Camphor modulates TRPV3 cation channels activity by interacting with critical pore-region cysteine residues

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Abstract: TRPV3 ion channels mediate thermo-transduction, nociception, inflammation and dermatitis in mammals. TRPV1-4 proteins have been shown to have conserved cysteine-residues in the pore-forming regions. These residues participate in channel activation via S-nitrosylation of channel proteins. Camphor is a commonly used ligand for TRPV3 channels. Thus the knowledge about the potential binding/interacting site(s) for camphor will help to design effective and potent analgesic compounds. In an overlap-extension PCR method, following primer-pairs were used to mutate conserved cysteine-residues in the pore-region of TRPV3 channels; GATTGAGAATcCTCCAAGGACAAAAAGGAC, TRPV3-C612S-Fw and GTCCTTGGAGgACTTCTCAATCAGTCAGTGAGG, TRPV3-C612S-Rv primers pair. And for TRPV3-C619S: GGACTCcAGTTCCTATGGCCAGC, TRPV3-C619S-Fw and GCTGGCCATAgGAACTGGAGTCC, TRPV3-C619S-Rv respectively. All cDNA constructs were confirmed by DNA-sequencing and used to make cRNAs. Oocytes expressing mTRPV3-C619S and mTRPV3-C612S mutant channels were challenged with 2-APB (1 mM), camphor (10 mM) and dihydrocarveol (10 mM) either at –40 mV or +40 mV holding potentials in voltage-clamp experiments. Responses of both mutants to 2-APB were similar to wild-type mTRPV3. Interestingly, responses to camphor were totally lost in mTRPV3-C619S mutant, while responses to dihydrocarveol remained intact. In contrast mTRPV3-C612S displayed slightly altered (16±2 % reduction) phenotype with respect to camphor sensitivity. It is concluded that pore-region cysteines play critical role in camphor sensitivity of TRPV3 ion channels.

Keywords: Camphor modulates, cysteine, nociception, inflammation, dermatitis, transient receptor potential (TRP).

INTRODUCTION

Transient receptor potential (TRP) channels work as molecular sensors for multiple environmental and endogenous stimuli (Clapham, 2003). These vanilloid receptors constitute a subgroup in a large super-family of nonselective cation channels. They share membrane topology with voltage-gated K^+ channels that have six transmembrane segments (S1–S6) and a pore-loop between S5 and S6. They also contain additional structural motifs in the two relatively large intracellular termini (the so called N- and C-terminus). Ankyrin repeats on the N-terminus and calcium binding domains and TRP-BOX towards C-terminus contribute to specific physiological functions associated with these proteins. TRPV3 channel is activated by modest thermal heating around 32°C (Peier *et al*., 2002, Smith *et al*., 2002, Xu *et al*., 2002). TRPV3 expression is robust in skin keratinocytes, tongue and nose epithelial cells. Its chemo-sensitivity underpins flavor sensations of plant origin. TRPV3 contributes to the genetic susceptibility to migraine headache in the Spanish population (Carreno *et al*., 2012).

Animals lacking TRPV3 are deficient in thermal sensation and heat-induced hyperalgesia, which supports the role of TRPV3 proteins in nociception and Thermosensation (Moqrich *et al*., 2005, Caterina, 2007). Keratinocytes obtained from TRPV3 knockout mice did not show any TRPV3 mediated currents and the animals suffer with a phenotype of wavy/curly whiskers and dermatitis. This shows that the functional channel is essential for normal hair maturation, epidermal-barrier patterning, and thermosensation (Moqrich *et al*., 2005 Cheng *et al*., 2010). Primary keratinocytes secrete messenger molecules like ATP and growth factors that transmit vital informations to proliferating and differentiating keratinocytes. Keratinocytes communicate with sensory neurons through these chemical messengers (Lee and Caterina, 2005, Lumpkin and Caterina, 2007, Mandadi *et al*., 2009, Cheng *et al*., 2010). This chemical communication is attenuated in TRPV3-null mice (Mandadi *et al*., 2009, Cheng *et al*., 2010). Additionally, autosomal-dominant TRPV3 mutants manifest constitutive cationic activity, steering the skin to hairlessness, dermatitis and inflammatory cutaneous lesions (Asakawa *et al*., 2006, Xiao *et al*., 2008b, Imura *et al*., 2009, Yoshioka *et al*., 2009).

Despite a sequence homology to other vanilloid receptors, TRPV3 exhibits unusual channel property known as sensitization. Due to sensitization repeated application of a stimulus leads to progressive increases in the responses of TRPV3 ion channels. The sensitization is observed both in native and expression systems regardless of the stimulating modality (Peier *et al*., 2002, Smith *et al*., 2002, Xu *et al*., 2002, Chung *et al*., 2004b, Chung *et al*., 2004a, Hu *et al*., 2006). One mechanism that has been put forward implicates Ca^{2+}-mediated interactions between calmodulin (CaM) and the channel (Xiao *et al*., 2008a, Phelps *et al*., 2010). In a calcium free extracellular

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solution no sensitization is observed and thus this phenotype is believed to be a contextual relevance (Sherkheli *et al*., 2009), rather than the intrinsic property of the channel protein (Liu *et al*., 2011). Calcium and membrane-derived lipids are physiological regulators of TRPV3 ion channels (Hu *et al*., 2006).

TRPV3 is a multimodal receptor ion channel, hence it is not surprising that it has built-in structural motifs specifically designed to handle a variety of impinging stimuli. The heat sensor is located in the 6^{th} transmembrane helix and adjacent extracellular loop within the pore region (Grandl *et al*., 2008). These specific mutant channels show normal responses to 2-aminoethyoxy diphenylborinate (2-APB) or camphor. Hu and co-workers (Hu *et al*., 2009) reported that two specific amino acid residues (H426 and R696) are crucial for TRPV3 responsiveness to 2-APB. These mutants show normal responses to camphor whereas sensitivity to 2-APB is completely lost. This clearly shows that camphor and 2-APB have different activation mechanisms. It is previously reported (Sherkheli *et al*., 2009) that camphor not 2-APB desensitizes TRPV3 ion channels. For rational design of lead therapeutic compounds, knowledge of binding/interacting domain on a target pharmacophore is critical.

Thus in the present investigation, elusive camphor interaction domain was searched. Using specifically designed primers, targeted cysteine resides (C612 and C619) in the pore region of TRPV3 ion channels were mutated to serine. The serine contains a hydroxyl group (-OH) in contrast to highly reactive thiol-group (-SH) in cysteine residues. The reactive thiol side chain often serves as a nucleophile in protein-protein interactions, resulting in the formation of disulphide cross-linkers. In subsequent voltage-clamp experiments, it could be confirmed that pore-region cysteine resides are critical for TRPV3 responsiveness to camphor.

MATERIALS AND METHODS

DNA constructs and site-directed point mutations in TRPV3 pore-loop regions

The wild type mouse TRPV3 (mTRPV3) cDNA was a generous gift from Dr. David Julius (UCSF, CA, USA). Human TRPV3 (NCBI accession # AF514998) was cloned in-house using standard molecular biology methods. mTRPV3 C619S and C612S mutations were carried out by overlap extension PCR method, using synthetic complementary oligonucleotides (Ho *et al*., 1989). All cDNA mutations were verified by DNA sequencing (ABI PRISM 3100; Applied Biosystems). All oligonucleotides (primers) were purchased from Invitrogen or MWG Biotech and solved in sterile water to a concentration of 100 pmol/µl. The following point mutations were generated in the pore region of TRPV3. GATTGAGAAGT**G**CTCCAAGGACAAAAAGGACT**G** CAGTTCCT Wild Type GATTGAGAAGT**c**CTCCAA GGACAAAAAGGACTGCAGTTCCT C612S mutant GATTGAGAAGTGCTCCAAGGACAAAAAGGACT**c**A GTTCCT, C619S mutant For TRPV3-C612S forward and reverse primers were; GATTGAGAATcCTCCAAGGAC AAAAAGGAC, TRPV3-C612 S-Fw, GTCCTTGGAGgA CTTCTCAATCAGTCAGTGAGG, TRPV3-C612S-Rv. And for TRPV3-C619S; GGACTCcAGTTCCTATGGC CAGC TRPV3-C619S-Fw, and GCTGGCCATAgGAAC TGGAGTCC, TRPV3-C619S-Rv respectively.

Synthesis and injection of TRP cRNA

For efficient expression in *Xenopus* oocytes, cDNA of wild type and mutant TRPV3 ion channels were subcloned by a PCR-based standard method into the oocyte expression vector pSGEM (Villmann *et al*., 1997). These sub-cloned cDNAs were subsequently used to make cRNA. The generation of cRNA was performed by standard methods as described elsewhere (Sherkheli *et al*., 2008, Sherkheli, 2010, Sherkheli *et al*., 2012, Sherkheli *et al*., 2010, Sherkheli, 2007). Linearized plasmids containing cloned cDNA of TRP ion channels were used as templates for *invitro* transcription. With the help of capping analogue m7G(5´)ppp(5´)G and AmpliCap-T7 MessageMaker Kit (Epicentre, Madison, WI), capped RNAs for each TRP ion channel were produced. RNA was cleaned with ethanol-precipitation method and re-dissolved in RNase-free water, affording a final concentration of 1 µg/µl. Ovarian lobes from mature anesthetized (by immersion in 0.15% 3-aminobenzoic acid ethyl ester) female *Xenopus laevis* were obtained through surgical procedures. Isolated ovarian tissue was placed in Barth´s solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO3)^2$, 0.41 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 5 mM Tris-HCl, pH 7.4; 100 U/ml penicillin, 50 µg/ml streptomycin). Isolated ovarian tissue was treated with collagenase (Type I, 4 mg/ml in Ca^{2+}-free Barth´s solution) for two hours at room temperature. The liberated oocytes were incubated overnight in fresh Barth´s solution (15°C). After a resting time of 24 hours, mature and smart looking oocytes (stage V to VI) were picked for cytoplasmic injection of cRNA (about 50 nl per oocyte; approximate cRNA concentration 1 µg/µl) with a sharp pipette and a pressure injector (npi PDES 04T, Tamm, Germany). Impregnated oocytes were placed in fresh Barth´s solution kept in an incubator at 16-18°C. Oocytes were investigated for functional expression of TRP channels at 3 to 5 days after injection.

Electrophysiological recordings in Oocytes

Two-electrode voltage-clamp recordings were performed to obtain current responses to the investigational substances. Ligands were diluted to the final concentration in either Ca^{2+}-containing (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 200 µM flufenamic acid, 10 mM HEPES, pH 7.4) or Ca^{2+}-free (115 mM NaCl, 2.5

mM KCl, 10 mM HEPES, 10 mM EGTA, pH 7.4) standard extracellular solution (SES) as depicted within respective figures. Ligands were applied through a multibarrel single tip superfusion device or by manual application. The duration of application was usually 10-20 seconds. Electrodes, pulled from borosilicate glass with the help of a Kopf vertical pipette puller, were backfilled with 3 M KCl. Oocytes were continuously held at - 40 mV with the help of a command potential from the amplifier. The evoked current signals were recorded with a two-electrode voltage-clamp amplifier (TURBO TEC-03, npi, Tamm, Germany) and acquired using the PCLAMP software (Axon Instruments, Sunnyvale, CA).

STATISTICAL ANALYSIS

All data was subjected to statistical analysis to check the significance using SigmaStat software (version 2.03). The p values<0.05=* p<0.005=** and p<0.001=*** indicate different levels of significance in mean values for each set of data. Data are expressed as mean ±S.E.M. of 6 to 8 independent measurements of responses under comparable experimental conditions unless otherwise stated.

RESULTS

Agonist specific responses of mTRPV3 in calcium free SES

TRPV3 is known to show increased responses to the same stimuli in the presence of extracellular calcium. Repeated application of 1 mM 2-APB resulted in slight sensitization similar to results reported in literature (fig. 1A). The same procedure was repeated with TRPV3 expressing oocytes using 1 mM 2-APB in Ca^{2+}-free SES and no sensitization or desensitization was observed (fig. 1B). In the next step 10 mM camphor was repeatedly applied (short-term ~15 seconds) to TRPV3 expressing oocytes. Initially no sensitization or desensitization was seen. But around 12th application of 10 mM camphor, magnitude of camphor induced currents started decreasing significantly as shown fig. 1C. mTRPV3 expressing oocytes also showed robust

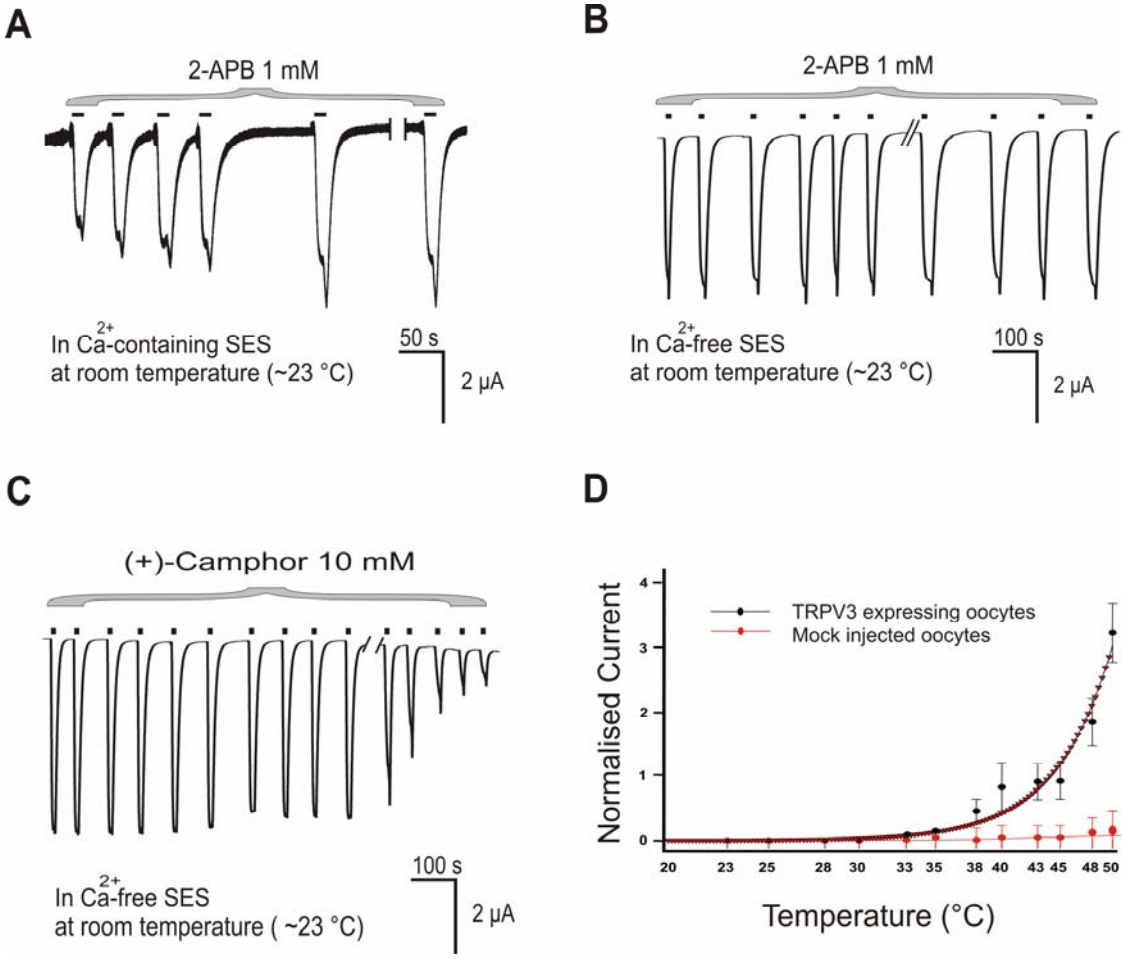

Fig. 1: (A) a representative trace showing TRPV3 response to repeated 2-APB exposure in Ca^{2+}-containing SES. (B) response to repeated 2-APB exposure in Ca^{2+}- free SES. (C) Response of TRPV3 to repeated exposure of camphor in Ca^{2+}-free SES. (D) Heat activation of TRPV3 in oocytes, the mock injected oocytes do not show any significant response to heat (n= 6 in each case).

responses to a heat-stimulus (fig. 1D), whereas corresponding mock injected oocytes remained non-responsive.

Different binding domains for 2-APB, camphor and dihydrocarveol

Since terpenoids are general agonists for TRPV3 activation (Vogt-Eisele *et al*., 2007), it was interesting to find out whether they bind to the same site as 2-APB or to a different binding site specific for monoterpenoids. The first indication of different binding domains for camphor and 2-APB is predicted by desensitization patterns of currents induced by either substance (Sherkheli *et al*., 2009). The evoked responses clearly show that camphor, but not 2-APB, desensitizes TRPV3. Additionally the effects of agonist mixes on the response properties of TRPV3 were tested (see fig 2). Different binding sites of monoterpenes and 2-APB would argue for a collaborative/ additive effect of both substances. Potentiations of 2-APB (3 mM) responses were observed when mixed with monoterpenes like camphor. This result is similar to that reported earlier by Hu and colleagues (Hu *et al*., 2006). The current-magnitudes increased to 294±37 % ($p<0.008$, n=6) for camphor (5 mM) plus 2-APB (3 mM) as compared to the magnitudes of current of individually applied 2-APB (taken as 100%). Similarly, mixes of dihydrocarveol (2 mM) and 2-APB increased current size to 194±31% ($p<0.01$, n=6). Interestingly, no such potentiation of currents was observed when mixes of dihydrocarveol and camphor were applied (46±25%, $p<0.49$, n=6).

The responses of singly applied camphor and dihydrocarveol were 30±12 % and 70±9%, respectively. This cross-potentiation property supports the idea that 2-APB and terpenoids interact at different domains on TRPV3 and hence activate the cannel through independent mechanisms.

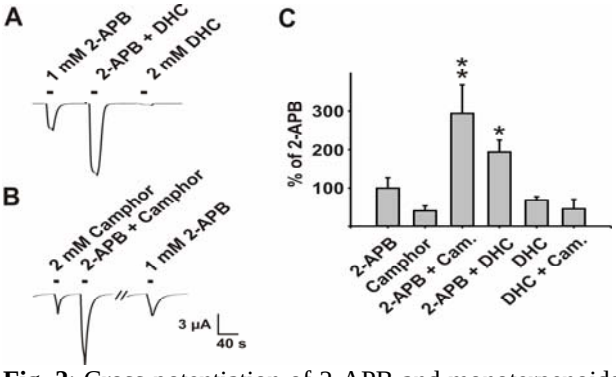

Fig. 2: Cross-potentiation of 2-APB and monoterpenoids. (A) 1 mM 2-APB or 2 mM DHC were applied singly or in mixture to oocytes expressing TRPV3. B) 1 mM 2-APB or 2mM capmhor were applied singly or in mixture to oocytes expressing TRPV3.(C) Quantified response amplitudes of camphor, DHC and the respective mixes relative to the average current evoked by 1 mM 2-APB (n=6).

Pore-loop mutations impair TRPV3 sensitivity for camphor

Vanilloid TRP cation channels like TRPV1, TRPV3 and TRPV4 are reported to contain cysteines conserved on the N-terminal side of the presumed pore-forming region (see fig 3) found between the fifth and sixth transmembrane domains (Yoshida *et al*., 2006). These csyteines play a decisive role in the channel-activation by nitiric oxide (NO) via induction of S-nitrosylation of the TRP channel proteins. Recently, Grandl and colleagues (2008) showed that pore-loop of the TRPV3 contains the putative temperature sensor. This later study highlights the importance of the pore-loop region for overall functional viability of these channels. Based upon the predictions of Yoshida's group, point mutations in the pore-loop of TRPV3 were generated to investigate whether these cysteines play any role in the terpenoid or 2-APB activation of the channel.

Consequently point mutations generated in conserved pore-loop cysteines afforded mutant (TRPV3-C619S) which is insensitive to camphor but its response to 2-APB is normal (like wild type). To study the general behavior of TRPV3 mutant TRPV3-C619S and TRPV3-C612S, oocytes expressing these mutant channels were challenged with 2-APB (1 mM), camphor (10 mM) and 10 mM DHC (dihydrocarveol) either at -40 mV or +40 mV holding potentials. Responses of both mutants to 2-APB were similar to wild-type TRPV3. Interestingly, responses to camphor were totally lost in TRPV3-C619S mutant, while responses to DHC remained intact. Whereas TRPV3-C612S showed only slightly reduced (16±2 %; $p<0.37$) sensitivity to camphor (figs. 4C & D).

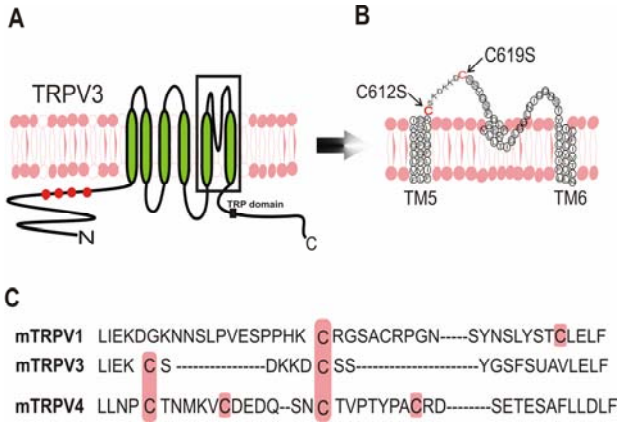

Fig. 3: Illustration of potential mutation sites of TRPV3 and other Vanilloid channels. (A) Putative topology of TRPV3 for depiction of point mutations in the poreloop of the channel. (B) Exact sequence of the pore-loop of TRPV3 showing conserved cysteines mutated to check camphor sensitivity. (C) Depiction of sequences of pore-loops of three TRPV channels showing highly conserved cysteine residues among vanilloid subfamily of TRP ion channels.

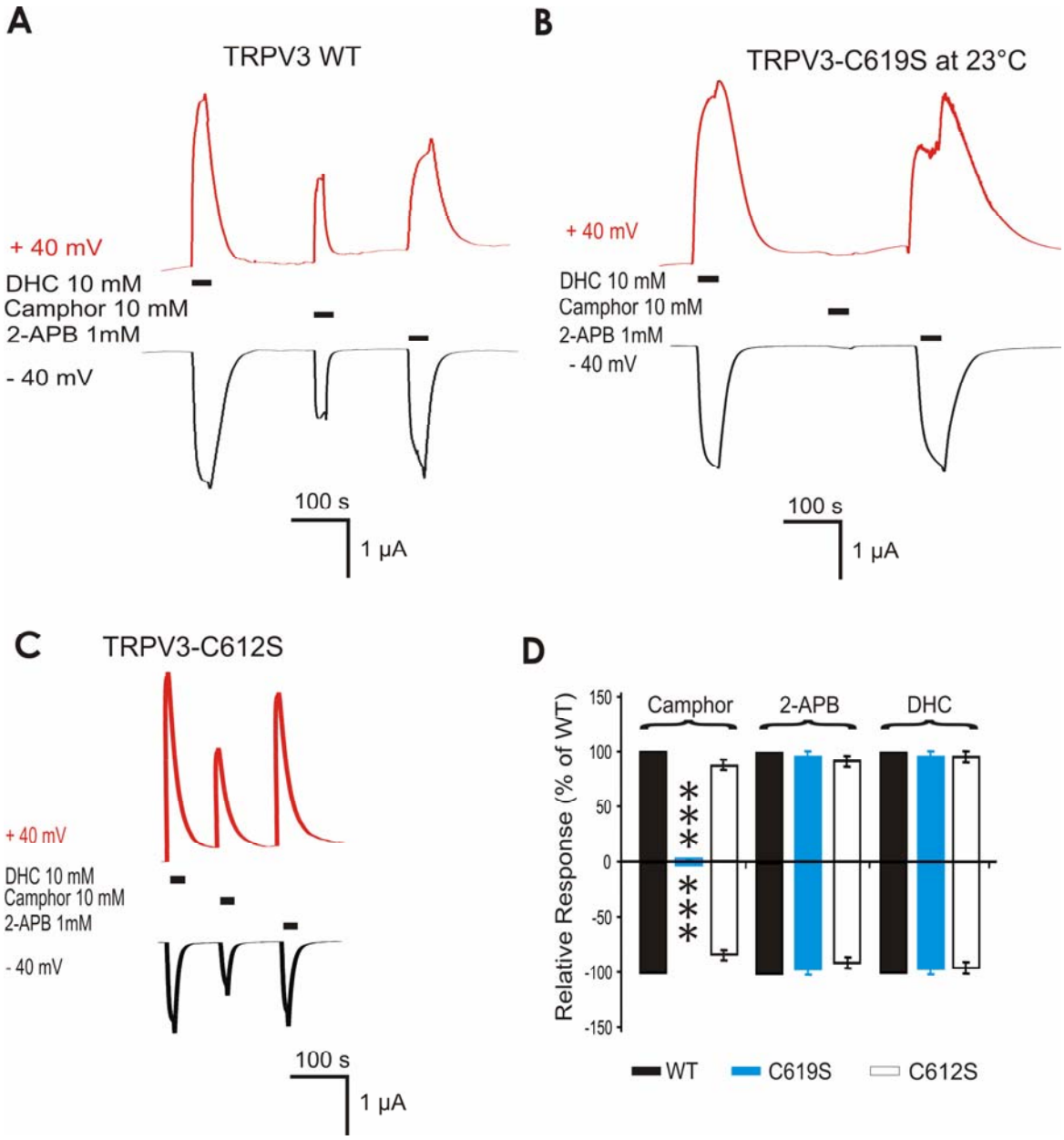

Fig. 4: Point-mutants and loss of camphor sensitivity of TRPV3. (A) an original trace showing WT TRPV3 response to 2-APB, camphor and DHC at two different holding potentials in the same oocyte clamped either at – 40 or + 40 mV. (B) An original trace showing loss of responsiveness to camphor by mutant TRPV3 (C619S). (C) Another cysteine mutant (C612S) shows least impairment to responsiveness to terpenoids. (D) Quantified data for wild-type and mutant TRPV3 channels. All experiments were carried out under Ca^{2+}-free SES at room temperature (~23°C). Data are expressed as mean ± SEM of six independent experiments

DISCUSSION

The members of TRP super-family of cation channels are implicated in a diverse array of patho-physiological processes. They play significant roles in inflammation, nociception, thermo-sensation, cell-proliferation and many other physiological processes (Clapham *et al*., 2001, Nilius, 2007). Substantial evidence suggests that TRP ion channels are allosterically modulated by chemical and thermal stimuli through independent and clearly distinct molecular mechanisms. Vanilloid substances interact either at intracellular or intramembranous loci in/or adjoining to S3 and S4 domains (Jordt and Julius, 2002, Gavva *et al*., 2004). A short molecular stretch in the C-terminal domain (Val686-Trp752) is responsible for heat sensitivity of TRPV1 channels. In a chimeric-swap this region was transplanted into the cold-sensitive TRPM8 channel. This chimeric-

switch converted cold-sensitive TRPM8 to behave like a heat sensor (Brauchi *et al*., 2007). Supplementary evidence that supports independent/distinguishable mechanistic and thermal regulation of TRPV channels has been reported in literature (Hu *et al*., 2009, Grandl *et al*., 2008, Yang *et al*., 2010, Phelps *et al*., 2010). Boukalova and coworkers reported (Boukalova *et al*., 2010) many mutant TRPV channels detailing additional mechanistic informations about functional aspects of distinct protein domains within a channel subunit. In this investigation TRPV3-Y565S responded normally to temperature and 2-APB, while the corresponding mutation led to a complete loss of function in TRPV1. Charge-replacing mutations, comparable to the most affected mutations in TRPV1, did not alter the heat- and 2-APB-induced activation in TRPV2 and TRPV3. Furthermore, neither hTRPV2-K527E nor hTRPV3-K581E mutants channels showed any aberration in 2-APB responses, which is a contrast to corresponding rat TRPV1-K571E mutant. Interestingly, even TRPV1/TRPV3 heteromeric channels have been reported (Cheng *et al*., 2011) that exhibit distinct temperature sensitivity activation thresholds and varied thermal sensitization to different stimuli. It was previously reported that bicyclic monoterpenoids can induce desensitization in TRPV3 mediated currents (also see fig 1A, B & C) but this phenotype is not observed either with 2-APB or monocyclic monoterpenoids like dihydrocarveol (Sherkheli *et al*., 2009). From this observation it is deduced that independent and distinguishable molecular mechanisms underpin the activation of individual TRPV channels; and these mechanisms are not conserved across the subfamily of TRP cation channels.

TRPV3 responses are potentiated when a mixture of agonists is applied (Hu *et al*., 2006). In the present investigation it is observed that this phenotype is not present in all kind of agonist mixes (see fig 2). Potentiation is marked with 2-APB and camphor mixture or 2-APB and dihydrocarveol mixture but virtually absent in case of camphor and dihydrocarveol mixture, where inhibition is apparent instead of potentiation. The simple conclusion from this experiment is that 2-APB and camphor do not share the interaction/binding domain on TRPV3 receptor molecules. This separate binding-sites hypothesis is in conformity with random mutagenesis studies by other groups (Grandl *et al*., 2008), in which mutants lack responses to 2-APB but display normal phenotype to camphor or heat. TRPV channels possess conserved cysteine residues in the pore-region (see fig 3), which are critical for activation of TRPV channels by nitric oxide through the process of S-nitrosylation (Yoshida *et al*., 2006). Grandl and co-workers (Grandl *et al*., 2008) analyzed about ~14,000 TRPV3 mutants generated in a random mutagenesis approach. However, this long list of mutant TRPV3 does not include these specific cysteine mutants. In the present investigation, specifically designed oligonucleotide primer-pairs were used to replace the thiol-group with less reactive hydroxyl group. One of these mutants C619S-TRPV3 completely lost its sensitivity to camphor (figs. 4 B & D). However, the same mutant displays normal TRPV3 phenotype to 2-APB and dihydrocarveol. The double mutant C612S/C619S-TRPV behaved similar to C619S-TRPV3 (data not shown). The mutant C612S-TRPV3 displayed normal phenotype to 2-APB, camphor and dihydrocarveol both at negative and positive holding potentials. The ligand potentiation experiments detailed in fig 2 and mutation experiments detailed in fig 4, clearly demonstrate that all three ligands of TRPV3 ion channels use different mechanisms to activate TRPV3 receptor molecules. These results are in conformity with earlier results (Sherkheli *et al*., 2009); where it is reported that only camphor induces agonist specific desensitization of TRPV3 ion channels after prolonged incubation. Whereas 2-APB, menthol or dihydrocarveol manifest no such property (figs. 1 A, B & C).

Natural products like camphor, carvacrol, menthol, dihydrocarveol, thymol and unsaturated fatty acids, membrane derived lipid-metabolites either activate or potentiate TRPV3 ion channels (Vogt-Eisele *et al*., 2007, Sherkheli, 2010, Sherkheli *et al*., 2009, Peier *et al*., 2002, Smith *et al*., 2002, Xu *et al*., 2002, Chung *et al*., 2004b, Chung *et al*., 2004a, Hu *et al*., 2006). Camphor, a traditional topical analgesic, is a non-specific TRPV3 ligand. Camphor is less potent than synthetic TRPV3 ligand 2-APB. 2-APB also has nonspecific actions on other channels; for example, antagonist action on IP3 receptors (Maruyama and Takeuchi, 1997) and blocking of gap-junctional channels (Harks *et al*., 2003). 2-APB also activates TRPV1 and TRPV2 and does not have desensitization properties like that of camphor (Sherkheli *et al*., 2009). Therefore, development of highly specific and potent TRPV3 ligands is an urgent clinical need. The knowledge of independent site of interaction(s) is critical for better design and optimization of therapeutic efficacy of useful natural and synthetic substances.

CONCLUSION

Thus it is **c**oncluded that, the knowledge about the binding/interacting domain of camphor on TRPV3 receptor proteins will help to develop ligands with better receptor affinity and pharmacological potency to be employed in treatment of diseases involving TRPV3 malfunctions.

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Vol. 26, No.3, May 2013

Vol. 26, No.3, May 2013

Vol. 26, No.3, May 2013

Vol. 26, No.3, May 2013

May 2013 Vol. 26, No.3, Pages 431-651

May 2013 Vol. 26, No.3, Pages 431-651

May 2013 Vol. 26, No.3, Pages 431-651