

# Apical K<sup>+</sup> Channels in *Necturus* Taste Cells

## *Modulation by Intracellular Factors and Taste Stimuli*

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**ABSTRACT** The apically restricted, voltage-dependent K<sup>+</sup> conductance of *Necturus* taste receptor cells was studied using cell-attached, inside-out and outside-out configurations of the patch-clamp recording technique. Patches from the apical membrane typically contained many channels with unitary conductances ranging from 30 to 175 pS in symmetrical K<sup>+</sup> solutions. Channel density was so high that unitary currents could be resolved only at negative voltages; at positive voltages patch recordings resembled whole-cell recordings. These multi-channel patches had a small but significant resting conductance that was strongly activated by depolarization. Patch current was highly K<sup>+</sup> selective, with a  $P_K/P_{Na}$  ratio of 28. Patches containing single K<sup>+</sup> channels were obtained by allowing the apical membrane to redistribute into the basolateral membrane with time. Two types of K<sup>+</sup> channels were observed in isolation. Ca<sup>2+</sup>-dependent channels of large conductance (135–175 pS) were activated in cell-attached patches by strong depolarization, with a half-activation voltage of approximately –10 mV. An ATP-blocked K<sup>+</sup> channel of 100 pS was activated in cell-attached patches by weak depolarization, with a half-activation voltage of approximately –47 mV. All apical K<sup>+</sup> channels were blocked by the sour taste stimulus citric acid directly applied to outside-out and perfused cell-attached patches. The bitter stimulus quinine also blocked all channels when applied directly by altering channel gating to reduce the open probability. When quinine was applied extracellularly only to the membrane outside the patch pipette and also to inside-out patches, it produced a flickery block. Thus, sour and bitter taste stimuli appear to block the same apical K<sup>+</sup> channels via different mechanisms to produce depolarizing receptor potentials.

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## INTRODUCTION

Taste buds are the specialized end organs of gustation. A taste bud contains 50–150 bipolar receptor cells that extend from the basal lamina to the surface of the tongue, where their apical microvilli protrude into the taste pore to sample the environment. Taste transduction is initiated when sapid chemicals interact with the apical membrane of receptor cells leading to the release of neurotransmitter from basolateral synapses onto gustatory afferents. Although the classic taste stimuli (sweet, sour, bitter, salty, and umami) depolarize taste cells, the precise mechanisms involved in taste transduction are not well understood (for review, see Kinnamon, 1988; Roper, 1989; Avenet and Lindemann, 1989a; Avenet and Kinnamon, 1991).

The lack of information about taste is due in part to the small size of the receptor cells and their relative inaccessibility for physiological recording in situ. Recently, methods were developed for the isolation of taste receptor cells for patch-clamp recording. These studies have revealed that taste cells are not passive transducers, but have voltage-dependent  $\text{Na}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{K}^+$  currents with properties similar to those of neurons (frog: Avenet and Lindemann, 1987b; Miyamoto, Okada, and Sato, 1988; mudpuppy: Kinnamon and Roper, 1988a; tiger salamander: Sugimoto and Teeter, 1990; rat: Akabas, Dodd, and Al-Awqati, 1988; Herness, 1989; Béhé, DeSimone, Avenet, and Lindemann, 1990; mouse: Spielman, Mody, Brand, Whitney, MacDonald, and Salter, 1989). Although the precise role these conductances play in taste transduction is unclear, taste cells can generate action potentials in response to taste stimulation (Avenet and Lindemann, 1987a; Kinnamon and Roper, 1988b; Béhé et al., 1990; Avenet and Lindemann, 1991). Patch-clamp studies have begun to reveal the diverse mechanisms these cells use for chemosensory transduction. Apically located, amiloride-sensitive  $\text{Na}^+$  channels mediate the transduction of  $\text{Na}^+$  salt taste (Avenet and Lindemann, 1988, 1989b), while ligand-gated receptors coupled to cation channels appear to mediate the transduction of the amino acid L-arginine in catfish (Teeter, Brand, and Kumazawa, 1990). Receptors coupled to adenylyl cyclase are thought to mediate sweet taste in rodents (Avenet, Hofmann, and Lindemann, 1988; Tonosaki and Funakoshi, 1988; Striem, Pace, Zehavi, Naim, and Lancet, 1989; Cummings, Avenet, Roper, and Kinnamon, 1991).

Previous studies have shown that taste cells in the mudpuppy, *Necturus maculosus*, respond to sour stimuli (acids) with rapidly activating receptor potentials or action potentials, and to the bitter stimulus quinine with slowly activating receptor potentials. Both types of taste stimuli decrease the voltage-dependent  $\text{K}^+$  current in these cells (Kinnamon and Roper, 1988a), which is carried by  $\text{K}^+$  channels that are restricted to the apical membrane of the taste cells (Kinnamon, Dionne, and Beam, 1988b; Roper and McBride, 1989). In this paper we show that this apical  $\text{K}^+$  current is carried by several different types of  $\text{K}^+$  channels. These channels, which differ in their voltage sensitivity and response to intracellular factors, are all blocked directly by sour and bitter stimuli, via different mechanisms, to elicit taste receptor potentials.

Preliminary accounts of this work have been published previously (Cummings and Kinnamon, 1989a, b; Kinnamon and Cummings, 1989).

## METHODS

*Preparation*

Adult specimens of *Necturus maculosus* were maintained at 10°C in filtered freshwater aquaria and fed minnows weekly. Taste cells were isolated from the surrounding nongustatory epithelium as described previously (Kinnamon, Cummings, and Roper, 1988a). Briefly, mudpuppies were killed and the lingual epithelium was bluntly dissected free of the underlying connective tissue. After a 30-min incubation in amphibian physiological saline (APS) containing collagenase (1 mg/ml; type 3; Worthington Biochemical Corp., Freehold, NJ), albumin (1 mg/ml), and glucose (5 mM), the mucosal layers of the epithelium were gently separated from the underlying lamina propria. The taste buds, which remained attached to the lamina propria, were dissociated in Ca<sup>2+</sup>-free APS containing BAPTA (1 mg/ml; Molecular Probes, Inc., Eugene, OR) for 10 min. Isolated taste cells were plated onto glass coverslips coated with Cell-Tak (Collaborative Research, Bedford, MA) and placed in the recording chamber.

Cells were viewed at 400× with a Nikon Diaphot inverted microscope. The apical ends of taste receptor cells were identified by the presence of a rounded cap at the tip of an elongate process distal to the nucleus, a procedure we confirmed in previous studies by labeling the apical surface of the tongue with fluorescein isothiocyanate-conjugated wheat germ agglutinin before dissociation (Kinnamon et al., 1988a, b).

For some experiments, cells were plated onto gridded coverslips and stored at 4°C for 3–12 h to allow the apical membrane to redistribute into the basolateral membrane. Taste cells became rounded after this lengthy incubation, and the position of the apical membrane could no longer be discerned. To determine the approximate location of the apical membrane on the rounded taste cells, drawings were made of taste cells before incubation at 4°C and the cells were later relocated using the grid maps on the coverslips. There was no effect of this procedure on the magnitude or time course of whole-cell currents, or on the properties of unitary K<sup>+</sup> currents in multi-channel patches. Patches containing single K<sup>+</sup> channels could be obtained more readily with this procedure.

*Patch-Clamp Techniques*

Single-channel K<sup>+</sup> currents were recorded from the apical membrane of taste receptor cells using cell-attached, inside-out and outside-out configurations of the patch-clamp technique (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). Patch pipettes were fabricated from microhematocrit tubes (Scientific Products, McGaw Park, IL) on a two-stage vertical puller (model PB-7; Narishige, Tokyo, Japan); they had resistances of 6–9 MΩ when filled with saline. Dental periphery wax (Surgident, Ltd., Los Angeles, CA) was used to insulate the shanks of the pipettes to reduce electrode capacitance during recording. When the pipette was filled with APS, high resistance seals on the apical membrane could not be obtained. Seals in the range of 1–4 GΩ could usually be obtained, however, when the pipette contained a high K<sup>+</sup> saline. Outside-out patches were extremely difficult to obtain, presumably because the apical microvilli or the extremely high channel density prevented membrane resealing.

We examined the effects of taste stimuli on K<sup>+</sup> channels in cell-attached patches using a pipette perfusion system adapted from Tang, Wang, Quandt, and Eisenberg (1990). A narrow inner pipette (o.d. = 100 μm) of fused silica tubing was inserted inside the recording pipette to within 500 μm of the tip. The other end of the tubing fit tightly into polyethylene tubing (PE 10), which exited the pipette holder through an airtight opening. The inner pipette system was filled with intracellular pipette saline and the open end was heat sealed. After GΩ seal formation, negative pressure in the pipette was equilibrated to ambient pressure and the distal,

sealed end of the polyethylene tubing was snipped open and inserted into one of several small reservoirs containing different taste stimuli or control solutions. Gentle suction applied to the pipette lumen pulled the solutions into the tubing and perfusion pipette. Diffusion across the 500- $\mu\text{m}$  gap between the perfusion pipette tip and the membrane occurred within 2 s to 2 min depending on the size of the stimulus molecule. Timing began when fast green, which was included in the experimental solutions, was visualized in the tip of the patch pipette. Recording during more than three exchanges of the pipette solution on one patch was hindered by the development of noise produced by a buildup of fluid in the patch pipette suction tubing.

Patch current was measured at room temperature using an Axopatch 1D patch-clamping amplifier (Axon Instruments, Inc., Foster City, CA). The signal was filtered at 2 kHz before being digitized at 100- $\mu\text{s}$  intervals by a laboratory computer system (Indec, Sunnyvale, CA). For multi-channel patches, unitary channel currents were measured by eye with the aid of a computer-driven cursor, because typical computer-derived amplitude histograms failed to separate adequately channels of different sizes. Unitary currents were recorded from inside-out patches at several negative potentials where individual openings were rare, but more distinct; average current values were obtained for each channel during the 3–5-s recording duration at each potential. These values were plotted on a graph and linear regression lines were fit by computer through 0 mV (the reversal potential for all channels in the patch). We then inspected the raw data and searched for corresponding points for each channel at less hyperpolarized potentials where single-channel currents were more similar in amplitude. The conductance of each channel was determined from the slope of the regression line. In patches containing a large number of channels with differing conductance (e.g., Fig. 1), unitary currents could only be resolved at potentials negative to  $-60$  mV.

Open probability for each patch was calculated as the total open duration divided by the total duration of the recording, which was always 5 s or greater for these experiments. In patches containing a single channel, open probability is referred to as  $P_o$ . In patches containing multiple channels of identical conductance, open probability is referred to as  $nP_o$ . In the complex situation where patches contained an unknown number of channels of different conductances, channel activity was measured by the probability that any channel was open in the patch; we referred to this as the channel activity index. Generally, the channel activity index gives an underestimate of the total channel activity in the patch at a given voltage, with the underestimate being proportional to the number of channels in the patch. We usually selected a holding potential that produced a channel activity index of  $\sim 0.5$ , so that modulations above or below that value could be observed easily. Channel activity often increased with time during the recording, particularly after patch excision, necessitating holding the patch at more hyperpolarized potentials.

### *Solutions*

APS contained (mM): 112 NaCl, 2 KCl, 8 CaCl<sub>2</sub>, and 3 HEPES (buffered to pH 7.2 with NaOH). The high Ca<sup>2+</sup> facilitated seal formation with the apical membrane of taste cells. Taste stimuli and other chemicals were dissolved in APS, except for acid stimulation, where no buffer was used. In some experiments NaCl was replaced with KCl to eliminate the resting potential of the cell.

Two pipette solutions were used for cell-attached and inside-out patches; one contained 114 mM Kgluconate, the other 114 mM KCl. Both also contained the following (mM): 2 CaCl<sub>2</sub>, 2 MgCl<sub>2</sub>, and 10 HEPES (buffered to pH 7.2 with KOH or TrisOH). "Intracellular" pipette solution for whole-cell recordings and outside-out patches contained (mM): 80 Kgluconate, 10 NaCl, 2 MgCl<sub>2</sub>, 10 HEPES (buffered to pH 7.2 with KOH), 0.09 CaCl<sub>2</sub>, and 1 BAPTA (yielding 10<sup>-8</sup> M free Ca<sup>2+</sup>).

"Intracellular" bath solution for inside-out patches contained (mM): 114 KCl, 2 MgCl<sub>2</sub>, 2

NaCl, 10 HEPES (buffered to pH 7.2 with KOH), 0.09 CaCl<sub>2</sub>, and 1 BAPTA. Free Ca<sup>2+</sup> concentration was varied by holding the BAPTA concentration constant at 1 mM and changing the CaCl<sub>2</sub> as follows (mM): 0.91 CaCl<sub>2</sub> for 10<sup>-6</sup> M free Ca<sup>2+</sup>, 0.50 CaCl<sub>2</sub> for 10<sup>-7</sup> M free Ca<sup>2+</sup>, and 0.70 CaCl<sub>2</sub> for 5 × 10<sup>-7</sup> M free Ca<sup>2+</sup>. In the selectivity experiments the 114 mM KCl was replaced with 80 mM NaCl and 36 mM KCl; TrisOH was used to titrate the solution to a pH of 7.2. All bath solutions included 0.05% phenol red to allow a continuous monitoring of pH during experimentation.

## RESULTS

### *Apical K<sup>+</sup> Channel Diversity*

Taste receptor cells acutely isolated from the mudpuppy lingual epithelium have a distinctive elongated shape with the nucleus located nearer to one end. The specialized apical membrane could often be identified as a distinct, rounded cap at the tip of the process located furthest from the nucleus. Gigaohm seals (1–4 GΩ) to this region of membrane could be made with high K<sup>+</sup> pipette solutions and prolonged gentle suction. Cell-attached and excised patches from an apical cap typically contained many channels with a variety of single-channel current amplitudes (Fig. 1). In contrast, patches from membrane adjacent to the apical cap sealed easily and rarely contained channels.

Apical K<sup>+</sup> channels were voltage dependent. At depolarized potentials, the high level of activity and the density of K<sup>+</sup> channels prevented resolving individual channel openings; the recording resembled a whole-cell recording. The reversal potential for the ensemble of channels in a multi-channel patch was evaluated as the voltage where no net current flow was observed in the patch; this value was always ~0 mV in symmetrical KCl solutions. Above this value current was outward, but unitary current amplitudes could not be resolved. Although most of the channels opened at hyperpolarized potentials as well as depolarized potentials, we found that the larger channels opened only at the more depolarized potentials. In 26 apical patches we found multiple channels ranging in conductance from 30 to 175 pS, with as many as 10 resolvable current levels per patch. We were unable to determine if all of these current levels represent separate channel proteins. In some cases the current stepped from one open level to another and then closed to baseline, suggesting that at least some of these current levels represent subconductance states (arrow, Fig. 1A). Because there were so many events in any given timespan, it was virtually impossible to do quantitative studies of these transitions to address this question.

### *Ion Selectivity*

The ion selectivity of apical K<sup>+</sup> channels was ascertained by bath-applying different cations to inside-out patches. Kgluconate rather than KCl was used in the pipette to separate the equilibrium potential of Cl<sup>-</sup> from that of K<sup>+</sup>, since the apical membrane is thought to contain a Cl<sup>-</sup> conductance (Roper and McBride, 1989; McBride and Roper, 1991). With Kgluconate in the pipette and KCl in the bath, unitary currents reversed at -15 mV (Fig. 2). This shift in the reversal potential resulted from a lowered K<sup>+</sup> activity in the Kgluconate solution relative to the KCl solution (D. Ewald,

unpublished data),<sup>1</sup> rather than a  $\text{Cl}^-$  conductance. Exposure of the inside of the membrane to elevated  $\text{Na}^+$  (80 mM NaCl, 36 mM KCl) caused the average reversal to shift  $+27 \pm 2.4$  mV ( $n = 7$ ), which is close to the +29-mV shift predicted by the Nernst equation for a purely  $\text{K}^+$ -selective conductance. From the Goldman-Hodgkin-Katz equation we calculated a  $P_{\text{K}}/P_{\text{Na}}$  permeability ratio of 28 for the apical  $\text{K}^+$  conductance. No differences were observed in the average reversal potential for the different sized channels.

#### *Voltage Dependence*

We examined the voltage dependence of current through the ensemble of channels in multi-channel patches of apical membrane. In most patches the channel activity index (see Methods) at rest (approximately  $-65$  mV; Kinnamon and Roper, 1988a) was  $\sim 0.05$  (Fig. 3). That is, at the resting potential there was a small but significant open probability for the ensemble of channels. This correlates well with the resting  $\text{K}^+$  conductance seen in whole-cell studies (Kinnamon and Roper, 1988a). Hyperpolarization by 30 mV or more was required to reduce the channel activity index to  $\sim 0.01$ . When apical patches were depolarized from rest, channel activity was greatly increased (Fig. 3 B). The smaller channels (115 pS and less) showed relatively larger increases in mean open time, as well as in the probability of being open, with depolarization. With strong depolarizations we were unable to distinguish individual channel openings. For most patches, inactivation was not observed for apical  $\text{K}^+$  channels even in response to prolonged depolarization, again consistent with observations in whole-cell clamp studies (Kinnamon and Roper, 1988a).

#### *Modulation by Intracellular Factors*

Using inside-out apical patches, we found that channel activity was modulated by two intracellular factors: ATP and  $\text{Ca}^{2+}$ . Fig. 4A shows that channel activity in a multi-channel patch changes as a function of bath-applied ATP and  $\text{Ca}^{2+}$ . Representative recordings for each of the bath conditions are illustrated in Fig. 4B. ATP reversibly blocked some channel activity, while  $\text{Ca}^{2+}$  reversibly increased the activity. In whole-cell recordings, the  $\text{K}^+$  current was unaffected by the presence of 10  $\mu\text{M}$  cAMP in the pipette, and by the membrane-permeable analogues 8-CPT cAMP and 8-bromo cAMP (1 mM) in the bath, unlike taste cells of the frog (Avenet et al., 1988).

It appeared that different  $\text{K}^+$  channels in these multi-channel patches were selectively modulated by the different intracellular factors. In the presence of ATP some channels appeared to be selectively blocked, and in elevated  $\text{Ca}^{2+}$ , other

<sup>1</sup> Potassium ion activity was measured in 114 mM KCl and 114 mM Kgluconate pipette solutions using K-selective microelectrodes filled with the K-selective liquid ion exchanger IE-90 (World Precision Instruments, Sarasota, FL). Potential change as a function of  $\text{K}^+$  concentration was measured in  $\text{K}^+$  standard solutions and test solutions.  $\text{K}^+$  activity of 114 mM Kgluconate was approximately half that of 114 mM KCl, suggesting incomplete dissociation of Kgluconate. This drop in  $\text{K}^+$  activity would cause a 19-mV hyperpolarizing shift in the  $\text{K}^+$  reversal potential, which is slightly more than the 15 mV we observed. This large shift was observed only in inside-out patches; in cell-attached patches  $\text{K}^+$  currents reversed near 0 mV with 114 mM Kgluconate in the pipette (Figs. 5 and 7). This correlates with previous studies in which we estimated intracellular  $\text{K}^+$  to be near 80 mM in *Necturus* taste cells (Kinnamon and Roper, 1987).

channels, particularly the larger conductance channels, appeared to be selectively activated. To investigate this further we developed a method to isolate single apical K<sup>+</sup> channels. When dissociated cells were stored in APS at 4°C for 3–12 h, the cells partially resorbed their apical caps and the channels began dispersing from the apical pole. Patching near, but not on, the apical pole with small pipettes allowed us to

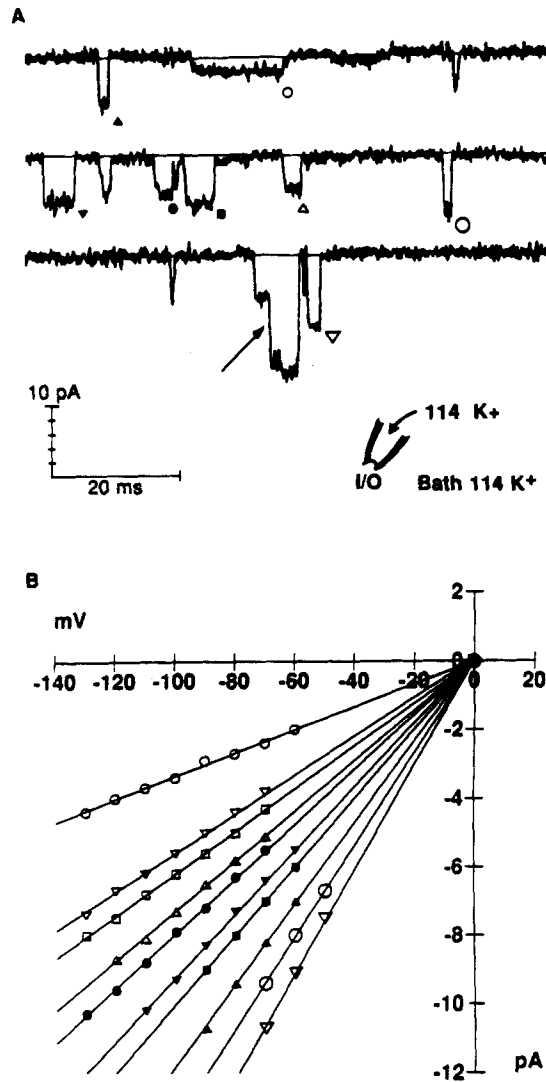


FIGURE 1. Data from an inside-out patch of apical membrane of a taste cell showing unitary K<sup>+</sup> currents from channels of different conductances. The patch was held at  $-70$  mV in symmetrical KCl (114 mM). (A) Unitary currents. Symbols denote different single-channel current amplitudes as designated below. Arrow denotes two possible subconductance states of a single channel. The solid line in each trace of this and subsequent figures represents the closed state. (B) Current-voltage ( $I$ - $V$ ) plot with 10 different unitary conductances for the K<sup>+</sup> channels seen in this patch. Individual channel amplitudes were measured as described in Methods. Linear regression lines were drawn through these points and through 0 mV, the observed reversal potential for all channels in the patch. The channel conductances observed in this patch had values of 34, 57, 62, 73, 80, 93, 100, 118, 134, and 151 pS.

record from single K<sup>+</sup> channels in some patches; most patches still contained multiple unitary current amplitudes. Similar patches from acutely dissociated cells rarely if ever contained K<sup>+</sup> channels.

The channel type observed most frequently in isolation had a large single-channel conductance (135–175 pS) and was Ca<sup>2+</sup> sensitive as well as voltage dependent (Fig.

5). Fig. 5 B shows the current–voltage plot for a 170-pS channel; Fig. 5 C illustrates the voltage and  $\text{Ca}^{2+}$  dependence for this same channel. The plot denoted by the open squares illustrates the voltage dependence of the channel in the cell-attached configuration; the 50% open time level ( $V_{1/2}$ ) of this channel is  $-10$  mV. When this cell-attached patch was excised and exposed to  $10^{-8}$  M free  $\text{Ca}^{2+}$  in the bath, there was little effect on the channel's open probability (Fig. 5 C, solid triangles). However, when the free  $\text{Ca}^{2+}$  concentration was increased to  $10^{-6}$  M, the channel became exceedingly active, requiring hyperpolarization to keep channel activity at an interpretable level (Fig. 5 C, solid squares). We found that the open probability of a  $\text{Ca}^{2+}$ -dependent channel in the cell-attached configuration was almost always identical to that in the inside-out configuration when the bath  $\text{Ca}^{2+}$  concentration was  $10^{-8}$  M. This suggests that the physiological level of free  $[\text{Ca}^{2+}]_i$  is at or near this value.  $\text{Ca}^{2+}$  concentrations of  $10^{-7}$  M caused only a slight increase in the activity of these channels, but at  $5 \times 10^{-7}$  M caused a large increase in activity (Fig. 6). Often, in

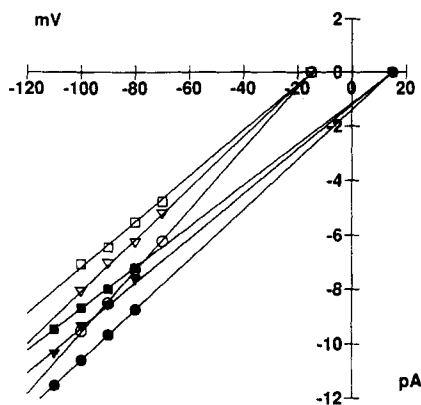


FIGURE 2.  $I$ - $V$  plots for three channels in an inside-out apical patch, exposed to different concentrations of cations in the bath. The pipette contained 114 mM Kgluconate and 2 mM NaCl. Open symbols denote unitary current amplitudes in 114 mM KCl/2 mM NaCl; closed symbols denote currents through the same channels in 36 mM KCl/80 mM NaCl. In the 114 KCl/2 NaCl bath the reversal potential was  $-15$  mV rather than 0 mV, due to the low  $\text{K}^+$  activity of Kgluconate in the pipette (see foot-

note 1). In 36 KCl/80 NaCl the reversal potential shifted by  $+27$  mV, which was close to the  $+29$ -mV shift predicted by the Nernst equation for a purely  $\text{K}^+$ -selective conductance.

experiments where  $10^{-6}$  M  $\text{Ca}^{2+}$  or higher concentrations were tested, the channel activity was so exuberant that the patch was lost.

In eight patches we observed  $\text{K}^+$  channels whose mean conductance was  $99.6 \pm 5.6$  pS. Generally, this class of channels exhibited a very strong voltage dependence and was blocked by intracellular ATP. Fig. 7 A illustrates the current–voltage plot for one of these channels in a cell-attached patch; its conductance was 95 pS (the cell was bathed in 114 KCl to eliminate the resting potential). Fig. 7, B and C, show that this channel became very active at voltages above  $-60$  mV and reached  $V_{1/2}$  at  $-47$  mV. With one exception, these 100-pS channels all had significantly lower half-activation voltages than their  $\text{Ca}^{2+}$ -dependent counterparts. Inside-out patches revealed that channels of this conductance class were blocked by intracellular ATP. Fig. 7 D is a recording from a patch containing two ATP-sensitive channels whose  $nP_o$  dropped from 0.28 to 0.05 in the presence of 5 mM ATP. When the ATP was washed out there appeared to be a rebound effect as the  $nP_o$  increased to 0.44. We observed the ATP block and rebound effect in all eight channels of this class. ATP sensitivity does not



appear to be restricted to channels of this class, however, since we also observed an ATP block in one patch containing a single 170-pS Ca<sup>2+</sup>-dependent channel (all other Ca<sup>2+</sup>-dependent channels tested were unaffected by ATP). The ATP block is not likely to be due to chelation of Ca<sup>2+</sup> by ATP because Ca<sup>2+</sup> was buffered to low levels (10<sup>-8</sup> M) by 1 mM BAPTA throughout the experiment. We did not determine whether hydrolysis of ATP is required to achieve the block.

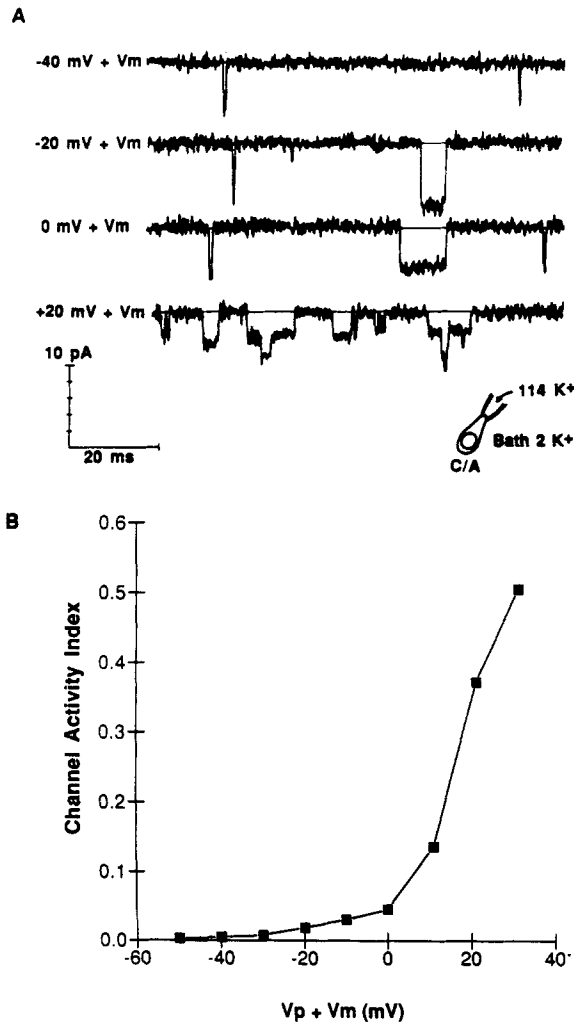


FIGURE 3. Voltage dependence of channel activity in a cell-attached multi-channel patch from the apical membrane. The bath contained APS (2 mM K<sup>+</sup>) and the pipette 114 mM Kgluconate. (A) Single-channel recordings at representative voltages; V<sub>m</sub> refers to the resting potential (which is unknown). (B) Estimated open probability of the channels in the patch (channel activity index) as a function of voltage applied to the patch. The channels occasionally opened at hyperpolarized potentials as well as at rest. When the cell was depolarized from rest, the open probability of the channels increased dramatically.

#### *Effects of Taste Stimuli*

In initial experiments several taste stimuli were screened for effects on the whole-cell K<sup>+</sup> current (data not shown). The K<sup>+</sup> current was elicited by a 1,500-ms pulse to +60 mV from a holding potential of -80 mV. The taste stimulus, dissolved in saline together with fast green dye, was applied 250 ms after the initiation of the voltage

pulse from a micropipette located 20–30  $\mu\text{m}$  from the apical cap of the taste cell. Citric acid (1 mM in unbuffered APS), tetraethylammonium chloride (TEA; 10 mM), quinine (10 mM), and  $\text{CaCl}_2$  (200 mM) all blocked the  $\text{K}^+$  current to varying degrees. The amino acid L-arginine (100 mM) and a cocktail of several amino acids (L-valine, L-phenylalanine, L-arginine, L-histidine, L-lysine, and L-glutamate; 50 mM each), all taste stimuli in the mudpuppy (McPheeters and Roper, 1985), had no effect. In addition, there was no effect of fast green per se on the  $\text{K}^+$  current. Since citric acid

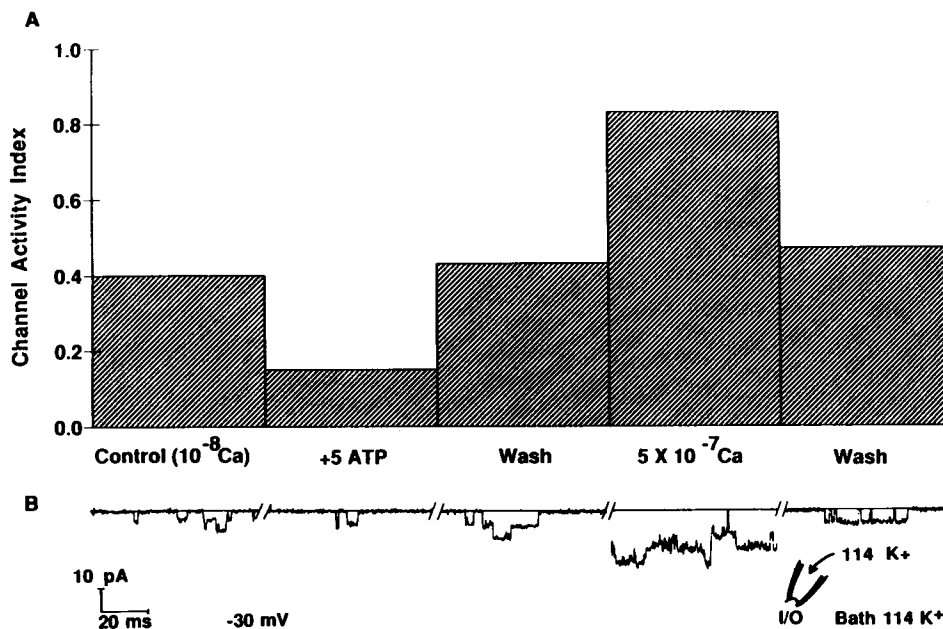


FIGURE 4. Effects of ATP and  $\text{Ca}^{2+}$  on channel activity in an inside-out multi-channel patch. The pipette contained 114 mM Kgluconate and the bath 114 mM KCl. The patch was clamped at  $-30$  mV. Changes in channel activity were measured in response to bath applied ATP (5 mM) and  $\text{Ca}^{2+}$  ( $5 \times 10^{-7}$  M). Control solutions contained  $10^{-8}$  M  $\text{Ca}^{2+}$  and no ATP. Bar graph of channel activity (A) and representative recordings (B) are shown for each of the bath concentrations tested. The duration of exposure to each test solution was as follows: control, 53 s; ATP, 5.3 min; wash, 1.8 min; elevated  $\text{Ca}^{2+}$ , 43 s; wash, 1.7 min. ATP reversibly blocked the activity of some channels, while  $\text{Ca}^{2+}$  reversibly increased the activity of other channels in the patch.

had the strongest effect and quinine the second strongest effect, we focused on these two taste stimuli in our single-channel studies.

To mimic the *in vivo* situation where taste stimuli have direct access to the  $\text{K}^+$  channels in the apical membrane, we applied taste stimuli to outside-out apical patches. Citric acid, bath-applied at 0.1 mM (pH 3.8), produced a nearly complete block of the  $\text{K}^+$  channels in three patches containing multiple  $\text{K}^+$  channels (Fig. 8A). The block was dose dependent, with a threshold of  $\sim 0.01$  mM citric acid (pH 4.8);

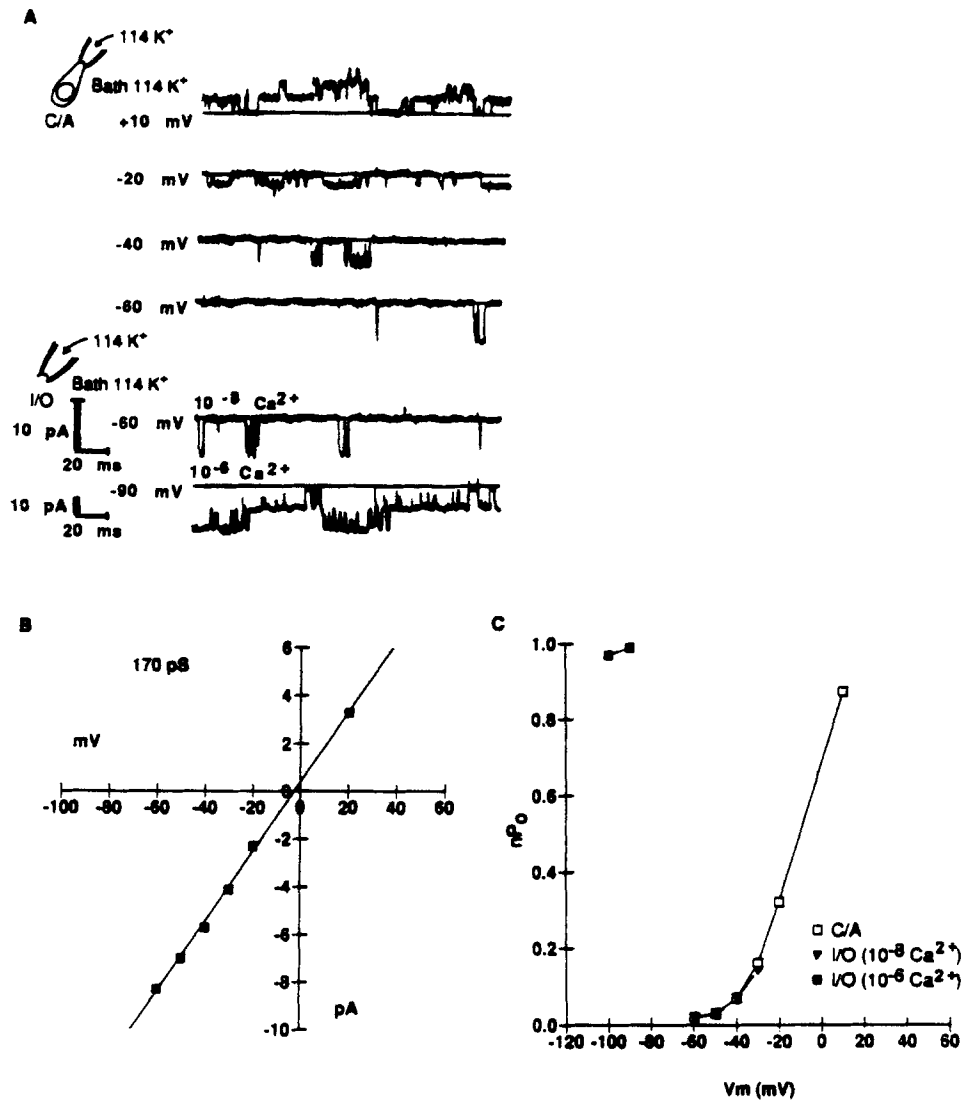


FIGURE 5. Voltage and Ca<sup>2+</sup> dependence of a class of larger conductance channels, ranging from 135 to 175 pS. The pipette contained 114 mM Kgluconate and the bath contained 114 mM KCl plus 10<sup>-8</sup> or 10<sup>-6</sup> M Ca<sup>2+</sup>. All plots are from the same patch that was initially cell attached and subsequently excised. (A) Channel activity before (first four traces) and after (last two traces) patch excision at different holding voltages. The patch contains two channels of equal conductance. Note the gain change in the bottom trace. (B) The *I-V* plot shows that the channels had a unitary conductance of 170 pS in the cell-attached configuration. (C) Open probability plot illustrates the voltage and Ca<sup>2+</sup> dependence of the channels. The plot denoted by the open squares illustrates the voltage dependence in the cell-attached configuration; the half-activation voltage was -10 mV. The filled triangles show that the open probability remained constant when the patch was excised and exposed to 10<sup>-8</sup> M Ca<sup>2+</sup>. When the bath was perfused with 10<sup>-6</sup> M Ca<sup>2+</sup> (filled squares), the channels became very active (only two points at negative voltages could be obtained).

Kinnamon and Roper, 1988a) and complete block occurring at 1 mM (pH 3.0; Fig. 9). The block did not appear to be voltage dependent, since block was observed at holding potentials varying from  $-90$  to  $0$  mV. The block was reversible (data not shown). Although these data suggest that protons block the channels directly, we could not rule out the possibility that changes in intracellular pH were mediating the effect, especially if the channels were slightly permeable to protons (Hille, 1984). To test for an indirect effect of acid on the channels, we bath-applied citric acid ( $0.1$  mM) to the cell while recording from apical  $K^+$  channels in a cell-attached patch. In this configuration the acid bathed the entire cell, including the apical membrane, yet it did not have direct access to the channels in the patch. Fig. 8 B shows that this application had no effect on the channels in the patch. In addition, channels in inside-out patches were not blocked by bath-applied citric acid ( $0.1$  mM; data not shown). These data indicate that intracellular pH does not mediate the block, and also rule out the involvement of a second messenger.

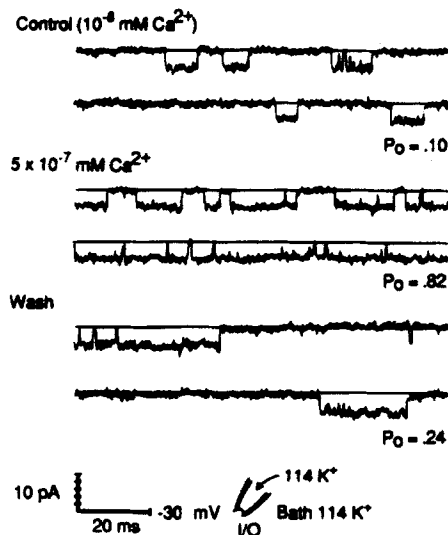


FIGURE 6. Effect of  $5 \times 10^{-7}$  M  $Ca^{2+}$  on channel activity in an inside-out patch containing a single  $135$ -pS channel. The pipette contained  $114$  mM Kgluconate and the bath  $114$  mM KCl and  $10^{-8}$  M  $Ca^{2+}$ . The patch was clamped at  $-30$  mV. The open probability jumped from  $0.1$  in  $10^{-8}$  M  $Ca^{2+}$  to  $0.82$  in  $5 \times 10^{-7}$  M  $Ca^{2+}$ .

Outside-out apical patches were extremely difficult to form, presumably because the high channel density prevented membrane resealing. In addition, data from this configuration were difficult to interpret because the multiple channels in this relatively larger area of membrane made it difficult to resolve individual channel openings. To study the effects of taste stimuli on channel activity, we used a pipette perfusion technique developed by Tang et al. (1990). We used it in the cell-attached configuration and controlled for endogenous membrane voltage shifts by depolarizing the cell with bath-applied  $114$  mM KCl. Not only did this permit observation of the channel/taste stimulus interaction, but it also isolated the applied stimulus to a small area of apical membrane in a similar manner to that occurring *in vivo* due to tight junctions in the intact taste bud.

The effects of two taste stimuli, citric acid and quinine, are shown in Fig. 9, on the same multi-channel perfused patch. Citric acid ( $1$  mM) quickly, completely, and

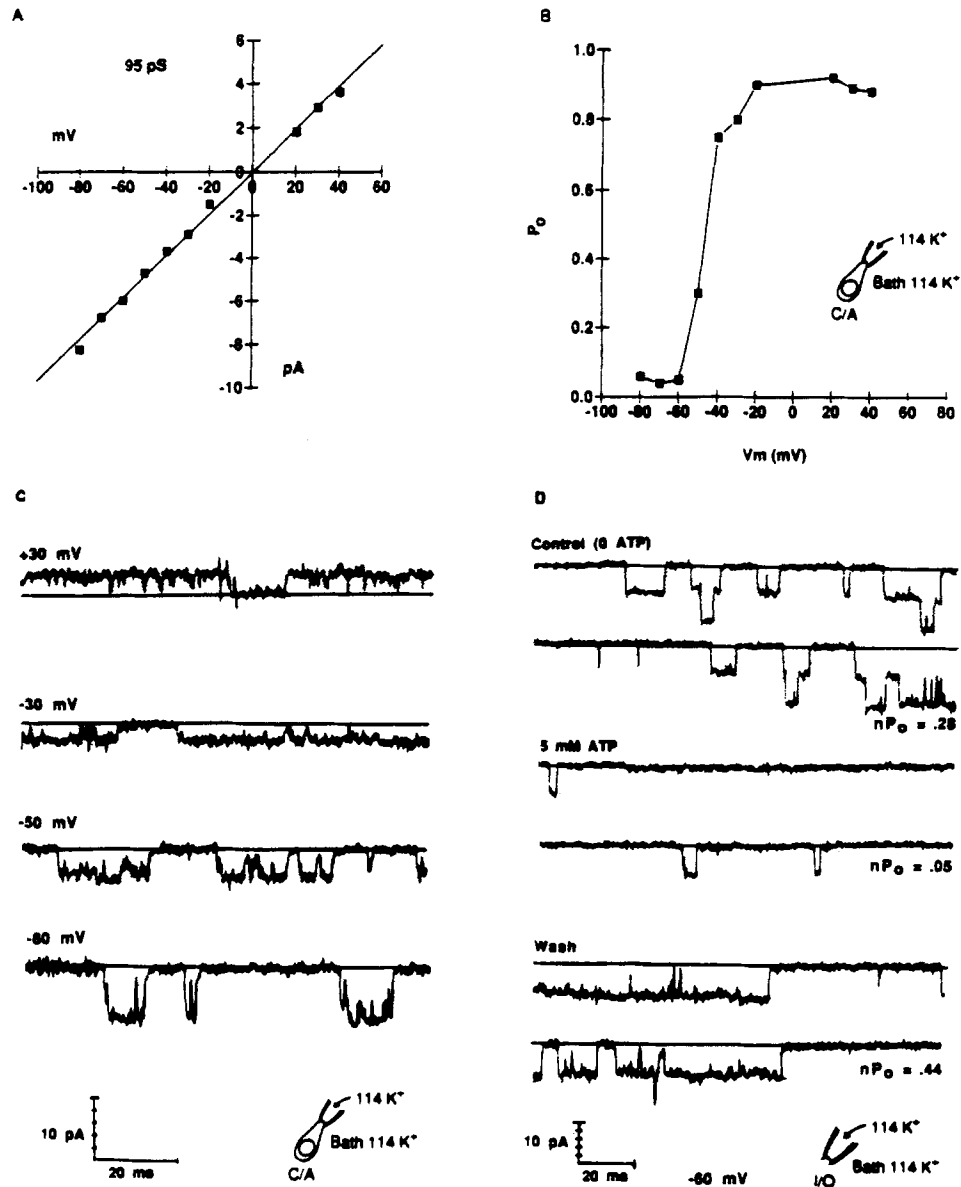


FIGURE 7. Voltage dependence and effect of ATP on a class of apical K<sup>+</sup> channels with a mean conductance of 100 pS. (A) *I-V* plot for one channel in a cell-attached patch; its conductance was 95 pS. The bath contained 114 mM KCl to zero the resting potential. The pipette contained 114 mM Kgluconate. (B) The open probability plot shows that this channel was strongly voltage dependent, with a half-activation voltage of -47 mV. (C) Representative traces of this channel at different holding potentials. (D) Unitary currents recorded from an inside-out patch containing two ATP-sensitive channels. Channels were reversibly blocked by 5 mM ATP, while the patch was clamped at -60 mV. Note the rebound effect after the wash.

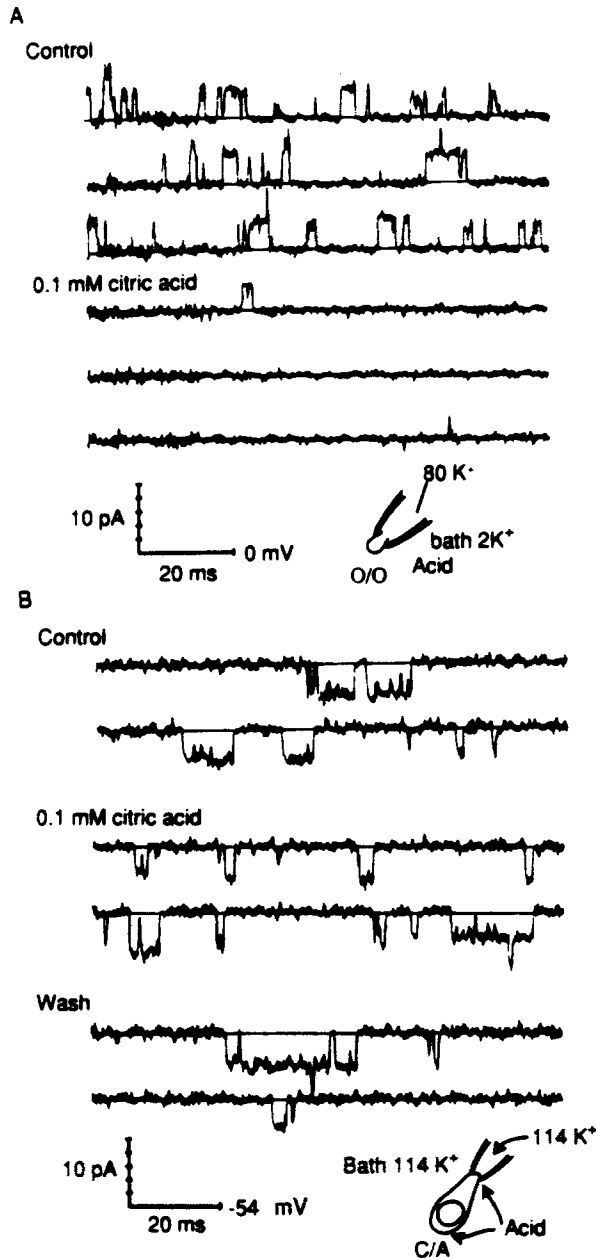


FIGURE 8. Citric acid blocked apical K<sup>+</sup> channels directly. (A) 0.1 mM citric acid (pH 3.8) almost completely blocked channel activity in an outside-out multi-channel patch held at 0 mV. The pipette contained 80 mM Kgluconate and the bath contained 2 mM K<sup>+</sup>. (B) Data recorded from a cell-attached apical patch. When citric acid (0.1 mM) was bath-applied to the cell, with no direct access to channels in the patch, it had no effect on K<sup>+</sup> channel activity. The pipette contained 114 mM Kgluconate and the bath 114 KCl to eliminate the resting potential of the cell.

reversibly blocked all the channels in the patch. Recovery of activity took much longer to effect than the onset of acid block, possibly because the test concentration was at least 10 times the concentration needed to achieve a complete block. Quinine also blocked these channels, although the time course of onset was considerably slower; and at 1 mM it did not completely block all the channels present in the patch.

An 85% reduction in channel activity took 2 min, or nearly 50 times the time required for citric acid to achieve a complete block. This difference in time course is due in part to the diffusion process, since quinine is a larger molecule than citric acid and would be expected to diffuse the 500- $\mu$ m gap much more slowly. Quinine may, however, block the channels via an indirect mechanism, which would account for some of the delay in onset of the effect. There is evidence for bitter taste stimuli activating second messengers in taste cells (Akabas et al., 1988). In addition, quinine is membrane permeant, suggesting that it may interact with the channels via their hydrophobic domains, or may block the channels from inside the cell.

The cell-attached experiment in Fig. 10 allowed us to differentiate between direct and indirect effects of quinine. The quinine was applied in two different ways: directly

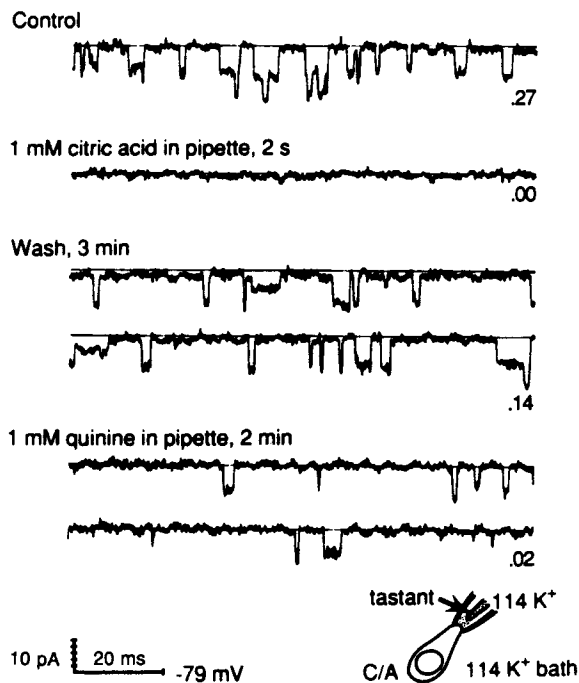


FIGURE 9. Effect of citric acid and quinine, applied via pipette perfusion, on apical K<sup>+</sup> channels in a cell-attached patch. The pipette contained 114 mM Kgluconate and the bath 114 mM KCl to eliminate the resting potential of the cell. The patch was clamped at -79 mV. Citric acid (1 mM; pH 3) blocked all channels quickly and completely. Quinine (1 mM) also blocked the channels, but the block was incomplete and the onset occurred much more slowly. The number at the right of each trace represents the channel activity index for each condition.

via the pipette perfusion apparatus to apical channels in the patch, and indirectly via bath exchange, which would allow access to channels in the patch only from the cytoplasm or from within the membrane. When 1 mM quinine was applied directly, a 58% reduction in channel activity occurred in 3 min. An indirect application of 1 mM quinine to the bath, while the quinine was still present in the pipette, caused a further drop in channel activity to 86% block. After an 8-min bath wash, channel activity returned to control levels. A pipette wash resulted in a rebound of the open probability to a much higher level. Subsequent direct application of 1 mM quinine again blocked the K<sup>+</sup> channels by 72%. Finally, direct application of 10 mM quinine in the pipette produced a complete block of all the channels in the patch. In two

other patches, indirect application of 10 mM quinine blocked all of the channels (data not shown).

Note that the direct block, where there was a simple reduction in the open probability, appeared to involve interference with the gating mechanism of the channels. In contrast, the indirect block caused a "flickering" of the open channels, suggesting a conductance blocking mechanism. This flickering type of block was also observed in channels in inside-out patches when quinine was bath applied. Fig. 11

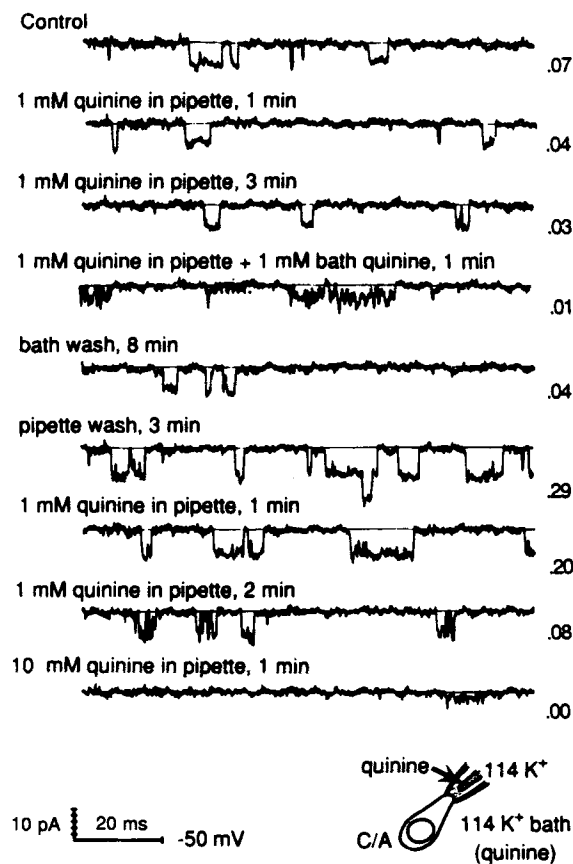


FIGURE 10. Effects of quinine applied directly, via pipette perfusion, and indirectly, via bath perfusion, on the activity of apical K<sup>+</sup> channels in a cell-attached patch. The pipette contained 114 mM Kgluconate and the bath 114 mM KCl. The patch was held at -50 mV. Quinine applied directly caused a 58% reduction in channel activity within 3 min. The addition of 1 mM quinine to the bath increased the block to 86% in 1 min. After 8 min of bath wash only, channel activity returned to the same value that was seen earlier with just 1 mM quinine in the pipette. After 3 min additional wash of the pipette, the channel activity rebounded to 0.29. Subsequent reapplication of 1 mM quinine to the pipette again blocked the channels by 72% in 2 min. Finally, direct application of 10 mM quinine in the pipette blocked all of the channels in the patch. Channel activity indexes are indicated at the right of each trace, as described for Fig. 9.

shows an inside-out multi-channel patch exposed to quinine. Quinine (0.1 mM) caused the open channels to flicker rapidly between the open and closed states, and at 1 mM caused a complete conductance block of the channels. These data suggest that the flickering block observed when quinine was applied to the entire cell (Fig. 10) was probably due to a direct effect of quinine on the inside of the channel, rather



than to activation of a second messenger. Thus, quinine appears to act on both sides of the membrane, via different mechanisms, to block the apical K<sup>+</sup> channels.

In all multi-channel patches tested, citric acid (1 mM) and quinine (10 mM) blocked all channels in the patch. Unlike the intracellular modulators, there appeared to be no differential effects of the taste stimuli on particular channel types. Therefore, we did not attempt to study the effects of taste stimuli on patches containing only a single channel type.

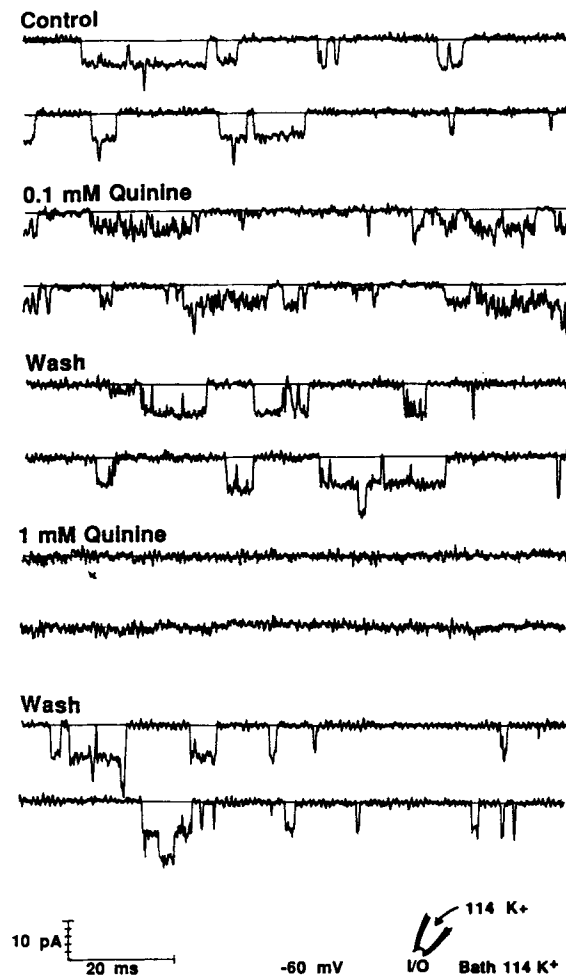


FIGURE 11. Effect of quinine on a multi-channel inside-out patch. Pipette contained 114 Kgluconate and the bath contained 114 KCl. The patch was held at  $-60$  mV. Quinine (0.1 mM) caused the open channel to flicker rapidly between open and closed states. A higher concentration of quinine caused a complete block of channel activity, and the effect was reversible.

#### DISCUSSION

This study provides the first description of channels that comprise the apically restricted K<sup>+</sup> conductance of *Necturus* taste receptor cells. Here we report that this conductance consists of several types of K<sup>+</sup> channels which differ in their unitary

conductance, voltage dependence, and sensitivity to intracellular  $\text{Ca}^{2+}$  and ATP. All of the channels are blocked directly by sour and bitter taste stimuli, using different mechanisms, to elicit characteristic taste receptor potentials.

These data agree well with previous results obtained from intracellular recordings of taste cells in intact lingual epithelia (Kinnamon and Roper, 1987; Roper and McBride, 1989) and from whole-cell and loose-patch recordings of isolated taste cells (Kinnamon and Roper, 1988a; Kinnamon et al., 1988b). Taken together, the data show that the apical  $\text{K}^+$  conductance in *Necturus* taste cells is voltage dependent, is partially activated at rest, shows little or no inactivation, is partially  $\text{Ca}^{2+}$  dependent, is blocked by external TEA, and, most importantly, is restricted to the apical membrane where it is modulated by sour and bitter taste stimuli. The apical  $\text{K}^+$  channels subserve a variety of functions in the taste cells including action potential repolarization, maintenance of the resting potential, and transduction of taste stimuli into receptor potentials. One difference we did observe between whole-cell and single-channel studies is in the voltage dependence of activation. In whole-cell recordings, outward currents activate at more depolarized potentials than in cell-attached patches. This difference may be due to the domination of whole-cell records by the large conductance class of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels which activate at more depolarized values (e.g., Fig. 5 C). Alternatively, or in addition, there may be dialysis of an intracellular factor or factors normally involved in channel activation in whole-cell recordings. The factor is not likely to be  $\text{Ca}^{2+}$ , however, since this study suggests that free intracellular  $\text{Ca}^{2+}$  is at or near  $10^{-8}$  M, the concentration used in our intracellular salines.

The large diversity of  $\text{K}^+$  channels found on the apical membrane was unexpected. Single  $\text{K}^+$  channels in taste cells have been described in only four previous studies. Two voltage-dependent  $\text{K}^+$  channels were observed in tiger salamander taste cells, a 21-pS channel and a 147-pS channel (Sugimoto and Teeter, 1990). In rat taste cells a 90-pS delayed rectifier channel and a 225-pS  $\text{Ca}^{2+}$ -dependent  $\text{K}^+$  channel were identified (Akabas, Dodd, and Al-Awqati, 1990). In isolated taste cells of the frog, three different  $\text{K}^+$  channels were predominant: a 22-pS voltage-independent channel, a 44-pS voltage-independent channel blocked by cAMP-dependent protein kinase, and a 74-pS  $\text{Ca}^{2+}$ -dependent channel (Avenet et al., 1988). Previous studies in our lab have shown a 56-pS inward rectifier  $\text{K}^+$  channel on the basolateral membrane of *Necturus* taste cells (Kinnamon and Cummings, 1989). In all of these studies, patches were obtained from basolateral membrane or from isolated cells in which the apical membrane could not be identified. In this study we detected several apical  $\text{K}^+$  channels with different conductances, but only a subset of these was observed in isolation. The best characterized channel types were a 100-pS ATP-blocked channel and several large conductance  $\text{Ca}^{2+}$ -activated channels. It is possible that these channels migrate from the apical cap more readily than the smaller  $\text{K}^+$  channels (33–80 pS), which we never observed in isolation. Alternatively, the smaller conductances in a multi-channel apical patch may represent substates of the larger conductance channels. If the smaller channels represent substates, however, it is not clear why substates were rarely observed when the larger channels were present in isolation. Perhaps substates become more predominant when channels are tightly packed in the membrane. Alternatively, the high density of channels may permit

interaction between different channel proteins, leading to a higher than expected number of simultaneous transitions, which in turn gives the appearance of substates. Strong coupling between open and closed transitions has been observed in ACh receptors on cultured myotubes (Yeramian, Trautmann, and Claverie, 1986). A similar clustering of channels exhibiting simultaneous transitions has been observed when membrane fragments from olfactory cilia (Labarca, Simon, and Anholt, 1988) and catfish taste epithelia (Teeter et al., 1990) were incorporated into lipid bilayers.

The Ca<sup>2+</sup>-dependent channels observed in *Necturus* taste cells resemble the maxi BK channels observed in many cell types (Latorre and Miller, 1983), including mammalian taste cells (Akabas et al., 1990). In *Necturus* they probably mediate at least a portion of the large, hyperpolarizing after-potential that follows excitation of these cells (Kinnamon and Roper, 1987; Roper and McBride, 1989).

ATP-blocked channels have not been reported previously in taste cells, but have been described in a variety of other cell types including cardiac cells (Noma, 1983), insulin-secreting pancreatic  $\beta$  cells (Cook and Hales, 1984), skeletal muscle fibers (Spruce, Standen, and Stanfield, 1985), smooth muscle cells (Gelband, Silberberg, Groschner, and van Breeman, 1990), and cultured neurons (Ashford, Sturgess, Trout, Gardner, and Hales, 1988). Although the voltage dependence and unitary conductance of the different ATP-sensitive channels differ, hydrolysis of ATP is not required for the blocking effect in most cell types. These channels are thought to provide a regulatory link between the metabolism of a cell and its excitability (for review, see Ashcroft, 1988). In taste cells, ATP-sensitive channels could be involved directly in adaptation to taste stimulation if, for example, continuous stimulation of a cell resulted in lowered cytoplasmic ATP levels. Any reduction in ATP would open the channel and hyperpolarize the cell.

The unique apical localization of these diverse K<sup>+</sup> channels strongly suggests that they are involved in the transduction of taste stimuli into receptor potentials. Since taste stimuli normally interact with the apical membrane of taste cells, an apical localization of channels insures that the entire conductance is exposed to the taste stimulus. This is important because we have shown in this study that protons and quinine directly block the channels. If K<sup>+</sup> channels were uniformly distributed, the depolarization produced by block of the apical channels would be shunted by voltage-dependent activation of basolateral channels. The K<sup>+</sup> channel type we have observed most frequently on the basolateral membrane is a 56-pS inward rectifier channel, which closes in response to membrane depolarization (Kinnamon and Roper, 1988a; Kinnamon and Cummings, 1989). Thus, the depolarization produced by taste stimuli that block the apical K<sup>+</sup> conductance is amplified by the depolarization-induced block of the basolateral inward rectifier K<sup>+</sup> channels.

The receptor potential induced by acids has a rapid onset and often elicits an action potential in the taste cell. In contrast, quinine induces a slow-onset, long-duration receptor potential that rarely elicits an action potential (Kinnamon and Roper, 1988a). The different temporal characteristics of the receptor potentials elicited by these two stimuli can be explained by their mechanisms of block of the apical K<sup>+</sup> conductance. Protons block the channels rapidly and directly. Low external pH has been shown to block K<sup>+</sup> channels by causing a depolarizing shift in their voltage dependence of activation in many cells (Hille, 1984), including taste cells

(Teeter, Sugimoto, and Brand, 1989); however, the origin of the taste cell channels in that study was unknown. In contrast, quinine blocks apical  $K^+$  channels by both direct and indirect mechanisms. The reduction in open probability produced by direct application of quinine to  $K^+$  channels in a patch had a much slower onset than block of the same channels by protons. Although this may be attributed in part to the slower diffusion of quinine from the perfused pipette to the membrane, we believe that the time course of the quinine block itself is slower as well. Using rapid applications that were not diffusion limited, we have observed that the onset of quinine block of the whole-cell  $K^+$  current is slower than the onset of proton block. Quinine has been shown to reduce the open probability of  $K^+$  channels in other tissues with a slow time course (Bokvist, Rorsman, and Smith, 1990; Fatherazi and Cook, 1991). It is possible that quinine block requires partitioning of the molecule into the membrane where it can interfere with the hydrophobic domains of the channel protein. The slow onset of the quinine block may account for the slow onset and rise time of the quinine taste receptor potential (Kinnamon and Roper, 1988a). The flickery block of apical  $K^+$  channels produced by indirect application of quinine to apical  $K^+$  channels is probably produced by quinine entering the cell and blocking the channels from the inner face of the membrane, since the same flickery block is produced when quinine is applied to inside-out patches. A similar flickery block in response to quinidine, a related molecule, was observed in inside-out patches containing  $K^+$  channels from dissociated smooth muscle cells (Wong, 1989). This indirect block may account for the long duration receptor potentials and prolonged taste of quinine. Quinine probably fails to elicit action potentials in taste cells because the slower depolarization inactivates the voltage-dependent  $Na^+$  conductance.

The rebound of  $K^+$  channel activity after application of quinine to the external membrane (Fig. 10) is interesting, and occurred in every patch exposed to quinine. This rebound of activity resembles the rebound typically observed after exposure of ATP-blocked channels to intracellular ATP. The functional significance of the increased  $K^+$  channel activity is unclear, but may represent a type of adaptation to repeated stimulation of quinine. Similar rebounds did not occur after exposure of patches to citric acid.

If the entire apical  $K^+$  conductance is blocked by both protons and quinine, is the mudpuppy able to discriminate between these tastes? Possibly the different durations of the receptor potentials provide the basis of taste discrimination. Alternatively, the animal may not discriminate. Recent data indicate that the bitter-tasting divalent cations also block this conductance (Bigiani and Roper, 1991), and behavioral studies suggest that these stimuli are all aversive to the mudpuppy (Bowerman, A. G., and S. C. Kinnamon, unpublished observations). It is possible that the apical  $K^+$  conductance provides an aversive warning signal for the mudpuppy to eject foods that are unpalatable.

It is unknown if block of an apically restricted  $K^+$  conductance is involved in taste transduction in mammals. Mammals clearly distinguish sour and bitter tastes, and these tastes are detected by taste buds residing in different papillae innervated by different afferent nerves. Bitter taste is detected primarily by taste cells in vallate and foliate papillae, while sour taste is detected by taste cells in fungiform, as well as vallate and foliate papillae (Frank, 1973; Hanamori, Miller, and Smith, 1988). Recent

studies show that K<sup>+</sup> channel blockers have little or no effect on the sour response in mammalian fungiform taste cells, suggesting that sour taste is probably transduced in these cells by a different mechanism (Gilbertson, Avenet, Kinnamon, and Roper, 1991). It remains possible, however, that taste cells of the vallate and foliate papillae have an apical K<sup>+</sup> conductance and that this conductance is involved in bitter taste transduction. Most K<sup>+</sup> channel blockers taste bitter to humans, and recent studies have shown that bitter-tasting compounds block K<sup>+</sup> currents in taste cells isolated from vallate taste buds (Spielman et al., 1989). Studies examining the distribution of K<sup>+</sup> channels on these cells will be required to ascertain their role in taste transduction.

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