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Resynthesis of phosphatidylinositol 4,5-bisphosphate mediates adaptation of the caffeine response in rat taste receptor cells

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Caffeine, a prototypic bitter stimulus, produces several physiological actions on taste receptor cells that include inhibition of K_{IR} and K_V potassium currents and elevations of intracellular calcium. These responses display adaptation, i.e. their magnitude diminishes in the sustained presence of the stimulus. Levels of the membrane lipid phosphatidylinositol-4,5-bisphosphate (PIP_2) are well known to modulate many potassium channels, activating the channel by stabilizing its open state. Here we investigate a putative relationship of K_{IR} and K_V with PIP_2 levels hypothesizing that inhibition of these currents by caffeine might be allayed by PIP_2 resynthesis. Using standard patch-clamp techniques, recordings of either potassium current from rat posterior taste receptor cells produced essentially parallel results when PIP_2 levels were manipulated pharmacologically. Increasing PIP_2 levels by blocking phosphoinositide-3 kinase with wortmannin or LY294002, or by blocking phospholipase C with U73122 all significantly increased the incidence of adaptation for both K_{IR} and K_V. Conversely, lowering PIP_2 synthesis by blocking PI4K or using the PIP_2 scavengers polylysine or bovine serum albumin reduced the incidence of adaptation. Adaptation could be modulated by activation of protein kinase C but not calcium calmodulin kinase. Collectively, these data support two highly novel conclusions: potassium currents in taste receptor cells are significantly modulated by PIP_2 levels and PIP_2 resynthesis may play a central role in the gustatory adaptation process at the primary receptor cell level.

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Adaptation, the reduced neurophysiological or psychophysical response in the presence of a constant stimulus, occurs in all sensory modalities. In gustation, it has been documented neurophysiologically at every level of the neuraxis, from receptor cells to central neurons, as well as in conscious perception. Underlying mechanisms for adaptation are not well understood, but are likely to include an amalgam of processes occurring at different sites (peripheral and central) as well as different taste qualities. Recent advances in the study of taste receptor cells (TRCs, e.g. Chandrashekar *et al.* 2006) have dramatically advanced our understanding of cellular transduction mechanisms underlying responses to tastant stimuli. Once canonical ideas of the role of cyclic nucleotides (Kinnamon & Margolskee, 1996; Gilbertson *et al.* 2000; Smith & Margolskee, 2001; Gilbertson & Boughter, 2003) have been displaced by a pathway that utilizes the *trp* channel TRPM5 (Perez *et al.* 2002; Zhang *et al.* 2003) activated downstream of the enzyme phospholipase C-β2 (PLCβ2, Rossler 1998; Zhang, 2003). This pathway, initiated by stimulation of members of the seven-transmembrane receptor families T1R or T2R, adequately explains how these receptor-expressing cells depolarize in response to an appropriate gustatory stimulus. These early, or primary, transduction events are subsequently followed by activation of later, or secondary, pathways involving cell-to-cell communication among the individual cells within the bud. At present, these secondary pathways are less well understood but they involve a number of neurotransmitters and neuropeptides (Herness *et al.* 2005; Roper, 2006). Quite probably, these primary pathways act in conjunction with the secondary pathways to ultimately excite the afferent nerve fibre and relay the presence of oral stimuli to the central nervous system.

The combination of the discovery of these new key signalling molecules in TRCs with advances in the knowledge of lipid regulation of ion channels has provided a new basis for investigation of adaptation at the primary receptor cell level. In particular, the understanding of lipid

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regulation by the phosphatidylinositiol 4,5-bisphosphate (PIP_2) of ion channels has advanced greatly in the last several years (Hilgemann *et al.* 2001; Suh & Hille, 2005, 2008). It is now known that many members of the inwardly rectifying (K_{IR}) and the delay-rectifying (K_V) potassium channels are activated by PIP_2, which interacts directly with the channel stabilizing its open state. Our previous work (Zhao *et al.* 2002) demonstrated that caffeine, a prototypic bitter stimulus, has multiple actions on TRCs that include inhibition of K_{IR}, inhibition of K_V and increases of intracellular calcium. Interestingly, all these responses display adaptation, i.e. the inhibition of the current or the elevation of calcium declines in magnitude during the continued present of caffeine. We hypothesize that PIP_2 resynthesis plays a critical role in relieving the inhibition of both K_{IR} and K_V produced by caffeine. This paper provides the first evidence that potassium channels in TRCs are modulated by changing levels of PIP_2 and suggests that the dynamic regulation of this lipid plays an important role in adaptation of the bitter stimulus caffeine.

Methods

Ethical approval

All procedures were approved by the University's Laboratory Animal Care and Use Committee and adhered to the NIH *Guide for the Care and Use of Laboratory Animals*.

Anaesthesia and tissue/cell preparation

Experiments were performed on adult male Sprague–Dawley rats. Animals were brought to a surgical level of anaesthesia by intraperitoneal injection of 0.09 ml $(100\ g\ BW)^{-1}$ ketamine ($91\ mg\ ml^{-1}$; Hospira Inc. Lake Forest, IL) acepromazine ($0.09\ mg\ ml^{-1}$; Boehringer Ingelheim Vetmedica, Inc, St. Joseph, MO) mixture prior to kill by decapitation and excision of foliate and circumvallate papillae. Isolated taste receptor cells were dissociated from excised tissue by incubation in cysteine-activated ($1\ mg\ ml^{-1}$) papain ($14\ U\ ml^{-1}$) divalent-free bicarbonate-buffered solution (in mM: 80 NaCl, 5 KCl, 26 $NaHCO_3$, 2.5 $NaH_2PO_4H_2O$, 20 D-glucose, 1 EDTA) as previously described (Herness, 1989). This solution maintains a pH of 7.2–7.4 in a 5% CO_2 incubator. After incubation, tissue blocks were transferred to a pseudo-extracellular fluid (ECF; in mM: 126 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 5 Na_2Hepes (4-(2-hydroxyethyl)-1-piperazine ethanesulphonic acid, disodium salt), 1.25 $NaH_2PO_4.H_2O$, 10 D-glucose, adjusted with HCl to pH 7.3) and the epithelium removed with fine forceps and subjected to mild agitation. Some papillae were maintained in an ice-cold ECF solution for later dissociation. Taste receptor cells were identified from other lingual cells by their emblematic morphology.

Reagents

Pharmacological reagents were obtained from commercial vendors. Bovine serum albumin (BSA), caffeine, heparin, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride (LY294002), 4α-phorbol-12,13-didecanoate (4α-PDD), phosphatidylinositol (4,5) bisphosphate (PIP_2), phosphatidylinositol (3,4,5) trisphosphate (PIP_3), phorbol 12-myristate 13-acetate (PMA), polylysine, and wortmannin were purchased from Sigma (St Louis, MO, USA). KN-93 (*N*-[2-[[[3-(4′-chlorophenyl)-2- propenyl] methylamino]methyl]phenyl]-*N*-(2-hydroxyethyl)-4′-methoxybenzenesulpho-namide phosphate salt) and U73122 (1-[6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl]-1*H*-pyrrole-2,5-dione) were purchased from Calbiochem (San Diego, CA, USA) and ionomycin was obtained from Fluka (St Louis, MO, USA). Some agents (ionomycin, KN-93, LY294002, 4α-PDD, PMA, U73122, and wortmannin) required solubilization in dimethyl sulfoxide (DMSO). These reagents were dissolved in stock solution aliquots of $20\ mg\ ml^{-1}$ and stored frozen. The highest tested concentration of these drugs was the equivalent of 1 : 1000 dilution of DMSO in ECF. We have previously tested ECF of standard composition and 1 : 1000 DMSO and noted no discernible effects on either sodium or potassium currents in whole cell configuration (Herness *et al.* 1997). PIP_3 was solubilized in $10\ mg\ ml^{-1}$ DMSO.

Electrophysiology

Potassium currents were recorded from voltage-clamped taste receptor cells isolated from rat foliate and circumvallate papillae using standard patch clamp recording techniques in the whole cell configuration as previously described (Herness, 2002). Microelectrode pipettes were pulled on a gas-cooled multistage puller from 1.5 mm (o.d.) borosilicate glass (World Precision Instruments, Sarasota, FL, USA). The composition of pseudo-intracellular fluid (ICF) used for recording K_{IR} and K_V consisted of (in mM) 130 potassium gluconate, 10 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA (ethylenebis (oxonitrilo)tetraacetate), 10 Hepes (sodium salt), and 4 ATP (disodium salt). Recording electrode resistances were typically 5–7 MΩ when filled with ICF and measured in ECF. The extracellular solution for recording K_{IR} consisted of the standard ECF recipe with the replacement of 25 mM NaCl by an equivalent amount of KCl (final extracellular potassium concentration of 30 mM). K_V currents were recorded using the standard ICF and ECF compositions. A few experiments were performed using the perforated patch configuration with amphotericin B as

the ionophore (400 μg ml^{-1} in the ICF). The composition of the perforated patch ICF was (in mM): 55 KCl, 75 K_2SO_4, 8 $MgCl_2$, and 10 Hepes. A period of approximately 30 min was required to reach a stable level of recording after gigaseal formation.

To achieve the whole cell recording configuration, the pipette tip was positioned to contact the cell membrane and negative pressure was applied to its interior to facilitate gigaseal formation. Junction potentials were corrected before the electrode contacted the cell. Seal resistances were on the order of several decades of gigaohms. Further negative pressure was applied to enter whole-cell recording mode. Fast and slow capacitance compensation was employed as necessary with amplifier controls. Cell membrane capacitance and uncompensated series resistance were adjusted to produce optimal transient balancing. Membrane capacitance was 3–6 pF; series resistance averaged 10 MΩ in conventional whole-cell mode and 20–50 MΩ in most amphotericin B-perforated patch-clamp recordings. Low-pass filtering due to resistance–capacitance coupling was considered minimal. The product of these factors produces a time constant of 30–300 μs or a cutoff frequency ($1/2\pi RC$, where R is resistance and C is capacitance) of 1.6–16.6 kHz.

A test concentration of 20 mM was chosen for the caffeine stimulus. We have previously examined the effects of both 10 and 20 mM caffeine as test concentrations (Zhao *et al.* 2002). There were no significant differences in their electrophysiological responses. These concentrations were chosen based on their effectiveness in producing both neural and psychophysical responses in the rat. Twenty millimolar caffeine is about a mid-range concentration for the glossopharyngeal neural response and is a test concentration that is highly avoided in a two-bottle choice assay (Iwasaki & Sato, 1981). It also compares well to the human psychophysical range for caffeine, which similarly is the millimolar range (Drewnowski, 2001; Keast & Roper, 2007).

Caffeine was focally applied through a pipette positioned approximately five hundred micrometres from the recorded taste receptor cell. Flow was directed towards the apical portion of the cell against a backflow of ECF that prevented further diffusion of the stimulus. The flow rate of ECF, from a gravity-based perfusion system, was approximately 1 ml min^{-1}. The dissociated cell preparation used in these studies potentially allows stimulation of apical and basolateral surfaces of the dissociated cell, a stimulation situation that for many tastant stimuli would be unlike that encountered under *in situ* conditions. In these studies we employed a stimulating technique that mostly restricts the stimulus application to the apical end of the taste receptor cell, thus mitigating the concern of basolateral exposure. Perhaps more importantly, it should be noted that basolateral exposure of the tastant stimulus caffeine most likely does occur *in situ*. Caffeine is membrane permeant and under *in situ* situations enters the taste receptor cell from the apical membrane and can reach intracellular and basolateral sites of the receptor cell. Peri *et al.* (2000) have demonstrated that a number of amphipathic bitter stimuli directly enter the taste receptor cell. Therefore since caffeine exposure to intracellular and basolateral sites occurs normally *in situ*, similar exposure *in vitro* is not considered problematic. Other assurances that specific transduction mechanisms are being studied include the observation that not all cells respond to a particular tastant, including caffeine, which agrees well with the heterogeneous distribution of transduction mechanisms across TRCs. Non-specific effects, such as the direct block of an ion channel or metabolic disturbances, would be expected to be common to all cells. As well, non-specific effects would not be blocked by specific pharmacological intervention. We have demonstrated, using this method, the PKC effects on caffeine as well as other studies including PKA mediation of potassium inhibition, the PKC effects on cycloheximide, β-blockers with noradrenaline stimulation, and CCK-A antagonists blocking the CCK effect. Finally, complimentary data, obtained with different techniques (patch clamp recordings or calcium imaging), are commonly impaired by pharmacological intervention of the transduction pathway. For example, tastants that produce measureable responses with both patch and fura-2 analysis, such as caffeine, are commonly blocked by agents that interfere with the IP_3 pathway, suggesting a common mechanistic origin to the response as opposed to a coincident artifact. Collectively, these observations further underscore that physiological responses to caffeine stimulation are being measured.

It should be emphasized the percentage of cells responding to caffeine in our assay is not, on a scientific basis, directly comparable to the percentage of T2R expression within the taste bud as observed by other techniques, such as immunocytochemistry or *in situ* hybridization. Although there are several reasons that preclude this direct comparison, the most important involves sampling differences. Cells chosen for electrophysiological analysis do not represent a random sample from taste buds and hence cannot be quantitatively compared to expression patterns determined with other techniques. First, sampling for electrophysiological analysis involves an unknown bias imposed by the dissociation procedure on isolated cells. Second, isolated cells selected for electrophysiological analysis are purposefully chosen by morphological criteria; specifically, cells with large round nuclei which facilitates proper placement of the patch clamp electrode are chosen (as well as other criteria such as the absence of blebs, smooth membrane, and cytoplasmic contrast). These imposed biases could result in over selection of a particular cell type (e.g. type II cells, which are known to express T1R

or T2R receptor members, have large round nuclei) that make quantitative comparison to other techniques invalid. Other considerations, such as that not all T2R expressing taste receptor cells might be responsive to caffeine or that all caffeine responsive cells necessarily require T2R expression, are further examined in the Discussion.

Data were acquired with a high-impedance amplifier (Axopatch 200A; Axon Instruments, Union City, CA, USA), a Pentium based computer, a 12-bit 330 kHz A/D converter (Digidata 1200; Axon Instruments), and a commercial software program (pCLAMP, v. 8.01; Axon Instruments). Recordings were made at room temperature. Membrane currents were acquired after low-pass filtering with a cut-off frequency of 5 kHz (at −3 dB). A software-driven digital-to-analog converter generated the voltage protocols. Data were elicited using ramp protocols. For both K_{IR} and K_V currents, ramps were driven from a holding potential of −50 mV. Ramps were generated from −140 mV to +10 mV for K_{IR} currents and from −100 mV to +100 mV for K_V currents. Command potential ramps were generated at $0.166\ V\ s^{-1}$ and $0.125\ V\ s^{-1}$ for measuring K_{IR} and K_V currents, respectively. Data were analysed off-line with a combination of software programs that included a software acquisition suite (pCLAMP, Axon Instruments) and a technical graphics/analysis program (Origin 7.5, OriginLab Corp., Northampton, MA, USA). K_{IR} currents were analysed at the peak inward current, which typically occurred around −120 mV or for outward current at +10 mV. K_V currents were analysed at peak outward current magnitudes, typically at +100 mV. A response was defined as adapting if its magnitude began to decline towards baseline (i.e. a reduction in the caffeine-induced inhibition) within the first 90 s during the sustained application of the stimulus and the decline continues within the next 4 min of stimulus presentation. This time period compares well with the decline of the neurophysiological response observed in the rat and psychophysical measures of adaptation in humans. Data are presented as means ± standard error of the mean. The value of current before drug application was normalized as 100%.

Statistics were performed using a one-tailed Chi-squared analysis used to evaluate the significance of the difference between control and experimental groups. Since there were no differences in the manner in which any of the control values were obtained (e.g. cell sampling, testing protocol, caffeine concentration), responses of control cells to caffeine were combined for all treatments since pooling control data in this fashion facilitates comparison as it yields a larger sample size for statistical comparison. Similarly, data obtained from foliate or circumvallate taste receptor cells were combined since our previous studies detailing ion currents in these cells have never demonstrated any significant differences between these groups. Values of $P < 0.05$ were considered to indicate statistical significance.

Results

Caffeine responses display adaptation

Focal stimulation of taste receptor cells with the bitter tastant caffeine results in two types of electrophysiological responses: inhibition of an inwardly rectifying potassium current (K_{IR}; Fig. 1*A*) and inhibition of a delayed-rectifier potassium current (K_V; Fig. 1*D*; Zhao *et al.* 2002). As expected for tastant stimulation, these inhibitions were easily reversible with washout of the stimulus. Also as expected, not all TRCs responded to caffeine. Over the varied number of control and test groups for K_{IR} and K_V, the percentage of cells unresponsive to caffeine ranged from 7 to 25%. When measuring K_{IR}, both inward (Fig. 1*A*, open squares) and outward (Fig. 1*A*, open circles) components of the current were inhibited by caffeine exposure. Stimulation resulted in an average of 62 ± 1.2% and 40 ± 1.0% remaining current magnitudes for inward and outward components, respectively. For K_V current, caffeine produced an average of 49 ± 2.4% remaining current. Many of these K_{IR} and K_V responses displayed adaptation. During the continued presence of caffeine, response magnitudes were maximal upon initial application of the stimulus and within the next few minutes declined back to prestimulus levels (Fig. 1*B* and *E*; K_{IR} and K_V, respectively). Some responses recorded from other cells did not display adaptation, i.e. the response magnitude was constant during protracted stimulus application (Fig. 1*C* and *F*; K_{IR} and K_V, respectively). Under control conditions, 58% of cells (100 of 171 cells) displayed adaptation when measuring K_{IR} current and 31% of cells (39 of 128 cells) displayed adaptation when measuring K_V.

There were no differences in the averaged magnitude of the caffeine inhibition when comparing adapting and non-adapting responses (data not shown). The similarity of the observations for these two types of potassium current suggests that a similar mechanism may underlie this phenomenon. A role for PIP_2, the precursor lipid for the second messenger IP_3 and DAG, in this adaptation process was investigated since it has been shown to be an important regulator of ion channels, such as potassium channels, and since it is thought to play a central role in gustatory transduction mechanisms. Our results, during sustained taste stimulation, suggest that dynamic changes in PIP_2 levels influence both K_{IR} and K_V potassium currents. The effects of a wide variety of pharmacological agents used in this study emerged only after the challenge of caffeine which implies study of a stimulus-dependent event. Specifically, after stimulus-evoked decline of PIP_2

levels, its resynthesis in TRCs can transform non-adapting responses into those displaying adaptation.

Increasing PIP_2 levels in TRCs enhances adaptation

In other cell types, PIP_2 is well known to modulate both K_{IR} and, to a lesser extent, K_V channels, where its resynthesis typically activates channel activity (or in some cases removes inactivation). K_{IR} channels typically display greater affinity for this phospholipid. Based on these observations, we hypothesized that increasing PIP_2 levels should enhance adaptation of the caffeine response by relieving the inhibition of the channel produced by caffeine stimulation. To test this hypothesis, two regulatory enzymes of PIP_2 were targeted: phosphatidylinositol 3-kinase (PI3K), which acts to decrease PIP_2 levels by converting it to PIP_3, and PLCβ2, which similarly acts to decrease PIP_2 levels by using it as a substrate for IP_3 and DAG production.

PI3K actions were tested with wortmannin, a cell-permeant fungal metabolite that acts as a potent and selective inhibitor of this enzyme. Wortmannin acts to block the catalytic activity of this kinase without affecting upstream signalling events. It also inhibits other kinases such as phosphoinositide 4-kinase (PI4K) at concentrations about 100-fold higher than that required for inhibition of PI3K (e.g. Suh & Hille, 2005; Etkovitz *et al.* 2007).

For both K_{IR} and K_V, treatment with wortmannin (1 μM) significantly increased the number of cells that responded to caffeine with adapting responses. In addition, for both currents, wortmannin treatment was able to transform a non-adapting response to an adapting response within a single cell, consistent with the notion that adaptation is promoted by wortmannin-induced PIP_2 accumulation. A sample recording from a single cell is presented in Fig. 2 (top) for K_{IR} current which illustrates a non-adapting response to 20 mM caffeine

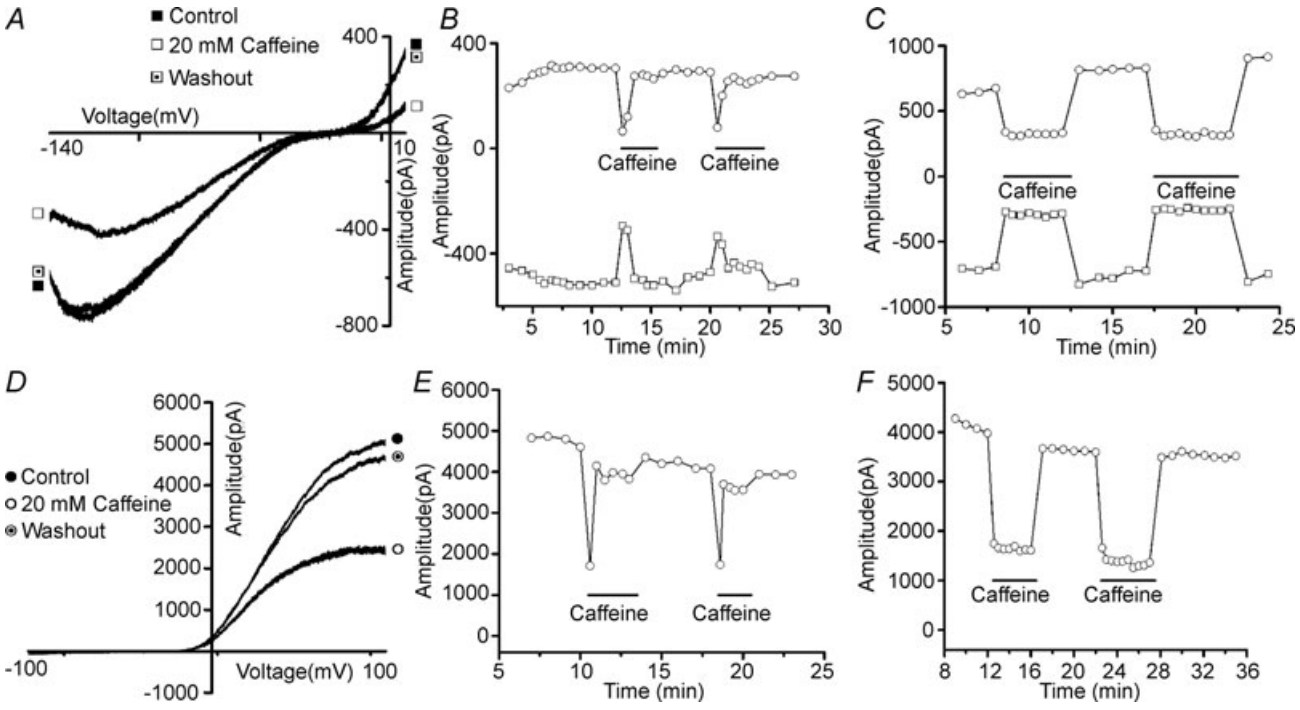

Figure 1. Sample recordings of both K_{IR} and K_V currents from dissociated rat taste receptor cells before, during and after application of 20 mM caffeine

Both K_{IR} (*A*) and K_V (*D*) currents, isolated for study, are inhibited by caffeine stimulation. When these responses are recorded during a prolonged continuous exposure to caffeine, some displayed adaptation, i.e. their response magnitude diminished over time. *B* and *E* illustrate adapting responses from K_{IR} and K_V, respectively. Note that the magnitude of the response, which is an inhibition of either current, diminishes over time and typically returns to baseline within 30 s although the stimulus remains present for 3 min. On the other hand, some electrophysiological responses to caffeine did not display adaptation. *C* and *F* illustrate non-adapting responses to K_{IR} and K_V, respectively, from two different cells. In these recordings the inhibition of either current was sustained throughout the stimulus presentation. In graphs representing data for K_{IR} (*B* and *C*), open squares represent the maximum magnitude of the inward portion of the potassium current and open circles represent the maximum magnitude of the outward portion of the K_{IR} current. In graphs representing data for K_V (*E* and *F*), open circles represent the magnitude of the maximal outward K_V current.

altered to an adapting response after the addition of 1 μM wortmannin. For K_{IR} about 85% (22 of 26 cells) of TRC responses to caffeine displayed adaptation after 1 μM wortmannin exposure compared to 56% of cells in the ECF control (100 of 171 cells; Fig. 2*B*). This increase was statistically significant ($P < 0.005$) when compared with a one-tailed chi-squared analysis. Wortmannin treatment did not influence the magnitude of the inhibition produced by caffeine ($38 \pm 0.95\%$ *versus* $35 \pm 2.36\%$, control *versus* treated cells, $P < 0.32$; *t* test). These observations are consistent with the notion of PI3K blockage by wortmannin resulting in increased PIP_2 levels. On the other hand, when the wortmannin concentration was increased to 50 μM, none of the tested cells displayed adaptation (0 of 15 cells), also statistically significant when compared to control cells (100 of 171 cells) using a one-tailed chi-squared analysis ($P < 0.0008$). These data suggest, in agreement with published data in other cell types, that in TRCs 1 μM wortmannin mainly inhibits PI3K activity, increasing PIP_2 and promoting adaptation, whereas at higher concentration (50 μM) it strongly blocks PI4K, decreasing PIP_2 and eliminating adaptation.

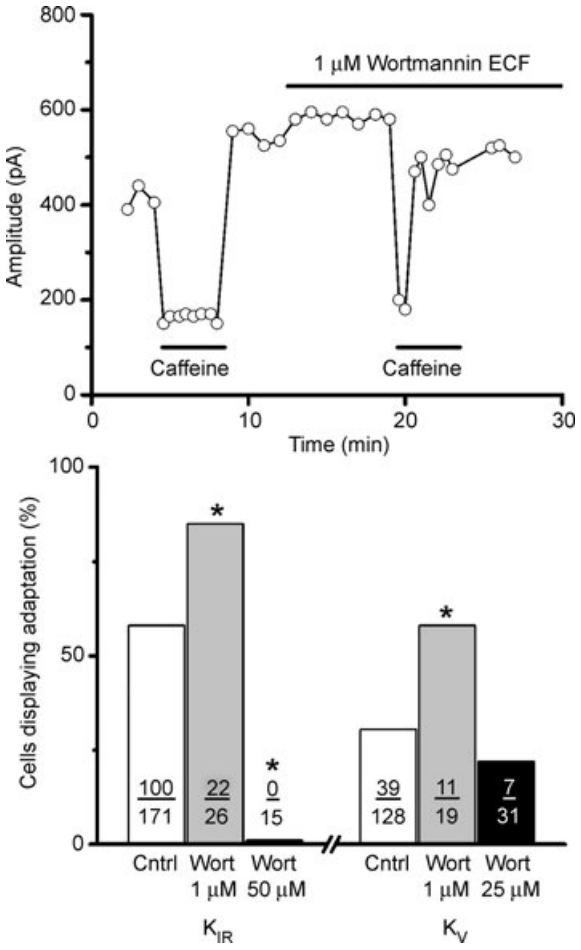

Figure 2. Adaptation of the caffeine response is strongly influenced by manipulations of endogenous levels of PIP_2
The application of 1 μM Wortmannin, an inhibitor of PI3K that acts to increase PIP_2 levels by blocking its conversion to PIP_3, significantly increased the number of caffeine responses displaying adaptation. Data from a representative cell are presented in the top panel. Wortmannin successfully transformed a non-adapting response to caffeine into an adapting response. In this cell, stimulation with 20 mM caffeine produced a sustained inhibition of K_V yet during the application of wortmannin the response to caffeine displayed adaptation, i.e. the magnitude of the inhibition declined though the caffeine stimulus was still present. Summarized data are presented in bar graph form in the bottom panel for both K_{IR} and K_V. Wortmannin, at 1 μM (grey bars), which acts to increase PIP_2 levels, significantly increased the number of cells displaying adaptation for both K_{IR} and for K_V when compared to their respective control groups. At higher concentration (black bars), where wortmannin is known to block PI4K, an enzyme in the biosynthetic pathway for PIP_2, and would hence decrease PIP_2 levels, wortmannin produced a significant decrease in the number of cells displaying adaptation for both K_{IR} and K_V. The number of cells displaying adaptation over the number of total cells tested is presented for each condition. Asterisks indicate statistical significance when compared to the control group ($P < 0.05$).

Similar effects of wortmannin were observed when recording K_V currents. A low concentration of wortmannin (1 μM) statistically increased the percentage of cells displaying adaptation from a control value of 31% (39 of 128 tested cells) to 58% (11 of 19 tested cells; $P < 0.0093$). As with K_{IR}, the magnitude of the inhibition of K_V current by caffeine ($49 \pm 2.4\%$) was unaltered by wortmannin treatment ($50 \pm 2.4\%$). Using a high concentration of wortmannin (25 μM), expected to inhibit both PI3K and PI4K, the percentage of cells displaying responses with adaptation to caffeine dropped to 22% (7 of 31 tested cells). Although the effect of wortmannin at high concentration was in the same direction as its effect on K_{IR} currents, this dosage failed to achieve statistical significance ($P < 0.1924$). Collectively, these data suggest that interfering with PIP_2 production either by blocking its conversion to PIP_3 or by limiting its production by PI4K produce results consistent with the hypothesis that PIP_2 modulates these potassium channels.

These effects of wortmannin were confirmed using additional pharmacological agents employed to manipulate endogenous PIP_2 levels. Two were tested: LY294002, a potent inhibitor of PI3K, and U73122, an inhibitor of PLCβ2. Treatment with either agent would be expected to increase PIP_2 levels since both enzymes use PIP_2 as a substrate and therefore either agent would be predicted to increase the incidence of adaptation of the caffeine response. Consistent with previous data, both agents acted to increase the number of cells producing adapting responses to caffeine. While recording K_{IR}, treatment with 50 μM LY294002, applied in the ICF, increased the number of caffeine responses displaying adaptation from 58% (100 of 171 cells) under control condition to 86% (12 of 14 cells; Fig. 3). Similarly, for caffeine mediated inhibition of K_V, adapting responses increased from 31% to 67% (29 of 43 cells). Both effects were statistically

significant when compared to control cells using a one-tailed chi-squared analysis ($P < 0.0225$, $P < 0.0001$, respectively). Similar to wortmannin treatment, the magnitude of the caffeine-induced inhibition of either K_{IR} or K_V was unaffected by LY294002 treatment. Remaining current for inward K_{IR} current was $63 \pm 3.3\%$ after LY294002 application compared to $62 \pm 1.0\%$ for control cells. For K_V measurements, the remaining current was $53 \pm 2.6\%$ compared to a control value of $49 \pm 2.4\%$. Thus inhibition of PI3K with either low concentrations of wortmannin or LY294002 produced essentially parallel results in increasing the incidence of adaptation to caffeine stimulation.

Inhibition of PLCβ2 produced results qualitatively similar to that produced by LY294002 thus providing additional confirmatory evidence that elevating membrane PIP$_2$ levels by blocking its hydrolysis facilitates the process of adaptation. While recording K_{IR}, treatment with 125 μM U73122, applied through the pipette, increased the number of adapting cells from 58% (100 of 171 cells) to 82% (14 of 17 cells). Similarly, while recording K_V, the number of adapting responses increased from 31% to 51% (10 of 17 tested cells). Both treatments were statistically significant ($P < 0.0027$; $P < 0.0101$, respectively). The magnitude of the inhibition produced by caffeine was unaffected by U73122 treatment. Remaining current for inward K_{IR} current was $62 \pm 2.6\%$ after U73122 application compared to $62 \pm 1.0\%$ for control cells. For K_V measurements, the remaining current was $51 \pm 3.4\%$ compared to a control value of $49 \pm 2.4\%$.

Reducing PIP$_2$ levels diminishes the incidence of caffeine adaptation

The previously described experiments produced data suggesting that pharmacological manipulations designed to increase endogenous levels of PIP$_2$ facilitate the mechanism of adaptation of the caffeine response. Here we test additional manipulations of PIP$_2$ designed to reduce rather than augment PIP$_2$ levels.

Polycations, such as neomycin, polylysine or BSA, are commonly employed as a practical treatment used for lowering PIP$_2$ levels. They act as scavengers to block the anionic head-groups of PIP$_2$ and consequently disrupt its ability to bind positive charges within target proteins. Here we tested polylysine and BSA on taste receptor cells hypothesizing it should effectively prevent elevations of PIP$_2$ levels during resynthesis thus preventing PIP$_2$ interactions with both K_{IR} or K_V channels and hence impeding the adaptation process. In preliminary experiments, different concentrations of polylysine applied via the ICF were tested. It was noted that stable recordings were difficult using high concentrations to the ICF (100 μg ml^{-1}) and that low concentrations (5 μg ml^{-1}) did not produce any functional changes. A mid-range concentration of 25 μg ml^{-1} polylysine was employed, which produced a strong and significant inhibitory effect on adaptation of the caffeine response, reducing the number of responses that displayed adaptation. For K_{IR}, the number of responses displaying adaptation decreased from 58% to 25% (7 of 28 cells, $P < 0.0005$, Fig. 4). For K_V, the number of adapting responses was reduced from 31% to 6% (1 of 16 cells; $P < 0.0001$). Polylysine treatment had minimal effects on the magnitude of the caffeine inhibition. For K_{IR} currents, the remaining current (treatment *versus* control) was $78 \pm 2.4\%$ *versus* $62 \pm 1.2\%$ and for K_V currents $47 \pm 4.3\%$ *versus* $49 \pm 4.2\%$. Overall, these data thus nicely counterbalance the previously described pharmacological manipulations of PIP$_2$ levels that demonstrated that elevations of PIP$_2$ in TRCs facilitated the process of adaptation to the caffeine.

Moreover, these data are corroborated by similar treatment with BSA. BSA virtually eliminated adaptation without any significant effect on the magnitude of the inhibition produced by caffeine. When recording K_{IR}, the number of caffeine responses displaying adaptation decreased from 58% to zero (0 of 42 cells, $P < 0.0001$; Fig. 4) after treatment with 0.5% BSA. For K_V, the number of adapting responses was reduced from 31% to zero (0 of

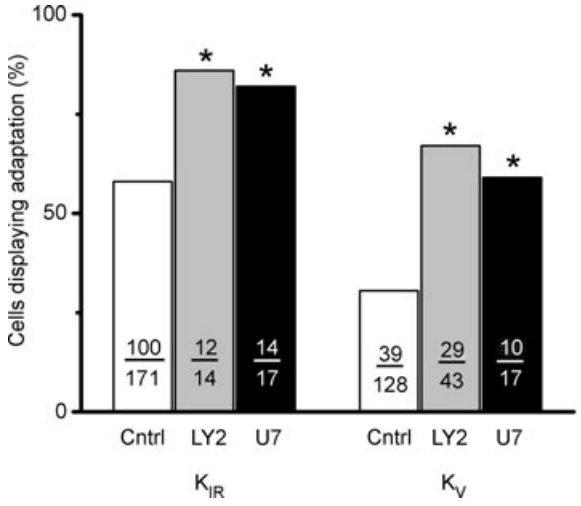

Figure 3. Adaptation is facilitated by elevations of endogenous levels of PIP$_2$
Summary data for two pharmacological treatments, LY294002 (LY2), a potent inhibitor of phosphatidylinositol 3-kinase, and U73122 (U7), an inhibitor of phospholipase C, are presented as a bar graph. Both antagonists act to increase endogenous levels of PIP$_2$, by blocking its phosphorylation to PIP$_3$ or its hydrolysis to IP$_3$ and DAG, respectively. Both treatments significantly increased the number of cells producing responses to caffeine with adaptation when compared to untreated control cells. These observations are consistent with the hypothesis that elevated levels of endogenous PIP$_2$ facilitate the process of adaptation. The number of cells contributing to each point is indicated. Asterisks indicate statistical significance ($P < 0.05$).

24 cells; $P < 0.0001$). The magnitude of inhibition of the K_{IR} by caffeine was unaffected for either K_{IR} ($63 \pm 2.9\%$ treated *versus* $62 \pm 1.2\%$ control) or K_V (40.3 ± 2.2 treated *versus* $49 \pm 4.2\%$ control) currents.

Applying PIP_2 or PIP_3 reduced responsiveness to caffeine

In separate experiments, the effect of direct application of exogenous PIP_2 or PIP_3 was tested on K_{IR} and K_V currents. PIP_2 was applied to cells via pipette administration during whole-cell patch recording. It is important to note that PIP_2 diffusion allows only steady increase of PIP_2 as it diffuses into the cytoplasm and subsequently incorporates into the plasma membrane. This technique, though practical, does not permit precise temporal control of PIP_2 concentration so that the decline and subsequent elevation occurring with resynthesis during tastant stimulation cannot be mimicked. Instead, pipette administration permits caffeine stimulation against a sustained elevated background level of PIP_2. Under these conditions the most striking result when recording either K_{IR} or K_V currents was a substantial decrease in the number of cells which responded to caffeine. When measuring K_{IR}, only 17% of TRCs ($n = 9$ of 52 tested cells) responded to caffeine in the presence of sustained PIP_2 levels (tested at either 10 or 50 μM) compared to about 75% of tested cells under control conditions. Of those cells that responded to caffeine, all displayed responses with adaptation compared to 58% adaptation under control conditions (Fig. 5, $P < 0.0311$ 10 μM; $P < 0.047$ 50 μM). Thus, sustained PIP_2 levels may act to stabilize K_{IR} channels, preventing their subsequent inhibition by caffeine.

On the other hand, caffeine stimulation during the sustained presence of PIP_2 while recording K_V was more complex. Sustained PIP_2 also reduced the number of cells that responded to caffeine to about 56% (10 and 50 μM; 34 of 60 tested cells). However, unlike K_{IR}, those cells that responded to caffeine did so either with or without adaptation. The number of adapting responses was not significantly different from control values. PIP_2, tested at 10 μM, produced 27% of adapting responses ($n = 5/18$ cells; $P < 0.41$) and, at 50 μM, 25% of responses displayed adaptation ($n = 4/16$ cells; $P < 0.33$).

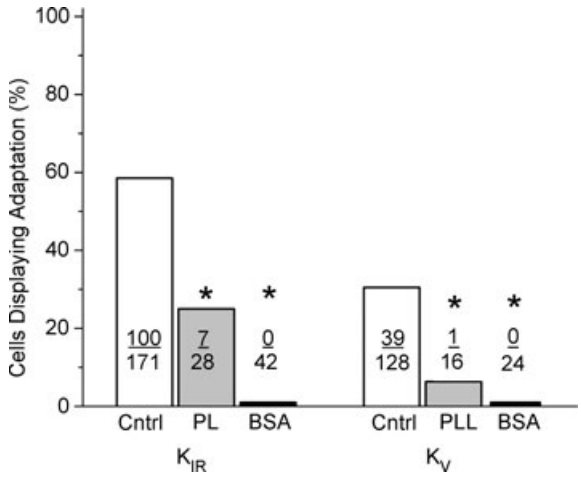

Figure 4. Reduction of PIP_2 levels reduces the incidence of adaptation of potassium current response to caffeine
The use of scavenger polycationic molecules, such as polylysine (PL) or bovine serum albumin (BSA), is known to decrease the effective concentration of PIP_2 by binding to its anionic head-group and consequently disrupting its ability to bind positive charges within target proteins, such as potassium channels. Treatment with polylysine (PL; grey bars) produced a strong inhibitory action of the adaptation of the caffeine response. For K_{IR} the incidence of adaptation was reduced from a control value of 58% to 25%. When recording K_V currents, the incidence of adaptation was reduced from 31% to 6%. Both effects were statistically significant, as indicated by asterisks. The effect produced by BSA treatment proved to be more robust than that of polylysine. BSA acted to eliminate adaptation of the caffeine response for both tested potassium currents. No cells were observed to elicit adapting responses to caffeine in the presence of BSA when measuring either K_{IR} or K_V currents. The number of cells contributing to each bar of the graph is indicated. Asterisks indicate statistical significance ($P < 0.05$).

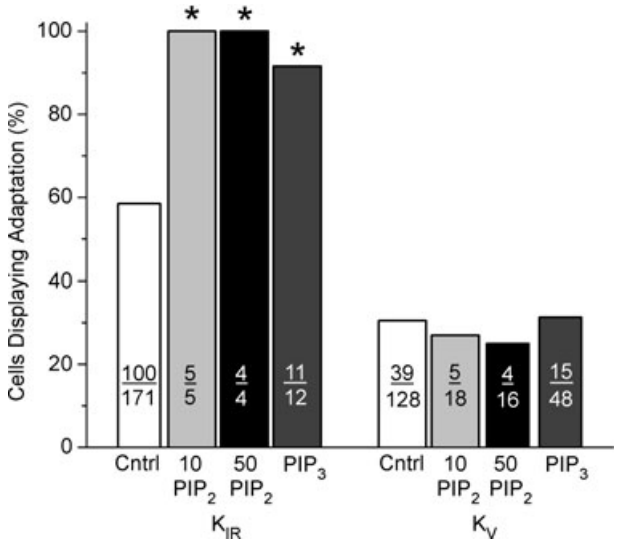

Figure 5. Exogenous application of PIP_2 or PIP_3 differentially affected caffeine inhibitions of K_{IR} or K_V
Sustained application of PIP_2, at either 10 μM or 50 μM, substantially increased the number of responses displaying adaptation when compared to control responses for KIR currents. At these same concentrations, exogenous PIP_2 application had little effect on the incidence of adaptation when measuring K_V currents. Similarly, PIP_3, another phosphoinositide signalling molecule, produced an unexpected increase in adaptation of K_{IR} but was without effect on K_V. PIP_3 increased the value for K_{IR} to 92% whereas that for K_V was the same as the control value (31%). The number of cells contributing to each bar is indicated. Asterisks indicate statistical significance ($P < 0.05$).

In additional experiments, the potential role of PIP_3 was tested. PIP_3 was chosen for investigation since, after the initial discovery that PIP_2 produced profound influence over a variety of ion channels, reports appeared that additional phosphoinositides, such as PIP_3, could produce similar actions (cf. Czech, 2000). These effects, though, can be quite diverse on different classes of channels. Exogenous PIP_3 was added to the patch recording pipette using the whole cell recording configuration. A test concentration of 1 μM was chosen as preliminary experiments using 5 μM failed to produce stable recordings. PIP_3 had a strong effect on the adaptation of the caffeine response on K_{IR} but was essentially without effect when recording K_V currents. For K_{IR} currents, the addition of PIP_3 in the pipette increased the incidence of adaptation in the caffeine response to 92% (11 of 12), a significant increase when compared to control cells (58%; $P < 0.0115$). On the other hand, application of PIP_3 was without effect on K_V currents; 31% (15 of 48) of tested cells displayed adaptation compared to 31% for control cells (Fig. 5). These data represent one of the few examples where analogous results for K_{IR} and K_V were not observed and might be due to the stronger influence that phosphoinositides exert over inwardly rectifying potassium channels *versus* outward potassium channels.

Adaptation of the caffeine response can be modulated by protein kinase C

Many of the enzymes influential in the regulation of PIP_2 levels are themselves subject to modulation. For example, since our present data demonstrated that PLCβ2 activity may modulate adaptation, potential regulatory actions of targets downstream of PLCβ2 were investigated. These included Ca^{2+}/calmodulin-dependent kinase II (CaMK-II) and PKC. To test for modulatory actions of PKC, phorbol 12-myristate 13-acetate (PMA), a well-known PKC activator, was employed. PMA is a phorbol ester that affects PKC by mimicking diacylglycerol and activates both group A (α, βI, βII, γ) and group B (δ, ε, η, θ) protein kinases in the 1–100 nM range. Application of PMA significantly enhanced adaptation when added either to the pipette ICF solution (1 μM) or applied extracellularly (1 μM) while recording K_{IR}. After PMA application, 84% of tested cells (26 of 31 cells) displayed adaptation compared to the control value of 58% (Fig. 8, $P < 0.0036$). Similar to all other pharmacological treatments, PMA had only modest effects on the magnitude of the inhibition produced by caffeine stimulation ($67 \pm 3.3\%$ treated cells *versus* $62 \pm 1.2\%$ for untreated cells). In addition to PMA, its inactive analogue 4α-phorbol-12,13-didecanoate (4α-PDD) was tested under the same conditions. It was without effect on adaptation, yielding a value of 60% (6 of 10 cells, $P > 0.4622$). It was also without effect on the magnitude of inhibition ($61 \pm 3.7\%$ treated cells *versus* $62 \pm 1.2\%$ untreated cells).

When measuring K_V, similar phorbol ester effects were observed. PMA, added either to the pipette (1 μM) or to the ECF (1 μM), produced adaptation of 64% (16 of 25) of tested cells compared to 31% (39 of 128) for control cells. A modest decrease in the magnitude of the inhibition was noted ($42 \pm 3.5\%$ inhibition *versus* $49 \pm 4.2\%$ in control cells). However, the inactive phorbol ester 4α-PDD, added to the ICF (1 μM), produced an unexpected 53% of responses with adaptation (9 of 17 tested cells) though no real effect was noted on the magnitude of inhibition ($45 \pm 4.2\%$ in treated cells *versus* $49 \pm 4.2\%$ in control cells). The unexpectedly large effect of 4α-PDD on the incidence of adaptation suggests that PKC modulation on these potassium channels cannot be convincingly concluded.

To test for CaMK-II activity, *N*-[2-[[[3-(4′-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-*N*-(2-hydroxyethyl)-4′-methoxybenzenesulphonamide phosphate salt (KN-93) a selective and cell permeant CaMK-II inhibitor, and its inactive analogue, 2-[*N*-(4′-methoxybenzenesulphonyl)]amino-N-(4′- chlorophenyl)-2-propenyl-*N*-methylbenzylamine phosphate (KN-92), were tested. Inhibition of CaMK-II by KN-93 was without effect on adaptation. For K_{IR} currents, 56% of tested cells displayed adaptation (9 of 16 cells; Fig. 6) compared to 58% (100 of 171) for control cells. The magnitude of inhibition was slightly depressed at $71 \pm 5.2\%$ remaining current compared to $62 \pm 1.2\%$ for controls cells. Similarly when recording K_V current, 26% of tested cells (8 of 31 cells) displayed adaptation compared to 31% (39 of 128 cells) for control cells. The magnitude of inhibition was unaffected ($50 \pm 2.1\%$ tested cells *versus* $49 \pm 4.2\%$ control cells). Thus neither current magnitude of inhibition was significantly affected by pharmacological manipulation of CaMK-II. Additionally, calmodulin itself appears to be vital to cell function, since 50 and 100 μM *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide hydrochloride (W-7) added into the pipette (25, 50, or 100 μM) quickly produced irreversible damage to the cell ($n = 7$; data not shown). KN-92, which served as a negative control, produced results similar to KN-93 (28%; 8 of 29 cells). Collectively, these data suggest that whereas calmodulin has important functions in taste receptor cells, its participation through CaMK-II does not occur in the adaptation process to caffeine.

Discussion

Collectively, these data strongly suggest that the response of TRCs to a gustatory stimulus is influenced by changing

levels of phosphatidylinositol 4,5-bisphosphate, the first such demonstration to our knowledge. They further document modulation of both K_{IR} and K_V currents by PIP_2 as an underlying mechanism for this phenomenon. PIP_2 has long been hypothesized to be important in taste transduction mechanisms as a substrate for the hydrolytic production of the second messengers IP_3 and DAG. As well, in other cell types PIP_2 is well known to influence the gating of potassium channels, both inwardly and delayed-rectifying types (e.g. Hilgemann *et al.* 2001; Suh & Hille, 2005, 2008). In this study data suggest a similar phenomenon occurs in TRCs. To date, most studies of taste transduction mechanisms have focused on events leading to the decline of PIP_2 while consequences of its resynthesis have remained essentially unstudied. Levels of PIP_2 can be changed by multiple enzymes involved in phosphoinositide metabolism, such as phosphoinositide kinases, phosphoinositide phosphatases, and phospholipase C. Since PIP_2 levels are low in most cells, estimated at only 1% of total plasma membrane phospholipid (e.g. Gamper & Shapiro, 2007), membrane proteins that are responsive to PIP_2 are highly sensitive to small changes in its levels, such as receptor-induced depletions and subsequent resynthesis even during prolonged stimulation. The data of the present communication suggest that potassium channels in TRCs are strongly influenced by PIP_2 levels and these events may influence later events occurring after the initial transduction mechanisms, namely adaptation.

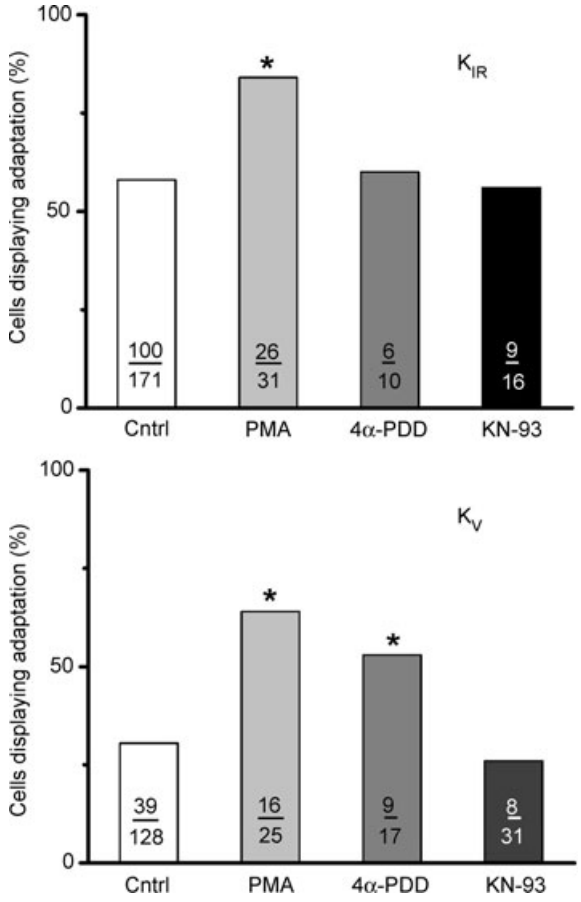

Figure 6. The activation of PKC could effectively increase the number of taste receptor cell responses to caffeine that displayed adaptation whereas activation of calcium–calmodulin was ineffective in modulating adaptation
When recordings K_{IR} current, the phorbol ester PMA, an activator of PKC, increased the number of cells displaying adaptation to caffeine from a control value of 58% to 84%. 4α-PDD, an inactive analogue of PMA, did not significantly differ from control values. KN-93, an activator of calcium–calmodulin, gave a value of 61%, which was not significantly different from control values. When recording K_V currents, both PMA and its inactive analogue 4α-PDD produced significant increases in the percentage of cells displaying adaptation responses to caffeine when compared to untreated control cells (64% or 53% *versus* 31% for PMA, 4α-PDD, or control cells, respectively). Hence we are unable to distinguish between the possibilities that the PMA effect is artifactual (since the inactive analogue is also effective) or that the PMA is effective (as for K_{IR}) but the inactive compound produces some artifactual response with K_V channels. The calcium calmodulin activator KN-93 gave a value of 36%, which was not different from control values, suggesting it to be inactive. The number of cells contributing to each treatment value are indicated. Asterisks indicate statistical significance ($P < 0.05$).

Actions of caffeine on TRCs

Our previous study on the effects of the bitter stimulus caffeine on TRCs (Zhao *et al.* 2002) suggested multiple effects occur including inhibition of potassium channels and elevation of intracellular calcium. To date the transduction steps for caffeine remain unresolved. In the canonical view of bitter taste transduction, PIP_2 levels would be expected to be initially reduced via a caffeine-initiated stimulation of the T2R–gustducin–PLCβ2 cascade. Alterations in membrane potential of the TRC subsequently result from depolarization via TRPM5 activation followed by subsequent activation of voltage-activated ion channels, such as sodium and potassium channels. Activation of PLCβ2, the PLC isoform expressed in TRCs (Rossler *et al.* 1998), and subsequent PIP_2 hydrolysis, is widely acknowledged as essential for the transduction of stimuli such as sweet and bitter, due, for example, to the inability of PLCβ2 knockout mice to respond, either neurophysiologically or behaviourally, to several compounds representing these qualities (Zhang *et al.* 2003). Steps leading to activation of the TRPM5 ion channel in this cascade at present remain obscure but are hypothesized to involve calcium. The release of calcium from intracellular stores by IP_3 production may be necessary to gate the TRPM5 channel (Zhang *et al.* 2007). An influx of a cation current through the TRPM5 channel fully depolarizes the TRC, which in turn activates voltage-gated ion channels within the cell to potentially trigger an action potential. The large

majority of the caffeine-responsive TRCs in our study (Zhao *et al.* 2002) expressed sodium currents, suggesting these TRCs are electrically excitable and capable of firing action potentials. Further caffeine application did not inhibit these sodium currents, thus not interfering with the cell's ability to fire action potentials.

Others have suggested that T2R-independent mechanisms may exist for bitter stimuli (e.g. Rosenzweig *et al.* 1999; Peri *et al.* 2000; Caicedo *et al.* 2003; Dotson *et al.* 2005; Nelson *et al.* 2005; Zubare-Samuelov *et al.* 2005). Support for this notion comes from observations that elimination of transduction elements such as α-gustducin or PLCβ2 did not completely eliminate bitter responses or that other pathways, such as cGMP may be involved. Our data do not directly address whether caffeine may operate via T2R-dependent or T2R-independent mechanisms. Although it is likely that caffeine operates through a receptor in the T2R family, to date such a receptor in the T2R family with caffeine as its ligand has yet to be identified. However, whereas T2R-dependent mechanisms may exist, T2R-indepdnent mechanisms for caffeine are also likely. Caffeine is known to enter cells directly and may interact with a variety of intracellular enzymes and/or ion channels. Further, caffeine was not tested on PLCβ2 knock-out mice so that a complete dependence of the caffeine response on PLCβ2 has not yet been established. As well, the caffeine response was not eliminated in an IP_3R knockout mouse (Hisatsune *et al.* 2007), suggesting that the PLCβ2-IP_3 canonical pathway may not be essential to caffeine transduction. Finally, in our data the inhibition of potassium current by caffeine was not eliminated by inhibition of PLCβ2, though adaption was effected. Caffeine may well have more than one transduction mechanism. As the avoidance of bitter stimuli is essential to survival, multiple bitter detection mechanisms would be evolutionarily advantageous.

The inhibition of potassium currents in TRCs by bitter stimuli has been observed in several organisms (e.g. Avenet & Lindemann, 1987; Cummings & Kinnamon, 1992; Chen & Herness, 1997; Seto *et al.* 1999; Zhao *et al.* 2002; Straub *et al.* 2003). Bitter stimuli inhibit potassium channels during both active (K_V) and resting (K_{IR}) states. The mechanistic steps connecting bitter stimulation and potassium channel inhibition is not known with certainty. K_V channels in TRCs are known to be inhibited by cAMP (Herness *et al.* 1997) and bitter stimuli may influence cyclic nucleotide levels. For example, quench-flow analysis has shown that caffeine stimulation produced not only measurable increases in IP_3 production (Spielman *et al.* 1994) but also more robust production of cGMP (Rosenzweig *et al.* 1999). Some K_{IR} channels may be inhibited by the $\beta\gamma$ subunit of G-proteins, which could occur after receptor-mediated G-protein stimulation. Regardless of the mechanism, inhibition of potassium currents by a tastant stimulus such as caffeine has several potential actions on the TRC. Like other cell types, K_{IR} contributes substantially to the resting potential of TRCs (Sun & Herness, 1996) and its inhibition would act to depolarize the cell, potentially stimulating voltage-gated channels, such as the sodium channel, and action potential production. Inhibition of K_V by either the potassium channel blocker TEA or by caffeine acts to broaden the action potential (Chen *et al.* 1996; Zhao *et al.* 2002) keeping the cell in a depolarized state for a longer period of time. The relaxation of caffeine's inhibition on these inhibitory potassium currents that we propose occurs by PIP_2 resynthesis thus acts to reduce the cell's excitability, as would occur during adaptation of a tastant response.

PIP_2 regulation of potassium channels

Our data demonstrate that two types of potassium currents, K_{IR} and K_V, are influenced by dynamic changes in PIP_2 levels in TRCs. A variety of pharmacological manipulations, all targeted to alter PIP_2 levels during stimulus-induced resynthesis, produced consistent results on the incidence of adaptation: increasing PIP_2 levels increased adaptation while decreasing PIP_2 levels produced the converse effect. These events are summarized in Fig. 7. Pharmacological manipulation of enzymes at a number of points in this synthetic/hydrolytic pathway of PIP_2 produced similar results. Three manipulations that would elevate PIP_2 levels were performed: blocking PI3K with either wortmannin or LY294002 or blocking PLCβ2 using U73122. All significantly enhanced adaptation, as

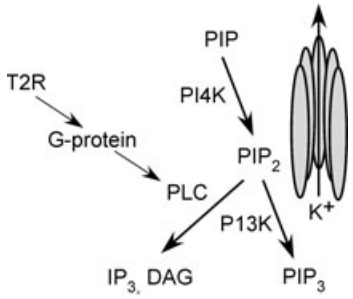

Figure 7. Summary of the pharmacologically targeted enzymes that influence PIP_2 interactions with potassium channels
The phospholipid PIP_2 is hypothesized to interact directly with either K_{IR} or K_V potassium channels where dynamic fluctuations in its level influence channel activity. Pharmacological inhibition of the enzymes PLC or PI3K, either of which acts to increase PIP_2 levels, resulted in an increase in the occurrence of adaptation of either potassium current to caffeine stimulation. On the other hand, treatments acting to decrease PIP_2 levels, such as pharmacologically blocking its synthetic enzyme, PI4K, or BSA or polylysine application, which interferes with PIP_2 interactions with the channels, resulted in a decrease in the occurrence of adaptation to caffeine stimulation. These findings suggest that potassium channels expressed in taste receptor cells are sensitive to fluctuations of PIP_2 and that PIP_2 resynthesis, which would occur after tastant stimulation, may act to restore channel activity.

would be predicted with elevated PIP_2 levels in the membrane. Conversely, blocking its synthetic enzyme PI4K with higher concentrations of wortmannin and thus reducing PIP_2 levels produced the expected results of decreasing adaption observed with K_{IR} and K_V. Similarly, reducing PIP_2 levels using a complimentary method of polylysine or BSA scavengers, which prevent the phospholipid from interacting with the channel, also diminished the incidence of adaptation of the caffeine response.

Two additional observations support the notion of relief of the caffeine inhibition by PIP_2 resynthesis. First, these effects emerged only after TRCs were challenged with a caffeine stimulus, suggesting stimulus-dependent resynthesis is required for the relaxation of the caffeine-induced inhibition. Pharmacological alterations of the potassium currents prior to caffeine stimulation were not noted. Second, none of these manipulations altered the initial magnitude of the caffeine-induced inhibition of the potassium current. Hence, their effects were noted only after stimulation, when resynthesis would occur, but not during the start of the caffeine-initiated inhibition. Collectively, they suggest that the inhibition of the potassium current induced by caffeine is mitigated by elevating levels of PIP_2 as would occur during its resynthesis subsequent to a stimulus-induced decline.

Direct application of phospholipid also affected the incidence of adaptation though these data represented one of the few examples where analogous results for K_{IR} and K_V were not observed. Both PIP_2 and PIP_3 had obvious effects on K_{IR} whereas both were much less effective on K_V. This difference may be explained by the stronger influence that phosphoinositides exert over inwardly rectifying potassium channels *versus* outward potassium channels (Suh & Hille, 2008). Structural differences of the K_{IR} (with two transmembrane domains) and K_V (with six transmembrane domains) are likely to play important roles. Polybasic domains (for K_{IR} just after the second transmembrane segment and for K_V just after the sixth transmembrane segment) are the putative interactive residues of the ion channels with PIP_2. This same phenomenon could underlie the greater incidence of adaption observed when measuring K_{IR} (58%) when compared to K_V (31%) currents in general.

The increased number of adaptive cells observed when levels of PIP_2 are elevated suggests that PIP_2 acts to stabilize both K_{IR} and K_V potassium channels, consistent with the notion that this phospholipid acts to maintain their open state (e.g. Logothetis *et al.* 2007; Tucker & Baukrowitz, 2008), thus mitigating the inhibition that otherwise would have resulted from caffeine stimulation. In general PIP_2 regulation of K_V channels is much less studied than regulation of K_{IR} (e.g. Bian & McDonald, 2007). PIP_2 regulation of K_V channels may differ in that both regulation of inactivation and regulation of activation could be occurring. In general it does not appear that regulation of inactivation of K_V is occurring in TRCs. Outward potassium currents in TRCs consist of inactivating and sustained components with time constants on the order of several seconds over several seconds (Chen *et al.* 1996). When caffeine was applied after having evoked outward K_V currents for an extended duration (e.g. 90 s), and hence recording only sustained potassium current, caffeine produced inhibitions with adaptation (personal observations). Since caffeine would be inhibiting potassium current with no inactivation under these conditions, it would suggest that removing inactivation is not the underlying mechanism for PIP_2 modulation. It has been demonstrated that with KCNQ channels, a type of delayed rectifier channel with very slow inactivation kinetics, PIP_2 acts to stabilize the open state of the channel (Loussouarn *et al.* 2003).

That PIP_2 and PIP_3 exerted parallel actions on K_{IR} adds to a growing list of ion channels where these two phosphoinositides act agonistically rather than antagonistically. Both PIP_2 and PIP_3 may promote PIP_2 synthesis by activating PI4P5-kinase (which synthesizes PIP_2) via small GTPase ADP ribosylation factor (ARF) proteins (Czech, 2000; Skippen *et al.* 2002). As well, both connect to pathways that tend to inhibit PI3K (Czech, 2000). Although it is difficult to make generalizations about the actions of PIP_2 and PIP_3 on different ion channels or transporters, both also have similar actions on many TRP channels, such as the TRPC6 channel where PIP_3 may activate TRPC6 current by displacing Ca^{2+}–calmodulin thereby increasing the current (Kwon *et al.* 2007). These results suggest that PIP2 and PIP3 regulation of potassium channels in TRCs may be more similar to their reported modulatory roles in TRP channels than to their reported modulatory roles for other types of ion channels. For example, in olfactory receptor neurons PIP_2 and PIP_3 have opposing actions on CNG channels (Zhainazarov *et al.* 2004; Brady *et al.* 2006). As well, application of exogenous PIP_2 must be affecting numerous membrane signalling pathways in the membrane that could differentially affect these types of potassium channels.

PIP_2 regulation may be subject to modulation since activation of the regulatory kinase PKC increased adaptation. The exact mechanism of PKC modulation of adaptation isn't yet understood. Based on other systems, it is likely that the catalytic activity of PLCβ2 is feedback regulated by PKC phosphorylation. PKC, once activated, would act to phosphorylate PLCβ2 in turn reducing its activity, and hence maintaining higher levels of PIP_2 with the continuance of adaptation. Additionally, PIP5K, synthetic to PIP_2, can be regulated positively by a PKC-mediated pathway (Park *et al.* 2001). These events will keep the PIP_2 levels high after PI4K was activated by calcium entry. Although activation of PKC

increased adaptation for both K_{IR} and K_V currents, control experiments with the inactive analogue of the PKC activation, 4α-PDD, failed when tested on K_V currents. Although this calls into question results with PKC modulation of PIP_2 activation with K_V currents, other experiments on K_V currents conducted with 50 nM bisindolylmaleimide, an antagonist of PKC, acted to eliminate adaptation (data not shown). These results suggest that 4α-PDD could produce unknown actions on K_V channels. Further study is required before any conclusion can be made on PKC modulation of the PIP_2 influence on adaption for K_V currents.

Finally, the molecular identity of the ion channels underlying these currents is not yet known. K_{IR} was among the first of any ion channels documented to be influenced by PIP_2 levels and remains the best analysed (e.g. Xie *et al.* 2007). Receptor-induced depletion of PIP_2 can inhibit K_{IR} channels and its resynthesis stabilize the channel in the open configuration by interacting with basic residues on the channel. Rundown is accelerated by manipulations that lower PIP_2 (e.g. PIP_2 antibodies, PLCβ2 activation, or polycations) and is slowed or reversed by manipulations that increase PIP_2 such as phosphatase inhibitors or PIP_2 itself. The channel types KIR1 (ROMK), $K_{IR}2$, $K_{IR}3$ (GIRK) and $K_{IR}6$ (KATP) are all activated by PIP_2. Although K_{IR} currents in TRCs have been characterized (Sun & Herness, 1996) the molecular identity of these channels remains unknown. The molecular identity of K_V type channels in TRCs is less well studied, thought $K_V1.5$ and $K_V3.1$ are well expressed (Liu *et al.* 2005). Whether these channel types are modulated by PIP_2 remains an open question. Outside of a report that interaction of PIP_2 with outwardly rectifying potassium channels can produce a functional change from inactivation to non-inactivation (i.e. A-type or delayed rectified type; Oliver *et al.* 2004) there is little in the literature on K_V type channels and PIP_2. Thus, our data may represent fertile ground for future study.

PIP_2 and adaptation in gustatory receptor cells

The mechanisms of gustatory adaptation at the periphery are incompletely understood. Peripheral adaptation was first documented with the first chorda tympani electrophysiological recording (Pfaffmann, 1941). Early studies on adaptation focused on the decline of the chorda tympani neural response to maintained gustatory stimulation (Smith *et al.* 1978). Time constants of the decline of the chorda tympani response and those describing the decline of perception were in close agreement suggesting the temporal pattern of action potentials did represent a neural correlate of perception. Although there was initial debate as to whether adaptation observed in the chorda tympani firing pattern was produced by adaptation within the primary receptor cell (or, e.g. at the receptor cell:nerve synapse), recordings of the gustatory action potential in taste receptor cells to sustained stimulation firmly established the phenomenon occurred in the receptor cell (e.g. Béhé *et al.* 1990). With more recent discoveries of primary transduction pathways, the study of adaptation has received comparatively little attention. Our results suggest that at least one component of the adaptation process resides in the TRC itself. However, since only one stimulus has been tested, it is premature to generalize these effects to other bitter or sweet stimuli.

Collectively the experiments presented here present a unified view for the role of PIP_2 resynthesis in the adaptation process to caffeine. Pharmacological manipulations that elevated PIP_2 levels enhanced adaptation whereas those manipulations that acted to decrease PIP_2 levels had the converse effect. It is probable that one mechanism does not account for the entire adaptation mechanistic scheme. Other events, such as receptor and/or enzyme phosphorylation or the influence of PIP_2 resynthesis on other target proteins (such as TRPM5, hemichannels, and/or the IP_3 receptor) are likely to participate in this process within the TRC. Additionally, the effects of PIP_2 are not restricted to adaptation. Nevertheless, potassium channels in TRCs are clearly modulated by PIP_2 resynthesis, a novel observation in itself, and restoring them to their open state appears a likely substrate for adaptation. The participation of extracellular calcium in this process and its route of ingress are the subjects for future investigation.

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