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Extracellular Quaternary Ammonium Blockade of Transient Receptor Potential Vanilloid Subtype 1 Channels Expressed in *Xenopus laevis* Oocytes

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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, whereas the neutral local anesthetic, benzocaine, does not, suggesting that a titratable amine is required for high-affinity inhibition. Consistent with this possibility, extracellular tetraethylammonium (TEA) and tetramethylammonium application produces potent, voltage-dependent pore block. Alanine substitutions at Phe649 and Glu648, 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 transient receptor potential 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 (TRPV1) channel, also known as the capsaicin receptor (Caterina et al., 1997). Subsequent identification and characterization of now more than 30 different subtypes of TRP channels has helped to categorize six receptor families and has demonstrated that these nonselective cation channels respond to a wide range of chemical and physical stimuli (Zhang et al., 2003; DeSimone and Lyall, 2006; Wu et al., 2010). On the basis of 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 probably consists of six transmembrane domains, with intracellular N and C termini and a reentrant 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, and temperature, and display a weak voltage-dependent activation (Clapham et al., 2005).

In this study, we focused 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 systems, with prominent expression in afferent pain-sensing neurons (A-δ and C fibers), dorsal root, and nodose and trigeminal ganglia (Szallasi et al., 1995; Caterina et al., 1997; Mezey et al., 2000; Tóth 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, somatostatin, and substance P, which in turn, regulate vascular, immune, and smooth muscle functions (Barthó et al., 2004, 2008). After tissue injury, an overabundance of these

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ABBREVIATIONS: TRP, transient receptor potential; TRPV1, transient receptor potential vanilloid subtype 1; Kv, voltage-gated potassium; LA, local anesthetic; QX-314, *N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium; TEA, tetraethylammonium; TMA, tetramethylammonium; QA, quaternary ammonium.

neuropeptides can contribute to the process of neurogenic inflammation by activation of proalgesic second messenger proteins and increased neuronal firing (Maggi, 1995; Holzer and Maggi, 1998), underlining the importance of TRPV1 as a physiological drug target.

In several studies, 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, *N*-(2,6-dimethylphenylcarbamoylmethyl)triethylammonium (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 were 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 Kv channels, that permanently charged quaternary ammonium compounds are able to potently block TRPV1 intracellularly, thus delimiting similarities among these seemingly disparate family members (Oseguera et al., 2007; Jara-Oseguera et al., 2008). However, it remains unclear whether TRPV1 is also susceptible to extracellular inhibition by quaternary ammonium compounds. LA molecules, which have both amphipathic and amphiphilic properties and maintain a dynamic equilibrium between charged and neutral forms, generally contain a titratable amine group attached to a hydrophobic aromatic moiety via an amide 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 whether 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 *X. laevis* oocytes, is potently inhibited by extracellular quaternary ammonium compounds. Furthermore, we demonstrate that the charged (or chargeable) amine of LAs 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 the inhibitory efficacy and affinity of these compounds for TRPV1 channels.

Materials and Methods

Chemical Reagents. All chemical reagents were obtained from Sigma-Aldrich Canada (Oakville, ON, 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 HEPES, and pH values were corrected to 7.4 with NaOH.

Molecular Biology. *X. laevis* oocytes were injected with rat TRPV1-capped mRNA (~1 μg/μl), which was synthesized using the T7 mMESSAGE mMACHINE kit (Ambion Canada, Streetsville, ON, Canada). Mutant channels, L647A, E648A, and F649A, were generated using site-directed mutagenesis, and correct incorporation of the mutations was confirmed with automated sequencing. After injection, oocytes were incubated for 1 to 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 $MgCl_2$, 0.5 mM $CaCl_2$, 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 A or indirect activation and phosphorylation of TRPV1-interacting enzymes recruited by phospholipase C (Vellani et al., 2001; Bhave et al., 2002). The pCLAMP 10.0 software suite (Molecular Devices) was used for data acquisition and offline analysis.

Statistical Analysis. Calculations for statistical comparisons were performed with Prism 5 (GraphPad Software, Inc., 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 approximate EC_{50} concentration of capsaicin (15 μM). All inhibition-response curves were fit using Hill four-parameter, nonlinear regressions. Continuous data were analyzed with Student's *t* test or repeated analysis of variance (analysis of variance; with Dunn's multiple comparison test for post hoc comparisons between individual groups) as appropriate. We considered $P \leq 0.05$ as statistically significant. Data are given as means ± S.E.M. where appropriate.

Results

Lidocaine Inhibition of TRPV1 Channels in *X. 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 titratable amine tail and a hydrophobic aromatic head, connected via an amide linker (Fig. 1C, inset). We expressed rat TRPV1 in *X. laevis* oocytes and coapplied an approximate 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 20% inhibition in inward current. Increasing the lidocaine concentration to 10 μM produced 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 reversal potential (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 at which inward currents could still be observed. Compared with the effects at

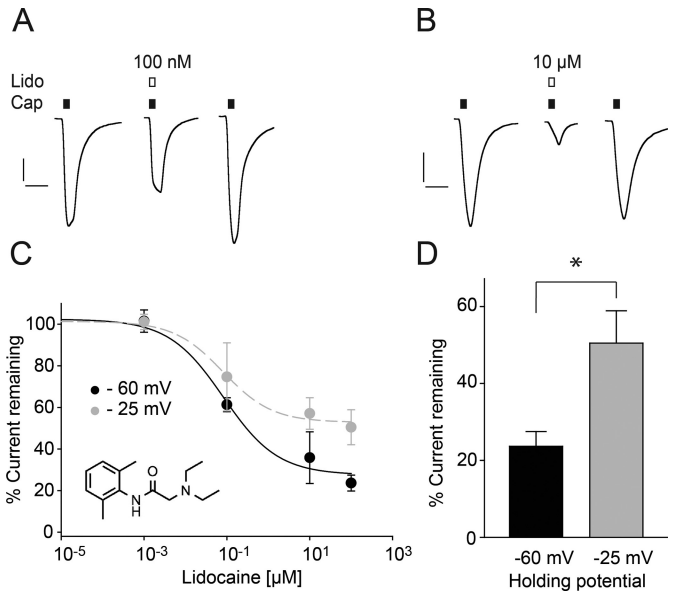

Fig. 1. Lidocaine inhibits capsaicin-evoked TRPV1 currents in *Xenopus laevis* oocytes in a concentration-dependent and reversible manner. The coapplication of 15 μM capsaicin with different concentrations of lidocaine was flanked by two control applications of 15 μM capsaicin to control for (de)sensitization. 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 and B, representative capsaicin-evoked current traces before and after the coapplication of capsaicin (Cap) with 100 nM (A) or 10 μM (B) lidocaine (Lido) 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 (●, n = 4–5) and −25 mV (◎, n = 4). Data points at each concentration represent the mean ± S.E.M. of remaining current, normalized to the mean peak current. Data were fit with the Hill equation (here and in all following fits: Hill four-parameter, nonlinear regression). D, bar graph comparing the inhibition of 100 μM lidocaine observed at a holding potential of −60 mV (■, 24 ± 4%) versus −25 mV (▨, 50 ± 8%) represented as percentage of current remaining. Efficacy of inhibition at −60 mV versus −25 mV displays significant voltage dependence (P = 0.02). The inset depicts the chemical structure of lidocaine. ∗, P > 0.05.

−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. 1, C and D). Complete inhibition of TRPV1 activation was not achieved, even at the highest concentrations of lidocaine that were used 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 *X. laevis* oocytes.

The Neutral LA Benzocaine Does Not Strongly Inhibit TRPV1 Channels in *X. laevis* Oocytes. 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 (pK_a, 2.5), because 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 with lidocaine or QX-314, does not significantly inhibit capsaicin-evoked TRPV1 currents in the range of 1 nM to 100 μM (analysis of variance, 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 benzocaine was coapplied with capsaicin (data not shown).

Quaternary Ammonium Compounds Potently Inhibit Capsaicin-Induced TRPV1 Currents in *X. laevis* 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 whether the charged ammonium moiety of lidocaine and QX-314 mediates inhibition of TRPV1. The quaternary ammonium (QA) compound, TEA, represents an ideal candidate to test this hypothesis because it mimics the charged amine tail group of QX-314 but does not contain the amino-amide linker or the lipophilic head. When coapplied 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, top and middle panels). The IC_{50} for TEA inhibition of TRPV1 was 41 ± 13 nM, with a Hill coefficient of 0.9 ± 0.1 (Fig. 3A, bottom panel). We next tested the smallest of the quaternary ammonium salts, tetramethylammonium (TMA). As shown in Fig. 3B, TMA also robustly inhibited capsaicin-induced TRPV1 currents in oocytes,

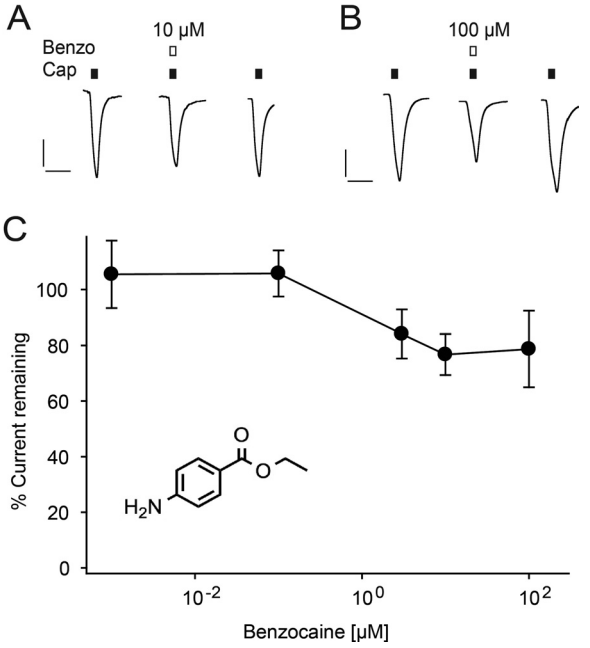

Fig. 2. Benzocaine does not strongly inhibit capsaicin-evoked TRPV1 currents in *Xenopus laevis* oocytes. A and B, representative capsaicin-evoked current traces before and after the coapplication of capsaicin (Cap) with 10 μM (A) or 100 μM (B) benzocaine (Benzo). Calibration bars: horizontal, 1 min; vertical, 0.1 μA. C, data points at each concentration represent the mean ± S.E.M. of the 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.

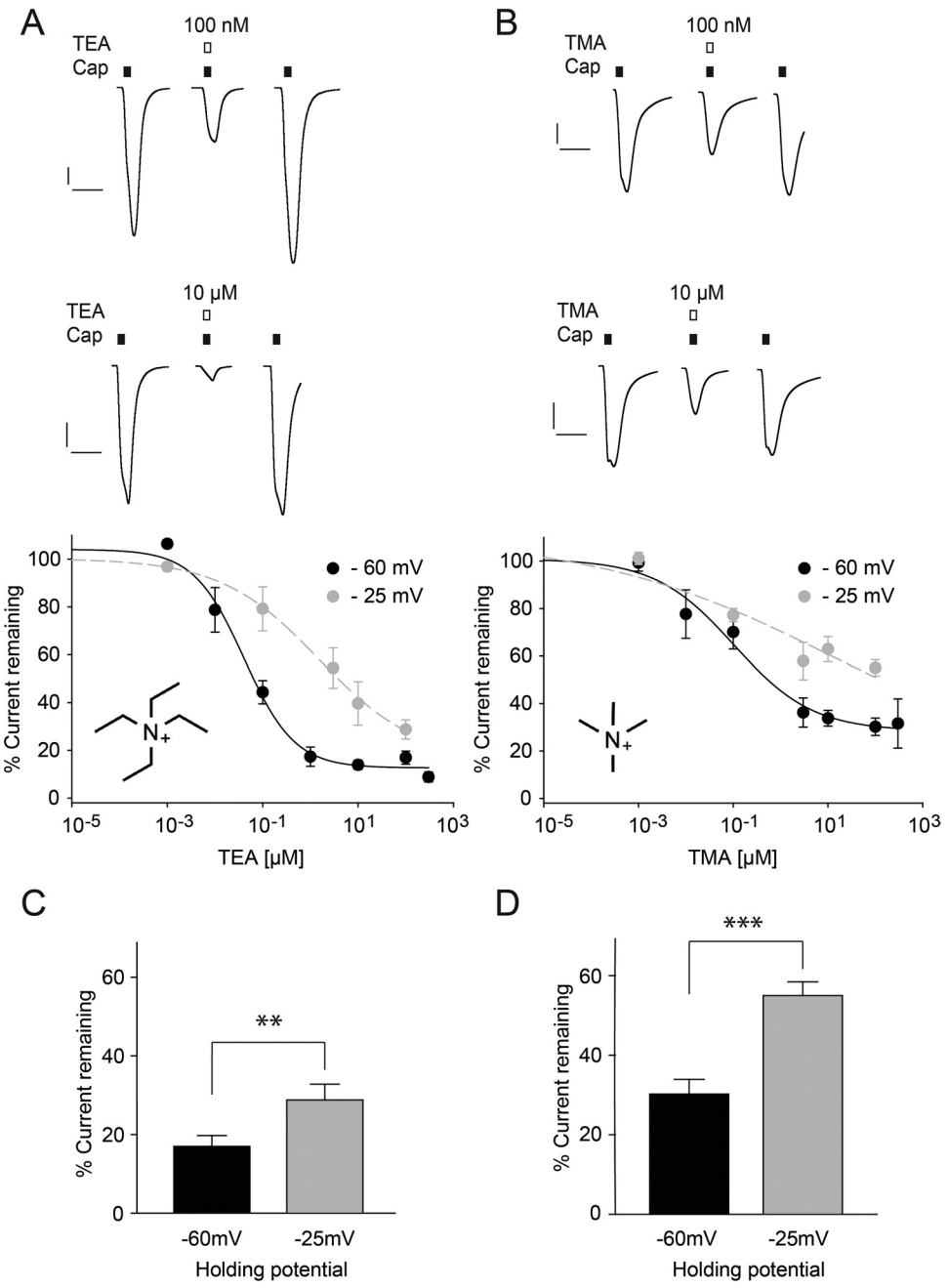

Fig. 3. The quaternary ammonium compounds, tetraethylammonium and tetramethylammonium, potently inhibit capsaicin-evoked TRPV1 currents. A and B, representative capsaicin (Cap)-evoked current traces before and after the coapplication of capsaicin with 100 nM (top panels) or 10 μM (middle panels) TEA (A) or TMA (B). Calibration bars: horizontal, 1 min; vertical, 0.1 μA. Bottom panels show concentration-response data for the inhibition of capsaicin-evoked inward currents by TEA (A) or TMA (B) at −60 mV (●, TEA, n = 10–20; TMA, n = 5–11) and −25 mV (◎, TEA, n = 4; TMA, n = 4). Data points at each concentration represent the mean ± S.E.M. of the remaining current, normalized to the mean peak current. C, bar graph comparing 100 μM TEA inhibition observed at a holding potential of −60 mV (■; 17 ± 3%) versus −25 mV (▨; 29 ± 4%). D, results with 100 μM TMA, observed at a holding potential of −60 mV (■; 30 ± 4%) versus −25 mV (▨; 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. *, $P > 0.05$; **, $P > 0.01$.

although with lower efficacy and affinity than TEA: at 100 nM and 10 μM, TMA produced mean TRPV1 current inhibitions of approximately 40 and 70%, respectively (Fig. 3B, top and middle panels). The corresponding IC_{50} for TMA inhibition of TRPV1 was 104 ± 60 nM, with a Hill coefficient of 0.5 ± 0.1 (Fig. 3B, bottom 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. 3, C and D). Administration of 100 nM TEA or TMA produced little to no reduction in currents elicited by capsaicin, whereas at 100 μM approximately 30% current remained present with TEA and approximately 60% with TMA (Fig. 3, C and 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 were not feasible, given the contaminating effects of the inherently 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 Weaken TEA Inhibition. Because TRPV1 shares sequence homology with Kv channels from the *Shaker* family at the putative selectiv-

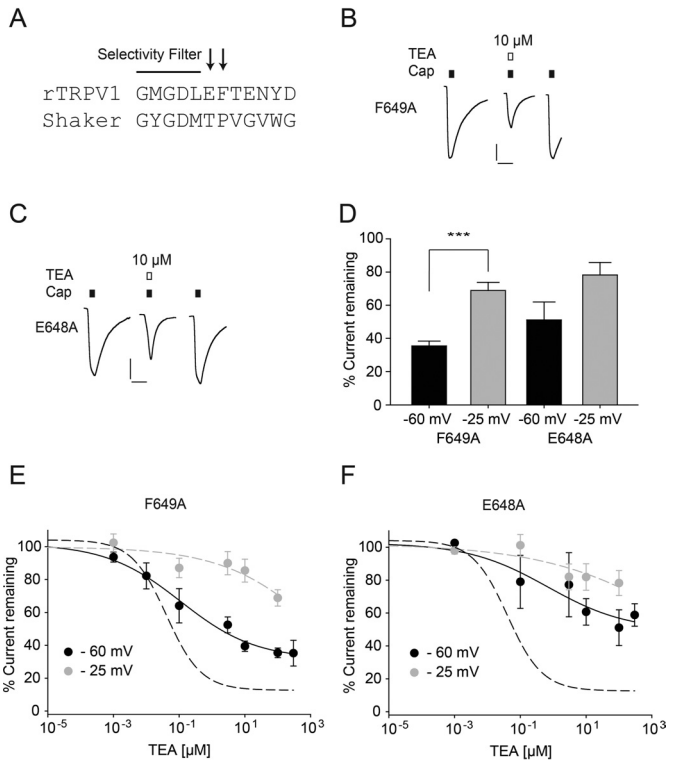

Fig. 4. Mutation of residues near the selectivity filter ameliorates TRPV1 inhibition by tetraethylammonium. A, sequence comparison between rTRPV1 and the *Shaker* voltage-gated potassium channel. Site-directed mutagenesis was performed on amino acid residues indicated by black arrows. B and C, representative capsaicin (Cap)-evoked current traces before and after the coapplication of capsaicin with 10 μM TEA for F649A (B) or E648A (C) (for details, see *Materials and Methods*). 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 (■; F649A, 35 ± 3%; E648A, 51 ± 11%) versus −25 mV (▨; F649A, 69 ± 5%; E648A, 78 ± 8%). In the presence of TEA, the F649A mutant but not the E648A mutant exhibits significant voltage-dependent relief of inhibition ($P = 0.001$). E and F, concentration-response data for the inhibition of capsaicin-evoked inward currents by TEA for (E) F649A at −60 mV (●; $n = 4–6$) and −25 mV (◍; $n = 4–5$) and (F) E648A at −60 mV (●; $n = 4–7$) and −25 mV (◍; $n = 4$); the black dashed regression lines represents wild-type TEA inhibition. A significant decrease in inhibition was observed between wild-type versus both F649A and E648A mutants at −60 mV ($P < 0.001$, data not shown). Data points at each concentration represent the mean ± S.E.M. of the remaining current, normalized to the mean peak current. ***, $P > 0.001$.

ity filter (Fig. 4A) (Owsianik et al., 2006; Moiseenkova-Bell et al., 2008), 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 nonfunctional. In terms of blockade, we found that compared with 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. 4, E and F), suggesting that both GluE648 and Phe649 could be involved in TEA inhibition of TRPV1. TEA blockade was also demonstrated to be voltage-dependent for the TRPV1 mutants, although a significant decrease between −60 mV versus −25 mV was only detected in the F649A mutant (Fig. 4D).

Discussion

In this study, we have demonstrated that TRPV1 channels, expressed in *X. 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 human embryonic kidney 293t cells, although with significantly lower potency (Leffler et al., 2008). Furthermore, we have recently shown that the extracellular administration of the quaternary lidocaine derivative, QX-314, inhibits TRPV1-mediated currents in *X. laevis* oocytes (Rivera-Acevedo et al., 2011). Taken 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 *X. laevis* oocytes. In particular, 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 to a possible contribution of the latter came from the observation that even at the highest lidocaine concentration used (100 μM), approximately 30% of the current remained (Fig. 1C). Indeed, the pharmacological efficacy of lidocaine, a weak amphipathic base with a pK_a of 7.9, and for that matter that of 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 the charged form of LAs 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 used to help define the structure and basic biophysical properties of the intracellular TRPV1 channel pore (Oseguera et al., 2007; Jara-Oseguera et al., 2008). In our current study, similar to results with 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, because we generally observed 10 to 15% residual current. This effect could potentially be attributed to TEA behaving like a partially permeant ion, as observed for other voltage-dependent inhibitors (Huang et al., 2000; Huang and Moczydlowski, 2001). In considering the size of TEA (~8 Å) and the estimated TRPV1 pore diameter of 7 Å (Owsianik et al., 2006), this seems possible. Consistent with this hypothesis, the smaller TMA (~6 Å) (Briend et al., 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, through TRPV1 channels (Chung et al., 2008). Indeed, this mechanism has been suggested to take place in explanted dorsal root ganglion neuron cultures

exposed to prolonged applications of QX-314 in the presence of capsaicin (Binshtok et al., 2007). Taken together, 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 with that at −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 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 (Thr449) near the *Shaker* voltage-gated potassium channel pore (MacKinnon and Yellen, 1990; Heginbotham and MacKinnon, 1992). Phenylalanine or tyrosine substitution at this site supports TEA block through cation-π interactions with the negative electrostatic surface potential at the face of the aromatic ring (mediated through the π-electron cloud) (Heginbotham and MacKinnon, 1992; Ahern et al., 2006). Given this precedent, we sought to test whether Phe649, a phenylalanine side chain that aligns with the residue adjacent to Thr449 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% versus F649A: 35% of current remaining at 300 μM TEA). However, because of the apparent intolerance of the TRPV1 channels at the Phe649 site to the in vivo nonsense suppression method, required for the expression of fluorinated phenylalanine residues, we were unable to directly test for a possible cation-π interaction between Phe649 and TEA (data not shown), similar to that demonstrated between lidocaine and a conserved phenylalanine residue in sodium channels (Ahern et al., 2008; Pless et al., 2011). Next, we turned our attention to Glu648, the residue that aligns directly with Thr449 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 Glu648 to alanine ameliorated TEA inhibition, suggesting that this negative charge interacts electrostatically with the positively charged amine found in TEA, TMA, and LAs. Of importance, both E648A and F649A channels displayed activation properties and reversal potentials similar to those of wild-type 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 Phe649 and acidic Glu648 may 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 (Lukacs et al., 2007; Novakova-Tousova et al., 2007; Chung et al., 2008). 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, protein kinase C, cAMP-dependent protein kinase A, phospholipase C, diacylglycerol, arachidonic acid metabolites, and polyunsaturated fatty acids (Bhave et al., 2002; Prescott and Julius, 2003; Matta et al., 2007; Pingle et al., 2007; Woo et al., 2008), and changes in the presence and concentrations of these molecules have been shown to alter TRP channel 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 notion is further supported by the observation that LAs and QAs can block TRPV1 in mammalian cells (Oseguera et al., 2007; Jara-Oseguera et al., 2008; Leffler et al., 2008).

In summary, we conclude that quaternary ammonium 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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Authorship Contributions

Participated in research design: Rivera-Acevedo, Pless, Schwarz, and Ahern.

Conducted experiments: Rivera-Acevedo.

Performed data analysis: Rivera-Acevedo.

Wrote or contributed to the writing of the manuscript: Rivera-Acevedo, Pless, Schwarz, and Ahern.

References

Ahern CA, Eastwood AL, Dougherty DA, and Horn R (2008) Electrostatic contributions of aromatic residues in the local anesthetic receptor of voltage-gated sodium channels. *Circ Res* **102:**86–94.

Ahern CA, Eastwood AL, Lester HA, Dougherty DA, and Horn R (2006) A cation-π interaction between extracellular TEA and an aromatic residue in potassium channels. *J Gen Physiol* **128:**649–657.

Barthó L, Benko R, Holzer-Petsche U, Holzer P, Undi S, and Wolf M (2008) Role of extrinsic afferent neurons in gastrointestinal motility. *Eur Rev Med Pharmacol Sci* **12 (Suppl 1):**21–31.

Barthó L, Benkó R, Patacchini R, Pethö G, Holzer-Petsche U, Holzer P, Lázár Z, Undi S, Illényi L, Antal A, et al. (2004) Effects of capsaicin on visceral smooth muscle: a valuable tool for sensory neurotransmitter identification. *Eur J Pharmacol* **500:**143–157.

Bhave G, Zhu W, Wang H, Brasier DJ, Oxford GS, and Gereau RW 4th (2002) cAMP-dependent protein kinase regulates desensitization of the capsaicin receptor (VR1) by direct phosphorylation. *Neuron* **35:**721–731.

Binshtok AM, Bean BP, and Woolf CJ (2007) Inhibition of nociceptors by TRPV1-mediated entry of impermeant sodium channel blockers. *Nature* **449:**607–610.

Briend LA, Peltre MJ, Man P, and Barthomeuf D (1993) Thermal stability of tetrapropylammonium (TPA) and tetra methylammonium (TMA) cations occluded in SAPO-37 molecular sieves. *Zeolites* **13:**201–211.

Butterworth JF 4th and Strichartz GR (1990) Molecular mechanisms of local anesthesia: a review. *Anesthesiology* **72:**711–734.

Caterina MJ, Leffler A, Malmberg AB, Martin WJ, Trafton J, Petersen-Zeitz KR, Koltzenburg M, Basbaum AI, and Julius D (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science* **288:**306–313.

Caterina MJ, Schumacher MA, Tominaga M, Rosen TA, Levine JD, and Julius D (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* **389:**816–824.

Chung MK, Güler AD, and Caterina MJ (2008) TRPV1 shows dynamic ionic selectivity during agonist stimulation. *Nat Neurosci* **11:**555–564.

Clapham DE, Julius D, Montell C, and Schultz G (2005) International Union of Pharmacology. XLIX. Nomenclature and structure-function relationships of transient receptor potential channels. *Pharmacol Rev* **57:**427–450.
Davis JB, Gray J, Gunthorpe MJ, Hatcher JP, Davey PT, Overend P, Harries MH, Latcham J, Clapham C, Atkinson K, et al. (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature* **405:**183–187.
DeSimone JA and Lyall V (2006) Taste receptors in the gastrointestinal tract. III. Salty and sour taste: sensing of sodium and protons by the tongue. *Am J Physiol Gastrointest Liver Physiol* **291:**G1005–G1010.
Heginbotham L and MacKinnon R (1992) The aromatic binding site for tetraethylammonium ion on potassium channels. *Neuron* **8:**483–491.
Holzer P and Maggi CA (1998) Dissociation of dorsal root ganglion neurons into afferent and efferent-like neurons. *Neuroscience* **86:**389–398.
Huang CJ and Moczydlowski E (2001) Cytoplasmic polyamines as permeant blockers and modulators of the voltage-gated sodium channel. *Biophys J* **80:**1262–1279.
Huang CJ, Favre I, and Moczydlowski E (2000) Permeation of large tetraalkylammonium cations through mutant and wild-type voltage-gated sodium channels as revealed by relief of block at high voltage. *J Gen Physiol* **115:**435–454.
Jara-Oseguera A, Llorente I, Rosenbaum T, and Islas LD (2008) Properties of the inner pore region of TRPV1 channels revealed by block with quaternary ammoniums. *J Gen Physiol* **132:**547–562.
Jordt SE, Tominaga M, and Julius D (2000) Acid potentiation of the capsaicin receptor determined by a key extracellular site. *Proc Natl Acad Sci USA* **97:**8134–8139.
Koplas PA, Rosenberg RL, and Oxford GS (1997) The role of calcium in the desensitization of capsaicin responses in rat dorsal root ganglion neurons. *J Neurosci* **17:**3525–3537.
Latorre R, Zaelzer C, and Brauchi S (2009) Structure-functional intimacies of transient receptor potential channels. *Q Rev Biophys* **42:**201–246.
Leffler A, Fischer MJ, Rehner D, Kienel S, Kistner K, Sauer SK, Gavva NR, Reeh PW, and Nau C (2008) The vanilloid receptor TRPV1 is activated and sensitized by local anesthetics in rodent sensory neurons. *J Clin Invest* **118:**763–776.
Lev S, Katz B, and Minke B (2012) The activity of the TRP-like channel depends on its expression system. *Channels (Austin)* **6:**86–93.
Liu H, Atkins J, and Kass RS (2003) Common molecular determinants of flecainide and lidocaine block of heart Na^+ channels: evidence from experiments with neutral and quaternary flecainide analogues. *J Gen Physiol* **121:**199–214.
Lukacs V, Thyagarajan B, Varnai P, Balla A, Balla T, and Rohacs T (2007) Dual regulation of TRPV1 by phosphoinositides. *J Neurosci* **27:**7070–7080.
MacKinnon R and Yellen G (1990) Mutations affecting TEA blockade and ion permeation in voltage-activated K^+ channels. *Science* **250:**276–279.
Maggi CA (1995) Tachykinins and calcitonin gene-related peptide (CGRP) as cotransmitters released from peripheral endings of sensory nerves. *Prog Neurobiol* **45:**1–98.
Matta JA, Miyares RL, and Ahern GP (2007) TRPV1 is a novel target for omega-3 polyunsaturated fatty acids. *J Physiol* **578 (Pt 2):**397–411.
Mezey E, Tóth ZE, Cortright DN, Arzubi MK, Krause JE, Elde R, Guo A, Blumberg PM, and Szallasi A (2000) Distribution of mRNA for vanilloid receptor subtype 1 (VR1), and VR1-like immunoreactivity, in the central nervous system of the rat and human. *Proc Natl Acad Sci USA* **97:**3655–3660.
Moiseenkova-Bell VY, Stanciu LA, Serysheva II, Tobe BJ, and Wensel TG (2008) Structure of TRPV1 channel revealed by electron cryomicroscopy. *Proc Natl Acad Sci USA* **105:**7451–7455.
Novakova-Tousova K, Vyklicky L, Susankova K, Benedikt J, Samad A, Teisinger J, and Vlachova V (2007) Functional changes in the vanilloid receptor subtype 1 channel during and after acute desensitization. *Neuroscience* **149:**144–154.
Oseguera AJ, Islas LD, García-Villegas R, and Rosenbaum T (2007) On the mechanism of TBA block of the TRPV1 channel. *Biophys J* **92:**3901–3914.
Owsianik G, Talavera K, Voets T, and Nilius B (2006) Permeation and selectivity of TRP channels. *Annu Rev Physiol* **68:**685–717.
Pingle SC, Matta JA, and Ahern GP (2007) Capsaicin receptor: TRPV1 a promiscuous TRP channel. *Handb Exp Pharmacol* **179:**155–171.
Pless SA, Galpin JD, Frankel A, and Ahern CA (2011) Molecular basis for class Ib anti-arrhythmic inhibition of cardiac sodium channels. *Nat Commun* **2:**351.
Prescott ED and Julius D (2003) A modular PIP2 binding site as a determinant of capsaicin receptor sensitivity. *Science* **300:**1284–1288.
Ries CR, Pillai R, Chung CC, Wang JT, MacLeod BA, and Schwarz SK (2009) QX-314 produces long-lasting local anesthesia modulated by transient receptor potential vanilloid receptors in mice. *Anesthesiology* **111:**122–126.
Rivera-Acevedo RE, Pless SA, Ahern CA, and Schwarz SK (2011) The quaternary lidocaine derivative, QX-314, exerts biphasic effects on transient receptor potential vanilloid subtype 1 channels in vitro. *Anesthesiology* **114:**1425–1434.
Szallasi A, Nilsson S, Farkas-Szallasi T, Blumberg PM, Hökfelt T, and Lundberg JM (1995) Vanilloid (capsaicin) receptors in the rat: distribution in the brain, regional differences in the spinal cord, axonal transport to the periphery, and depletion by systemic vanilloid treatment. *Brain Res* **703:**175–183.
Tóth A, Boczán J, Kedei N, Lizanecz E, Bagi Z, Papp Z, Edes I, Csiba L, and Blumberg PM (2005) Expression and distribution of vanilloid receptor 1 (TRPV1) in the adult rat brain. *Brain Res Mol Brain Res* **135:**162–168.
Vellani V, Mapplebeck S, Moriondo A, Davis JB, and McNaughton PA (2001) Protein kinase C activation potentiates gating of the vanilloid receptor VR1 by capsaicin, protons, heat and anandamide. *J Physiol* **534 (Pt 3):**813–825.
Woo DH, Jung SJ, Zhu MH, Park CK, Kim YH, Oh SB, and Lee CJ (2008) Direct activation of transient receptor potential vanilloid 1(TRPV1) by diacylglycerol (DAG). *Mol Pain* **4:**42.
Wu LJ, Sweet TB, and Clapham DE (2010) International Union of Basic and Clinical Pharmacology. LXXVI. Current progress in the mammalian TRP ion channel family. *Pharmacol Rev* **62:**381–404.
Zhang Y, Hoon MA, Chandrashekar J, Mueller KL, Cook B, Wu D, Zuker CS, and Ryba NJ (2003) Coding of sweet, bitter, and umami tastes: different receptor cells sharing similar signaling pathways. *Cell* **112:**293–301.

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