Extracellular quaternary ammonium blockade of transient receptor potential vanilloid subtype 1 channels expressed in *Xenopus laevis* oocytes

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Running title page

Running title: Extracellular quaternary ammonium block of TRPV1

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List of non-standard abbreviations:

TRPV1, transient receptor potential vanilloid subtype 1;
TRP, transient receptor potential;
TEA, tetraethylammonium;
TMA, tetramethylammonium;
Kv, voltage-gated potassium;
CGRP, calcitonin gene-related peptide;
PKA, cAMP-dependent protein kinase;
PLC, phospholipase C;
LA, local anesthetic;
E_{rev}, reversal potential;
DRG, dorsal root ganglionic neurons;
QA, quaternary ammonium;
PIP_{2}, phosphatidylinositol 4,5-bisphosphate;
DAG, diacylglycerol
Abstract

Transient receptor potential vanilloid subtype 1 (TRPV1) channels are essential nociceptive integrators in primary afferent neurons. These nonselective cation channels are inhibited by local anesthetic compounds through an undefined mechanism. Here, we show that lidocaine inhibits TRPV1 channels expressed in *Xenopus laevis* oocytes, while the neutral local anesthetic, benzocaine, does not, suggesting a titratable amine is required for high affinity inhibition. Consistent with this possibility, extracellular tetraethylammonium (TEA) and tetramethylammonium (TMA) application produces potent, voltage-dependent pore block. Alanine substitutions at F649 and E648, residues in the putative TRPV1 pore region, significantly abrogated the concentration-dependent TEA inhibition. The results suggest that large cations, shown previously to enter cells through activated TRP channels, can also act as channel blockers.
Introduction

Transient receptor potential (TRP) channels play an essential role as environmental sensors throughout the human body. The involvement of TRP channels in chemical and physical sensation was first demonstrated with the discovery of the vanilloid-type 1 TRP channel, TRPV1, also known as the capsaicin receptor (Caterina et al., 1997). Subsequent identification and characterization of now more than 30 different subtypes of transient receptor potential channels has helped to categorize six receptor families and has demonstrated that these non-selective cation channels respond to a wide range of chemical and physical stimuli (DeSimone and Lyall, 2006; Wu et al., 2010; Zhang et al., 2003). Based on their sequence homology with voltage-gated potassium (Kv) channels, it has been proposed that TRP channels are arranged as tetramers around a central, ion-conducting pore. Each of the four domains likely consists of six transmembrane domains, with intracellular N- and C-termini and a re-entrant pore loop between domains 5 and 6 containing the selectivity filter (Clapham et al., 2005; Moiseenkova-Bell et al., 2008). Many TRP channels display polymodal characteristics, that is, they are activated and/or modulated by a wide range of stimuli, including endogenous and exogenous ligands, pH, temperature; and display a weak voltage-dependent activation (Clapham et al., 2005).

In this study, we focus on the TRPV1 channel subtype, which has been implicated in nociception, inflammation, and hyperalgesia (Caterina et al., 2000; Davis et al., 2000). The TRPV1 channel is widely distributed throughout the peripheral and central nervous system, with prominent expression in afferent pain sensing neurons (A-delta and C fibers), dorsal root, nodose and trigeminal ganglia (Caterina et al., 1997;
Mezey et al., 2000; Szallasi et al., 1995; Toth et al., 2005). Under normal physiological conditions, activation of capsaicin-sensitive sensory nerve fibers elicits the release of the neuropeptides, neurokinin A, calcitonin gene-related peptide (CGRP), somatostatin, and substance P, which in turn, regulate vascular, immune, and smooth muscle functions (Bartho et al., 2008; Bartho et al., 2004). Following tissue injury, an overabundance of these neuropeptides can contribute to the process of neurogenic inflammation by activation of pro-algesic second messenger proteins and increased neuronal firing (Holzer and Maggi, 1998; Maggi, 1995), underlining the importance of TRPV1 as a physiological drug target.

Recently, local anesthetics (LAs) such as lidocaine have been demonstrated to interact with TRPV1 channels. For example, application of the TRPV1 agonist, capsaicin, has been shown to allow transmembrane passage of the permanently charged lidocaine derivative, QX-314, presumably via the ‘dilated’ TRPV1 permeation pathway (Binshtok et al., 2007; Chung et al., 2008; Ries et al., 2009). Subsequently, lidocaine, and also QX-314, have been demonstrated to directly interact with TRPV1 channels in various preparations, exhibiting both activating and inhibiting properties (Leffler et al., 2008; Rivera-Acevedo et al., 2011). In *Xenopus laevis* oocytes, we have found that lidocaine and QX-314 exert biphasic effects on TRPV1 channels, inhibiting capsaicin-evoked TRPV1 currents at lower (micromolar) concentrations and activating TRPV1 channels at higher (millimolar) concentrations. Whereas the mechanisms are unknown, studies into the permeation and gating pathway of TRPV1 have found - similar to results obtained with voltage-gated potassium (Kv) channels - that permanently charged quaternary ammonium compounds are able to potently block TRPV1
intracellularly, thus delimiting similarities amongst these seemingly disparate family members (Jara-Oseguera et al., 2008; Oseguera et al., 2007). However, it remains unclear if TRPV1 is also susceptible to extracellular inhibition by quaternary ammonium compounds. Local anesthetic (LA) molecules, which have both amphipathic and amphiphilic properties and maintain a dynamic equilibrium between charged and neutral forms, generally contain a tritratable amine group linked to a hydrophobic aromatic moiety via an amine or ester linkage. It has been shown previously in sodium channels that these different chemical moieties have distinct pharmacological effects (Butterworth and Strichartz, 1990; Liu et al., 2003).

On the basis of these findings, we sought to determine if the inhibition of TRPV1 channels is a general characteristic of LAs, and, if so, to define the molecular determinants of this blockade. In this study, we provide evidence that TRPV1, expressed in *Xenopus laevis* oocytes, is potently inhibited by extracellular quaternary ammonium compounds. Furthermore, we demonstrate that it is the charged (or chargeable) amine of LAs that is critical for the inhibition of TRPV1. Finally, in an attempt to delineate a possible binding site for quaternary ammonium compounds, we show that point mutations near the putative TRPV1 pore region reduce these compounds’ inhibitory efficacy and affinity for these channels.
Materials and Methods

Chemical Reagents. All chemical reagents were obtained from Sigma-Aldrich Canada (Oakville, Ontario, Canada). Capsaicin (8-methyl-N-vanillyl-6-nonenamide) was dissolved in absolute ethanol to produce a stock solution of 100 mM and stored at −20°C. Lidocaine HCl, benzocaine, tetraethylammonium chloride (TEA), and tetramethylammonium chloride (TMA) were directly dissolved in the extracellular solution (see below). The solutions were buffered using 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and pH values corrected to 7.4 with NaOH.

Molecular Biology. *Xenopus laevis* oocytes were injected with rat TRPV1 capped mRNA (~1 µg/µl), which was synthesized using the T7 mMessage mMMachine kit (Ambion Canada, Streetsville, Ontario, Canada). Mutant channels, L647A, E648A, and F649A, were generated using site-directed mutagenesis, and correct insertion of the mutations was confirmed with automated sequencing. Post-injection, oocytes were incubated for 1-2 days to obtain expression levels between 0.1 and 3 μA.

Electrophysiology. We recorded voltage-clamped TRPV1 currents from oocytes with the two-electrode voltage-clamp technique using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The holding potential was −60 mV, unless specified otherwise. Microelectrodes were pulled from borosilicate glass tubes (Frogy 1001; Harvard Apparatus, Holliston, MA) and backfilled with 3 M KCl. Ringer’s solution (116 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 0.5 mM CaCl₂, and 5 mM HEPES; pH 7.4) was used as the standard external solution. All control and test solutions were applied for 10 s with...
an automated fast-switching perfusion system (ValveBank8, AutoMate Scientific, Inc., Berkeley, CA) in a custom-made 30-µl bath chamber. We standardized drug applications to 10 s with a 2 min washout period to minimize Ca\(^{2+}\) induced desensitization (Koplas et al., 1997); as well as potentiation associated with extended capsaicin administration due to direct channel phosphorylation by cAMP-dependent protein kinase (PKA) or indirect activation and phosphorylation of TRPV1 interacting enzymes recruited by phospholipase C (PLC) (Bhave et al., 2002; Vellani et al., 2001). The pCLAMP 10.0 software suite (Molecular Devices, Sunnyvale, CA) was used for data acquisition and offline analysis.

Statistical Analysis. Calculations for statistical comparisons were performed with Prism 5 (GraphPad, San Diego, CA) and Sigma Plot 10 (Systat Software Inc., Chicago, IL). In single drug application experiments, baseline capsaicin activation was calculated from the mean of the control and recovery inward currents recorded in response to an EC\(_{50}\) concentration of capsaicin (15 μM). All inhibition-response curves were fit using Hill four-parameter, non-linear regressions. Continuous data were analyzed with Student’s \(t\) test or repeated analysis of variance (ANOVA; with Dunn’s Multiple Comparison Test for post hoc comparisons between individual groups) as appropriate. We considered a \(P\) value \(\leq 0.05\) statistically significant. Data are given as mean ± SEM where appropriate.
Results

Lidocaine inhibition of TRPV1 channels in *Xenopus laevis* oocytes. To define the molecular determinants of LA-mediated blockade of TRPV1 channels, we first determined the effects of lidocaine, which contains both a tritratable amine tail and a hydrophobic aromatic head, connected via an amine linkage (see inset of Fig. 1C). We expressed rat TRPV1 in *Xenopus laevis* oocytes and co-applied an ~EC$_{50}$ concentration of capsaicin (15 μM) with increasing concentrations of lidocaine, flanked by two control applications of capsaicin alone. As shown in Fig. 1A, addition of 100 nM lidocaine produced a 20% inhibition in inward current. Increasing the lidocaine concentration to 10 μM produced a 70% inhibition of capsaicin-elicited currents (Fig. 1B). The resulting IC$_{50}$ for lidocaine inhibition of TRPV1 was 82 ± 65 nM, with a Hill coefficient of 0.7 ± 0.5. We characterized the voltage-dependence of lidocaine inhibition by investigating blockade at a more depolarized holding potential. The E$_{rev}$ for our system, determined from current-voltage plots, was −20 mV (data not shown). To test the voltage-dependence of lidocaine inhibition, we examined blockade at −25 mV, the closest membrane potential where inward currents could still be observed. Compared with the effects at −60 mV, we observed a significant decrease in the efficacy of lidocaine current inhibition when oocytes were held at −25 mV, consistent with a charged pore blocking mechanism whereby the blocking site is within the electric field. (Fig. 1C,D). Complete inhibition of TRPV1 activation was not achieved, even at the highest concentrations of blockers that were employed in this study; and a residual inward current remained even at high (100 μM) lidocaine concentrations. Our data thus clearly show that extracellular lidocaine
produces concentration-dependent and reversible inhibition of capsaicin-induced inward currents in *Xenopus laevis* oocytes.

The neutral LA benzocaine does not strongly inhibit TRPV1 channels. To test the role of cationic charge in the inhibition of TRPV1 by lidocaine and QX-314, we investigated the blocking abilities of the neutral LA, benzocaine (pKₐ, 2.5), as less than 1% of benzocaine molecules are charged under our experimental conditions (pH 7.4). As shown in Fig. 2A, administration of 10 μM benzocaine produced little effect on TRPV1 currents, and 100 μM resulted in a modest inhibition that did not reach statistical significance (Fig. 2B). Analysis of the concentration-dependent effects of benzocaine demonstrated that benzocaine, in contrast to lidocaine or QX-314, does not significantly inhibit capsaicin-evoked TRPV1 currents in the range of 1 nM to 100 μM (ANOVA, P > 0.05; Fig. 2C). In supplemental experiments with very high benzocaine concentrations (> 1 mM), we similarly did not observe significant TRPV1 inhibition but noted a small degree of channel potentiation when co-applied with capsaicin (data not shown).

Quaternary ammonium compounds potently inhibit capsaicin-induced TRPV1 currents in oocytes. We previously showed that the quaternary lidocaine derivative, QX-314, inhibits capsaicin activated TRPV1 channels (Rivera-Acevedo et al., 2011). Benzocaine, on the other hand, which features a lipophilic aromatic moiety that is shared with both lidocaine and QX-314 but lacks a nitrogen that can be charged under physiological conditions, did not inhibit TRPV1 currents (see above). On this basis, we
next sought to determine if it is the charged ammonium moiety of lidocaine and QX-314 which mediates inhibition of TRPV1. The quaternary ammonium (QA) compound, TEA, represents an ideal candidate to test this hypothesis as it mimics the charged amine tail group of QX-314 but does not contain the amino-amide linker or the lipophilic head. When co-applied with capsaicin, we found that TEA potently inhibits TRPV1 even at nanomolar concentrations: 100 nM TEA reduced peak current amplitudes by more than 50% and TEA concentrations of 10 μM almost completely inhibited capsaicin-elicited TRPV1 currents (Fig. 3A, upper and middle panel). The IC$_{50}$ for TEA inhibition of TRPV1 was 41 ± 13 nM, with a Hill coefficient of 0.9 ± 0.1 (Fig. 3A, lower panel). We next tested the smallest of the quaternary ammonium salts, TMA. As shown in Fig. 3B, TMA also robustly inhibited capsaicin-induced TRPV1 currents in oocytes, although with lower efficacy and potency than TEA: at 100 nM and 10 μM, TMA produced mean TRPV1 current inhibitions of approximately 40% and 70%, respectively (Fig. 3B, upper and middle panel). The corresponding IC$_{50}$ for TMA inhibition of TRPV1 was 104 ± 60 nM, with a Hill coefficient of 0.5 ± 0.1 (Fig. 3B, lower panel).

**TRPV1 inhibition by quaternary ammonium compounds is voltage-dependent.**

Similar to lidocaine, we characterized the voltage-dependence of TEA and TMA inhibition by investigating blockade at a holding potential of −25 mV. Compared with the effects at −60 mV, we observed a marked decrease in both TEA and TMA current inhibition (Fig. 3C/D). Administration of 100 nM TEA or TMA produced little to no reduction in currents elicited by capsaicin, while at 100 μM approximately 30% current remained present with TEA, and approximately 60% with TMA (Fig. 3C/D). Compared
with the effects at −60 mV, both QAs produced an inhibition-response curve that was shifted to the right and had a decreased slope at −25 mV. The IC$_{50}$ for TEA increased to 1.5 ± 0.8 μM; with a Hill coefficient of 0.4 ± 0.1 (Fig. 3A). For TMA, the IC$_{50}$ increased to >1 mM (Fig. 3B). Attempts to more thoroughly characterize this voltage-dependence where not feasible given the contaminating effects of the inherent weak voltage-dependence of gating and strong desensitization at depolarized potentials. Nonetheless, the data indicate that TRPV1 inhibition by QAs is more effective at hyperpolarized potentials, consistent with the notion that TEA and TMA enter the electric field during blockade, possibly by occupying a binding site in the selectivity filter.

**Mutations near the selectivity filter abrogate TEA inhibition.** As TRPV1 shares sequence homology with Kv channels from the *Shaker* family at the putative selectivity filter (Fig. 4A) (Moiseenkova-Bell et al., 2008; Owsianik et al., 2006), we mutated amino acids close to the putative selectivity filter of TRPV1 to investigate their possible role in TEA inhibition. The F649A and E648A mutations resulted in channels with detectable expression, normal gating and sensitivity to capsaicin, however the L647A mutation was non-functional. In terms of blockade, we found that compared to the robust inhibition by 10 μM TEA observed with wild type TRPV1 (Fig. 3A), the mutant channels F649A (Fig. 4B) and E648A (Fig. 4C) displayed a significant reduction in inhibition levels ($P < 0.001$; data not shown). These effects were consistent over a wide range of TEA concentrations (Fig. 4E/F), suggesting that both E648 and F649 could be involved in TEA inhibition of TRPV1. TEA block was also demonstrated to be voltage-dependent
for the TRPV1 mutants, although a significant decrease between −60 mV vs. −25 mV was only detected in the F649A mutant (Fig. 4D).
Discussion

In this study, we have demonstrated that TRPV1 channels, expressed in *Xenopus laevis* oocytes, are potently inhibited by extracellular application of local anesthetics and quaternary ammonium compounds. These findings are in good agreement with an earlier observation that capsaicin-evoked TRPV1 inward currents can be inhibited by extracellular lidocaine in mammalian HEK293t cells, although with significantly lower potency (Leffler et al., 2008). Further, we have recently shown that the extracellular administration of a quaternary lidocaine derivative, QX-314, inhibits TRPV1-mediated currents in *Xenopus laevis* oocytes (Rivera-Acevedo et al., 2011). Together, these results suggest that it is a general trait of titratable LAs to inhibit TRPV1-mediated inward currents. Here, we sought to test which specific structural aspects of LA molecules mediate extracellular TRPV1 inhibition in *Xenopus laevis* oocytes. Specifically, we assessed the individual contributions of the ubiquitous lipophilic aromatic head and linker regions found in all LA compounds and that of the often charged (or chargeable) amine tail groups. A first hint towards a possible contribution of the latter came from the observation that even at the highest lidocaine concentration employed (100 μM), approximately 30% of the current remained (Fig. 1C). Indeed, the pharmacological efficacy of lidocaine, a weak amphipathic base with a pKa of 7.9, and for that matter LAs in general, is linked to pH (Butterworth and Strichartz, 1990). The protonatable amine group of lidocaine, attached to a lipophilic head by an amide linker, exists in a dynamic equilibrium between charged and uncharged states and it is generally accepted that it is the charged form of LAs that is pharmacologically active, e.g., responsible for blocking voltage-gated sodium channels (Butterworth and
Strichartz, 1990). To more directly assess the role of charge in TRPV1 inhibition, we characterized extracellular block by the quaternary permanently charged amines, TEA and TMA, which have been instrumental in the structure-function study of potassium channel pore regions. More recently, these compounds have been successfully employed to help define the structure and basic biophysical properties of the intracellular TRPV1 channel pore (Jara-Oseguera et al., 2008; Oseguera et al., 2007). In our current study, similar to both lidocaine and QX-314, extracellular application of TEA produced a reduction in capsaicin-evoked inward currents, albeit with substantially higher affinity (Fig. 3A). Of note, even a saturating (1 µM) concentration of TEA did not result in complete TRPV1 inhibition, as we generally observed 10-15% of residual current. This effect could potentially be attributed to TEA behaving like a partially permeant ion, as observed for other voltage-dependent inhibitors (Huang, 2001; Huang et al., 2000). Considering the size of TEA (~8 Å) and the estimated TRPV1 pore diameter of 7 Å (Owsianik et al., 2006), this seems entirely possible. Consistent with this hypothesis, the smaller TMA (~6 Å) (Briend, 1993) displayed significantly lower efficacy than TEA (Fig 3B): the TMA-mediated inhibition also saturated near 1 µM but more than 35% of the current remained. Moreover, it has previously been shown that large organic cations can permeate, albeit relatively slowly, TRPV1 channels (Chung et al., 2008). Indeed, this mechanism has been suggested to take place in explanted DRG cultures exposed to prolonged applications of QX-314 in the presence of capsaicin (Binshtok et al., 2007). Collectively, the data available to date indicate that QAs may act as permeant blockers of TRP channels. Consistent with this possibility, TEA and TMA displayed strong voltage dependent relief of inhibition at −25 mV compared to −60 mV, similar to the extracellular
open channel block of voltage-gated potassium channels and intracellular pore block in TRPV1 channels by other QAs (Jara-Oseguera et al., 2008).

On the basis of the predicted structural homology between TRPV1 and Shaker-like potassium channels (Latorre et al., 2009), we investigated potential molecular binding sites in the area surrounding the selectivity filter. It has been established that TEA produces extracellular block through the actions of a specific residue (T449) near the Shaker voltage-gated potassium channel pore (Heginbotham and MacKinnon, 1992; MacKinnon and Yellen, 1990). Phenylalanine or tyrosine substitution at this site supports TEA block through cation-pi interactions with the negative electrostatic surface potential at the face of the aromatic ring (mediated through the pi-electron cloud) (Ahern, 2006; Heginbotham and MacKinnon, 1992). Given this precedent, we sought to test if F649, a phenylalanine side chain that aligns with the residue adjacent to T449 in Shaker plays a similar role in TEA inhibition of TRPV1. Mutation of this residue to a nonaromatic alanine dramatically altered TEA inhibition, but did not abolish it completely (wild type: 14% vs F649A: 35% of current remaining at 300 µM TEA). However, due to the TRPV1 channels apparent intolerance at the F649 site of the in vivo nonsense suppression method, required for the expression of fluorinated phenylalanine residues, we were unable to directly test for a possible cation-pi interaction between F649 and TEA (data not shown), which has been demonstrated to occur between lidocaine and a conserved phenylalanine residue in sodium channels (Ahern et al., 2008; Pless et al., 2011). Next, we turned our attention to E648, the residue that aligns directly with T449 in Shaker, and has also been shown to be critical for proton-evoked TRP channel activation without affecting heat or capsaicin sensitivity (Jordt et al., 2000). Mutation of E648 to alanine
ameliorated TEA inhibition, suggesting that this negative charge interacts electrostatically with the positively charged amine found in TEA, TMA, and LAs. Importantly, both E648A and F649A channels displayed activation properties and reversal potentials similar to WT channels (data not shown), suggesting that these substitutions were tolerated and did not result in general dysfunction of the selectivity filter region. Taken together, these results indicate that both the aromatic F649 and acidic E648 contribute to inhibition of TRPV1 by compounds with charged (or chargeable) amines.

We choose the *Xenopus* oocyte expression system because it is highly amenable for high-throughput pharmacologic characterization of drug-receptor activity, and for many years has been successfully used to investigate the molecular properties of a diverse range of ion channels. It is important to note that although general TRPV1 channel activity is conserved throughout various expression systems, differences in the constitution of membrane lipids, intracellular ions, second messenger molecules and experimental conditions do appear to affect the efficacy of agonist and antagonist activity of TRP channels (Chung et al., 2008; Lukacs et al., 2007; Novakova-Tousova et al., 2007). The polymodal nature of TRPV1 channels makes them particularly sensitive to regulation by a wide range of endogenous lipids such as phosphatidylinositol 4,5-bisphosphate (PIP2), PKC, PKA (cAMP-dependent protein kinase), PLC, diacylglycerol (DAG), arachidonic acid metabolites, and PUFAs (poly unsaturated fatty acids) (Bhave et al., 2002; Matta et al., 2007; Pingle et al., 2007; Prescott and Julius, 2003; Woo et al., 2008), and changes in the presence and concentrations of these molecules have been shown to alter TRP channels activity (Lukacs et al., 2007). We thus believe that
although the specific drug-receptor affinity may change depending on the expression system, (Lev et al., 2012) the observation of QA blockade will be universal, regardless of the cell system used. This is further supported by the observation that LAs and QAs can block TRPV1 in mammalian cells (Jara-Oseguera et al., 2008; Leffler et al., 2008; Oseguera et al., 2007).

In summary, we conclude that quaternary amine moieties underlie the potent LA-mediated TRPV1 inhibition. Mutations of residues near the TRPV1 pore significantly alter the potency of QA-mediated inhibition, providing a starting point to further elucidate the extracellular binding site for these molecules. Ultimately, the results introduce a basic molecular model for the observed, but so far unexplained, phenomenon of LA-mediated TRPV1 inhibition in vitro and serve as a basis for understanding the complex LA-mediated effects on TRPV1 channels.
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Participated in research design: Rivera-Acevedo, Pless, Schwarz and Ahern.
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Contributed new reagents or analytical tools: none
Performed data analysis: Rivera-Acevedo.
Wrote or contributed to the writing of the manuscript: Rivera-Acevedo, Pless, Schwarz and Ahern.
References


Footnote

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Figure legends

**Figure 1.** Lidocaine inhibits capsaicin-evoked TRPV1 currents in *Xenopus laevis* oocytes in a concentration-dependent and reversible manner. The co-application of 15 μM capsaicin with different concentrations of lidocaine was flanked by two control applications of 15 μM capsaicin to control for (de)sensitization. Only one drug application was performed per oocyte and 2 min washout intervals were used between all applications. Capsaicin-evoked currents were not observed in uninjected oocytes (data not shown). (A) & (B) Representative capsaicin-evoked current traces before and after the co-application of capsaicin with 100 nM (A) or 10 μM (B) lidocaine at a holding potential of −60 mV. Calibration bars: horizontal = 1 min; vertical = 0.1 μA. (C) Concentration-response data for the inhibition of capsaicin-evoked inward currents by lidocaine at −60 mV (black, n = 4–5) and −25 mV (grey, n = 4). Data points at each concentration represent mean ± SEM of remaining current, normalized to the mean peak current. Data was fit with the Hill equation (here and in all following fits: Hill four-parameter, non-linear regression). (D) Bar graph comparing the inhibition of 100 μM lidocaine observed at a holding potential of −60 mV (black, 24 ± 4 %) vs. −25 mV (grey, 50 ± 8 %) represented as % current remaining. Efficacy of inhibition at −60 mV vs. −25 mV displays significant voltage-dependence (*P* = 0.02). The inset depicts the chemical structure of lidocaine. * *P* > 0.05

**Figure 2.** Benzocaine does not strongly inhibit capsaicin-evoked TRPV1 currents in *Xenopus laevis* oocytes. (A) & (B) Representative capsaicin-evoked current traces
before and after the co-application of capsaicin with 10 µM (A) or 100 µM (B) benzocaine. Calibration bars: horizontal = 1 min; vertical = 0.1 µA. (C) Data points at each concentration represent mean ± SEM of remaining current, normalized to the mean peak current (n = 4–5). Note that data means are connected by straight lines. The inset depicts the chemical structure of benzocaine.

Figure 3. The quaternary ammonium compounds, tetraethylammonium and tetramethylammonium, potently inhibit capsaicin-evoked TRPV1 currents. (A) & (B) Representative capsaicin-evoked current traces before and after the co-application of capsaicin with 100 nM (upper panels) or 10 µM (middle panels) tetraethylammonium (TEA; A) or tetramethylammonium (TMA; B). Calibration bars: horizontal = 1 min; vertical = 0.1 µA. Lower panels show concentration-response data for the inhibition of capsaicin-evoked inward currents by TEA (A) or TMA (B) at −60 mV (black; TEA, n = 10–20; TMA, n = 5–11) and −25 mV (grey; TEA, n = 4; TMA, n = 4). Data points at each concentration represent mean ± SEM of remaining current, normalized to the mean peak current. (C) Bar graph comparing 100 µM TEA inhibition observed at a holding potential of −60 mV (black; 17 ± 3 %) vs. −25 mV (grey; 29 ± 4 %). (D) Results with 100 µM TMA, observed at a holding potential of −60 mV (black, 30 ± 4 %) vs. −25 mV (grey, 55 ± 3 %). Both TEA and TMA exhibit significant voltage-dependence of TRPV1 inhibition (P = 0.03 and 0.002, respectively). Insets depict the chemical structures of TEA and TMA. * P > 0.05 ** P > 0.01
Figure 4. Mutation of residues near the selectivity filter ameliorates TRPV1 inhibition by tetrathyaminmonium. (A) Sequence comparison between rTRPV1 and the Shaker voltage-gated potassium channel. Site-directed mutagenesis was carried out on amino acid residues indicated by black arrows. (B) & (C) Representative capsaicin-evoked current traces before and after the co-application of capsaicin with 10 μM tetrathyaminmonium (TEA) for F649A (B), or E648A (C; details, see body text). Calibration bars: horizontal = 1 min; vertical = 0.1 μA). (D) Bar graph comparing 100 μM TEA inhibition observed for the TRPV1 mutants at a holding potential of –60 mV (black; F649A, 35 ± 3 %; E648A, 51 ± 11 %) vs. –25 mV (grey; F649A, 69 ± 5 %; E648A, 78 ± 8 %). In the presence of TEA, the F649A mutant exhibits significant voltage dependent relief of inhibition (P = 0.001), but not the E648A mutant. (E) Concentration-response data for the inhibition of capsaicin-evoked inward currents by TEA for F649A at –60 mV (black; n = 4–6) and –25 mV (grey; n = 4–5), and (F) E648A at –60 mV (black; n = 4–7) and –25 mV (grey; n = 4); the black dashed regression lines represents wild type TEA inhibition. A significant decrease in inhibition was observed between wild type vs. both F649A and E648A mutants at –60 mV (P < 0.001, data not shown). Data points at each concentration represent mean ± SEM of remaining current, normalized to the mean peak current. *** P > 0.001
Figure 3

A

B

C

D

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