Dual actions of caffeine on voltage-dependent currents and intracellular calcium in taste receptor cells

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Dual actions of caffeine on voltage-dependent currents and intracellular calcium in taste receptor cells. Am J Physiol Regulatory Integrative Comp Physiol 283: R115–R129, 2002; 10.1152/ajpregu.00410.2001.—Although the numerous stimuli representing the taste quality of bitterness are known to be transduced through multiple mechanisms, recent studies have suggested an unpredicted complexity of the transduction pathways for individual bitter stimuli. To investigate this notion more thoroughly, a single prototypic bitter stimulus, caffeine, was studied by using patch-clamp and ratiometric imaging techniques on dissociated rat taste receptor cells. At behaviorally relevant concentrations, caffeine produced strong inhibition of outwardly and inwardly rectifying potassium currents. Caffeine additionally inhibited calcium current, produced a weaker inhibition of sodium current, and was without effect on chloride current. Consistent with its effects on voltage-dependent currents, caffeine caused a broadening of the action potential and an increase of the input resistance. Caffeine was an effective stimulus for elevation of intracellular calcium. This elevation was concentration dependent, independent of extracellular calcium or ryanozide, and dependent on intracellular stores as evidenced by thapsigargin treatment. These dual actions on voltage-activated ionic currents and intracellular calcium levels suggest that a single taste stimulus, caffeine, utilizes multiple transduction mechanisms.

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of phospholipase found in taste cells, phospholipase C-β2 (46). Indeed, bitter compounds such as caffeine, denatonium, and strychnine stimulate inositol trisphosphate (IP_{3}) production (53). Thus the α-subunit of gustducin may couple to phosphodiesterase, lowering cyclic nucleotides, whereas the βγ-complex may stimulate phospholipase C-β2, which in turn elevates IP_{3} (60).

In addition to the modulation of second messengers such as cAMP and IP_{3} by two arms of the gustducin protein, other transduction mechanisms for bitter stimuli may exist. Modulation of second messengers may occur in a receptor-independent manner via direct stimulatory interactions on G proteins that further activate downstream transduction cascades (36, 41) or by direct interaction with enzymes that degrade cyclic nucleotides (28, 42). Particularly bitter stimuli (caffeine and theophylline) were measured to increase cGMP, whereas others (strychnine and denatonium) were ineffective. Ion channels are also thought to be direct targets for some bitter stimuli, such as the direct block of potassium channels by quinine in rat taste receptor cells (9) or the activation of a cationic channel directly gated by bitter stimuli, such as quinine or denatonium, which results in an inward depolarizing current in frog taste receptor cells (55, 56). This investigation seeks to better clarify one widely used bitter compound in particular, caffeine.

Caffeine (1,3,7-trimethylxanthine) is an alkaloid and is closely related to other methylxanthines such as theophylline (1,3-dimethylxanthine) and theobromine (3,7-dimethylxanthine). All are naturally occurring compounds in cocoa beans, cola nuts, coffee, and tea and are reported as bitter in humans. Although caffeine is commonly employed in gustatory science as a bitter stimulus, its underlying transduction mechanisms are essentially unstudied. However, as a pharmacological tool, caffeine has been well studied in other cell types. Its actions are mostly circumscribed to three mechanisms (37): mobilization of intracellular calcium, inhibition of phosphodiesterases, and antagonism of adenosine receptors. Stimulus-related activation of all three cellular signaling components has been reported in taste receptor cells. Elevations of intracellular calcium have been reported for several bitter stimuli, such as denatonium (3, 7, 39), as well as for certain sweet stimuli (4). Various phosphodiesterases have been reported to be expressed in taste receptor cells (32) and may be substrates for particularly bitter stimuli (28, 42) or via activation of the gustducin pathway. Moreover, a role for stimulus-induced activation of adenosine receptors has been proposed in taste sensations (49, 50), where activation of adenosine receptor, by caffeine or other methylxanthines, may act to intensify the perception of certain sweeteners. Thus it is possible that taste receptor cells could utilize analogous mechanisms to transduce caffeine stimulation. The purpose of the present study is to gain insight into the cellular actions of caffeine by using patch-clamp recording and ratiometric calcium-imaging techniques. Such observations are prerequisite to understanding more fully caffeine's transduction mechanisms in taste receptor cells.

METHODS

All experiments were performed on isolated taste receptor cells dissociated from rat circumvallate and foliate papillae (Sprague-Dawley, 180–350 g) by using whole cell patch-clamp recording procedures (conventional and perforated-patch methods) or ratiometric calcium imaging with the fluorophore fura 2. Recordings were conducted at room temperature.

**Dissociation procedure.** Taste receptor cells were dissociated from the posterior rat tongue as previously described (16). Briefly, lingual tissue (circumvallate and foliate papillae) was excised from the tongue after the animal reached a surgical level of anesthesia achieved with intramuscular injection of ketamine-acepromazine at 0.09 ml/100 g body wt [91 mg/ml ketamine (Parke-Davis), 0.09 mg/ml acepromazine (Vedco)]. Papillae were blocked from tongue tissue and incubated in a cysteine-activated (1 mg/ml) papain (14 U/ml)-divalent-free bicarbonate-buffered solution for several hours at 32°C in 5% CO_{2}-95% air. Cells were dissociated in a pseudoextracellular fluid by mild agitation. Some papillae were maintained in ice-cold extracellular fluid (ECF) solution for later dissociation. Dissociated taste receptor cells were easily identified by their elongated or bipolar morphology (16).

**Solutions.** The divalent-free solution for enzymatic incubation was composed of (in mM) 80 NaCl, 5 KCl, 26 NaHCO_{3}, 2.5 NaH_{2}PO_{4}, H_{2}O, 20 d-glucose, and 1 EDTA. The standard ECF solution used for the dissociation procedure included (in mM) 126 NaCl, 1.25 NaH_{2}PO_{4}, H_{2}O, 5 KCl, 5 NaHEPES, 2 MgCl_{2}, 2 CaCl_{2}, and 10 glucose, with pH adjusted to 7.2–7.4 with HCl. Most experiments were performed by using the perforated-patch configuration of the patch-clamp technique with amphoterin B as the ionophore [400 μg/ml in the intracellular fluid (ICF)]. The composition of the ICF for filling the pipette consisted of (in mM) 55 KCl, 75 K_{2}SO_{4}, 8 MgCl_{2}, and 10 HEPES. During recording of calcium and chloride current, KCl and K_{2}SO_{4} were replaced by an equivalent amount of CsCl and Cs_{2}SO_{4}. The composition of ICF for recording inwardly rectifying potassium (K_{IR}) current in conventional whole cell configuration recording mode consisted of (in mM) 140 KCl, 2 MgCl_{2}, 1 CaCl_{2}, 11 ethylenebis(oxy)tri(ethanotri)tetraacetate (EGTA), 10 HEPES, and 4 ATP (magnesium salt). The extracellular solution for recording K_{IR} current consisted of the standard ECF recipe with the replacement of 95 mM NaCl by an equivalent amount of KCl (final extracellular potassium concentration of 100 mM). When recording chloride currents, a potassium-free extracellular solution was employed for the bath solution; it consisted of (in mM) 126 NaCl, 5 HEPES, 2 CaCl_{2}, 2 MgCl_{2}, and 10 glucose with a final chloride concentration of 134 mM. The pH of the extracellular recording solution was adjusted to 7.4 with Tris base. The ECF solution for recording calcium current consisted of (in mM) 140 NaCl, 20 tetraethylammonium chloride (TEA), 10 HEPES, 5 glucose, 10 KCl, 10 CaCl_{2}, 2 MgCl_{2}, and 5 4-aminopyridine (4-AP), with pH adjusted to 7.4. In some experiments, 10 mM CaCl_{2} in ECF was replaced by 20 mM BaCl_{2}.

**Whole cell electrophysiological recording.** Micropipettes used for whole cell recording were pulled on a gas-cooled multistage puller from 1.5-mm-OD borosilicate glass (World Precision Instruments, Sarasota, FL). Resistances were typically 4–7 MΩ when filled with ICF and measured in ECF. Junction potentials were corrected before the electrode contacted the cell. The pipette tip was positioned to contact the cell membrane, and negative pressure was applied to its interior to facilitate gigaseal formation. Seal resistances were typically several decades of gigahms. After gigaseal
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formation, it was necessary to apply further negative pressure to enter conventional whole cell recording mode. For amphotericin B perforated-patch recordings, ~30 min were required to reach a stable level of recording after gigaseal formation. Fast and slow capacitance compensation was employed as necessary with amplifier controls in both recording situations. 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 (1/2πRC, where R is resistance and C is capacitance) of 1.6–16.6 kHz.

Data were acquired with a high-impedance amplifier equipped by using a high-resistance feedback head stage (Axopatch-1B, Axon Instruments), a Pentium-based 450-MHz computer, a 12-bit 500-kHz analog-to-digital converter (Digi-Data 1320, Axon Instruments), and a commercially available software program (pCLAMP, version 7.0 or 8.01, Axon Instruments). Membrane currents were acquired after low-pass filtering with a cutoff frequency of 10 kHz (at ~3 dB). A software-driven digital-to-analog converter generated the voltage protocols. In most situations, currents were measured with voltage protocols by using standard command frequencies (1/2

RESULTS

Caffeine was applied with a pipette perfusion system that allowed focal application of stimulus by using an eight-barreled pipette controlled by Teflon valves and channeled into a quartz pipette that was positioned close to the cell with a micromanipulator (ValueLink 8, Automate Scientific, Oakland, CA). Stimuli were presented against a slow background perfusion of ECF. This procedure allowed quick focal application and removal of the stimulus (<10 s). Ratios (340 nm/380 nm) before, during, and after stimulus presentation were taken to reflect changes of intracellular calcium in response to the stimulus. Exposure levels at 340- and 380-nm excitation wavelengths were chosen to produce images well below saturated levels and to optimize ratios. A 60-min loading time of fura 2-AM generally required exposure times of 0.03 s at 340 nm and 0.01 s at 380 nm that subsequently resulted in a baseline ratio close to 0.7. This allowed optimal crossing over of 340- and 380-nm wavelengths during times of elevated calcium and, hence, maximum ratios. Ratios were calculated from the mean intensity of pixels within a software-defined region of interest (ROI) within the cell. In these experiments, the ROI was chosen from the somatot al region of the taste receptor cell. Corrected ratios were background subtracted. Mean intensity from an ROI of background subtraction was subsequently applied to gray-scale images of the resulting ratio values calculated from background-subtracted 340- and 380-nm images. Baseline ratio values, with which stimulated ratio values were compared, were calculated as mean values of five to seven data points acquired before stimulus application at the rate of one point per minute.

The actions of caffeine were tested on a variety of voltage-dependent ionic currents isolated from dissociated posterior taste receptor cells. We previously performed biophysical characterizations of a number of ion currents in these cells never demonstrated any significant differences between these groups. The value of current before application of drugs was normalized as 100%. Pooled one-tailed Student's t-test was used to evaluate the statistical significance of the difference between means. P < 0.05 was considered to indicate statistical significance. Values are means ± SE.

Calcium imaging. Intracellular calcium levels in dissociated taste receptor cells were monitored by using standard ratiometric techniques with the membrane-permeable calcium-sensitive dye fura 2 and a commercially available software package for data acquisition and analysis (SimplePCI, Compix, Cranberry Twp, PA). Dissociated posterior taste receptor cells were loaded with fura 2-AM (5 μM, dissolved in DMSO) in the presence of a dispersing reagent, 0.05% Pluronic F-127 (dissolved in DMSO), and 1% bovine serum albumin for ~60 min and then washed with normal ECF for ≥20 min. Images were acquired with a charge-coupled device camera (Hamamatsu Orka, Hamamatsu Photonic KK, Hamamatsu City, Japan) through an oil-immersion ×40 objective lens on an inverted microscope. For dual-wavelength ratiometric calcium measurements, pairs of fluorescent images were recorded at 340- and 380-nm excitation. Excitation wavelengths were produced with a software-driven monochromator (Polychrome II, Photonics, Applied Scientific Instrumentation, Eugene, OR), and light was collected through a 510-nm emission filter. Paired images were obtained once every 10 s during the stimulation period and once per minute during baseline measurements.

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these currents: voltage-dependent sodium currents (19), \( K_{IR} \) currents (54), outward potassium currents (10, 21), and chloride currents (20). These currents are heterogeneously distributed on a cell-by-cell basis in expression and magnitude. Of the currents tested in this communication, caffeine had its strongest effect on potassium and calcium currents. Small inhibitions were noted on sodium currents, and caffeine was without effect on chloride currents. In addition, caffeine evoked calcium release from intracellular stores in a concentration-dependent manner.

**Potassium currents.** The bath application of caffeine to dissociated posterior taste receptor cells produced inhibitions of evoked outward potassium current that were concentration dependent and reversible. Figure 1A illustrates a representative family of current traces evoked using whole cell voltage-clamp recordings from a holding potential of \(-80 \text{ mV}\) with the perforated-patch technique. Inward sodium and outward potassium components are clearly evident. Application of 20 mM caffeine to this cell resulted in an immediate and significant inhibition of the outward potassium currents with little effect on the inward sodium currents. These inhibitions were quickly reversed with rinse of the bathing solution. Previous characterization of these outward potassium currents (10) demonstrated that they are composed of multiple components, including delayed-rectifier, A-type, and calcium-activated potassium currents, and are similar in voltage sensitivity and inactivation properties to neuronal potassium channels. Only a very small component of this outward current is carried by chloride (20). The inhibition of these outward currents by caffeine application is very similar to that reported by quinine application, including its magnitude, temporal onset, and reversibility (9).

The inhibition of caffeine on the potassium currents displayed some voltage dependence. Figure 1B illustrates the current-voltage (I-V) relationship of the currents illustrated in Fig. 1A. Caffeine caused little inhibition of the sodium current, whereas the effect on potassium currents was profound. Although inhibition of potassium current could be measured at all suprathreshold potentials, it was most evident at more depolarized command steps, indicating some voltage dependence to this effect. The small inhibition of sodium currents was without effect on its activation, voltage sensitivity, or reversal potential. As expected for a taste stimulus, caffeine effects were concentration dependent. Data from a different taste receptor cell stimulated with four concentrations of caffeine are illustrated in Fig. 1C. In this cell, response magnitudes for potassium and sodium currents were recorded over a 60-min period, during which it was stimulated with an ascending concentration series of caffeine (1, 5, 10, and 20 mM stimulated for 5.8, 5.5, 4, and 4.2 min, respectively). For potassium currents, little inhibition was evident at 1 mM, although higher concentrations produced successively higher-magnitude inhibitions. In contrast, for sodium current, slight inhibitions were noted at \( \geq 10 \text{ mM} \). All inhibitions were reversible. In other cells (data not shown), a single concentration of caffeine was repeatedly presented and produced stable and reversible inhibitions. Summarized data for the
inhibition of potassium currents by different caffeine concentrations are presented in Fig. 1D.

Caffeine effects on outward potassium currents were tested at different holding potentials as an initial assessment of which components of the outward current might be influenced by this bitter stimulus. For example, the transient and delayed-rectifier potassium currents are significantly inactivated at more depolarized holding potentials (e.g., −50 mV), whereas the calcium-activated potassium current is not. When inhibitions of outward current produced by 10 mM caffeine were compared at different holding potentials from the same cell, inhibitions occurred at both holding potentials (Fig. 2A). Summarized data are presented in Fig. 2B. Current was inhibited 26 ± 5% (n = 9) and 39 ± 4% (n = 11) at −80 and −50 mV, respectively. These results are similar to inhibition of outward currents in these cells at these same holding potentials produced by serotonin (17). Similar effects of caffeine could be demonstrated with the use of the potassium channel inhibitors TEA and 4-AP. We previously established that the concentrations of TEA and 4-AP used in this study differentially affect sustained and transient current in taste receptor cells (10); therefore, pretreatment with either agent may provide some evidence into potassium current subtype affected by caffeine. In particular, TEA inhibition of outward potassium currents may include cAMP-sensitive outward current as well as sustained outward current, whereas 4-AP inhibition (at this concentration) is more specific for transient outward current. As shown in Fig. 2C, caffeine was capable of further reducing the magnitude of the outward current in the presence of 1 mM 4-AP or 10 mM TEA. Summarized data are presented in Fig. 2D for TEA and in Fig. 2E for 4-AP. Bath application of 10 mM TEA inhibited potassium current to 31.3% of its original magnitude (n = 5), and, thereafter, 10 mM caffeine with 10 mM TEA could further inhibit the remaining potassium current to 14.0% (n = 5). Bath application of 1 mM 4-AP reduced potassium to 52.7% of its original magnitude (n = 7), and 10 mM caffeine with 1 mM 4-AP further decreased potassium current to 25.2% (n = 7). All actions of TEA and caffeine were reversible. Collectively, these data suggest that caffeine may affect multiple components of the outward potassium current. A role for calcium-activated potassium current is most strongly supported by these data, particularly given caffeine’s increased effectiveness at a holding potential of −50 mV. However, because inhibition could be demonstrated in all three paradigms, it seems likely, but not directly proven, that caffeine may exert an inhibitory action at a site common to potassium channels.

To determine whether caffeine-induced effects on potassium currents were dependent on or related to the elevations of intracellular calcium that are also produced by caffeine stimulation (see below), experiments with the calcium chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA) were performed. BAPTA, via pipette administration, and the membrane-permeant ester BAPTA-AM were employed with similar results. Caffeine alone inhibited outward potassium current to 47.8 ± 4% of its original magnitude (n = 26). With the use of whole cell recording with 10 mM BAPTA in the pipette, 20 mM caffeine reduced the same current to 51.9 ± 3% of its original magnitude.

![Fig. 2. Caffeine inhibits several types of potassium currents. A: magnitude of caffeine inhibition is influenced by the holding potential. Outward potassium currents were inhibited at holding potentials of −80 and −50 mV. At −50 mV, which greatly enriches the contribution of calcium-activated potassium current, caffeine (10 mM) inhibition is evident. B: summarized data at holding potentials of −50 and −80 mV. C: calcium-activated potassium current may additionally be enriched by application of potassium channel blockers tetraethylammonium (TEA, 10 mM) or 4-amino-pyridine (4-AP, 1 mM), which are most effective on delayed-rectifier and transient potassium currents. In the presence of either potassium channel blocker, caffeine (Caff) persisted as an effective inhibitor of the remaining potassium current. D and E: summarized data for TEA and 4-AP, respectively. Total inhibition of the potassium current is reported for potassium channel blocker or potassium channel blocker plus caffeine. **P < 0.01. Nos. in parentheses, no. of cells.](http://ajpregu.physiology.org/)
In separate experiments, with 10–15 min of preincubation in 10 μM BAPTA-AM, 20 mM caffeine inhibited currents to 53.7 ± 3% of original magnitude \((n = 21)\). Thus BAPTA appeared to have no significant influence on the caffeine-induced inhibition of outward potassium current. As a positive control, BAPTA-AM was tested on calcium-activated potassium current to ensure that its administration was successful.

**Caffeine inhibits K\textsubscript{IR} current.** K\textsubscript{IR} current is carried by a class of potassium channels distinct in their electrophysiological and molecular properties from those that produce outward potassium current. This current, which has its highest conductance at negative potentials, is ubiquitously distributed across taste receptor cells and contributes with other conductances to the resting potential \((54)\). In normal extracellular potassium concentrations, its conductance is generally 1–2 nS. In the present study, bath application of 10 or 20 mM caffeine inhibited K\textsubscript{IR} current \((recorded in 100 mM extracellular potassium)\). These actions persisted during the application of caffeine, and washout of caffeine from the bath solution reversed them. Representative K\textsubscript{IR} current traces from a single taste receptor cell are presented before, during, and after application of 10 mM \((6.6-	ext{min application})\) or 20 mM \((6.7 \text{ min})\) caffeine in Fig. 3A. Currents were inhibited at all potentials, and inhibition displayed little voltage dependence. Data from a different taste receptor cell for 10 and 20 mM caffeine are presented in Fig. 3B, which illustrates the time course of reversibility and a simple dose dependence of inhibition between the tested concentrations. In most cells, K\textsubscript{IR} current was tested more than twice with stable and reversible inhibitions. Summarized data of K\textsubscript{IR} current inhibition to these two concentrations of caffeine are presented in Fig. 3C.

Unlike outward potassium currents, K\textsubscript{IR} current is not sensitive to inhibition by cAMP but can be inhibited by G-protein analogs such as guanosine 5’-O-(3-thiotriphosphate) \((\text{personal observations})\). These observations, considered with the quick reversibility of these effects, suggest that K\textsubscript{IR} current inhibition may not be cyclic nucleotide mediated but could be receptor linked via G-protein activation. Alternatively, direct block of K\textsubscript{IR} current by caffeine has been suggested in other cell types \((11, 57)\). By either mechanism, inhibition of K\textsubscript{IR} current would be expected to depolarize the resting potential, allowing the resting potential to drift away from the potassium equilibrium potential as a result of leak conductances. Additionally, because of the rectifying nature of this conductance, inhibition would augment its ability to prevent shunting of current during depolarization.

**Caffeine reversibly inhibits calcium currents.** In addition to its action on potassium currents, caffeine also inhibited calcium currents. Calcium currents are heterogeneously distributed across taste receptor cells, which express a mixture of T-type, L-type, and/or T- and L-type calcium currents. Calcium currents were recorded in ECF with an elevated calcium concentration of 10 mM and a combination of 20 mM TEA and 5 mM 4-AP to inhibit outward potassium current. Additionally, potassium was replaced with equimolar cesium in the ICF to inhibit potassium channels internally. Because these experiments were performed with perforated-patch-clamp recording, the possibility of decreasing calcium currents as a result of rundown was...
minimized. Application of 10 mM caffeine inhibited calcium current significantly. A representative family of calcium currents is presented in Fig. 4A before and during caffeine application (6 min). These currents contain transient and persistent components. In most cases, this inhibition was rapidly reversed with rinse. The I-V relationship from this cell is presented in Fig. 4B. Caffeine was noted to inhibit calcium current with little voltage dependence; inhibition was evident over all suprathreshold potentials. Data from a different cell are presented in Fig. 4C. The inhibition produced by caffeine was reversible when the stimulus was rinsed from the bathing solution. (Typical with the perforated-patch technique, the early portion of the record demonstrates the settling of the current magnitude as the series resistance is established.) Summarized data are presented in Fig. 4D.

Caffeine does not affect chloride currents. Chloride currents in rat posterior taste receptor cells have been previously characterized (20). As occurs in other epithelial cells, taste receptor cells express a heterogeneous array of chloride currents that display strong outward rectification and contain calcium-dependent and calcium-independent components. Chloride conductance is ubiquitously distributed across taste receptor cells, and its magnitude is small compared with other ionic conductances (typically 1 nS). These currents are additionally subject to enhancement by adrenergic agents and likely contribute with KIR current to the resting potential of taste receptor cells. In the present experiments, chloride currents were recorded using the perforated-patch technique; no significant differences were noted in any of the critical features previously characterized using the whole cell technique, including tail currents and rectifying in the positive direction. Currents were isolated by recording in potassium-free solutions and holding at zero-current potential to inactivate most voltage-dependent conductances. The cell was held at 0 mV, and a series of depolarizing or hyperpolarizing command pulses, in 20-mV increments, were applied to final voltages of −140 or +120 mV. Bath application of 10 or 20 mM caffeine did not affect the chloride current amplitude, temporal course, or duration for positive or negative evoked currents. A continuous record from a single cell is presented in Fig. 5A over a period of 80 min. Current magnitudes to a depolarizing or hyperpolarizing test pulse were stable when 10 mM (7.4 min) or 20 mM (7.3 min) caffeine was added to the bathing solution. In 12 tested cells (Fig. 5B), the current magnitude to an outward test current was 102.5 ± 1.8% of its original magnitude. Similarly, the magnitude of these currents in the presence of 20 mM caffeine was 101.4 ± 1.8% of the current magnitude before caffeine application (n = 11).

Effects of caffeine on membrane resistance and the gustatory action potential. These effects of caffeine on voltage-dependent ionic conductances, particularly those observed on outward potassium currents, suggest that caffeine may alter membrane resistance and membrane voltage in a manner that would be physiologically significant. To test this possibility, input resistance of the membrane and the gustatory action potential were recorded.

Input membrane resistance was measured in current-clamp recording by injection of a series of hyper-
polarizing and depolarizing currents of 80-ms duration. Figure 6A illustrates a series of current-clamp recordings and the extrapolated I-V plot from a taste receptor cell before, 5.5 min after caffeine application, and 3.5 min after initiation of rinse by using the perforated-patch technique. Depolarizing current injections were often suprathreshold and successfully elicited action potentials. Caffeine significantly increased the input resistance of the cell in a reversible manner. In this cell, the input resistance was increased by ~25%. The caffeine measurement was taken after 5.5 min of caffeine superfusion, and the rinse sample after 3.5 min of ECF superfusion. Summarized data are presented in Fig. 6B. The membrane resistance was increased to 112 ± 3% of its original value (n = 13) by application of 10 mM caffeine and to 124 ± 5% of its original value (n = 12) by 20 mM caffeine. Both changes were statistically significant (P < 0.01). These results are consistent with the strong suppression of outward potassium current by caffeine.

The previously observed inhibitory effects of caffeine on voltage-dependent currents additionally predict that one should observe changes in the waveform of the gustatory action potential. To test this prediction, gustatory action potentials were elicited by current injection in current-clamp mode. Application of 10 or 20 mM caffeine caused modulation of the waveform of the action potential. A representative cell is presented in Fig. 7A. In this cell, the latter two of five current injections elicited action potentials that had obvious afterhyperpolarization components. Action potentials in Fig. 7A were elicited 2.7 min after caffeine application or 3.6 min after rinse. We previously established that posterior taste cells exhibiting action potentials express at least two possible subtypes, designated fast and slow (10); the action potentials of Fig. 7A are an example of slow action potentials.
example of fast action potentials. Bath application of 20 mM caffeine decreased the amplitude of the action potential, prolonged its duration (measured at half-amplitude), and reduced the amplitude of the afterhyperpolarization potential. These changes were reversible with washout of caffeine from the bathing medium. Summarized data to two concentrations of caffeine on the amplitude of the gustatory action potential are presented in Fig. 7B. The amplitude was reduced to $83 \pm 5\%$ ($n = 14$) or $81 \pm 6\%$ ($n = 10$) of its original magnitude by 10 and 20 mM caffeine application, respectively. Summarized data to two concentrations of caffeine on the duration (measured at half-amplitude) of the gustatory action potential are presented in Fig. 7C. The duration was increased to $115 \pm 4\%$ ($n = 13$) or $136 \pm 7\%$ ($n = 10$) of its original magnitude by 10 and 20 mM caffeine application, respectively. No noticeable differences were observed on the effect of caffeine on the waveform of the action potential when fast and slow action potentials were compared.

Caffeine increases intracellular calcium in taste receptor cells. Caffeine is widely recognized as a releasing agent of calcium from intracellular pools. With the use of standard ratiometric procedures with the fluoroprobe fura 2, six concentrations of caffeine, ranging from 0.01 to 20 mM, were tested in 109 taste receptor cells. Caffeine elevated intracellular calcium in a majority of tested cells. A typical response, recorded over an 11-min period, is illustrated in Fig. 8. In Fig. 8A, the time-lapse digital images of a single taste receptor cell represent background-subtracted ratio values of the raw 340 nm/380 nm images to which a pseudocolor scheme has been applied. Caffeine was applied to this cell at 250 s for 4 min. In Fig. 8B, the raw intensity values for the individual wavelengths are presented. The baseline intensities were adjusted before data recording through their exposure times so that the intensity recorded at 380-nm excitation exceeded that at 340-nm excitation. This procedure resulted in maximal crossover (and, hence, ratio value) when intracellular calcium levels increased. Crossover of the individual wavelength intensities was indeed dramatic when 10 mM caffeine were applied to this cell. The resulting ratio values of these individual wavelength intensities are presented in Fig. 8C. The rise in intracellular calcium, as indicated by the ratio values, was abrupt at the onset of stimulus presentation and subsequently declined to a tonic value that was maintained until washout. On washout, ratio values returned to baseline. The action of caffeine on intracellular free calcium was reversible. The kinetics of the calcium response caused by caffeine displayed phasic and tonic components. Typically, intracellular calcium increases produced by caffeine reached maximum levels at the initial application of the stimulus and then gradually decreased to a level above baseline, although the caffeine was still present in the solution. After caffeine was removed, the intracellular calcium level would return to baseline. In general, for repeatable response magnitudes to occur, an interstimulus interval of $\approx 5$ min was required. Ratio responses from one cell to six concentrations of caffeine are presented in Fig. 9A. This cell was presented with 0.01, 0.1, 1.0, 5.0, 10, and 20 mM caffeine in an ascending series over an 80-min test period. Only four concentrations, 1.0, 5.0, 10, and 20 mM, resulted in measurable increases of intracellular calcium. Summarized data for four suprathreshold concentrations of caffeine are presented in Fig. 9B. Taste receptor cells responded to caffeine with elevations of intracellular calcium in a dose-dependent man-

Fig. 7. Caffeine altered the waveform of the gustatory action potential. A: representative voltage traces from a single taste receptor cell before, during, and after bath application of 20 mM caffeine. Current injection protocol used to evoke these voltage perturbations is shown below middle panel. B: summarized data for 10 and 20 mM caffeine and amplitude of the gustatory action potential (AP). C: summarized data for 10 and 20 mM caffeine on duration (measured at half-amplitude) of the gustatory action potential. $*P < 0.05$; $**P < 0.01$. Nos. in parentheses, no. of cells.
Data are presented as averaged ratio values for the baseline period before stimulus presentation (typically ~5 min) and peak ratio value during stimulus presentation (Fig. 8C). Ratio values were 1.0 ± 0.19, 1.6 ± 0.12, 1.7 ± 0.12, and 1.9 ± 0.08 (mean ± SE) to 0.1, 1, 5, and 10 mM caffeine, respectively. Not all cells responded to caffeine, but the percentage of responsive cells also increased with increasing caffeine concentration. The numbers of responsive cells were 3 of 25 (12%), 12 of 28 (43%), 13 of 28 (46%), and 39 of 50 (78%) tested cells for 0.1, 1, 5, and 10 mM caffeine, respectively.

Caffeine response requires intracellular calcium. A final set of experiments was performed to determine whether elevations of intracellular calcium were dependent on extracellular calcium or intracellular stores of calcium.

Experiments to determine whether extracellular calcium is required for caffeine-induced elevations of cytoplasmic calcium were performed by using a calcium-free ECF with EGTA buffer. A sample response is presented in Fig. 10A. A caffeine-sensitive cell was tested with six presentations of 5 mM caffeine (all at 1 min each) over an 80-min period before and during bath application of calcium-free ECF. Caffeine continued to elevate intracellular calcium in the absence of extracellular calcium. Note the diminution of response magnitude during repeated stimulus presentation in calcium-free ECF that began to reverse with readdition of extracellular calcium. Figure 10B illustrates summarized data for caffeine stimulation in calcium-free ECF.

The average response magnitude, normalized to prestimulation baseline (as 100), was 287.51 ± 22.74 in normal ECF and 248.89 ± 26.32 in calcium-free ECF (n = 7; pooled data to 5 and 10 mM caffeine). The presence of calcium-free ECF did not affect baseline ratio measurements (96.9 ± 2). Thus removal of extracellular calcium did not significantly alter the ability of caffeine to elevate intracellular calcium levels.

To test whether caffeine-stimulated elevation of intracellular calcium is dependent on intracellular stores, taste receptor cells were treated with 1 μM thapsigargin, an inhibitor of calcium transport into intracellular stores. Cells were typically perfused with thapsigargin for 25–40 min before caffeine application (Fig. 10C). A caffeine-sensitive taste receptor cell was stimulated with 10 mM caffeine and exposed to 1 μM thapsigargin over a period of 120 min. Caffeine was completely ineffective as a stimulus after exposure to thapsigargin. This inhibition of the caffeine response was long lasting. Results from an additional cell, not exposed to thapsigargin but tested with caffeine over the same duration, are presented in Fig. 10D to demonstrate that taste receptor cells are able to maintain responsiveness over this test period. Summarized data with thapsigargin treatment are presented in Fig. 10E. Before thapsigargin treatment, the caffeine average response magnitude was 288.26 ± 42.79, whereas after thapsigargin treatment it declined to 117.78 ± 17.64 (n = 5; pooled data to 5 and 10 mM caffeine). Thapsigargin treatment alone did not significantly affect baseline ratio measurements (98.3 ± 5%).
Given that caffeine-induced calcium elevations in taste receptor cells require intracellular stores, the possible involvement of ryanodine receptors was investigated. Three concentrations of ryanodine were tested; none resulted in alterations of intracellular calcium. Responses, normalized to baseline ratios, were 99.1 (n = 11) of prestimulated levels to 50 nM ryanodine, 103.6 (n = 5) to 20 μM ryanodine, and 101.8 (n = 7) to 100 μM ryanodine. Ryanodine application was also tested to determine whether it could block caffeine-induced calcium elevations. Normalized to caffeine responses before ryanodine application, the caffeine responses after 10–15 min of exposure were 102.5 (n = 8) to 50 nM ryanodine, 100.3 (n = 5) to 20 μM ryanodine, and 75.7 (n = 7) to 100 μM ryanodine. These data suggest a lack of ryanodine receptors in rat taste receptor cells and agree well with observations reported in mud-puppy taste cells (39).

Collectively, these data suggest that caffeine-induced elevations of intracellular calcium are dependent on intracellular calcium stores and do not require extracellular calcium or ryanodine-sensitive intracellular stores. The decline in response magnitude to repeated stimulus presentations in the absence of extracellular calcium likely reflects the inability of the cell to restore intracellular pools in the absence of extracellular calcium. Similarly, small declines in the caffeine response in the presence of ryanodine are more likely attributed to sensitization, as observed with repeated caffeine exposures.

DISCUSSION

The present study establishes that taste receptor cells respond to the presence of the bitter stimulus caffeine in a multifaceted manner. Voltage-dependent ionic currents as well as intracellular calcium levels of posterior rat taste receptor cells were affected. In particular, caffeine application produced an inhibition of outward potassium current, KIR current, and calcium current, whereas chloride currents were unaffected. In addition, caffeine application was also noted to produce...
significant elevations of intracellular calcium. All of these effects were rapid in onset, persisted during the period of application of caffeine, and recovered completely on washout, consistent with the expected consequences of taste stimulation. Moreover, these effects occurred at concentrations known to produce gustatory responses by using electrophysiological or behavioral measures. For example, the rat threshold for whole nerve integrated responses is reported to be 10 mM for the glossopharyngeal nerve and 10–30 mM for the chorda tympani (24). This same study reported 10 mM as the behavioral threshold for aversion in a two-bottle preference test. Thus the concentrations used in this study, 1–20 mM, compare well with previous data in rat. In fact, the lowest concentration of caffeine observed to elicit a response was 0.1 mM, which elevated calcium in 12.0% of the tested cells. Most studies of internal calcium mobilization use higher caffeine concentrations, typically 5–20 mM, suggesting that taste receptor cells may possess more specialized mechanisms for the detection of this chemical. By comparison, the lowest concentration to inhibit potassium currents in this study was 1 mM. Overall, it appears that caffeine’s action on potassium currents and intracellular calcium occurs via independent mechanisms.

**Caffeine effects on potassium currents.** Caffeine inhibited at least two distinct types of potassium currents in posterior taste receptor cells: an outward potassium current and a KIR current. Outward potassium currents in posterior rat taste receptor cells are multifaceted. They are composed of at least three distinct components: delayed rectifier type, transient A-type, and calcium-activated potassium currents (10). KIR current, on the other hand, is carried by a class of potassium channel distinct from those carrying outward currents. These channels possess unique electrophysiological, pharmacological, and molecular properties (54). We previously reported that ~25–30% of the outward potassium current can be inhibited by cAMP, cAMP analogs, such as 8-(4-chlorophenylthio)-cAMP, or the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (21). This inhibition is sufficient to alter the waveform of the action potential, causing its broadening. It is also kinase dependent, inasmuch as kinase inhibitors, such as H-8 [N-[2-(methylamino)ethyl]-5-isouquinolinesulfonyamide] or protein kinase inhibitor (a peptide fragment of the regulatory subunit of protein kinase A), prevented inhibition of potassium currents that would have otherwise been produced by 8-(4-chlorophenylthio)-cAMP or 3-isobutyl-1-methylxanthine. These observations are particularly relevant to the inhibition of potassium currents observed in this communication by caffeine.

At concentrations used in this study, caffeine is a potent inhibitor of phosphodiesterase. Hence, it is plausible to assume that inhibitions of outward potassium current produced by caffeine or cAMP (e.g., via phosphodiesterase inhibition) could operate by similar mechanisms. In fact, the situation is likely more complicated. First, caffeine has multiple effects on second-messenger systems in taste cells. Caffeine has been shown to be a potent stimulator of cGMP production in murine posterior taste receptor cells (44). With the use of quench-flow analysis, stimulation of taste receptor cells with 25 mM caffeine was demonstrated to increase cGMP within 50 ms. Additionally, with the use of the same methods, caffeine (10 mM) was also demonstrated to increase levels of IP3 (53). Data for cAMP accumulation due to caffeine stimulation are not available. Thus caffeine may increase several second-messenger molecules; the downstream actions of these second messengers are incompletely understood. Our preliminary study of cGMP has shown that its time course is similar to that of cAMP, but, more importantly, the percentage of cells tested that responded to cGMP was much less than the percentage that responded to caffeine (unpublished results). Second, there exists a discrepancy in the time course of the outward potassium current inhibition mediated by cyclic nucleotide and that mediated by caffeine (personal observations). Cyclic nucleotide inhibition is slow in onset and slowly reversible, whereas that produced by caffeine is rapid in onset (occurring without discernable latency with bath application) and easily reversed. In fact, the time course of inhibition of caffeine more closely resembled that observed with quinine on these cells (9) or by forskolin (21). Quinine and forskolin act as direct blockers of potassium channels. Caffeine, too, has been reported to directly inhibit potassium channels in a number of cell types, including mammalian ventricular myocytes (48), vascular smooth muscle (38), dissociated chick autonomic ganglion neurons and pineal cells (43), and rat anterior pituitary cells (26).

The biophysical properties of the caffeine-sensitive potassium channels are clearly different in these cells, suggesting a direct effect on conserved pore regions of the channel molecule. It may well be that caffeine operates like forskolin, which operates via two mechanisms to inhibit potassium currents: a direct block and cyclic nucleotide inhibition of potassium channels. Additionally, experiments with TEA and 4-AP (Fig. 2) suggest that calcium-activated potassium current may be a target of caffeine inhibition, a target different from that assumed for the cAMP-mediated inhibition of potassium current. Taken collectively, a more likely possibility is that the predominant action of caffeine on potassium channels may be an occlusion of its channel pore in a manner similar to that of TEA and 4-AP, because these effects were cumulative. Furthermore, caffeine did not change the inactivation kinetics of potassium channel significantly (personal observations).

In addition to outward potassium currents, caffeine also inhibited KIR current. This effect may be considered exceptional, inasmuch as KIR current has previously proven to be recalcitrant to most tested modulations. KIR current is of particular interest, because, in most cell types, it is a major participant in the determination of the cell’s resting potential. Hence, inhibition of KIR current acts to depolarize the membrane potential and, in excitable cells, may elicit an action potential. In posterior taste cells, it has been demon-
strated that $K_{IR}$ current is a major contributor to the resting potential (54). In taste cells, the $K_{IR}$ conductance is ~50% maximal at values of the potassium equilibrium potential, and barium, an effective inhibitor of $K_{IR}$ current, depolarizes the zero-current potential, whereas TEA, a weak blocker, is only mildly effective and 4-AP, an ineffective blocker of $K_{IR}$ current, is without effect. Hence, its inhibition would be suspected to be a prime target for initial transduction cascades to alter the membrane potential and subsequently elicit an action potential. Surprisingly, however, many tested tastants did not alter this current (personal observations). Additionally, the application of cAMP, a proposed transduction agent in bitter and sweet cascades, was similarly ineffective in altering $K_{IR}$ current (personal observations). As with outward potassium currents, direct block of $K_{IR}$ channels by caffeine has also been reported (11, 57). In isolated guinea pig ventricular myocytes, 10 mM caffeine consistently reduced the slope of the $I-V$ relation of $K_{IR}$ current. Loading cells with BAPTA to suppress intracellular calcium increase did not prevent this effect of caffeine. Thus caffeine, which produced a large and easily reversible inhibition of $K_{IR}$ current, could easily depolarize the taste receptor cell through its action on this current, possibly via a direct block of the inwardly rectifying channel.

**Caffeine actions on intracellular calcium.** Among the more complicated effects of caffeine stimulation on taste receptor cells are its modulations of cellular calcium levels. An inhibition of calcium current and a release of intracellular calcium were observed. So little is known of regulatory mechanisms of intracellular calcium in taste receptor cells that it is premature to speculate regarding mechanism. One of the first issues to be resolved for a more complete understanding of caffeine’s actions on calcium is determination of the type of intracellular calcium store that is calcium sensitive in taste receptor cells. Our data suggest that taste receptor cells have IP$_3$-sensitive calcium stores but do not possess ryanodine-sensitive calcium stores. Caffeine is a well-known agonist of the latter, whereas it has actually been reported to inhibit the former (6, 52). For example, in salivary and pancreatic acinar cells, caffeine has been reported to activate ryanodine receptors, leading to calcium release, but to inhibit IP$_3$ receptors and calcium release from IP$_3$-sensitive calcium stores (51). Some precedent data are available in mudpuppy taste cells, where different bitter stimuli have been demonstrated to operate via IP$_3$-sensitive calcium stores or from a novel IP$_3$- and ryanodine-insensitive store (39). The latter was observed with concomitant inhibition of voltage-gated ionic currents, not unlike those observed in this communication with caffeine. Hence, calcium stores in taste receptor cells are likely to be complex, if not novel.

Caffeine’s seemingly opposite actions on intracellular calcium, releasing calcium from intracellular stores, while simultaneously causing an inhibition of calcium influx from extracellular sources, have been observed in other cell types and may represent an integrated mechanism for caffeine regulation of total intracellular calcium from plasma membrane and endoplasmic reticulum. Caffeine-mediated transient increase in intracellular calcium, similar to that observed in taste receptor cells, has been reported in a multitude of other cell types and reflects immediate calcium release from intracellular stores (13, 14, 40). Calcium release from caffeine-sensitive and IP$_3$-gated stores inactivates calcium channels in other cell types, such as ventricular myocytes (1), pituitary GH3 cells (26), and chromaffin cells (29). Caffeine similarly inhibits calcium current and increases intracellular calcium in rod photoreceptors (27). In these cells, it was concluded that an intracellular calcium-dependent mechanism, triggered by caffeine, led to suppression of the calcium current, although a smaller component could be attributed to a direct effect of caffeine’s action on the calcium channel. One key component was that, if barium, rather than calcium, were used as a charge carrier through voltage-gated calcium channels, then caffeine was without effect on the calcium current. The entrance of barium, rather than calcium, is without effect on intracellular stores or in priming ryanodine receptors. We performed the same experiment on taste receptor cells and found the same result: that using barium as a current carrier prevented any inhibition of the calcium current by caffeine (personal observations). This suggests that calcium release from the intracellular calcium pool might mediate the suppression of the calcium channel by caffeine. Together, the degree of interregulation of plasma membrane and endoplasmic reticulum calcium channels and the degree of store loading suggest that these coincident events may be a regulatory mechanism of caffeine’s action. In neurons, these two membrane systems have been proposed as a binary membrane system for overall calcium regulation (5). An important factor in determining the sensitivity of IP$_3$ or ryanodine receptors is the content of calcium in the lumen of the endoplasmic reticulum. These receptors become primed via previous entry of calcium from the plasma membrane voltage-gated calcium channels. Hence, the degree of store loading is dependent on previous activation of the cell, a form of “short-term memory.” Thus the complicated interplay among calcium release from caffeine-sensitive stores, calcium influx through plasma membrane calcium channels, and prior caffeine stimulation may play critical roles in determining the response to this stimulus.

**Putative transduction mechanisms of caffeine.** To transduce the presence of caffeine, taste receptor cells appear to utilize the same signal transduction mechanisms that serve as major cellular targets of caffeine’s actions in other cell types. Most prominently, these include caffeine’s inhibition of cyclic nucleotide phosphodiesterases and caffeine’s release of calcium from intracellular stores. Additionally, the direct blockage of potassium channels by caffeine may play an important role. The inhibition of phosphodiesterase and release of intracellular calcium are well established in the transduction mechanisms for bitter stimuli. As such, it
seems reasonable that these known actions of caffeine on other cell types and the role of these same mechanisms in bitter transduction likely serve as the cellular basis for the multiple effects of caffeine observed on taste receptor cells in this study.

A general notion that has received empirical and theoretical support is the closure of a resting potassium channel conductance by the taste cells as an early transduction mechanism, resulting in depolarization of the membrane potential on stimulus arrival. The membrane depolarization subsequently affects more dynamic changes in conductance, such as eliciting an action potential, which in turn cause the activation of calcium required for synaptic transmission. Additionally, the action potential may be modulated by changes in voltage-gated ionic currents. The present data would agree with such an interpretative view for caffeine.

One level of complication in interpreting steady-state alterations of current is distinguishing among those effects on voltage-dependent conductances that might be employed to transduce early events in the transduction cascade, such as the arrival of the stimulus and those changes in voltage-gated conductances that might be responsible for later events, such as transmitter release. The inhibition of KIR current by caffeine could clearly serve as a mechanism for the former. The roles that modulation of second-messenger systems may play in the transduction mechanisms associated with caffeine stimulation of taste receptor cells await further study. Direct evidence exists for two such second-messenger systems. Quench-flow analysis has shown that caffeine stimulation produced measurable increases in IP3 production (53) as well as cGMP production (44). Of these, elevation of cGMP was more robust. However, IP3 may play a significant role in releasing intracellular calcium from the caffeine-sensitive intracellular store. The actions of cGMP and intracellular calcium stores in taste receptor cells are not well understood.

In overview, the transduction mechanisms underlying the various effects observed in this study suggest a complex interplay of multiple transduction pathways. Caffeine’s multiple actions on electrical properties of taste receptor cells may be exemplary of tasters in general. One set of mechanisms may act in early events to depolarize the membrane potential and produce an action potential. Other actions, such as second-messenger production and internal calcium release, may act to modulate, perhaps in a taster-specific manner, the subsequent production of a train of action potentials. This view suggests that action potentials play essential signaling mechanisms within the bud, likely for activating the afferent nerve as well as cell-to-cell communication within the bud.

REFERENCES


CAFFEINE EFFECTS ON TASTE RECEPTOR CELLS


