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Canonical transient receptor potential 4 and its small molecule modulators

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Canonical transient receptor potential 4 (TRPC4) forms non-selective cation channels that contribute to phospholipase C-dependent Ca^{2+} entry into cells following stimulation of G protein coupled receptors and receptor tyrosine kinases. Moreover, the channels are regulated by pertussis toxin-sensitive $G_{i/o}$ proteins, lipids, and various other signaling mechanisms. TRPC4-containing channels participate in the regulation of a variety of physiological functions, including excitability of both gastrointestinal smooth muscles and brain neurons. This review is to present recent advances in the understanding of physiology and development of small molecular modulators of TRPC4 channels.

TRPC4 channel, non-selective cation channel, G protein coupled receptors, small molecular modulators, G proteins, excitation, contraction, Ca^{2+} signaling

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The transient receptor potential (TRP) protein superfamily consists of a diverse group of non-selective cation channels, which are implicated in multiple signal transduction pathways, with particular importance in sensory physiological responses to temperature, light, smell, taste, as well as mechanical and painful chemical stimuli [1,2]. The canonical subfamily of TRP (TRPC) channels are closely related to the prototypical *Drosophila* TRP protein, with 30%–40% amino acid sequence identity [3]. Seven mammalian TRPC proteins (TRPC1–7) have been identified and based on amino acid sequence similarity, they are further divided into three groups: TRPC1/C4/C5, TRPC3/C6/C7 and TRPC2 [4]. Channels formed by TRPC proteins are composed of four subunits, which are either identical or different. It is well accepted that TRPC members within the same group can form heterotetrameric channels [5], but evidence also exists for heterotetramers formed between members from different groups. For example, TRPC3 and TRPC4 can associate to form a redox-sensitive cation channel in endothelial cells [6]. Notably, TRPC1 has been shown to partner not only with members of the TRPC subfamily, e.g., TRPC3, TRPC4, and TRPC5, but also with other TRP proteins, e.g., TRPP2, TRPV4, and TRPV6 [7–10].

The first full-length TRPC4 cDNA sequence was reported for a clone isolated from bovine adrenal gland [11]. Since then, several TRPC4 orthologues, including splice variants, had been isolated from a number of other species such as rat, mouse and human, and some of them had been functionally examined [11,12]. In rat, mouse and human, the most abundant transcripts of TRPC4 appear to be

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TRPC4α and TRPC4β. The two isoforms vary only at the C-terminus, in which the TRPC4β variant lacks a region containing 84 amino acids as compared to TRPC4α [13,14] (Figure 1). While TRPC4 is predominately expressed in the brain, it is also found in diverse tissues including endothelia, adrenal gland, smooth muscles of the gastrointestinal track, placenta and testis [19].

1 Structure and biological relevance of TRPC4

Like other TRP channels, a single TRPC4 subunit has six transmembrane segments (S1–S6) with a putative pore-forming region (P-loop) between the fifth (S5) and sixth (S6) segments and intracellularly localized N- and C-termini [2]. Within the cytoplasmic N-terminus of TRPC4, four ankyrin-like repeats, a calmodulin (CaM) binding site, a coiled-coil domain and a caveolin-binding site have been identified [2,4]. In the cytoplasmic C-terminus, there are a TRP box (a stretch of relatively conserved six residues found in all members of TRPCs, TRPMs, and TRPVs [1] and in the case of TRPCs is EWKFAR), a second coiled-coil domain, a conserved protein 4.1-binding domain, and a shared binding site for CaM and inositol 1,4,5-trisphosphate receptors (IP_3Rs) [15,16,20]. Interestingly, the CaM and IP_3R binding (CIRB) site also binds to SESTD1, a protein that contains a SEC14-like lipid binding domain and two spectrin domains and binds phosphoinositides in a Ca^{2+}-dependent fashion [21]. It also appears to be critical for the stimulatory effect of $G\alpha_{i/o}$ proteins on TRPC4 channels [22]. These suggest that the CIRB site may be the converging point of TRPC4 channel gating by multiple factors, such as Ca^{2+}-CaM, IP_3Rs, $G_{i/o}$ proteins, phosphoinositides, and cytoskeleton (Figure 1). Additionally, the region immediately downstream of the TRPC4 CIRB site also binds spectrins [23].

The last three C-terminal amino acids (TRL) of TRPC4 comprise a PDZ-interacting domain. PDZ domains were identified as 80–100 amino acid repeated sequences in the synapse-associated protein PSD-95, the human homolog of the *Drosophila* Dlg protein (hdlg) and the epithelial tight-junction protein zona occludens-1 (ZO-1) [24]. Within the TRPC subfamily, this TRL motif is specific for TRPC4 and TRPC5. The PDZ-binding motif TRL interacts with regulatory factor of the Na^+/H^+ exchanger (NHERF), also known as ezrin/radixin/moesin-50 (ERM50). The PDZ motifs of NHERF/EBP50 bind to the TRPC4/C5 channels and phospholipase Cβ (PLCβ), and link the lipase and the channels to the actin cytoskeleton [25], via the actin-binding membrane-cytoskeletal adaptors, ezrin, radixin, and moesin (ERM) [26]. As a result, the PDZ-interacting domain regulates the localization and surface expression of TRPC4 [26].

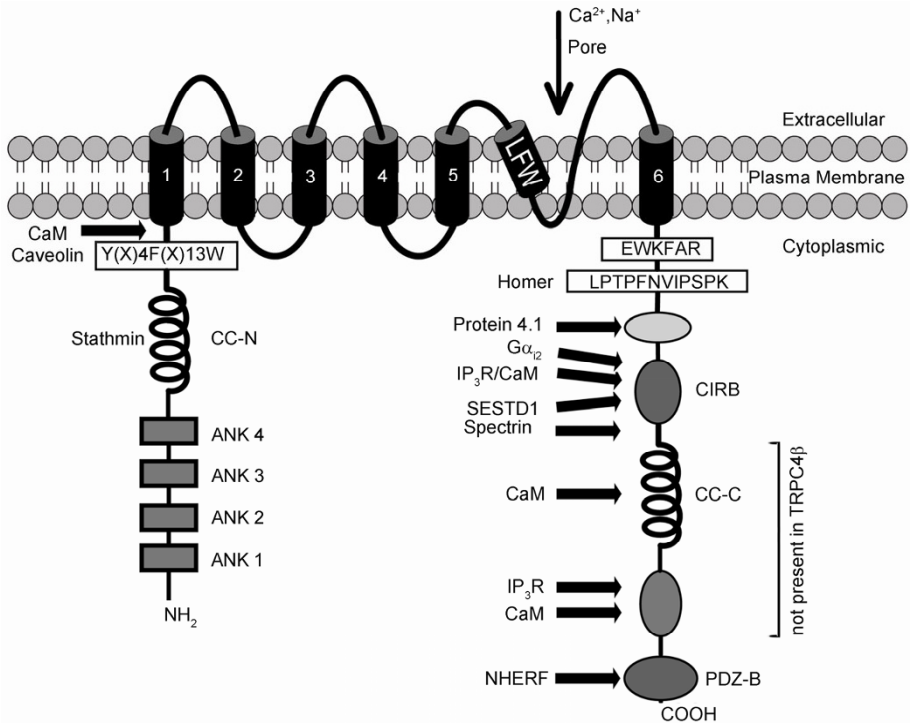


Figure 1 Structural features of TRPC4α, which contains an extra region with 84 amino acids as compared to TRPC4β. Within this region, two calmoudlin (CaM) binding motifs and one IP_3 receptor binding domain had been experimentally identified [15,16]. Homer had been suggested to bind to the proline-rich region of the related TRPC1 [17]. Stathmin was shown to bind to the N-terminal coiled-coil domains (CC-N) of TRPC4 and TRPC5 [18]. Other domains and interacting proteins are described in the text. ANK 1–4, ankyrin-like repeats 1–4; CC-C, C-terminal coiled-coil domains, CIRB, calmodulin/IP_3 receptor binding region; PDZ-B, PDZ binding domain; LFW, amino acid motif conserved in the hydrophobic putative pore region; EWKFAR, sequence of the TRP box. Adapted from [4] with modifications.

TRPC4 shares an overall 65% sequence identity with TRPC5. However, the similarity is not evenly distributed throughout the full-length sequences between the two proteins. TRPC4 and TRPC5 are quite similar at the N-termini, transmembrane domains, and the beginning of the C-termini encompassing the TRP motifs (83% identity for aa1–665 of TRPC4 and aa1–669 of TRPC5), as well as the CIRB sites and the spectrin-binding sites (77% identity for aa695–754 of TRPC4 and aa702–761 of TRPC5). However, the rest of the C-termini, encompassing >200 residues (aa755–974 of TRPC4α and aa762–975 of TRPC5), bear virtually no homology, except for the final convergence of the last five residues, VTTRL, in which the last three residues are critical for binding to NHERF, as explained above. Therefore, although it has often been thought that TRPC4 and TRPC5 are regulated similarly, differences should exist between the two channels.

In terms of physiological functions, channels formed by TRPC4 are often distinct from those composed of TRPC5. TRPC4 has been suggested to be a key determinant of endothelial Ca^{2+} signaling and of endothelial cell functions, such as nitric oxide release and barrier stability [27,28]. Vascular endothelial cells from TRPC4$^{-/-}$ mice lacked store-operated Ca^{2+} currents [27]. Therefore, TRPC4 appeared to be an indispensable component of store-operated channels in vascular endothelial cells and these channels directly provide a Ca^{2+} entry pathway essential for the regulation of blood vessel tone [27]. Similarly, lung vascular endothelial cells isolated from TRPC4$^{-/-}$ mice showed reduced Ca^{2+} responses to thrombin and PAR-1 peptide [28]. These were associated with a lack of actin stress fiber formation, deficiencies in the endothelial cell retraction response and an increase in vascular permeability [28]. In neuroendocrine cells, activation of transiently expressed TRPC4 via stimulation of co-expressed histamine receptor type 1 (H_1R) provided enough Ca^{2+} influx to trigger a robust secretory response comparable to that activated by a train of depolarizing pulses [29]. TRPC4 was also shown to be critically involved in the Ca^{2+} entry pathway needed for serotonin-induced dendritic release of γ-aminobutyric acid (GABA) from interneurons onto thalamic relay neurons [30]. In addition, TRPC1-TRPC4 heteromeric channels are thought to mediate the plateau potential and epileptiform discharge evoked by agonists of group 1 metabotropic glutamate receptors (mGluRs) in lateral septal and CA1 hippocampal neurons [31–33]. These channels are also involved in neurodegeneration induced by severe epileptic seizures in these brain regions [32]. Likewise, the TRPC1-TRPC4 channels are important for neurotransmission at dendritic-dendritic synapses between mitral/tufted cells and granule cells in the olfactory bulb, which manifests as long lasting depolarization and sustained Ca^{2+} influx in the granule cells [34]. In the gastrointestinal system, comparison of the properties between native currents in interstitial cells of Cajal (ICC) and ionic currents of heterologously expressed TRPC4 in HEK293 cells revealed a very similar current-voltage (*I-V*) relationship, indicating that TRPC4 is a strong candidate of the pacemaker channel in ICC [35,36]. Similarly, in intestinal smooth muscle cells, TRPC4 forms a 55-pS cation channel and underlies at least 80% of the muscarinic agonist-elicited cation currents (mI_{CAT}) [37]. In TRPC4-deficient ileal myocytes the carbachol-induced membrane depolarization was diminished greatly and the atropine-sensitive contraction elicited by acetylcholine released from excitatory motor neurons was markedly reduced as well [37]. TRPC4 and to a lesser extent, TRPC6, channels couple muscarinic receptors to depolarization of intestinal smooth muscle cells and activation of voltage-gated Ca^{2+} channels to mediate Ca^{2+} influx and contraction, and thereby accelerate motility of small intestine *in vivo* [37]. Additional functions of TRPC4 also include formation of normal-sized myotubes during postnatal human myogenesis [38], keratinocyte differentiation [39], neurite extension of human postmitotic neurons [40]. In single-nucleotide polymorphism (SNP) studies, variants of TRPC4 have been associated with photoparoxysmal response/idiopathic generalized epilepsies [41]; a gain-of-function variant (I957V) of TRPC4 was found to be associated with a lower incidence of myocardial infarction in diabetic patients [42].

For functions that appear to overlap with TRPC5, both TRPC1-TRPC4 and TRPC5 channels may be involved in seizure-induced neuronal death in mouse hippocampus [32]. In certain populations of proopiomelanocortin neurons, channels composed of TRPC1, TRPC4, and/or TRPC5 may mediate the effect of leptin acting at the long-form leptin receptor [43]. In neurons of lateral amygdala, TRPC4 seems to mediate the postsynaptic responses triggered by the activation of either group 1 mGluRs or cholecystokinin 2 receptors. This effect is involved in innate fear responses just like that mediated by TRPC5 [44,45].

2 Activation mechanism and electrophysiological properties

It is widely agreed that the opening of TRPC4 channels requires activation of receptors coupled to the $G_{q/11}$ family of G proteins, which communicate with PLCβ, or receptor tyrosine kinases, which stimulate PLCγ [13,46]. The activation of PLC leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$), producing diacylglycerols (DAG) and inositol 1,4,5-trisphosphate (IP_3). Intracellular dialysis of $PI(4,5)P_2$ was shown to inhibit TRPC4α but not TRPC4β [47]. However, TRPC4β activation seems to require $PI(4,5)P_2$, as not only manipulations that reduced $PI(4,5)P_2$ availability inhibited the channel activation, but also supplementation of exogenous $PI(4,5)P_2$ to the cytoplasmic side suppressed channel desensitization [22,48]. It

would be interesting to know if the $PI(4,5)P_2$ requirement depends on the lipid itself, as in the case of several members of the TRPM subfamily [49–52] or one of its hydrolysis products, as suggested for the TRPC3/C6/C7 subgroup [53].

However, different from the TRPC3/C6/C7 subgroup, TRPC4 (and TRPC5) is not activated by DAGs. Uniquely, it becomes activated following the stimulation of receptors that couple to pertussis toxin (PTX) sensitive $G_{i/o}$ proteins [22]. The muscarinic cation current (mI_{CAT}) in intestinal smooth muscle cells represents the best characterized native channel activity of TRPC4 [37]. It has long been recognized that both $G_{i/o}$-coupled M2 and $G_{q/11}$-coupled M3 muscarinic receptors are involved in the activation of mI_{CAT} [54]. Not surprisingly, treatment with PTX inhibited both native mI_{CAT} and heterologously expressed TRPC4 channels no matter the activation was induced by a muscarinic agonist or GTPγS [47,55]. Also interesting is that the effect of $G_{i/o}$ appears to be mediated by the Gα but not Gβγ subunits and the study using GTPase-deficient $G\alpha_{i/o}$ subunits revealed for a preference of $G\alpha_{i2}$ for TRPC4 and $G\alpha_{i3}$ for TRPC5 [22]. Intriguingly, the same study also found that the critical region on TRPC4 for interacting and functional coupling by $G\alpha_i$ proteins overlaps with the C-terminal CIRB site [22], implying a pivotal role of this motif in overall gating of TRPC channels. Furthermore, the CIRB motif of TRPC4 and TRPC5 had previously been shown to bind to SESTD1, through which the channel could be regulated by phosphoinositides, Ca^{2+} and cytoskeleton [21]. Therefore, sorting out the interplay among Ca^{2+}, CaM, IP_3Rs, $G\alpha_i$'s, SESTD1, phosphoinositides, and cytoskeleton at the CIRB motif should be of great significance in elucidating molecular mechanism(s) of TRPC4 channel activation.

In addition to the mechanisms discussed above, TRPC4 channels had also been shown to respond to mercury compounds [56] and nitric oxide (NO) [57]. Early studies had implied a role of TRPC4-containing channels in store-operated Ca^{2+} entry [27,28,58,59]; however, it is unclear whether this represented a direct gating by STIM1, which senses the store depletion signal [60,61], or an indirect effect of intracellular Ca^{2+} rise resulting from either Ca^{2+} release from internal stores or Ca^{2+} influx mediated by "real" store-operated channels formed by Orai proteins [62]. The latter argument is possible because activation of TRPC4 channels is dependent on intracellular Ca^{2+} rise [13].

In whole-cell recordings, the agonist-induced currents of TRPC4 have a nonlinear *I-V* relationship, typically with an outward rectification at positive potentials and a U- or V-shaped *I-V* relation at negative potentials [13,47]. The currents reverse at close to 0 mV, but there is a flat region between 0 and +40 mV (Figure 2), which can be eliminated by the removal of Mg^{2+} from both sides of the membrane [13,14]. In excised inside-out patches, TRPC4 has a reported single channel conductance of 42 pS [13].

3 Pharmacology

3.1 TRPC4 activators

To date, no specific activator of TRPC4 has been reported. In most studies, TRPC4-containing channels, either native or heterologously expressed, are commonly activated by the stimulation of G-protein coupled receptors or receptor tyrosine kinases [13,37,47,54]. Sometimes, intracellular dialysis of GTPγS or NaF was used to activate TRPC4 through direct stimulation of G proteins [13,47,63]. In inside-out membrane patches, TRPC4 currents were strongly increased by application to the cytoplasmic side of calmidazolium, a CaM antagonist, or a peptide that represented the TRPC-binding domain of the type 3 IP_3R [15].

Lanthanides are the most commonly used inhibitors of nonselective cation channels and Ca^{2+} channels and they do inhibit TRPC3, C6, and C7. However, in the case of TRPC4 and TRPC5, high micromolar concentrations of lanthanides potentiate their currents [64]. Neutralization of negatively charged amino acid residues, E543, E595 and E598, situated close to the segments S5 and S6 of TRPC5, resulted in a loss of potentiation by lanthanides [64]. These residues are conserved in TRPC4. Because other TRPC isoforms and native cation channels are inhibited by lanthanides, the potentiation of TRPC4 and TRPC5 by micromolar La^{3+}/Gd^{3+} represents a unique property that is useful for establishing the contribution of these TRPC isoforms in native tissues [43,65].

3.2 TRPC4 inhibitors

ML204 has been reported as a novel, potent, and selective TRPC4 channel inhibitor [66]. ML204 inhibited intracellular Ca^{2+} rise mediated by TRPC4β with an IC_{50} value of approximately 1 μmol L^{-1} and it exhibited a 19-fold selectivity for TRPC4 over TRPC6 [66]. Results from whole-cell patch clamp recordings suggested that ML204 most likely exerted a direct inhibitory effect on the TRPC4 channel rather than acted through an interference with the signal transduction pathways [66]. Selectivity studies showed that ML204 had no appreciable inhibition on TRPV1, TRPV3, TRPA1, and TRPM8, or native voltage-gated sodium, potassium, and calcium channels in mouse dorsal root ganglion neurons. Therefore, ML204 appears to be an excellent chemical tool for modulating TRPC4 or TRPC5 channels [66]. Recently, ML204 has been successfully used to identify the functions of native TRPC4 and/or TRPC5 channels in visceral pain [67], kidney filtration barrier deregulation [68], and neuronal excitability regulation [69].

Flufenamic acid (FFA), mefenamic acid (MFA), niflumic acid (NFA) and diclofenac sodium (DFS) have been shown to inhibit TRPC4 and TRPC5 channels in a concentration-dependent manner [70]. However, these drugs are known to have other targets [71,72] and they are not potent

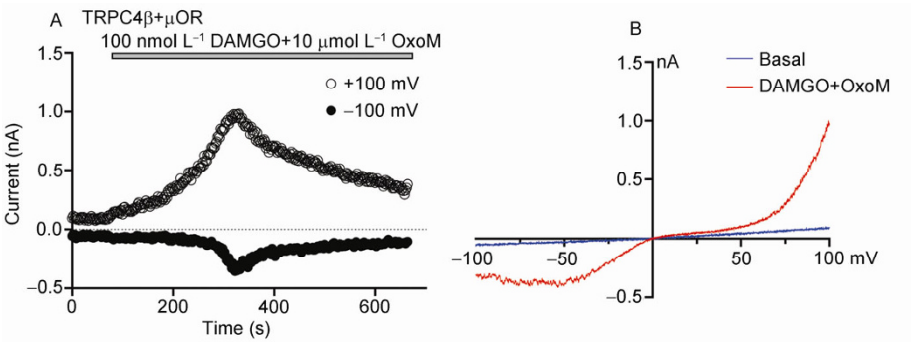


Figure 2 Agonist-activated currents in a HEK293 cell that co-expressed mouse TRPC4β and μ-opioid receptor (μOR). A, For whole-cell experiments, the standard extracellular solution contained (in mmol L^{-1}): 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.4 adjusted with NaOH. The intracellular solution consisted of (in mmol L^{-1}): 140 CsCl, 0.1 EGTA, 1 $MgCl_2$, 10 HEPES, pH 7.2 adjusted with CsOH. Currents were measured using 500-ms voltage ramps from +100 mV to −100 mV in 2-s intervals with the holding potential of 0 mV. Shown are time courses of currents at +100 mV (open circles) and −100 mV (filled circles), in response to the application of 10 μmol L^{-1} Oxo-M and 100 nmol L^{-1} DAMGO to simultaneously activate the muscarinic (endogenously expressed) and μ-opioid (transfected) receptors, respectively. B, Current-voltage relationships recorded from the same cell by the voltage ramp collected before agonist application (basal, blue line) and at the peak of the agonist response (red line).

on TRPC4. TRPC4 was blocked by FFA, MFA, NFA and DFS with IC_{50} values of 55±5, 84±8, 102±9, and 138±7 μmol L^{-1}, respectively [70]. The potency of inhibition was shown to depend on modifications of the 2-phenylaminobenzoate skeleton and a structure-activity relationship has been described for FFA analogues with modifications of the phenylamino ring [70]. Interestingly, fenamate analogues with differential effects on TRPC4 and TRPC5 channels, showing inhibition with acute but potentiation with long exposure, have also been identified [70]. SKF-96365 and 2-aminoethoxydiphenyl borate (2APB) are non-specific blockers commonly used to inhibit TRPC channels. Receptor-activated TRPC4 was blocked by 50 μmol L^{-1} SKF-96365 [73,74] or by 75 μmol L^{-1} 2APB [73,75]. However, the lack of specificity is a major concern for the use of these drugs in establishing the contribution of TRPC4-containing channels in native systems.

The steroid hormone progesterone was found to inhibit TRPC4 channel activity with an IC_{50} of 6.2 μmol L^{-1} [76]. However, this effect appears to be common for most TRPC channels, except TRPC5, suggesting that at the high gestational levels of progesterone, TRPC channels could be inhibited. It was conducive that this effect may be important for minimizing uterine contractility and immunosuppression during pregnancy [76]. Table 1 shows the structures and potency of small molecules that have been shown to inhibit TRPC4 channels.

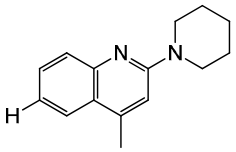
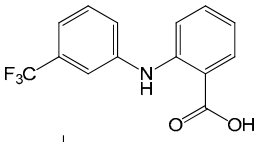
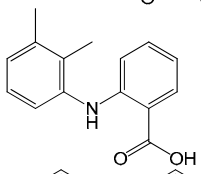
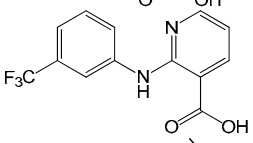
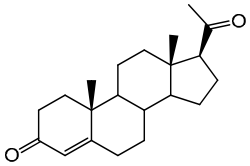
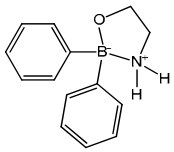
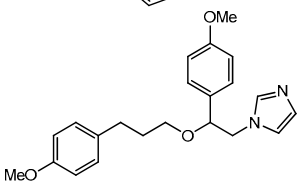
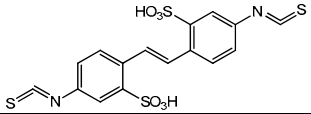
In whole-cell recordings, receptor-evoked activation of TRPC4α, but not TRPC4β, was strongly inhibited by the application of diC8 form of $PI(4,5)P_2$ through pipette dialysis. In contrast, several other phosphoinositides, including $PI(3,4)P_2$, $PI(3,5)P_2$, and $PI(3,4,5)P_3$, did not mimic the inhibitory effect of $PI(4,5)P_2$; some of them even potentiated the activity of TRPC4α [47]. $PI(4,5)P_2$ bound to the C terminus of TRPC4α but not that of TRPC4β *in vitro* and its inhibitory action was abolished by the treatment with cytochalasin D or by the deletion of the C-terminal PDZ-binding motif, indicative of a dependence on the association of TRPC4α with actin cytoskeleton [47]. On the other hand, as mentioned above, $PI(4,5)P_2$ has been suggested to be an essential lipid for sustaining the open state of TRPC4β [48]. Furthermore, intracellular dialysis of a short peptide, EQVTTRL, representing the last seven C-terminal amino acids of TRPC5 with the PDZ-binding motif, inhibited the carbachol-induced plateau potentials in entorhinal cortical neurons, which were thought to be mediated by channels formed by TRPC4 and/or TRPC5 [77].

4 Outlook and challenges

TRPC4 isoforms form nonselective cation channels that integrate signaling pathways activated from stimulation of G protein-coupled receptors and receptor tyrosine kinases. It remains debated about the activation mechanism of TRPC4 channels. A number of early studies supported the view that TRPC4 was essential for store-operated or Ca^{2+} release-activated Ca^{2+} (CRAC) channels [27,28,58,59]. However, subsequent studies showed that only neurotransmitter-induced receptor-operated channels were impaired in the TRPC4 knockout mice [37,45]. Because of the recent demonstration of the involvement of STIM and Orai proteins in CRAC channel [62], physical and functional interactions between STIM1/Orai1 and with TRPC1 or TRPC4 channels to contribute to store-operated Ca^{2+} entry have been suggested [60,61]. Further studies are warranted to examine the interaction between TRPC4 and STIM/Orai and define the role of TRPC4 in store-operated Ca^{2+} entry.

TRPC4 proteins are abundantly expressed in brain neurons and smooth muscle cells, where they form Ca^{2+}-permeable nonselective cation channels implicated in di-

Table 1 Summary of TRPC4 channel inhibitors

Compound name	Structure	Concentration effect on TRPC4	Selectivity on channels	Refs
ML204		IC_{50}=0.96 μmol L^{-1}	No effect on TRPV1, TRPV3, TRPA1 and TRPM8 or native voltage-gated Na^+, K^+, Ca^{2+} channels	[66]
Flufenamic acid (FFA)		IC_{50}=55±5 μmol L^{-1}	Effect on Ca^{2+}- activated Cl^- channels, voltage-gated Na^+, K^+ or Ca^{2+} channels, and other TRP channels	[70]
Mefenamic acid (MFA)		IC_{50}=84±8 μmol L^{-1}	Effect on Ca^{2+}- activated Cl^- channels, voltage-gated Na^+, K^+ or Ca^{2+} channels, and other TRP channels	[70]
Niflumic acid (NFA)		IC_{50}=102±9 μmol L^{-1}	Effect on Ca^{2+}- activated Cl^- channels, voltage-gated Na^+, K^+ or Ca^{2+} channels, and other TRP channels	[70]
Progesterone		IC_{50}=6.2 μmol L^{-1}	Effect on TRPC3, TRPC5, TRPC6 channels	[76]
2-aminoethoxydiphenyl borate (2APB)		Blocked at 75 μmol L^{-1}	Inhibits IP_3Rs, TRPC and TRPM channels, activates TRPA1 and some TRPV channels	[73,75]
SKF-96365		Blocked at 50 μmol L^{-1}	Nonselective for TRPC channels, store-operated Ca^{2+} entry, and voltage-gated Ca^{2+} channels	[73,74]
DIDS (4,4′-dixothiocyanatositbene-2,2′-disulfonic acid)		Blocked at 10 μmol L^{-1}	Inhibits Cl^- uptake, activates voltage-gated K^+ channels	[36]

verse physiological functions, including smooth muscle contractility and synaptic transmission [30,34,37,45]. In addition, genetic analyses have found association of TRPC4 variants with human disease [41,42]. It is thus foreseeable that TRPC4 channels have important physiological significance and are valuable therapeutic drug targets. However, functional characterization of TRPC4 channels in native tissues has been hampered by the lack of specific pharmacological tools. Efforts have been undertaken to screen for small molecular modulators using a cell-based fluorescence assay. Uniquely, co-expression of TRPC4β with the μ opioid receptor in HEK293 cells resulted in an intracellular Ca^{2+} response to stimulation by the μ agonist, DAMGO, which was lacking with the expression of either TRPC4 or the opioid receptor alone [66]. The very low background of this system allowed for fluorescence-based high throughput screening using cells loaded with the calcium specific fluorescent indicator, such as fluo-4. The DAMGO-induced intracellular Ca^{2+} concentration rise was almost entirely dependent on TRPC4β-mediated Ca^{2+} entry. This approach had led to identification of multiple lead compounds, which were further tested to remove those that targeted the μ opioid receptor and G proteins. Promising lead compounds have been confirmed using patch-clamp recordings, the gold standard for ion channel drug screening [66]. It is anticipated that more new small molecular probes for TRPC4 will be

uncovered in the near future and some of them will possess the desired high potency and selectivity and be suitable for development of drug therapies targeting at TRPC4.

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