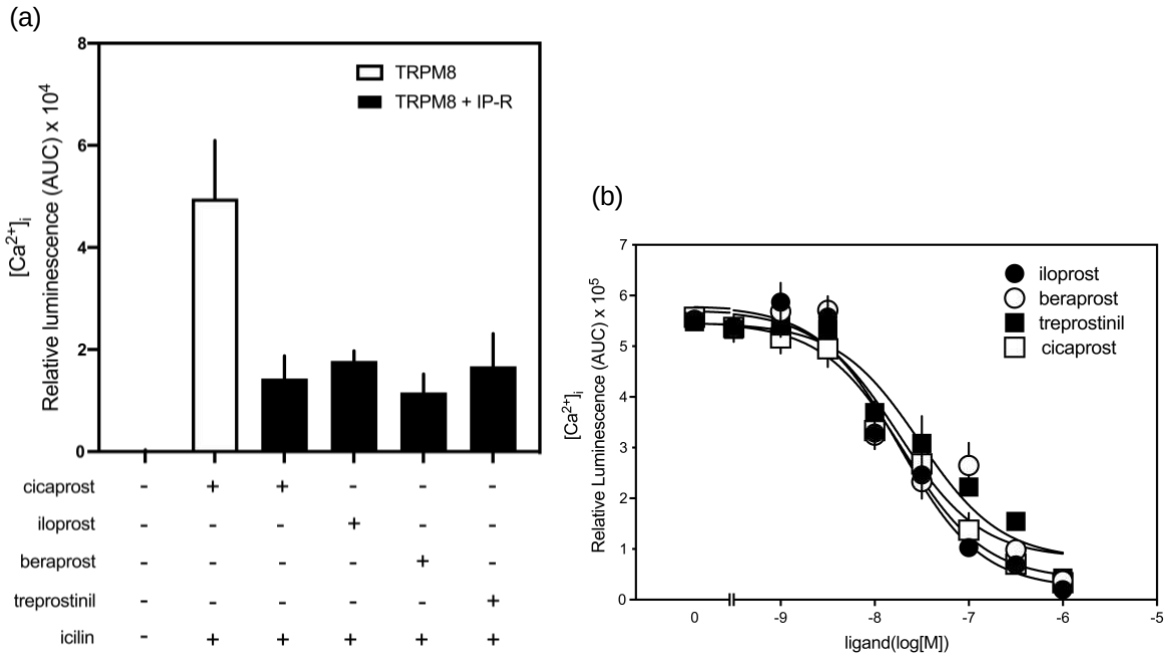

Figure 14. (a) Impact of synthetic IP-R on icilin-induced TRPM8 activation. HEK293T cells co-expressing TRPM8 channels with IP receptors and G5A were treated with 1 µM of the designated prostacyclin analogues for 10 minutes, followed by TRPM8 activation with 1 µM icilin. Data shown as means ± SEM; n = 16; (b) Effect of increasing concentrations of synthetic IP-R agonists on icilin-induced TRPM8 activation. Data shown as means ± SEM; n = 5. Adapted from Trif et al., 2024

Based on the previous data showing that TRPM8 function is unaffected by elevated intracellular cAMP levels or Gs proteins, we sought to explore the role of the Gq/11 protein-mediated pathway in the negative regulation of TRPM8 channels by IP receptors. We pre-stimulated HEK293T cells expressing both TRPM8 and IP receptors with 1 μM cicaprost for 10 minutes and then treated the cells with the Gq/11 blocker YM254890.

The pretreatment with YM254890 effectively restored TRPM8 activity in response to icilin, as shown in Figure 15 (a). This indicates that the inhibitory effect of IP receptor activation on TRPM8 is mediated through Gq/11 proteins. By blocking the Gq/11 pathway, we could negate the negative regulation imposed by the IP receptors on TRPM8 channels.

This experiment further investigated the role of the Gq/11 protein-mediated pathway by evaluating the effects of various pharmacological inhibitors (Figure 15 b) on the downregulation of TRPM8 channels via IP receptors, pre-activated with 1 μM cicaprost for 10 minutes. In HEK293T cells expressing TRPM8 and IP receptors, pretreatment with gallein, an inhibitor of G protein βγ-subunits, did not reverse the inhibition of TRPM8 function induced by cicaprost through IP receptor agonists. Similarly, blocking PLCβ with U-73122 and inhibiting protein kinases with PP2 did not prevent the suppression of TRPM8 function by IP receptor agonists. Conversely, the Gq/11 inhibitor YM254890 could fully restore TRPM8 activity in response to icilin, consistent with previous experimental findings (Figure 40). Furthermore, YM254890 could restore TRPM8 response to icilin in a dose-dependent manner, as shown in Figure 16. The EC_{50} for this experiment, performed under the same experimental conditions, was 100 nM.

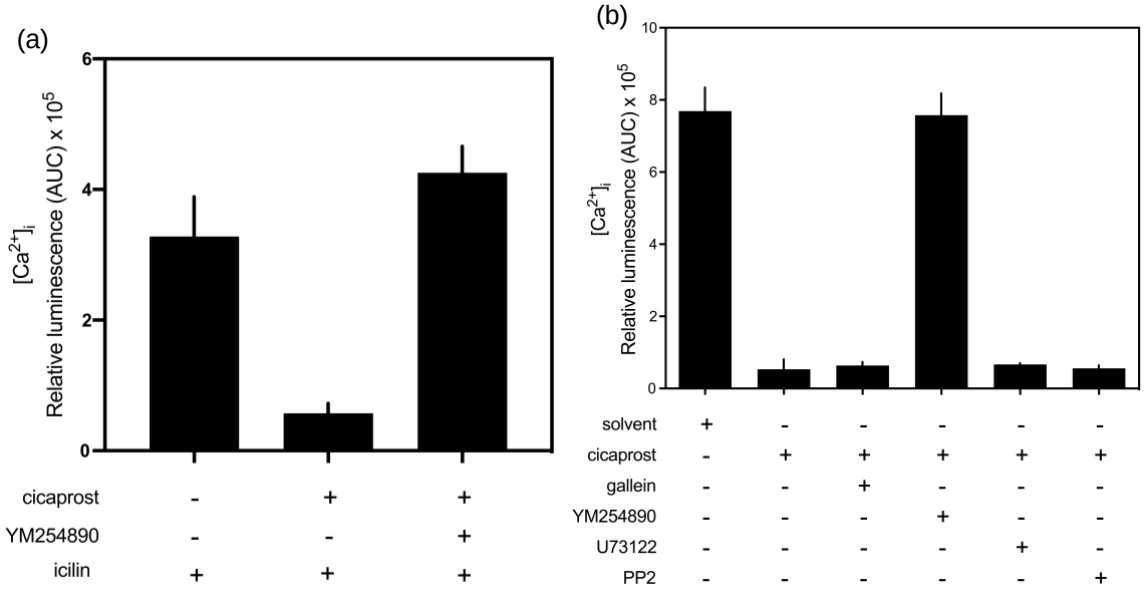

Figure 15. (a) Effect of pretreatment with the Gq/11 inhibitor YM254890 on the icilin-induced response of TRPM8 to 1 μM cicaprost in HEK293T cells expressing TRPM8 and IP, together with

the calcium probe G5A. Data presented as means ± SEM; n = 16; Impact of various pharmacological inhibitors on the suppression of TRPM8 channels by IP receptor agonist. HEK293T cells co-expressing TRPM8 channels with IP receptors and G5A were treated with the designated inhibitors for 30 minutes prior to a 10-minute incubation with 1 μM cicaprost. Subsequently, cells were stimulated with 1 μM icilin, and intracellular calcium ($[Ca^{2+}]_i$) levels were measured. The concentrations of inhibitors used were as follows: Gallein = 30 μM, YM254890 = 1 μM, U73122 = 30 μM, PP2 = 300 μM. Data presented as means ± SEM; n = 5. Adapted from Trif et al., 2024

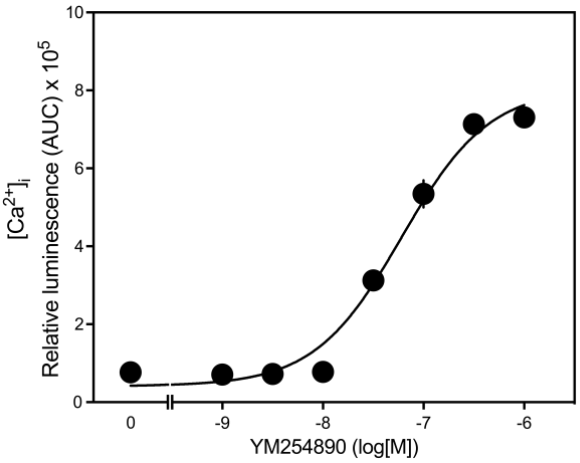

Figure 16. Effect of increasing concentrations of YM254890 on icilin-induced intracellular calcium increase in HEK293T cells co-expressing TRPM8, IP-R, and G5A, following a 10-minute pre-incubation with 1 μM cicaprost. Data shown as means ± SEM; n = 5. From Trif et al., 2024

To explore the role of Gq/11 proteins in modulating TRPM8 activity upon IP receptor activation, we assessed the responses of a TRPM8 mutant (TRPM8-TM) with three mutations which prevent Gq protein binding (X. Zhang, 2019). This analysis was performed on HEK293T cells co-expressing IP receptors, pre-treated with 1 μM cicaprost and subsequently stimulated with 1 μM icilin. The results were compared to those obtained from cells expressing wild-type TRPM8 channels.

In an initial experiment depicted in Figure 17 (a), cells expressing the TRPM8-TM mutant appeared unaffected by IP receptor activation, implying that Gq/11 proteins mediate the inhibitory effect. However, subsequent analyses conducted by collaborators indicated that cicaprost could still inhibit TRPM8 activity in cells expressing TRPM8-TM. As illustrated in Figure 17 (b), while cicaprost significantly inhibited wild-type TRPM8 channels co-expressed with IP receptors, its effectiveness was considerably reduced in the TRPM8-TM mutant.

Notably, although the inhibition was greatly diminished, higher concentrations of cicaprost still significantly inhibited TRPM8-TM activation by icilin.

These findings suggest a significant role for Gq/11 proteins in the signaling cascade that leads to TRPM8 inhibition upon IP receptor activation or a yet unidentified mediator in the inhibition process.

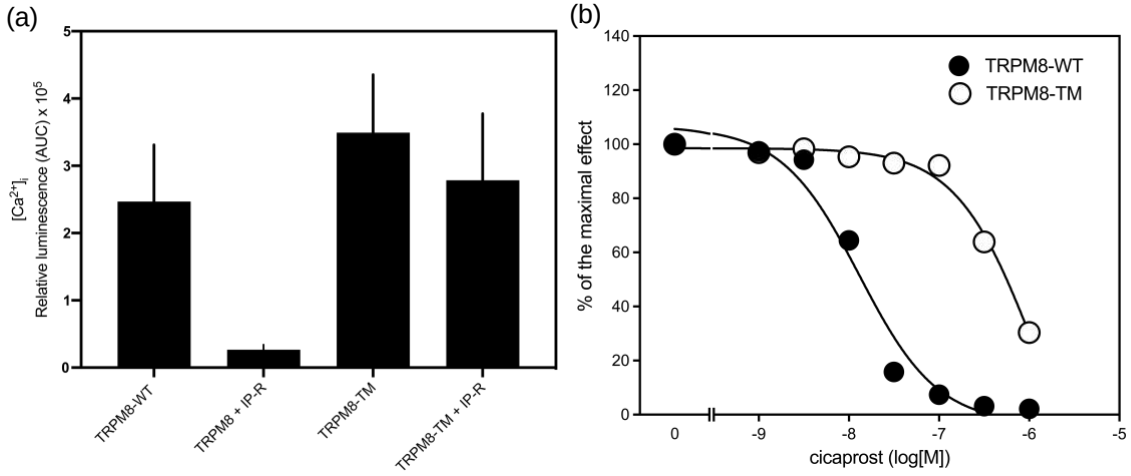

Figure 17. (a) Effect of 1 μM cicaprost on HEK293T cells co-expressing wild-type TRPM8 or triple mutant TRPM8 and IP receptors, following stimulation with 1 μM icilin. Data shown as means ± SEM; n = 5; (b) Effect of increasing concentrations of cicaprost in HEK293T cells co-expressing TRPM8-WT or TRPM8-TM following stimulation with 1 μM icilin. Data shown as means ± SEM; n = 5. Adapted from Trif et al., 2024

TRPM8 channels play a crucial role in cellular responses to cooling stimuli. To investigate whether cicaprost-mediated activation of IP receptors affects this process, we examined the impact on cooling-induced TRPM8 activation. Our collaborators at the Faculty of Biology conducted these experiments, as shown in Figure 18. Their findings indicate that pretreating HEK293T cells co-expressing TRPM8 and IP receptors with cicaprost markedly decreased the activation of TRPM8 in response to cooling ramps.

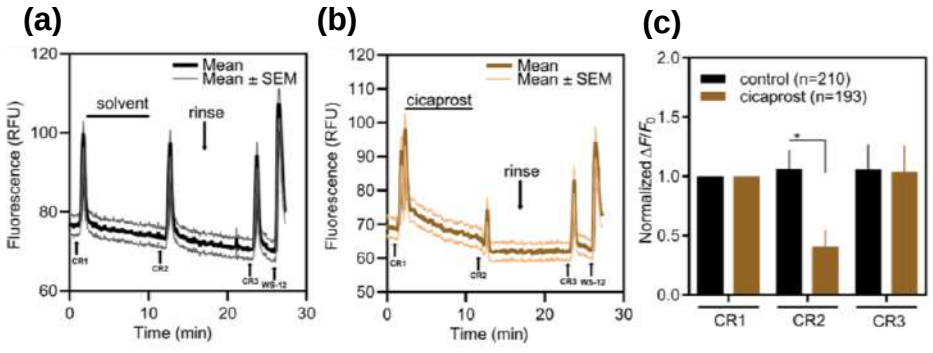

Figure 18. Calcium microfluorimetry was conducted in HEK293T cells expressing TRPM8 channels and IP receptors, subjected to three cooling ramps (CR1–3). Measurements were made in the absence (a, n =

104) and presence (b, n = 72) of 1 μM cicaprost. At the end of each experiment, the selective TRPM8 channel agonist WS-12 (5 μM) was applied to confirm the functional expression of TRPM8 channels. The data presented as continuous traces represent the means, while dotted traces indicate the SEM. (c) Mean responses to cooling ramps CR2 and CR3 are quantified as ΔF/F0 and normalised to the response elicited by the first cooling ramp (CR1). Data are shown as means ± SEM. * $P \leq 0.05$, significantly different as indicated; Student's two-tailed t-test. From Trif et al., 2024.

To investigate whether IP receptors influence the activity of TRPM8 channels within a more physiologically relevant framework, they examined the potential functional interaction between these proteins in mouse dorsal root ganglion (DRG) neurons, which are implicated in pain and inflammatory responses.

Our collaborators at the Faculty of Biology first identified neurons expressing TRPM8 based on their response to the selective TRPM8 agonist WS-12 (Sherkheli & Hatt, 2008). Exposure of these neurons to icilin elicited a substantial intracellular calcium response, as illustrated in Figure 19 a,e. However, preincubation with 1 μM of cicaprost for 8 minutes significantly reduced the calcium transients induced by icilin (Figure 19 b,e). This reduction was effectively counteracted by employing a maximal concentration of the IP receptor antagonist Cay10441, suggesting that the inhibitory effect of cicaprost was mediated through IP receptors co-expressed with TRPM8 channels in mouse DRG neurons (Figure 19 c,e). In line with findings from the heterologous expression system, pretreatment of DRG neurons with the Gq/11 inhibitor YM254890 (1 μM) fully restored TRPM8 channel responses to icilin in neurons pretreated with 1 μM cicaprost for 10 minutes (Figure 19 d,e). These findings substantiate the hypothesis that the Gq/11-dependent inhibition of TRPM8 channels by IP receptor agonists is also evident in DRG neurons.

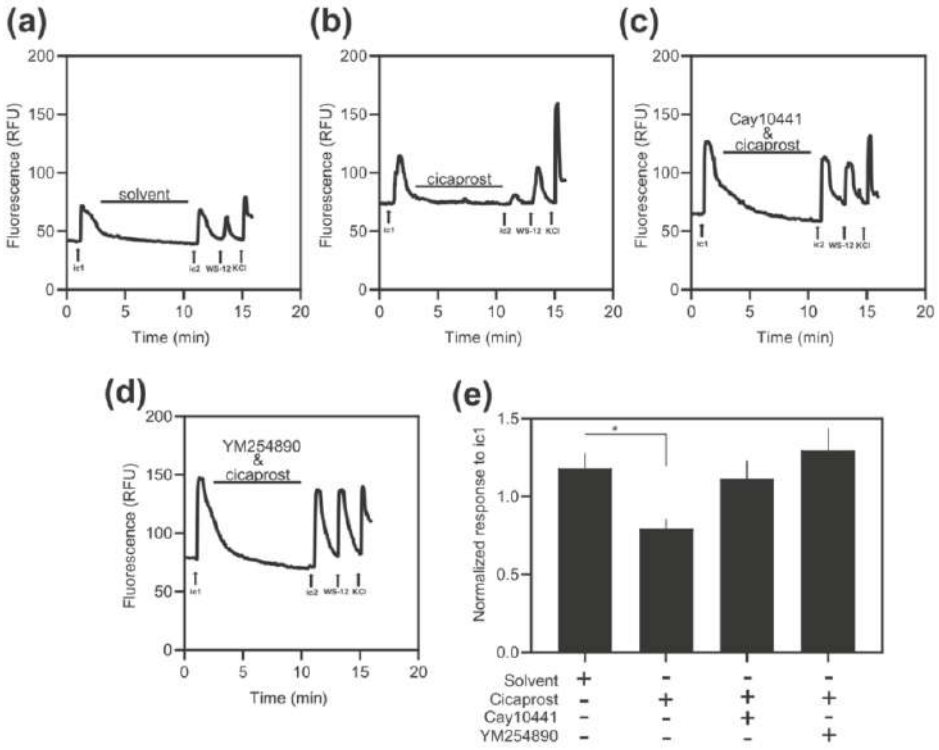

Figure 24. Inhibition of TRPM8 by IP receptor in DRG neurons. (a–d) recordings of calcium transients in DRG-derived neurons, subjected to two rounds of 1 μM icilin stimulation (ic1 and ic2), followed by stimulation with the selective TRPM8 channel agonist WS-12 (5 μM). The recordings were performed either in the absence (a) or in the presence of 1 μM cicaprost (b–d), with or without 30 μM Cay10441 (c) or 1 μM YM254890 (d). At the end of each experiment, a high potassium solution (KCl, 50 mM) was applied to depolarise the neurons and confirm their viability. (e) - quantification of the response (ΔF/F0) induced by the second icilin stimulation (ic2), normalised to the response from the first icilin stimulation (ic1), performed in the absence and the presence of cicaprost, 30 μM Cay10441, and 1 μM YM254890. Data are expressed as means ± SEM, n = 26 (solvent), n = 34 (cicaprost), n = 29 (Cay10441), and n = 27 (YM254890). Statistical significance is denoted by * $P \leq 0.05$, as determined by Student's two-tailed t-test. From Trif et al., 2024.

CONCLUSIONS AND PERSPECTIVES

This study delves into the intricate relationship between GPCRs and transient TRP channels, focusing specifically on the interaction between the TRPM8 ion channel and IP receptors. Our research aimed to optimise assays designed initially for assessing GPCR activity and subsequently apply these assays to investigate non-GPCR targets and elucidate the functional interaction between TRPM8 and IP receptors.

During the optimisation phase, we effectively measured cAMP and intracellular calcium levels, with HTS metrics calculated for each experiment, leading to the validation of the assays' robustness and reliability for subsequent applications.

Our findings confirmed that IP receptors can inhibit TRPM8 channels. This inhibition occurs independently of the Gs-cAMP pathway and involves Gq/11 coupling. Notably, the inhibition of TRPM8 by IP receptors was observed in heterologous systems (HEK293T cells) and neurons isolated from murine DRGs.

The results underscore a novel signaling pathway where IP receptors, upon activation by selective agonists such as cicaprost, beraprost, and iloprost, inhibit TRPM8 channel function via Gq/11 proteins. This atypical signaling mechanism enhances our understanding of the regulatory roles of TRPM8 channels and IP receptors in inflammatory pain.

The implications of our findings are significant for the field of pain and inflammation management. Identifying a Gq/11-mediated pathway inhibiting TRPM8 by IP receptors offers new insights into the molecular mechanisms underlying inflammatory pain.

Future research should focus on several key areas:

1. In vivo validation: While our study demonstrated the interaction between TRPM8 and IP receptors in vitro and isolated neurons, further research is necessary to validate these findings in vivo. Animal models of inflammation and pain can provide valuable insights into the physiological relevance of this interaction.

2. Pathway elucidation: Detailed studies are needed to elucidate the molecular mechanisms governing the Gq/11-mediated inhibition of TRPM8. Understanding the downstream signaling pathways and interacting partners will enhance our knowledge of this regulatory mechanism.

3. Therapeutic development: The identifying compounds that can selectively modulate the TRPM8-IP receptor interaction hold promise for developing novel analgesics and anti-inflammatory drugs. High-throughput screening of chemical libraries could identify potential candidates for therapeutic development.

4. Broader implications: Investigating whether similar interactions occur between other TRP channels and GPCRs could broaden our understanding of cellular signaling networks. This knowledge could reveal additional therapeutic targets for various pain-related conditions.

In conclusion, our study provides a foundation for future research into the complex interactions between TRPM8 channels and IP receptors. The novel signaling pathway identified herein opens new avenues for understanding and potentially treating inflammatory pain, highlighting the importance of TRPM8 and IP receptors in cellular signaling and drug discovery.

Aknowledgements

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Lastly, I would like to acknowledge myself for the resilience and determination that enabled me to complete this significant chapter of my life.

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List of published papers

1. Cosmin Trif***, <u>Alexandra-Maria Banica</u>***, Alexandra Manolache, Sorina Andreea Anghel, Debora-Elena Huţanu, Teodora Stratulat, Rodica Badea, George Oprita, Tudor Selescu, Stefana M. Petrescu, Marco Sisignano, Stefan Offermanns, Alexandru Babes, Sorin Tunaru, Inhibition of TRPM8 function by the prostacyclin receptor requires coupling to Gq/11 proteins. Br J Pharmacol. **2023**. https://doi.org/10.1111/bph.16295. **IF**: **9.40**
2. Sendetski, M., Wedel, S., Furutani, K., Hahnefeld, L., Angioni, C., Heering, J., Zimmer, B., Pierre, S., **<u>Banica, A.-M.</u>**, Scholich, K., Tunaru, S., Geisslinger, G., Ji, R.-R., Sisignano, M., Oleic acid released by sensory neurons inhibits TRPV1-mediated thermal hypersensitivity via GPR40, ISCIENCE **(2024)**, doi: https://doi.org/10.1016/j.isci.2024.110552. **IF: 4.60**

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