Ethanol Modulates the VR-1 Variant Amiloride-insensitive Salt Taste Receptor. II. Effect on Chorda Tympani Salt Responses

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ABSTRACT The effect of ethanol on the amiloride- and benzamil (Bz)-insensitive salt taste receptor was investigated by direct measurement of intracellular Na^+ activity ($[Na^+]_i$) using fluorescence imaging in polarized fungiform taste receptor cells (TRCs) and by chorda tympani (CT) taste nerve recordings. CT responses to KCl and NaCl were recorded in Sprague-Dawley rats, and in wild-type (WT) and vanilloid receptor-1 (VR-1) knockout mice (KO). CT responses were monitored in the presence of Bz, a specific blocker of the epithelial Na^+ channel (ENaC). CT responses were also recorded in the presence of agonists (resiniferatoxin and elevated temperature) and antagonists (capsazepine and SB-366791) of VR-1 that similarly modulate the Bz-insensitive VR-1 variant salt taste receptor. In the absence of mineral salts, ethanol induced a transient decrease in TRC volume and elicited only transient phasic CT responses. In the presence of mineral salts, ethanol increased the apical cation flux in TRCs without a change in volume, increased transepithelial electrical resistance across the tongue, and elicited CT responses that were similar to salt responses, consisting of both a phasic component and a sustained tonic component. At concentrations <50%, ethanol enhanced responses to KCl and NaCl, while at ethanol concentrations >50%, those CT responses were inhibited. Resiniferatoxin and elevated temperature increased the sensitivity of the CT response to ethanol in salt-containing media, and SB-366791 inhibited the effect of ethanol, resiniferatoxin, and elevated temperature on the CT responses to mineral salts. VR-1 KO mice demonstrated no Bz-insensitive CT response to NaCl and no sensitivity to ethanol. We conclude that ethanol increases salt taste sensitivity by its direct action on the Bz-insensitive VR-1 variant salt taste receptor.

KEY WORDS: resiniferatoxin • SB-366791 • capsazepine • salt taste • Na^+ imaging

INTRODUCTION

Recent studies demonstrate that in rat and mouse fungiform taste receptor cells (TRCs), the amiloride- and benzamil (Bz)-insensitive salt taste receptor is a constitutively active nonselective cation channel that is derived from the vanilloid receptor-1 (VR-1) gene. It accounts for all of the amiloride- and Bz-insensitive chorda tympani (CT) taste nerve responses to Na^+ salts and part of the response to K^+, NH_4^+, and Ca^{2+} salts (Lyall et al., 2004b, 2005a). The amiloride-insensitive salt taste receptor is activated by the vanilloids resiniferatoxin (RTX) and capsaicin (CAP) and by elevated temperature (>38°C). It is blocked by VR-1 antagonists capsazepine (CZP) and N-(3-methoxyphenyl)-4-chlorocinnamide (SB-366791). VR-1 knockout (KO) mice lack the amiloride- and Bz-insensitive component of the NaCl CT response and demonstrate no sensitivity to RTX, CAP, or elevated temperature (Lyall et al., 2004b, 2005a). Since the amiloride- and Bz-insensitive salt taste receptor demonstrates many biochemical, pharmacological, physiological, and functional similarities with the cloned VR-1, we hypothesize that endocannabinoids, lipoxygenase metabolites of arachidonic acid, nicotine, lipid derivatives, and intracellular second messengers that either activate or sensitize the VR-1 receptor channel (Davis et al., 2002; Gunthorpe et al., 2002; Geppetti and Trevisani, 2004; Heck et al., 2005; Liu et al., 2004), will also have similar effects on the amiloride- and Bz-insensitive salt taste receptor.

Ethanol activates primary sensory neurons from trigeminal or dorsal root ganglia, as well as VR-1 expressing HEK-293 cells (Trevisani et al., 2002; Geppetti and Trevisani, 2004). In rat gastric epithelial cells, ethanol decreases cell viability by acting directly on the VR-1 nonspecific cation channel (Kato et al., 2003). Ethanol potentiated the response of VR-1 to CAP, H^+, and heat and lowered the temperature threshold for heat activation of VR-1 (Trevisani et al., 2002). Ethanol not only affects receptors in the central nervous system, but is also a potent gustatory stimulus (Diamant et al., 1963; Hellekant, 1965a,b; Hellekant et al., 1997; Sako

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Abbreviations used in this paper: Bz, benzamil; CAP, capsaicin; CT, chorda tympani; CZP, capsazepine; ENaC, epithelial Na^+ channel; KO, knockout; RTX, resiniferatoxin; RVI, regulatory volume increase; SB-366791, *N*-(3-methoxyphenyl)-4-chlorocinnamide; TRC, taste receptor cell; VR-1, vanilloid receptor-1; WT, wild-type.

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and Yamamoto, 1999; Danilova and Hellekant, 2000; Lyall et al., 2005c). Stimulating the tongue with ethanol elicits neural responses in rat, mice, dog, cat, monkey, and humans (Diamant et al., 1963; Hellekant 1965a,b; Hellekant et al., 1997; Sako and Yamamoto, 1999; Danilova and Hellekant, 2000; Lyall et al., 2005c). Ethanol also produces mixture interactions when applied together with sweet, bitter, salty, and sour taste stimuli (Hellekant et al., 1997; Sako and Yamamoto, 1999). We have recently demonstrated that ethanol enhances the Bz-insensitive unilateral Na^+ flux across the apical membranes of polarized rat TRCs and enhances the Bz-insensitive NaCl rat CT response (Lyall et al., 2005b,c).

In this study, we investigated if ethanol modulates salt taste responses via its action on the amiloride- and Bz-insensitive salt taste receptor. The effect of ethanol on the Bz-insensitive salt taste receptor was investigated by direct measurement of intracellular Na^+ activity ($[Na^+]_i$) using fluorescence imaging in polarized rat fungiform TRCs and by the CT taste nerve recordings (Simon, 2002; Lyall et al., 2005c). The CT responses were monitored in two animal models, a rat model and a VR-1 KO mouse model (Caterina et al., 2000; Lyall et al., 2004b, 2005a). CT responses were recorded while the tongue was stimulated with ethanol, mineral salts, and mixtures of ethanol plus mineral salts. In addition, CT responses were also monitored in the absence and presence of specific agonists (RTX and elevated temperature) and antagonists (CZP and SB-366791) of the VR-1 receptor that also modulate the VR-1 variant salt taste receptor (Lyall et al., 2004b, 2005a,c). The results indicate that ethanol specifically enhances the Bz-insensitive Na^+ flux across the apical membrane of polarized fungiform TRCs and modulates the Bz-insensitive CT responses to KCl and NaCl. At concentrations $<50\%$, ethanol behaves as an agonist of the CT response to 100 mM NaCl. Both CZP and SB-366791 inhibited the effects of ethanol on the apical Na^+ flux and on the CT responses to KCl and NaCl. VR-1 KO mice lacked the amiloride- and Bz-insensitive component of the NaCl CT response and were insensitive to ethanol. We conclude that ethanol modulates salt responses by its action on the VR-1 variant cation channel in rat and mice fungiform TRCs. Preliminary reports of this study have been published as abstracts (Lyall et al., 2005b; Vinnikova et al., 2005).

MATERIALS AND METHODS

CT Taste Nerve Recordings

Animals were housed in the Virginia Commonwealth University animal facility in accordance with institutional guidelines. All in vivo and in vitro animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Commonwealth University. Female Sprague-Dawley rats (150–200 g) were anesthetized by intraperitoneal injection of pentobarbital (60 mg/Kg) and supplemental pentobarbital (20 mg/kg) was administered as necessary to maintain surgical anesthesia. The animal's corneal reflex and toe-pinch reflex were used to monitor the depth of surgical anesthesia. Body temperatures were maintained at 37°C with a Deltaphase Isothermal PAD (Model 39 DP; Braintree Scientific, Inc.). The left CT nerve was exposed laterally as it exited the tympanic bulla and placed onto a 32G platinum/iridium wire electrode. The CT responses were recorded under zero current-clamp and voltage-clamp conditions and analyzed as described previously (Ye et al., 1991, 1993; Lyall et al., 2001, 2004a,b, 2005a,c).

The anterior lingual surface was stimulated with deionized H_2O rinse and with ethanol solutions ranging in concentration from 0 to 100%. In addition, the lingual surface was stimulated with a rinse solution (10 mM KCl) and with salt solutions (10 mM KCl + 100 mM NaCl) containing ethanol varying in concentration between 0 and 60%. CT responses were recorded in the presence of benzamil (Bz; 5 μM), a specific and potent blocker of the apical epithelial Na^+ channel (ENaC). CT responses were also recorded in the presence of the VR-1 agonists RTX (0.25 μM or 0.50 μM) or elevated temperatures (23°C–55.5°C), and VR-1 antagonists CZP (25 μM) or *N*-(3-methoxyphenyl)-4-chlorocinnamide (SB-366791; 1 μM) (Lyall et al., 2004b, 2005a,c). All drugs were purchased from Sigma-Aldrich. Typically, stimulus solutions remained on the tongue for 2 min. Control stimuli consisting of 300 mM NaCl and 300 mM NH_4Cl, applied at the beginning and at the end of the experiment, were used to assess preparation stability.

To investigate the effect of temperature on the CT response to ethanol and to mixtures of ethanol + mineral salts, the lingual surface was superfused (8 ml/min) with salt solutions using syringe pumps and heating coils maintained between 23°C and 55.5°C. The CT response was plotted as a function of the temperature (°C) of the stimulus solution delivered to the tongue surface, and the data were fitted using a modified Hill equation of the form

$$r = q + \frac{at^n}{k^n + t^n + b^m t^m}, \quad (1)$$

where r is the CT response and t is the temperature. The quantities a, b, k, m, n, and q are parameters used to fit the data according to least squares criteria as described before (Lyall et al., 2004b, 2005a).

CT responses were also monitored in wild-type (WT; C57BL/6J) and homozygous VR-1 KO (B6.129S4-Trpv1^{tm1Jul}) mice (The Jackson Laboratory). Mice (30–40 g) were anesthetized by intraperitoneal injection of pentobarbital (30 mg/kg), and supplemental pentobarbital (10 mg/kg) was administered as necessary to maintain surgical anesthesia. The rest of the procedure was the same as described above for rats (Lyall et al., 2004b, 2005c). At the end of the experiment, the animals were humanely killed by an intraperitoneal overdose of pentobarbital (~195 mg/kg body weight for rats and 150 mg/kg body weight for mice).

$[Na^+]_i$ Measurement in Polarized Fungiform TRCs

Relative changes in intracellular Na^+ activity ($[Na^+]_i$) were monitored in polarized TRCs by loading the tissue with sodium-green (Molecular Probes) as described in detail earlier (Lyall et al., 2005c). Changes in TRC $[Na^+]_i$ were monitored in the presence and absence of Bz, CZP, or SB-366791. This was done to distinguish between the apical Na^+ flux through the Bz-sensitive ENaCs and the Bz-insensitive VR-1 variant nonspecific cation channels in fungiform TRCs (Lyall et al., 2004b, 2005a). The relative changes in TRC $[Na^+]_i$ were expressed as percent change in F_{490}

of sodium-green relative to apical zero Na^+ concentration. In individual taste buds, the data were presented as the mean ± SEM of *n*, where *n* represents the number of regions of interest within the taste bud. The data were also presented as the mean ± SEM of *N*, where *N* represents the number of individual taste buds studied. Student's *t* test was employed to analyze the differences between sets of data.

RESULTS

In Vitro Studies

Na^+ enters TRCs across the apical membrane via two pathways. One pathway is blocked by amiloride or Bz, and represents the Na^+ flux through apical epithelial Na^+ channels, ENaCs. The second pathway is insensitive to amiloride or Bz, and represents the Na^+ flux through an apical CZP-sensitive VR-1 variant nonspecific cation channel (Lyall et al., 2004b, 2005a,c). We hypothesize that ethanol modulates CT salt responses through its direct action on the Bz-insensitive VR-1 variant nonspecific cation channel in the apical membrane of fungiform TRCs. To test this hypothesis, we first studied the effect of ethanol stimulation on the unilateral apical Na^+ flux in polarized TRCs.

Effect of Ethanol on the Unilateral Apical Na^+ Flux in Polarized Fungiform TRCs. Fig. 1 shows the effect of ethanol on the F_{490} of Na-green–loaded TRCs in the presence and absence of external Na^+. Consistent with previous studies (Lyall et al., 2005c), in a lingual epithelial preparation perfused on both sides with Na^+-free Ringer's solution (pH 7.4), perfusing the apical membrane with Na^+-free Ringer's solution containing 10, 20, 30, and 40% ethanol (ETH) produced a dose-dependent increase in F_{490} (Fig. 1 A). At each ethanol concentration, the increase in F_{490} was transient (*d–e*) and was followed by a spontaneous partial recovery toward the control level (*e*–f). In contrast, increasing apical Na^+ concentration from 0 to 150 mM in the presence of Bz produced a sustained increase in F_{490} (*a–b–c*).

As discussed earlier (Lyall et al., 2005c), in the absence of external Na^+, the increase in F_{490} due to ethanol stimulation most likely does not represent changes in TRC $[Na^+]_i$ but rather represents changes in cell volume. A decrease in cell volume will result in an increase in dye concentration inside the cells and an increase in F_{490} of Na-green, even though there are no changes in cell Na^+ (Xu et al., 1995). In the continuous presence of ethanol, the spontaneous recovery of F_{490} to baseline indicates that following osmotic cell shrinkage, TRCs exhibit regulatory volume increase (RVI).

Fig. 1 B also shows that perfusing apical membrane with Na^+-free Ringer's solution containing 40% ethanol (ETH) produced a transient increase in F_{490} (Fig. 1 B, *c–d*) that spontaneously recovered to its control level (*d–e*). Subsequently, perfusing control Ringer's solution containing 150 mM NaCl + 5 μM Bz + 40% ETH

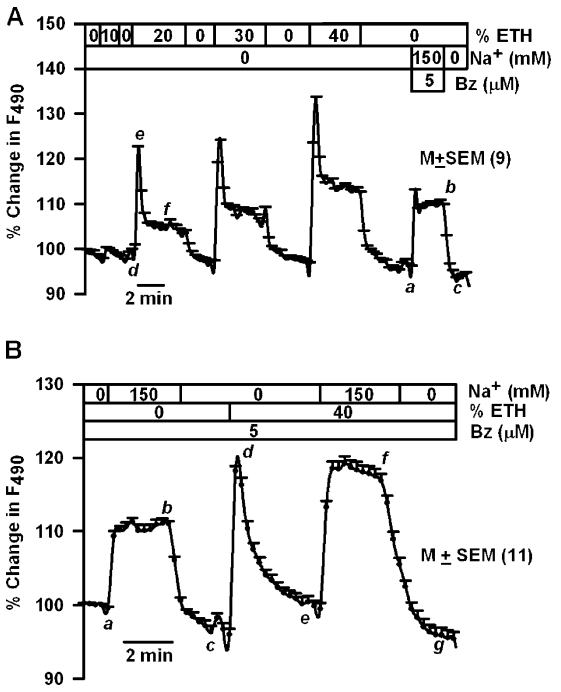

FIGURE 1. Effect of ethanol on TRC $[Na^+]_i$. A polarized epithelial preparation was initially perfused on both sides with 0 Na^+-Ringer's solution containing 150 mM NMDG-Cl (pH 7.4). (A) Temporal changes in F_{490} of Na-green–loaded TRCs were monitored while the apical membrane was perfused with 0 Na^+-Ringer's solution containing 10, 20 (*d–e–f*), 30, 40% ETH and with Ringer's solution containing 150 mM NaCl + 5 μM Bz (*a–b–c*). (B) Temporal changes in F_{490} of Na-green–loaded TRCs were monitored while the apical membrane was perfused with Ringer's solution containing 150 mM NaCl + 5 μM Bz (*a–b–c*), 0 Na^+ Ringer's solution containing 40% ETH (*c–d–e*), and with Ringer's solution containing 150 mM NaCl + 5 μM Bz + 40% ETH (*e–f–g*). The relative changes in $[Na^+]_i$ are presented as percent changes in F_{490} relative to bilateral 0 Na^+ and are expressed as mean ± SEM of *n*, where *n* = number of regions of interest within the taste bud.

produced a sustained increase in F_{490} (*e–f*) that was completely reversible (*f–g*). In the presence of ethanol, the magnitude of the sustained increase in F_{490} (*e–f–g*) was significantly greater relative to the corresponding increase in F_{490} with NaCl alone (*a–b–c*). A sustained increase in F_{490} suggests that ethanol does not induce significant changes in cell volume when presented with apical Na^+, a cation that permeates the Bz-insensitive cation channel (Lyall et al., 2005c).

The results presented in Fig. 1 B further indicate that ethanol increases the unilateral Bz-insensitive Na^+ flux across the apical membrane of fungiform TRCs. As reported earlier, ethanol (10–40%) increased Na^+ flux across the apical membrane of TRCs in a dose-dependent manner, and the increase in Na^+ flux was blocked by the VR-1 antagonists CZP or SB-366791 (Lyall et al., 2005c). VR-1 agonists (RTX, CAP, elevated temperature, ATP) increase the apical membrane conductance

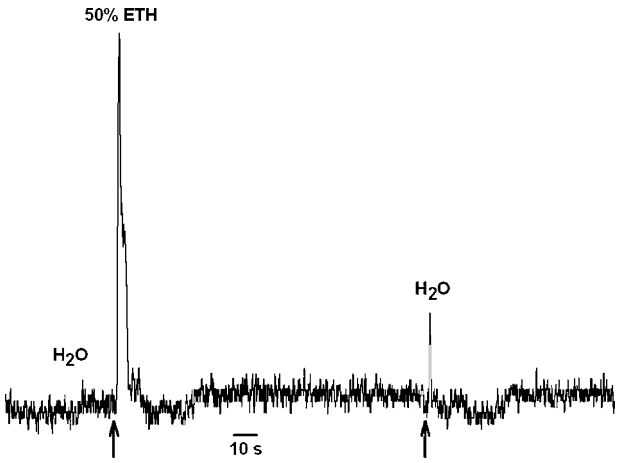

FIGURE 2. Effect of ethanol on the CT responses. Rat tongue was stimulated with 50% ethanol (50% ETH), and CT responses were recorded with reference to H_2O rinse at 23°C. The time period at which the tongue was superfused with different solutions is indicated by arrows.

and enhance the flux of Na^+, NH_4^+, and Ca^{2+} across the apical membrane of fungiform TRCs in a dose-dependent manner (DeSimone et al., 2001; Lyall et al., 2004b, 2005a). Thus, the effect of ethanol is similar to the other VR-1 agonists (Trevisani et al., 2002; Kato et al., 2003; Lyall et al., 2005c).

In Vivo Studies

VR-1 agonists and antagonists that modulate the Bz-insensitive apical membrane cation conductance and the apical cation flux in fungiform TRCs also modulate the Bz-insensitive CT responses to mineral salts (Lyall et al., 2004b, 2005a). We hypothesize that ethanol also modulates the Bz-insensitive CT responses to mineral salts. We further hypothesize that in the absence of ions, ethanol will produce CT responses that are different from salt responses but will be dependent upon ethanol-induced osmotic cell shrinkage (Lyall et al., 2005c).

Effect of Ethanol on CT Responses in the Absence of Mineral Salts. A rat tongue was initially rinsed with deionized H_2O and then stimulated with 50% ethanol dissolved in deionized H_2O (Fig. 2). Both the rinse and the stimulating solutions were maintained at room temperature (~23°C). Ethanol stimulation elicited a CT response composed of only a transient phasic component. As shown earlier (Lyall et al., 2005c), the transient phasic response was not affected by varying the ethanol concentration between 40 and 100%, addition of 0.1 μM SB-366791 to ethanol solutions, or by increasing the temperature of the ethanol solution to 42°C. However, the transient phasic responses to ethanol were inhibited by preshrinking TRCs in vivo by topical lingual application of hypertonic mannitol (Lyall et al., 2005c). This strongly suggests that in the absence of ions, ethanol produces transient phasic CT responses that are linked to osmotic shrinkage of TRCs.

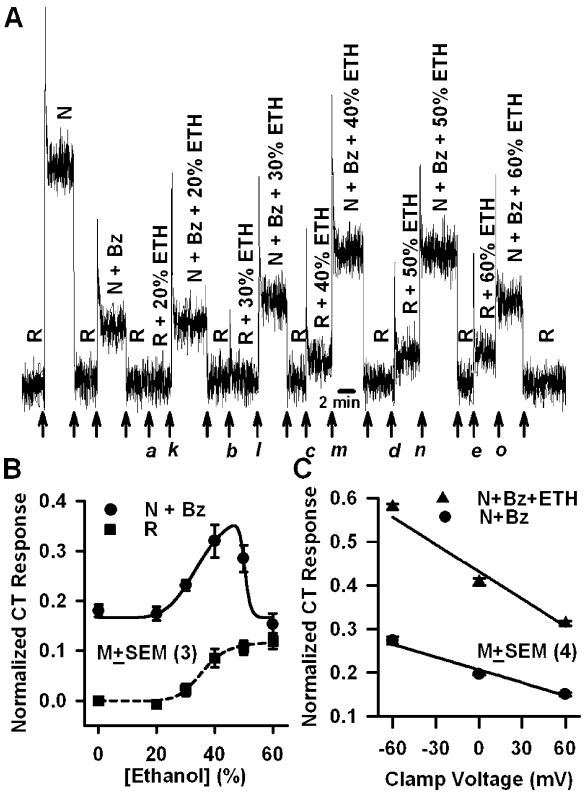

FIGURE 3. Effect of ethanol on the CT response in the presence of mineral salts. (A) Rat tongue was stimulated with ethanol (ETH; 20–60%) solutions containing either 10 mM KCl (R), 100 mM NaCl + 10 mM KCl (N), or 100 mM NaCl + 10 mM KCl + 5 μM Bz (N + Bz) maintained at room temperature (23°C). The time period at which the rat tongue was superfused with different solutions is indicated by arrows. The magnitude of the net NaCl CT response was obtained by the difference between the stimulating solution (N + Bz + ETH) and the corresponding rinse solution (R + ETH) at a specific ETH concentration. (B) The mean ± SEM values of the CT responses from three animals (*N*) are plotted as a function of ethanol concentration. (C) CT responses were recorded during superfusion of the tongue with R and then with N + Bz or with N + Bz + 40% ETH at zero current clamp and at −60 and +60 mV lingual voltage clamp. In each case the NaCl CT responses were normalized to the corresponding CT responses obtained with 300 mM NH_4Cl. Each point represents the mean ± SEM of four animals.

Effect of Ethanol on CT Responses in the Presence of 10 mM KCl. In the next series of experiments, we investigated if CT responses to ethanol are altered in the presence of mineral salts. A rat tongue was first rinsed with 10 mM KCl (R) and then stimulated with 10 mM KCl + ethanol (ETH). Ethanol concentration in the stimulating solutions was varied between 20 and 60%. Data summarized in Fig. 3 A show that R + 20% ETH did not increase the CT response above baseline relative to R alone (no transient phasic response; *a*). Stimulating with R + 30% ETH produced only a transient phasic

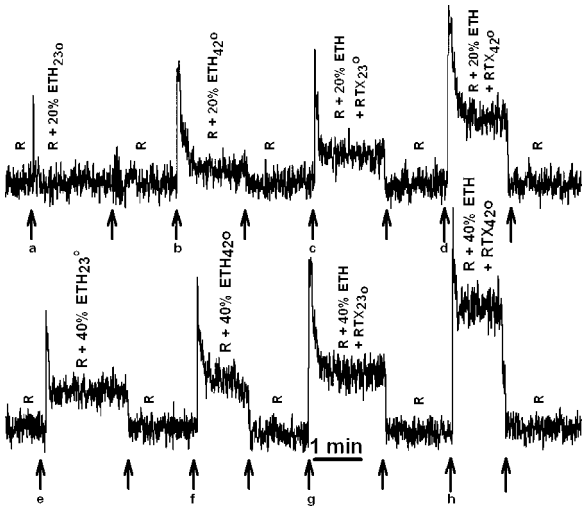

FIGURE 4. Effect of ETH, elevated temperature, and RTX on the CT responses to 10 mM KCl. Rat tongue was stimulated with 10 mM KCl (R), 10 mM KCl + ETH (R + ETH), or 10 mM KCl + ETH + 0.5 μM RTX (R + ETH + RTX). The ETH concentration was either 20 or 40%. CT responses were monitored at 23°C or 42°C. The time period at which the rat tongue was superfused with different solutions is indicated by arrows.

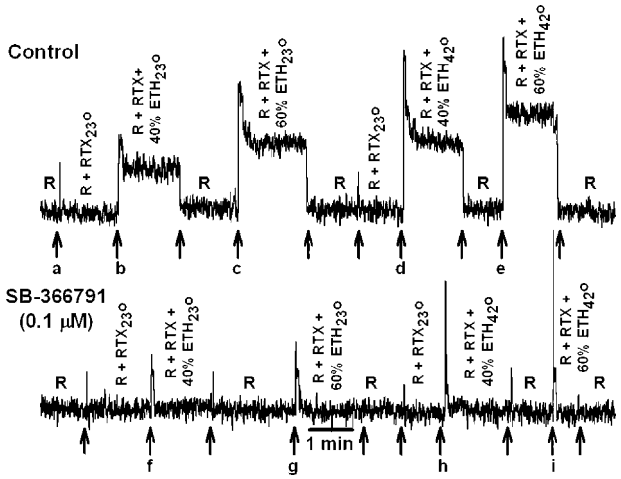

FIGURE 5. Effect of SB-366719 on the CT responses to 10 mM KCl. Rat tongue was stimulated with 10 mM KCl (R), 10 mM KCl + 0.5 μM RTX (R + RTX), or 10 mM KCl + 0.5 μM RTX + ETH (R + ETH + RTX). The ETH concentration was either 40 or 60%. The time period at which the rat tongue was superfused with different solutions is indicated by arrows. CT responses were monitored at 23°C or 42°C in the absence (Control) and presence of 0.1 μM SB-366791.

response (*b*). However, stimulating with R + 40% ETH produced a CT response composed of a transient phasic response that was followed by a sustained tonic response (*c*). Similarly, both phasic and tonic components of the CT response were observed following stimulation of the tongue with R + 50% ETH (*d*) and with R + 60% ETH (*e*). Essentially identical results were obtained when the tongue was superfused with stimulating solutions containing 10 mM NaCl + increasing concentrations of ethanol (unpublished data). Data from three animals are summarized in Fig. 3 B (■). The results indicate that the presence of even a small amount of a mineral salt in a mixture with ethanol alters the CT response profile relative to ethanol alone. In the presence of 10 mM KCl, ethanol at concentrations >30% induced an increase in the tonic component of the CT response. The maximum increase in the tonic response was achieved between 50 and 60% ethanol concentration (Fig. 3 B, ■).

Effect of RTX, Ethanol, and SB-366791 on CT Responses in the Presence of 10 mM KCl. We have previously shown that the Bz-insensitive NaCl CT responses are insensitive to external pH (pH_o) and ATP. However, in the presence of a low suprathreshold concentration of RTX, a VR-1 and VR-1 variant cation channel agonist, the Bz-insensitive NaCl CT responses become sensitive to both pH_o and ATP (Lyall et al., 2004b, 2005a). Therefore, in the next series of experiments, we tested if a subthreshold concentration of RTX (0.5 μM) modulates the effects of ethanol and elevated temperature on the Bz-insensitive NaCl CT response.

As shown in Fig. 4, superfusing the tongue with 10 mM KCl + 20% ETH at 23°C (R + 20% $ETH_{23°}$) produced only a transient phasic response (*a*). Stimulating with 10 mM KCl + 20% ETH at 42°C (R + 20% $ETH_{42°}$) gave a CT response composed of a transient phasic response followed by a sustained tonic component (*b*). Stimulating with 10 mM KCl + 20% ETH + 0.5 μM RTX at 23°C (R + 20% ETH + $RTX_{23°}$) also produced a CT response consisting of both a phasic component and a sustained tonic component (*c*). Increasing the temperature of the stimulating solution to 42°C (R + 20% ETH + $RTX_{42°}$) enhanced the magnitude of the tonic component by 50% (*d*) relative to 23°C (*c*).

Fig. 4 also shows that stimulating the tongue with 10 mM KCl + 40% ETH at 23°C (R + 40% $ETH_{23°}$) gave a CT response comprising of both a phasic and a tonic component (*e*). The magnitude of the response was enhanced when the stimulating solution was presented at 42°C (*f*) relative to 23°C (*e*). Stimulating with R + 40% ETH + 0.5 μM RTX further enhanced the response at 23°C (*g*) and at 42°C (*h*) relative to its corresponding magnitude at *e* and *f*, respectively.

In the above experiment, the apical membrane was first treated with ethanol and then with RTX. In the next series of experiments, the apical membrane was first treated with RTX and then with ethanol. Data summarized in Fig. 5 (Control; upper trace) show that stimulating the tongue with 10 mM KCl + 0.5 μM RTX at 23°C (R + $RTX_{23°}$) gave only a transient phasic response (*a*). This suggests that at low concentration of

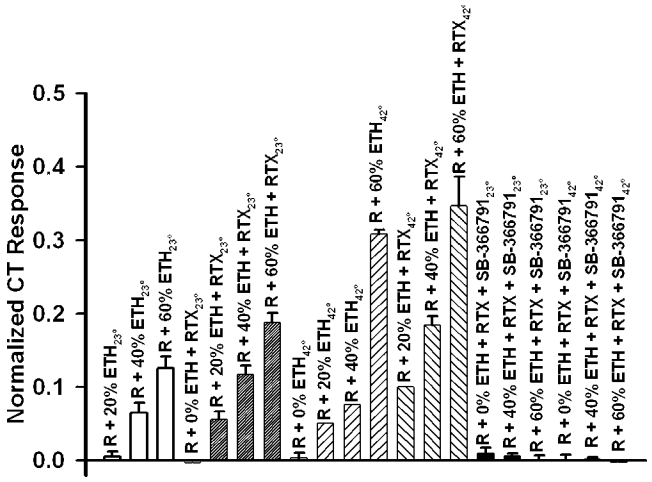

FIGURE 6. Effect of ETH, temperature, RTX, and SB-366719 on the CT responses to 10 mM KCl. Summary of data from experiments shown in Figs. 4 and 5. Each bar represents the mean ± SEM of the normalized CT response from three animals (*N*).

KCl, the RTX-induced increase in apical K^+ flux in TRCs is not sufficient to enhance the magnitude of the CT response. However, in the presence of RTX, stimulating the tongue at 23°C with either 40% (*b*) or 60% (*c*) ethanol, increased the tonic component of the CT response in a dose-dependent manner. Increasing the temperature of the stimulating solutions to 42°C increased the magnitude of the CT response to 40% ETH (*d* versus *b*) and 60% ETH (*e* versus *c*) relative to 23°C.

Superfusing the tongue with stimulating solutions containing RTX + ETH + SB-366791 (0.1 μM) completely inhibited the tonic component of the CT response, and thus only transient phasic responses were observed at 23°C and 42°C in the presence of ETH and RTX (Fig. 5, SB-366791, lower trace, *f*, *g*, *h*, and *i*). The data from several such experiments described in Figs. 4 and 5 are summarized in Fig. 6. Taken together, the results indicate that both RTX and elevated temperature modulate the effect of ethanol on the KCl CT response and that these effects are inhibited by SB-366791.

Effect of Ethanol on the Temperature Threshold of the CT Responses in the Presence of 10 mM KCl. Next, we tested if RTX modulates the temperature threshold of the VR-1 variant cation channel. The CT responses to 10 mM KCl + ethanol were monitored while the temperature of the solution was varied between 23°C and 55.5°C. As shown in Fig. 7 A, stimulating the tongue with 10 mM KCl + 60% ethanol (R + 60% ETH) produced a sharp increase in the CT response around 38°C and gave a maximum enhancement of the CT response at 42°C, and the response decreased above 42°C (Fig. 7 A, ■). Stimulating with 10 mM KCl + 60% ETH + 0.5 μM RTX (R + 60% ETH + RTX) enhanced the CT response at 23°C and at elevated temperatures (Fig. 7 A, ●). In three animals, the mean temperature at which

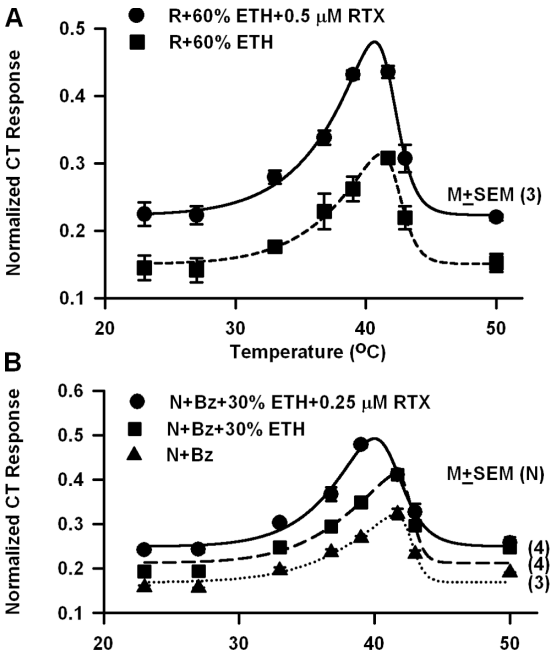

FIGURE 7. Effect of temperature on the CT response to mineral salts. (A) CT responses were monitored while the rat tongues were first rinsed with 10 mM KCl (R) at 23°C and then stimulated with 10 mM KCl + 60% ETH (R + 60% ETH) or 10 mM KCl + 60% ETH + 0.5 μM RTX (R + 60% ETH + 0.5 μM RTX) maintained at temperatures between 23°C and 55.5°C. (B) CT responses were monitored while the rat tongues were first rinsed with 10 mM KCl (R) at 23°C and then stimulated with 10 mM KCl + 100 mM NaCl + 5 μM Bz (N + Bz), 10 mM KCl + 100 mM NaCl + 5 μM Bz + 30% ETH (N + Bz + 30% ETH), or 10 mM KCl + 100 mM NaCl + 5 μM Bz + 30% ETH + 0.25 μM RTX (N + Bz + 30% ETH + 0.25 μM RTX) maintained at temperatures between 23°C and 55.5°C. The values are expressed as mean ± SEM from three animals (*N*). Fitted curves in each case were drawn using Eq. 1.

the CT response to 10 mM KCl + 60% ETH was enhanced by 50% ($t_{0.5}$) was 38.3 ± 0.3°C. In the presence of 0.5 μM RTX, the $t_{0.5}$ was decreased to 36.7 ± 0.3°C ($P < 0.05$; $N = 3$; paired). Thus, RTX shifts the temperature threshold of the KCl CT response to the left.

Effect of Ethanol on the CT Responses in the Presence of 100 mM NaCl. The above experiments were performed with 10 mM KCl. However, to test whether ethanol modulates salt taste, we investigated the effect of ethanol on the CT responses to 100 mM NaCl. The CT responses were monitored while the rat tongue was stimulated with a rinse solution (R) containing 10 mM KCl + ETH (20–60%) and then with stimulating solutions containing 10 mM KCl + 100 mM NaCl + 5 μM Bz + ETH (20–60%). In each case, the ETH-induced change in the net magnitude of the NaCl response was calculated as the difference between the CT response with 100 mM NaCl + 10 mM KCl + 5 μM Bz + a particular concentration of ETH and the rinse response (10 mM KCl + the corresponding ETH concentration). Data summarized in Fig. 3 A also show that stimulating the

tongue with 100 mM NaCl + 5 μM Bz + ETH produced a dose-dependent increase in the magnitude of the Bz-insensitive NaCl CT response between 20 and 40% ETH concentration (*k*, *l*, and *m*, respectively). The Bz-insensitive NaCl CT response achieved its maximum value between 40 (*m*) and 50% (*n*) ethanol concentration. The magnitude of the Bz-insensitive NaCl CT response decreased at 60% ethanol concentration (*o*) relative to 50% ethanol (*n*). Data from three animals are also summarized in Fig. 3 B. The results indicate that similar to other VR-1 agonists, RTX, CAP, and temperature, the relationship between the Bz-insensitive NaCl CT response and ethanol concentration is bell shaped (Fig. 3 B, ●) (Lyall et al., 2004b, 2005a). Thus, ethanol, depending upon its concentration, acts both as an agonist and an antagonist of the Bz-insensitive NaCl CT response.

To investigate if ethanol produces changes in the Bz-insensitive NaCl CT response by modulating the apical membrane conductance in TRCs, we monitored the sensitivity of the NaCl CT responses to applied lingual potential difference. Fig. 3 C shows the CT response to 100 mM NaCl + 5 μM Bz in the absence (N + Bz) and presence of 40% ethanol (N + Bz + ETH) as a function of the applied lingual potential at −60, 0, and +60 mV. As reported earlier (Lyall et al., 2004b, 2005a), the rat CT responses to 100 mM NaCl + 5 μM Bz were slightly enhanced at −60 mV lingual voltage clamp (referenced to the oral cavity) and slightly suppressed at +60 mV. In the presence of 40% ethanol, the same voltages exerted significantly larger effects on the response (Fig. 3 C). In the presence of 40% ethanol, the slope of the response $(-2.1 \pm 0.2) \times 10^{-3}$ response units/mV was greater relative to its value $(-9.7 \pm 0.5) \times 10^{-4}$ response units/mV in the absence of ethanol ($P < 0.05$; $N = 4$; paired). The observation that the size of the response at zero voltage and the slope of the response (i.e., the response conductance) are proportional (Lyall et al., 2004b, 2005a) suggests that in TRCs that are sensitive to ethanol, the increase in the Bz-insensitive NaCl CT response is due to an increase in the apical membrane conductance to Na^+. This is consistent with the observation that in in vitro experiments, ethanol increases the unilateral apical Na^+ flux in polarized fungiform TRCs (Fig. 1 B) (Lyall et al., 2005c).

Next we tested the effect of RTX and ethanol on the temperature threshold of the VR-1 variant cation channel in the presence of 100 mM NaCl. The CT responses to 10 mM KCl + 100 mM NaCl + 5 μM Bz (N + Bz) were monitored in the presence and absence of ETH (N + Bz + ETH) and 0.25 μM RTX (N + Bz + ETH + RTX), while the temperature of the solution was varied between 23°C and 55.5°C (Fig. 7 B). Ethanol (30%) enhanced the NaCl CT response at room temperature and at elevated temperatures without a change in the

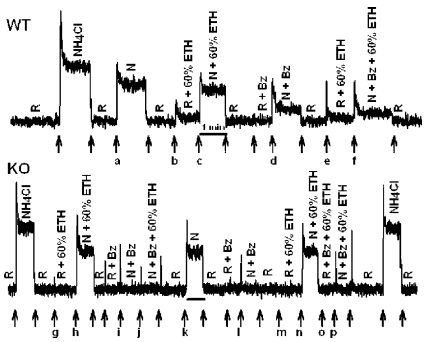

FIGURE 8. Effect of ETH on the CT responses in WT and VR-1 KO mice. CT responses were monitored in WT and KO mice while the tongues were rinsed with 10 mM KCl (R) and then stimulated with 10 mM KCl + 60% ETH (R + 60% ETH), 100 mM NaCl (N), 100 mM NaCl + 5 μM Bz (N + Bz), 100 mM NaCl + 60% ETH (N + 60% ETH), and 100 mM NaCl + 5 μM Bz + 60% ETH (N + Bz + 60% ETH). The time period at which the rat tongue was superfused with different solutions is indicated by arrows.

temperature threshold of the CT response (Fig. 7 B, ■) relative to N + Bz (Fig. 7 B, ▲). The addition of 0.25 μM RTX to 30% ETH solution also enhanced the NaCl CT response and shifted the temperature curve to the left (Fig. 7 B, ●). The mean temperature at which the CT response was enhanced by 50% ($t_{0.5}$) in the presence of 10 mM KCl + 100 mM NaCl + Bz (N + Bz), 10 mM KCl + 100 mM NaCl + Bz + 30% ETH (N + Bz + 30% ETH), and 10 mM KCl + 100 mM NaCl + Bz + 30% ETH + 0.25 μM RTX (N + Bz + 30% ETH + 0.25 μM RTX) was 37.4 ± 0.57°C ($N = 3$), 37.9 ± 0.25°C ($N = 4$; $P > 0.05$), and 36.4 ± 0.19°C ($N = 4$), respectively. The results indicate that ethanol enhances the CT response to NaCl without affecting the temperature threshold of the amiloride-insensitive salt taste receptor. In contrast, a mixture of ethanol + RTX enhanced the magnitude of the NaCl CT response at all temperatures and shifted the temperature curve to the left ($P < 0.01$; $N = 4$; paired). The results further suggest that ethanol and RTX act on different sites on the VR-1 variant cation channel.

Studies with the VR-1 KO Mice

To investigate whether ethanol modulates the NaCl CT responses via the VR-1 variant cation channel, the effect of ethanol was investigated on the CT responses in WT and VR-1 KO mice. Consistent with our earlier observations (Lyall et al., 2004b, 2005a), in WT mice (Fig. 8, WT), stimulating the tongue with 100 mM NaCl (N) produced a CT response (*a*), and a significant part of the CT response was Bz insensitive (*d*). Similar to the case in rats, in WT mice, stimulation with 10 mM KCl + 60% ETH (R + 60% ETH) elicited a CT response relative 10 mM KCl (R; *b* and *e*). In addition, stimulating the tongue with 100 mM NaCl + 5 μM Bz + 60% ETH

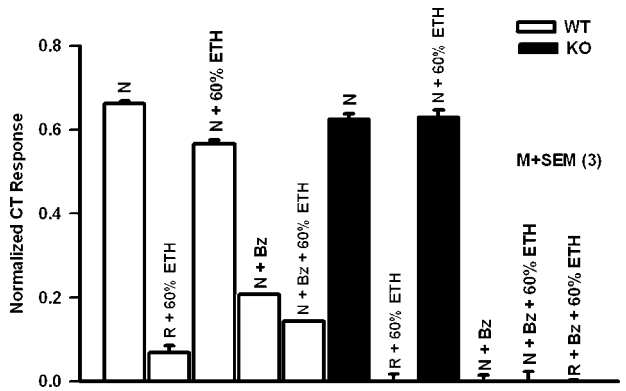

FIGURE 9. CT responses in WT and VR-1 KO mice. Summary of data from three WT and three VR-1 KO mice. Each bar represents the mean ± SEM of the normalized CT response from three animals (*N*). R = 10 mM KCl; N = 100 mM NaCl + 10 mM KCl + 5 μM Bz; ETH = ethanol.

(N + Bz + 60% ETH) inhibited the CT response relative to 100 mM NaCl + 5 μM Bz (N + Bz) (*f* versus *d*).

In contrast, VR-1 KO mice (Fig. 8, KO) demonstrated no Bz-insensitive NaCl CT response component (*i* and *l*), no CT response to R + 60% ETH (*g* and *m*), no effect of 60% ETH on the NaCl CT response (*h* and *n*) relative to NaCl alone (*k*), and no effect of 60% ETH on the Bz-insensitive NaCl CT response (*j* and *o*). These results indicate that the NaCl CT responses in VR-1 KO mice are insensitive to ethanol. The data from three WT and three VR-1 KO mice are summarized in Fig. 9. The results show that in VR-1 KO mice, the magnitude of the CT response to R + 60% ETH, N + Bz, N + Bz + 60% ETH, and R + Bz + 60% ETH was not significantly different from zero ($P > 0.05$; $N = 3$). These results are consistent with the observations that the Bz-insensitive NaCl CT responses in VR-1 KO mice are also insensitive to other VR-1 agonists (e.g., RTX, elevated temperature, and RTX + elevated temperature) (Lyall et al., 2004b, 2005a).

DISCUSSION

Ethanol is a potent gustatory stimulus and modulates CT taste nerve responses (Diamant et al., 1963; Hellekant, 1965a,b; Hellekant et al., 1997; Sako and Yamamoto, 1999; Danilova and Hellekant, 2000; Lyall et al., 2005c). Results presented in this study demonstrate that CT responses due to ethanol in water mixtures are quite distinct from ethanol–mineral salt mixtures. Ethanol–water mixtures gave only a transient phasic response, while ethanol–mineral salt mixtures produced CT responses with both phasic and tonic characteristics. At concentrations <50%, ethanol enhanced, and at concentrations >50%, it inhibited the Bz-insensitive CT response to 100 mM NaCl. At 10 mM KCl and 10 mM NaCl, the CT response increased as a saturating function of ethanol concentration with saturation occurring between 50 and 60% ethanol. Our data show that the phasic-only responses observed in ethanol–water mixtures and the tonic responses observed in ethanol–mineral salt solutions arise from entirely diverse transduction processes. Investigation of these transduction mechanisms at the cellular level shows that ethanol elicited two major effects on fungiform TRCs. In the absence of permeable cations, ethanol induced transient osmotic cell shrinkage. In the presence of permeable cations, ethanol increased the Bz-insensitive cation flux across the apical membrane of polarized fungiform TRCs without a significant change in cell volume. In each case, the above cellular events initiated transduction mechanisms that produced distinct CT responses. In the former case (i.e., in the absence of permeable cations), stimulating the tongue with ethanol produced only a transient phasic CT response, and in the latter case (i.e., in the presence of mineral salts), ethanol produced CT responses consisting of both a transient phasic component and a sustained tonic component. The relationship between ethanol-induced changes in TRCs, CT response profiles and the possible involvement of the VR-1 variant salt taste receptor will be discussed below.

The results shown in Figs. 1 and 2 suggest that in the absence of permeable ions, the ethanol-induced osmotic decrease in TRC volume is related to the transient phasic CT response (Lyall et al., 2005c). Strong evidence for this comes from our studies with mixtures containing ethanol and hypertonic mannitol. Hypertonic mannitol solutions also elicit a transient phasic CT response, providing a link to a decrease in TRC volume (Lyall et al., 1999, 2005c). Second, preshrinking TRCs in vivo with hypertonic mannitol reduced the magnitude of the ethanol-induced transient phasic CT response (Lyall et al., 2005c). Although, at present, the cellular mechanism(s) that link a decrease in cell volume to the transient phasic CT response are not known, it is likely that membrane conductance(s) activated during cell shrinkage (Schwiebert et al., 1994; Koch and Korbmacher, 2000; Lyall et al., 2005c) are involved in generating the phasic CT response.

In the presence of mineral salts, ethanol enhanced the apical entry of cations (Fig. 1 B) and produced CT responses that are similar to salt responses; i.e., in the presence of mineral salts, ethanol produced both a transient phasic component and a sustained tonic component of the CT response (Figs. 3–5). As reported earlier (Lyall et al., 2005c), the rate of stimulation determines if both phasic and tonic components are observed in the neural recordings. At a low rate of lingual stimulation, only a slowly rising tonic phase of the CT response is observed. However, it is important to note that at a lower rate of flow, the slowly rising tonic phase

attained the same magnitude as with a relatively high flow rate (Lyall et al., 2001, 2005c).

In polarized fungiform TRCs loaded with Na-green, ethanol induced a monotonic and sustained increase in F_{490} in the presence of 150 mM NaCl + 5 μM Bz (Fig. 1 B). This indicates that ethanol increases the apical Bz-insensitive Na^+ flux in fungiform TRCs. A maintained increase in F_{490} is consistent with the notion that in the presence of apical Na^+ (a membrane permeable cation), an increase in apical Na^+ flux is not accompanied by transient changes in cell volume (Lyall et al., 2005c). This suggests that in addition to providing the basis for the CT response, the flux of cations through the Bz-insensitive nonspecific cation pathway also serves to mitigate the osmotically induced decrease in cell volume. It is likely that VR-1 and VR-1 variant nonspecific cation channels may have a similar role in other tissues.

The results shown in Fig. 3 C indicate that ethanol increases the Bz-insensitive Na^+ conductance in the apical membrane of TRCs in vivo. This was demonstrated directly by our measurement of CT responses under lingual voltage clamp. Ethanol not only increased the magnitude of the Bz-insensitive NaCl CT response at −60, 0, and +60 mV, but it also increased the slope of the relationship between clamp voltages and the CT response (Fig. 3 C). An increase in the slope is indicative of an increase in Na^+ conductance of the apical membrane of TRCs in vivo (Lyall et al., 2004b, 2005a). This indicates that in TRCs, the VR-1 cation channel is constitutively active at the resting membrane potential. Its activity is further enhanced at negative applied potentials and is diminished at positive applied potentials. At present, the exact mechanism by which ethanol activates the VR-1 variant cation channel is not known. However, it is likely that like other agonists of TRPV1, ethanol functions by modifying the voltage-dependent properties of the channel. In HEK-293 cells expressing TRPV1, in the absence of CAP, the channel demonstrated activation at positive voltages. CAP induced a leftward shift in the activation curve. Thus, in the presence of CAP, the channel became active at physiological membrane potentials (Voets et al., 2004). Alternately, ethanol may stabilize the receptor channel in the open state (Voets et al., 2004; Zuo et al., 2004). This can also explain how the Bz-insensitive Na^+ apical flux from a constitutively active VR-1 varinat cation channel can be further potentiated by ethanol.

Several studies indicate that ethanol modulates the CT responses to sweet, sour, and bitter stimuli (Hellekant et al., 1997; Sako and Yamamoto, 1999). In contrast, in both a primate model (Hellekant et al., 1997) and rat model (Sako and Yamamoto, 1999) ethanol was reported to have no effect on the CT responses to NaCl. In NaCl-best fibers (N-fibers), the NaCl/ethanol mixture responses were about the same as for the NaCl alone. Consistent with the above result, in our study, ethanol also had no effect on the Bz-sensitive NaCl responses (Lyall et al., 2005c). Taken together, the above results suggest that ethanol does not affect the amiloride- or Bz-sensitive ENaCs in the apical membrane of fungiform TRCs.

Our results suggest that instead of producing a general effect on the apical membrane channels involved in Na^+ transport, ethanol specifically affects only the Bz-insensitive VR-1 variant nonspecific cation channels. No effect of ethanol was observed on the Bz-sensitive (ENaC) component of the NaCl CT response (Hellekant et al., 1997; Sako and Yamamoto, 1999; Lyall et al., 2005c). In our studies, TRCs perfused with Na^+-free Ringer's solution demonstrated RVI after ethanol-induced transient osmotic cell shrinkage (Fig. 1 A) and regulatory volume decrease following ethanol washout (Lyall et al., 2005c). These results further suggest that ethanol does not affect the Na^+-independent volume regulatory mechanisms in TRC membranes. In both rats (Fig. 3) and WT mice (Figs. 8 and 9), stimulating the tongue repeatedly with NaCl solutions containing increasing ethanol concentrations gave reproducible CT responses that were completely reversible. This suggests that TRCs are able to maintain a favorable electrochemical gradient for Na^+ across the apical membrane. Since the Na^+ gradient is maintained by the Na^+-K^+ ATPase, the results indirectly suggest that under our experimental conditions, stimulating the tongue repeatedly with increasing ethanol concentrations does not inhibit the Na^+ pump.

As discussed earlier (Lyall et al., 2005c), ethanol is membrane permeable and may get to the basolateral membrane from the apical side when applied topically to the lingual surface by crossing tight junctions. Alternately ethanol could also permeate across the apical membrane via the VR-1 variant cation channel. It was recently demonstrated that HEK-293T cells expressing TRPV1 rapidly take up styryl dyes, such as FMI-43, when the channel is opened and not when the channel is pharmacologically blocked (Meyers et al., 2003). This suggests that organic cationic dyes are able to pass through TRPV1. However, at this point, it is not known if ethanol also permeates the VR-1 variant cation channels in TRCs.

However, it is also possible that ethanol produces its effect not by acting on the apical membrane, but rather on the basolateral membrane of TRCs. In our studies, simultaneous topical lingual application of 0.1 μM SB-366791 (a specific blocker of the VR-1 variant cation channel) with VR-1 agonists completely inhibited the increase in the tonic CT response to mineral salts induced by ethanol, RTX, and elevated temperature (Fig. 5). The low concentration of SB-366791 and the obser-

vation that the responses of ethanol, RTX, and elevated temperature are inhibited without a delay suggest that SB-366791 acts on the apical membrane to inhibit ethanol responses. Under these conditions, it is most unlikely that SB-366791 crosses tight junctions and reaches the basolateral membrane at a concentration sufficient to inhibit ethanol responses (Lyall et al., 2005c). Furthermore, ethanol produced completely different effects on TRCs in vitro (Fig. 1 A) and on CT responses (Fig. 2) in the absence of apical cations in vivo. These results strongly suggest that the effects of ethanol reported here occur at the apical membrane and that the tonic taste nerve responses observed with ethanol–salt mixtures involve specifically the Bz-insensitive salt taste receptor.

In our studies, ethanol increased the unilateral Bz-insensitive Na^+ flux across the apical membrane of polarized rat fungiform TRCs (Fig. 1 B) and specifically enhanced the Bz-insensitive component of the NaCl CT response (Lyall et al., 2005c). An important question is whether ethanol acts directly on the VR-1 cation channel or indirectly by inducing the release of peptides (Simon et al., 2003) or other activators of VR-1 from nerve fibers. As discussed earlier (Lyall et al., 2005c), our studies with isolated taste bud fragments strongly suggest that ethanol directly modulates the VR-1 variant cation channel in TRCs.

Ethanol Activates the Apical VR-1 Variant Cation Channel in TRCs

In vitro studies on polarized fungiform TRCs and in vivo CT responses made under lingual voltage clamp indicate that the ethanol-induced increase in the cation flux across the apical membrane of fungiform TRCs is the basis of altered neural responses to salt stimulation in mixtures containing mineral salts and ethanol. Bz-insensitive NaCl CT responses to mineral salts demonstrate many functional similarities with cloned VR-1. Both receptors are nonspecific cation channels that are activated by vanilloids (RTX and CAP) and elevated temperature (>38°C), and are blocked by the VR-1 antagonists (CZP and SB-366791). This indicates that the Bz-insensitive component of the CT response may be a VR-1 variant cation channel that functions as the amiloride-insensitive salt taste receptor in the apical membranes of fungiform TRCs (Lyall et al., 2004b, 2005a).

We have previously shown that VR-1 agonists RTX, CAP, and elevated temperature modulate the Bz-insensitive NaCl CT response. Stimulating the tongue with increasing concentrations of RTX and CAP and increasing the temperature of the stimulating solution in a stepwise manner elicited biphasic dose–response relationships (Lyall et al., 2004b, 2005a). In the presence of 100 mM NaCl + Bz, ethanol also produced a biphasic concentration–response relationship (Fig. 3 B; ●). In our earlier studies (Lyall et al., 2004b), both RTX and CAP desensitize the Bz-insensitive NaCl CT response at concentrations >1 μM and 40 μM, respectively. Bz-insensitive NaCl CT responses were completely inhibited at RTX and CAP concentrations of 10 μM and 200 μM, respectively. Our preliminary studies indicate that desensitization of the channel is most likely related to changes in TRC $[Ca^{2+}]_i$ (Heck et al., 2005). An increase in TRC $[Ca^{2+}]_i$ reduced the response magnitude in the agonist dose–response curve at all agonist concentrations and a decrease in $[Ca^{2+}]_i$ increased the response magnitude in the agonist dose–response curve at all agonist concentrations and significantly inhibited the decline from maximal response at the higher agonist concentrations seen under control conditions. In these studies, no Ca^{2+} was present in the stimulating solutions. Consistent with this hypothesis, ethanol has been demonstrated to increase $[Ca^{2+}]_i$ in gastric mucosal cells (Mustonen et al., 2005). An increase in TRC $[Ca^{2+}]_i$ may result in the activation of the Ca^{2+}-activated K^+ channels in the basolateral membrane (Mustonen and Kivilaakso, 2003; Mustonen et al., 2004). In our preliminary studies, an increase in TRC $[Ca^{2+}]_i$ and the blockage of Ca^{2+}-activated K^+ channels by verrucologen altered the RTX-induced activation–inactivation kinetics of the VR-1 variant nonspecific cation channel (Heck et al., 2005).

Similar to VR-1, the amiloride-insensitive salt taste receptor can integrate the effect of multiple stimuli (Lyall et al., 2004b, 2005a). The data shown in Figs. 3–6 demonstrate that the CT responses to 10 mM KCl are enhanced in the presence of RTX and at elevated temperatures. This indicates that both RTX and elevated temperature sensitize the KCl CT response to ethanol stimulation (Fig. 6). Similar effects of temperature and RTX were observed on the Bz-insensitive CT responses to 100 mM NaCl in the presence of ethanol (Fig. 7 B). It is suggested that the increase in temperature activates the TRPV1 channel by shifting the voltage dependence of activation to more electronegative voltages, resulting in the activation of the channel at resting physiological voltages (Voets et al., 2004). As mentioned above, ethanol may also stabilize the receptor channel in the open state (Zuo et al., 2004) and further enhance the channel activity at elevated temperatures. Consistent with previous studies, the increase in the tonic CT response to mineral salts induced by RTX and elevated temperature (Lyall et al., 2004b, 2005a) and ethanol (Fig. 5) were completely blocked by SB-366791. Similarly, ruthenium red, the pore-blocking antagonist, completely inhibited heat responses in oocytes expressing hTRPV1 and hTRPV1b (Lu et al., 2005).

The data further indicate that in the presence of RTX, ethanol increased 10 mM KCl CT responses at

TABLE I

Differences and Similarities between TRPV1 and the VR-1 Variant Nonspecific Cation Channel in Fungiform TRCs

	TRPV1	VR-1 variant cation channel
Activity at 23°C in the absence of agonists	Not active	Constitutively active
Nonselective cation channel	Yes	Yes
Activation by RTX, CAP, ETH, and temperature	Yes	Yes
Desensitization at high concentrations of the agonists	Yes	Yes
Temperature threshold of activation	44°C	38°C
Leftward shift in the temperature threshold by RTX and CAP	Yes	Yes
Leftward shift in the temperature threshold by ETH	Yes	No
Activation by acidic pH	Yes	No
Inhibition by SB-366791, CZP, and ruthenium red	Yes	Yes
Sensitized by ATP	Yes	Yes
Activation by cetyl-pyridinium chloride	No	Yes

room temperature and at elevated temperatures without altering the mean temperature at which the CT response was enhanced by 50% ($t_{0.5}$) (Fig. 7 A). Similarly, 30% ethanol also increased the CT response to 100 mM NaCl at room temperature and at elevated temperatures without altering the $t_{0.5}$ of the Bz-insensitive NaCl CT response. In this regard, the effect of ethanol on the fungiform TRCs differs from its effect on the primary sensory neurons from trigeminal or dorsal root ganglia, as well as, VR-1–expressing HEK-293 cells (Geppetti and Trevisani, 2004). Ethanol potentiated the response of VR-1 to CAP, H^+, and heat and lowered the temperature for heat activation of VR-1 (Trevisani et al., 2002). In TRCs, the effect of ethanol is similar to the effect of nicotine on the VR-1 receptor. Nicotine increased the CAP-activated current in both trigeminal neurons and in cells heterologously expressing VR-1 receptors without altering the temperature threshold of the heat-activated currents (Liu et al., 2004). Since the $t_{0.5}$ of the Bz-insensitive NaCl CT response is not affected by ethanol but is decreased by RTX (Fig. 7 B), the data suggest that RTX and ethanol produced their effect by acting at different sites on the VR-1 variant cation channel.

The specificity of ethanol as a salt taste modulator is further demonstrated by the observation that VR-1 KO mice, that lack the Bz-insensitive component of the NaCl CT response, also do not respond to ethanol, RTX, and elevated temperatures. The data indicate that ethanol produces its effect on salt responses via the amiloride-insensitive VR-1 variant cation channel in fungiform TRCs. However, the exact mechanism by which ethanol modulates the VR-1 variant cation channel in TRCs remains to be established.

The additional evidence, that ethanol acts specifically via the VR-1 receptor, is provided by studies in rat gastric epithelial cells (Kato et al., 2003). Gastric mucosal epithelial cells express a VR-1 that is 99.8% identical with cloned VR-1. Exposing cells to 10% ethanol decreased cell viability. The cell damage induced by ethanol was dose dependently prevented by pretreatment with CAP or RTX. In addition, ethanol-induced cell damage was totally abolished when cells were simultaneously exposed to ethanol solutions containing CZP or ruthenium red. These finding suggest that VR-1 is expressed peripherally in gastric mucosal epithelial cells and plays a cellular protective role during ethanol toxicity (Kato et al., 2003). In a similar study, CAP also induced protection against ethanol-induced oxidative injury in the gastric mucosa of rats (Park et al., 2000). As stated above, the presence of VR-1 or the VR-1 variant nonspecific cation channels in other nonneuronal cells may also be involved in RVI and offer protection to cells from osmotic damage.

From this and previous studies (Lyall et al., 2004b, 2005a,c), it emerges that the VR-1 variant cation channel in TRCs while having many similarities with the classical TRPV1 channel also differs significant from it. The similarities and dissimilarities between the two channels are summarized in Table I. Both channels are nonselective cation channels (Grant et al., 2002; Lyall et al., 2004b, 2005a). An important difference between the TRPV1 expressed in HEK-293 cells and the VR-1 variant cation channel present in TRCs is that unlike TRPV1 (Caterina et al., 2000; Davis et al., 2002; Gunthorpe et al., 2002; Voets et al., 2004), the VR-1 variant cation channel is constitutively active in the absence of resiniferatoxin or CAP (Lyall et al., 2004b, 2005a,c). While both channels are activated at elevated temperatures, the VR-1 variant cation channel has a lower activation temperature (around 38°C) (Lyall et al., 2004b, 2005a) relative to 42°C for the TRPV1 (Lu et al., 2005). In contrast to TRPV1 (Trevisani et al., 2002), the temperature threshold of the VR-1 variant cation channel was not affected by ethanol (Fig. 7 B). The VR-1 variant cation channel in TRCs is only activated by low pH in the presence of an agonist (Lyall et al., 2004b, 2005a). In contrast, the TRPV1 channel is activated by

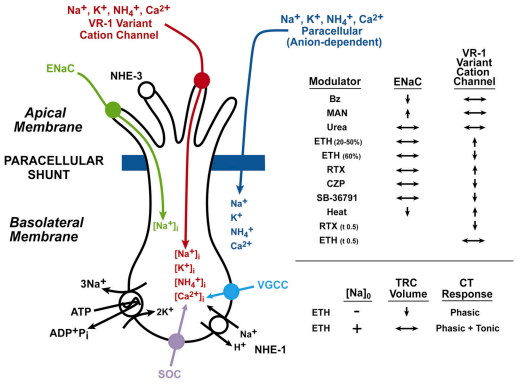

FIGURE 10. Proposed model for Na^+ transport in fungiform TRCs and salt taste transduction in the anterior tongue. The abbreviations used in the figure are as follows: ENaC, amiloride-sensitive epithelial Na^+ channel (green); VR-1, vanilloid receptor-1 (red); NHE-1, basolateral Na^+-H^+ exchanger-1; NHE-3, apical Na^+-H^+ exchanger-3; MAN, mannitol; Bz, benzamil; ETH, ethanol; CZP, capsazepine; RTX, resinifieratoxin; SB-366791, *N*-(3-methoxyphenyl)-4-chlorocinnamide; $[Na]_o$, external Na^+; CT, chorda tympani; $t_{0.5}$, mean temperature at which the CT response was enhanced by 50%; paracellular shunt (dark blue); VGCC, voltage-gated Ca^{2+} channels (light blue); SOC, store-operated Ca^{2+} channel (purple); no change (↔); increase (↑); decrease (↓). See text for details.

low pH in the absence of an agonist (Davis et al., 2002; Gunthorpe et al., 2002). In addition, cetylpyridinium chloride is an agonist of the VR-1 variant cation channel in TRCs but is not an activator of the cloned TRPV1 (Lyall et al., 2004b, 2005a). However, in spite of these important differences, both channels are modulated by ATP and by changes in intracellular calcium (Tominaga et al., 2001; Lyall et al., 2004b, 2005a; Heck et al., 2005).

In humans the major mechanism mediating salt taste is amiloride insensitive (Halpern, 1998; Feldman et al., 2003). It has been suggested that in humans there exists a relationship between alcohol and salt consumption (Van de Walle et al., 1991). In some subjects as the alcohol consumption increased, the proportion of subjects who salted their food without tasting was greater, and conversely, the proportion of subjects who virtually never added salt to their food decreased. This study suggests that there exists a correlation between alcohol consumption level and pattern of salt consumption. However, it is not clear if there is a relationship between the amount of salt consumed and the level of alcohol consumption. To understand the chronic effects of alcohol on human salt taste, further studies are needed to evaluate the chronic effect of ethanol on the VR-1 variant cation channel in animal models.

The main points of this study can be integrated into a model of Na^+ transport in fungiform TRCs and salt taste transduction mechanism in the anterior tongue (Fig. 10). In fungiform TRCs, Na^+ sodium transport occurs through both cellular and transcellular pathways. Na^+ ions enter TRCs across the apical membrane by at least two pathways. One is Na^+ specific and involves Na^+ entry via the apical amiloride- and Bz-sensitive ENaCs (light green). The second is nonspecific and involves Na^+ transport through VR-1 variant nonspecific cation channels (red). The latter channels are insensitive to both amiloride and Bz and are permeable to Na^+, K^+, NH_4^+, and Ca^{2+} ions (Lyall et al., 2004b, 2005a,c). The entry of Na^+ depolarizes the receptor potential leading to the activation of membrane voltage-gated Ca^{2+} channels (VGCCs), an increase in $[Ca^{2+}]_i$, and subsequent release of neurotransmitter. It is also likely that similar to bitter compounds, ethanol also induces capacitative Ca^{2+} entry into TRCs via the store-operated Ca^{2+} channels (SOCs) in TRC membranes (Perez et al., 2003). The Na^+ flux through ENaCs is responsible for the amiloride- and Bz-sensitive component of the NaCl CT response, and the Na^+ flux through the VR-1 variant nonspecific cation channels is responsible for the amiloride- and Bz-insensitive component of the NaCl CT response. The exit of Na^+ from TRCs occurs via the basolateral Na^+-K^+ ATPase. An additional Na^+ transport mechanism involves the basolateral Na^+-H^+ exchanger isoform 1 (NHE-1) (Vinnikova et al., 2004). The apical NHE-3 seems to be quiescent (Vinnikova et al., 2004). The transcellular transport of Na^+, K^+, NH_4^+, and Ca^{2+} ions also occurs via the paracellular shunt mechanism and is anion dependent (Ye et al., 1991). The VR-1 variant channel is nonfunctional in VR-1 KO mice. It is modulated by RTX, CAP, cetylpyridnium chloride, and temperature, and is inhibited by VR-1 antagonists CZP and SB-366791. In rat fungiform TRCs, the VR-1 variant cation channel accounts for all of the Bz-insensitive CT responses to Na^+ salts and part of the CT response to K^+, NH_4^+, and Ca^{2+} salts (Lyall et al., 2004b, 2005a).

In the absence of permeable ions, ethanol induces a transient decrease in TRC volume and elicits only transient phasic CT responses. In the presence of mineral salts, ethanol increases the Bz-insensitive apical cation flux in TRCs without changes in cell volume and elicits CT responses that are similar to salt responses, consisting of both a phasic component and a sustained tonic component. Below 50% concentration ethanol enhanced and above 50% concentration it inhibited CT responses to 100 mM NaCl. At 10 mM KCl and NaCl, ethanol increased responses according to a saturating monotonic function of ethanol concentration. Stimulating the tongue with mixtures of RTX and ethanol or stimulating with ethanol solutions at elevated temperature increased the sensitivity of the CT response to ethanol. Because the effects of ethanol on mineral salts are blocked by CZP and SB-366791 and because VR-1 KO mice are insensitive to ethanol, RTX, and temperature,

we conclude that ethanol produces these taste effects by direct action on the Bz-insensitive VR-1 variant salt taste receptor.

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