Ethanol Modulates the VR-1 Variant Amiloride-insensitive Salt Taste Receptor. I. Effect on TRC Volume and Na^+ Flux

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ABSTRACT The effect of ethanol on the amiloride- and benzamil (Bz)-insensitive salt taste receptor was investigated by the measurement of intracellular Na^+ activity ($[Na^+]_i$) in polarized rat fungiform taste receptor cells (TRCs) using fluorescence imaging and by chorda tympani (CT) taste nerve recordings. CT responses were monitored during lingual stimulation with ethanol solutions containing NaCl or KCl. CT responses were recorded in the presence of Bz (a specific blocker of the epithelial Na^+ channel [ENaC]) or the vanilloid receptor-1 (VR-1) antagonists capsazepine or SB-366791, which also block the Bz-insensitive salt taste receptor, a VR-1 variant. CT responses were recorded at 23°C or 42°C (a temperature at which the VR-1 variant salt taste receptor activity is maximally enhanced). In the absence of permeable cations, ethanol induced a transient decrease in TRC volume, and stimulating the tongue with ethanol solutions without added salt elicited only transient phasic CT responses that were insensitive to elevated temperature or SB-366791. Preshrinking TRCs in vivo with hypertonic mannitol (0.5 M) attenuated the magnitude of the phasic CT response, indicating that in the absence of mineral salts, transient phasic CT responses are related to the ethanol-induced osmotic shrinkage of TRCs. In the presence of mineral salts, ethanol increased the Bz-insensitive apical cation flux in TRCs without a change in cell volume, increased transepithelial electrical resistance across the tongue, and elicited CT responses that were similar to salt responses, consisting of both a transient phasic component and a sustained tonic component. Ethanol increased the Bz-insensitive NaCl CT response. This effect was further enhanced by elevating the temperature from 23°C to 42°C, and was blocked by SB-366791. We conclude that in the presence of mineral salts, ethanol modulates the Bz-insensitive VR-1 variant salt taste receptor.

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

INTRODUCTION

Ethanol is a potent gustatory stimulus. In rat, dog, cat, monkeys, and humans, recordings from whole chorda tympani (CT) nerve, glossopharyngeal nerve, and individual taste nerve fibers demonstrate that ethanol elicits neural responses when applied to the tongue (Diamant et al., 1963; Hellekant, 1965a,b; Hellekant et al., 1997; Sako and Yamamoto, 1999; Danilova and Hellekant, 2000). Recordings from single CT fibers of rhesus monkey (*Macaca mulatta*) suggest that ethanol stimulates primarily the sucrose-best (S) fibers. In mixtures of ethanol and sucrose, ethanol affected both the temporal pattern and impulse activity. It prolonged the phasic and increased the tonic part of the response (Hellekant et al., 1997). In mixtures of ethanol and quinine, ethanol suppressed the responses to quinine in bitter-best (Q) taste fibers. In ethanol/citric acid mixtures, ethanol also suppressed the response to citric acid in acid-best (H) fibers (Hellekant et al., 1997; Sako and Yamamoto, 1999). In NaCl-best (N) fibers, ethanol at 1 M concentration enhanced and at 3 M concentration suppressed the mean responses to NaCl. However, due to the large scatter in the data, the overall changes in the responses in the presence and absence of ethanol were reported to be not statistically significant (Hellekant et al., 1997; Sako and Yamamoto, 1999). The above studies indicate that ethanol produces taste mixture interactions when presented along with bitter, sweet, sour, and salt taste stimuli. However, at present, the action of ethanol at the level of taste receptor cells (TRCs) and the complex cascade of intracellular signaling events that result in taste nerve responses and mixture interactions among different taste modalities have not been investigated in detail.

Ethanol at concentrations >1.8% is transiently hypertonic. Although ethanol permeates cell membranes easily, it transiently decreases cell volume in epithelial cells (Mustonen and Kivilaakso, 2003; Mustonen et al.,

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Abbreviations used in this paper: BZ, benzamil; CT, chorda tympani; CZP, capsazepine; ENaC, epithelial Na^+ channel; FIR, fluorescence intensity ratio; NHE-1, Na^+-H^+-exchanger-1; ROI, region of interest; SBFI-AM, 4,4′-[1,4,10-trioxa-7,13-diazacyclo-pentadecane-7,13-diylbis (5-methoxy-6,12-benzo-furandiyl]-bistetrakis(acetyloxy) methyl ester; TRC, taste receptor cell; VR-1, vanilloid receptor-1.

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2004, 2005). Ethanol is a well-established "barrier breaker" in gastric mucosa. It decreased shunt and apical cell membrane resistance and partially closed gap junctions in gastric epithelial cells (Mustonen and Kivilaakso, 2003; Mustonen et al., 2004, 2005). In the Caco-2 cell monolayers (a cell culture model of the intestinal epithelium), ethanol transiently decreased the transepithelial electrical resistance, increased paracellular permeability, and disrupted epithelial tight junctions (Rao et al., 2004). Luminal ethanol increased intracellular Ca^{2+} and opened Ca^{2+}-dependent K^+-selective channels in the basolateral membrane via Ca^+ signaling pathway, with resultant shrinkage of cells (Mustonen and Kivilaakso, 2003; Mustonen et al., 2004, 2005). In intestinal epithelial cells, the effects of ethanol on tight junctions and paracellular permeability most likely occur through a tyrosine kinase–dependent mechanism (Rao et al., 2004). However, at present, it is not known if ethanol also modulates TRC volume and shunt resistance.

In both mice and rats, the amiloride- and benzamil (Bz)-insensitive salt taste receptor shares many biochemical, pharmacological, physiological, and functional similarities with the cloned vanilloid receptor-1 (VR-1) (Lyall et al., 2004b, 2005c). Ethanol activates primary sensory neurons from trigeminal or dorsal root ganglia, as well as TRPV1 expressing HEK-293 cells. Ethanol potentiated the response of VR-1 to capsaicin, protons, and heat and lowered the threshold for heat activation of VR-1 from ~42°C to ~34°C (Trevisani et al., 2002; Geppetti and Trevisani, 2004). Treating rat gastric epithelial cells with 10% ethanol decreased cell viability by acting directly on the VR-1 nonspecific cation channel (Kato et al., 2003). It is likely that ethanol also modulates the Bz-insensitive salt taste receptor in TRCs. Therefore, in this study, we investigated if ethanol elicits its effects on the gustatory system, in part, via its interactions with the amiloride- and Bz-insensitive salt taste receptor. The effect of ethanol was investigated on cell volume and intracellular Na^+ activity ($[Na^+]_i$) in polarized rat fungiform TRCs using fluorescence imaging in vitro. In parallel in vivo experiments, we monitored rat CT nerve responses to mineral salts in the presence and absence of ethanol (Simon, 2002). The results suggest that in the absence of permeable cations, ethanol induces a transient osmotic decrease in TRC volume and elicits only transient phasic CT responses. In the presence of NaCl, ethanol increased the Bz-insensitive Na^+ flux across the apical membrane of polarized TRCs without a change in cell volume and elicited CT responses consisting of both a phasic and a tonic component, and increased the Bz-insensitive CT responses by modulating the VR-1 variant salt taste receptor. Preliminary reports of this study have been published as abstracts (Lyall et al., 2005a; Vinnikova et al., 2005).

MATERIALS AND METHODS

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

Rats were anesthetized by exposing them to an inhalation anesthetic, isoflurane (1.5 ml), in a desiccator. When rats were fully unconscious, a midline incision was made in the chest wall and the aorta severed. The tongues were then rapidly removed and stored in ice-cold Ringer's solution. The Ringer's solution contained (in mM) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Na-pyruvate, 10 glucose, 10 HEPES, pH 7.4. The lingual epithelium was isolated by collagenase treatment. A small piece of the anterior lingual epithelium containing a single fungiform papilla was mounted in a special microscopy chamber as described earlier (Lyall et al., 2001, 2002a,b, 2004b). Relative changes in $[Na^+]_i$ were monitored in polarized TRCs by loading the tissue with Na^+-sensitive fluoroprobes 1,3-benzenedicarboxylic acid, 4,4′-[1,4,10-trioxa-7,13-diazacyclo-pentadecane-7,13-diylbis(5-methoxy-6,12-benzo-furandiyl]-bistetrakis(acetyloxy) methyl ester (SBFI-AM), or sodium-green-AM (both from Molecular Probes). Tissues were loaded with sodium-green-AM (30 μM) at 4°C for 2 h. The tissues were loaded with SBFI (10 μM) in the presence of 0.15% pluronic at room temperature for 4 h. Before an experiment was started, the tissue was perfused on both sides with control solution for 15 min. The tissue was continuously perfused at a rate of 1 ml/min, and the solution changes in the apical compartment were made using three-way miniature solenoid valves. The TRCs in the taste bud were visualized from the basolateral side through a Carl Zeiss MicroImaging, Inc. 40× (0.8 NA) or 60× (0.95 NA) objective with a Carl Zeiss MicroImaging, Inc. Axioskop 2 plus upright fluorescence microscope and imaged with a set up consisting of a cooled CCD camera attached to an image intensifier, an epifluorescent light source, and appropriate dichroic beam splitters and emission filters for SBFI and Na-green. In sodium-green–loaded cells, the changes in $[Na^+]_i$ were monitored by exciting the cells at 490 nm, and the emitted light was imaged at 535 nm at 15-s intervals. Na-green is ideal for studying the effects of drugs that emit strongly when excited with UV light. Since it is a single wavelength dye, its emission can be affected, however, by factors such as dye bleaching, dye leakage from the cells, changes in focal plane, and variations in cell volume (Lyall et al., 2002b), effects that can be minimized by using the ratiometric dye, SBFI. SBFI-loaded TRCs were alternately excited at 340 and 380 nm and imaged at 15-s intervals. The emitted light was detected with a set up containing a 430-nm dichroic beam splitter and a 510-nm emission filter (20-nm band pass; both from Omega Optical). Small regions of interest (ROIs) in the taste bud (diameter 2–3 μm) were chosen in which the changes in fluorescence intensity at 490 nm (F_{490}; Na-green) or the fluorescence intensity ratio (FIR) (F_{340}/F_{380}; SBFI) were analyzed using imaging software (TILLvisIon v 4.0.7.2; TILL Photonics). Each ROI contained two to three receptor cells. Thus, the F_{490} or F_{340}/F_{380} recorded for an ROI represents the mean value from two to three receptor cells within the ROI. In a typical experiment, the fluorescence intensity measurements were made in an optical plane in the taste bud containing at least six ROIs (~18 cells). The background and autofluorescence at 340, 380, and 490 nm were corrected from images of a taste bud without the dye. All experiments were done at room temperature (~23°).

The lingual epithelial preparations were initially perfused on both apical and basolateral sides with a Na^+-free Ringer's solution (containing in mM): 150 NMDG-Cl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.4. It is expected that in the absence of external Na^+, TRC $[Na^+]_i$ will decrease to near zero. Temporal changes in TRC $[Na^+]_i$ were monitored as a response to a unilateral increase in apical Na^+ concentration from 0 to 150 mM. Under these conditions, the temporal changes in TRC $[Na^+]_i$ represent the maximum unilateral apical Na^+ flux. This was achieved

by switching from Na^+-free Ringer's solution to control Ringer's solution (containing in mM): 150 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 HEPES, pH 7.4, in the apical compartment. The changes in TRC $[Na^+]_i$ were monitored in the presence and absence of Bz, capsazepine (CZP), or SB-366791. This was done to distinguish between the apical Na^+ flux through the Bz-sensitive epithelial Na^+ channels (ENaCs) and the Bz-insensitive VR-1 variant nonspecific cation channels in fungiform TRCs (Lyall et al., 2004b, 2005c). In some experiments, changes in TRC $[Na^+]_i$ were monitored in the presence of basolateral ouabain, a Na^+-K^+-ATPase blocker. The relative changes in TRC $[Na^+]_i$ were expressed as percent change in F_{490} of sodium green or as a change in FIR (F_{340}/F_{380}) of SBFI relative to apical zero Na^+ concentration.

$[Na^+]_i$ Measurement in Isolated Fungiform Taste Bud Fragments

In some studies, taste buds were harvested from rat fungiform papillae, aspirated with a micropipette (Vinnikova et al., 2004), and individually transferred onto coverslips, coated with CELL-TAK (BD Biosciences). The glass coverslips formed the bottom of the recording/perfusion chamber (Model RC-26GLP) that was held in a chamber platform (Model P-1; both from Warner Instrument Corp.) and placed on the stage of an upright Carl Zeiss MicroImaging, Inc. Axioskop 2 plus upright fluorescence microscope. TRCs were loaded with sodium-green or SBFI and imaged as described above. Before the experiment was started, the cells were perfused on both sides with control solution for 15 min. All measurements were performed at room temperature. Initially, TRCs were perfused with a Na^+-free Ringer's solution. The changes in relative TRC $[Na^+]_i$ were monitored upon switching from a Na^+-free Ringer's solution to control Ringer's solution (pH 7.4). In these experiments, the control solution contained, in addition, 5 μM Bz and 1 μM zoniporide (a specific blocker of the basolateral Na^+-H^+- exchanger -1 [NHE-1]). Zoniporide was a gift from Pfizer Inc. This was done to inhibit Na^+ flux via the apical ENaCs and the basolateral NHE-1 (Lyall et al., 2004a). All experiments were done at room temperature (~23°C).

Data Analysis

In Na-green–loaded TRCs, changes in $[Na^+]_i$ were expressed relative to the fluorescence intensity (F_{490}) under control conditions or in SBFI-loaded TRCs as changes in FIR (F_{340}/F_{380}) relative to control. F_{490} under control conditions for each ROI was taken as 100%. In individual taste buds, the data were presented as the mean ± SEM of *n*, where *n* represents the number of ROIs 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.

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. An indifferent electrode was placed in nearby tissue. Neural responses were differentially amplified with an optically coupled Isolated Bio-Amplifier (ISO-80; World Precision Instruments). For display, responses were filtered using a band pass filter with cutoff frequencies 40 Hz to 3 kHz and fed to an oscilloscope. Responses were then full-wave rectified and integrated with a time constant of 1 s. Integrated neural responses and current and voltage changes were recorded on a chart recorder and also captured on disk using Labview software (National Instruments) and analyzed offline. Stimulus solutions were injected into a Lucite chamber (3 ml; 1 ml/s) affixed by vacuum to a 28 mm^2 patch of anterior dorsal lingual surface. In some experiments, the solutions were injected into the chamber at the rate of 0.13 ml/s. The chamber was fitted with separate Ag-AgCl electrodes for measurement of current and potential. These electrodes served as inputs to a voltage–current clamp amplifier that permitted the recording of neural responses with the chemically stimulated receptive field under zero current clamp or voltage clamp. The clamp voltages were referenced to the mucosal side of the tongue (Ye et al., 1991; 1993).

To investigate the effect of ethanol on the CT response, the anterior lingual surface was rinsed with deionized H_2O and then stimulated with ethanol solutions ranging in concentration from 0 to 100%. To investigate the effect of ethanol on the CT responses to mineral salts, the lingual surface was stimulated with a rinse solution containing 10 mM KCl and with a stimulus solution containing 10 mM KCl + 100 mM NaCl in the presence of ethanol at concentrations varying between 0 and 60%. CT responses were recorded in the presence of 5 μM Bz. CT responses were also recorded at room temperature (~23°C) and at 42°C, a temperature at which the VR-1 variant nonspecific cation channel is maximally active (Lyall et al., 2004b), and in the presence of VR-1 antagonists, capsazepine (CZP; 25 μM) or *N*-(3-methoxyphenyl)-4-chlorocinnamide (SB-366791; 0.1 μM), that also inhibit the VR-1 variant salt taste receptor (Lyall et al., 2004b, 2005c). 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. The data were digitized and analyzed offline. The numerical value of a tonic integrated CT response was obtained in the quasi-steady-state part of the response as the area under the integrated CT response curve for a time interval of 1 min measured from the end of a typical 2-min stimulation period (Lyall et al., 2002b). The change in area under the integrated CT response curves to various stimuli under different conditions was normalized to the response observed in each animal to 300 mM NH_4Cl. This ratio of areas was averaged across the number of animals in each group (*N*) and expressed as the mean ± SEM of *N*. Student's *t* test was employed to analyze the differences between sets of data. To quantify peak phasic ethanol responses in water or in mannitol solution, in each animal the peak phasic response to a given stimulus was normalized to the mean tonic response to 300 mM NH_4Cl. This ratio was averaged across the number of animals in each group (*N*) and expressed as the mean ± SEM of *N* and analyzed statistically using Student's *t* test.

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 at 23°C or 42°C (Lyall et al., 2004b, 2005c).

RESULTS

In Vitro Studies

SBFI Loading. Fig. 1 A shows the transmitted image of a fungiform papilla containing a single taste bud

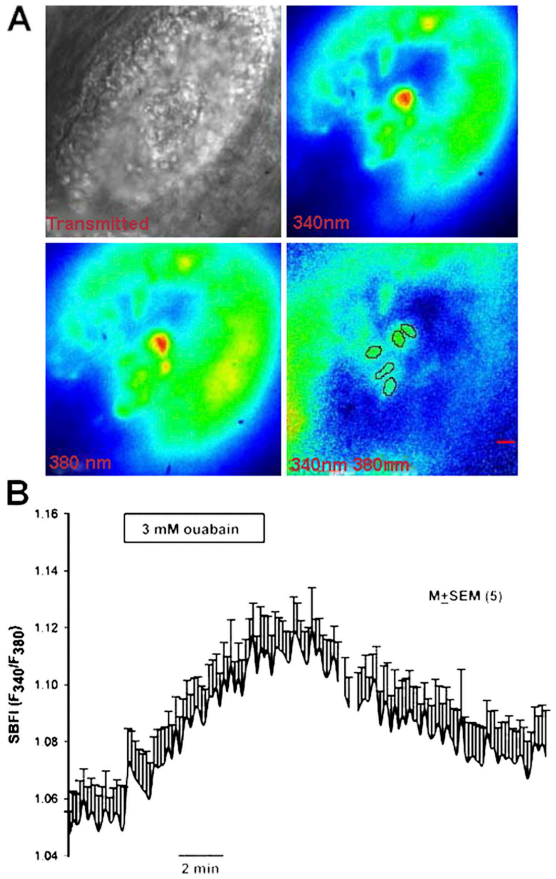

FIGURE 1. SBFI loading and the effect of ouabain on TRC $[Na^+]_i$. (A) An isolated piece of rat anterior lingual epithelium containing a single fungiform papilla was mounted in a special microscopy chamber and was perfused with Ringer's solution containing SBFI-AM. The taste bud was viewed from the basolateral side with a 60× magnification. The figure shows the transmitted image of the taste bud (Transmitted), fluorescence image of the same taste bud excited at 340 nm (340 nm), fluorescence image of the same taste bud excited at 380 nm (380 nm), and the fluorescence intensity ratio image (340 nm/380 nm) with five ROIs. Bar, 10 μm. (B) A polarized epithelial preparation was initially perfused on both sides with control Ringer's solution containing 150 mM NaCl (pH 7.4). Temporal changes in F_{340}/F_{380} (FIR) of SBFI-loaded TRCs were monitored while the basolateral membrane was perfused with Ringer's solution containing 3 mM ouabain. Values are expressed as mean ± SEM of *n*, where *n* = number of ROIs within the taste bud.

mounted in the microscopy chamber. The taste bud was viewed from the basolateral side with a 60× water immersion objective (Transmitted). Fig. 1 also shows the fluorescence images of an optical plane through the same taste bud excited at 340 and 380 nm and the ratio image (340 nm/380 nm) showing FIR in five ROIs within the taste bud where cells could be easily identified. SBFI is specifically taken up by TRCs within the papilla; however, squamous epithelial cells on the papillary periphery also take up the dye. In a taste bud initially perfused with control Ringer's solution (pH 7.4), perfusing the basolateral membrane with Ringer's solution containing 3 mM ouabain reversibly increased FIR (Fig. 1 B; F_{340}/F_{380}). At constant external Na^+ concentration and pH, the increase in $[Na^+]_i$ is due to the ouabain-induced inhibition of the basolateral Na^+-K^+-ATPase.

The results demonstrate that SBFI is intracellular and measures changes in $[Na^+]_i$ in the cytosolic compartment of TRCs. Even after loading with SBFI for 4 h at room temperature, TRCs remain functional. Since TRCs maintain the Na^+ gradient across the basolateral membrane via the Na^+-K^+-ATPase, the result with ouabain signifies that under our in vitro conditions TRCs continue to synthesize and utilize ATP to sustain a nonequilibrium steady state, a necessary condition for cell viability. Similar to SBFI, the single wavelength dye, Na-green, was also specifically taken up by the TRCs within the papilla (Lyall et al., 2002b). Consistent with this, we have previously shown that TRCs similarly loaded with Na-green or SBFI spontaneously regulate intracellular pH (pH_i) via the basolateral NHE-1 (Lyall et al., 2002a, 2004a; Vinnikova et al., 2004). In addition, changes in pH_i regulate ENaC activity and the apical Na^+ flux (Lyall et al., 2002b). This provides additional evidence that TRCs loaded with Na-green or SBFI maintain a nonequilibrium membrane Na^+ gradient.

Effect of Apical Na^+, Bz, and CZP on the Unilateral Apical Na^+ Flux in Polarized Fungiform TRCs. The relative changes in $[Na^+]_i$ were monitored in polarized fungiform TRCs loaded with Na-green. In Fig. 2 A, a lingual epithelial preparation was initially perfused on both sides with Na^+-free Ringer's solution containing 150 mM NMDG-Cl (pH 7.4). Perfusing the apical membrane with control Ringer's solution containing 150 mM NaCl increased F_{490} (*a*–*b*), indicating an increase in TRC $[Na^+]_i$. Perfusing the apical membrane with control Ringer's solution containing 25 μM Bz decreased F_{490} (*b*–*c*). In the continuous presence of Bz, switching the apical solution from control Ringer's solution to Na^+-free Ringer's solution further decreased F_{490} (*c*–*d*) to baseline. The Bz-induced decrease in resting TRC $[Na^+]_i$ (*b*–*c*) reflects the inhibition of Na^+ flux via the apical Bz-sensitive ENaCs. A further decrease in TRC $[Na^+]_i$ (*c*–*d*), induced by lowering apical Na^+ from 150 mM to 0 in the presence of Bz, reflects Na^+ efflux via a Bz-insensitive pathway (Lyall et al., 2002b).

Fig. 2 B again demonstrates that raising the apical Na^+ concentration from 0 to 150 mM increased TRC $[Na^+]_i$ (*a*–*b*) and Bz inhibited a significant part of apical Na^+ flux (*b*–*c*). In the presence of Bz, perfusing the apical membrane with Ringer's solution containing 150 mM NaCl + 25 μM CZP decreased F_{490} to baseline (Fig. 2 B, *c*–*d*), indicating that CZP inhibits the Bz-insensitive component of the apical Na^+ flux. In another preparation, in the continuous presence of Bz + CZP, lowering

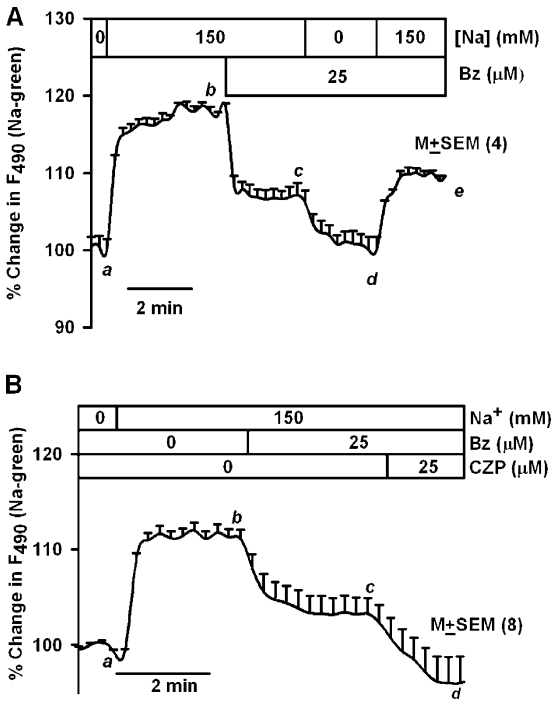

FIGURE 2. Effect of apical Na^+, Bz, and CZP on TRC $[Na^+]_i$. (A) A polarized epithelial preparation was initially perfused on both sides with 0 Na^+ Ringer's solution containing 150 mM NMDG-Cl (pH 7.4). Temporal changes in F_{490} of Na-green–loaded TRCs were monitored while the apical membrane was perfused with Ringer's solution containing 150 mM NaCl (*a–b*), 150 mM NaCl + 25 μM Bz (*b–c*), and 150 mM NMDG-Cl + 25 μM Bz (*c–d*). (B) A polarized epithelial preparation was initially perfused on both sides with 0 Na^+ Ringer's solution containing 150 mM NMDG-Cl (pH 7.4). Temporal changes in F_{490} of Na-green were monitored while the apical membrane was perfused with Ringer's solution containing 150 mM NaCl (*a–b*), 150 mM NaCl + 25 μM Bz (*b–c*), and 150 mM NaCl + 25 μM Bz + 25 μM CZP (*c–d*). 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 the of *n*, where *n* = number of ROIs within the taste bud.

apical Na^+ concentration from 150 mM to 0 produced only a small additional decrease in F_{490} (unpublished data). The results indicate that Na^+ enters TRCs across the apical membrane via two pathways. One pathway is blocked by apical amiloride or Bz, and represents the Na^+ flux through apical ENaC; the second pathway is insensitive to amiloride or Bz, and represents the Na^+ flux through an apical CZP-sensitive VR-1 variant cation channel (Lyall et al., 2004b, 2005c).

Effect of Ethanol on the Unilateral Apical Na^+ Flux in Polarized Fungiform TRCs. Ethanol elicits and potentiates nociceptor responses in trigeminal or dorsal root ganglia and decreases cell viability in gastric epithelial cells through VR-1 (Trevisani et al., 2002; Kato et al., 2003). The amiloride- and Bz-insensitive VR-1 variant cation channel in fungiform TRCs demonstrates many functional similarities with VR-1 (Lyall et al., 2004b, 2005c). Therefore, we investigated if ethanol also modulates the apical Na^+ flux through the VR-1 variant nonspecific cation channel in fungiform TRCs. Data presented in Fig. 3 show the effect of ethanol on the F_{490} of Na-green–loaded TRCs in the presence and absence of external Na^+.

Effect of Ethanol in the Absence of Apical Na^+. Initially, a lingual epithelial preparation was perfused on both apical and basolateral sides with Na^+-free Ringer's solution (pH 7.4). Perfusing the apical membrane with Na^+-free Ringer's solution containing 10% ethanol (ETH) produced a transient increase in F_{490} (Fig. 3 A, *f–g*) that spontaneously decreased to near its control level within 2 min (*g–h*). Perfusing the apical membrane with Na^+-free Ringer's solution containing 20% ETH also produced a transient increase in F_{490} (Fig. 3 A, *a–b*) that spontaneously decreased to near its control level within 2 min (*b–c*). In the presence of 20% ETH, the magnitude of the transient increase in F_{490} (*a–b*) was greater relative to its value in 10% ETH (*f–g*). Switching from Na^+-free Ringer's solution containing either 20% ETH (*e–f*) or 10% ETH (*j–k*) to Na^+-free Ringer's solution without ethanol (0 ETH) produced a decrease in F_{490}. The magnitude of the decrease in F_{490} was greater during a switch from 20% ETH to 0 ETH relative to the corresponding decrease in F_{490} observed during a switch from 10% ETH to 0 ETH (Fig. 3 A, *e–f* > *j–k*). In another lingual epithelial preparation (Fig. 3 B), perfusing the apical membrane with 40% ETH in the absence of Na^+ (*a–b–c*) and its washout (*e–f–g*) produced similar effects on F_{490}. The magnitudes of the transient increase in F_{490} were greater relative to either 20 or 10% ETH (Fig. 3 A). In four polarized TRC preparations initially perfused with apical 0 Na^+-Ringer's solution and then with 0 Na^+-Ringer's solution containing 10, 20, and 40% ETH produced a mean transient increase in F_{490} of 2.4 ± 0.6%; 3.8 ± 0.5%, and 13.8 ± 2.9% ($P < 0.01$; $N = 4$; paired) relative to 0 ETH, respectively.

In Fig. 3, because TRCs were initially bathed on both sides with 0 Na^+ Ringer's solution, it is expected that $[Na^+]_i$ should have decreased to a value close to zero. Therefore, in the absence of external Na^+, the increase in F_{490} due to ethanol stimulation or the decrease in F_{490} due to ethanol washout, most likely, do not represent changes in TRC $[Na^+]_i$. Sodium-green is a single wavelength dye, and its fluorescence is affected by changes in cell volume (Xu et al., 1995). In the absence of external Na^+, an increase in F_{490} of Na-green–loaded TRCs is consistent with a decrease 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^+. In the continuous presence of ethanol, the spontaneous recovery of F_{490} toward baseline suggests that the decrease in volume is transient and TRCs spontane-

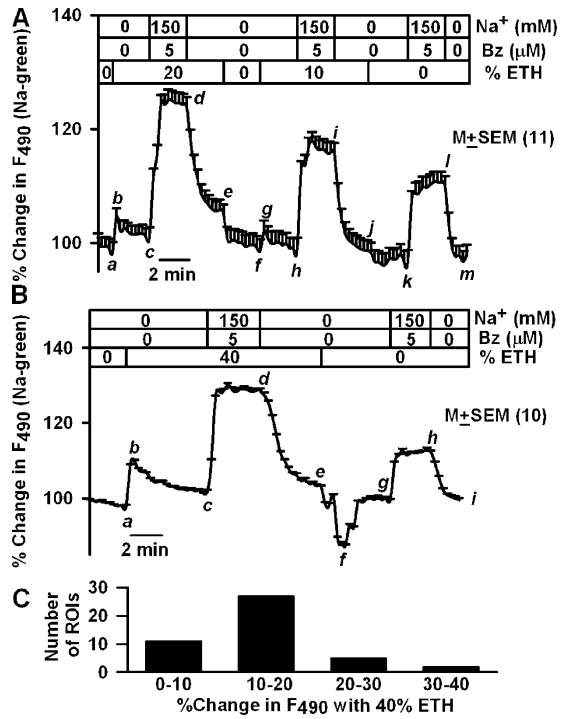

FIGURE 3. Effect of ethanol on TRC $[Na^+]_i$. A polarized epithelial preparation was initially perfused on both sides with 0 Na^+ Ringer's solution containing 150 mM NMDG-Cl (pH 7.4). (A) Temporal changes in F_{490} of Na-green–loaded TRCs were monitored while the apical membrane was perfused with 0 Na^+ Ringer's solution containing 20% (*a–b–c*) or 10% (*f–g–h*) ETH and with Ringer's solution containing 150 mM NaCl + 5 μM Bz + ETH at 20% ETH (*c–d–e*), 10% ETH (*h–i–j*), and 0 ETH (*k–l–m*). (B) 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 40% ETH (*a–b–c*) and with Ringer's solution containing 150 mM NaCl + 5 μM Bz + 40% ETH (*c–d–e*) and 0 ETH (*g–h–i*). The relative changes in $[Na^+]_i$ are presented as percent changes in F_{490} relative to bilateral 0 Na^+ and are expressed as the mean ± SEM of *n*, where *n* = number of ROIs within the taste bud. (C) Ethanol-induced changes in F_{490} in different ROIs within the taste buds. Data are plotted from five individual polarized TRC preparations containing 47 ROIs. In each case, the apical membrane was first perfused with 0 Na^+ Ringer's solution + 40% ETH and then with control Ringer's solution containing 150 mM NaCl + 5 μM Bz + 40% ETH. The histogram shows the number of ROIs that fall within a given interval corresponding to 0–10, 10–20, 20–30, and 30–40% increase in F_{490}.

ously recover their volume to near control levels. The data shown in Fig. 3 B further demonstrate that the washout of ethanol causes an increase in cell volume that also recovers spontaneously to near control levels.

Effect of Ethanol in the Presence of Apical Na^+. In the second part of the experiment, we investigated the effect of ethanol on the Bz-insensitive apical Na^+ flux in polarized fungiform TRCs. The relative Na^+ flux was measured as an increase in F_{490} of Na-green–loaded TRCs in response to an increase in apical Na^+ concentration from 0 to 150 mM. In the presence of Bz, increasing the apical Na^+ concentration from 0 to 150 mM produced a reversible increase in F_{490} (Fig. 3 A, *k–l–m*). Under isosmotic conditions, an increase in F_{490} indicates an increase in TRC $[Na^+]_i$ (Lyall et al., 2002b). Switching from 0 Na^+ Ringer's solution + 10% ETH (at *h*) to control Ringer's solution containing 150 mM NaCl + 5 μM Bz + 10% ETH in the apical compartment reversibly increased F_{490} (Fig. 3 A, *h–i–j*). The magnitude of the increase in F_{490} in the presence of 10% ETH (*h–i–j*) was greater than its corresponding value in the absence of ETH (*k–l–m*). Switching from 0 Na^+ Ringer's solution + 20% ETH (at *c*) to control Ringer's solution containing 150 mM NaCl + 5 μM Bz + 20% ETH in the apical compartment produced a reversible increase in F_{490} (Fig. 3 A, *c–d–e*). The magnitude of the change in F_{490} represented by *c–d–e* was greater than the corresponding increases in F_{490} in the presence of either 10% ETH (*h–i–j*) or 0 ETH (*k–l–m*).

In another polarized TRC preparation (Fig. 3 B), switching from 0 Na^+ Ringer's solution + 40% ETH (at *c*) to control Ringer's solution containing 150 mM NaCl + 5 μM Bz + 40% ETH in the apical compartment produced a greater increase in F_{490} (Fig. 3 B, *c–d–e*) relative to 0 ETH (Fig. 3 B, *g–h–i*). In the presence of 40% ETH, the magnitude of the increase in F_{490} relative to 0 ETH was greater than the corresponding increase in F_{490} shown in Fig. 3 A with 20 and 10% ETH.

In five individual polarized fungiform taste bud preparations, switching from 0 Na^+ Ringer's solution + 40% ETH to control Ringer's solution containing 150 mM NaCl + 5 μM Bz + 40% ETH in the apical compartment produced variable increases in F_{490} in all 47 ROIs within the taste buds relative to 0 ETH (Fig. 3 C). The increase in F_{490} in the presence of 40% apical ethanol ranged between 0 and 40% among individual ROIs. Among 47 ROIs, 11 ROIs (23.4%) demonstrated an increase in F_{490} between 0 and 10%, 27 ROIs (57.4%) showed an increase between 10 and 20%, 5 ROIs (10.6%) demonstrated an increase between 20 and 30%, and 4 ROIs (8.5%) responded with an increase in F_{490} between 30 and 40% (Fig. 3 C). These results indicate that within the taste bud, TRCs are heterogeneous and can be separated into subgroups based on their response to ethanol stimulation. It is likely that the cell population that responds to ethanol stimulation with a maximum increase in the Bz-insensitive Na^+ flux is the most important cell population for determining the ethanol-modulated salt taste threshold. Using a similar approach, and by monitoring the distribution of resting pH_i values in different ROIs within the taste buds, we identified two distinct subpopulations of TRCs within fungiform taste buds involved in sour taste transduction (Vinnikova et al., 2004).

In the above experiments, the apical membrane was first treated with ethanol in 0 Na^+ Ringer's solution and

then stimulated with Ringer's solution containing 150 mM NaCl + ethanol. In the next series of experiments, we investigated the effect of 150 mM NaCl + ethanol without first exposing the apical membrane to ethanol in 0 Na^+ Ringer's solution. In Fig. 4 A, a lingual epithelial preparation was initially perfused on the basolateral side with Na^+-free Ringer's solution and on the apical side with Na^+-free Ringer's solution + 5 μM Bz. Perfusing the apical membrane with Ringer's solution containing 150 mM NaCl + 5 μM Bz + 10% ETH produced a sustained increase in F_{490} (Fig. 4 A, *c–d*). The magnitude of the increase in F_{490} (*c–d*) was greater relative to control (0 ETH; *e–f*). Increasing ETH concentration to 40% also produced a sustained increase in F_{490} (Fig. 4 A, *a–b*). The magnitude of the increase in F_{490} in the presence of 40% ETH (Fig. 4 A, *a–b*) was greater relative to its corresponding value in the presence of 10% ETH (Fig. 4 A, *c–d*) or 0 ETH (Fig. 4 A, *e–f*).

Fig. 4 B shows that stimulating the apical membrane with 150 mM NaCl + 5 μM Bz containing 0 (*a–b*), 20 (*b–c*), and 40% (*c–d*) ETH in a stepwise manner also produced a dose-dependent increase in F_{490} in polarized TRCs loaded with Na-green. Fig. 4 C summarizes the mean data from 17 individual polarized TRC preparations loaded with Na-green in which the ethanol-induced increase in the unilateral apical Na^+ flux was monitored as described in Fig. 3 (A–C) and Fig. 4 (A and B). The results indicate that ethanol, at a concentration between 10 and 40%, increased the unilateral Bz-insensitive Na^+ flux across the apical membrane of fungiform TRCs in a dose-dependent manner. We have previously shown that VR-1 agonists (RTX, CAP, elevated temperature, ATP) increase the apical membrane conductance 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, 2005c). Thus, the effect of ethanol on the apical membrane cation conductance is similar to the other VR-1 agonists.

It should be noted that in Fig. 4, stimulating the apical membrane with Ringer's solution containing 150 mM NaCl + ethanol produced sustained increases in F_{490}. However, a similar increase in ethanol in Na^+-free Ringer's solution containing 150 mM NMDG-Cl produced only transient changes in F_{490} that recovered spontaneously to near baseline (Fig. 3). The results suggest that ethanol induces a transient decrease in TRC volume in the presence of apical $NMDG^+$, a cation that does not permeate the Bz-insensitive pathway. However, ethanol does not decrease cell volume when presented with apical Na^+, a cation that permeates the Bz-insensitive pathway.

SBFI is a ratiometric dye and its FIR is not affected by changes in cell volume (Xu et al., 1995). In a polarized TRC preparation loaded with SBFI and perfused on both sides with 0 Na^+ Ringer's solution, switching to 0 Na^+ Ringer's solution + 40% ETH in the apical compartment produced a transient increase in FIR (Fig. 5 A, *a–b*) followed by a spontaneous decrease of FIR toward baseline (Fig. 5 A, *b–c*). Upon washout of ethanol, a decrease in FIR was also observed (*e–f*). As stated above, since the tissue was initially bathed on both sides with 0 Na^+ Ringer's solution, it is expected that TRC $[Na^+]_i$ should have decreased to a value close to zero. The ethanol-induced increase in FIR (*a–b*) in the absence of external Na^+ may be due to an increase in the residual $[Na^+]_i$ still present in cell subcompartments. Alternately, at low intracellular Na^+ concentration, the increase in FIR may reflect the response of SBFI to a

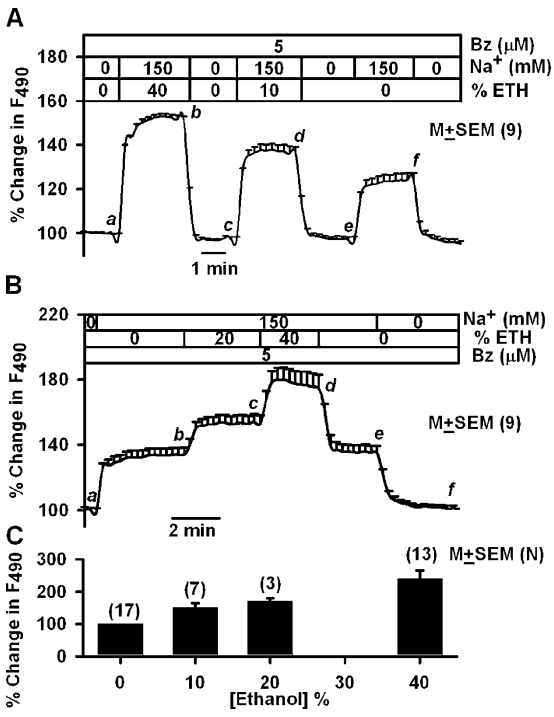

FIGURE 4. Effect of ethanol on apical Na^+ flux. A polarized epithelial preparation was initially perfused on both sides with 0 Na^+ Ringer's solution containing 150 mM NMDG-Cl (pH 7.4). (A) Temporal changes in F_{490} of Na-green–loaded TRCs were monitored while the apical membrane was perfused with Ringer's solution containing 150 mM NaCl + 5 μM Bz with 40% ETH (*a–b*), 10% ETH (*c–d*), and 0 ETH (*e–f*). (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 + ETH at 0 (*a–b*), 20% (*b–c*), and 40% (*c–d*) concentration. The relative changes in $[Na^+]_i$ are presented as percent changes in F_{490} relative to bilateral 0 Na^+ and are expressed as the mean ± SEM of *n*, where *n* = number of ROIs within the taste bud. (C) Summary of data from 17 TRC preparations. The mean percent changes in F_{490} at different ethanol concentrations were normalized to F_{490} values at 0 ETH. The number in parenthesis indicates the number of individual TRC preparations (*N*) investigated for a particular ETH concentration. All values were significantly greater ($P < 0.01$) than control (0 ETH).

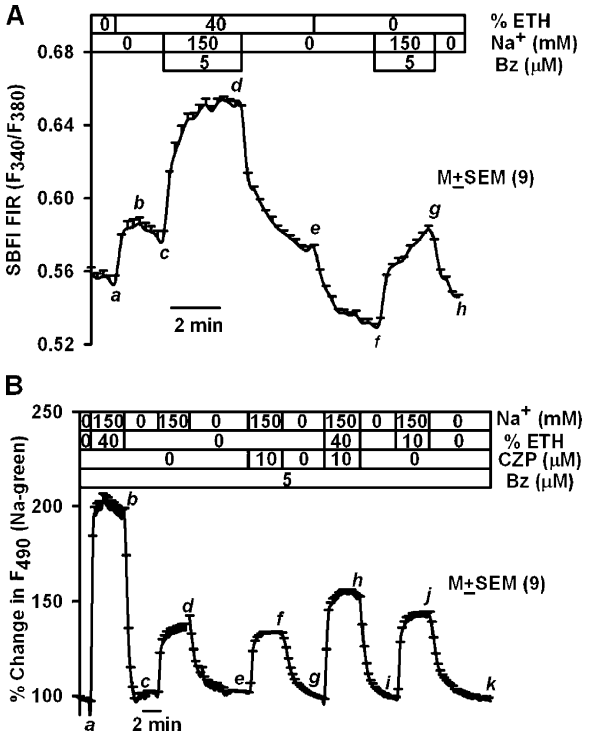

FIGURE 5. Effect of ethanol on apical Na^+ flux. (A) A polarized epithelial preparation was initially perfused on both sides with 0 Na^+ Ringer's solution containing 150 mM NMDG-Cl (pH 7.4). Temporal changes in FIR (F_{340}/F_{380}) of SBFI-loaded TRCs were monitored while the apical membrane was perfused with Ringer's solution containing 150 mM NMDG-Cl + 40% ETH (*a–b–c*) or with 150 mM NaCl + 5 μM Bz + 40% ETH (*c–d–e*) and 0 ETH (*f–g–h*). The relative changes in $[Na^+]_i$ are presented as changes in FIR relative to bilateral 0 Na^+ and are expressed as the mean ± SEM of *n*, where *n* = number of ROIs within the taste bud. (B) A polarized epithelial preparation was initially perfused on both sides with 0 Na^+ Ringer's solution containing 150 mM NMDG-Cl (pH 7.4). 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 + ETH at 40% (*a–b–c*), 10% (*i–j–k*), and 0 ETH (*c–d–e*) concentration, and with Ringer's solution containing 150 mM NaCl + 5 μM Bz + 10 μM CZP + ETH at 40% (*g–h–i*) or 0 (e–*f–g*) concentration. The relative changes in $[Na^+]_i$ are presented as percent changes in F_{490} relative to bilateral 0 Na^+ and are expressed as the mean ± SEM of *n*, where *n* = number of ROIs within the taste bud.

cell shrinkage–induced increase in K^+ concentration inside TRCs ($[K^+]_i$). Similarly, following ethanol washout, a decrease in FIR may reflect the response of SBFI to a decrease in residual $[Na^+]_i$ or $[K^+]_i$ induced by cell swelling. Taken together, the fluorescence changes observed in TRCs loaded with Na-green or SBFI suggest that ETH induces transient changes in cell volume in the presence of apical $NMDG^+$. Consistent with the data shown in Fig. 3, 40% ETH induced a sustained increase in FIR in response to an increase in apical NaCl concentration from 0 to 150 mM (Fig. 5 A, *c–d*). The increase in FIR in the presence of 40% ETH (*c–d–e*) was significantly greater relative to its corresponding magnitude in 0 ETH (Fig. 5 A, *f–g–h*).

Effect of Ethanol on the Unilateral Apical Na^+ Flux in the Presence of VR-1 Antagonists. To investigate if ethanol increases apical Na^+ flux via the VR-1 variant nonspecific cation channel, further experiments were performed in the presence of the VR-1 antagonists CZP and SB-366791. In a lingual epithelial preparation (Fig. 5 B), perfusing the apical membrane with Ringer's solution containing 150 mM NaCl + 5 μM Bz + 40% ETH + 10 μM CZP produced a significantly smaller increase in F_{490} (*g–h*) relative to stimulation with 150 mM NaCl + 5 μM Bz + 40% ETH (Fig. 5 B, *a–b*). In the presence of CZP, 40% ETH gave only a slightly bigger response (*g–h*) relative to 10% ETH alone (*i–j*). In the absence of CZP (Fig. 5 B), 10% (*i–j–k*) and 40% (*a–b–c*) ETH produced similar changes in F_{490} relative to 0 ETH as also shown above in Fig. 4 A. In another polarized fungiform taste bud preparation initially perfused with 0 Na^+ Ringer's solution, switching to control Ringer's solution containing 150 mM NaCl + 5 μM Bz + 1 μM SB-366791 + 40% ETH produced the same magnitude of increase in F_{490} as with 150 mM NaCl + 5 μM Bz + 0 ETH (unpublished data). Thus in the presence of SB-366791, the expected increase in F_{490} with ethanol stimulation was not observed. These results indicate that VR-1 antagonists inhibit the ethanol-induced increase in the Bz-insensitive Na^+ flux across the apical membrane of fungiform TRCs.

Effect of Ethanol on Isolated Fungiform TRCs. To investigate if ethanol acts directly on TRCs or produces its effects via a secondary mechanism involving the release of peptides or other activators of VR-1 from nerve fibers (Simon et al., 2003) or nontaste cells, the effect of ethanol was investigated in isolated fungiform taste bud fragments. Fig. 6 A shows that isolated fungiform TRCs take up Na-green readily. Upon switching from 0 Na^+ Ringer's solution (F_{490} 0 Na) to control Ringer's solution containing 150 mM NaCl (F_{490} 150 Na), all TRCs responded with an increase in fluorescence intensity (F_{490}). Isolated TRCs are exposed to changes in Na^+ on both apical and basolateral membranes. Across the basolateral membrane, Na^+ flux is coupled to the NHE-1 activity and is accompanied by changes in TRC pH_i (Lyall et al., 2002a, 2004a; Vinnikova et al., 2004). Since NHE-1 is ubiquitously expressed in all TRCs, it is expected that all TRCs will respond to an increase in external Na^+ with an increase in $[Na^+]_i$. It follows that under our in vitro conditions, isolated TRCs also remain functional, they maintain a Na^+ gradient and regulate pH_i via the basolateral NHE-1.

Isolated TRCs loaded with Na-green were perfused with Na^+-free Ringer's solution containing 150 mM NMDG-Cl + 5 μM Bz + 10 μM zoniporide or with control Ringer's solution containing 150 mM NaCl + 5 μM

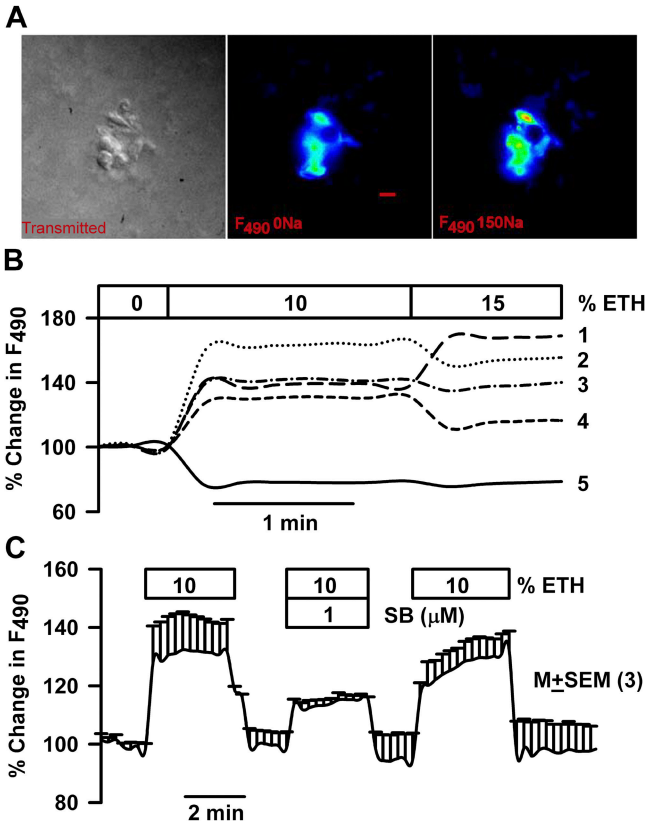

FIGURE 6. Effect of ethanol on isolated TRCs. (A) Na-green loading in isolated fungiform TRCs. Isolated fungiform taste bud fragments were placed onto coverslips coated with CELL-TAK, which were attached to the bottom of the recording/perfusion chamber. Taste bud fragments were perfused with control Ringer's solution containing Na-green-AM for 2 h. The taste buds were imaged at 60× magnification. Bar, 10 μm. The figure shows the transmitted image of a taste bud fragment in an optical plane (Transmitted), and the fluorescence image of the same taste bud in the same optical plane excited at 490 nm perfused with 0 Na^+ Ringer's solution (F_{490} 0Na) and with Ringer's solution containing 150 mM Na (F_{490} 150Na). Bar, 10 μm. (B) Initially, an isolated fungiform taste bud fragment with five individual TRCs was perfused with 0 Na^+ Ringer's solution containing 150 mM NMDG-Cl + 5 μM Bz + 10 μM zoniporide (pH 7.4). Zoniporide was added to block the activity of basolateral NHE-1. Temporal changes in F_{490} of Na-green–loaded TRCs were monitored while the taste bud fragment was perfused with Ringer's solution containing 150 mM NaCl + 5 μM Bz + 10 μM zoniporide + ethanol at 10 or 15% concentration. The relative changes in $[Na^+]_i$ in each TRC are presented as percent changes in F_{490} relative to bilateral 0 Na^+. (C) Initially an isolated fungiform taste bud fragment with three individual TRCs was perfused with 0 Na^+ Ringer's solution containing 150 mM NMDG-Cl + 5 μM Bz + 10 μM zoniporide (pH 7.4). Temporal changes in F_{490} of Na-green–loaded TRCs were monitored while the taste bud fragment was perfused with Ringer's solution containing 150 mM NaCl + 5 μM Bz + 10 μM zoniporide + 10% ethanol or with 150 mM NaCl + 5 μM Bz + 10 μM zoniporide + 10% ethanol + 1 μM SB-366791. The relative changes in $[Na^+]_i$ are expressed as the mean ± SEM of *n*, where *n* = number of TRCs within the taste bud.

Bz + 10 μM zoniporide + ethanol (ETH; 5–15%; pH 7.4). Zoniporide was added to block the Na^+ flux via the basolateral NHE-1 (Lyall et al., 2004a; Vinnikova et al., 2004). Fig. 6 B shows the effect of 10 and 15% ethanol on a fungiform taste bud fragment containing five TRCs. All five TRCs gave variable responses to ETH stimulation. In one cell (cell 5), 10% ethanol produced a decrease in F_{490}, while the other four TRCs (cells 1–4) responded with an increase in F_{490}. The increase in F_{490} among individual TRCs varied between 30 and 60%. Only one cell (cell 1) responded with a dose-dependent increase in F_{490} when stimulated with 15% ETH, while in the other three cells (cells 2–4), 15% ETH induced a decrease in F_{490} relative to 10% ETH stimulation. In 19 individual TRCs studied, eight cells (42%) demonstrated a dose-dependent increase in F_{490} with 5, 10, and 15% ETH stimulation. In the other 58% of the cells, increasing the ethanol concentration did not show any effect on resting F_{490} or the fluorescence intensity decreased with increasing ethanol concentration. In three additional TRCs (Fig. 6 C), stimulating the cells with control Ringer's solution containing 10% ETH + 1 μM SB-366791 reversibly inhibited the increase in F_{490} relative to 10% ETH alone. Similar results were also obtained with taste buds isolated from the circumvallate papillae (unpublished data). These results suggest that within a taste bud, TRCs are heterogeneous and demonstrate different sensitivities to ethanol stimulation. The results further suggest that ethanol acts directly on TRCs to increase the Bz-insensitive Na^+ flux via the SB-366791–sensitive VR-1 variant cation channel (Lyall et al., 2004b, 2005c).

In Vivo Studies

Results presented in Figs. 3–6 suggest that in TRCs, ethanol modulates the Bz-insensitive apical Na^+ flux via the VR-1 variant cation channel (Lyall et al., 2004b, 2005c). 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, 2005c). We hypothesize that ethanol also modulates the Bz-insensitive CT responses to mineral salts. We also reasoned 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.

Effect of Ethanol on CT Responses in the Absence of Mineral Salts. We first monitored CT responses to ethanol alone with reference to H_2O rinse. As shown in Fig. 7 A, a rat tongue was initially rinsed with deionized H_2O and then stimulated with ethanol at concentrations varying between 40 and 100%. Both the rinse and stimulating solutions were maintained at room temperature (~23°). At the time period indicated by the arrows at *a*,

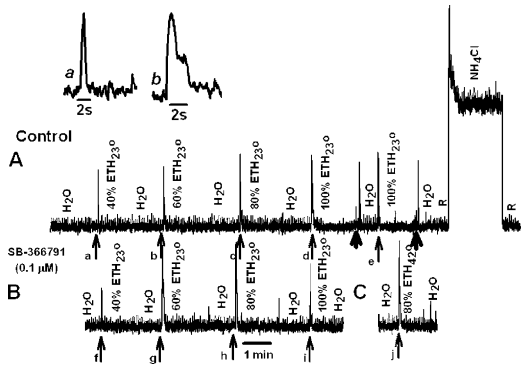

FIGURE 7. Effect of ethanol on the CT responses. Rat tongue was stimulated with 40, 60, 80, and 100% ethanol (ETH) solutions containing either 0 (A) or 0.1 μM SB-366791 (B). CT responses were recorded at 23°C (A and B) and at 42°C (C) with reference to H_2O rinse at 23°C. The time period at which the rat tongue was superfused with different solutions is indicated by arrows. Following 100% ETH stimulation, the tongue was rinsed with deionized H_2O (A; thick arrows). The tongue was rinsed with 10 mM KCl (R) and then stimulated with 300 mM NH_4Cl (NH_4Cl) to obtain a reference CT response. The data were normalized to 0.3 M NH_4Cl CT response in each animal.

b, c, d, and e, the tongue was stimulated with 40, 60, 80, and 100% ETH, respectively. The ethanol-induced CT responses were composed of only a transient phasic component (Lyall et al., 2005b). We did not observe a sustained tonic component of the CT response at any ethanol concentration. The top part of Fig. 7 A shows the transient phasic responses to 40 and 60% ethanol stimulation in an extended time scale. Ethanol, at a concentration of 40%, induced a transient phasic response consisting of a rapid rising phase that spontaneously returned to baseline within ~1 s (*a*). Similar to 60% ETH (*b*); most of the transient phasic responses to ethanol stimulation at a concentration of 60% and above were of longer duration (>2 s), consisting of a rapid rising phase that spontaneously decreased to a pseudo-steady-state level for ~1 s before falling to baseline. The magnitude of the transient phasic response remained invariant with increasing ethanol concentration. In two cases, following stimulation with 100% ETH, rinsing the tongue with deionized H_2O (short thick arrows) also elicited transient phasic responses. Stimulating the tongue with ethanol solutions containing 0.1 μM SB-366791, a potent and specific blocker of the VR-1 variant cation channel (Lyall et al., 2004b, 2005c), did not affect the transient phasic responses to ethanol concentrations between 40 and 100% relative to control (Fig. 7 B, *f, g, h,* and *i*).

Effect of Temperature on the CT Response to Ethanol. The apical VR-1 variant cation channel in TRCs is activated by elevated temperature (Lyall et al., 2004b, 2005c). To test if ethanol responses are also modulated at elevated temperatures, the tongue was stimulated with ethanol solutions maintained at 42°C, and the CT responses were monitored with reference to the H_2O rinse at 23°. Increasing the temperature to 42°C (Fig. 7 C, *j*) had no effect on the CT response to 80% ETH stimulation relative to 23°C (Fig. 7 A, *c*, and Fig. 7 B, *h*).

Effect of Osmolarity on the CT Response to Ethanol. In the above experiments, ethanol was used at concentrations between 40 (6.8 M) and 100% (17.0 M). These are extremely hypertonic solutions. We hypothesize that ethanol-induced transient phasic CT responses are an indirect effect due to hyperosmolarity. In the absence of mineral salts (i.e., in deionized water), stimulating the lingual surface with ethanol produced only transient phasic CT responses (Fig. 7). In in vitro studies, in polarized TRCs bathed in 0 Na^+ Ringer's solution containing the nonpermeant cation, $NMDG^+$, ethanol stimulation caused a transient decrease in TRC volume (Fig. 3). We hypothesize that ethanol-induced transient phasic CT responses are due to cell shrinkage. To test this hypothesis, we applied a hypertonic solution of mannitol to the lingual surface to preshrink TRCs in vivo and then monitored CT responses to 50% ETH. We reasoned that if ethanol produces a transient phasic CT response by inducing cell shrinkage, preshrinking TRCs with a hypertonic solution should inhibit the phasic response. Consistent with previous studies (Lyall et al., 1999), superfusing the tongue with 0.5 M mannitol (M at the arrow) produced a transient phasic response (Fig. 8 A). Mannitol increases transepithelial resistance across the lingual epithelium and decreases TRC volume (Lyall et al., 1999). This suggests that the mannitol-induced transient phasic CT response is related to cell shrinkage. Subsequently, superfusing the tongue with 50% ethanol solution containing 0.5 M mannitol (50% ETH + M) produced a transient phasic CT response whose magnitude was 40% smaller than its corresponding value before mannitol treatment (50% ETH). In three animals (Fig. 8 B), pretreating the tongue with 0.5 M mannitol decreased the ethanol-induced transient phasic response by 38.5 ± 2.7% relative to control ($P < 0.01$; $N = 3$). These results strongly suggest that in the absence of mineral salts, a decrease in cell volume contributes significantly to the ethanol-induced transient phasic CT response.

Effect of Ethanol on the CT Response in the Presence of 100 mM NaCl. Fig. 9 A shows the effect of ethanol on the NaCl CT response. Stimulating the tongue with 10 mM KCl + 100 mM NaCl (R + N) gave a CT response relative to 10 mM KCl rinse (R). About 70% of the NaCl CT response was inhibited by superfusing the tongue with 10 mM KCl + 100 mM NaCl + 5 μM Bz (R + N + Bz). Rinsing the lingual surface with a rinse solution containing 40% ethanol (10 mM KCl + 40% ETH; R + ETH) and then with NaCl solution containing ethanol

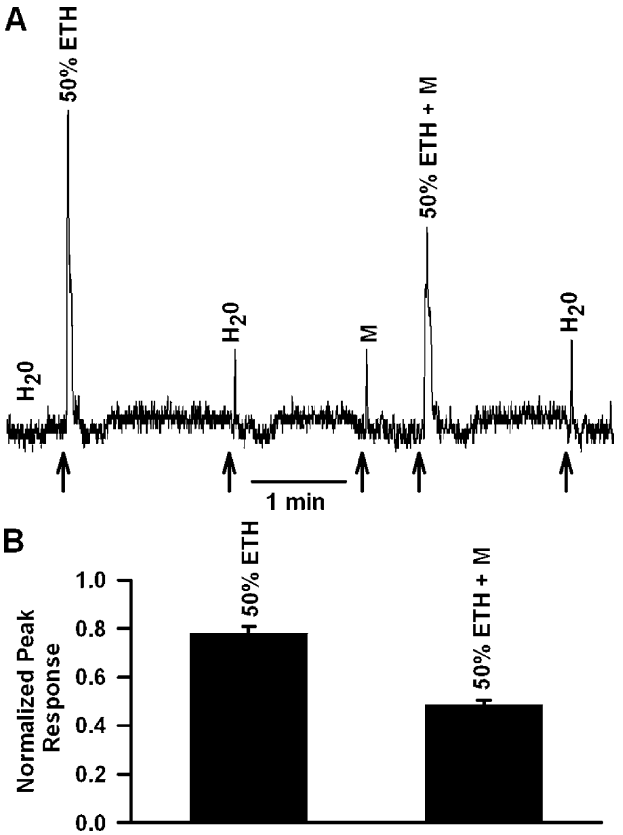

FIGURE 8. Effect of mannitol on the ethanol-induced transient phasic CT response. (A) CT response was monitored while the rat tongue was first rinsed with deionized H_20 and then with 50% ethanol (50% ETH). The CT responses were also recorded when the rinse and the ethanol solutions contained 0.5 M mannitol (M). The time period at which the rat tongue was superfused with different solutions is indicated by arrows. (B) Summary of data from three such experiments. Each bar represents the mean ± SEM of the normalized peak response from three animals (*N*).

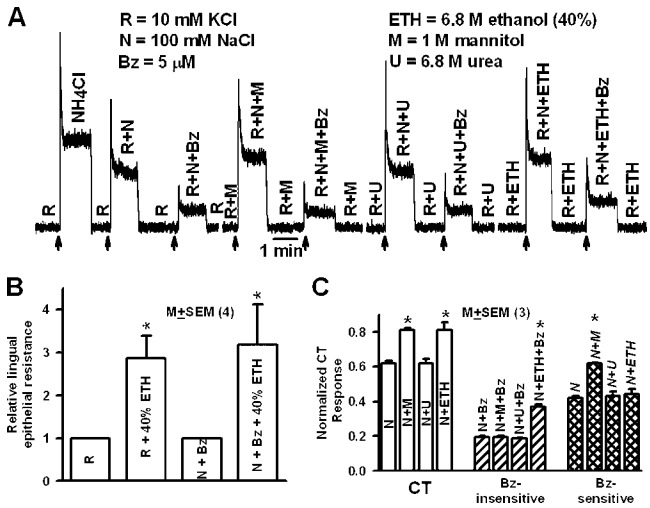

FIGURE 9. Effect of mannitol (M), urea (U), and ethanol (ETH) on the NaCl CT response. (A) CT response was monitored while the rat tongue was first rinsed with 10 mM KCl (R) and then stimulated with 10 mM KCl + 100 mM NaCl (R + N) and with 10 mM KCl + 100 mM NaCl + 5 μM Bz (R + N + Bz). The CT responses were also recorded when the rinse and the stimulating solutions contained 1 M mannitol (M), 6.8 M urea (U), or 6.8 M ethanol (40% ETH). The arrows represent the time period when the tongue was stimulated with the various NaCl stimulating solutions. The tongue was stimulated with 300 mM NH_4Cl to obtain a reference CT response. The data were normalized to the 0.3 M NH_4Cl CT response in each animal. (B) Effect of ethanol on the transepithelial electrical resistance across the tongue. The changes in the transepithelial electrical resistance in the presence of 40% ETH were represented relative to 10 mM KCl (R) or 100 mM NaCl + 5 μM Bz (N + Bz). The values are expressed as the mean ± SEM from four animals (*N*). *, $P < 0.05$ (paired). (C) Summary of data from three individual animals. The open bars (left) represent the CT response in the presence of NaCl (N), NaCl + 1 M mannitol (N + M), NaCl + 6.8 M Urea (N + U), and NaCl + 6.8 M ethanol (N + ETH). The hatched bars (middle) represent the Bz-insensitive component of the CT response to N + Bz, N + M + Bz, N + U + Bz, and N + ETH + Bz. The cross-hatched bars (right) represent the Bz-sensitive component of the CT response to N, N + M, N + U, and N + ETH. The Bz-sensitive component was obtained by subtracting the Bz-insensitive response from the corresponding CT response in the absence of Bz. The data were normalized to the 0.3 M NH_4Cl CT response in each animal. The values are expressed as the mean ± SEM from three animals (*N*). *, $P < 0.05$ (paired).

(10 mM KCl + 100 mM NaCl + 40% ETH; R + N + ETH) produced a CT response whose magnitude was greater relative control. In solutions containing 40% ethanol, subsequently superfusing the tongue with NaCl solution containing Bz (R + N + ETH + Bz) inhibited the CT response. In the presence of ethanol, the magnitude of the Bz-insensitive component of the NaCl CT response was greater relative to control. Data summarized in Fig. 9 C show that ethanol enhanced the NaCl CT response by activating the Bz-insensitive component of the NaCl CT response (N + ETH + Bz; $P < 0.05$, $N = 3$; paired). Ethanol at this concentration had no effect on the Bz-sensitive ENaC component of the CT response.

Addition of 40% ethanol (6.8 M) to 10 mM KCl or 100 mM NaCl also produced a significant increase in the transepithelial electrical resistance across the tongue (Fig. 9 B). This result is in contrast to the effect of ethanol reported on the intestinal epithelial cells. In the Caco-2 cell monolayer (Rao et al., 2004) and gastric mucosa (Mustonen and Kivilaakso, 2003; Mustonen et al., 2004), ethanol was reported to produce a decrease in the transepithelial electrical resistance, suggesting that in the intestinal epithelium, ethanol increases paracellular permeability and disrupts epithelial tight junctions. In spite of the differences regarding the transepithelial electrical resistance, in cultured rabbit gastric epithelial cells (Mustonen et al., 2004; 2005) and in isolated *Necturus* gastric mucosa (Mustonen and Kivilaakso, 2003), ethanol treatment resulted in epithelial cell shrinkage.

To distinguish between the direct effect of ethanol on the VR-1 variant cation channel and a possible indirect effect due to hyperosmolarity, we monitored NaCl CT responses and the relative changes in the transepithelial resistance across the tongue in the presence of 1 M mannitol or 6.8 M urea. Consistent with our earlier studies (Lyall et al., 1999), 10 mM KCl + 1 M mannitol produced only a transient phasic response relative to 10 mM KCl (R) alone. Mannitol also increased the transepithelial potential and transepithelial electrical resistance across the tongue (unpublished data). An increase in transepithelial resistance is consistent with an osmotically induced decrease in cell volume (Lyall et al., 1999). As also shown in Fig. 9 A, mannitol (M) increased the NaCl CT response (R + N + M) relative to the control (R + N). Mannitol had no effect on the Bz-insensitive part of the CT response (R + N + M + Bz) relative to control (R + N + Bz). The Bz-sensitive (ENaC) component of the CT response was obtained by subtracting the Bz-insensitive CT response from the CT response in the absence of Bz. The results from three animals (Fig. 9 C) indicate that mannitol (M) specifically enhanced the Bz-sensitive NaCl CT response (N+M; $P < 0.05$; $N = 3$; paired). The increase in the Bz-sensitive NaCl CT response is most likely due to the activation of apical ENaCs due to cell shrinkage (Ji et al., 1998; Lyall et al., 1999).

In contrast, 6.8 M urea had no effect on the NaCl CT response in the absence (R + N + U) and presence of Bz (R + N + U + Bz) relative to control (R + N) (Fig. 9 A). This indicates that urea affects neither the Bz-sensitive nor the Bz-insensitive component of the NaCl CT response (Fig. 9 C) (Lyall et al., 1999). Although both urea and ethanol, with similar reflection coefficients, readily penetrate the lingual epithelium, at equivalent molar concentrations, only ethanol enhanced the Bz-insensitive NaCl CT response. Second, a hypertonic solution containing mannitol, which induces cell shrinkage, increases the Bz-sensitive NaCl CT response (ENaC) only. This suggests that the Bz-insensitive NaCl CT response is not affected by an increase in osmolarity per se but is enhanced due to the specific effect of ethanol on the apical VR-1 variant cation channel in TRCs. This is consistent with the reports that among the TRPV channel family, VR-1 receptor (TRPV1) is not sensitive to osmolarity (Gunthorpe et al., 2002; Clapham, 2003; Clapham et al., 2003).

In Ringer's solution containing 150 mM NaCl, stimulating the apical membrane with ethanol produced monotonic increase in $[Na^+]_i$ (Figs. 3–6). This suggests that cell volume changes do not occur when TRCs are stimulated with ethanol in the presence of a permeant cation, Na^+. We reasoned that if volume changes do not occur under these conditions, then preshrinking TRCs in vivo with mannitol should have no effect on the Bz-insensitive NaCl CT responses in the presence of ethanol. Data presented in Fig. 9 show that 0.5 M mannitol by itself does not affect the Bz-insensitive CT response to 100 mM NaCl. In addition, in three animals, stimulating the tongue with 100 mM NaCl + 5 μM Bz + 50% ethanol + 0.5 M mannitol (with reference to a rinse solution containing 10 mM KCl + 0.5 M mannitol) produced CT responses that were not different from the CT responses obtained with 100 mM NaCl + 5 μM Bz + 50% ethanol (with reference to a rinse solution containing 10 mM KCl) (unpublished data). These results suggest that in the presence of external Na^+, the effect of ethanol on the Bz-insensitive NaCl CT response is not affected by changes in TRC volume.

Effect of SB-366791 on the CT Response to Mineral Salts in the Presence of Ethanol. In our in vitro studies, VR-1 antagonists (SB-366791 or CZP) inhibited the ethanol-induced increase in the unilateral Na^+ flux across the apical membrane of polarized TRCs (Figs. 5 and 6). We next tested if the cation flux through the SB-366791–sensitive nonspecific cation channel is related to the ethanol-induced increase in the CT response to mineral salts. As shown in Fig. 10 A, the tongue was first rinsed with 10 mM KCl (R) and then with 10 mM KCl + 40% ethanol (R + ETH). The results demonstrate that similar to the case with 100 mM NaCl (Fig. 9 A), in the presence of 10 mM KCl, ethanol also elicited CT response comprising both a fast phasic component and a slow sustained tonic component. These results indicate that ethanol activates a nonspecific cation channel that is permeable to Na^+ as well as K^+. Superfusing the tongue with solution containing 10 mM KCl + 40% ethanol + 0.1 μM SB-366791 (R + ETH + SB) completely inhibited the tonic component of the CT response, and only the transient phasic response was observed. Similar results were obtained with stimulating solutions containing 100 mM NaCl (unpublished data). These results indicate that ethanol modulates CT responses to mineral salts and that these effects are inhibited by SB-366791.

Effect of Flow Rate on the CT Response to Ethanol. Stimulating the tongue with ethanol in the absence of mineral salts induced only transient phasic CT responses (Fig. 7). In the presence of mineral salts, ethanol produces CT responses composed of both a phasic and a tonic component (Fig. 9 A and Fig. 10, A and B). In contrast, earlier studies (Hellekant, 1965a; Sako and Yamamoto, 1999) reported that in rat, cat, and dog, ethanol elicited tonic responses with small or negligible phasic responses. We hypothesize that the differences between CT profiles reported in this and earlier studies could be explained due to differences in the experimental conditions employed. One important factor that determines if the phasic component of the CT response is observed or not is the rate at which the

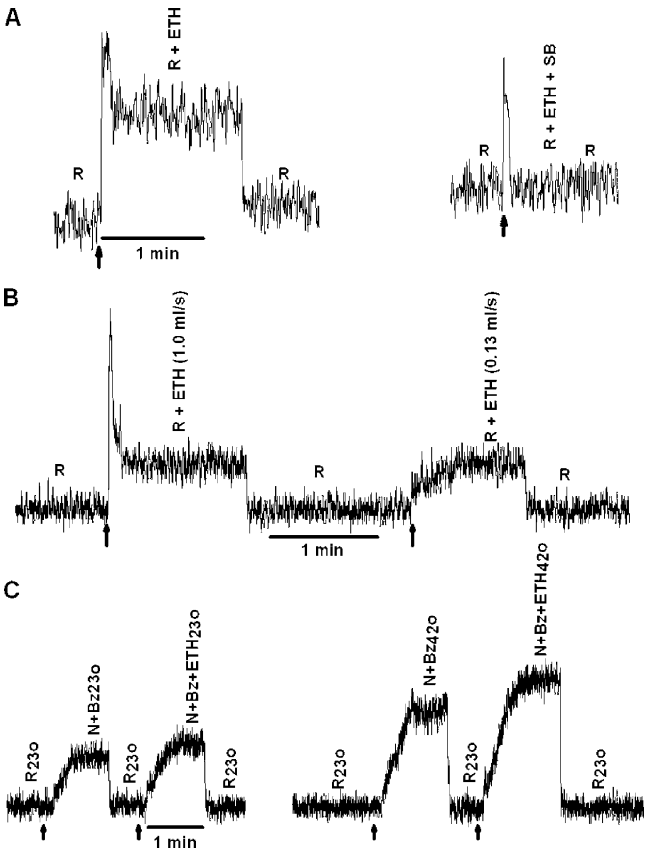

FIGURE 10. Effect of SB-366791, flow rate, and temperature on the CT response to mineral salts. (A) Rat tongue was stimulated with 10 mM KCl (R), 10 mM KCl + 40% ethanol (R + ETH), or 10 mM KCl + 40% ethanol + 0.1 μM SB-366791 (R + ETH + SB). The time period at which the rat tongue was superfused with different solutions is indicated by arrows. (B) CT response was monitored while the rat tongue was first rinsed with 10 mM KCl (R) and then stimulated with 10 mM KCl + 60% ETH (R + ETH). The tongue was superfused with the rinse solution at the rate of 1 ml/s while the stimulating solutions were perfused at the rate of 1 ml/s or 0.13 ml/s. The tongue was stimulated with 300 mM NH_4Cl (1 ml/s) to obtain a reference CT response. The data were normalized to the 0.3 M NH_4Cl CT response in each animal. (C) Rat tongue was stimulated with 10 mM KCl maintained at 23°C ($R_{23°}$) and then with 10 mM KCl + 100 mM NaCl + 5 μM Bz (N + Bz) or with 10 mM KCl + 100 mM NaCl + 30% ethanol (N + Bz + ETH) maintained at 23°C or 42°C. The time period at which the rat tongue was superfused with different solutions is indicated by arrows.

tongue is stimulated with the test solutions (Lyall et al., 2001). To test this hypothesis, the tongue was superfused with salt solutions at the rate of 1 ml/s or at 0.13 ml/s. In the experiment shown in Fig. 10 B, the tongue was first stimulated with a rinse solution containing 10 mM KCl (R) and then with 10 mM KCl + 60% ethanol (R + ETH). In the presence of 10 mM KCl, ethanol again elicited CT responses that were comprised of both a fast phasic component and a slow tonic component. These two components were observed when the flow rate was 1 ml/s. When the flow rate was reduced to 0.13 ml/s, stimulating the tongue with R + ETH elicited a tonic response with small or negligible phasic response. It should be noted that at the flow rate of 0.13 ml/s, the tonic phase achieved the same maximum amplitude as during the flow rate of 1 ml/s (Lyall et al., 2001).

Effect of Temperature on the CT Response to Mineral Salts in the Presence and Absence of Ethanol. The lingual surface was superfused with salt solutions using syringe pumps and heating coils to maintain the temperature at 23°C or 42°C. Similar to the data shown in Fig. 10 B, superfusing the tongue with 100 mM NaCl + 5 μM Bz (23°C) at the rate of 0.13 ml/s elicited only the tonic component of the Bz-insensitive NaCl CT response (N + $Bz_{23°}$). Superfusing the tongue with 100 mM NaCl + 5 μM Bz + 30% ethanol at 23°C (N + Bz + $ETH_{23°}$) enhanced the CT response relative to NaCl + Bz (N + $Bz_{23°}$). Stimulating the tongue with 100 mM NaCl solution maintained at 42°C (N + $Bz_{42°}$) increased the CT response relative to 23°C. In the final step, stimulating with 100 mM NaCl + 5 μM Bz + 30% ethanol at 42°C (N + Bz + $ETH_{42°}$) further enhanced the CT response relative to NaCl at 23°C (N + $Bz_{23°}$) or 42°C (N + $Bz_{42°}$). In three animals, increasing the temperature of 100 mM NaCl + 5 μM Bz solution from 23°C to 42°C increased the normalized CT response from 0.16 ± 0.005 to 0.32 ±0.012. In solutions containing 100 mM NaCl + 5 μM Bz + 30% ethanol, increasing the temperature from 23°C to 42°C increased the normalized CT response from 0.20 ± 0.002 to 0.42 ±0.02 ($P < 0.01$; paired; $N = 3$). The results indicate that both the elevated temperature and ethanol produce synergistic effects on the Bz-insensitive NaCl CT response (Lyall et al., 2005b). A similar increase in the CT responses to 10 mM KCl or 10 mM KCl + ethanol was observed at elevated temperature (Lyall et al., 2005a). VR-1 agonists (resiniferatoxin and capsaicin) and elevated temperature also produced additive effects on the Bz-insensitive NaCl CT response (Lyall et al., 2004b, 2005c).

DISCUSSION

Ethanol elicits neural responses in several species, including humans (Diamant et al., 1963; Hellekant, 1965a,b; Hellekant et al., 1997; Sako and Yamamoto, 1999; Danilova and Hellekant, 2000). The temporal profiles of the CT responses to ethanol stimulation differ widely among species. In rat, cat, and dog, ethanol increased tonic responses with small or negligible phasic responses (Hellekant, 1965a; Sako and Yamamoto, 1999). In cat and dog, ethanol was shown to produce an initial depression in the CT response followed by the slow onset of discharge (Hellekant, 1965a). In contrast, in a primate model, the response of ethanol consisted of a phasic part followed by a sustained tonic response. The tonic response demonstrated no declina-

tion at ethanol concentrations >3 M and increased with time (Hellekant et al., 1997). While some of the above differences in the CT response profile can certainly be attributed to variations among species, it is likely that differences in the CT response profiles also arise due to the different experimental conditions employed to investigate ethanol responses in the above studies. In some studies, the CT responses were monitored after adapting the tongue to H_2O rinse (Sako and Yamamoto, 1999) and in others to the artificial saliva (Hellekant et al., 1997). In some studies, ethanol responses were investigated in a mixture with NaCl, sucrose, quinine-HCl, citric acid, or HCl containing varying concentrations of ethanol (Hellekant et al., 1997; Sako and Yamamoto, 1999). In yet other studies, the tongue was stimulated with ethanol solutions at temperatures varying between 29°C and 33°C (Hellekant, 1965a), at 33°C (Hellekant et al., 1997), or at room temperature (25°C) (Sako and Yamamoto, 1999). While in some studies, the tongue was stimulated with ethanol solutions at the rate of 0.5 ml/s (Sako and Yamamoto, 1999) and in others at 1.6 ml/s (Hellekant, 1965a). In this study, we demonstrate that CT response profiles to ethanol stimulation differ significantly depending upon the ethanol concentration, presence or absence of mineral salts, temperature, flow rate, osmolarity, and the presence or absence of specific agonists and antagonists of the VR-1 receptor. The specific effect of each condition, as it relates to the effect of ethanol on the CT response profile is discussed below.

Effect of Ethanol in the Absence of Mineral Salts

Stimulating the tongue with ethanol solutions diluted with deionized H_2O (i.e., in the absence of permeable ions) elicited only transient phasic CT responses with duration of <2 s. The magnitude of the phasic response was unaffected by ethanol concentration, temperature, and presence or absence of SB-366791 (Fig. 7). The ethanol-induced transient phasic responses were quite variable and were not observed in all preparations. In some preparations, transient phasic responses were also obtained with water rinses following lingual stimulation with 100% ethanol (Fig. 7 A). The in vitro studies in polarized fungiform TRCs suggest that the transient phasic responses in the absence of ions or in the presence of nonpermeable cations, such as, $NMDG^+$, are related to ethanol-induced transient decrease in cell volume, given the fact that solutions containing ethanol at concentrations between 10 (1.7 M) and 60% (10.2 M) are extremely hyperosmotic. This hypothesis is supported by the observations that stimulating the tongue with ethanol increases the transepithelial electrical resistance across the tongue in vivo (Fig. 9 B). Consistent with this, stimulating the tongue with hypertonic mannitol solutions decreased TRC volume and increased transepithelial electrical resistance across the tongue in vivo (Lyall et al., 1999).

The effect of ethanol on TRC volume was demonstrated by our studies in polarized TRCs in vitro. In TRCs loaded with Na-green and perfused on both sides with 0 Na^+ Ringer's solution containing $NMDG^+$, stimulating the apical membrane with ethanol caused a transient dose-dependent increase in F_{490} that recovered spontaneously toward baseline (Fig. 3). As stated earlier, in the absence of Na^+, the transient increase in F_{490} induced by ethanol is indicative of a transient decrease in cell volume. A decrease in TRC volume will increase dye concentration within the cell and an increase in the fluorescence intensity, without an apparent change in $[Na^+]_i$. A transient increase in FIR (F_{340}/F_{380}) was also observed with SBFI. As stated earlier, the FIR of SBFI is insensitive to changes in cell volume. However, in this case, the increase in FIR is most likely the result of a cell shrinkage–induced elevation in the residual TRC $[Na^+]_i$ or an increase in $[K^+]_i$. An increase in $[K^+]_i$ in the presence of very low $[Na^+]_i$ may interact with SBFI to increase FIR. Since changes in fluorescence demonstrated spontaneous recovery toward baseline (Fig. 3 and Fig. 5 A), it suggests that cell volume also recovers spontaneously in the absence of external Na^+. The data further suggest that Na^+-independent transport mechanisms in TRC membranes are involved in regulatory volume increase following stimulation with ethanol.

Similarly, in the absence of external Na^+, washout of ethanol produced transient decreases in F_{490} in TRCs loaded with Na-green that spontaneously increased toward baseline (Fig. 3). This suggests that the increase in cell volume upon ethanol washout also recovers spontaneously in the absence of external Na^+. The data further suggest that Na^+-independent transport mechanisms in TRC membranes are involved in regulatory volume decrease (RVD) following the washout of ethanol. Urea, which also readily permeates the cell membranes, produces a transient decrease in TRC volume (Lyall et al., 1999). However, at present, the identities of specific Na^+-independent volume regulatory mechanisms in TRCs membranes are unknown.

The evidence that in the absence of permeable ions the ethanol-induced decrease in TRC volume is related to the transient phasic CT response is provided by our studies with mannitol. Consistent with previous studies (Lyall et al., 1999), hypertonic mannitol solutions elicited a transient phasic CT response (Fig. 8 A), indicating a link to a decrease in TRC volume. Second, preshrinking TRCs with 0.5 M mannitol reduced the magnitude of the ethanol-induced transient phasic CT response. At present, the cellular mechanisms that link a decrease in cell volume to the transient phasic CT response are unknown. We hypothesize that osmotic cell shrinkage induces changes in one or more membrane

conductances that are responsible for generating the transient phasic response (Schwiebert et al., 1994; Koch and Korbmacher, 2000).

Consistent with our results, low luminal ethanol (5%) decreased cell volume in epithelial cells by opening basolateral Ca^{2+}-dependent K^+-selective channels via Ca^{2+} signaling pathway (Mustonen and Kivilaakso, 2003; Mustonen et al., 2004, 2005) In addition, ethanol has been shown to modify F-actin content in rat pancreatic acinar cells (Siegmund et al., 2004). Thus, it is likely that ethanol-induced changes in cell volume may also involve changes in the cytoskeleton of TRCs. However, at present, the exact mechanisms involved in ethanol-induced changes in TRC volume are not known.

In our studies, both ethanol (Fig. 9 B) and mannitol (Lyall et al., 1999) increased the relative lingual epithelial resistance. In contrast to this, in both isolated *Necturus* gastric mucosa (Mustonen and Kivilaakso, 2003) and Caco-2 cell monolayers (Rao et al., 2004), ethanol produced a transient decrease in transepithelial electrical resistance, indicating that ethanol increases paracellular permeability. This is consistent with the role of ethanol as a "barrier breaker" in gastric mucosa (Mustonen and Kivilaakso, 2003; Mustonen et al., 2004, 2005). Disruption of the gastrointestinal barrier function and the diffusion of luminal toxins and pathogens into the systemic circulation are central to the pathogenesis of a number of diseases (Rao et al., 2004). In this respect, the effect of ethanol in TRCs is different from its effect on the gastrointestinal epithelial cells.

Effect of Ethanol in the Presence of Mineral Salts

In the presence of mineral salts, ethanol enhanced the apical entry of cations and produced CT responses that are similar to salt responses; i.e., in the presence of mineral salts, ethanol produced both a phasic component and a sustained tonic component of the CT response (Lyall et al., 2005b). An important factor that determines if both phasic and tonic components are observed in CT recordings is the rate at which the tongue is stimulated with ethanol (Fig. 10, B and C). Stimulating the tongue with a low flow rate (0.13 ml/s) demonstrated only a slowly rising tonic phase of the CT response. In contrast, stimulating the tongue with a higher flow rate of 1 ml/s demonstrated a rapid phasic response that decreased to a sustained tonic phase. It is possible that differences between studies in which ethanol was reported to increase tonic responses with small or negligible phasic responses (Hellekant, 1965a; Sako and Yamamoto, 1999) and the present study may be due to differences in the rate at which the tongue was stimulated with ethanol solutions. However, it is important to note that at a lower rate, the slowly rising tonic phase attains the same magnitude as with a relatively high flow rate (Lyall et al., 2001).

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 (Figs. 3–6). 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. Further support for this idea comes from the studies with SBFI. Ethanol also produced a monotonic increase in FIR (F_{340}/F_{380}) in SBFI-loaded TRCs (Fig. 5 A). Since changes in FIR are independent of volume changes, the increase in FIR reflects an increase in TRC $[Na^+]_i$. The results indicate that ethanol increases the Bz-insensitive Na^+ conductance in the apical membrane of TRCs.

Ethanol induced transient changes in TRC volume in the presence of $NMDG^+$, an ion that does not permeate the Bz-insensitive VR-1 variant cation channel (Lyall et al., 2004b). Since it did not induce volume changes in the presence of apical Na^+ (Fig. 3), an ion that readily permeates the channel, it suggests that during ethanol stimulation, the apical entry of Na^+ prevents a change in cell volume. Thus, apical Na^+ entry through the Bz-insensitive VR-1 variant cation channel in TRCs may serve as a volume regulatory mechanism during stimulation with ethanol and other VR-1 agonists. In agreement with this, ethanol produced sustained CT responses consisting of both a phasic and a tonic response in the presence of mineral salts (Figs. 9 and 10), and these responses were not affected by preshrinking TRCs in vivo by pretreating the lingual surface with hypertonic mannitol rinse solutions (unpublished data).

Ethanol reversibly increased the unilateral Bz-insensitive Na^+ flux across the apical membrane of polarized fungiform TRCs (Figs. 3–6) and specifically enhanced the Bz-insensitive component of the NaCl CT response (Fig. 9) without affecting the Bz-sensitive ENaC component of the NaCl CT response. Ethanol and elevated temperature produced additive effects on the Bz-insensitive NaCl CT response (Fig. 10 C). VR-1 antagonists CZP or SB-366791 inhibited both the ethanol-induced increase in apical Na^+ flux and the increase in the Bz-insensitive NaCl CT response (Fig. 10 A). Taken together, these results indicate that ethanol increases the Bz-insensitive NaCl CT response by modulating the apical VR-1 variant nonspecific cation channel in TRCs (Lyall et al., 2004b, 2005c). Consistent with this study in rat gastric epithelial cells, which also express VR-1, ethanol produced cell damage by interacting directly with the VR-1 nonselective cation channel (Kato et al., 2003).

Ethanol is membrane permeable and may get to the basolateral membrane from the apical side across tight

junctions. Thus, it is possible that ethanol produces its effects at the basolateral membrane of TRCs. Ethanol specifically increased the unilateral Bz-insensitive Na^+ flux across the apical membrane of polarized TRCs (Figs. 3–5), and simultaneous topical lingual application of ethanol + 0.1 μM SB-366791 completely inhibited the ethanol-induced increase in the tonic CT response to NaCl (Fig. 10 A). The low concentration of SB-366791 used and the fact that SB-366791 inhibited ethanol responses reversibly without a delay suggests that SB-366791 acts on the apical membrane to inhibit the ethanol response. Under these conditions, it is most unlikely that SB-366791 crosses tight junctions and reaches basolateral membrane in sufficient concentration to inhibit ethanol effects on the basolateral membrane. Furthermore, in the absence of apical cations, ethanol produced completely different effects on TRCs in vitro (Fig. 3) and on the CT responses in vivo (Fig. 8). These results strongly suggest that ethanol effects occur at the apical membrane.

The lingual epithelial preparation contains, in addition to an intact fungiform taste bud, nontaste cells, such as squamous epithelial cells, cells surrounding the taste buds, truncated nerve fibers, and connective tissue. It is, therefore, possible that ETH may produce some of its effects via a secondary mechanism that involves the release of peptides or other activators of VR-1 from nerve fibers or nontaste cells. Neuropeptide effects on taste responses have been convincingly demonstrated (Simon et al., 2003). While indirect effects cannot be ruled out entirely, the fact that ethanol causes an increase in the Na^+ flux into TRCs in isolated taste bud fragments, devoid of nerve fibers and nontaste cells, indicates that direct action of ethanol on TRCs is a distinct mode of taste cell modulation by ethanol. This conclusion is strengthened by the fact that Na^+ flux measurements were performed on taste bud fragments during continuous perfusion with the Ringer's solution, so that a significant accumulation of secretory products from any source would be unlikely. Finally, the ethanol-induced increase in Bz-insensitive Na^+ flux in TRCs in isolated fungiform taste bud fragments was blocked by SB-366791 (Fig. 6 C). This is an indication that the likely locus of ethanol interaction with TRCs is the apical membrane VR-1 variant taste receptor.

At present, the exact mechanism of how ethanol modulates the VR-1 variant cation channel is not known. However, it is likely that ethanol, like other agonists of the TRPV1 channel, functions as a gating modifier of the channel. It was recently demonstrated that TRPV1 is activated by depolarization. Increases in temperature resulted in a graded shift of its voltage-dependent activation curve. In addition, the activation of the channel by capsaicin shifted the channel activation curve toward physiological membrane potentials (Voets et al., 2004). Thus, it is likely that ethanol also shifts the equilibrium of the VR-1 cation channel in the open state, resulting in increased channel activity at more physiological voltages in TRCs.

It is important to note that isolated TRCs are not polarized and when stimulated, both apical and basolateral membranes are exposed to ethanol. In contrast, in polarized TRCs, ethanol specifically stimulates the VR-1 variant cation channels in the apical membrane of TRCs. Therefore, it is likely that cell volume changes and ethanol dose–response relationships may be quite different from those obtained in polarized TRCs.

In summary, the results suggest that in the absence of permeable cations, ethanol decreases TRC volume, increases transepithelial electrical resistance across the lingual epithelium, and elicited only transient phasic CT responses. In the presence of NaCl or KCl, ethanol produced CT responses that are similar to salt responses, comprised of both a phasic and a tonic component. At concentrations between 10 and 50% ethanol enhanced the Bz-insensitive Na^+ flux across the apical membrane of polarized TRCs without a change in cell volume and enhanced the magnitude of the Bz-insensitive NaCl CT response. Elevating the temperature from 23°C to 42°C further increased the Bz-insensitive NaCl CT response in the presence of ethanol. Both the ethanol-induced increase in the Bz-insensitive apical Na^+ flux and the increase in the Bz-insensitive NaCl CT response were blocked by the VR-1 antagonists SB-366791 and CZP. We conclude that ethanol modulates the VR-1 variant nonspecific cation channel in the apical membrane of TRCs.

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