Changes in Taste Receptor Cell $[\text{Ca}^2+]_i$ Modulate Chorda Tympani Responses to Bitter, Sweet and Umami Taste Stimuli

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RUNNING TITLE: Regulation of bitter, sweet and umami CT responses by $[\text{Ca}^2+]_i$

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ABSTRACT

Relationship between taste receptor cell (TRC) Ca\textsuperscript{2+} ([Ca\textsuperscript{2+}]i) and rat chorda tympani (CT) nerve responses to bitter (quinine and denatonium), sweet (sucrose, glycine and erythritol) and umami (monosodium glutamate (MSG) and MSG+inosine 5' monophosphate (IMP)) taste stimuli was investigated before and after lingual application of ionomycin (Ca\textsuperscript{2+} ionophore)+Ca\textsuperscript{2+}, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-(acetoxyethyl ester) (BAPTA-AM, Ca\textsuperscript{2+} chelator), U73122 (phospholipase C blocker), thapsigargin (Ca\textsuperscript{2+}-ATPase blocker) and diC8-PIP\textsubscript{2} (synthetic phosphatidylinositol-4,5-bisphosphate). The phasic CT response to quinine was indifferent to changes in [Ca\textsuperscript{2+}]i. However, a decrease in [Ca\textsuperscript{2+}]i inhibited the tonic part of the CT response to quinine. The CT responses to sweet and umami stimuli were indifferent to changes in TRC [Ca\textsuperscript{2+}]i. However, a decrease in [Ca\textsuperscript{2+}]i attenuated the synergistic effects of ethanol on the CT response to sweet stimuli and of IMP on the glutamate CT response. U73122 and thapsigargin inhibited the phasic and tonic CT responses to bitter, sweet and umami stimuli. While diC8-PIP\textsubscript{2} increased the CT response to bitter and sweet stimuli, it did not alter the CT response to glutamate but inhibited the synergistic effect of IMP on the glutamate response. The results suggest that bitter, sweet and umami taste qualities are transduced by [Ca\textsuperscript{2+}]i-dependent and [Ca\textsuperscript{2+}]i-independent mechanisms. Changes in TRC [Ca\textsuperscript{2+}]i in the BAPTA-sensitive cytosolic compartment regulate quality specific taste receptors and ion channels which are involved in the neural adaptation and mixture interactions. Changes in TRC [Ca\textsuperscript{2+}]i in a separate sub-compartment, sensitive to inositol trisphosphate and thapsigargin, but
inaccessible to BAPTA and ionomycin+Ca^{2+} are associated with neurotransmitter release.

**Key words:** Ionomycin, BAPTA, U73122, benzamil, diC8-PIP₂
INTRODUCTION

Several studies suggest that taste receptor cells (TRCs) respond with an increase in intracellular Ca$^{2+}$ ([Ca$^{2+}$]$_i$) when stimulated with sweet [Rebello and Medler 2010], bitter [Akabas et al. 1988; Caicedo and Roper 2001; Ogura et al. 2002; Medler 2010; Rebello and Medler 2010], umami [Narukawa et al. 2006; Rebello and Medler, 2010], and a mixture containing sweet and bitter [Huang and Roper 2010] taste stimuli. In Type II cells binding of tastants to G-protein coupled receptors (GPCRs) for sweet, umami and bitter taste activates a signal transduction pathway that involves, G proteins gustducin or G$\alpha_i$ leading to the release of the G$\beta\gamma$ subunits and the subsequent activation of phospholipase C $\beta_2$ (PLC$\beta_2$), hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP$_2$) to inositol trisphosphate (IP$_3$) and diacylglycerol (DAG), binding of IP$_3$ to IP$_3$ receptor (IP$_3$R$_3$), release of [Ca$^{2+}$], from intracellular stores and activation of TRPM5 channels. Activation of TRPM5 channels leads to Na$^+$ entry, membrane depolarization and activation of voltage-gated cation channels (VGCCs) in TRCs resulting in the release of ATP through pannexin and connexin hemichannels. ATP binds to ionotropic purinergic receptors on the taste nerve fibers and provides the linkage between TRCs and the nerve fibers [Roper 2007; Yasuo et al. 2008]. In addition to its role in the neurotransmitter release, alterations in TRC [Ca$^{2+}$], have been shown to modulate chorda tympani (CT) taste nerve responses to salty [DeSimone and Lyall 2008; Lyall et al. 2009; DeSimone et al. 2012] and sour stimuli [Lyall et al. 2002, 2004, 2006; DeSimone et al. 2012]. Although an increase in cytosolic [Ca$^{2+}$], seems to be critical for normal responses in Type II TRCs [Medler 2010], at present, a detailed
understanding of the relationship between TRC [Ca\(^{2+}\)]\(_i\) and neural responses to taste stimuli representing bitter, sweet and umami taste qualities is lacking.

The objective of this study was to investigate the effects of changes in TRC [Ca\(^{2+}\)]\(_i\) on rat CT responses to representative bitter, sweet and umami taste stimuli. TRC [Ca\(^{2+}\)]\(_i\) was either increased or decreased \textit{in situ} by topical lingual application of a Ca\(^{2+}\) ionophore ionomycin+Ca\(^{2+}\) or a membrane permeable Ca\(^{2+}\) chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis-(acetoxymethyl ester) (BAPTA-AM), respectively. The effects of BAPTA loading in TRCs were reversed by saturating the intracellular Ca\(^{2+}\)-binding sites on intracellularly trapped BAPTA by treating rat lingual TRCs \textit{in vivo} with ionomycin+Ca\(^{2+}\) [DeSimone et al. 2012]. The data presented in this study suggest that changes in TRC [Ca\(^{2+}\)]\(_i\) in two cytosolic sub-compartments, namely an ionomycin- and BAPTA-sensitive sub-compartment and an intracellular IP\(_3\)- and thapsigargin-sensitive sub-compartment play an important role in regulating the neural responses to bitter, sweet and umami stimuli. The increase in cytosolic [Ca\(^{2+}\)]\(_i\) in the later sub-compartment is most likely dependent upon the internal Ca\(^{2+}\) stores. Changes in [Ca\(^{2+}\)]\(_i\) in the cytosolic compartment regulate taste receptors and downstream intracellular signaling intermediates/ion channels involved in the taste transduction mechanism and are involved in neural adaptation or in mixture interactions between different taste qualities. Changes in TRC [Ca\(^{2+}\)]\(_i\) in a separate sub-compartment, inaccessible to Ca\(^{2+}\)-buffering by BAPTA, are associated with neurotransmitter release and subsequent neural response.
MATERIALS AND METHODS

In vivo studies

CT taste nerve recordings. Animals were housed in the VCU (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 VCU. A total of 43 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°C 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. 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, Sarasota, FL, USA). For display, responses were filtered using a band pass filter with cutoff frequencies 40 Hz-3 KHz and fed to an oscilloscope. Responses were then full-wave rectified and integrated with a time constant of 1s. Integrated neural responses and lingual current and voltage changes were recorded on a chart recorder and also captured on disk using Labview software (National Instruments, Austin, TX, USA) and analyzed off-line. Stimulus solutions were injected into a Lucite chamber (3 ml; 1 ml/s) affixed by vacuum to a 28 mm² patch of anterior dorsal lingual surface. Typically, stimulus solutions remained on the tongue for 1-2 min. Control stimuli consisting of 300 mM NH₄Cl and 300 mM NaCl
(Table 1) applied at the beginning and at the end of experiment were used to assess
preparation stability (Figs. 3D and 7). The preparation was considered stable only if the
difference between the magnitude of the control stimuli at the beginning and at the end
of the experiment was less than 10% [Lyall et al. 2009a, 2010a]. The neural responses
were recorded and analyzed as described before [Lyall et al. 2009a, 2010a; DeSimone
et al. 2012].

Solutions. The composition of the rinse and stimulating solutions is shown in Table 1.
For the majority of the CT recordings the rinse (R) and stimulating solutions were used
without added CaCl₂. However, only in those experiments in which we used ionomycin
to load the cells with Ca²⁺, 1 or 10 mM CaCl₂ was added to the rinse and stimulating
solutions. In Table 1 and in the text these rinse and stimulating solutions are identified
with a superscript (†; Table 1).

TRCs were loaded in vivo with Ca²⁺ using 150 µM ionomycin (a Ca²⁺ ionophore; Sigma) in DMSO for 45 min. In addition, rinse and stimulating solutions (Table 1) used
for CT recordings post-ionomycin treatment contained either 1 or 10 mM CaCl₂ [Lyall et
al. 2002a; 2004a; 2009; DeSimone et al. 2012]. TRCs were loaded in vivo with a Ca²⁺
chelator, 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid tetrakis-(acetoxymethyl ester) (BAPTA-AM; Sigma), by dissolving BAPTA-AM directly in
dimethyl sulfoxide (DMSO) and applying topically to the tongue for 45 min at a
concentration either 13 or 33 mM. BAPTA-AM is membrane permeable, and once inside
the cell, the acetoxymethyl (-AM) group is hydrolyzed by intracellular non-specific
esterases, and the free acid form is released. BAPTA-acid chelates free intracellular
Ca²⁺ and decreases resting TRC [Ca²⁺]. In addition, any increase in TRC [Ca²⁺] during
taste transduction, due either to the release of Ca\textsuperscript{2+} from intracellular stores or the influx of Ca\textsuperscript{2+} through VGCCs in TRC membranes, is buffered by intracellular BAPTA. The effects of BAPTA loading in TRCs were reversed by saturating the Ca\textsuperscript{2+}-binding sites on the intracellularly trapped BAPTA by treating the cells with ionomycin+Ca\textsuperscript{2+}. To rat tongues already treated with BAPTA-AM, we topically applied 150 μM ionomycin to the lingual surface for 30 min. Following ionomycin treatment the lingual surface was perfused for 10 min with the rinse solution containing 10 mM KCl+10 mM CaCl\textsubscript{2} and then perfused with 10 mM KCl rinse solution without CaCl\textsubscript{2} for another 10 min. This was done to remove ionomycin and external CaCl\textsubscript{2} [DeSimone et al. 2012].

As in our previous studies [Lyall et al. 2002a; 2004a; 2006], TRCs were loaded in vivo with Ca\textsuperscript{2+} using ionomycin+10 mM extracellular CaCl\textsubscript{2} for the following reasons: (i) Topical lingual application of ionomycin alone or just adding 10 mM CaCl\textsubscript{2} to the rinse and stimulating solutions did not alter CT responses to taste stimuli [Lyall et al. 2002a; 2004a]; (ii) Topical lingual application of ionomycin+10 mM CaCl\textsubscript{2} maximally inhibited the Bz-sensitive NaCl CT response [DeSimone et al. 2012] and the CT responses to acidic stimuli [Lyall et al. 2004a]; (iii) Post BAPTA-AM treatment, topical lingual application of ionomycin+10 mM CaCl\textsubscript{2} induced 100% recovery in the CT responses to NaCl, HCl, CO\textsubscript{2} and acetic acid [DeSimone et al. 2012]; (iv) When the rinse and NaCl solutions contained 1 mM CaCl\textsubscript{2}, ionomycin treatment had no effect on the NaCl CT responses [DeSimone et al. 2012]; and (vi) Most importantly, in rats first treated with BAPTA-AM and then with Post-BAPTA-post-ionomycin+Ca\textsuperscript{2+} treatment the CT responses to the control stimuli (300 mM NH\textsubscript{4}Cl) did not differ by more than 2-5% at the beginning and the end of the experiment.
Changes in TRC [Ca\(^{2+}\)] were also altered by either blocking PLC activity through topical lingual application of 250 μM U73122 or by depleting intracellular Ca\(^{2+}\) stores with 250 μM thapsigargin (both from Sigma). In sweet, bitter or umami sensing Type II TRCs, activation of specific GPCRs is linked to the activation of PLCβ2, hydrolysis of phosphatidylinositol 4, 5-bisphosphate (PIP\(_2\)) into inositol 1,4,5-trisphosphate (IP\(_3\)) + diacylglycerol (DAG) and a decrease in membrane PIP\(_2\) levels. In some experiments, we also tested the effect of increasing membrane PIP\(_2\) levels in TRC membranes directly by the topical lingual application of 250 μM diC8-PIP\(_2\), a short chain synthetic PIP\(_2\) (Echelon, Salt Lake City, Utah, USA) [Lyall et al. 2010a]. In some experiments we added 250 μM diC8-PIP\(_2\) directly into the stimulating solutions. An equivalent amount of DMSO was added to the control stimulating solutions.

**Data Analysis.** The numerical value of an integrated tonic 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 30 seconds measured from the end of a typical 2 min stimulation period. The changes in the area under the integrated CT response curves to various stimuli under different experimental conditions were normalized to the responses observed in each animal to 300 mM NH\(_4\)Cl and were expressed as the mean±standard error of the mean of n; where n represents the number of animals in each group; M±SEM (n). In some experiments we also quantified the phasic part of the CT response. The height of the stimulus-induced maximum CT response relative to baseline response was divided by the mean steady-state (tonic) response to 0.3M NH\(_4\)Cl [Lyall et al. 2010a; DeSimone et al. 2012]. Student’s t-test was employed to analyze the differences between sets of data.
RESULTS

Effect of changes in TRC [Ca^{2+}] on the CT responses to bitter taste stimuli

*Studies with ionomycin+Ca^{2+}*

Consistent with previous studies [Lyall et al. 2002; 2004; DeSimone et al. 2012] treating the rat tongue with ionomycin+10 mM CaCl_2 decreased the magnitude of the CT response to 20 mM HCl relative to control (Fig. 1). In contrast, no effect of ionomycin+Ca^{2+} was observed on the CT responses to 10 mM quinine or 10 mM denatonium relative to control (Fig. 1). These results suggest that increasing [Ca^{2+}], above the resting level in TRCs produces a differential effect on taste stimuli. While an increase in TRC [Ca^{2+}] inhibits CT response to sour taste stimuli (HCl), it does not affect CT responses to bitter (quinine and denatonium) taste stimuli.

*Studies with BAPTA-AM*

Topical lingual application of 13 mM and 33 mM BAPTA inhibited the tonic CT response to quinine in a dose-dependent manner (Figs. 2A-2C). At 33 mM BAPTA, the tonic CT response to quinine was inhibited close to the rinse baseline level. No effect of BAPTA (33 mM) was observed on the transient (phasic) component of the quinine CT response (Fig. 2D).

*Studies with post-BAPTA-post-ionomycin+Ca^{2+}*

Titrating the Ca^{2+} binding sites on the intracellularly trapped BAPTA with ionomycin+Ca^{2+} restored the tonic CT response to quinine close to its control value (Figs. 2A-2C; Post-BAPTA-post-ionomycin+Ca^{2+}). Post-BAPTA-post-ionomycin+Ca^{2+} treatment did not alter the phasic component of the quinine CT response. These results suggest that an increase in TRC cytosolic [Ca^{2+}] is essential for eliciting the tonic
component of the CT response to quinine. The phasic component of the quinine CT
response is [Ca\(^{2+}\)]-insensitive (Figs. 2A, 2B and 2D).

Loading TRCs with BAPTA enhanced the NaCl CT response and post-BAPTA-
post-ionomycin+Ca\(^{2+}\) treatment decreased the response to its control level (Fig. 3A-3C).
BAPTA treatment (Fig. 3D) or post-BAPTA-post-ionomycin+Ca\(^{2+}\) treatment (data not
shown) had no effect on the CT response to 300 mM NH\(_4\)Cl or 100 mM KCl [DeSimone
et al. 2012]. These results suggest that changes in TRC cytosolic Ca\(^{2+}\) modulate neural
responses to different taste stimuli in a quality- and stimuli-specific manner.

Effect of changes in TRC [Ca\(^{2+}\)]\(_i\) on the CT responses to sweet taste stimuli in the
absence and presence of ethanol.

Studies with ionomycin+Ca\(^{2+}\)

In the raw CT recordings shown in Fig. 1 and Figs. 4A-4C the tonic CT
responses to glycine, sucrose and erythritol did not show significant differences before
and after treating the tongue with ionomycin+Ca\(^{2+}\). The mean normalized tonic CT
responses to erythritol (Fig. 4D), glycine (Fig. 4D) and sucrose (data not shown) were
not altered after ionomycin+Ca\(^{2+}\) treatment (p >0.05; unpaired). We also observed no
significant differences in the mean normalized phasic CT response to glycine (Fig. 4E),
erythritol (data not shown), and sucrose (data not shown) before and after
ionomycin+Ca\(^{2+}\) treatment.

We have previously shown that ethanol added to the rinse solution (10 mM KCl)
elicits CT responses in a dose-dependent manner in Sprague-Dawley rats, NP rats and
P rats [Lyall et al. 2005; Coleman et al. 2011]. In Sprague-Dawley rats, ethanol
increased the tonic CT response significantly from control between 40% and 60%. The
maximum increase in the tonic CT response was obtained at the ethanol concentration between 50% and 60% [Lyall et al. 2005]. Consistent with these earlier observations, at the concentration used here (30%), ethanol, by itself, did not elicit a CT response (Fig. 4C). In mixtures containing sweet stimuli (glycine, sucrose and erythritol) plus 30% ethanol (ETOH), ethanol enhanced the tonic CT responses to sweet stimuli (Figs. 4A-4D). The magnitudes of the tonic CT responses to sweeteners in the presence of 30% ethanol were significantly bigger than the sum of tonic CT responses to sweeteners alone and ethanol alone. The tonic CT responses to sweet stimuli containing 30% ethanol were not significantly affected by treating the tongue with ionomycin+Ca^{2+} relative to control (Fig. 4A-4D). Ethanol also enhanced the phasic CT response to glycine (Fig. 4E; p = 0.01; unpaired) and erythritol (data not shown). No effect of ionomycin+Ca^{2+} was observed on the phasic CT response to glycine (Fig. 4E) and erythritol (data not shown) in the presence of ethanol relative to control. These results suggest that increasing [Ca^{2+}], above the resting levels in TRCs does not modulate CT responses to sweet taste stimuli or the synergistic effects of ethanol on the CT responses to sweet taste stimuli.

**Studies with BAPTA-AM**

No significant effect of BAPTA was observed on the phasic or tonic CT responses to SC45647 (Fig. 5A), erythritol (Fig. 5B), and sucrose (Figs. 5C-5E) (p >0.05; unpaired). However, the synergistic effect of ethanol on the tonic CT response to sucrose (Fig. 5D; p = 0.0063), SC45647 (Fig. 5A) and erythritol (Fig. 5B) were significantly attenuated after BAPTA treatment. Ethanol also produced a synergistic effect on the phasic CT response to sucrose under control conditions and after BAPTA-
AM treatment (Fig. 5E; *p = 0.02; unpaired). However, the magnitude of the phasic CT response in the presence of sucrose+ethanol was not different before and after BAPTA treatment. These results suggest that CT responses to sweet stimuli involve both \([Ca^{2+}]\)-dependent and \([Ca^{2+}]\)-independent transduction mechanisms and produce differential effects on the phasic and tonic CT responses to sweet taste stimuli.

**Effect of changes in TRC \([Ca^{2+}]\), on the CT responses to umami taste stimuli**

*Studies with ionomycin+Ca\(^{2+}\)*

IMP (1 mM) enhanced the phasic and tonic CT response to glutamate (MSG+Bz+SB), however the tonic and phasic CT responses to MSG+Bz+SB or MSG+Bz+SB+IMP were not affected by treating the tongue with ionomycin+Ca\(^{2+}\) relative to control (Figs. 6A and 6B). These results suggest that IMP produces synergistic effects on both the phasic and tonic CT responses to glutamate and that increasing \([Ca^{2+}]\) levels above the resting levels in TRCs has no effect on the CT response to glutamate alone or the synergistic effect of IMP on the glutamate CT response.

*Studies with BAPTA-AM*

No effect of BAPTA was observed on the CT response to MSG+Bz+SB (Figs. 6C-6E). However, BAPTA treatment (Figs. 6D and 6E) inhibited the synergistic effect of IMP on the glutamate CT response (Fig. 6E; p = 0.022; unpaired). These results suggest that CT responses to umami stimuli involve both \([Ca^{2+}]\)-dependent and \([Ca^{2+}]\)-independent transduction mechanisms.

**Effect of U73122 on the CT responses to bitter, sweet and umami taste stimuli**
Topical lingual application of U73122, a non-specific PLC blocker, did not alter the phasic CT response to quinine, sucrose and SC45647 (Figs. 7A-7C). However, the tonic component of the CT responses to the above stimuli was inhibited by U73122 treatment [Lyall et al. 2010a]. U73122 inhibited tonic CT responses to quinine, SC45647, MSG+Bz+SB and MSG+Bz+SB+IMP (Fig. 7D). Consistent with previous studies [Lyall et al. 2010a], U73122 did not inhibit the control CT response to 0.3 M NH₄Cl but inhibited the CT response to 300 mM NaCl (Fig. 7D). These studies are consistent with the observations that in Type II cells PLCβ₂ enzyme is an essential intracellular signaling intermediate in bitter, sweet and umami taste transduction [Zhang et al. 2003].

**Effect of diC8-PIP₂ on CT responses to bitter and sweet taste stimuli**

Increasing membrane PIP₂ levels in TRCs by the topical lingual application of 250 μM diC8-PIP₂ for 30 min enhanced the tonic CT response to 500 mM sucrose (Figs. 8B and 9C) and 250 mM glycine (Figs. 8C and 9C). However, no significant effects of diC8-PIP₂ were observed on the CT response to 10 mM quinine (Figs. 8A and 9C). DiC8-PIP₂ also enhanced the tonic CT response to sucrose and SC45647 when presented as a mixture along with the sweet stimuli (Fig. 8D).

**Effect of diC8-PIP₂ on CT responses to MSG and MSG+IMP**

Although the phasic and tonic CT response to glutamate were unaffected by treating the tongue with diC8-PIP₂ relative to control (Fig. 9A), it significantly inhibited the tonic CT response to MSG+IMP relative to control (Figs. 9B and 9C; p <0.014; unpaired; n = 3). These results suggest that increasing membrane PIP₂ levels do not
affect the CT response to glutamate but inhibits the synergistic effects of IMP on the tonic CT response to glutamate.

**Effect of thapsigargin on the CT responses to sweet, bitter and umami taste stimuli**

Topical lingual application of 250 μM thapsigargin for 30 min inhibited the phasic (data not shown) and tonic CT responses to 300 mM NH₄Cl, 300 mM NaCl, 10 mM quinine, 500 mM sucrose, MSG+Bz+SB and MSG+Bz+SB+IMP relative to control (Fig. 10). These data suggest that bitter, sweet and umami taste transduction mechanisms are dependent upon the release of Ca²⁺ from intracellular stores.
DISCUSSION

The main effects of changes in TRC cytosolic $\text{[Ca}^{2+}\text{]}_i$ on the CT responses to bitter, sweet and umami taste stimuli along with the proposed taste receptor and intracellular signaling intermediates regulated by $\text{[Ca}^{2+}\text{]}_i$ are summarized in Table 2. The results suggest that bitter, sweet and umami taste qualities are transduced by $\text{[Ca}^{2+}\text{]}_i$-dependent and $\text{[Ca}^{2+}\text{]}_i$-independent mechanisms. Changes in TRC $\text{[Ca}^{2+}\text{]}_i$ in the two cytosolic sub-compartments, one accessible by ionomycin+$\text{Ca}^{2+}$ and BAPTA-AM and a separate sub-compartment, dependent upon intracellular $\text{Ca}^{2+}$ stores and modulated by $\text{IP}_3$ and thapsigargin, play important roles in neural responses to bitter, sweet and umami stimuli.

Changes in cytosolic $\text{[Ca}^{2+}\text{]}_i$ regulate CT responses to bitter taste stimuli

Bitter taste stimuli induce an increase in TRC $\text{[Ca}^{2+}\text{]}_i$ [Akabas et al. 1988; Caicedo and Roper 2001; Caicedo et al. 2002; Medler 2010; Rebello and Medler 2010]. However, in an earlier study [Zhang et al. 2003], TRPM5 channel activity was unaffected by changes in $\text{[Ca}^{2+}\text{]}_i$, $\text{IP}_3$, or thapsigargin-mediated depletion of internal stores, suggesting that bitter taste transduction does not involve $\text{[Ca}^{2+}\text{]}_i$ as an intracellular signal. In our studies, the phasic CT response to quinine was independent of changes in TRC $\text{[Ca}^{2+}\text{]}_i$ (Figs. 1, 2A, 2B and 2D). We have previously shown that similar to the WT mice TRPM5 KO mice elicit a phasic CT response to quinine [Oliveira-Maia et al. 2009a]. In addition, blocking PLC activity with U73122 did not affect the phasic CT response to quinine (Fig. 7A). It is most likely that the decision regarding both the tastant identification and a measure of its palatability are made by the animals during the initial phasic part of the neural response [Carleton et al. 2010]. This initial
phasic CT response seems to be transduced via a Ca^{2+}-, PLCβ_2- and TRPM5-independent mechanism, suggesting that changes in cytosolic [Ca^{2+}]_i in Type II cells involved in bitter taste transduction are not associated with the neurotransmitter release.

The tonic CT response to quinine is nearly abolished when changes in TRC [Ca^{2+}] are prevented by BAPTA loading in a dose-dependent manner (Figs. 2A-2C). Blocking PLC activity with U73122 also inhibited the tonic CT response to quinine (Fig. 7A). In addition, increasing PLC activity by increasing TRC membrane PIP_2 levels with diC8-PIP_2 enhanced the tonic CT response to quinine (Figs. 8A and 9C) [Lyall et al. 2010b]. Taken together, the above results suggest that the tonic CT response to quinine is dependent upon the activation of PLCβ_2, an increase in TRC Ca^{2+} and activation of TRPM5 [Oliveira-Maia et al. 2009a]. It is likely that similar to the case of NaCl and acidic stimuli [Lyll et al. 2002, 2004; DeSimone et al. 2012], an increase in cytosolic [Ca^{2+}]_i during the tonic part of the neural response is involved in bitter taste adaptation by way of a separate molecular adaptation mechanism.

In some inbred strains, ethanol has both a sweet taste quality as well as a bitter taste quality and, is thus, expected to interact with both sweet and bitter taste transduction pathways. In a two bottle choice test, even at a concentration of 5%, ethanol is aversive, and is most likely bitter [Coleman et al. 2011]. Recent studies [Oliveira-Maia et al. 2009a,b] suggest that some bitter stimuli, namely, nicotine, acetylcholine and ethanol elicit CT responses that are both TRPM5-dependent and TRPM5-independent. The TRPM5-independent transduction mechanism for nicotine, acetylcholine and ethanol is dependent upon nicotinic acetylcholine receptors expressed in a subset of TRCs within the taste buds. At present the relationship
Changes in cytosolic $[\text{Ca}^{2+}]_i$ regulate CT responses to sweet taste stimuli

Changes in TRC $[\text{Ca}^{2+}]_i$ produced either only a small change (e.g. in erythritol CT response; Fig. 4D) or no change in the CT responses to representative sweet stimuli (glycine, sucrose; Figs. 1, 4A, 4B, 4D and 5). These results suggest that both the phasic and the tonic CT responses to sweet stimuli are independent of changes in TRC $[\text{Ca}^{2+}]_i$. The tonic CT response to sweet stimuli is inhibited by blocking PLC activity with U73122 (Figs. 7B and 7C) and enhanced by increasing PLC activity by increasing its substrate concentration (the cell membrane PIP$_2$ levels) with diC8-PIP$_2$ (Figs 8 and 9C) [Lyall et al. 2010b]. It is important to note that diC8-PIP$_2$ also enhanced the CT response to sweet stimuli when added directly to the sweet stimulus (Fig. 8D). These results suggest that in Type II cells involved in sweet taste transduction the tonic CT response to sweet stimuli is dependent upon PLC$\beta_2$ activation.

Relative to NP rats, P rats demonstrated increased levels of both T1R3 mRNA and protein in the fungiform taste receptive field, greater responses to sucrose, greater sensitivity to lower ethanol concentrations, greater enhancement in the sucrose tonic CT response in the presence of ethanol and a leftward shift in the relationship between ethanol concentration and the sucrose CT response [Coleman et al. 2011]. These data suggest that ethanol enhances the CT response to sucrose by interacting with T1R3, a component of the dimeric sweet taste receptor (T1R2+T1R3). Here we show that similar to the case in P and NP rats, ethanol enhances CT responses to sweet stimuli in Sprague-Dawley rats (Figs. 4 and 5). Inhibiting sweet taste transduction by blocking
PLCβ2 with U73122 not only inhibited the CT response to SC45647 but also eliminated the enhancement in the SC45647 response in the presence of ethanol [Coleman et al. 2011]. While ethanol modulates sweet taste responses primarily by interacting with T1R3, a component of the sweet-taste receptor [Bachmanov et al. 2001, 2002; Inoue et al. 2004; Lu et al. 2005; Nelson et al. 2001], other intracellular effectors, such as cell Ca2+, also play a role in mixture interactions. Chelating TRC [Ca2+] with BAPTA loading, did not affect the tonic CT responses to sucrose, but inhibited the subsequent increase in the CT response in the presence of ethanol (Fig. 5D). These results suggest that the modulation of the tonic CT nerve responses to sweet stimuli by ethanol is dependent upon an increase in TRC Ca2+. We hypothesize that the synergistic effects of ethanol on tonic CT response to sucrose involves the Ca2+-activated non-selective cation channel, TRPM5 [Talavera et al. 2005]. In contrast the ethanol-induced increase in the phasic CT response of sucrose does not seem to be Ca2+-dependent (Fig. 5E), suggesting that the transduction mechanisms for the phasic and tonic CT responses to sweet stimuli are different.

McCaughey (2007) recorded taste-evoked responses to sweeteners in the nucleus of the solitary tract in C57BL/6ByL and 129P3/J mice that have different alleles of Tas1r3. In these studies the initial phasic response monitored 600 ms following stimulation did not differ between the two strains and was not observed preferentially in neurons classified as sugar responsive. In contrast the tonic response monitored 5s following stimulation differed between the two strains and affected primarily the sweet responsive cells. These results suggest that phasic and tonic responses are associated with divergent neural pathways that originate with different transduction mechanisms
and are sent to different nucleus of the solitary tract cells within each strain. It is suggested that the phasic response to sweeteners provides a cue that could drive ingestion by Tas1r3-null mice, especially in long-term tests involving post-ingestive conditioning [Damak et al. 2003]. In the companion paper [DeSimone et al. 2012] and in the earlier work published from our lab [Lyall et al. 2002, 2004 and 2006] we have provided strong evidence that in the case of acidic stimuli phasic and tonic responses have different transduction mechanisms.

**Changes in cytosolic [Ca\textsuperscript{2+}], regulate CT responses to umami taste stimuli**

Altering TRC [Ca\textsuperscript{2+}] levels produced no changes in the phasic or tonic CT responses to glutamate (Fig. 6), suggesting that both the phasic and the tonic CT response to umami stimuli are independent of changes in TRC [Ca\textsuperscript{2+}]. In our studies, IMP enhanced both the phasic and tonic CT responses to glutamate (Fig. 6). The enhancement in the CT response to glutamate by IMP was inhibited after treatment with BAPTA (Fig. 6C). These results indicate that the synergistic effect of IMP on the glutamate CT response depends upon an increase in cytosolic [Ca\textsuperscript{2+}]. It is interesting to note that in contrast to IMP, the synergistic effects of N-geranylcyclopropylcarboximide (NGCC) on the glutamate CT response were inhibited when TRCs were loaded *in vivo* with Ca\textsuperscript{2+} using ionomycin [Dewis et al. 2006]. Thus both [Ca\textsuperscript{2+}]-dependent and [Ca\textsuperscript{2+}]-independent mechanisms are involved in umami taste transduction.

Blocking PLC activity with U73122 partially inhibited the CT response to glutamate and the synergistic effect of IMP on the glutamate response (Fig. 7D). These results suggest that both PLC\(\beta_2\)-dependent and PLC\(\beta_2\)-independent mechanisms are involved in umami taste transduction. Another difference between umami taste stimuli
and the sweet taste stimuli was observed with respect to diC8-PIP$_2$. While increasing TRC membrane PIP$_2$ levels enhanced the CT response to sweet taste stimuli (Fig. 8), it did not produce any significant effect on the CT response to glutamate (Fig. 9). Most importantly, diC8-PIP$_2$ inhibited the synergistic effect of IMP on the glutamate CT response (Fig. 9). We speculate that PIP$_2$ inhibits the IMP effects by competing for the binding site of IMP on the umami receptor [Lyall et al. 2010b].

Recent studies suggest that in the TRCs glutamate is detected by multiple receptors and transduction pathways [Yasuo et al. 2008; Yasumatsu et al. 2011]. The candidate umami receptors include: a variant of brain-expressed metabotropic glutamate receptor 4 (taste mGluR4), a heteromer T1R1+T1R3 and a variant of the type 1 metabotropic glutamate receptor that has a truncated N-terminal domain (truncated mGluR1). Taste-mGluR4 is expressed in rat circumvallate taste buds in the posterior tongue innervated by the glossopharyngeal nerve and binds glutamate with reduced affinity [Chaudhari et al. 1996, 2000]. The T1R1+T1R3 receptor is expressed in both anterior and posterior taste receptive fields. The mouse T1R1+T1R3, heterologously expressed in human embryonic kidney (HEK) cells, responds to many amino acids. In contrast the human type heteromer preferentially responds to glutamate and its response is synergistically enhanced by IMP [Nelson et al. 2002; Li et al. 2002]. Truncated mGluR1 is expressed in rat circumvallate taste buds and demonstrates low binding affinity to glutamate.

T1R3 KO mice demonstrate greatly diminished behavioral preferences and CT responses to umami stimuli and the synergism between MSG and IMP, suggesting that the T1R1+T1R3 heterodimer receptor plays an important role in umami taste in the
anterior taste receptive field [Zhao et al. 2003; Damak et al. 2003]. However, in T1R3
KO mice the glossopharyngeal taste nerve responses to umami taste stimuli are not
affected [Damak et al. 2003], suggesting that T1R1+T1R3 heterodimer receptor
independent umami transduction mechanism in the circumvallate TRCs. In a recent
study, the umami-responsive single CT fibers in WT mice were classified into sucrose-
best responsive S-type and umami best responsive M-type fibers. S-type showed a
large synergistic effect on responses to MSG or monopotassium glutamate when IMP
was present. In T1R3-KO mice, S-type fibers showing the large synergism were absent,
but M-type fibers with slight or no synergism still remained [Yasumatsu et al. 2006,
2011; Yasuo et al. 2008]. These data suggest that T1R3 KO mice lack the signal elicited
by MSG in the anterior tongue that are not umami specific and may be similar to those
elicited by sweet compounds (S-type cell and fibers).

In several studies mice lacking the intracellular signaling effectors such as Gα
gustducin, PLCβ₂, type III inositol-1,4,5-triphosphate (IP₃R₃) and TRPM5 also do not
show large deficits in the behavioral and CT responses to umami taste stimuli [He et al.
2004; Hisatsune et al. 2007; Talavera et al. 2005; Damak et al. 2006]. Taken together,
the above results suggest that in the anterior tongue two pathways are involved in
umami taste transduction. One pathway involves T1R3, Gα gustducin, IP₃R₃ and
TRPM5 and the second pathway is independent of the above intracellular signaling
molecules. The second pathway most likely is also present in the circumvallate taste
receptive field [Yasuo et al. 2008]. More recent studies [Yasumatasu et al. 2011]
suggest that the CT response to monopotassium glutamate (MPG) is carried by single
fibers of two major types, sucrose best (S) and MPG-best (M) fibers. In addition, each
fiber type has two subtypes, one type shows synergism between MPG and IMP (S1, M1), and the second type shows no synergism (S2, M2). T1R3 and TRPM5 KO mice demonstrated the absence of only S1-type fibers while the other fiber types (S2, M1 and M2) were still present in these KO mice. Yasumatasu et al. (2011) further demonstrated that mGluR antagonists selectively suppressed MPG responses of M1 and M2 type. We speculate that in our study the Ca\(^{2+}\)-independent CT response to glutamate may arise from its interactions with mGluRs. The Ca\(^{2+}\)-independent glutamate neural responses in the absence of IMP may occur via PLC\(\beta\)\(_2\)- and a TRPM5-independent mechanism, possibly by the interaction of glutamate with the metabotropic glutamate receptors (mGluRs) [Yasuo et al. 2008].

**IP\(_3\)- and thapsigargin-dependent [Ca\(^{2+}\)]\(_i\) sub-compartment**

An increase in [Ca\(^{2+}\)]\(_i\) is required for the release of neurotransmitter from the presynaptic terminals in all neurons [Llinas et al. 1992; Llinas and Moreno 1998; Beierlein et al. 2004]. An increase in [Ca\(^{2+}\)]\(_i\) in the microdomains against the cytoplasmic surface of the plasmalemma during transmitter release, suggests that the synaptic vesicle fusion responsible for transmitter release is triggered by the activation of a low-affinity Ca\(^{2+}\)-binding site at the active zone [Llinas et al. 1992; Llinas and Moreno 1998]. However, the relationship between changes in [Ca\(^{2+}\)]\(_i\) in the cytosolic compartment and the synaptic regions of the TRCs is not known at present. Our results with U73112 (Fig. 7), are consistent with the hypothesis that in Type II TRCs, down-stream of GPCRs (T1Rs or T2Rs) the activation of PLC\(\beta\)\(_2\) and the formation of IP\(_3\) is necessary for the generation of the tonic CT response to sweet, umami and bitter taste stimuli [Zhang et al. 2003; Yasuo et al. 2008]. These studies are supported by the observations that
depleting intracellular Ca\textsuperscript{2+} stores with thapsigargin greatly reduces CT responses to sweet, bitter and umami taste stimuli (Fig. 10).

Recent studies [Huang et al. 2010] suggest that in Type II TRCs membrane voltage initiated by TRPM5 channels is required for ATP secretion during taste reception. However, even in the absence of TRPM5 channel activity, ATP release could be triggered by depolarizing TRCs with KCl. It is suggested that taste-stimuli evoked increase in [Ca\textsuperscript{2+}], in Type II TRCs has a dual role. It results in the opening of TRPM5 channels to depolarize receptor cells and in concert with membrane potential increase in [Ca\textsuperscript{2+}]\textsubscript{i} opens ATP-permeable gap junction hemichannels [Huang et al. 2010]. However, if sufficiently depolarized, Type II TRCs can release transmitter even in the absence of [Ca\textsuperscript{2+}], [Romanov et al. 2008]. Taste stimuli depolarize TRCs to increase the Ca\textsuperscript{2+} influx through VGCCs that is involved in the vesicular release of the neurotransmitters serotonin and noradrenaline [Huang et al. 2008]. In IP\textsubscript{3}R\textsubscript{3}-deficient [Hisatsune et al. 2007] and PLC\textbeta\textsubscript{2} KO mice [Zhang et al. 2003], the CT and glossopharyngeal nerve responses to bitter, sweet and umami taste stimuli were greatly reduced but not abolished relative to WT mice. The above results indicate that IP\textsubscript{3}R\textsubscript{3} is a crucial mediator of bitter, sweet and umami taste perception [Hisatsune et al. 2007]. The results further suggest that bitter, sweet and umami tastes are transduced by both IP\textsubscript{3}-dependent and IP\textsubscript{3}-independent pathways.

In summary, the data presented in this paper and the companion paper [DeSimone et al. 2012], suggest that the transduction of all taste qualities involves both Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent mechanisms. The phasic component of the CT response to sweet, bitter and umami stimuli is independent of changes in cytosolic
[Ca^{2+}]; (Table 2) but must involve an increase in [Ca^{2+}] in a separate cytosolic sub-compartment dependent upon internal Ca^{2+} stores. However, the tonic part of the CT response to quinine is strongly dependent upon an increase in cytosolic [Ca^{2+}], and is most likely involved in the neural adaptation to bitter taste. While the tonic component of the CT response to sweet and umami stimuli is independent of changes in cytosolic [Ca^{2+}], the synergistic effects of ethanol and IMP on the CT response to sweet and umami stimuli, respectively, are dependent upon an increase cytosolic [Ca^{2+}].

Surprisingly, in contrast to sweet, bitter and umami taste, a decrease in [Ca^{2+}] induced by loading TRCs with BAPTA enhanced the amiloride- and Bz-sensitive epithelial Na^{+} channel (ENaC)-dependent and the putative TRPV1t-dependent neural responses. In addition, a decrease in TRC [Ca^{2+}] inhibited the tonic CT response to acidic stimuli by increasing the adaptation rate via the activation of the basolateral Na^{+}-H^{+} exchanger. Chelating [Ca^{2+}], with BAPTA abolished the tonic CT response to acidic stimuli while leaving the phasic response intact [DeSimone et al. 2012]. This suggests that for some taste qualities the transduction mechanisms for phasic and tonic neural responses are different [McCaughey 2007]. Our results further suggest that simply monitoring changes in cytosolic Ca^{2+} levels in isolated taste cells does not give enough information about the transduction mechanism of taste stimuli since one or more components of the neural response may be regulated by changes in cytosolic Ca^{2+}. 
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Fig. 1. Effect of ionomycin+Ca\textsuperscript{2+} on the CT response to HCl, quinine, sucrose and denatonium. Shows a representative CT response recorded while the rat tongue was first superfused with a rinse solution (10 mM KCl+10 mM CaCl\textsubscript{2}; R+Ca\textsuperscript{2+}; Table 1) and then with 20 mM HCl, 10 mM quinine, 500 mM sucrose and 10 mM denatonium, each containing, in addition, 10 mM CaCl\textsubscript{2} (Table 1) under control conditions (Control+Ca\textsuperscript{2+}) and after the topical lingual application of 150 μM ionomycin (Post-ionomycin+Ca\textsuperscript{2+}) for 45 min. The arrows represent the time period for which the tongue was superfused with different taste stimuli.

Fig. 2. Effect of BAPTA and Post-BAPTA-post-ionomycin+Ca\textsuperscript{2+} on the CT response to quinine. Shows representative CT responses recorded in two separate rats while their tongues were superfused with a rinse solution (10 mM KCl; R; Table 1) and then with 10 mM quinine under control conditions (Control), after topical lingual application of 13 mM BAPTA (A) or 33 mM BAPTA (B). Following this in both rats the tongue was treated with 150 μM ionomycin for 45 min. The tongue was then rinsed with the rinse solution containing 10 mM CaCl\textsubscript{2} for another 10 min. The CT responses were then recorded after stimulating the tongue with 10 mM quinine solutions (Post-BAPTA-post-ionomycin+Ca\textsuperscript{2+}). (C) Shows the mean changes in the quinine tonic CT response under control conditions (0 BAPTA; open bar), after treatment with BAPTA (Post-BAPTA; open bars; 13 mM or 33 mM BAPTA) and after post-BAPTA-post-ionomycin+Ca\textsuperscript{2+} treatment (grey bars). In each case the quinine tonic CT responses were normalized to the corresponding tonic CT responses obtained with 300 mM NH\textsubscript{4}Cl. The number of rats in each group is given in the parenthesis above the each
bar. (D) Shows the mean±SEM change in the quinine phasic CT response in 3 rats under control conditions (Control; open bar) and after treatment with 33 mM BAPTA-AM (Post-BAPTA (33 mM); grey bar). In each case the quinine phasic CT responses were normalized to the corresponding tonic CT responses obtained with 300 mM NH₄Cl.

**Fig. 3. Effect of Post-BAPTA and Post-BAPTA-post-ionomycin+Ca²⁺ on the CT responses to NaCl, HCl and quinine.** Shows representative CT traces in which the rat tongue was first stimulated with R and then with 300 mM NaCl, 20 mm HCl and 10 mM quinine under control conditions (Control; A), following topical lingual application of 13 mM BAPTA (Post-BAPTA; 13 mM; B) and following post-BAPTA-post-ionomycin+Ca²⁺ treatment (Post-BAPTA-post-ionomycin+Ca²⁺; C). (D) Shows a representative CT response to 300 mM NH₄Cl under control conditions (Control) and after topical lingual application of 33 mM BAPTA-AM (Post-BAPTA; 33 mM). The arrows represent the time periods when the rat tongue was superfused with the rinse solution and the stimulating solutions.

**Fig. 4. Effect of ionomycin+Ca²⁺ on the CT response to sweet taste stimuli.** Shows representative CT traces in which the rat tongue was first stimulated with rinse solution containing 10 mM CaCl₂ (†R) and then with 250 mM glycine (A), 500 mM sucrose (B) and 250 mM erythritol (C) containing 10 mM CaCl₂ with and without 30% ethanol (ETOH) (Table 1) under control conditions (Control+Ca²⁺) and after topical lingual application of 150 µM ionomycin for 30 min (Post-ionomycin+Ca²⁺). The arrows represent the time periods when the rat tongue was superfused with the rinse solution and the stimulating solution. (D and E) Show the summary of the normalized tonic CT response to erythritol and glycine (D) and the normalized phasic CT response to glycine.
(E), respectively, in the absence and in the presence of 30% ethanol (ETOH) under control conditions (Control+Ca²⁺; open bars) and after ionomycin treatment (Post-ionomycin+Ca²⁺; grey bars). The values are represented as M±SEM of n, where n represents the number of animals in each group. In each animal the CT responses under different experimental conditions were normalized to the tonic CT response to 300 mM NH₄Cl. It is important to note that ETOH by itself when added to the rinse solution did not elicit a CT response (C). *p = 0.047; **p = 0.0125; ***p = 0.0024; #p = 0.0004 (unpaired).

**Fig. 5. Effect of BAPTA on the CT responses to sweet taste stimuli in the absence and presence of ethanol (ETOH).** Shows representative CT responses recorded while the rat tongue was superfused with 10 mM KCl (rinse solution; R), 8 mM SC456437 and 8 mM SC45647+30% ETOH (A), 250 mM erythritol and 250 mM erythritol+30% ETOH (B) and 500 mM sucrose and 500 mM sucrose+30% ETOH (C) (Table 1) under control conditions (Control) and after the topical application of 33 mM BAPTA-AM (Post-BAPTA; 33 mM). The arrows represent the time period for which the tongue was superfused with the sweet or umami stimuli. It is important to note that ethanol (30%) when added to the rinse solution did not elicit a tonic CT response (C). (D and E) Show the summary of the normalized tonic (D) and phasic (E) CT responses to 500 mM sucrose in the absence and in the presence of 30% ethanol (ETOH) before (Control; open bars) and after BAPTA treatment (Post-BAPTA; grey bars). The values are represented as M±SEM of n, where n represents the number of animals in each group. In each animal the CT responses under different experimental conditions were normalized to the tonic CT response to 300 mM NH₄Cl. *p = 0.02; **p = 0.0001
The tonic CT response to sucrose+ETOH was significantly decreased after BAPTA treatment ($p = 0.0063$; unpaired).

**Fig. 6. Effect of ionomycin+Ca$^{2+}$ and BAPTA-AM treatment on the CT responses to umami taste stimuli in the absence and presence of IMP.** *(A and B)* The CT responses were recorded while the rat tongue was superfused first with a rinse solution (10 mM KCl+10 mM CaCl$_2$; R+Ca$^{2+}$; Table 1) and then with 100 mM MSG+5 μM Bz+1 μM SB (MSG+Bz+SB) and MSG+Bz+SB+1 mM IMP, each containing, in addition, 10 mM CaCl$_2$, under control conditions (Control+Ca$^{2+}$) and after topical lingual application of 150 μM ionomycin+10 mM CaCl$_2$ for 30 min (Post-ionomycin+Ca$^{2+}$). The values of the tonic and phasic CT responses are represented as M±SEM of 3 animals. In each animal the CT responses under different experimental conditions were normalized to the tonic CT response to 300 mM NH$_4$Cl. *$p = 0.048$; **$p = 0.0065$; ***$p = 0.0042$ (unpaired). *(C-D)* Show representative CT responses recorded while the rat tongue was superfused first with 10 mM KCl (rinse solution; R) and then with 100 mM MSG+5 μM Bz+1 μM SB (MSG+Bz+SB) and MSG+Bz+SB+1 mM IMP under control conditions (Control; C) and after topical lingual application of 33 mM BAPTA-AM for 30 min (Post-BAPTA; 33 mM; D). *(E)* Shows the summary of the normalized tonic CT response to MSG+Bz+SB in the absence and in the presence of 1 mM IMP before (Control; open bars) and after BAPTA treatment (Post-BAPTA; grey bars). The values are represented as M±SEM of 3 animals. In each animal the CT responses under different experimental conditions were normalized to the tonic CT response to 300 mM NH$_4$Cl. *$p = 0.025$; **$p = 0.0015$ (unpaired). The CT response to MSG+Bz+SB+IMP was significantly reduced after BAPTA treatment ($p = 0.022$; unpaired).
Fig. 7. Effect of U73122 on the CT response to bitter, sweet and umami taste stimuli. Shows representative CT responses recorded while the rat tongue was first superfused with a rinse solution (10 mM KCl; R), and then with 20 mM quinine (A), 500 mM sucrose (B) and 8 mM SC45647 (C) under control conditions (Control) and after the topical application of 250 μM U73122 (Post-U73122; Table 1). The arrows represent the time period for which the tongues were superfused with different taste stimuli. (D) Summary of the normalized CT data from 3 rats of 300 mM NH₄Cl, 300 mM NaCl, 20 mM quinine, 8 mM SC45647, 100 mM MSG+5 μM Bz+1 μM SB and 100 mM MSG+5 μM Bz+1 μM SB+1 mM IMP under control conditions (Control; open bars) and after the topical application of 250 μM U73122 (Post-U73122; grey bars). In each case the tonic CT responses were normalized to the corresponding CT responses obtained with 300 mM NH₄Cl. *p = 0.0044; **p = 0.0025 (unpaired; n = 3).

Fig. 8. Effect of diC₈-PIP₂ on the CT responses to bitter and sweet taste stimuli. Shows representative CT responses recorded while the rat tongue was first superfused with a rinse solution (10 mM KCl; R), and then with 10 mM quinine (A), 500 mM sucrose (B) and 250 mM glycine (C) under control conditions (Control) and after topical lingual application of 250 μM diC₈-PIP₂ for 30 min (Post-diC₈-PIP₂). (D) Shows a representative CT responses recorded while the rat tongue was first superfused with a rinse solution (10 mM KCl; R), and then with 300 mM sucrose, 3 mM SC45647, 300 mM sucrose+250 μM diC₈-PIP₂ and 3 mM SC45647+250 μM diC₈-PIP₂. The arrows represent the time period for which the tongue was superfused with the sweet stimuli with and without diC₈-PIP₂.
**Fig. 9. Effect of diC8-PIP2 on the CT responses to umami, sweet and bitter taste stimuli.** (A and B) Show representative CT responses recorded while the rat tongue was first superfused with a rinse solution (10 mM KCl; R), and then with 100 mM MSG+5 μM Bz+1 μM SB (MSG+Bz+SB) and 100 mM MSG+5 μM Bz+1 μM SB+1 mM IMP (MSG+Bz+SB+IMP) under control conditions (Control) and after topical lingual application of 250 μM diC8-PIP2 for 30 min (Post-diC8-PIP2). The arrows represent the time period for which the tongue was superfused with the umami stimuli with and without diC8-PIP2. (C) Shows the summary of the normalized CT response to 20 mM quinine, 500 mM sucrose, 250 mM glycine, MSG+Bz+SB and MSG+Bz+SB+IMP before (Control; open bars) and after topical lingual application of 250 μM diC8-PIP2 for 30 min (Post-diC8-PIP2; grey bars). The values are represented as M±SEM of n, where n represents the number of animals in each group given in parenthesis above each bar. In each animal the CT responses under different experimental conditions were normalized to the tonic CT response to 300 mM NH4Cl. *p = 0.0203; ††p = 0.0136; †††p = 0.0005 (unpaired).

**Fig. 10. Effect of intracellular Ca\(^{2+}\) store depletion on the CT response to sweet, bitter and umami taste stimuli.** CT responses were recorded while the tongue was first superfused with a rinse solution (10 mM KCl; R), and then with 300 mM NH4Cl, 300 mM NaCl, 20 mM quinine, 500 mM sucrose, 100 mM MSG+5 μM Bz+1 μM SB (MSG+Bz+SB) and 100 mM MSG+5 μM Bz+1 μM SB+1 mM IMP (MSG+Bz+SB+IMP) before (Control; open bars) and after topical lingual application of 250 μM thapsigargin for 40 min (Post-thapsigargin; filled bars). In each animal the CT responses under different experimental conditions were normalized to the CT response to 300 mM NH4Cl.
at the beginning of the experiment and expressed as mean±SEM of 3 rats. *p = 0.0087;

**p = 0.0037 and ***p = 0.0001 (unpaired).
### Table 1
Composition of solutions used in CT experiments

<table>
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<tr>
<th>Rinse</th>
<th>Stimulus solution</th>
<th>(mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>†R</td>
<td>†Sucrose</td>
<td>10 KCl+500 sucrose</td>
</tr>
<tr>
<td>†R</td>
<td>†Glycine</td>
<td>10 KCl+250 glycine</td>
</tr>
<tr>
<td>†R</td>
<td>†Erythritol</td>
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<tr>
<td>†R</td>
<td>†SC45647</td>
<td>10 KCl+5 SC45647</td>
</tr>
<tr>
<td>†R+ETOH</td>
<td>†Sucrose+ETOH</td>
<td>10 KCl+30% ETOH+500 sucrose</td>
</tr>
<tr>
<td>†R+ETOH</td>
<td>†Glycine+ETOH</td>
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<td>†Erythritol+ETOH</td>
<td>10 KCl+30% ETOH+250 erythritol</td>
</tr>
<tr>
<td>†R+ETOH</td>
<td>†SC45647+ETOH</td>
<td>10 KCl+30% ETOH+5 SC45647</td>
</tr>
<tr>
<td>†R</td>
<td>†MSG</td>
<td>10 KCl+100 MSG+0.005 Bz+0.001 SB</td>
</tr>
<tr>
<td>†R</td>
<td>†MSG+IMP</td>
<td>10 KCl+100 MSG+0.005 Bz+0.001 SB+1 IMP</td>
</tr>
<tr>
<td>†R</td>
<td>†Quinine</td>
<td>10 KCl+10 quinine hydrochloride</td>
</tr>
<tr>
<td>†R</td>
<td>†Denatonium</td>
<td>10 KCl+10 denatonium benzoate</td>
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<tr>
<td>†R</td>
<td>†NaCl</td>
<td>10 KCl+100 NaCl</td>
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<td>R</td>
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</tr>
<tr>
<td>R</td>
<td>Control-2</td>
<td>300 NaCl</td>
</tr>
</tbody>
</table>

R = 10 mM KCl; ETOH = ethanol (30%).

†Only in experiments in which ionomycin was used both rinse and stimulating solutions contained, in addition, 10 mM CaCl₂. Ionomycin (150 μM) was directly dissolved in
dimethylsulfoxide (DMSO) and applied topically to the tongue for 30 min. DMSO [Lyall et al. 1999] or ionomycin (data not shown), by themselves, did not alter CT responses to taste stimuli.

MSG = Monosodium glutamate; IMP = inosine 5’-monophoshate; Bz = benzamil; SB (SB-366791; N-(3-methoxyphenyl)-4-chlorocinnamide). Bz and SB were added to the stimulating solutions to block Na⁺ entry into TRCs through ENaC and putative TRPV1t cation channel.

In some studies, we topically applied 250 μM U73122, a non-specific blocker of phospholipase Cs (PLCs) and its inactive analogue, U73343 [Coleman et al. 2011], 250 μM thapsigargin, a non-competitive inhibitor of sarco/endoplasmic reticulum Ca²⁺-ATPase (SERCA) [Rogers et al. 1995] and diC8-PIP₂, a synthetic phosphatidylinositol-4,5-bisphosphate (PIP₂) [Lyall et al. 2010a,b].
Table 2
Summary of the effects of BAPTA, ionomycin+Ca^{2+}, U73122, diC8-PIP_{2} and thapsigargin on the CT responses to bitter, sweet and umami taste stimuli

<table>
<thead>
<tr>
<th>Taste Stimuli</th>
<th>Response Measured</th>
<th>Ionomycin +Ca^{2+}</th>
<th>BAPTA</th>
<th>DiC8-PIP_{2}</th>
<th>U73122</th>
<th>Thapsigargin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bitter</td>
<td>Phasic CT response</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
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<tr>
<td>Bitter</td>
<td>Tonic CT Response</td>
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<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Sweet</td>
<td>Phasic CT response</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
</tr>
<tr>
<td>Sweet</td>
<td>Tonic CT response</td>
<td>↔</td>
<td>↔</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>ETOH+ (Sweet Stimuli)</td>
<td>Tonic CT response</td>
<td>↔</td>
<td>↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Umami</td>
<td>Phasic CT response</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
</tr>
<tr>
<td>Umami</td>
<td>Tonic CT response</td>
<td>↔</td>
<td>↔</td>
<td>↔</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>umami+ IMP</td>
<td>Tonic CT response</td>
<td>↔</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

↓ (decrease); ↑ (increase); ↔ (no change); Bitter stimuli = quinine or denatonium; Sweet stimuli = sucrose, SC45647, glycine or erythritol; Umami stimuli = monosodium glutamate (MSG) or MSG+inosine 5’ monophosphate (IMP); ETOH = ethanol.