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Transient Receptor Potential and K^+ channel Heteromerization: How to mix and match for new properties

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Abstract Ion channels are essential proteins that control a huge diversity of physiological processes. Structurally, they are formed from multiple subunits that function together as multimers. Usually, the multimers consist of several identical subunits (homomers), although heteromerization - the formation of multimers from different subunits - is also possible and has been characterized for more than one class of ion channels. The methods employed for the study of heteromerization are diverse. First, the presence of these hybrids can be confirmed by using specific antibodies, labeling techniques or advanced microscopy. Their biophysical characterization can be made possible through the use of varied heterologous expression methods or by constructing complex DNA plasmids, known as concatemers, that encompass all the distinct subunits, leading to the fusion of the different subunits comprising the putative heteromeric channel. Finally, the heteromeric channels are investigated using pharmacological and electrophysiological approaches. *Transient receptor potential* (TRP) channels play a crucial role in sensory transduction in the peripheral nervous system, being critically involved in pain and temperature sensing. In addition, different members of this family have been identified throughout the body, playing various roles in different tissues. Even though most TRP channels seem to form homomers, several studies have identified and confirmed heteromeric complexes with diverse properties. Similar findings have been reported for several types of potassium channels. This work aims to describe in detail the methods employed in studying heteromeric ion channels and to provide an updated perspective on the latest findings regarding this topic - focusing on TRP and potassium channels.

Keywords: TRP channels, heteromerization, potassium channels, concatemers

Introduction

Ion channels usually operate as multimers (Gamper and Shah, 2022), as their functional structures are generally composed of two or more monomers which are frequently inserted into the plasma membrane, allowing the passage of ions in the right settings (Alberts et al. 2002). Traditionally, the characterization of ion channels has been successfully conducted using the heterologous expression system, where cloned DNA is transfected in various cell lines, followed by biophysical and pharmacological inquiries of the target protein. Primary tissue cultures and *in vivo* experiments, however, reveal a more complex landscape in which different ion channels co-exist and interact (Vierra and Trimmer, 2022; Bhuiyan *et al.*, 2024). This is also the case for *Transient Receptor Potential* (TRP) channels, a superfamily of ion channels which play essential roles in sensory transduction and nociception (Julius, 2013; Kashio and Tominaga, 2022). In addition to their well-established functional expresssion in the peripheral nervous system, increasing evidence indicates their key involvement in other tissues and cells types, both under physiological (Sawamura *et al.*, 2017; Wu *et al.*, 2023) and pathological conditions (Alptekin *et al.*, 2015; Stokłosa *et al.*, 2020; Chinigò *et al.*, 2021). Given that cells rarely express only one type of TRP channel, it is plausible that different monomers can directly interact and form working hetero-multimers with distinct properties. This work aims to describe the mechanisms underlying heteromerization by reviewing well established examples, the preferred techniques used in these types of studies, as well as recent papers on TRP heteromerization. Understanding these interactions can be relevant for designing new drugs targeting chronic pain and other TRP-related pathological conditions. This is not a new area of interest, since previous reviews have already documented certain aspects of this topic (Cheng, Sun and Zheng, 2010; Berg, Patwardhan and Akopian,

2012). However, our approach aims to update these perspectives, highlighting the evidence accumulated over the recent years.

The study of heteromerization

There are several methods generally used in investigating the possible heteromerization of ion channels, all employed with varying degrees of success. Antibodies are an indispensable tool in modern day biology and the specificity they provide in identifying their targets is unmatched. This way, different subunits can be labeled and first-hand interactions can be confirmed via co-immunoprecipitation. In rodent dorsal root ganglia (DRGs), co-labeling of TRPV1 and TRPV2 has revealed a population of neurons which express both channels, while brain lysates and co-immunoprecipitation showed the presence of heteromers (Liapi and Wood, 2005). In the same year, a similar methodological approach was used to report the co-immunoprecipitation of TRPV1-TRPA1 heteromers in rat DRG neurons (Rutter *et al.*, 2005). Similarly, TRPV5 and TRPV6 have been identified in nephrons and co-immunoprecipitated to show their direct physical interaction (Hoenderop *et al.*, 2003).

Fluorescence resonance energy transfer (FRET) is a method developed in the 60s, (Stryer and Haugland, 1967) used to measure the distance between a pair of different fluorescent molecules. In short, the donor can activate the acceptor only if the distance between them is small enough, thus indicating whether two constructs are in proximity. Linking the pair of FRET fluorophores to different cellular or protein subunits is ideal for investigating the formation of heteromers. Using this approach, Hellwig et al. determined that, among TRPV subunit combinations, TRPV1-TRPV2 and TRPV5-TRPV6 were the most favorable pairs. The latter even showed a FRET efficiency larger than that of the corresponding homomers, but the authors made no further comments in this regard (Hellwig *et al.*, 2005). Interestingly, TRPV5-TRPV6 have been shown to interact with a TRP channel subunit from a different subfamily, TRPML3 (Guo *et al.*, 2013). One other group investigated potential interactions between TRPV1-TRPV2-TRPV3-TRPV4 pairs and reported that the FRET efficiencies of heteromers are not significantly lower than the ones exerted by homomers (Cheng *et al.*, 2007). A 2010 report confirmed the existence of physical TRPA1-TRPV1 interactions, which were further functionally explored by the same laboratory (Staruschenko, Jeske and Akopian, 2010).

Atomic force microscopy is a type of scanning microscopy with a resolution which is high enough to enable the detection of protein subunits (Pleshakova *et al.*, 2018). This lends itself to the identification of heteromers; for example, one group confirmed TRPV2-TRPP2 hybrids bound by specific antibodies (Stewart *et al.*, 2010), while another imaged TRPV1-TRPA1 concatemer products (Fischer *et al.*, 2014). Recently, cryo-electron microscopy proved to be an essential tool for elucidating ion channel structures and the formation of heteromers (Won *et al.*, 2024).

DNA transfection and immortalized cell lines are an integral tool of ion channel study and, for more than 30 years (Lester, 1988; Hu *et al.*, 2018) the most important step in elucidating the properties of such a protein is the cloning of its coding sequence. This method allows different plasmid DNA to be used, thus allowing the expression of various cloned monomers or subunits in adjustable proportions. Given that plasmid DNA is highly customizable, this approach opens a vast array of possibilities for the investigation of heteromer formation. Firstly, various tags can be added to the desired subunits, making their detection much more easily achieved by using specific antibodies or different fluorophores. Secondly, one important characteristic of a plethora of ion channels, is their capability to be chemically modulated, either through activation or inhibition. These ligand-channel interactions enable the pharmacological characterization of heteromerization, as well as the documentation of potential modified relationships to various endogenous or exogenous compounds.

Concatemerization is a technique that involves the covalent linkage of different coding sequences in the same DNA plasmid. Thus, with variable and adjustable proportions of monomers, the generated plasmids can be subsequently expressed in heterologous systems and thoroughly characterized. Several studies have made use of this method and it has become a relatively common-practice in this field of research. A more extensive review on the topic is provided by Sack, Shamotienko and Dolly (2008), where the authors describe, among other aspects, the limitations of this method. One earlier report indicates the difficulties regarding the linker domains and their size restrictions. If the linker is too long or too short the channel becomes dysfunctional (Baumann, Baur and Sigel, 2003). Another aspect one should be aware of is the inherent possible structures of subunit assembly. One cautionary tale is that of the cyclic-nucleotide gated channels. Initially, based on concatemerization experiments, it was thought that the channels could be comprised of more than one β subunit (Shapiro and Zagotta, 1998), but later reports provided convincing evidence that functional channels contain at most a single β subunit (Weitz *et al.*, 2002).

Generally, studies focused on the topic of heteromerization use one or more of the aforementioned techniques, first to establish the presence of the hybrid channels and then to investigate their novel properties (Fig 1).

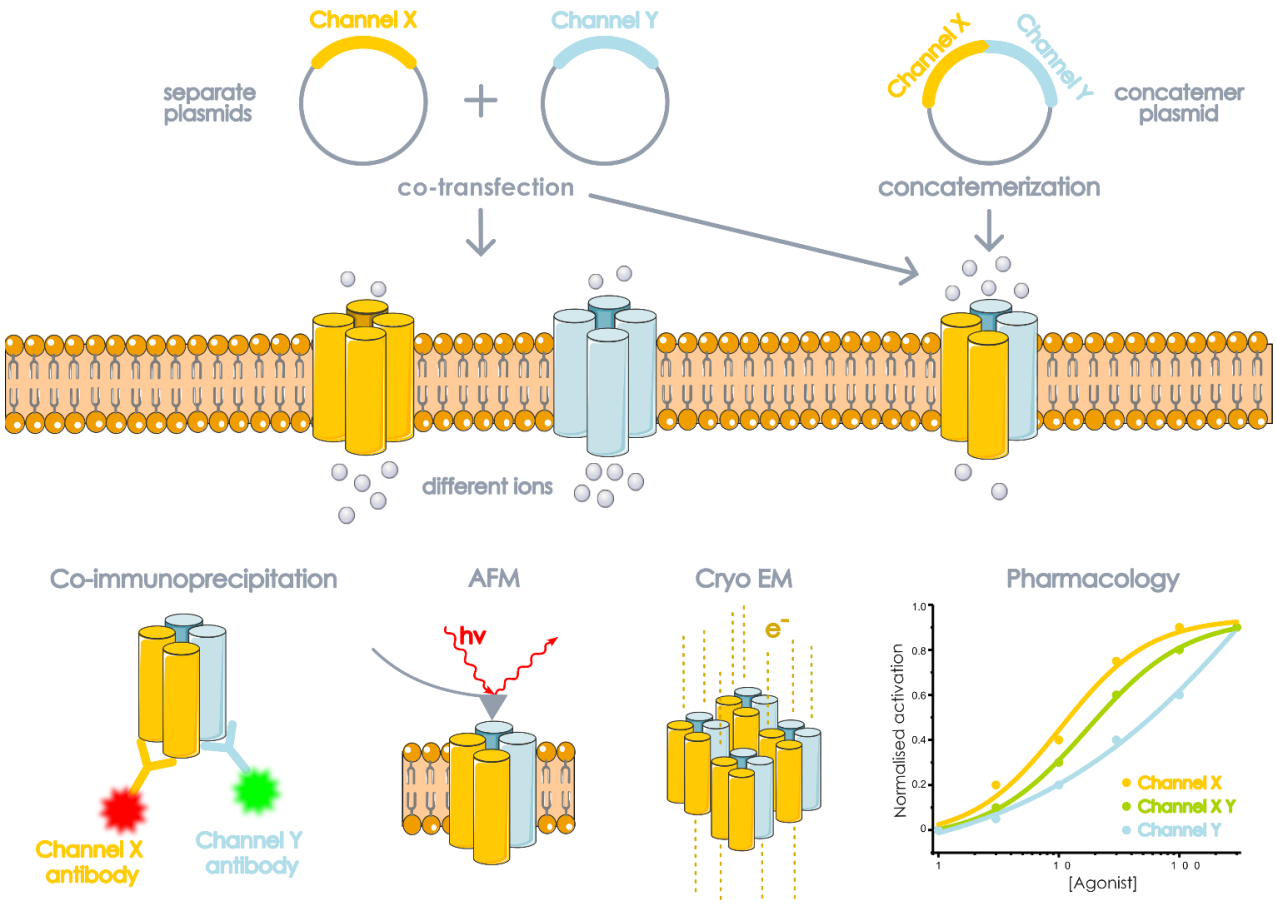

Fig. 1: *In vitro* methods for investigating heteromeric ion channels. In heterologous systems, controlled expression of heteromeric ion channels can be achieved in two ways: either by co-transfection of cells with two plasmids each encoding a different subunit of the channel, or by using a single plasmid that encodes the cDNAs for both ion channel subunits, in the form of a concatemer. Once expressed in the plasma membrane of the transfected cells, the ion channel's functional properties can be investigated using pharmacological techniques. To confirm heteromeric channels formation and analyze their structural features, several methods are commonly used, including co-immunoprecipitation, atomic force microscopy (AFM), and cryo-electron microscopy (cryo-EM).

Heteromerization of K⁺ voltage-gated ion channels

Out of all ion channel families, K^+ channels represent the most diverse group. Based on their structure, they can be divided into three major classes. The first class is represented by tetrameric inward rectifying K^+ channels (K_{ir}), each subunit containing two transmembrane (TM) domains and one pore region. The second class consists of the two-pore domain K^+ channels: they are dimers and their subunits contain four TM domains and two P-loops each, hence the name of the family. The last family is comprised of K^+ channels that have six TM domains per subunit, and can be further subdivided according to their biophysical properties into: voltage gated K^+ channels (K_v), small conductance calcium activated K^+ channels (SK) and large conductance calcium-activated K^+ channels (BK, Maxi-K or Slo) K^+ channels (González *et al.*, 2012).

K_{ir2} channels are part of the K_{ir} family, which represents the subclass with the most potent inward rectification. These channels are predominantly expressed in neurons (Papanikolaou, Lewis and Butt, 2019) and cardiomyocytes (Karle *et al.*, 2002), where they play a crucial role in maintaining the membrane resting potential (Nichols and Lopatin, 1997). In 2002, using co-immunoprecipitation, Preisig-Müller et al. discovered that the co-expression of human $K_{ir2.1}$ and $K_{ir2.2}$ in HEK293 cells led to the formation of heterodimers. When co-expressed in *Xenopus* ovocites, $K_{ir2.1}$ and $K_{ir2.2}$ resulted in a Ba^{2+}-sensitive inwardly rectifying potassium current with an IC_{50} different from that observed in cells expressing only Kir2.1 or Kir2.2 individually, proving that the two channels can form functionally different heteromeric structures (Preisig-Müller *et al.*, 2002). A mutant variant of the guinea pig $K_{ir2.1}$ (which differs from the human variant in only one amino-acid) has been linked to the Andersen-Tawil Syndrome, a congenital disorder which causes cardiac arrhythmia and periodic paralysis (Veerapandiyan, Statland and Tawil, 2019). Interestingly, it was shown that homomeric mutant $K_{ir2.1}$ channels exhibit greatly reduced inwardly-rectifying currents. Moreover, the mutant is able to form heteromers with $K_{ir2.x}$ wild type channels, resulting in a similar reduction of the inwardly-rectifying current (Preisig-Müller *et al.*, 2002). These results indicate that even when only one of the subunits composing heteromeric K_{ir} channels is mutated; their partner's function is also modulated, proving that heteromerization leads to functional proteins with novel characteristics.

Two-pore domain K^+ channels (K_{2P}) are primarily responsible for leak (background) K^+ currents. They play a crucial role in controlling the resting membrane potential and regulating the duration of action potentials during neuronal excitation (Talley *et al.*, 2000). Based on their modulatory factors, they are classified into two groups: TWIK (**T**andem of pore domains in a **W**eakly **I**nwardly rectifying **K**$^+$ channel), which are modulated by pH fluctuations and protein kinase C (Lesage *et al.*, 1996; Chavez *et al.*, 1999), and TREK (**T**WIK-**RE**lated **K**$^+$ channel), which are activated by mechanical stimuli and unsaturated fatty acids (Fink *et al.*, 1998; Herrera-Pérez and Lamas, 2023). The TASK subfamily (**T**WIK-related **A**cid-**S**ensitive **K**$^+$) comprises special K^+ channels inhibited by extracellular acidification (Rajan *et al.*, 2001). The expression and electrophysiological analysis of TASK1 and TASK3 concatemers in *Xenopus* oocytes revealed an intermediate pH sensitivity compared to the monomers, as well as an intermediate Ruthenium Red-dependent inhibition. These results were confirmed by the coexpression of these two channels (Czirják and Enyedi, 2002).

Another pair of K^+ channels heterodimers, that exhibit distinct properties, consists of TREK1-TREK2 subunits. TREK1 is activated by the alkalinization of extracellular pH and inhibited by acidification (Sauter *et al.*, 2016). Conversely, TREK2 behaves in the opposite manner to changes in extracellular pH (Bang, Kim and Kim, 2000). The TREK1-TREK2 heterodimer has a distinct response to pH: extracellular acidification from 8.5 to 7.4 leads to heterodimer inhibition characteristic of TREK1, and further acidification to a pH of 6.5 leads to an activation characteristic of TREK2 (Levitz *et al.*, 2016). These results show that, within the K^+ family, heteromerization is common and may play an integral role in their *in vivo* functions.

Voltage gated potassium channels (Kv) are integral for neuronal function, with their structure composed of four monomers which assemble into a tetrameric complex. Extensive research has been conducted in order to elucidate the properties of these channels, doubled by investigations regarding the heteromerization of subunits. One report revealed the pharmacological implications of Kv1 channel heteromers. Briefly, κM-RIIIJ, a cone snail toxin is much more potent in inhibiting heteromers with only three Kv1.2 subunits as compared to Kv1.2 homomers (Cordeiro *et al.*, 2019). Another investigation highlighted how heteromeric channels formed by Kv1.1 and Kv1.2 modify key electrophysiological properties, including activation and inactivation kinetics, as well as susceptibility to blockers such as tetraethylammonium (Al-Sabi *et al.*, 2013). Kv7 members are also capable of heteromerization. Kv7.2 and Kv7.3 have been shown to exhibit mutations in the voltage sensing domain involved in epilepsy. Mutant subunits can heteromerize with wild-type subunits and alter the latter's sensitivity to voltage and the membrane expression (Miceli *et al.*, 2015). Apart from these classic wet lab approaches, mathematical models were proposed for various working Kv heteromers (McGahan and Keener, 2022), proving a continuous interest in this topic.

TRP channels

The superfamily of TRP ion channels, usually assembled into tetramers, consists of several distinct homologically similar families; TRPV – vanilloid, TRPA – ankyrin, TRPM – melastatin, TRPC – canonical, TRPML – mucolipin, TRPP – polycystin. The 2021 Nobel Prize in Physiology and Medicine was awarded to researchers whose primary focus was on TRP channels and their upmost importance for detecting the environmental changes in temperature at the level of the peripheral nervous system. TRPV1 was cloned more than 25 years ago (Caterina *et al.*, 1997) and ever since it has been a key player in nociception (Julius, 2013; Basso and Altier, 2017) research due to its essential role in pain-related afflictions and its sensitivity to noxious heat (Basbaum *et al.*, 2000). TRPV1 is not the sole culprit in these pathological pain states: several other TRP channels have been shown to play a critical role in the initiation and maintenance of neuropathic pain, namely TRPA1 (Souza Monteiro de Araujo *et al.*, 2020), TRPM3 (Behrendt, 2019), and TRPC5 (Khare *et al.*, 2024).

Apart from their role in nociception and temperature sensing (Julius, 2013; Kashio and Tominaga, 2022), TRP channels are also important for other physiological processes: immune function (Wu *et al.*, 2023), different central nervous system activities (Sawamura *et al.*, 2017) and regulation of skin cell proliferation and differentiation (Tóth *et al.*, 2014), to name just a few. In addition to their normal function, TRP channels are frequently found to be involved in pathological conditions, especially different types of cancer (Alptekin *et al.*, 2015; Stokłosa *et al.*, 2020; Chinigò *et al.*, 2021). Despite ongoing investigations, out of the current 28 members of the superfamily, some still exhibit elusive characteristics and their definite roles are unclear. Even the plasma membrane localization is still debated, as some of the proteins seem to be sequestered to other organelles or subcellular compartments (Tian *et al.*, 2022). Taken into consideration their wide variety of functions and subcellular localization, it is no surprise that TRP channels have been identified virtually everywhere (Clapham, 2003). This ubiquity is also overlapped by varied forms of co-expression and interactions which will be discussed in more detail below. In terms of heteromerization, TRPV1 and TRPA1 have been the most studied subunits. Using concatemers, Fischer et al showed that TRPA1:TRPV1 heteromeric constructs have significantly altered properties compared to each original homomer: the sensitivity of TRPV1 to low pH, as well as its current-voltage relationship of capsaicin dependent activation are greatly reduced, while the heat sensing properties are not modulated. It is not entirely clear if the TRPA1 subunits are functional or

severely inhibited, since the authors were not able to elicit any calcium influx upon challenging the cells with carvacrol, a non-electrophilic TRPA1 agonist (Fischer *et al.*, 2014). The Akopian group showed prolonged interest for this interaction. In 2007, they reported functional interactions between TRPA1 and TRPV1 when co-expressed in heterologous expression systems, as well as when natively expressed in DRG neurons, mainly involving cross-desensitization, but no direct evidence of heteromerization was provided (Akopian *et al.*, 2007). Three years later, single channel activity measured in TRPV1-TRPA1-transfected CHO cells were compared to similar activity in cells expressing either TRPA1 or TRPV1. The report focused on calcium and revealed that low extracellular ion concentrations are more prone to triggering channels activity in cells expressing both channels compared to cells expressing just one subunit, while high calcium concentrations increase the open probability of homomeric but presumably not heteromeric channels. Moreover, intracellular calcium has the same impact on the combination as on TRPA1 (but not TRPV1) alone, very likely due to a higher probability of channel opening (Patil, Jeske and Akopian, 2010). These properties were further validated in a paper published in the same year, which confirmed the modulation of TRPA1 activity by TRPV1 in sensory neurons (Staruschenko, Jeske and Akopian, 2010). Furthermore, in 2015 the involvement of an accessory protein, TMEM100, which binds and controls the TRPV1-TRPA1 complexes, was discovered. More importantly, the same work revealed phenotypic effects: mice which lack the TMEM100 protein are resistant to TRPA1-dependent pain (e.g., mechanical stimulation after the injection of mustard oil, a selective TRPA1 agonist), while TRPV1-mediated pain is not altered (e.g., hot plate assays) (Weng *et al.*, 2015).

TRPV1-TRPV3 heteromers obtained through concatemerization also exhibit intermediary properties. As single channel units, TRPV1-TRPV3 channels are revealed to exhibit a greater conductance than homomeric TRPV1 and less than TRPV3. Additionally, their pharmacological profiles of ligand dependent activation and inhibition differ: capsaicin is less potent in opening the heteromers and ruthenium red is less effective at blocking them. In terms of heat activation kinetics, the temperature threshold of TRPV1-TRPV3 concatemers was 33°C, compared with the thresholds for homomeric TRPV3 and TRPV1 channels of 30 and, respectively, 38°C. (Cheng *et al.*, 2012).

The TRPV5-TRPV6 combination briefly mentioned before has been previously characterized in a 2003 paper which demonstrated the intermediary biophysical properties of TRPV5-TRPV6 heteromers regarding barium permeability, ruthenium red block and calcium dependent inactivation (Hoenderop *et al.*, 2003). TRPV5 and TRPV6 seem to be rather nonselective in terms of heteromerization, as one report found evidence of pairing with TRPML3. Throughout the paper, the authors found intermediary properties of the heteromeric channel (obtained through co-transfection) but notably also novel sodium conductance properties (Guo *et al.*, 2013).

TRPC channels also allow the formation of heteromers and TRPC1 seems to be a key player in these interactions. One example comes from a 2023 paper which describes TRPC1-TRPC4 heteromerization and how these complexes differ from their homomeric counterparts. It is yet unclear if TRPC1 functions as a homomeric ion channel, so the researchers focused on how TRPC4 is modulated in the presence of TRPC1, and what they uncovered was a change in the outward rectifying current-voltage (IV) curve. This aspect might be relevant for cellular excitability, given that TRPC4 homomers do not exhibit any outward current at slightly positive potentials (0-40 mV), while the heteromers do (Park *et al.*, 2023). TRPC1-TRPC4 also gathered attention due to their involvement in renal cell carcinoma treatment with Englerin. One report investigated carcinoma cell lines which seem to natively express both ion channels as well as the heteromers (confirmed by the similarity of their IV properties with those of a concatemer expressed in HEK293 cells). Using RNA interference to knock down each channel individually, the authors confirmed that the heteromer is necessary for Englerin-induced cytotoxicity (Ludlow *et al.*, 2017). The structure of TRPC1-TRPC4 heteromers has also been resolved with cryo-electron microscopy, revealing a definite subunit ratio of 1:3 and a notable decrease in ion permeability for calcium, both in terms of influx and efflux (Won *et al.*, 2024).

Apart from TRPC1-TRPC4, another 2001 paper identified TRPC1-TRPC5 heteromers in rat brains and briefly discussed their properties in heterologous expression systems. Single channel recordings revealed significantly less outward rectifying conductance for the hybrid as opposed to the TRPC5 homomer (Strübing *et al.*, 2001). A 2022 paper confirmed the presence of TRPC1 and TRPC5 heteromerization in rodent brains and characterized hybrid multimers of the aforementioned TRP channels with the metabotropic glutamate receptor 1 (Kollewe *et al.*, 2022). Pharmacologically TRPC1-TRPC5 constructs also require GTPγS in order to be activated by carbachol, while the TRPC5 homomers do not (Kang and So, 2024).

Finally, members of the TRPP family, also known as polycystic kidney disease (PKD) channels have been found to form heteromers that act as sour taste receptors. The authors confirmed the necessity of both PKD1L3 and PKD2L1 for citric acid activation, as neither alone exhibited significant activity in the presence of this compound. Moreover, the selectivity for sourness was established by testing numerous other tastant molecules (Ishimaru *et al.*, 2012).

Conclusions

Heteromerization is a well-documented phenomenon among ion channels and its investigation uses established techniques such as generation of concatemers, co-transfections, co-immunoprecipitation and lately cryo-electron microscopy or atomic force microscopy. Transient receptor potential channels are nearly ubiquitous and their activity is important for a variety of physiological processes, extending beyond peripheral sensitivity. The TRP channel superfamily, for example, is subclassified in families based on homology, but a thorough review of the literature might undermine this clear-cut differentiation. As evidence for TRP channel heteromerization continues to accumulate, some important questions arise. How are the canonical functions of these proteins modulated by the presence of these hybrid constructs? How many cells actually express only homomeric TRP channels and to what extent are all of the possible heteromers actually formed *in vivo*? Do these heteromeric TRP channels, with mainly intermediary properties, lead to cell populations with more nuanced characteristics? Exhaustive studies have not been conducted in these directions, and as of now, this aspect is on the fringe of TRP channel research. Undoubtedly, interest in this area of research is growing, and a recent review summarized the information concerning the implications of TRP heteromerization in lung inflammation, with special focus given to TRPC and TRPV families (Zergane, Kuebler and Michalick, 2021). Significant gaps in knowledge remain; for instance, while TRPV1 and TRPV2 have been shown to interact and possibly form heteromers (Liapi and Wood, 2005; Rutter *et al.*, 2005), as of now there are no functional investigations. Even more strikingly, no data has been provided regarding the Melastatin family. Despite being a relatively narrow niche of research, heteromerization studies always reveal intriguing emerging properties of non-standard TRP tetramers, which might be impactful in the right context, be it normal physiology or pathology.

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