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REVIEW

The TRPM4 channel inhibitor 9-phenanthrol

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The phenanthrene-derivative 9-phenanthrol is a recently identified inhibitor of the transient receptor potential melastatin (TRPM) 4 channel, a Ca^{2+}-activated non-selective cation channel whose mechanism of action remains to be determined. Subsequent studies performed on other ion channels confirm the specificity of the drug for TRPM4. In addition, 9-phenanthrol modulates a variety of physiological processes through TRPM4 current inhibition and thus exerts beneficial effects in several pathological conditions. 9-Phenanthrol modulates smooth muscle contraction in bladder and cerebral arteries, affects spontaneous activity in neurons and in the heart, and reduces lipopolysaccharide-induced cell death. Among promising potential applications, 9-phenanthrol exerts cardioprotective effects against ischaemia-reperfusion injuries and reduces ischaemic stroke injuries. In addition to reviewing the biophysical effects of 9-phenanthrol, here we present information about its appropriate use in physiological studies and possible clinical applications.

Abbreviations

ABC, ATP binding cassette; AGS cells, human gastric adenocarcinoma cell line; BK_{Ca}, large conductance Ca^{2+}-activated K^+ current; CFTR, cystic fibrosis transmembrane conductance regulator; DSM, detrusor smooth muscle; EADs, early after depolarizations; H-89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamidedihydrochloride hydrate; HCN, hyperpolarization and cyclic nucleotide gated channel; $I_{Ca,L}$, L-type Ca^{2+} current; I_K, delayed outward rectifyer K^+ current; K_{ATP}, ATP sensitive K^+ channel; K_{IR}, inward rectifier K^+ current; K_V, voltage-gated K^+ current; MKN-45 cells, human gastric cancer cell line; MPB-104, 5-butyl-7-chloro-6-hydroxybenzo[c]quinolizinium chloride; NSC_{Ca}, Ca^{2+}-activated non-selective cation channels; SUR, sulfonylurea receptor; TRP, transient receptor potential channels; TRPC, transient receptor potential canonical; TRPM, transient receptor potential melastatin

Introduction

9-Phenanthrol, also called 9-hydroxyphenanthrene or phenanthrene-9-ol in the IUPAC (International Union of Pure and Applied Chemistry) nomenclature, has been known for more than a century (Pschorr and Schroter, 1902; Moriconi *et al.*, 1959). It is a benzo[c]quinolizinium derivative composed of three fused benzene rings (Figure 1). Despite primary results indicating fungitoxicity of the molecule (Rich and Horsfall, 1954), it was, until recently, only used as a biomarker of anthropogenic compound pollution. Indeed, 9-phenanthrol may originate from other hydroxylated polycyclic aromatic hydrocarbons (PAH). Those molecules come from incomplete combustion of organic matter, resulting, in part, from human activities. PAHs found in polluted soil or water can be ingested by organisms like fish. One

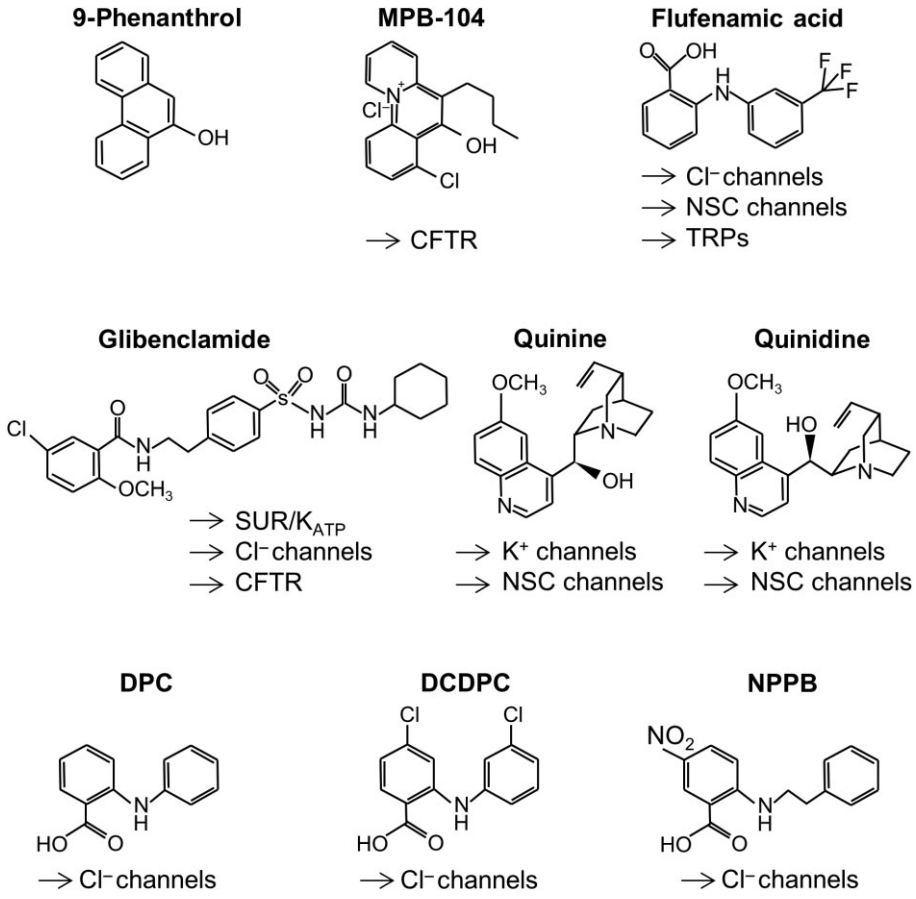

Figure 1

Chemical structures of pharmacological inhibitors of TRPM4 (9-phenanthrol, MPB-104, flufenamic acid, glibenclamide, quinine, quinidine) and NSC_{Ca} currents (DPC, DCDPC, NPPB). Under each compound, arrows indicate its other main targets among ion channels, in addition to TRPM4 and NSC_{Ca} currents. The list indicates typical targets, but is not exhaustive.

of these, the phenanthrene, is partly metabolized to form molecules such as 9-phenanthrol, which can become concentrated in tissues because of its hydrophobicity (Escartin and Porte, 1999; Koenig *et al.*, 2013). As PAHs such as phenanthrene are highly toxic, 9-phenanthrol may be applicable for depollution processes because it has a lower toxicity and can be produced after phenanthrene biodegradation by proteobacteria (Feng *et al.*, 2012).

Far from these environmental concerns, we observed in 2008 that 9-phenanthrol inhibits the transient receptor potential melastatin (TRPM) 4 channel (Grand *et al.*, 2008). TRPM4 forms a Ca^{2+}-activated non-selective cation (NSC_{Ca}) channel widely expressed in tissues from several mammalian species including humans (Launay *et al.*, 2002). The physiological roles for the TRPM4 channel were difficult to identify until the development of knockout mice and appropriate pharmacological tools (Guinamard *et al.*, 2011). Regarding pharmacology, it has been confirmed that 9-phenanthrol specifically targets the TRPM4 channel (Table 1) and its use in a variety of biological preparations has unmasked the contributions of the TRPM4 channel in physiological processes.

Here we review 9-phenanthrol as a TRPM4 channel inhibitor. We summarize the identification of 9-phenanthrol and document its specificity among ion channels. Further, we review physiological processes modulated by 9-phenanthrol and recommend appropriate applications of the drug, while acknowledging their caveats and limitations.

Looking for TRPM4 channel pharmacological inhibitors

The TRPM4 channel belongs to the transient receptor potential (TRP) protein family, whose members form non-selective cation channels (Alexander *et al.*, 2013). It shares with its closest relative, the TRPM5 channel, a lack of selectivity for monovalent cations (i.e. equal permeability for Na^+ and K^+), and while these channels are Ca^{2+} impermeable their activation mechanism is simultaneously sensitive to internal Ca^{2+} concentration (Launay *et al.*, 2002; Guinamard *et al.*, 2011). In addition, both channels have a higher activity at depolarized voltages. Such NSC_{Ca} currents were recognized in native preparations from a variety of tissues (Teulon, 2000). Unmasking their physiological roles has depended on pharmacological tools. Several pharmacological agents inhibit TRPM4 channels and NSC_{Ca} currents more generally (Figure 1). The most commonly used NSC_{Ca} channel inhibitor is the non-steroidal anti-inflammatory drug flufenamic acid

Table 1

Effect of 9-phenanthrol on recombinant ion channels

Current	Model	Effect	Concentration in mol·L^{-1}	References
TRPM4	HEK-293	Inhibition	$IC_{50} = 1.7 \times 10^{-5}$	Grand *et al.*, 2008
TRPM5	HEK-293	None	10^{-4}	Grand *et al.*, 2008
TRPC3	HEK-293	None	3×10^{-5}	Gonzales *et al.*, 2010b
TRPC6	HEK-293	None	3×10^{-5}	Gonzales *et al.*, 2010b
CFTR	CHO	None	2.5×10^{-4}	Grand *et al.*, 2008

When known, the IC_{50} is presented, otherwise, the highest concentration tested is indicated.

that inhibits both TRPM4 and TRPM5 channels (Ullrich *et al.*, 2005). This molecule is advantageous because it rapidly affects channel activity and is reversible. However, it has a large spectrum of targets, particularly among ion channels (Guinamard *et al.*, 2013). The bitter compound quinine and its stereoisomer quinidine similarly inhibit TRPM4 and TRPM5 channels (Talavera *et al.*, 2008), but once again are not specific (White, 2007). Spermine is another NSC_{Ca} antagonist without specificity for underlying channel type (Nilius *et al.*, 2004). The sulfonylurea glibenclamide was shown to inhibit native NSC_{Ca} currents and the TRPM4 channel (Demion *et al.*, 2007), but it is also known to interact with other channels, mainly those belonging to the ATP binding cassette (ABC) protein family and the ATP-dependent K^+ channel, which is a multimer of inwardly rectifying K^+ channel subunits and the sulfonylurea receptor (SUR; Alexander *et al.*, 2013). Note that the co-assembly of the TRPM4 channel with the SUR1, such as occurs in acute CNS injuries, potentiates its sensitivity to glibenclamide (Woo *et al.*, 2013b). TRPM4-like currents are also inhibited by the closely related chloride channel blockers diphenylamine-2-carboxylic acid, 3′,5′-dichlorodiphenylamine-2-carboxylic acid and 5-nitro-2-(3-phenylpropyl-amino)-benzoic acid in a variety of tissues (Gögelein and Pfannmüller, 1989; Chraïbi *et al.*, 1994; Teulon, 2000). Even though all of these molecules target the TRPM4 channel, none is a specific antagonist. Moreover, except for the presence of phenol rings, they share few (if any) chemical determinants (Figure 1).

Looking for new candidates to inhibit the TRPM4 channel, we were intrigued by several similarities between TRPM4 and ABC proteins. Both are sensitive to ATP and hold ATP binding sites within their amino acid sequence (Ullrich *et al.*, 2005; Frelet and Klein, 2006). In addition, as referenced earlier, the antidiabetic sulfonylurea glibenclamide modulates the TRPM4 channel (Demion *et al.*, 2007) as well as ABC proteins including SURs and the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel (Sheppard and Welsh, 1992; Frelet and Klein, 2006). We hypothesized that other modulators of ABC proteins might also affect the TRPM4 channel. Indeed this proved correct for the CFTR activator 5-butyl-7-chloro-6-hydroxybenzo[c]-quinolizinium chloride (MPB-104) (Figure 1), which inhibits the TRPM4 channel with an IC_{50} of ~2×10^{-5} mol·L^{-1} (Grand *et al.*, 2008). However, MPB-104 is not specific for the TRPM4 channel, as it also modulates the CFTR channel. Therefore, in searching for other related compounds, we tested 9-phenanthrol (Figure 1), which lacks several components necessary for CFTR channel activation. As expected, 9-phenanthrol did not modulate the CFTR channel, yet it inhibited TRPM4 channels heterologously expressed in HEK-293 cells (Grand *et al.*, 2008).

Inhibition of recombinant TRPM4 channel by 9-phenanthrol

As mentioned earlier, and shown in concentration–response curves (Figure 2), 9-phenanthrol inhibited human TRPM4 channel activity in patch recordings from transfected HEK-293 cells (Grand *et al.*, 2008). In the conventional whole-cell configuration, superfusion of 9-phenanthrol in the bath inhibited the TRPM4-mediated current with an IC_{50} of 1.7×10^{-5} mol·L^{-1} (Table 1). The inhibition was also observed in nystatin perforated whole-cell membrane currents from TRPM4-transfected COS-7 cells (Woo *et al.*, 2013a). Similar results were observed in the inside-out configuration (Grand *et al.*, 2008; Woo *et al.*, 2013a). These data indicate that the molecule may interact with the channel on both sides, or more likely, that the molecule is able to cross the membrane because of its hydrophobicity. The interaction site of 9-phenanthrol within the channel structure is not known. However, the glycosylation state of the protein is not involved. Indeed, the N988Q substitution, at an extracellular location near the pore-forming loop between the transmembrane segments 5 and 6, which results in the disappearance of the N-glycosylated forms of the protein, did not modify its inhibition by 9-phenanthrol (Woo *et al.*, 2013a). Because channel inhibition occurs within seconds in the inside-out configuration, but within a minute in the whole-cell configuration, a reasonable starting assumption is that 9-phenanthrol interacts with the TRPM4 channel intracellularly.

The Hill coefficient of the concentration–response curve is close to 1, indicating no cooperation in 9-phenanthrol interactions with the channel. The concentration–response curves performed in the inside-out configuration at positive and negative voltages showed no evidence of voltage-dependent inhibition (Grand *et al.*, 2008).

Whether recorded in inside-out patches or the whole-cell configuration, TRPM4 channel inhibition by 9-phenanthrol is reversible, even though complete recovery can take several minutes after channel exposure to high concentrations (~10^{-4} mol·L^{-1}).

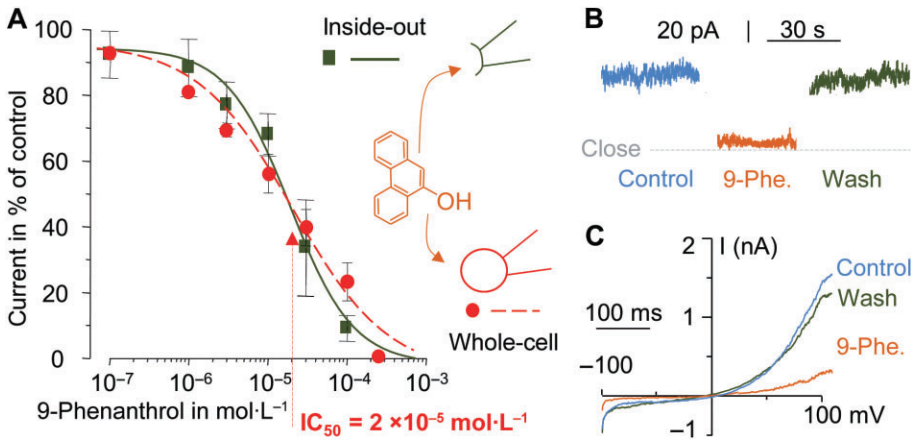

Figure 2

TRPM4 channel inhibition by 9-phenanthrol. Effect of 9-phenanthrol on TRPM4 currents after recombinant expression of the human *TRPM4* gene in HEK-293 cells. (A) Concentration–response curve for the effects of 9-phenanthrol in the inside-out configuration (green squares) or whole-cell configuration (red circles). Fitting the Hill equation to the data points indicates a similar IC_{50} at 2×10^{-5} mol·L^{-1} and a Hill coefficient close to 1. The chemical structure of 9-phenanthrol is provided on the right. (B) Effect of 10^{-4} mol·L^{-1} 9-phenanthrol applied to the inside of the membrane, on a representative current recording in the inside-out configuration (V_m = +40 mV). Around 80 channels were present in the patch. (C) Effect of 10^{-4} mol·L^{-1} 9-phenanthrol applied to the outside of the membrane, on a representative whole-cell recording. The voltage command is a ramp from V_m = –100 to +100 mV. Note that the effects of 9-phenanthrol are reversible. See Grand *et al.* (2008) for protocol.

Inhibition of endogenous TRPM4 currents by 9-phenanthrol

After its identification as a recombinant TRPM4 channel inhibitor, the effects of 9-phenanthrol were tested on endogenous TRPM4-like currents from several native tissues (Table 2).

The TRPM4 channel is highly expressed in epithelia such as kidney. HEK-293 cells obtained from this type of tissue weakly express an endogenous TRPM4-like current, which is inhibited by 80% by 10^{-4} mol·L^{-1} 9-phenanthrol in whole-cell recordings (Amarouch *et al.*, 2013).

The TRPM4 channel is also expressed in various smooth muscles cells (Earley, 2013). A TRPM4-like transient inward current is attenuated by 9-phenanthrol with an IC_{50} of 1×10^{-5} mol·L^{-1} in perforated patches, and totally inhibited by 3×10^{-5} mol·L^{-1} in whole-cell recordings from rat cerebral artery smooth muscle cells (Gonzales *et al.*, 2010b; Gonzales and Earley, 2012). 9-Phenanthrol, at 3×10^{-5} mol·L^{-1}, inhibits 40% of the same current during perforated-patch recordings from guinea pig and rat detrusor smooth muscle (DSM) cells (Parajuli *et al.*, 2013; Smith *et al.*, 2013a,b). In addition, 10^{-4} mol·L^{-1} 9-phenanthrol attenuates TRPM4-like currents in inside-out patch recordings from monkey colonic smooth muscle cells (Dwyer *et al.*, 2011).

We have reported the functional expression of TRPM4 channels in cardiomyocytes, including human cardiac tissue (Guinamard *et al.*, 2004; Simard *et al.*, 2013). Endogenous TRPM4 channel activity in inside-out patch recordings from mouse isolated atrial cardiomyocytes was inhibited by 80% in response to 10^{-5} mol·L^{-1} 9-phenanthrol (Simard *et al.*, 2013).

Purkinje cells from mouse cerebellum express a calcium-dependent depolarization-induced slow current whose properties match those of TRPM4 and TRPM5 channels (Kim *et al.*, 2013). The current is smaller in *Trpm4* null mice, but is not affected in *Trpm5* null mice, which indicates that the calcium-dependent inward current probably corresponds to a TRPM4 current. This current is totally abolished by 10^{-5} mol·L^{-1} 9-phenanthrol (Kim *et al.*, 2013).

The effects of 9-phenanthrol were also tested on TRPM4-like endogenous currents in two human gastric adenocarcinoma cell lines, AGS and MKN-45. In both cell types, 3×10^{-5} mol·L^{-1} 9-phenanthrol reduced the current by 90% (Kim *et al.*, 2012).

9-phenanthrol effects on other ion channels

To evaluate specificity of 9-phenanthrol for the TRPM4 channel, its effects were also tested on other recombinant ion channels as well as native currents (Tables 1 and 2). Concentrations at or exceeding 3×10^{-5} mol·L^{-1} were used in all cases. Note that 9-phenanthrol concentrations greater than 10^{-4} mol·L^{-1} may be difficult to test precisely because the molecule partly precipitates at such levels even if dissolved in DMSO.

9-Phenanthrol was tested on several members of the TRP channel family responsible for a large variety of non-selective cationic currents. First of all, it was tested on the TRPM5 channel, the closest TRPM4 relative within the family. A recombinant TRPM5 current was not affected by 10^{-4} mol·L^{-1} 9-phenanthrol in HEK-293 cells, which is an encouraging sign regarding its high selectivity for the TRPM4 channel (Grand *et al.*, 2008). This lack of effect on the TRPM5 channel was confirmed in a preparation of isolated lingual taste cells from mouse, which exhibit native NSC_{Ca} activated by linoleic acid. This NSC_{Ca} current was abolished by *Trpm5* gene disruption or application of the TRPM5 channel-specific inhibitor triphenylphosphine oxide, but not by 10^{-4} mol·L^{-1} 9-phenanthrol (Liu *et al.*, 2011). These data substantiate the effectiveness of 9-phenanthrol at discriminating TRPM4 from TRPM5 currents in native cells, which is challenging because TRPM4 and TRPM5 channels share biophysical and regula-

Table 2

Effects of 9-phenanthrol on endogenous currents

Current	Model	Effect	Concentration in mol.L^{-1}	References
TRPM4-like	HEK-293	Inhibition 80%	10^{-4}	Amarouch *et al.*, 2013
TRPM4-like transient inward	Rat cerebral arteries smooth muscle cells	Inhibition	$IC_{50} = 10^{-5}$	Gonzales *et al.*, 2010b
TRPM4-like transient inward	Monkey colonic smooth muscle cells	Inhibition 80%	10^{-4}	Dwyer *et al.*, 2011
TRPM4-like transient inward	Rat, guinea pig DSM cells	Inhibition 40%	3×10^{-5}	Parajuli *et al.*, 2013; Smith *et al.*, 2013a,b
TRPM4-like	Mouse atrial cardiomyocytes	Inhibition 80%	10^{-5}	Simard *et al.*, 2013
TRPM4-like	Human adenocarcinoma AGS cells	Inhibition 90%	3×10^{-5}	Kim *et al.*, 2012
TRPM4-like	Human adenocarcinoma MKN-45 cells	Inhibition 90%	3×10^{-5}	Kim *et al.*, 2012
TRPM4-like depolarization-induced slow	Mouse Purkinje cells from cerebellar slices	Inhibition 100%	10^{-4}	Kim *et al.*, 2013
TRPM5-like	Mouse tongue taste cells	None	10^{-4}	Liu *et al.*, 2011
TRPM7-like	Human adenocarcinoma AGS cells	None	3×10^{-5}	Kim *et al.*, 2012
TRPM7-like	Human adenocarcinoma MKN-45 cells	None	3×10^{-5}	Kim *et al.*, 2012
TRPM7-like	Mouse intestinal cells of Cajal	None	3×10^{-5}	Kim *et al.*, 2011
Voltage-dependent K^+	Rat cerebral arteries smooth muscle cells	None	3×10^{-5}	Gonzales *et al.*, 2010b
BK_{Ca}	Rat cerebral arteries smooth muscle cells	None	3×10^{-5}	Gonzales *et al.*, 2010b
K_{IR}	Rat cerebral arteries smooth muscle cells	None	3×10^{-5}	Gonzales *et al.*, 2010b
K_v	Rat cerebral arteries smooth muscle cells	None	3×10^{-5}	Gonzales *et al.*, 2010b
$I_{Ca,L}$	Rat cerebral arteries smooth muscle cells	None	3×10^{-5}	Gonzales *et al.*, 2010b
$I_{Ca,L}$	Rat ventricular cardiomyocyte	None Inhibition 50%	10^{-5} 10^{-4}	Simard *et al.*, 2012a
I_K	Rat ventricular cardiomyocyte	None Inhibition 40%	10^{-5} 10^{-4}	Simard *et al.*, 2012a

When known, the IC_{50} is presented, otherwise, the concentration tested is indicated with the corresponding % of inhibition.

tory properties as well as sensitivity to intracellular Ca^{2+} yet lack of Ca^{2+} permeability, as mentioned earlier (Guinamard *et al.*, 2011). It is interesting to note that the main difference between TRPM4 and TRPM5 channels is ATP sensitivity; TRPM4 is blocked by internal ATP, while TRPM5 is not (Ullrich *et al.*, 2005). This discrepancy is probably due to the presence of only one ATP binding site within the TRPM5 channel that appears to be inaccessible, while TRPM4 channel possesses four ATP binding sites (Ullrich *et al.*, 2005). This disparity between TRPM4 and TRPM5 might provide some insights into the mechanism(s) involved in selective TRPM4 inhibition by 9-phenanthrol.

Thirty micromolar 9-phenanthrol did not modulate transient receptor potential canonical (TRPC) 3 or TRPC6 currents after recombinant expression in HEK-293 cells (Gonzales *et al.*, 2010b). This lack of effect is important because TRPC3 and TRPC6 channels often co-express with the TRPM4 channel, particularly in cardiac and smooth muscle preparations.

In addition to non-selective cation channels, 9-phenanthrol was tested on two recombinant chloride channels. Firstly, the effects of 9-phenanthrol were assessed via radioactive iodide efflux on recombinant CFTR Cl^- channel in CHO cells. Even at 2.5×10^{-4} mol·L^{-1}, 9-phenanthrol had no effect (Grand *et al.*, 2008). Once again, TRPM4 and CFTR may be expressed in the same tissue. Secondly, an inhibitory effect of 9-phenanthrol on recombinant TMEM16A channels expressed in HEK-293 was reported in a recent abstract (Burris *et al.*, 2013), but the results have not yet been scrutinized. However, pharmacological parity with TRPM4 is conceivable, because the TMEM16A channel also supports a Ca^{2+}-activated chloride current (Terashima *et al.*, 2013) and is widely

co-expressed with the TRPM4 channel in smooth muscle cells (Bulley and Jaggar, 2013).

9-Phenanthrol was tested on several native ionic currents with properties different from TRPM4 current. A TRPM7-like endogenous current from human gastric adenocarcinoma cell lines AGS and MKN-45 was found to be insensitive to 3×10^{-5} mol·L^{-1} 9-phenanthrol (Kim *et al.*, 2012) as was a similar current from mouse interstitial cells of Cajal (Kim *et al.*, 2011).

In cerebral artery smooth muscle cells from rats, neither voltage-gated K^+ currents (K_V), large conductance Ca^{2+}-activated K^+ currents (BK_{Ca}), inward rectifyer K^+ currents (K_{IR}), nor L-type Ca^{2+} currents ($I_{Ca,L}$) were modulated by 3×10^{-5} mol·L^{-1} 9-phenanthrol (Gonzales *et al.*, 2010b). However, it is more likely that higher concentrations of the drug are needed to have an effect on these channels. Indeed, as reported in mouse isolated ventricular cardiomyocytes, 10^{-5} mol·L^{-1} 9-phenanthrol has no effect on any of these currents, but 10^{-4} mol·L^{-1} 9-phenanthrol partially inhibits L-type Ca^{2+} currents and the delayed outward rectifier K^+ current (I_K) (Simard *et al.*, 2012a).

In dopamine neurons of the substantia nigra from mice that expressed both TRPM4 and hyperpolarization and cyclic nucleotide-gated (HCN) channels (which gives rise to hyperpolarization-activated mixed cation current I_h in neurons), 10^{-4} mol·L^{-1} 9-phenanthrol and the HCN channel blocker ZD7288 have distinct effects on rhythmic neuronal activity (Mrejeru *et al.*, 2011). While indirect, these data suggest that 9-phenanthrol, even at high concentrations, does not affect HCN channels.

To be exhaustive, even if it is not in the field of ion channels, we have to mention the results from a study indicating a potent inhibitory effect of 9-phenanthrol, with other phenanthrene derivatives, on bovine heart cyclic-AMP-dependent PKA catalytic subunits (Wang *et al.*, 1994). However, the experiments were only conducted *in vitro* by biochemical assays of the reaction medium, and this inhibitory effect on PKA catalytic subunits has not been demonstrated in model cells of native tissues. To the best of our knowledge, this biochemical experiment from the 1990s has never been repeated or confirmed. On the contrary, the effects of 9-phenanthrol in cardiac preparations were not precluded by the simultaneous application of the PKA inhibitor H-89, which argues against an effect of 9-phenanthrol via PKA inhibition (Simard *et al.*, 2012a; 2013).

Altogether, these results indicate that 9-phenanthrol is a selective TRPM4 channel inhibitor. Nonetheless, as shown in cardiomyocytes, some side effects may be observed at concentrations at or exceeding 10^{-4} mol·L^{-1}.

Smooth muscle cell contraction

Identifying 9-phenanthrol as a TRPM4 channel-specific inhibitor has provided the opportunity to distinguish TRPM4 currents from currents evoked through TRPM5 and it has provided a powerful tool to reveal the role(s) of TRPM4 channels in physiological processes. Through its modulation of TRPM4 channels, 9-phenanthrol affects smooth muscle behaviour, spontaneous firing in neurons and cardiomyocytes, as well as protecting against cardiac or vascular injuries (see later and Table 3).

A preliminary series of studies focused on arterial smooth muscles that are known to express TRPM4 (Earley, 2013). Arteries respond to pressure overload by a vasoconstriction in order to autoregulate blood flow. In an experimental model of isolated cerebral arteries from rat, 9-phenanthrol dilated arteries that were previously pressurized (Gonzales *et al.*, 2010b). This occurred with an EC_{50} of 2.6×10^{-6} mol·L^{-1} indicating a high sensitivity to 9-phenanthrol. Interestingly, down-regulation of *Trpm4* expression using antisense oligodeoxynucleoides also affected vasoconstriction in this preparation, which further implicates the TRPM4 channel in this tissue (Earley *et al.*, 2004). Arterial contraction requires L-type Ca^{2+} current activation. Thus, control of membrane potential is a critical factor that influences the phenomenon. Using intracellular microelectrode recordings, 3×10^{-5} mol·L^{-1} 9-phenanthrol was shown to hyperpolarize vascular smooth muscle cells in pressurized cerebral arteries. The mechanism of action probably involves the inhibition of a TRPM4-depolarizing current via a mechanism explained later (Gonzales *et al.*, 2010b). 9-Phenanthrol was also shown to inhibit a depolarizing transient inward cationic current in the same preparation; this effect was prevented if the cells were treated with *Trpm4* small interfering RNA (Gonzales *et al.*, 2010a; Gonzales and Earley, 2012). The putative mechanism for vasoconstriction of cerebral arteries in response to pressure-dilatation involves a mechanosensitive membrane protein coupled to PLC, whose activation produces inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to its receptor on the sarcoplasmic reticulum to induce calcium release and thus TRPM4 channel activation ensues. The TRPM4 channel is an inward charge carrier (with a reversal potential ~0 mV) that induces cell depolarization in favour of L-type Ca^{2+} current activation, producing inward Ca^{2+} currents and cell contraction (Earley, 2013). The phenomenon also depends on PKC to promote translocation of TRPM4 channels to the plasma membrane. Small interfering *Trpm4* RNA or treatment with 2×10^{-5} mol·L^{-1} 9-phenanthrol similarly abolish the PKC-induced cerebral artery vasoconstriction (Crnich *et al.*, 2010). Cerebral artery pressure-induced constriction involves sphingosine-kinase-1 translocation from the cytosol to the plasma membrane, which produces sphingosine-1-phosphate following membrane depolarization. In an experimental model of rabbit posterior cerebral arteries, 5×10^{-6} mol·L^{-1} 9-phenanthrol attenuated this pressure-induced translocation (Lim *et al.*, 2012).

Among smooth muscles that express the TRPM4 channel, an effect of 9-phenanthrol was also demonstrated in DSM that controls urinary bladder contraction. The expression level of *Trpm4* mRNA is 2.6-fold greater in DSM cells than in cerebral artery myocytes (Parajuli *et al.*, 2013). The intracellular signalling that leads to DSM contraction remains to be determined, but it may resemble the one described earlier for cerebral arteries. Variations in the intracellular Ca^{2+} concentrations activate a transient inward cationic current that is consistent with an NSC_{Ca}. This inward current causes cell membrane depolarization, inducing L-type Ca^{2+} current activation, which leads to significant accumulation of intracellular Ca^{2+} that then stimulates contraction (Smith *et al.*, 2013b). 9-Phenanthrol affects several of these parameters in DSM. The transient inward cationic current with TRPM4-like properties was inhibited by 3×10^{-5} mol·L^{-1} 9-phenanthrol in both

Table 3

Effects of 9-phenanthrol on physiological processes

Physiological processes	Model	Effect	Concentration in mol·L^{-1}	References
Artery constriction	Rat cerebral arteries	Inhibition	$IC_{50} = 2.6 \times 10^{-6}$	Gonzales *et al.*, 2010b
Smooth muscle contraction	Rat, guinea pig isolated detrusor muscle	Inhibition	$IC_{50} = 3 \times 10^{-6}$	Parajuli *et al.*, 2013; Smith *et al.*, 2013a,b
Cardiac beating rate	Mouse, rat, rabbit isolated right atrium	Reduction	$EC_{50} = 7.8 \times 10^{-6}$	Hof *et al.*, 2013
Cardiac atrial action potential duration	Mouse isolated atrium	Reduction	$EC_{50} = 2.2 \times 10^{-5}$	Simard *et al.*, 2013
Hypoxia-reoxygenation induced cardiac arrhythmias	Mouse isolated ventricle	Reduction 60% Reduction 100%	10^{-5} 10^{-4}	Simard *et al.*, 2012a
Hypoxia-reoxygenation preconditioning	Rat isolated whole heart	Cardioprotection	3×10^{-5}	Wang *et al.*, 2013
LPS-induced cell death	Endothelial cells HUVECs	Reduction 50%	10^{-6}	Becerra *et al.*, 2011
Cell viability	Adrenocarcinoma cells AGS and MKN-45	None	3×10^{-5}	Kim *et al.*, 2012
Capillary structure formation	Endothelial cells HUVECs	Stimulation	5×10^{-6}	Loh *et al.*, 2013
Neuronal bursting activity	Mice coronal midbrain slice	Reduction	10^{-4}	Mrejeru *et al.*, 2011
Neuronal firing activity	Mice olfactory bulb slice	Reduction 50%	10^{-4}	Shpak *et al.*, 2012
Neuronal firing activity	Rat inhibitory prepositus hypoglossy nucleus neurons	Reduction 60%	10^{-4}	Saito and Yanagawa, 2013

When known, the IC_{50} is presented, otherwise, the concentration tested is indicated with the corresponding % of reduction.

rat and guinea pig freshly isolated DSM cells (Smith *et al.*, 2013a,b). The same concentration of 9-phenanthrol also reduced the baseline intracellular Ca^{2+} level measured by Ca^{2+}-sensitive fluorescent dyes (Smith *et al.*, 2013a). Because this effect on baseline Ca^{2+} is reduced by nifedipine, but not by carbachol, it is attributed to a decrease in Ca^{2+} influx via L-type channels subsequent to a decrease in channel activation due to the lack of membrane depolarization (Smith *et al.*, 2013a). Contractions of the DSM are also affected by 9-phenanthrol. Whereas pharmacological tools such as carbachol increase basal tension and spontaneous contractions, 9-phenanthrol reduces the force, amplitude and frequency of carbachol-induced contractions from rat isolated DSM strips with an IC_{50} ranging from 1.7 to 3.3×10^{-6} mol·L^{-1} (Smith *et al.*, 2013b). DSMs are activated by parasympathetic inputs that induce bladder voiding behaviour. This neurogenic control can be mimicked by electrical field stimulation, which induces DSM strips to contract. During such recordings, 9-phenanthrol attenuates the amplitude of the contraction and muscle force with an IC_{50} of 2.3 and 2.8×10^{-6} mol·L^{-1} respectively (Smith *et al.*, 2013b). These results obtained using 9-phenanthrol indicate that the TRPM4 channel is a major contributor to excitation–contraction coupling in the DSM.

Membrane current regulation has also been demonstrated in smooth muscle cells of the colon. The resting membrane potential is generally less negative than the K^+ equilibrium potential as a result of a spontaneous inward current with non-selective cation permeability, which shares single-channel properties with TRPM4 (Dwyer *et al.*, 2011). In freshly dispersed colonic smooth muscle cells from monkeys, this TRPM4-like current is inhibited by 10^{-4} mol·L^{-1} 9-phenanthrol. No further experiments were conducted using 9-phenanthrol in this preparation, even though the authors demonstrated that La^{3+} and Gd^{3+} both modulated the baseline membrane potential, presumably by attenuating the same TRPM4-like current (Dwyer *et al.*, 2011).

Modulation of heart function

Investigating the role of the TRPM4 channel in cardiac physiology and pathophysiology is of great importance, firstly, because the heart is among the foremost TRPM4-expressing tissues (Launay *et al.*, 2002) and secondly, because the only known channelopathy related to TRPM4 dysfunction in humans perturbs cardiac electrical activity: for example, congenital conduction block and Brugada syndrome (Kruse *et al.*, 2009; Liu *et al.*, 2010; 2013). Endogenous TRPM4-like currents have been thoroughly characterized in cardiac preparations (see review, Guinamard *et al.*, 2011), but demonstrating their specific roles in cardiac tissues has only become possible following the discovery of 9-phenanthrol as a TRPM4 channel inhibitor. 9-Phenanthrol revealed the roles of the TRPM4 channel in both basal cardiac activity and in pathological-like conditions.

Cardiac rhythm initiates in the specific pacemaker tissue dubbed the sinoatrial node at the flank of the right atrium. Intracellular microelectrodes can record spontaneous activity of isolated right atrium preparations suspended in a superfusion bath. The rate of spontaneous action potentials from mouse and rat preparations is reversibly reduced by

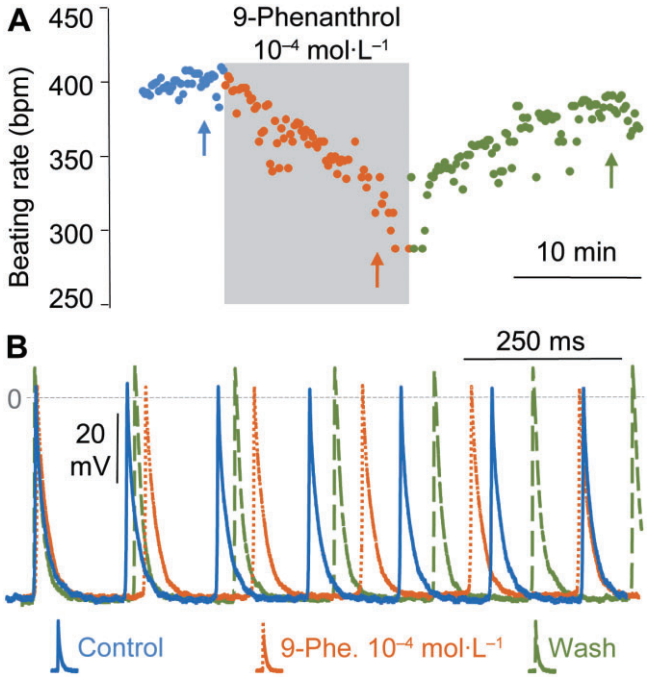

Figure 3

Effects of 9-phenanthrol on heart rhythm in a mouse isolated right atrium. Spontaneous action potentials were recorded using an intracellular microelectrode, while the right isolated atrium was superfused with oxygenated physiological solution. (A) Time course of the effect of 10^{-4} mol·L^{-1} 9-phenanthrol on beating rate measured every 10 s as beats min^{-1} (bpm). The application of 9-phenanthrol is indicated by the grey shading. Note that the effects of 9-phenanthrol were reversible. (B) Representative recordings for control, 9-phenanthrol, and washout, as indicated in (A) by arrows. See Hof *et al.* (2013) for protocols.

9-phenanthrol with an IC_{50} of 7.8×10^{-6} mol·L^{-1} (Figure 3; Hof *et al.*, 2013). A particular characteristic of sinoatrial node cells is the presence of a slow diastolic depolarization (absent in other cardiac myocytes) that drives the membrane potential over the threshold for Ca^{2+} current activation, and is responsible for the action potential upstroke. While a major part of this slow diastolic depolarization is due to the hyperpolarization-activated funny current (I_f) (Monfredi *et al.*, 2010), which is equivalent to I_h in neurons, an additional component of the slow diastolic depolarization is due to Ca^{2+}-activated depolarizing currents triggered by cytoplasmic Ca^{2+} oscillations. In the rabbit isolated sinoatrial node, 10^{-5} mol·L^{-1} 9-phenanthrol reduces both the beating rate and the slope of the slow diastolic depolarization, with no other effects on sinoatrial node action potentials. This 9-phenanthrol-induced reduction in sinoatrial node beating rate is absent in *Trpm4*$^{-/-}$ mice (Hof *et al.*, 2013) and TRPM4 currents were characterized in isolated sinoatrial node cells from wild-type mice (Demion *et al.*, 2007), which clearly demonstrates that the TRPM4 channel is involved in regulating the rate of beating. Interestingly, the ability of 9-phenanthrol to reduce the beating rate in mouse and rat isolated right atrium is more pronounced at low frequencies (Hof *et al.*, 2013), which suggests that the TRPM4 channel could protect against bradycardia and, this might be modulated by 9-phenanthrol.

The results summarized earlier indicate that 9-phenanthrol acts in sinoatrial node cells to directly slow the diastolic depolarization through inhibiton of TRPM4 channels. However, a study conducted on the HL-1 cell line derived from mouse atrial cells revealed that 9-phenanthrol also modulates cytoplasmic calcium oscillations (Burt *et al.*, 2013). HL-1 cells are derived from atrial cardiomyocytes, but not specifically sinoatrial node cells, and they acquire properties similar to those of pacemaker cells and develop free beating activity, with intracellular calcium oscillations being present in about 40% of the cells (Wondergem *et al.*, 2010). Fluorescent Ca^{2+} recordings using Fura-2 revealed that 10^{-5} mol·L^{-1} 9-phenanthrol abolishes these Ca^{2+} oscillations leading to a transient increase in cytoplasmic Ca^{2+}, attributed to the release of Ca^{2+} from an intracellular pool different from the sarcoplasmic reticulum and thus, is most likely to be mitochondrial Ca^{2+} (Burt *et al.*, 2013). These Ca^{2+} oscillations could participate in the spontaneous activity, since the application of 10^{-5} mol·L^{-1} 9-phenanthrol in cell-attached recordings from HL-1 cells abolished the depolarizing inward ionic currents that contribute to the slow diastolic depolarization (Burt *et al.*, 2013).

In addition to its effect on spontaneous beating, 9-phenanthrol also modulates action potentials evoked in mouse atrial cardiomyocytes; recorded using intracellular microelectrodes. 9-Phenanthrol reduced action potential duration, with an IC_{50} of 2.2×10^{-5} mol·L^{-1}, without affecting other action potential parameters or the resting membrane potential (Simard *et al.*, 2013). This effect of 9-phenanthrol on action potential duration is probably as a results of its inhibitory effect on TRPM4 channels because (i) a TRPM4 current can be recorded from mouse isolated atrial cells and this endogenous current is inhibited by 9-phenanthrol (Simard *et al.*, 2013); (ii) the effect of 9-phenanthrol is strongly reduced in ventricular tissue, which is known to express lower levels of the TRPM4 channel compared with the atrium (Guinamard *et al.*, 2006; Liu *et al.*, 2010; Simard *et al.*, 2013); and (iii) the 9-phenanthrol effect is completely absent in atria from *Trpm4*$^{-/-}$ mice (Simard *et al.*, 2013). While these experiments were done in mice, their relevance probably extends to humans as human isolated atrial cardiomyocytes have been reported to express functional TRPM4 channels (Guinamard *et al.*, 2004).

9-Phenanthrol does not only modulate basal cardiac activity, but has also been shown to prevent cardiac dysfunction induced by ischaemia-reperfusion episodes. Ischaemia, such as that observed in coronary occlusion, is a major source of cardiac damage. It produces electrical perturbations that lead to arrhythmias, it decreases contractility and it induces apoptosis. Although it corrects oxygen defects, reperfusion paradoxically exacerbates ischaemia-related perturbations, mainly through disturbance of internal Ca^{2+} homeostasis (Murphy and Steenbergen, 2008). As the TRPM4 channel is an ATP-sensitive Ca^{2+}activated channel and because it is highly expressed in heart, it was thought to be involved in ischaemia-related perturbations. Therefore, the TRPM4 channel inhibitor 9-phenanthrol was tested for possible cardioprotective effects. In an initial study, the effect of 9-phenanthrol against arrhythmias was investigated in a

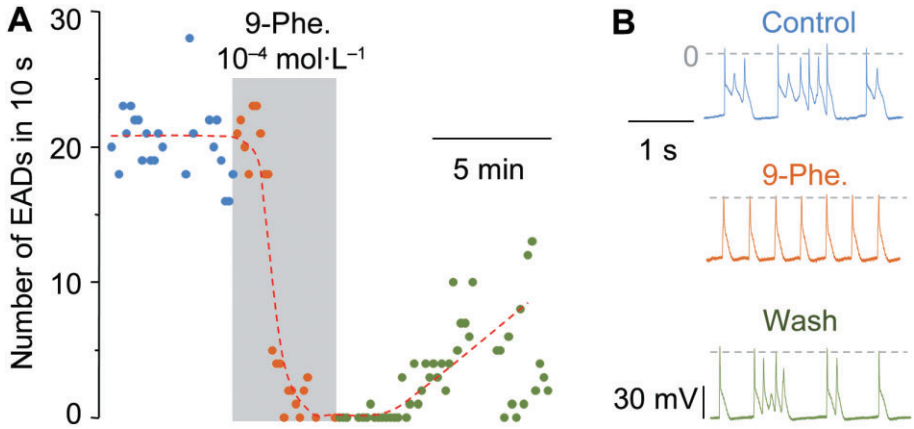

Figure 4

Cardioprotective effect of 9-phenanthrol. Anti-arrhythmic effects of 9-phenanthrol in a model of hypoxia-reoxygenation induced arrhythmias in mouse isolated ventricle. An isolated right ventricle was submitted to a hypoxic episode and then reoxygenated, which induces EADs. In this model, spontaneous beating was thought to arise from Purkinje fibres. (A) Time course of the effects of 10^{-4} mol·L^{-1} 9-phenanthrol on the occurrence of EADs. The number of EADs was measured in successive 10 s windows. The application of 9-phenanthrol is indicated by the grey shading. Note the total and reversible abolition of arrhythmias after the application of 9-phenanthrol. (B) Representative recordings for control, 9-phenanthrol and washout. While several EADs were present in one-fifth of the action potentials in control, the rhythm was perfectly regular in the presence of 9-phenanthrol. See Simard *et al.* (2012a) for protocols.

mouse isolated ventricle subjected to hypoxia-reoxygenation protocols, in order to mimic ischaemia-reperfusion conditions (Simard *et al.*, 2012a). During hypoxia, as well as during reoxygenation, arrhythmias were observed in the form of early after depolarizations (EADs). Superfusion of 10^{-5} mol·L^{-1} 9-phenanthrol during reoxygenation reduced the occurrence of EADs by 60% while 10^{-4} mol·L^{-1} totally abolished the EADs (Simard *et al.*, 2012a). Figure 4 shows an example of such anti-arrhythmic effects in ventricular myocytes. As in the atrial tissue, 9-phenanthrol does not regulate the resting membrane potential at the ventricular level. In this model, it was hypothesized that EADs were formed in the conductive tissue such as Purkinje fibres, where TRPM4 channels are highly expressed (Liu *et al.*, 2010), and were transmitted to the ventricular cells. In a second study, the cardioprotective effect of 9-phenanthrol was tested in a rat isolated whole heart perfused using a Langendorff apparatus (Wang *et al.*, 2013). Ischaemia-reperfusion was mimicked by switching off and on the perfusion of physiological solution in the coronary vessels. The preconditioning effect of 2×10^{-5} mol·L^{-1} 9-phenanthrol was tested by applying the drug 3 min before a 30 min ischaemia episode, which was then followed by reperfusion. 9-Phenanthrol prevented the decrease in contractile function, and the occurrence of ventricular fibrillation or tachycardia. In addition, as a proof of prophylaxis against cellular damage, 9-phenanthrol reduced lactate dehydrogenase activity and the size of the infarcted area (Wang *et al.*, 2013). As this effect of 9-phenanthrol was not prevented by addition of the ATP-sensitive K^+ channel inhibitor 5-hydroxydecanoate, its effects were attributed to the TRPM4 channel inhibition (Wang *et al.*, 2013). These two complementary studies revealed 9-phenanthrol to be a new and potentially powerful cardioprotective agent against ischaemia-reperfusion injuries. The rapid kinetics of 9-phenanthrol's antiarrhythmic effects is consistent with the direct involvement of the TRPM4 channel in cardiac action potentials (Simard *et al.*, 2012a). Inversely, the slow kinetics of 9-phenanthrol in the preconditioning model suggests an additional subcellular process subsequent to TRPM4 channel inhibition (Wang *et al.*, 2013).

Prevention of cell death

The TRPM4 channel is involved to some extent in cell death (Schattling *et al.*, 2012; Simard *et al.*, 2012b). Oxidative stress induces HeLa cell death by necrosis, but not apoptosis, through TRPM4 channel activation (Simon *et al.*, 2010). The effect of 9-phenanthrol on this process was investigated in a model of HUVECs, which endogenously express TRPM4 channels, but not TRPM5 channels (Becerra *et al.*, 2011). In those cells, LPS induces cell death by promoting Na^+ overload leading to cell depolarization and cell volume increase. LPS-induced cell death was estimated by lactate dehydrogenase release, thiazolyl blue tetrazolium bromide (MTT) salt colorimetric assay and trypan blue. Treatment with 10^{-6} mol·L^{-1} 9-phenanthrol reduced cell death by half (Becerra *et al.*, 2011). These protective effects of 9-phenanthrol were reproduced by glibenclamide (Becerra *et al.*, 2011), which is also known to inhibit the TRPM4 channel (Demion *et al.*, 2007). The LPS-induced Na^+-current is inhibited by glibenclamide, as expected, but the effect of 9-phenanthrol on this current was not tested (Becerra *et al.*, 2011). Note that in control conditions 9-phenanthrol at concentrations ranging from 10^{-7} to 5×10^{-6} mol·L^{-1} does not induce cell death in HUVECs, although higher concentrations may have deleterious effects (Loh *et al.*, 2013).

The prophylactic effect of 9-phenanthrol observed in HUVECs is probably not a widespread phenomenon, as application of 9-phenanthrol, even at 4×10^{-5} mol·L^{-1} does not protect the H9c2 cardiomyocyte cell line against peroxide-induced damage (Wang *et al.*, 2013).

The effects of 9-phenanthrol, at 1, 2 and 3×10^{-5} mol·L^{-1}, on cell viability were investigated in human gastric adenocarcinoma cell lines AGS and MKN-45 by use of the MTT colorimetric assay. None of these concentrations affected cell viability (Kim *et al.*, 2012).

Stimulation of angiogenesis after ischaemic stroke

Ischaemic stroke increases an ATP-sensitive NSC_{Ca} current in neurovascular cells and neuroendothelia (Simard *et al.*, 2006). This current is supported by a combination of SUR-1 and the TRPM4 channel, whose activation leads to oncotic death of endothelial cells and consequently, capillary fragmentation (Simard *et al.*, 2010; Woo *et al.*, 2013b). Ischaemic stroke conditions were mimicked in cultures of HUVECs by incubating the cells in a hypoxic solution and depriving them of glucose and serum (Loh *et al.*, 2013). Such deprivation increases cell death after 24 h. While 5×10^{-6} mol·L^{-1} 9-phenanthrol did not reduce cell death, it did promote the formation of capillary structures on Matrigel, as detected by light microscopy (Loh *et al.*, 2013). This effect was attributed to TRPM4 channel inhibition because 24 h of oxygen and glucose deprivation produces TRPM4 overexpression at the mRNA and protein level and activates a TRPM4-like current that is inhibited by 10^{-4} mol·L^{-1} 9-phenanthrol (Loh *et al.*, 2013). These results show that 9-phenanthrol, via TRPM4 channel inhibition, improves the functionality of endothelial cells under conditions that mimic ischaemic stroke.

Modulation of neuronal activity

9-Phenanthrol modulates electrical activity in neurons and neural systems. NSC_{Ca} current with the hallmarks of the TRPM4 channel are present in the CNS where they participate in generating bursting activity in networks such as the breathing-related rhythmogenic neurons from the pre-Bötzinger complex (Pace *et al.*, 2007; Mironov, 2008; Mironov and Skorova, 2011) and in neurons of the substantia nigra pars compacta (Mrejeru *et al.*, 2011). Glutamate receptor activation gives rise to cytoplasmic Ca^{2+} that activates the depolarizing NSC_{Ca} current, favouring rhythmic burst generation. The effect of 9-phenanthrol on this activity was tested in a coronal midbrain slice model from juvenile mice. Electrical bursting in nigrostriatal dopamine-releasing neurons was recorded under NMDA stimulation. Burst frequency was reversibly reduced by 10^{-4} mol·L^{-1} 9-phenanthrol, which changed the burst-firing pattern into one characterized by tonic firing (Mrejeru *et al.*, 2011).

Accessory olfactory bulb neurons involved in transmitting pheromonal stimuli also express *Trpm4* mRNA and exhibit a TRPM4-like current activated by afferent sensory fibre stimulation. In mice brain slices, inhibition of this current by 10^{-4} mol·L^{-1} 9-phenanthrol reduces their ability to discharge repetitively (i.e. persistent firing) (Shpak *et al.*, 2012).

The prepositus hypoglossi nucleus of the brainstem is involved in maintaining horizontal gaze. Inhibitory neurons from this structure exhibit NSC_{Ca} channels (Saito and Yanagawa, 2010). In slice preparations from the rabbit brainstem, 10^{-4} mol·L^{-1} 9-phenanthrol was shown to reduce firing rate by 60% in these neurons (Saito and Yanagawa, 2010; 2013).

The appropriate use of 9-phenanthrol in physiological studies

Our meta-analysis of the studies presented earlier suggests that 9-phenanthrol modulates physiological processes mainly through TRPM4 channel inhibition. (i) In most models, the drug reduces an NSC_{Ca} in *Trpm4*-expressing cells (Gonzales *et al.*, 2010a; Simard *et al.*, 2013; Smith *et al.*, 2013a,b). (ii) When tested, the effect of 9-phenanthrol is not reproduced in *Trpm4*$^{-/-}$ mice (Hof *et al.*, 2013; Simard *et al.*, 2013) or its effects can be mimicked by small interfering *Trpm4* RNA treatment (Crnich *et al.*, 2010). (iii) The IC_{50} values for the effects of 9-phenanthrol on physiological processes are in the same range as those measured for TRPM4 channel inhibition in recombinant HEK cells (Tables 1–3). (iv) The effect of 9-phenanthrol can be reproduced by other TRPM4 inhibitors such as flufenamic acid (Gonzales *et al.*, 2010a; Saito and Yanagawa, 2010; Mrejeru *et al.*, 2011; Shpak *et al.*, 2012; Simard *et al.*, 2012a; 2013; Amarouch *et al.*, 2013; Burt *et al.*, 2013; Kim *et al.*, 2013) and glibenclamide (Becerra *et al.*, 2011; Kim *et al.*, 2013).

We conclude that 9-phenanthrol can confidently be used as a specific TRPM4 channel inhibitor. Nevertheless, high concentrations (at or exceeding 10^{-4} mol·L^{-1}) must be used with caution for several reasons.

Firstly, 9-phenanthrol may precipitate at high concentrations, thus the final diluted concentration may be overestimated. 9-Phenanthrol is usually diluted in DMSO in a stock solution at 10^{-1} mol·L^{-1} and then diluted in the physiological perfusion solution, such that the maximal DMSO ratio of 0.01% is not exceeded even when the final concentration of 9-phenanthrol reaches 10^{-4} mol·L^{-1}. This concentration of DMSO in the bathing solution has no effect on the TRPM4 channel (Grand *et al.*, 2008). Because 9-phenanthrol partly precipitates at higher concentrations, one can increase the final DMSO concentration if a relatively high concentration of 9-phenanthrol is called for. Nevertheless, in general, this use of DMSO should be avoided because a high concentration of DMSO may introduce new side effects. DMSO inhibits non-selective cation channels such as NMDA and AMPA glutamate receptors when used at or above 0.5% (Lu and Mattson, 2001), and it inhibits whole-cell non-selective cationic currents in erythrocytes at or above 1% (Nardid *et al.*, 2013).

Secondly, in rat cardiomyocytes, 10^{-4} mol·L^{-1} 9-phenanthrol affects both L-type Ca^{2+} currents and delayed outward rectifier K^+ currents (Simard *et al.*, 2012a). Even if these effects on active membrane properties do not explain the modulation of electrical activity by 9-phenanthrol in cardiomyocytes, the effects on Ca^{2+} and K^+ channels must to be considered carefully because of the ubiquity and widespread influence of these channels on voltage trajectory and intracellular signalling in many types of excitable cells.

Thirdly, 9-phenanthrol autofluoresces under UV light (Moriconi *et al.*, 1959) in particular at 340 nm, which can induce artefacts when measuring Ca^{2+} or voltage using fluorescent dyes (Burt *et al.*, 2013). This can cause problems when using high concentrations.

Fourthly, while the effects of 9-phenanthrol are reversible in most studies, the recovery is more problematic at 10^{-4} mol·L^{-1}, which is probably due to the accumulation

of 9-phenanthrol in membranes, because of its high hydrophobicity.

The most appropriate concentrations of 9-phenanthrol for physiological studies range from 1×10^{-5} to 3×10^{-5} mol·L^{-1}. The lower limit corresponds closely to the IC_{50} for TRPM4 channel inhibition, while the upper limit produces an 80% inhibition of the current. The 3×10^{-5} mol·L^{-1} upper limit is sufficient to strongly affect recombinant TRPM4 current (Grand *et al.*, 2008) as well as physiological processes, and perhaps more importantly, is devoid of side effects. Although several studies have demonstrated the physiological effects of 9-phenanthrol, they are less apparent at lower concentrations.

Note that, while 9-phenanthrol dissolved in DMSO can be stored at –20°C, it degrades with time. We would thus recommend that once it is in solution it should be used within a month.

9-Phenanthrol, a TRPM4 channel inhibitor for what purpose?

Our reading and laboratory experience indicates that 9-phenanthrol is an appropriate tool to investigate the functional significance of the TRPM4 channel in physiological and pathological processes. The drug is suitable for a variety of physiological approaches such as: (i) electrophysiological recordings using patch-clamp pipettes in single-channel or whole-cell configurations; (ii) transmembrane potential recording in multicellular preparations using an intracellular microelectrode; (iii) calcium measurements by fluorescent dyes; (iv) measurements of smooth muscle contraction; and (v) cell culture (which is amenable to the same set of measuring tools listed earlier).

A particular strength of 9-phenanthrol is its ability to distinguish TRPM4 from TRPM5 channels, which are otherwise quite similar (Ullrich *et al.*, 2005; Guinamard *et al.*, 2011). Because these two ion channels hold ionic currents with similar properties, the discovery of 9-phenanthrol has provided a convenient tool to differentiate one from the other in native preparations. A variety of NSC_{Ca} reported in native cells remain to be identified at the molecular level. 9-Phenanthrol thus might help resolve whether the underlying channels are TRPM4, TRPM5, or another variety of TRP channel. This issue is especially relevant in kidney tissue, which is known to express high levels of *Trpm4* mRNA (Launay *et al.*, 2002) and a native NSC_{Ca} current, whose identity is still not known (Chraïbi *et al.*, 1994; Teulon, 2000). This disparity also applies for neurons of the pre-Bötzinger complex that express both *Trpm4* and *Trpm5* mRNA, and a NSC_{Ca} current implicated in rhythmic cellular and network bursting that serves to generate inspiratory breathing movements (Crowder *et al.*, 2007; Pace *et al.*, 2007; Mironov, 2008; Mironov and Skorova, 2011).

The TRPM4 channel has already been shown to be involved in a variety of physiological processes, such as cerebral artery constriction (Earley, 2013), insulin secretion by pancreatic beta cells (Cheng *et al.*, 2007), immune responses (Launay *et al.*, 2004; Vennekens *et al.*, 2007; Barbet *et al.*, 2008), DSM contraction (Smith *et al.*, 2013a,b) and cardiac action potentials (Hof *et al.*, 2013; Simard *et al.*, 2013). Moreover, the channel is implicated in pathologies such as autoimmune encephalomyelitis (Schattling *et al.*, 2012), ischaemia and ischaemia-reperfusion injuries in the brain or the heart (Simard *et al.*, 2012a; Loh *et al.*, 2013), cardiac hypertrophy (Guinamard *et al.*, 2006), as well as human cardiac-genetic diseases (Kruse *et al.*, 2009; Liu *et al.*, 2010; 2013). It is tempting to speculate that 9-phenanthrol, through its effects on TRPM4 current, may modulate such pathological processes and could potentially ameliorate or correct their perturbations. This has already been observed in several studies, as described earlier. By modulating cardiac rhythm, 9-phenanthrol might function as a bradycardic agent (Hof *et al.*, 2013). Also, as it reduces cardiac injuries induced by hypoxia-reoxygenation 9-phenanthrol may have cardioprotective properties (Simard *et al.*, 2012a; Wang *et al.*, 2013). By promoting angiogenesis, the drug may inhibit cerebral oedema damage after ischaemic stroke (Loh *et al.*, 2013). Its inhibitory effect on the detrusor muscle might mean it has potential as a drug for the treatment of overactive bladder (Smith *et al.*, 2013a,b). By preventing LPS-induced endothelial cell death, 9-phenanthrol may benefit the treatment of endotoxaemia-derived sepsis (Becerra *et al.*, 2011). However, while these possibilities are exciting and provocative, it is still too early to predict specific clinical applications because all of the studies reviewed earlier were performed *in vitro* or on isolated cells or tissues. Among the barriers that must be overcome before going further in that direction is the ability to reach the sufficiently high levels of circulating drug *in vivo* to inhibit the TRPM4 channel. In that regard, the low solubility of 9-phenanthrol might be an obstacle. In addition, the toxicity of 9-phenanthrol has to be carefully evaluated as PAH are known to have consistent toxic effects (Feng *et al.*, 2012). This last point was highlighted by results from a very recent study using *in vitro* biochemical assays, which indicated that 9-phenanthrol inhibits the biosynthesis of androgen and oestrogen in subcellular fractions of carp gonads (Fernandes and Porte, 2013).

Conclusion

The identification of 9-phenanthrol as a TRPM4 channel inhibitor opens up new ways to discover the role(s) of the TRPM4 channel and provides a specific and potent pharmacological tool to examine the ion channel-level mechanisms underlying physiological and pathophysiological processes. The applicability of this molecule or related drugs for therapeutic purposes is a new prospect that remains to be explored.

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Conflicts of interest

The authors state no conflict of interest.

References

Alexander SP, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Spedding M, Peters JA, Harmar AJ; CGTP Collaborators (2013). The Concise Guide to PHARMACOLOGY 2013/14: Ion Channels. Br J Pharmacol 170: 1607–1651.

Amarouch M-Y, Syam N, Abriel H (2013). Biochemical, single-channel, whole-cell patch clamp, and pharmacological analyses of endogenous TRPM4 channels in HEK293 cells. Neurosci Lett 541: 105–110.

Barbet G, Demion M, Moura IC, Serafini N, Léger T, Vrtovsnik F *et al.* (2008). The calcium-activated nonselective cation channel TRPM4 is essential for the migration but not the maturation of dendritic cells. Nat Immunol 9: 1148–1156.

Becerra A, Echeverría C, Varela D, Sarmiento D, Armisén R, Nuñez-Villena F *et al.* (2011). Transient receptor potential melastatin 4 inhibition prevents lipopolysaccharide-induced endothelial cell death. Cardiovasc Res 91: 677–684.

Bulley S, Jaggar JH (2013). Cl(-) channels in smooth muscle cells. Pflugers Arch [Epub ahead of print].

Burris S, Neeb ZP, Jaggar J (2013). 9-Phenanthrol inhibits TMEM16A channels. FASEB J 27. doi: 10.1007/s00424-013-1357-2.

Burt R, Graves BM, Gao M, Li C, Williams DL, Fregoso SP *et al.* (2013). 9-Phenanthrol and flufenamic acid inhibit calcium oscillations in HL-1 mouse cardiomyocytes. Cell Calcium 54: 193–201.

Cheng H, Beck A, Launay P, Gross SA, Stokes AJ, Kinet J-P *et al.* (2007). TRPM4 controls insulin secretion in pancreatic beta-cells. Cell Calcium 41: 51–61.

Chraïbi A, Van den Abbeele T, Guinamard R, Teulon J (1994). A ubiquitous non-selective cation channel in the mouse renal tubule with variable sensitivity to calcium. Pflügers Arch Eur J Physiol 429: 90–97.

Crnich R, Amberg GC, Leo MD, Gonzales AL, Tamkun MM, Jaggar JH *et al.* (2010). Vasoconstriction resulting from dynamic membrane trafficking of TRPM4 in vascular smooth muscle cells. Am J Physiol Cell Physiol 299: C682–C694.

Crowder EA, Saha MS, Pace RW, Zhang H, Prestwich GD, Del Negro CA (2007). Phosphatidylinositol 4,5-bisphosphate regulates inspiratory burst activity in the neonatal mouse pre Bötzinger complex. J Physiol 582: 1047–1058.

Demion M, Bois P, Launay P, Guinamard R (2007). TRPM4, a Ca2+-activated nonselective cation channel in mouse sino-atrial node cells. Cardiovasc Res 73: 531–538.

Dwyer L, Rhee P-L, Lowe V, Zheng H, Peri L, Ro S *et al.* (2011). Basally activated nonselective cation currents regulate the resting membrane potential in human and monkey colonic smooth muscle. Am J Physiol Gastrointest Liver Physiol 301: G287–G296.

Earley S (2013). TRPM4 channels in smooth muscle function. Pflügers Arch Eur J Physiol 465: 1223–1231.

Earley S, Waldron BJ, Brayden JE (2004). Critical role for transient receptor potential channel TRPM4 in myogenic constriction of cerebral arteries. Circ Res 95: 922–929.

Escartin E, Porte C (1999). Biomonitoring of PAH pollution in high-altitude mountains lakes through the analysis of fish bile. Environ Sci Technol 33: 406–409.

Feng T-C, Cui C-Z, Dong F, Feng Y-Y, Liu Y-D, Yang X-M (2012). Phenanthrene biodegradation by halophilic Martelella sp. AD-3. J Appl Microbiol 113: 779–789.

Fernandes D, Porte C (2013). Hydroxylated PAHs alter the synthesis of androgens and estrogens in subcellular fractions of carp gonads. Sci Total Environ 447: 152–159.

Frelet A, Klein M (2006). Insight in eukaryotic ABC transporter function by mutation analysis. FEBS Lett 580: 1064–1084.

Gonzales AL, Earley S (2012). Endogenous cytosolic Ca(2+) buffering is necessary for TRPM4 activity in cerebral artery smooth muscle cells. Cell Calcium 51: 82–93.

Gonzales AL, Amberg GC, Earley S (2010a). Ca2 + release from the sarcoplasmic reticulum is required for sustained TRPM4 activity in cerebral artery smooth muscle cells. Am J Physiol Cell Physiol 299: C279–C288.

Gonzales AL, Garcia ZI, Amberg GC, Earley S (2010b). Pharmacological inhibition of TRPM4 hyperpolarizes vascular smooth muscle. Am J Physiol Cell Physiol 299: C1195–C1202.

Gögelein H, Pfannmüller B (1989). The nonselective cation channel in the basolateral membrane of rat exocrine pancreas. Inhibition by 3′,5-dichlorodiphenylamine-2-carboxylic acid (DCDPC) and activation by stilbene disulfonates. Pflügers Arch Eur J Physiol 413: 287–298.

Grand T, Demion M, Norez C, Mettey Y, Launay P, Becq F *et al.* (2008). 9-Phenanthrol inhibits human TRPM4 but not TRPM5 cationic channels. Br J Pharmacol 153: 1697–1705.

Guinamard R, Chatelier A, Demion M, Potreau D, Patri S, Rahmati M *et al.* (2004). Functional characterization of a Ca(2+)-activated non-selective cation channel in human atrial cardiomyocytes. J Physiol 558: 75–83.

Guinamard R, Demion M, Magaud C, Potreau D, Bois P (2006). Functional expression of the TRPM4 cationic current in ventricular cardiomyocytes from spontaneously hypertensive rats. Hypertension 48: 587–594.

Guinamard R, Sallé L, Simard C (2011). The non-selective monovalent cationic channels TRPM4 and TRPM5. Adv Exp Med Biol 704: 147–171.

Guinamard R, Simard C, Del Negro C (2013). Flufenamic acid as an ion channel modulator. Pharmacol Ther 138: 272–284.

Hof T, Simard C, Rouet R, Sallé L, Guinamard R (2013). Implication of the TRPM4 nonselective cation channel in mammalian sinus rhythm. Heart Rhythm 10: 1683–1689.

Kim BJ, Nam JH, Kim SJ (2011). Effects of transient receptor potential channel blockers on pacemaker activity in interstitial cells of Cajal from mouse small intestine. Mol Cells 32: 153–160.

Kim BJ, Kim S-Y, Lee S, Jeon J-H, Matsui H, Kwon YK *et al.* (2012). The role of transient receptor potential channel blockers in human gastric cancer cell viability. Can J Physiol Pharmacol 90: 175–186.

Kim YS, Kang E, Makino Y, Park S, Shin JH, Song H *et al.* (2013). Characterizing the conductance underlying depolarization-induced slow current in cerebellar Purkinje cells. J Neurophysiol 109: 1174–1181.

Koenig S, Porte C, Solé M, Sturve J (2013). Biliary PAH and alkylphenol metabolites, biomarker enzyme activities, and gene expression levels in the deep-sea fish *Alepocephalus rostratus*. Environ Sci Technol 47: 2854–2861.

Kruse M, Schulze-Bahr E, Corfield V, Beckmann A, Stallmeyer B, Kurtbay G *et al.* (2009). Impaired endocytosis of the ion channel TRPM4 is associated with human progressive familial heart block type I. J Clin Invest 119: 2737–2744.

Launay P, Fleig A, Perraud AL, Scharenberg AM, Penner R, Kinet JP (2002). TRPM4 is a Ca2+-activated nonselective cation channel mediating cell membrane depolarization. Cell 109: 397–407.

Launay P, Cheng H, Srivatsan S, Penner R, Fleig A, Kinet J-P (2004). TRPM4 regulates calcium oscillations after T cell activation. Science 306: 1374–1377.

Lim M, Choi S-K, Cho Y-E, Yeon S-I, Kim E-C, Ahn D-S *et al*. (2012). The role of sphingosine kinase 1/sphingosine-1-phosphate pathway in the myogenic tone of posterior cerebral arteries. PLoS ONE 7: e35177.

Liu H, El Zein L, Kruse M, Guinamard R, Beckmann A, Bozio A *et al*. (2010). Gain-of-function mutations in TRPM4 cause autosomal dominant isolated cardiac conduction disease. Circ. Cardiovasc Genet 3: 374–385.

Liu H, Chatel S, Simard C, Syam N, Salle L, Probst V *et al*. (2013). Molecular genetics and functional anomalies in a series of 248 Brugada cases with 11 mutations in the TRPM4 channel. PLoS ONE 8: e54131.

Liu P, Shah BP, Croasdell S, Gilbertson TA (2011). Transient receptor potential channel type M5 is essential for fat taste. J Neurosci Off J Soc Neurosci 31: 8634–8642.

Loh KP, Ng G, Yu CY, Fhu CK, Yu D, Vennekens R *et al*. (2013). TRPM4 inhibition promotes angiogenesis after ischemic stroke. Pflugers Arch 466: 563–576.

Lu C, Mattson MP (2001). Dimethyl sulfoxide suppresses NMDA- and AMPA-induced ion currents and calcium influx and protects against excitotoxic death in hippocampal neurons. Exp Neurol 170: 180–185.

Mironov SL (2008). Metabotropic glutamate receptors activate dendritic calcium waves and TRPM channels which drive rhythmic respiratory patterns in mice. J Physiol 586: 2277–2291.

Mironov SL, Skorova EY (2011). Stimulation of bursting in pre-Bötzinger neurons by Epac through calcium release and modulation of TRPM4 and K-ATP channels. J Neurochem 117: 295–308.

Monfredi O, Dobrzynski H, Mondal T, Boyett MR, Morris GM (2010). The anatomy and physiology of the sinoatrial node – a contemporary review. Pacing Clin Electrophysiol 33: 1392–1406.

Moriconi EJ, Wallenberger FT, O'Connor WF (1959). A new synthesis of 9-phenanthrol; absorption spectra of the quinhydrone-type molecular compound between 9-phenanthrol and phenanthrenequinone. J Org Chem 24: 86–90.

Mrejeru A, Wei A, Ramirez JM (2011). Calcium-activated non-selective cation currents are involved in generation of tonic and bursting activity in dopamine neurons of the substantia nigra pars compacta. J Physiol 589: 2497–2514.

Murphy E, Steenbergen C (2008). Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. Physiol Rev 88: 581–609.

Nardid OA, Schetinskey MI, Kucherenko YV (2013). Dimethyl sulfoxide at high concentrations inhibits non-selective cation channels in human erythrocytes. Gen Physiol Biophys 32: 23–32.

Nilius B, Prenen J, Voets T, Droogmans G (2004). Intracellular nucleotides and polyamines inhibit the Ca2+-activated cation channel TRPM4b. Pflügers Arch Eur J Physiol 448: 70–75.

Pace RW, Mackay DD, Feldman JL, Del Negro CA (2007). Inspiratory bursts in the pre Bötzinger complex depend on a calcium-activated non-specific cation current linked to glutamate receptors in neonatal mice. J Physiol 582: 113–125.

Parajuli SP, Hristov KL, Sullivan MN, Xin W, Smith AC, Earley S *et al*. (2013). Control of urinary bladder smooth muscle excitability by the TRPM4 channel modulator 9-phenanthrol. Channels Austin Tex 7: 537–540.

Pschorr R, Schroter J (1902). 9-Aminophenanthrene. Berl Ber Chem Ges 35: 2726–2729.

Rich S, Horsfall JG (1954). Relation of polyphenol oxidases to fungitoxicity. Proc Natl Acad Sci U S A 40: 139–145.

Saito Y, Yanagawa Y (2010). Synaptic mechanism for the sustained activation of oculomotor integrator circuits in the rat prepositus hypoglossi nucleus: contribution of Ca2+-permeable AMPA receptors. J Neurosci Off J Soc Neurosci 30: 15735–15746.

Saito Y, Yanagawa Y (2013). Ca(2+)-activated ion currents triggered by ryanodine receptor-mediated Ca(2+) release control firing of inhibitory neurons in the prepositus hypoglossi nucleus. J Neurophysiol 109: 389–404.

Schattling B, Steinbach K, Thies E, Kruse M, Menigoz A, Ufer F *et al*. (2012). TRPM4 cation channel mediates axonal and neuronal degeneration in experimental autoimmune encephalomyelitis and multiple sclerosis. Nat Med 18: 1805–1811.

Sheppard DN, Welsh MJ (1992). Effect of ATP-sensitive K + channel regulators on cystic fibrosis transmembrane conductance regulator chloride currents. J Gen Physiol 100: 573–591.

Shpak G, Zylbertal A, Yarom Y, Wagner S (2012). Calcium-activated sustained firing responses distinguish accessory from main olfactory bulb mitral cells. J Neurosci Off J Soc Neurosci 32: 6251–6262.

Simard C, Sallé L, Rouet R, Guinamard R (2012a). Transient receptor potential melastatin 4 inhibitor 9-phenanthrol abolishes arrhythmias induced by hypoxia and re-oxygenation in mouse ventricle. Br J Pharmacol 165: 2354–2364.

Simard C, Hof T, Keddache Z, Launay P, Guinamard R (2013). The TRPM4 non-selective cation channel contributes to the mammalian atrial action potential. J Mol Cell Cardiol 59: 11–19.

Simard JM, Chen M, Tarasov KV, Bhatta S, Ivanova S, Melnitchenko L *et al*. (2006). Newly expressed SUR1-regulated NC(Ca-ATP) channel mediates cerebral edema after ischemic stroke. Nat Med 12: 433–440.

Simard JM, Kahle KT, Gerzanich V (2010). Molecular mechanisms of microvascular failure in central nervous system injury – synergistic roles of NKCC1 and SUR1/TRPM4. J Neurosurg 113: 622–629.

Simard JM, Woo SK, Gerzanich V (2012b). Transient receptor potential melastatin 4 and cell death. Pflügers Arch Eur J Physiol 464: 573–582.

Simon F, Leiva-Salcedo E, Armisén R, Riveros A, Cerda O, Varela D *et al*. (2010). Hydrogen peroxide removes TRPM4 current desensitization conferring increased vulnerability to necrotic cell death. J Biol Chem 285: 37150–37158.

Smith AC, Hristov KL, Cheng Q, Xin W, Parajuli SP, Earley S *et al*. (2013a). Novel role for the transient potential receptor melastatin 4 channel in guinea pig detrusor smooth muscle physiology. Am J Physiol Cell Physiol 304: C467–C477.

Smith AC, Parajuli SP, Hristov KL, Cheng Q, Soder RP, Afeli SAY *et al*. (2013b). TRPM4 channel: a new player in urinary bladder smooth muscle function in rats. Am J Physiol Renal Physiol 304: F918–F929.

Talavera K, Yasumatsu K, Yoshida R, Margolskee RF, Voets T, Ninomiya Y *et al*. (2008). The taste transduction channel TRPM5 is a locus for bitter-sweet taste interactions. FASEB J Off Publ Fed Am Soc Exp Biol 22: 1343–1355.

Terashima H, Picollo A, Accardi A (2013). Purified TMEM16A is sufficient to form Ca2+-activated Cl- channels. Proc Natl Acad Sci U S A 110: 19354–19359.

Teulon J (2000) Ca2+-activated nonselective cation channels. In: Endo M, Kurachi Y, Mishina M (eds). Pharmacology of Ionic Channel Function: Activators and Inhibitors. Springer-Verlag: Berlin, pp. 625–649.

Ullrich ND, Voets T, Prenen J, Vennekens R, Talavera K, Droogmans G *et al*. (2005). Comparison of functional properties of the Ca2+-activated cation channels TRPM4 and TRPM5 from mice. Cell Calcium 37: 267–278.

Vennekens R, Olausson J, Meissner M, Bloch W, Mathar I, Philipp SE *et al*. (2007). Increased IgE-dependent mast cell activation and anaphylactic responses in mice lacking the calcium-activated nonselective cation channel TRPM4. Nat Immunol 8: 312–320.

Wang BH, Ternai B, Polya GM (1994). Specific inhibition of cyclic AMP-dependent protein kinase by the antimalarial halofantrine and by related phenanthrenes. Biol Chem Hoppe Seyler 375: 527–535.

Wang J, Takahashi K, Piao H, Qu P, Naruse K (2013). 9-Phenanthrol, a TRPM4 inhibitor, protects isolated rat hearts from ischemia-reperfusion injury. PLoS ONE 8: e70587.

White NJ (2007). Cardiotoxicity of antimalarial drugs. Lancet Infect Dis 7: 549–558.

Wondergem R, Graves BM, Ozment-Skelton TR, Li C, Williams DL (2010). Lipopolysaccharides directly decrease Ca2 + oscillations and the hyperpolarization-activated nonselective cation current If in immortalized HL-1 cardiomyocytes. Am J Physiol Cell Physiol 299: C665–C671.

Woo SK, Kwon MS, Ivanov A, Geng Z, Gerzanich V, Simard JM (2013a). Complex N-glycosylation stabilizes surface expression of transient receptor potential melastatin 4b. J Biol Chem 288: 36409–36417.

Woo SK, Kwon MS, Ivanov A, Gerzanich V, Simard JM (2013b). The sulfonylurea receptor 1 (Sur1)-transient receptor potential melastatin 4 (Trpm4) channel. J Biol Chem 288: 3655–3667.