EFFECT OF NICOTINE ON CHORDA TYMPANI RESPONSES TO SALTY AND SOUR STIMULI

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ABSTRACT

The effect of nicotine on the benzamil (Bz)-insensitive (transient receptor potential vanilloid-1 variant cation channel, TRPV1t) and the Bz-sensitive (epithelial Na\(^+\) channel, ENaC) salt taste receptors and sour taste was investigated by monitoring intracellular Na\(^+\) and H\(^+\) activity (pHi) in polarized fungiform taste receptor cells (TRCs) and the chorda tympani (CT) nerve responses to NaCl, KCl and HCl. CT responses in Sprague-Dawley rats, wildtype and TRPV1 knockout (KO) mice were recorded in the presence and absence of agonists (resiniferatoxin and elevated temperature) and an antagonist (SB-366791) of TRPV1t, the ENaC blocker (Bz), and varying apical pH (pH\(_o\)). At concentrations <0.015M, nicotine enhanced and >0.015M, it inhibited CT responses to KCl and NaCl. Nicotine produced maximum enhancement in the Bz-insensitive NaCl CT response between pH\(_o\) 6 and 7. Resiniferatoxin and elevated temperature increased the sensitivity of the CT response to nicotine in salt-containing media, and SB-366791 inhibited these effects. TRPV1 KO mice demonstrated no Bz-insensitive CT response to NaCl and no sensitivity to nicotine, resiniferatoxin, and elevated temperature. We conclude that nicotine modulates salt responses by direct interaction with TRPV1t. At pH\(_o\) >8, the apical membrane permeability of nicotine was increased significantly, resulting in increase in TRC pHi and volume, activation of ENaC, and enhancement of the Bz-sensitive NaCl CT response. At pH\(_o\) >8, nicotine also inhibited the phasic component of the HCl CT response. We conclude that the effects of nicotine on
ENaC and the phasic HCl CT response is due to increase in TRC pH and volume.

**Keywords:** Salt taste, sour taste, benzamil, resiniferatoxin, SB-366791
INTRODUCTION

Nicotine is found in tobacco leaves and thus is a component of tobacco products. Nicotine is a gustatory stimulus. It elicits responses in both glossopharyngeal and chorda tympani (CT) taste nerves (Dahl et al. 1997) and in neurons of the nucleus of the solitary tract (Lemon and Smith 2005). Nicotine also elicited responses in single gustatory neurons from the insular cortex in monkeys (Scott et al. 1999). Nicotine is perceived as predominantly bitter by humans but its effects on other taste qualities are presently unknown. Two additional properties of nicotine suggest that it could be a potential modulator of both salty and sour taste. First, nicotine has been shown to modulate the transient receptor potential vanilloid-1 receptor (TRPV1) (Liu et al. 2004). Accordingly, we tested the hypothesis that nicotine modulates the CT taste nerve responses to NaCl and other mineral salts by interacting with the amiloride- and benzamil (Bz)-insensitive TRPV1 variant salt taste receptor, (TRPV1t) (Lyall et al. 2004b, 2005a,b,c; Simon and De Araujo 2005). The above hypothesis is based on the observations that in rat and mouse fungiform TRCs TRPV1t is a constitutively active non-selective cation channel present in the apical membrane of TRCs and is derived from the TRPV1 gene. All of the Bz-insensitive CT taste nerve responses to Na⁺ salts, and part of the response to K⁺, NH₄⁺, and Ca²⁺ salts is elicited by cation flux through TRPV1t (Lyall et al. 2004b; 2005a,b,c). However, TRPV1 knockout (KO) mice maintain normal salt detection performance, suggesting that there may be other amiloride- and Bz-insensitive salt transduction mechanisms in taste receptor fields other than the anterior
tongue (Ruiz et al. 2006; Treesukosol et al. 2007). TRPV1t is activated by vanilloids: resiniferatoxin (RTX) and capsaicin (CAP) and by elevated temperatures (>38°), and is inhibited by the TRPV1 antagonists (capsazepine and N-(3-methoxyphenyl)-4-chlorocinnamide (SB-366791)). TRPV1t demonstrates many similarities with the cloned TRPV1 (Lyall et al. 2005c). Therefore, it is expected that agonists that either activate or sensitize the TRPV1 channel, such as, endocannabinoids, lipoxygenase metabolites of arachidonic acid, lipid derivatives, nicotine, ethanol, H⁺, and intracellular second messengers (Davis et al. 2002; Trevisani et al. 2002; Geppetti and Trevisani 2004; Gunthorpe et al. 2002; Liu et al. 2004; Lyall et al. 2004b; 2005a,b,c) will also have similar effects on TRPV1t. To test if nicotine interacts specifically with TRPV1t, additional studies were conducted to determine if nicotine also affected the amiloride- and Bz-sensitive epithelial Na⁺ channels (ENaCs) in the apical membrane of TRCs.

Second, in solution nicotine behaves as a base and has the potential to alter cell pH. Accordingly, we also tested the hypothesis that upon entry into TRCs, nicotine alkalinizes resting intracellular pH (pHi) and inhibits CT responses to acidic stimuli. This hypothesis is based on the observations that the proximate stimulus for sour taste transduction is an acid-induced decrease in pHi in a subset of taste bud cells, the acid-sensing TRCs (DeSimone and Lyall 2006). PKD2L1, a polycystic-kidney-disease-like ion channel, expressed in a subset of TRCs is most likely involved in pH sensing (Huang et al. 2006; Ishimaru et al. 2006; LopezJimenez et al. 2006). In particular, transduction mechanism for the
phasic component of the CT response to acidic stimuli involves a decrease in pH, that results in TRC shrinkage through the change in F-actin to G-actin equilibrium. Cell shrinkage activates a flufenamic acid-sensitive non-specific cation conductance in the basolateral membrane of TRCs that is responsible for the elicitation of the phasic response to acid stimulation (Lyall et al. 2006). Nicotine can potentially modulate one or more steps in this transduction pathway.

The interactions between nicotine and the amiloride-insensitive and amiloride-sensitive salt taste receptors were investigated by measurement of TRC Na⁺ ([Na⁺]ᵢ) and H⁺ (pHᵢ) activity in polarized rat fungiform TRCs and by the CT taste nerve recordings (Lyall et al. 2005c). The CT responses were monitored in two animal models, a rat model and a TRPV1 KO mouse model (Caterina et al. 2000; Lyall et al. 2004b, 2005a,c). CT responses were recorded while the tongue was stimulated with nicotine alone and in mixtures with mineral salts in the absence and presence of specific agonists (RTX and elevated temperature) and antagonists (SB-366791) of TRPV1t (Lyall et al. 2004b, 2005a,c) and in the presence of Bz, a specific ENaC blocker. The interactions between nicotine and the acidic stimuli were investigated by the measurement of TRC pHᵢ and cell volume in polarized rat fungiform TRCs and by the CT recordings while stimulating the tongue with HCl (Lyall et al. 2006). The results presented in this paper support the conclusion that nicotine modulates the CT responses to mineral salts by interacting with both TRPV1t and ENaC and it modulates CT responses to acidic stimuli by inducing intracellular alkalinization and cell swelling.
MATERIALS AND METHODS

In this study we used the combined methodology of in vitro imaging and in vivo neural recordings to investigate the interaction of nicotine with TRPV1t and ENaC in the apical membranes of fungiform TRCs (Simon 2002).

In vitro studies

pH$_i$ and cell volume measurements in polarized fungiform TRCs.

Simultaneous measurement of cell volume changes and intracellular pH (pH$_i$) were made using the pH-sensitive dye BCECF (Molecular Probes, Eugene, OR, USA) and recording at both the pH-sensitive and pH-insensitive (isosbestic) wavelengths (Lyall et al. 2006). Changes in pH$_i$ were monitored as variations in the fluorescence intensity ratio (FIR; $F_{490}/F_{440}$) of BCECF (Lyall et al. 2001). At the end of each experiment, the changes in TRC pH$_i$ were calibrated by bilateral perfusion of high K$^+$ solutions containing 10X10$^{-6}$ M nigericin adjusted to pHs between 6.5 and 8.0 (Table 1; HK). The relative changes in TRC volume were monitored at the isosbestic wavelength of 440 nm ($F_{440}$). The fluorescence intensity at this wavelength is independent of pH and reflects the dye concentration inside the cell (Lyall et al. 2006).

[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 the single wavelength dye sodium green or the ratiometric dye 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) (SBFI) (both from Molecular Probes) as described earlier (Lyall et al. 2005a). Changes in TRC [Na+]i were monitored in the presence and absence of Bz or SB-366791. This was done to distinguish between the apical Na+ flux through the Bz-sensitive ENaCs and the Bz-insensitive TRPV1t in fungiform TRCs (Lyall et al. 2004b; 2005a,b). The relative changes in TRC [Na+]i were expressed as percent change in fluorescence intensity (F490) of sodium green or as changes in FIR (F340/F380) relative to apical zero Na+ concentration. In individual taste buds, the data were presented as the mean±standard error (M±SEM) of n, where n represents the number of regions of interest (ROIs) within the taste bud. The data were also presented as the M±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. Since we are comparing the fluorescence intensity, FIR and pH, values before and after nicotine treatment in the same polarized taste bud preparation, paired t-test was used to evaluate statistical significance.

Solutions. The composition of various solutions used in the in vitro experiments is given in Table 1.

In vivo studies

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 gm) 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° with a Deltaphase Isothermal PAD (Model 39 DP; Braintree Scientific, Inc., Braintree, MA, USA). 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 referenced to the oral cavity (Lyall et al. 2005a,b).

The anterior lingual surface was stimulated with a rinse solution (R; 0.01M KCl) and with NaCl solutions (0.01 M KCl+0.1M NaCl; N) containing nicotine (Nic; 0-0.1M). In some experiments both rinse (R) and NaCl solutions (N) with or without nicotine contained, in addition, 0.01M 4-(2-hydroxyethyl)-piperazine-1-ethanesulphonic acid (HEPES) or 0.01M Tris[hydroxymethyl]-aminomethane (TRIS) and were adjusted to different pHs with 1N NaOH or HCl. CT responses were recorded in the presence of benzamil (Bz; 5X10⁻⁶ M), a specific and potent blocker of the apical ENaC. CT responses were also recorded in the presence of the TRPV1 agonists, resiniferatoxin (RTX; 0.25X10⁻⁶ M) or elevated temperatures (23°-55.5°), and a TRPV1 antagonist, SB-366791 (0.25X10⁻⁶-1X10⁻⁶ M) (Lyall et al. 2004b; 2005a,c). All drugs were purchased from Sigma, St. Louis, MO, USA. Typically, stimulus solutions remained on the tongue for 2 min. Control stimuli
consisting of 0.3M NaCl and 0.3M NH₄Cl, applied at the beginning and at the end of the experiment were used to assess preparation stability. To compute the phasic or transient component of the response, we first obtained the area under the CT response curve from the onset of the stimulus to the onset of the tonic or time invariant part of the response. To normalize this result, this area was divided by the area under the 0.3 M NH₄Cl response curve over the final 30s of the tonic response period. The normalized data were reported as mean±SEM of the number of animals (N). Student’s t-test was employed to analyze the differences between sets of data. Since we are comparing the normalized CT responses before and after nicotine treatment in the same CT preparation, paired t-test was used to evaluate statistical significance.

To investigate the effect of temperature on the CT response to nicotine and to mixtures of nicotine+mineral salts, the lingual surface was superfused (8 ml/min) with salt solutions using syringe pumps and heating coils maintained between 23° and 55.5° as described before (Lyall et al. 2004b, 2005a).

CT responses were also monitored in Wildtype (WT; C57BL/6J) and homozygous TRPV1 KO mice (B6.129S4-Trpv1<sup>tmjul</sup>, The Jackson Laboratory, Bar Harbor, ME, USA). Mice (30-40 gm) 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 (approximately 195 mg/Kg body weight for rats and 150 mg/Kg body weight for mice).
RESULTS

In vitro studies

Effect of nicotine on TRC pH$_i$ and volume

Nicotine has two pKs 6.16 and 10.96. Nicotine is a base and upon entering TRCs it is expected to produce intracellular alkalinization. Accordingly, in a lingual epithelial preparation perfused on both sides with control solution (Table 1; C; pH 7.4) increasing nicotine concentration in the basolateral compartment (BL$_{\text{Nic}}$) in a stepwise manner from 0.0005 to 0.01M produced a dose-dependent increase in TRC pH$_i$ (Fig. 1A). The relationship between basolateral nicotine concentration and TRC pH$_i$ was almost linear ($\text{pH}_i = 0.028 \ [\text{Nic}] + 7.24; r^2 = 0.95; n=8$). In contrast, perfusing the apical membrane with 0.025M nicotine at pH 7.4 produced only a small increase in TRC pH$_i$ over a 5 min period (Fig. 1B). The increase in pH$_i$ was not reversed upon nicotine washout. Essentially similar results were obtained in 2 additional experiments. The results suggest that at physiological pH, the nicotine permeability of the TRC basolateral membrane is significantly greater than that of the apical membrane.

The membrane permeability of nicotine was strongly dependent upon pH$_o$. At pH 6.5 (BL$_{\text{pH}}$), basolateral nicotine (0.005M) produced a significantly smaller increase in TRC pH$_i$ (Fig. 1C; a-b-c) relative to pH 7.4 (Fig. 1C; d-e-f). At apical pH of 6.5 (AP$_{\text{pH}}$), apical nicotine (0.025M) produced a small decrease in pH$_i$ (Fig. 1D; a-b-c) that was completely reversible. In contrast, at apical pH of 9.0, nicotine produced a rapid increase in pH$_i$ (Fig. 1D; d-e-f). Essentially similar results were obtained in 2 additional experiments. The results suggest that at alkaline pH, the
Apical membrane permeability to nicotine is significantly enhanced. Upon nicotine washout TRC pH\textsubscript{i} did not return to baseline, suggesting that at this concentration the nicotine effects are not completely reversible.

Nicotine-induced alkalinization was accompanied by a decrease in F\textsubscript{440}, indicating cell swelling (Fig. 2A). Cell swelling will cause a decrease in dye concentration inside the cells and a decrease in F\textsubscript{440}. Nicotine-induced increase in pH\textsubscript{i} and a concomitant decrease in F\textsubscript{440} was also observed when TRCs were perfused on both sides with 0 Na solution (Table 1; 0Na; pH 7.4), 0Na-0K solution (Table 1; ONa-0K; pH 7.4) and 0Na-0K-0Cl solution (Table 1; ONa-0K-0Cl; pH 7.4) (data not shown). These results support the conclusion that nicotine-induced intracellular alkalinization is due to the entry of nicotine across the apical or basolateral membrane \textit{per se} and does not involve the activation of Na\textsuperscript{+}, K\textsuperscript{+}, Cl\textsuperscript{-} or HCO\textsubscript{3}\textsuperscript{-}-dependent pH regulatory mechanisms. Since in the absence of Na\textsuperscript{+} TRC pH\textsubscript{i} becomes acidic (Lyall et al. 2004a), it suggests that the membrane permeability to nicotine is not modulated by changes in pH\textsubscript{i}. The results further suggest that nicotine-induced cell swelling is also the result of an increase in TRC pH\textsubscript{i} \textit{per se} and does not involve secondary activation of Na\textsuperscript{+}, K\textsuperscript{+}, Cl\textsuperscript{-} or HCO\textsubscript{3}\textsuperscript{-}-dependent transport mechanisms (Lyall et al. 2006).

**Effect of nicotine on the unilateral apical Na\textsuperscript{+} flux in polarized fungiform TRCs**

Na\textsuperscript{+} enters TRCs across the apical membrane via two pathways. One pathway is blocked by apical amiloride or Bz, and represents Na\textsuperscript{+} flux through
ENaC. The second pathway is insensitive to amiloride or Bz, and represents Na$^+$ flux through the TRPV1t cation channel and is inhibited by SB-366791 (Lyall et al. 2002b, 2004b, 2005a). Figure 2B shows the effect of apical nicotine ($\text{AP}_{\text{Nic}}$) on the Bz-insensitive Na$^+$ flux across the apical membrane of TRCs. A lingual epithelial preparation loaded with SBFI was initially perfused on both sides with Na$^+$-free solution (Table 1; 0Na; pH 7.4). Perfusing the apical membrane with control solution containing 0.15M NaCl+5X10$^{-6}$ M Bz+0.015 M nicotine (Table 1; C; pH 7.4) increased FIR ($a$-$b$). Subsequently perfusing the control solution with Bz but without nicotine decreased FIR ($b$-$c$). Nicotine also produced an increase in Bz-insensitive apical Na$^+$ flux in isolated taste bud fragments in which the basolateral Na$^+$-H$^+$ exchanger-1 (NHE-1) was blocked by 10X10$^{-6}$ M zoniporide (Lyall et al. 2004a) (data not shown). The nicotine-induced increase in the Bz-insensitive apical Na$^+$ flux was blocked in the presence of 1X10$^{-6}$ M SB-366791 (data not shown). Under these conditions no effect of nicotine was observed on the Bz-sensitive unilateral Na$^+$ flux (data not shown). These results suggest that nicotine produces a greater unilateral Bz-insensitive apical Na$^+$ flux relative to control through the TRPV1t cation channel (Lyall et al. 2005b).

Figure 2C shows the effect of basolateral nicotine ($\text{BL}_{\text{Nic}}$) on the Bz-sensitive Na$^+$ flux across the apical membrane of TRCs. A lingual epithelial preparation loaded with Na-green was initially perfused on the apical side with Na$^+$-free solution (Table 1; 0Na; pH 7.4) and on the basolateral side with control solution (Table 1; C; pH 7.4). Perfusing the apical membrane with control solution containing 0.15M NaCl+1X10$^{-6}$ M SB-366791 reversibly increased $F_{490}$ ($a$-$b$-$c$).
SB-366791 inhibits Na⁺ flux through the Bz-insensitive pathway. Thus, the increase in $F_{490}$ represents the Na⁺ flux exclusively through ENaCs. Following this the basolateral membrane was perfused with control solution+0.015 M nicotine (BLNic). In the continuous presence of basolateral nicotine, perfusing the apical membrane with control solution+1X10⁻⁶ M SB-366791 produced a significantly greater increase in $F_{490}$ relative to control ($p<0.01$; n=7; paired). The basolateral membrane of TRCs is readily permeable to nicotine (Figs. 1A and 1C) and upon entering TRCs it produce intracellular alkalinization resulting in the activation of apical ENaC (Lyall et al. 2002b). Taken together the results support the conclusion that nicotine produces differential effects on ENaC and TRPV1t depending upon $pH_o$ and the cell membrane at which TRCs are exposed to nicotine.

**In vivo studies**

**Effect of nicotine on the CT response to 0.01M KCl**

We first investigated if CT responses to 0.01M KCl are modulated by nicotine in a dose-dependent fashion. A rat tongue was initially rinsed with 0.01M KCl+0.01M HEPES (R; pH 6.1) and then with R containing varying concentrations of nicotine (0-0.1M; pH 6.1). Figure 3A shows that in the presence of 0.01M KCl (R) nicotine at 0.005, 0.01, and 0.015M elicited phasic CT nerve response with a rapid onset and decay. However, the rate of return to a steady-state level varied with the nicotine concentration above 0.015M. This is illustrated in the insert above Fig. 3A using an expanded time scale for 0.005M and 0.05M.
nicotine. The nicotine-induced variation in the phasic CT response (phasic 0.01M KCl CT response/0.3M NH₄Cl CT response) in 3 animals is shown in Fig. 4A. The increased response seen at 0.025, 0.05, and 0.1M nicotine is the result of the delayed return to steady-state levels at these concentrations. As seen in Fig. 3A, nicotine presented in R, also produced tonic responses, starting at about 0.025M concentration. The concentration dependence of the tonic response (0.01MKCl/0.3M NH₄Cl) in 3 animals is shown in Fig. 4B (●; 0 SB). Tonic responses peaked at 0.05M nicotine.

Effect of nicotine on the Bz-insensitive part of the CT response to 0.1M NaCl

Liu et al. (2004) showed that nicotine induced an increase in CAP-activated currents in cells with heterologously expressed TRPV1 cation channel, indicating that nicotine sensitizes the channel to activation by vanilloids. We hypothesize that nicotine either activates or serves as a modulator of TRPV1t. Figure 3A shows the effect of nicotine on the tonic CT response to 0.1M NaCl+5X10⁻⁶ M Bz+0.01M HEPES, pH 6.1 (N+Bz). The results show that nicotine enhanced the Bz-insensitive response to NaCl in a dose-dependent manner. The response to N+Bz+0.015M nicotine was 72% greater than the response to N+Bz alone. On the other hand, the response to N+Bz+0.05M nicotine was inhibited by 19% relative to N+Bz. In 3 animals nicotine produced a bell shaped dose-response relationship on the Bz-insensitive CT response to 0.1M NaCl (Fig. 4C; ● 0 SB).
To investigate if nicotine 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 the applied voltage across the receptive field. Figure 3C shows the CT response to 0.1M NaCl+5X10⁻⁶ M Bz in the absence (R+N+Bz) and presence of 0.015M nicotine (R+N+Bz+Nic) as a function of the applied voltage across the receptive field at -60 mV, 0 and +60 mV. As reported earlier (Lyall et al., 2004b; 2005a; 2005c), the rat CT responses to 0.1M NaCl+5X10⁻⁶ M Bz were slightly enhanced at -60 mV and slightly suppressed at +60 mV. In the presence of 0.015M nicotine (N+Bz+Nic), the same voltages exerted significantly larger effects on the response (Fig. 3C). The changes in the CT response at 0, -60 mV and +60 mV in the absence and presence of nicotine from 3 animals are summarized in Fig. 4D. In each case the slope of the relation between CT response and voltage was calculated as described previously (Lyall et al. 2004b). Nicotine (0.015M) increased the slope from (6.37±0.92)x10⁻⁴ response units/mV (●) under control conditions to (13.39±1.36)x10⁻⁴ response units/mV (■; p < 0.05; N = 3; paired). This suggests that in nicotine-sensitive TRCs 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 nicotine increased the unilateral apical Na⁺ flux in polarized fungiform TRCs (Fig. 2).

**Modulatory effect of nicotine is blocked by SB-366791**

To determine if the effects of nicotine on the Bz-insensitive NaCl CT
response are exerted through TRPV1t, we obtained the nicotine concentration versus the Bz-insensitive NaCl CT response function in the presence of SB-366791, a selective competitive inhibitor of both TRPV1 (Gunthorpe et al. 2004) and the TRPV1t (Lyall et al. 2004b, 2005a,b,c).

**Effect of SB-366791 on the CT response to 0.1M NaCl**

Figure 3B shows a typical CT response to 0.1M NaCl+5X10⁻⁶ M Bz (R+N+Bz) obtained in the presence of 0.05X10⁻⁶ M SB-366791. Note that nicotine below 0.025M has no effect on the CT response. Increasing nicotine to 0.05M and 0.1M produced an increase in the CT response. In contrast, under control conditions (Fig. 3A) at these concentrations the response decreased. The results from 3 animals are also summarized in Fig. 4C and show that at a concentration as low as 0.05X10⁻⁶ M, SB-366791 shifts the nicotine concentration versus the tonic response function to the right along the nicotine concentration axis. This is consistent with the idea that SB-366971 acts as a competitive inhibitor of nicotine on the TRPV1t. At 0.25X10⁻⁶ M SB366971 (Fig. 4C; ▲), the rightward shift of the nicotine concentration versus the tonic response function is increased. As reported previously (Lyall et al. 2004b), at this higher concentration of SB-366971, the constitutive activity of the TRPV1t cation channel is also partially blocked.

In the presence of 0.5X10⁻⁶ M SB-366791, the CT response to 0.1M NaCl+5X10⁻⁶ M Bz+0.015M nicotine (R+N+Bz+Nic) was not different from baseline and there was no sensitivity of the NaCl CT responses to the applied voltage across the receptive field (data not shown). This suggests that SB-
366791 not only inhibits the constitutively active Bz-insensitive apical membrane Na\(^+\) conductance but also the nicotine-induced increase in the Na\(^+\) conductance.

*Effect of SB-366791 on the CT response to 0.01M KCl*

The effect of SB-366791 was also observed on the tonic response to nicotine when presented with 0.01M KCl (R). The data shown in Fig. 3B demonstrate that SB-366791 at 0.05X10\(^{-6}\) M had no effect on the CT response to R+nicotine relative to R alone. The mean data from 3 animals are summarized in Fig. 4B (○ versus ●). However, upon increasing SB-366791 to 0.25X10\(^{-6}\) M, the nicotine concentration versus tonic response function was shifted significantly to the right along the nicotine concentration axis (Fig. 4B; ▲ versus ○ or ●).

*Effect of external pH on the CT response to nicotine in 0.01M KCl or 0.1M NaCl*

We have previously shown that changes in pH\(_o\) modulate the effect of vanilloids on TRPV1\(t\) (Lyall et al. 2004b, 2005a). We hypothesize that the effect of nicotine on TRPV1\(t\) will also be modulated by changes in pH\(_o\). In these experiments we held the nicotine concentration at 0.015M (the concentration that produces the maximal enhancement of the tonic response in the medium containing 0.01M KCl+0.1M NaCl+5X10\(^{-6}\) M Bz). Consistent with previous studies (Lyall et al. 2002b), in the absence of nicotine the Bz-insensitive NaCl CT responses were insensitive to pH\(_o\) (Fig. 5A; insert). Nicotine (R+N+Bz+Nic) enhanced the Bz-insensitive NaCl CT response at all pHs relative to (R+N+Bz) alone and produced a bell-shaped tonic CT response function in the presence of
0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz at varying pH₀ values (Fig. 5A). The mean pH₀ versus CT response profile obtained in 3 animals is shown in the insert. In the presence of 0.01M KCl (R), the response to 0.015M nicotine was not significantly affected by changes in pH₀ (insert). The results support the conclusion that the effect of nicotine on TRPV1t in the presence of 0.1M NaCl is modulated by changes in pH₀ and that nicotine enhances the activity of TRPV1t optimally at pH 6-7.

**Effect of elevated temperature on the CT response to nicotine in the presence of 0.1M NaCl**

Next we tested the effect of nicotine on the temperature threshold of the TRPV1t cation channel in the presence of 0.1M NaCl. CT responses to 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz (R+N+Bz) were monitored in the presence and absence of 0.01M nicotine (R+N+Bz+Nic), while the temperature of the solutions was varied between 23° and 55.5°. The tongue was superfused with the rinse solution (R₂₃°; 0.01M KCl), 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz solution (R+N+Bz₂₃°) and R+0.1M NaCl+5X10⁻⁶ M Bz+0.01M nicotine solution (R+N+Bz+Nic₂₃°) maintained at room temperature (23°) at the rate of 3 ml/s. The NaCl solutions at higher temperatures were superfused at the rate of 8 ml/min. At the slower rate of superfusion (8 ml/min) the phasic component of the NaCl CT response is not observed (Lyall et al. 2004b, 2005b). An increase in temperature produced a sharp increase in the CT response to 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz (R+N+Bz) around 38° and gave a maximum enhancement of the CT
response around 42°, and the response decreased above 42° (Fig. 5B). Stimulating with 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz+0.01M nicotine (R+N+Bz+Nic) enhanced the CT response at 23° and at elevated temperatures (Fig. 5B). In 3 animals the temperature at which nicotine produced maximum increase in the CT responses was 42.2°±0.3°, a value not different from R+N+Bz alone (41.9°±0.2°). This is consistent with the idea that nicotine enhances the CT response to NaCl without affecting the temperature threshold of the TRPV1t cation channel.

**Nicotine-RTX interactions with the TRPV1t salt taste receptor**

The Bz-insensitive NaCl CT responses are insensitive to pH₀ and ATP. However, in the presence of a sub-threshold concentration of RTX, a TRPV1 and TRPV1t agonist, the Bz-insensitive NaCl CT responses become sensitive to both pH₀ and ATP (Lyall et al. 2004b, 2005a). Therefore, we tested if a sub-threshold concentration (0.25X10⁻⁶ M) of RTX modulates the effects of nicotine and elevated temperature on the Bz-insensitive NaCl CT response.

Figure 5C shows that superfusing the tongue with 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz+0.25X10⁻⁶ M RTX at 23° (R+N+Bz+RTX) produced a small increase in the Bz-insensitive NaCl CT response relative to 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz (R+N+Bz). Subsequently, superfusing the tongue with 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz+0.25X10⁻⁶ M RTX+0.015M nicotine at 23° (R+N+Bz+RTX+Nic) produced a CT response whose magnitude was significantly greater than 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz+0.015M nicotine at 23°
These results suggest that in the presence of a sub-threshold concentration of RTX, nicotine produces a greater increase in the CT response relative to either RTX or nicotine alone.

We also monitored the sensitivity of the NaCl CT responses to ±60 mV applied voltage across the receptive field in the presence of RTX alone and RTX+Nic. The applied voltages exerted significantly larger effects on the 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz+0.25X10^{-6} M RTX+0.015 M nicotine (R+N+Bz+RTX+Nic) CT response relative to 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz+0.015M nicotine (R+N+Bz+Nic) (Figs. 3C and 4D). The mean data from 3 such experiments are also summarized in Fig. 4D. In the presence of 0.015M nicotine+0.25X10^{-6} M RTX, the slope of the response (21.53±2.38)x10^{-4} response units/mV (■) was greater relative to its value (8.09±2.14)x10^{-4} response units/mV in the presence of RTX alone (●; p < 0.05; N = 3; paired). These results suggest that in the presence of 0.25X10^{-6} M RTX, nicotine produces a greater increase in the apical Bz-insensitive Na^+ conductance relative to 0 RTX.

Next we tested the effect of RTX and nicotine on the temperature threshold of the TRPV1t cation channel in the presence of 0.1M NaCl. The CT responses to 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz (R+N+Bz) were monitored in the presence and absence of 0.01M nicotine (R+N+Bz+Nic) and 0.25X10^{-6} M RTX (R+N+Bz+Nic+RTX), while the temperature of the solution was varied between 23° and 55.5° (Fig. 5C). The addition of 0.25X10^{-6} M RTX to 0.01M nicotine solution enhanced the NaCl CT response and shifted the temperature curve to the left. The mean temperature at which the CT response gave the
maximum response in the presence of 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz+0.015M nicotine (R+N+Bz+Nic) and 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz+0.015M nicotine+0.25X10^{-6} M RTX (R+N+Bz+Nic+RTX) was 42.2^\circ \pm 0.3^\circ and 39.3^\circ \pm 0.1^\circ (N = 3; p <0.05), respectively. The results suggest that nicotine enhances the CT response to NaCl without affecting the temperature threshold of the TRPV1t cation channel. In contrast, a mixture of nicotine and a sub-threshold concentration of RTX, enhanced the magnitude of the NaCl CT response and shifted the temperature curve to the left. The results further suggest that nicotine and RTX act on different sites on the TRPV1t cation channel.

**Effect of nicotine on salt responses in WT and TRPV1 KO mice**

To confirm that nicotine produces its effect on the Bz-insensitive NaCl CT responses by modulating the activity of the TRPV1t, we monitored the effect of nicotine on the CT response to NaCl in WT and TRPV1 KO mice. In WT mice (Fig. 6A), stimulating the tongue with 0.01M KCl+0.1M NaCl (R+N) produced a CT response and a significant part of the CT response was Bz-insensitive (0.01M KCl+0.1M NaCl+5X10^{-6} M Bz; R+N+Bz). Stimulating the tongue with 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz+nicotine (Nic; 0-0.015M) produced a dose-dependent increase in the CT response. The data from 3 WT mice are summarized in Fig. 6B and suggest that CT responses in WT mice are qualitatively similar to the responses observed in rats (Fig. 3) within the range of 0-0.015 M nicotine concentration.
In contrast, TRPV1 KO mice (Fig. 6C) demonstrated no CT response to 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz containing 0.005M (R+N+Bz+0.005M Nic), 0.01M (R+N+Bz+0.01M Nic) or 0.015 M nicotine (R+N+Bz+0.015 Nic). Essentially similar results were obtained in 2 additional KO mice. These results are consistent with the observations that CT salt responses in TRPV1 KO mice are insensitive to TRPV1 agonists (Lyall et al. 2004b, 2005a,b,c).

**Effect of nicotine-induced intracellular alkalinization and cell swelling on the CT responses to NaCl and HCl**

Figure 7A shows the effect of nicotine on the Bz-sensitive and Bz-insensitive NaCl CT responses as a function of apical pH. At pH 6.5 stimulating the tongue with 0.01M KCl+0.1M NaCl+0.015 M Nicotine (R+N+Nic_{6.5}) produced a bigger CT response (e-f) relative to 0.01M KCl+0.1M NaCl (R+N_{6.5}; a-b). At pH 6.5 the increase in the NaCl CT response could be accounted for by the increase in the Bz-insensitive component of the NaCl CT response ((g-h) > (c-d)). At pH 6.5, nicotine produced no effect on the Bz-sensitive component of the NaCl CT response ((f-g) = (b-c)). At pH 9, NaCl (R+N_{9.0}) produced a bigger CT response (i-j) relative to pH 6.5 (R+N_{6.5}; a-b). The increase in the NaCl CT response could be accounted for by the increase in the Bz-sensitive component of the NaCl CT response ((j-k) > (b-c)). At pH 9, superfusing the tongue with R+NaCl+0.015M nicotine (N+Nic_{9.0}), further enhanced the magnitude of the CT response ((m-n) > (i-j)). The increase in the NaCl CT response could be accounted for by the increase in the Bz-sensitive component of the NaCl CT response ((n-o) > (j-k)).
At pH 9, nicotine produced no effect on the Bz-insensitive component of the NaCl CT response \((\Delta o-p = \Delta k-l)\). In 3 animals (Fig. 7C), R+NaCl at pH 9 and R+NaCl+0.015M nicotine at pH 9 increased the Bz-sensitive NaCl CT response by 23.5±4.5% and 57.6±5.2%, respectively, relative to pH 6.5 (p<0.01; N = 3). At pH 6.5, nicotine produced no significant increase in the Bz-sensitive NaCl response but specifically increased the Bz-insensitive component of the NaCl CT response (p<0.01; paired; N = 3; Fig. 7C). These results suggest that at pH 6.5, nicotine specifically affects the Bz-insensitive part of the NaCl CT response and at pH 9, it affects only the Bz-sensitive ENaC component of the response.

As reported earlier (Lyall et al. 2002b), a decrease in pH\(_i\) of the acid sensing TRCs is the proximate signal for the elicitation for both the phasic and tonic parts of the CT response. We further tested if nicotine entry into TRCs and the subsequent increase in pH\(_i\), will interfere with the CT response to acidic stimuli. CT responses to HCl were monitored in the presence and absence of nicotine at pH values that facilitate apical nicotine entry into TRCs (Fig. 1D). Figure 7B shows that when the rinse solution (0.01M KCl+0.01M HEPES) was maintained at pH 6 (R\(_6\)), stimulating the tongue with 0.02M HCl (HCl) elicited a fast phasic response \((a)\) that slowly declined to the tonic response level \((b)\). At a rinse pH of 6 (R\(_6\)), the magnitude of the phasic response to two 0.02M HCl stimulations is represented by \((a-b)\) and \((k-l)\) at the beginning and at the end of the CT record. At a rinse pH of 9 (0.01M KCl+0.01M Tris; R\(_9\)), the phasic CT response during two stimulations with 0.02M HCl \((c-d\) and \(e-f)\) was smaller relative to at pH 6. In the final step, rinsing the tongue with rinse solution at pH 9
containing 0.015M nicotine (R+Nic₉) further inhibited the magnitude of the CT response during two stimulations with 0.02M HCl (g-h and i-j). In contrast to the phasic response, the magnitude of the tonic part of the CT response to 0.02M HCl was not altered when the rinse solution was at pH 9 or when the rinse solution contained, in addition, 0.015 M nicotine. Essentially similar results were obtained in 2 additional experiments. These results suggest that at alkaline pH nicotine specifically attenuates the magnitude of the phasic response to acidic stimulation.
DISCUSSION

Results presented in this study support the conclusion that nicotine modulates both the Bz-insensitive (ENaC) and Bz-sensitive (TRPV1t) salt taste receptors. Whether nicotine will stimulate TRPV1t or ENaC is largely determined by changes in the apical pH (pHₐ) or TRC pHᵢ, respectively. Nicotine at 0.015M had an enhancing effect on TRPV1t (as demonstrated by the increase in the CT response to NaCl+Bz) at all pHₐ values tested ranging from 3.1 and 9.0. However, the magnitude of the enhancement varied with pHₐ as a bell-shaped function (Fig. 5A, insert). At pHₐ values between 6 and 7, nicotine produced maximum enhancement of the apical TRPV1t cation channel. Above pH 7 the CT response declined and at pHₐ values above 8, the CT response was about the same as at pH 3.1. At pH values less than 8, the permeability of the apical membrane to nicotine is negligible (Fig. 1). This suggests that changes in TRC pHᵢ do not play a role in the nicotine-induced activation of TRPV1t. The physiological implication of the interactions of nicotine and RTX with TRPV1t will be discussed below. In contrast, at pH values above 8, the permeability of the apical membrane to nicotine is increased (Fig. 1). Its entry into TRCs caused intracellular alkalinization that in turn activated ENaCs (Figs. 7A and 7C). At pH values above 8, TRPV1t is much less responsive to nicotine than at pH 6-7 (Fig. 5A, insert). Above pH 8, nicotine primarily affects ENaC. The basolateral application of nicotine induced rapid intracellular alkalinization and cell swelling, cellular responses consistent with its properties as a ‘base’ (Fig. 1). The effects
of nicotine entry from the apical membrane at alkaline pH on TRC pHi, volume, and on salty and sour taste modalities will also be discussed below.

Modulation of TRPV1t by nicotine

Results presented in this paper demonstrate that nicotine modulates CT responses to mineral salts. The effect of nicotine on the CT responses to mineral salts was observed even at low salt concentrations (0.01M KCl). At low salt concentration increasing nicotine concentration increased the duration of the phasic response (Figs. 3A and 4A) and at higher concentrations elicited both phasic and tonic CT responses (Figs. 3A, insert and 4B). The increase in the duration of the phasic response and the appearance of the tonic response is related to the increase in the unilateral apical cation flux in the presence of nicotine (Fig. 2B and 2C). The increase in the magnitude of the CT response to 0.01M KCl in the presence of nicotine was dose-dependent indicating that the cation flux also varies with nicotine concentration.

There is a bell shaped relationship between nicotine concentration and the CT response to 0.1M NaCl (Figs. 3A and 4C). In our previous studies, a similar bell shaped dose-response relation was observed with other TRPV1t agonists, such as, RTX, CAP, cetylpyridinium chloride (Lyall et al. 2004b) and ethanol (Lyall et al. 2005c). RTX and CAP produced maximum enhancement of the Bz-insensitive NaCl CT response at 1X10^{-6} M and 40X10^{-6} M and completely inhibited the CT response at 10X10^{-6} M and 200X10^{-6} M, respectively (Lyall et al. 2004b, 2005a). By comparison, nicotine is a far less potent modulator of TRPV1t,
since it caused a maximum enhancement in the Bz-insensitive NaCl CT response at 0.015M and inhibited the response at 0.1M by 80% relative to N+Bz alone (Fig. 4C). There are additional important quantitative differences between nicotine and other TRPV1t agonists.

Nicotine induced a mean maximum enhancement of the CT response in the presence of 0.1M NaCl+5X10^{-6} M Bz of 72%. In contrast RTX, CAP, CPC, and ethanol increased the maximum enhancements in the NaCl+Bz CT response of more than 100%. Secondly, the maximum suppression of the CT response to 0.1M NaCl+5X10^{-6} M Bz by nicotine (19%) is less than that of the other agonists, which can suppress the response by as much as 100% (Lyall et al. 2004b, 2005a,c). Nonetheless, nicotine produces a concentration-response function that is qualitatively similar to that of a typical TRPV1t agonist.

Second, in the case of TRPV1, nicotine is not itself an agonist of the receptor, but it significantly sensitizes TRPV1 to the vanilloid, CAP (Liu et al. 2004). Thus, nicotine has no effect on TRPV1 unless it is presented with capsaicin. In the case of the TRPV1t, nicotine itself is an agonist (Figs. 3A and 4C). This difference may be due to the fact that, unlike TRPV1, which requires heat or a chemical agonist to become active, TRPV1t is constitutively active, so that nicotine modulates the receptor ion channel that is already in a conducting state.

Third, TRPV1 is activated by a decrease in pH\(_0\) (Davis et al. 2002; Geppetti and Trevisani 2004; Gunthorpe et al. 2002). In contrast, in the absence of a TRPV1t agonist, the Bz-insensitive NaCl CT response is not affected by
changes in pHo. Similarly, in the absence of an agonist, changes in pHo between 4.7 and 9.7 had no effect on the temperature threshold of the Bz-insensitive NaCl CT response. This indicates that the constitutively active TRPV1t is not affected by changes in pHo (Lyall et al. 2004b, 2005a). However, the CT responses activated by a TRPV1t agonist was strongly dependent on pHo. In our studies, CT response to NaCl+Bz+RTX was bell-shaped with the maximal response around pH 6 with lower responses observed both above and below pH 6 (Lyall et al. 2004b, 2005a). In addition, changing pHo from 6.0 to either 4.7 or 9.7 in the presence of RTX decreased the temperature threshold of the CT response (Lyall et al. 2004b, 2005a). Taken together, the data indicate that the TRPV1t cation channel in TRCs is only affected by pHo in the presence of an agonist (Lyall et al., 2004b; 2005a) such as nicotine (Fig. 5A). At constant pHo, changes in TRC pHi do not affect salt responses in the presence of RTX (Lyall et al. 2004b, 2005a). Therefore, it is likely that H+ ions bind to an external site on the TRPV1t channel protein and modulate the affinity of nicotine and RTX to their respective intracellular binding sites on the channel protein.

Nicotine enhanced the NaCl CT response without a shift in the temperature threshold of the CT response (Fig. 5B). In this regard the effect of nicotine on TRPV1t is similar to that reported on the cloned TRPV1 receptor. Nicotine did not alter the threshold temperature of heat activated currents in trigeminal ganglion neurons expressing TRPV1 (Liu et al. 2004).

SB-366791 inhibited the constitutive activity of TRPV1t and caused a rightward shift in the concentration-response to nicotine (Fig. 4C). Topical application
of SB-366791 also inhibited RTX-, CAP-, temperature-, and ethanol-induced increase in Bz-insensitive NaCl CT responses (Lyall et al. 2004b, 2005a,c). Similarly, SB-366791 inhibits TRPV1 when activated by H\(^+\), elevated temperatures, CAP, and ethanol. SB-366791 produced similar rightward shift in concentration-response to CAP in hTRPV1 expressing cells (Gunthorpe et al. 2004). This suggests that SB-366791 may also act as a competitive antagonist of TRPV1t cation channel. Since at present the detailed structure of TRPV1t is not known, it is not clear how SB-366791 competitively blocks the effects of nicotine, RTX, CAP, ethanol and temperature on the receptor cation channel. However, based on the effect of SB-366791 on TRPV1 receptor channel two mechanisms of inhibition have been proposed. One possibility is that SB-366791 binds at the CAP-binding site on the TRPV1 receptor and affects the gating mechanism of the cation channel. SB-366791 can work both as a true competitive antagonist as well as inhibit the agonist properties of RTX, CAP, ethanol, and temperature by an allosteric effect on the cation channel (Gunthorpe et al. 2004). The second possibility is that SB-366791 is a pure allosteric effector of TRPV1t and produces an agonist-independent inhibition of the channel at an independent allosteric site (Gunthorpe et al. 2004). However, the above two mechanisms are based on the assumption that all agonists produce effects on TRPV1 receptor by a common mechanism that involves gating of the receptor by shifting the temperature threshold of channel activation to lower temperatures. However, nicotine sensitizes both cloned TRPV1 and TRPV1t without a shift in the temperature threshold of the channel.
In HEK-293 cells expressing TRPV1, CAP increased the outward currents at room temperature, and induced a leftward shift in the voltage-dependent activation curve in a dose-dependent manner (Voets et al. 2004). This suggests that TRPV1 agonists function as gating modifiers. Since the Bz-insensitive NaCl CT response is observed in the absence of any agonists and demonstrates significant voltage-sensitivity but not voltage-dependence (Fig. 3C), it suggests that TRPV1t is constitutively active at room temperature in the absence of agonists (Figs. 3-6). It follows that TRPV1t is active at resting TRC membrane potential. Nicotine not only increased the Bz-insensitive NaCl CT response but also increased the Bz-insensitive apical Na\(^+\) conductance. In addition, making the TRC potential more negative enhanced the CT response to 0.1M NaCl (Figs. 3C and 4D). Taken together, these results suggest that RTX, CAP, ethanol and nicotine induced activation of TRPV1t most likely does not involve a leftward shift in the voltage-dependent activation curve of the channel. It is likely that TRPV1t agonists increase salt responses by stabilizing the channel in the open state, and thereby further potentiating the Bz-insensitive Na\(^+\) flux across the apical membrane of TRCs. This results in the enhancement of the CT responses to mineral salts.

At present the exact mechanism of how nicotine modulates the activity of TRPV1 or TRPV1t is not known. However, several hypotheses have been put forward as possible mechanism of nicotine-induced sensitization of the TRPV1 channel (Liu et al. 2004). These include: nicotine induced increase in the number of functional channels that increases the probability of the channel to remain in
its open state (Hui et al. 2003; Kwak et al. 2000) or alterations of the cell membrane’s mechanical properties that can in turn affect its binding to phosphatidylinositol-4,5-bisphosphate (PIP₂) (Hui et al. 2003; Prescott and Julius 2003).

Nicotine also demonstrated interactions with RTX (Figs. 3C and 4D). In the presence of a sub-threshold concentration of RTX (0.25X10⁻⁶ M) the nicotine-induced increase in the Bz-insensitive NaCl CT response was greater than that observed with RTX or nicotine alone. This increase in the response is related to the corresponding increase in the Bz-insensitive apical Na⁺ conductance in the presence of nicotine and RTX (Fig. 4D). In addition, in the presence of 0.25X10⁻⁶ M RTX, nicotine enhanced the CT response at room temperature and at elevated temperatures and shifted the temperature threshold of the channel to the left (Fig. 5C). These results suggest that the binding of RTX to either the intracellular binding site for CAP or to another ligand-independent allosteric site alters the conformation of the channel and sensitizes the channel to further stimulation with nicotine.

The specificity of nicotine as a salt taste modulator is further demonstrated by the observation that TRPV1 KO mice, that lack the Bz-insensitive component of the NaCl CT response, also do not respond to nicotine (Fig. 6C), ethanol, RTX and elevated temperatures (Lyall et al. 2005c). The data support the conclusion that nicotine produces its effect on salt responses via the amiloride- and Bz-insensitive TRPV1t cation channel in fungiform TRCs. However, the exact
mechanism of how nicotine modulates the TRPV1t cation channel in TRCs remains to be established.

**Possible consequences of nicotine-induced intracellular alkalinization and cell swelling in TRCs.**

Changes in TRC pH_i and cell volume are involved in modulating salt taste (Lyall et al. 1995, 1999, 2002b) and acid taste transduction (Lyall et al. 2001, 2002a,b, 2006). Consistent with this our data indicate that at pH values above 8, nicotine enters TRCs across the apical membrane causing intracellular alkalinization and activation of ENaC. This results in an increase in Bz-sensitive NaCl CT response (Figs. 7A and 7C). The pH of saliva secreted by the unstimulated human parotid gland ranges from 5.45 to 6.06. On stimulation the pH of parotid saliva becomes alkaline and increases to a maximum of 7.8 (Davenport 1982). It is likely that under these conditions the apical entry of nicotine into TRCs is enhanced.

In a sub-set of TRCs an acid-induced decrease in pH_i is the proximate signal for sour taste transduction (Lyall et al. 2001, 2002a,b, 2004a). In acid-sensing TRCs, a decrease in pH_i may be sensed via PKD2L1 channel (Huang et al. 2006; Ishimaru et al. 2006; LopezJimenez et al. 2006). A decrease in TRC pH_i is responsible for the elicitation of both the phasic and the tonic parts of the CT response to acid stimulation (Lyall et al. 2001, 2002b). The nicotine-induced intracellular alkalinization is accompanied by cell swelling (Fig. 2A). This results in a significant inhibition of the phasic part of the CT response to acids.
In addition, cell shrinkage activates Bz-sensitive ENaCs (Ji et al. 1998; Lyall et al. 1999, 2005c). Indeed, hypertonic mannitol- or cellobiose-induced TRC shrinkage in in vivo and increased the Bz-sensitive NaCl CT responses in rats (Lyall et al. 1999). The activity of the rat $\alpha\beta\gamma$ subunits of the epithelial Na$^+$ channel expressed in *Xenopus* oocytes was not affected by cell swelling or mechanically induced changes of membrane tension (Awayda and Subramanyam 1998). However, in another study (Ji et al. 1998), in hypotonic media, rat $\alpha\beta\gamma$ subunits of the epithelial Na$^+$ channel expressed in *Xenopus* oocytes demonstrated a decrease in current amplitude through the expressed channel. It is likely that the nicotine induced cell swelling will inhibit Na$^+$ flux through apical Bz-sensitive ENaCs and attenuate CT responses to NaCl. Thus, in the presence of nicotine, both an increase in TRC pH$_i$ and cell swelling will affect NaCl CT responses. However, under these conditions there was a net increase in the Bz-sensitive NaCl CT response (Figs. 7A and 7C). This indicates that in TRCs the activation of ENaC by intracellular alkalinization is significantly greater than its inhibition by cell swelling.

The results presented in this study demonstrate that nicotine produces specific effects on the CT taste nerve responses to salty and sour taste stimuli. These effects are taste quality specific and occur through specific taste receptors and downstream intracellular effectors. Nicotine effects are dependent upon pH$_o$ and nicotine concentration and are modulated by temperature and the presence of other agonists. In the case of both salty and sour taste, although an increase in pH$_i$ and cell volume are common intracellular effectors, they inhibit phasic
responses to HCl but increase the ENaC activity resulting in enhanced Bz-sensitive NaCl CT response. In contrast, nicotine is an agonist of TRPV1t, and depending upon its concentration is both an agonist and antagonist of the Bz-insensitive NaCl CT response. These effects of nicotine are modulated by temperature, pH0, and the presence of other TRPV1t agonists, such as, RTX. This is in contrast to the nicotine induced suppression of all gustatory responses (sucrose, NaCl, citric acid, monosodium glutamate, and quinine) of neurons in the nucleus of the solitary tract. This effect is most likely due to a nicotinic acetylcholine receptor-mediated excitation of trigeminal afferents that inhibit nucleus of the solitary tract units centrally (Simons et al. 2006).

The main points of this study can be integrated into a model of Na+ transport and salt taste transduction mechanism in the anterior tongue (Fig. 8). In fungiform TRCs Na+ transport occurs through both cellular and transcellular pathways. Na+ ions enter TRCs across the apical membrane by at least two pathways. One pathway involves Na+ entry through the apical amiloride- and Bz-sensitive ENaCs and is responsible for the amiloride- and Bz-sensitive CT component of the NaCl CT response. The second pathway involves TRPV1t, a non-selective cation channel that is not only permeable to Na+ but also to K+, NH4+ and Ca2+ ions (Lyall et al. 2004b, 2005a,b). In rat fungiform TRCs it accounts for all of the Bz-insensitive CT responses to Na+ salts and part of the CT response to K+, NH4+, and Ca2+ salts (Lyall et al. 2004b, 2005a). TRPV1t is maximally active around pH 6. The entry of Na+ depolarizes the receptor potential leading to the activation of membrane voltage-gated Ca2+ channels
(VGCC), an increase in $\text{[Ca}^{2+}\text{]}_i$, and subsequent release of the neurotransmitter. 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 Na$^+-$H$^+$ exchanger isoform 3 (NHE-3) seems to be quiescent in TRCs (Vinnikova et al., 2004a). 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).

In the presence of mineral salts nicotine increases the Bz-insensitive apical cation flux in TRCs and elicits CT responses that are similar to salt responses, comprised of both a phasic component and a sustained tonic component. Below 0.015M nicotine enhanced and above 0.015M it inhibited CT responses to 0.1M NaCl. Nicotine produced maximum enhancement of the CT response between pH 6 and 7. Stimulating the tongue with solutions containing RTX and nicotine at elevated temperature increased the sensitivity of the CT response to nicotine. Because the effects of nicotine on mineral salts are blocked by SB-366791 and because TRPV1 KO mice are insensitive to nicotine, ethanol, RTX, and temperature, we conclude that nicotine produces these taste effects by direct action on the Bz-insensitive TRPV1t salt taste receptor. Above pH 8 or when nicotine levels increase in the blood, nicotine permeates the TRC membranes ([Nic]) and alkalinizes TRCs. The increase in TRC pH$_i$, in turn, increases ENaC activity and the magnitude of the Bz-sensitive NaCl CT response.
An acid-induced decrease in a subset of TRCs is a proximate signal for both the phasic and tonic components of the CT response to acidic stimuli (DeSimone and Lyall, 2006). A decrease in pH leads to cell shrinkage and the activation of a flufenamic acid-sensitive membrane conductance that gives rise to the phasic CT response to acids. Nicotine increases resting TRC pH and volume and thus leads to the inhibition of the phasic component of the CT response to acid stimulation. However, at present it is not clear if nicotine affects salty or sour taste transduction mechanisms residing in type II (receptor cells) or type III (presynaptic cells) (Huang et al., 2007).

These conclusions are consistent with the observations that in human non-users of smokeless tobacco, use of smokeless tobacco reduces perceived intensity of salty, sour and bitter stimuli (Mela 1989). In humans the amiloride-sensitive component contributes about 20% to the taste response to NaCl (DeSimone and Lyall, 2006). Thus, it is likely that in humans the predominant effect of nicotine on salt taste is via the amiloride-insensitive component of the taste response to NaCl.
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Table 1
Composition of solutions used in *in vitro* experiments

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aNaPy = sodium pyruvate; R = Ringer’s solution; C = Control solution; NMDG = N-methyl-D-glucamine; Glu = gluconate; 0Na = Na⁺-free solution; 0Na-0K = Na⁺- and K⁺-free solution; 0Na-0K-0Cl = Na⁺-, K⁺-, and Cl⁻-free solution; bHK = high K⁺ solutions containing 0.01M nigericin; In some experiments the control solution pH was adjusted to 6.5; cIn some solutions Tris[hydroxymethyl]aminomethane was used instead of HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulphonic acid) and the pH of the solution was adjusted to 9 with 1N HCl.
Fig. 1. Effect of nicotine on TRC pH and volume. A polarized epithelial preparation loaded with BCECF was initially perfused on both sides with control Ringer’s solution, pH 7.4. Temporal changes in pH and cell volume were monitored as changes in FIR (F490/F440) and changes in the fluorescence intensity at the isosbestic wavelength 440 nm (F440). (A) At the time period indicated by the top horizontal bar the basolateral membrane (BLNic) was perfused with Ringer’s solution containing 0.0005, 0.001, 0.0025, 0.005 or 0.01M nicotine (Nic) and (B) the apical membrane was perfused with 0.025M nicotine. (C) Shows the effect of 0.005M nicotine in basolateral solution at pH 6.5 or 7.4. (D) Shows the effect of 0.025M nicotine in apical solution at pH 6.5 or 9.0. Values are presented as M±SEM of n, where n = number of ROIs within the taste bud.
Fig. 2. Effect of nicotine on TRC volume and the apical $\text{Na}^+$ flux. (A) Shows the effect of 0.01M basolateral nicotine (BLNic) on pH$_i$ and F$_{440}$. Values are presented as M±SEM of $n$, where $n =$ number of ROIs within the taste bud. (B) Shows the effect of apical nicotine (APNic) on the Bz-insensitive unilateral apical $\text{Na}^+$ flux in polarized TRCs. A lingual epithelial preparation loaded with SBFI was initially perfused on both sides with 0 Na$^+$ Ringer's solution, pH 7.4. Temporal changes in FIR (F$_{340}$/F$_{380}$) were monitored while the apical membrane was perfused with control Ringer's solution (pH 7.4) with (a-b) and without (b-c) 0.015M nicotine. The relative changes in [Na$^+$]$_i$ are presented as changes in FIR relative to apical 0 Na$^+$. Values are presented as M±SEM of $n$, where $n =$ number of ROIs within the taste bud. (C) Shows the effect of basolateral nicotine (BLNic) on the Bz-sensitive unilateral apical $\text{Na}^+$ flux in polarized TRCs. A lingual epithelial preparation
loaded with Na-green was initially perfused on both sides with 0 Na⁺ Ringer’s solution, pH 7.4. Temporal changes in fluorescence (F₄₉₀) were monitored while the apical membrane was perfused with control Ringer’s solution containing 1X10⁻⁶ M SB-366791 (pH 7.4) in the absence (a-b-c) and presence (d-e-f) of 0.015M nicotine in the basolateral compartment. SB-366791 was added to the apical control Ringer’s solution to block Na⁺ entry through TRPV1t. The relative changes in [Na⁺], are presented as changes in F₄₉₀ relative to apical 0 Na⁺. Values are presented as M±SEM of n, where n = number of ROIs within the taste bud.
Fig. 3. Effect of SB 366791 and applied voltage on the nicotine-induced changes in the CT responses to mineral salts. (A) Rat tongue was stimulated with nicotine (Nic; 0-0.1M) solutions containing either 0.01M KCl+0.01M HEPES (R; pH 6.1) or 0.01M KCl+0.1M NaCl+0.01M HEPES+5x10⁻⁶ M Bz (R+N+Bz; pH 6.1) maintained at room temperature (23°C). The fast onset and offset CT responses to R+0.005M Nic and R+0.05M Nic are shown in an expanded time scale in the insert. (B) Rat tongue was stimulated with 0.01M KCl+0.01M HEPES (R; pH 6.1) and then with 0.01M KCl+0.1M NaCl+0.01M HEPES+5x10⁻⁶ M Bz (R+N+Bz; pH 6.1) containing a fixed concentration of SB-366791 (SB; 0.05x10⁻⁶ M) and increasing concentrations of nicotine (0-0.1M). The corresponding control responses to 0.3M NH₄Cl are shown at the beginning and end of each experiment and did not differ by more than 1%. (C) Rat tongue was stimulated with a rinse solution (R; 0.01M KCl) and then with 0.01M KCl+0.1M NaCl+5x10⁻⁶ M Bz (R+N+Bz), 0.01M KCl+0.1M NaCl+5x10⁻⁶ M
Bz+0.25X10^{-6} M RTX (R+N+Bz+RTX), 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz+0.015 M nicotine (R+N+Bz+Nic) or with 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz+0.25X10^{-6} M RTX+0.015 M nicotine (R+N+Bz+RTX+Nic) under zero current clamp. During the tonic phase, a transepithelial voltage of -60 mV or +60 mV was applied to the lingual receptive field and the deflections in the tonic CT response were recorded relative to zero current clamp (0 mV).
Fig. 4. Summary of the data on the effects of SB-366791 and applied voltage on the CT responses to 0.01M KCl or 0.1M NaCl in the presence of nicotine. (A) Shows the mean phasic CT response (0.01M KCl/0.3M NH₄Cl) to R containing varying concentrations of nicotine in 3 individual animals. The data is presented as M ± SEM of the number of animals (N = 3). (B) Effect of SB-366719 on the CT responses to 0.01M KCl in the presence of nicotine. Rat tongue was stimulated with 0.01M KCl+0.01M HEPES (R; pH 6.1; ●), R+0.05X10⁻⁶ M SB-366791 (SB; ○), or R+0.25X10⁻⁶ M SB-366791 (SB; ▲) solutions containing nicotine (Nic; 0-0.1M). Each point represents the M ± SEM of the normalized tonic CT (0.01M KCl/0.3M NH₄Cl) response from 3 animals (N). (C) Dose response relationship between SB-366719 concentration (SB) and the CT responses to 0.1M NaCl in the presence of nicotine. Rat tongue was stimulated with 0.01M KCl+0.01M HEPES (R; pH 6.1) and then with 0.01M KCl+0.1M NaCl+0.01M HEPES+5X10⁻⁶ M Bz (R+N+Bz; pH 6.1) containing 0 (●), 0.05X10⁻⁶ M (SB; ○) or 0.25X10⁻⁶ M SB-366791 (SB; ▲) and increasing concentrations of nicotine (0-0.1M). Each point represents the M ± SEM of the normalized CT response (0.1M NaCl/0.3M NH₄Cl) from 3 animals (N). (D) Summary of data from applied voltage experiments in 3 individual animals, such as shown in Fig. 3C. The relationship between the response and the slope of the tonic CT response (0.1M NaCl/0.3M NH₄Cl) with applied voltage was calculated as described previously (Lyall et al. 2004b).
Fig. 5. Effect of the applied changes in extracellular pH (pHe) and temperature on the CT responses to KCl and NaCl in the presence of nicotine and RTX. (A) Rat tongue was stimulated with 0.01M KCl+0.01M HEPES (R) and then with 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz+0.01M HEPES or 0.01M TRIS-HCl (R+N+Bz) adjusted to pHs between 3 and 9 containing a fixed nicotine concentration (Nic; 0.015 M). Insert shows the relation between pHe and tonic CT response/0.3 M NH₄Cl to R+0.015M Nic (○), R+N+Bz (△), and R+N+Bz+0.015M Nic (●). Each point represents M±SEM from 4 animals. (B) CT responses were monitored while the rat tongues were first rinsed with 0.01M KCl (R) at 23° and then stimulated with 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz (R+N+Bz) or with 0.01M KCl+0.1M NaCl+5X10^{-6} M Bz+0.01M nicotine (R+N+Bz+ Nic) maintained at temperatures between 23° and 55.5°. (C) CT responses were monitored while the rat tongues were first rinsed with 0.01M KCl (R) at 23° and then stimulated with 0.01M KCl+0.1M NaCl+5X10^{-6}
M Bz+0.01M nicotine (R+N+Bz+Nic) or with 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz+0.01M nicotine+0.25X10⁻⁶ M RTX maintained at temperatures between 23° and 55.5°. The rinse solution (R_{23°}), R+NaCl+5X10⁻⁶ M Bz solution at 23° (R+N+Bz_{23°}) and R+NaCl+5X10⁻⁶ M Bz+nicotine solution at 23° (R+N+Bz+Nic_{23°}) were superfused at the rate of 3 ml/s. The NaCl solutions at higher temperatures were superfused at the rate of 8 ml/min.
Fig. 6. Effect of nicotine on the CT responses to 0.1M NaCl in WT and KO mice. In a WT (A) or KO (C) mouse the tongue was stimulated with a rinse solution (R; 0.01M KCl) and then with 0.01M KCl+0.1M NaCl (R+N), 0.01M KCl+0.1M NaCl+5X10⁻⁶ M Bz (R+N+Bz) containing 0.005M (R+N+Bz+0.005M Nic), 0.01M (R+N+Bz+0.01M Nic) or 0.015M (R+N+Bz+0.015M Nic) nicotine (Nic). (B) Summary of data from 3 WT mice from (A). Each point represents the M±SEM of the normalized CT response from 3 animals (N).
Fig. 7. Effect of pH on the CT response to nicotine in the presence of 0.15M NaCl or 0.02M HCl. (A) Rat tongue was stimulated with a rinse solution (R6.5; 0.01M KCl+0.01M HEPES; pH 6.5) and then with 0.01M KCl+0.1M NaCl+0.01M HEPES; pH 6.5) (R+N6.5), 0.01M KCl+0.1M NaCl+5X10^-6 M Bz+0.01M HEPES; pH 6.5) (R+N+Bz6.5) containing 0.015M (R+N+Bz+Nic6.5) nicotine concentration (Nic). In the second part of the experiment CT responses were monitored with both rinse and NaCl solutions containing 0.01M Tris[hydroxymethyl]aminomethane at pH 9.0. (B) Rat tongue was stimulated with a rinse solution (R; 0.01M KCl) and then with rinse solution containing HCl (0.01M KCl+0.02M HCl). In the first step, the rinse solution was adjusted to pH 6 (R6; 0.01M KCl+0.01M HEPES; pH 6). In the second step, the rinse solution was adjusted to pH 9 (R9; 0.01M KCl+0.01M Tris; pH 9). In the third step, the rinse solution was R9+0.015 M nicotine (R+Nic9). In each case the rinse solution was replaced by 0.01M KCl+0.02M HCl (HCl). The magnitude of the phasic response for pH 6, pH 9, and pH 9+0.015 M nicotine are represented by (a-b and k-l), (c-d and e-f), and (g-h and i-j), respectively. (C) Shows mean data from 3 animals in (A). Each point represents the M±SEM of the normalized CT response from 3 animals (N). Open bars and hatched bars represent changes in the Bz-sensitive and the Bz-insensitive component of the NaCl CT response under different conditions, respectively. *p<0.05, paired.
Fig. 8. Proposed model for Na⁺ transport in fungiform TRCs and salt taste transduction in the anterior tongue. The abbreviations used in the figure are: amiloride-sensitive epithelial Na⁺ channel (ENaC); Transient Receptor Potential Variant salt taste receptor-1 (TRPV1t); NHE-1 (basolateral Na⁺-H⁺ exchanger-1); NHE-3 (apical Na⁺-H⁺ exchanger-3); benzamil (Bz); nicotine (Nic); ethanol (ETH); resiniferatoxin (RTX); N-(3-methoxyphenyl)-4-chlorocinnamide (SB-366791); external Na⁺ ([Na]₀); tight junction; increase (↑); and decrease (↓). See text for details.