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Patch-Clamp Study of Isolated Taste Receptor Cells of the Frog

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Summary. Taste discs were dissected from the tongue of *R. ridibunda* and their cells dissociated by a collagenase/low Ca/mechanical agitation protocol. The resulting cell suspension contained globular epithelial cells and, in smaller number, taste receptor cells. These were identified by staining properties and by their preserved apical process, the tip of which often remained attached to an epithelial (associated) cell. When the patch pipette contained 110 mM KCl and the cells were superfused with NaCl Ringer's during whole-cell recording, the mean zero-current potential of 22 taste receptor cells was −65.2 mV and the slope resistance 150 to 750 MΩ. Pulse-depolarization from a holding voltage of −80 mV activated a transient TTX-blockable inward Na current. Activation became noticeable at −25 mV and was half-maximal at −8 mV. Steady-state inactivation was half-maximal at −67 mV and complete at −50 mV. Peak Na current averaged −0.5 nA/cell. The Ca-ionophore A23187 shifted the activation and inactivation curve to more negative voltages. Similar shifts occurred when the pipette Ca was raised. External Ni (5 mM) shifted the activation curve towards positive voltages by 10 mV. Pulse depolarization also activated outward K currents. Activation was slower than that of Na current and inactivation slower still. External TEA (7.5 mM) and 4-aminopyridine (1 mM) did not block, but 5 mM Ba blocked the K currents. K-tail currents were seen on termination of depolarizing voltage pulses. A23187 shifted the $I_K(V)$-curve to more negative voltages. Action potentials were recorded when passing pulses of depolarizing outward current. Of the frog gustatory stimulants, 10 mM Ca caused a reversible 5- to 10-mV depolarization in the current-clamp mode. Quinine (0.1 mM, bitter) produced a reversible depolarization accompanied by a full block of Na current and, with slower time-course, a partial block of K currents. Cyclic AMP (5 mM in the external solution or 0.5 μM in the pipette) caused reversible depolarization (to −40 to −20 mV) due to partial blockage of K currents, but only if ATP was added to the pipette solution. Similar responses were elicited by stimulating the adenylate cyclase with forskolin. Blockage of cAMP-phosphodiesterase enhanced the response to cAMP. These results suggest that cAMP may be one of the cytosolic messengers in taste receptor cells. Replacement of ATP by AMP-PNP in the pipette abolished the depolarizing response to cAMP. Inclusion of ATP-γ-S in the pipette caused slow depolarization to −40 to −20 mV, due to partial blockage of K currents. Subsequently, cAMP was without effect. The remaining K currents were blockable by Ba. These results suggest that cAMP initiates phosphorylation of one set of K channels to a nonconducting conformation.

Key Words sensory cells · taste- and chemoreception · gustatory senses · patch-clamp whole-cell recording · cyclic AMP · forskolin · AMP-PNP · ATP-γ-S

Introduction

In the mouth of vertebrates the epithelium covering the tongue contains secondary cells [14] which monitor the chemical composition of food with respect to a few basic gustatory qualities. These taste receptor cells (TRCs) are, apparently, differentiated bipolar epithelial cells which contact afferent nerve fibers through chemical synapses. In the taste buds of frogs the receptor cells reach the mucosal surface with a slim process which typically terminates in a conspicuous, large microvillus—the rod—containing a dense cylindrical bundle of microfilaments [15, 21]. Several investigators found that TRCs, when impaled with a microelectrode and stimulated with a current pulse, are capable of generating action potentials [25, 29, 55]. Furthermore, mucosal $CaCl_2$, which is one of the taste agents in amphibia, was recently shown to depolarize the basolateral membrane of *Necturus* taste cells. Beginning at a threshold potential of −50 mV action potentials are then fired [7]. However, it is not clear how the action potentials are involved in taste transduction, and the sensory transduction mechanism is essentially unknown.

Recently, attempts to patch-clamp amphibian taste cells were made in different laboratories [5, 6, 28]. Here we report initial progress with isolated gustatory receptor cells from the frog. Following dissociation from the taste bud, the chemo-sensory cells were identified by shape and by staining of the

microvillus, and patch-clamped at the basolateral membrane. In whole-cell recordings transmembrane currents and the blockage of K currents by cyclic AMP were characterized. Part of our results appeared in abstract form [5, 6].

Materials and Methods

Isolation of Taste Cells

Frogs of the species *R. ridibunda* were maintained at about 10°C with access to tap water. Animals were sacrificed by decapitation and pithing and their tongues removed. In the frog, taste cells are found on fungiform papillae (taste buds) of the tongue mucosa. This location makes them particularly suitable for isolation. From one tongue about 100 taste buds, 100 to 200 μm in diameter, were cut off at the stem with fine scissors under binocular magnification of 10 to 80×. The forward and middle parts of the tongue's surface were used predominantly. By means of Pasteur pipettes, the dissected taste discs were first rinsed with Ringer's for 10 min in order to remove mucous, then with Ringer's containing 0.5 to 1 mg/ml of collagenase.

The discs were then transferred into a small centrifuge tube containing Ringer's plus 0.5 to 1 mg/ml of collagenase and magnetically stirred for 60 min to loosen the basement membrane. Following centrifugation for 10 min at 1500 × g, the supernatant was replaced by Ca-free Ringer's containing 1 mM EGTA, to open cell-to-cell contacts. After 60 min in this solution, the surface-cells had a rounded, swollen appearance under the microscope, but were not yet dissociated. The discs were washed briefly in Ca-containing Ringer's transferred into the experimental chamber and, in Ca-containing Ringer's repeatedly sucked into and pushed out of a capillary (patch pipette glass) with an opening of about 100 μm. This mechanical agitation dissociated the taste bud epithelium into single cells and small groups of cells. The use of polyethylene capillaries rather than glass capillaries did not improve the yield of sensory cells.

The dissociated cells settled on the bottom of the chamber, where they attached within 30 min. The chamber had a volume of about 0.1 ml and consisted of a standard glass slide (bottom) onto which a cured silicon ring of 1-mm thickness and 10-mm inner diameter was pressed. Prior to recording, cells were kept in their chambers in a storage solution (Na-Ringer's with 5 mM glucose and 5 mM pyruvate) at 4°C. For patch-clamping, the chamber was placed on the stage of an inverted microscope (Olympus IMT2-F), and the cells superfused at 1.2 ml/min with appropriate solutions (without glucose and pyruvate) at room temperature (20 to 22°C). The resulting equilibration time was 10 to 20 sec. The cells were viewed with Nomarski optics at a total magnification of 400×.

Fluorescence Microscopy

The inverted microscope IMT2-F (Olympus) was used with a 100 W mercury lamp and standard dichroic mirror blocks (epifluorescence). Images were recorded through a 40× long-distance objective of 0.5 numerical aperture or a 100× (oil immersion) objective of 1.3 numerical aperture, using either a television camera (RCA TC 1040/H with intensified silicon-intensifier target) or photographic film (Ilford XP1 400 or Kodacolor VR 1000, both exposed at 1000 ASA).

Electrical Recording

The procedure for whole-cell recording closely followed the description by Hamill et al. [19]. Patch pipettes were pulled from thick-walled borosilicate glass capillaries (Jencons Scientific Ltd., Bedfordshire, UK, outer diameter 2 mm, inner diameter 1 to 1.25 mm) on a two-stage puller designed in the laboratory. After fire-polishing, the tip resistances were 7 to 10 MΩ when filled with Ringer's. For recordings with high time resolution the final tapers were coated with Sylgard® before polishing, where necessary.

The pipettes were manipulated with a hydraulic 3-D manipulator (Narishige MO-103N-L). Gentle coaxial approach onto the cell surface was achieved with a small dc-powered motor drive mounted on the manipulator at an angle of 35 degrees. During the approach, the pipette interior was maintained under positive pressure. Patch-clamp currents were recorded with an amplifier of our own design or with the EPC-7 amplifier (List-electronic, Darmstadt, FRG). Current and voltage signals were digitized at 40 kHz (16 bits) with an audio-PCM processor (Sony 501 ES, modified to pass zero frequency) and stored on a video recorder.

Sealing of the pipette on the cell surface was aided by gentle suction, yielding seal resistances in the order of 10 GΩ. With some preparations the formation of giga-seals was difficult, but could be improved by superfusing the cells with Ringer's containing 10 mM Ca. This solution was washed away once the seal had formed. After compensating the capacitance and adjusting the pipette (holding) voltage to −80 mV, the patch was broken by a burst of 200-mV, 10-msec clamp voltage-command pulses, or by suction. Upon breakage of the patch the input capacitance increased to 10 to 20 pF and current and noise level became larger. Using 10 mV voltage command pulses, the capacitive transient cancellation (subtraction) was then adjusted. It worked well for small voltage pulses, but with larger pulses nonlinear capacitance effects became noticeable. The pipette resistance was usually not compensated, and leakage current subtraction was not done.

Solutions and Reagents

The extracellular Ringer's solution contained (concentrations in mM): NaCl 110, $CaCl_2$ 1, $MgCl_2$ 0.5, K phosphate buffer 3.5 (pH 7.2). Where indicated, Na ions were replaced by K or N-methyl-D-glucamine (NMDG, Sigma). The phosphate buffer was replaced by 10 mM Tris-HEPES (pH 7.2) when Ca or Ba was used as the main external cation, or when the K concentration was modified. The pipette filling solution for whole-cell recording contained KCl 110, $MgCl_2$ 2, EGTA (Sigma) 0.1 ($pCa \geq 7$), HEPES-KOH buffer 10 (pH 7.2), or modifications as indicated. Osmolarities were determined with a vapor pressure osmometer (Wescor 5100C).

The following chemicals (abbreviation, type, source in brackets) were used in addition: collagenase (type I, Sigma) tetrodotoxin (TTX, Sigma), tetraethyl-ammonium (TEA, Sigma), 4-amino-pyridine (4AP, E.G.A.), amiloride (Sharp & Dhome GMBH, FRG), A23187 (Lilly), D600 (Knoll A.G.), 3-isobutyl-1-methylxanthine (IBMX), quinine, L-threonine, L-serine, L-leucine, L-glutamate, GTP, ATP (di-Na or Tris salt, Sigma), cy-

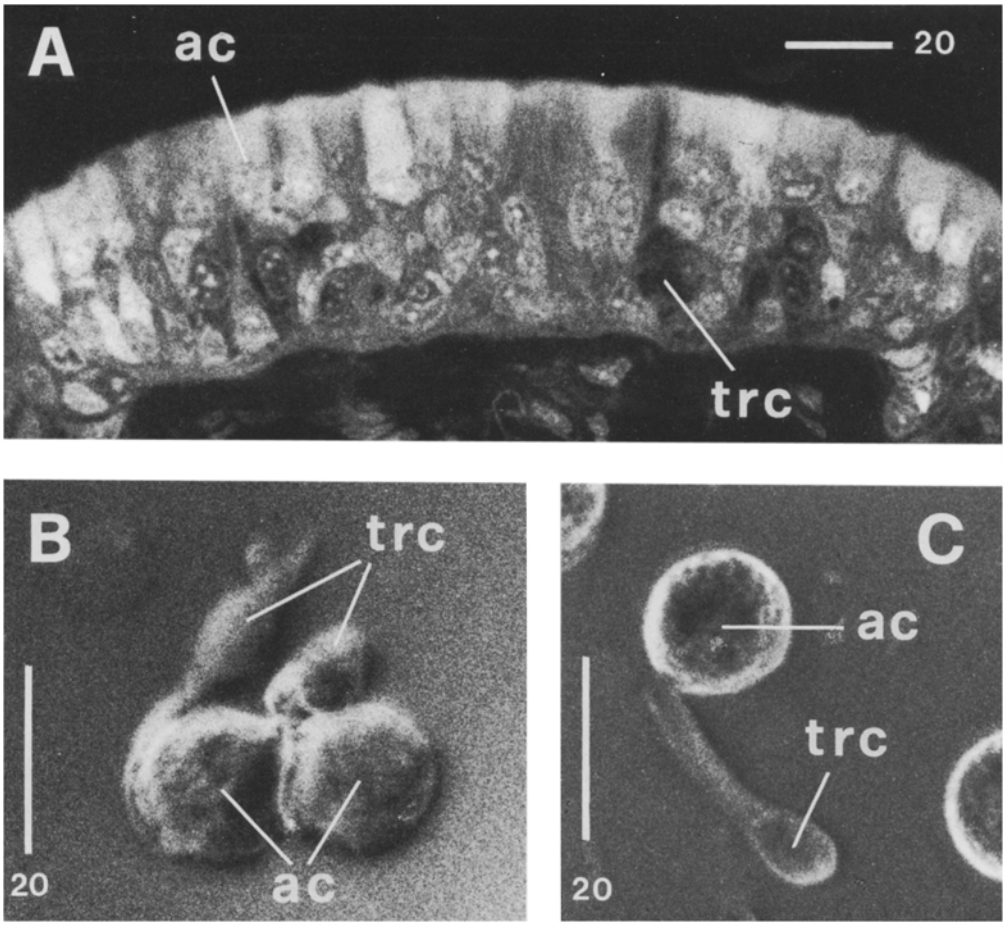

Fig. 1. (*A*) Taste disc of the frog in vertical cross section. The mucosal border faces upwards. Associated surface cells (*ac*) and taste receptor cells (*trc*) can be distinguished. Semi-thin section stained with toluidine blue. Print from a color slide (the darkly stained structures appear bright). Bar indicates 20 μm. (*B*, *C*) Taste receptor cells (*trc*) and associated surface cells (*ac*) after dissociation of the taste bud. Nomarski optics. Scales indicate 20 μm. Panel (*B*) depicts two spindle-shaped TRCs and panel (*C*) one flask-shaped TRC. Focussing showed a pair of adjacent parallel processes attached to the flask-shaped TRC

clic-AMP (cAMP), forskolin, Triton X-100 (Sigma), AMP-PNP (Li salt, Serva), ATP-γ-S (Li salt, Serva), trypan blue (Chroma Ges.), methylene blue (type B, Merck), merocyanin 540 (Serva), RH-414 (Molecular Probes), tetramethylrhodaminyl-phalloidin (Rh-phalloidin, Molecular Probes).

Results

Identification of Isolated Taste Receptor Cells

The light microscope shows in cross-sections of the frog's taste disc (Fig. 1*A*) one superficial layer of mucous-secreting epithelial cells, often called "associated" cells. Below them the somata of epithelial taste receptor cells (TRCs) are seen. These cells send slim apical processes, so-called dendrites, to the mucosal surface of the tissue. The dendrites are closely associated with sheet-like processes of wing cells [15]. After the collagenase/low Ca treatment, the majority of dissociated epithelial cells derived from taste discs consisted of associated cells (wing cells and mucous cells) which rounded off when contact to their neighbors was lost. Less numerous were ciliated epithelial cells from the rim of the sensory disc. Regular beating of the cilia was used as a criterion for the viability of the preparation.

Dispersed among these types of rounded cells were occasional flask- and spindle-shaped cells with one, seldomly two adjacent neck-like protrusions, the tips of which were often still in contact with an associated cell (Fig. 1*B*, *C*). At the other pole of the cell a short process was usually seen. Shape and size of these cells resembled that of the bipolar TRCs in the intact epithelium, even though the ma-

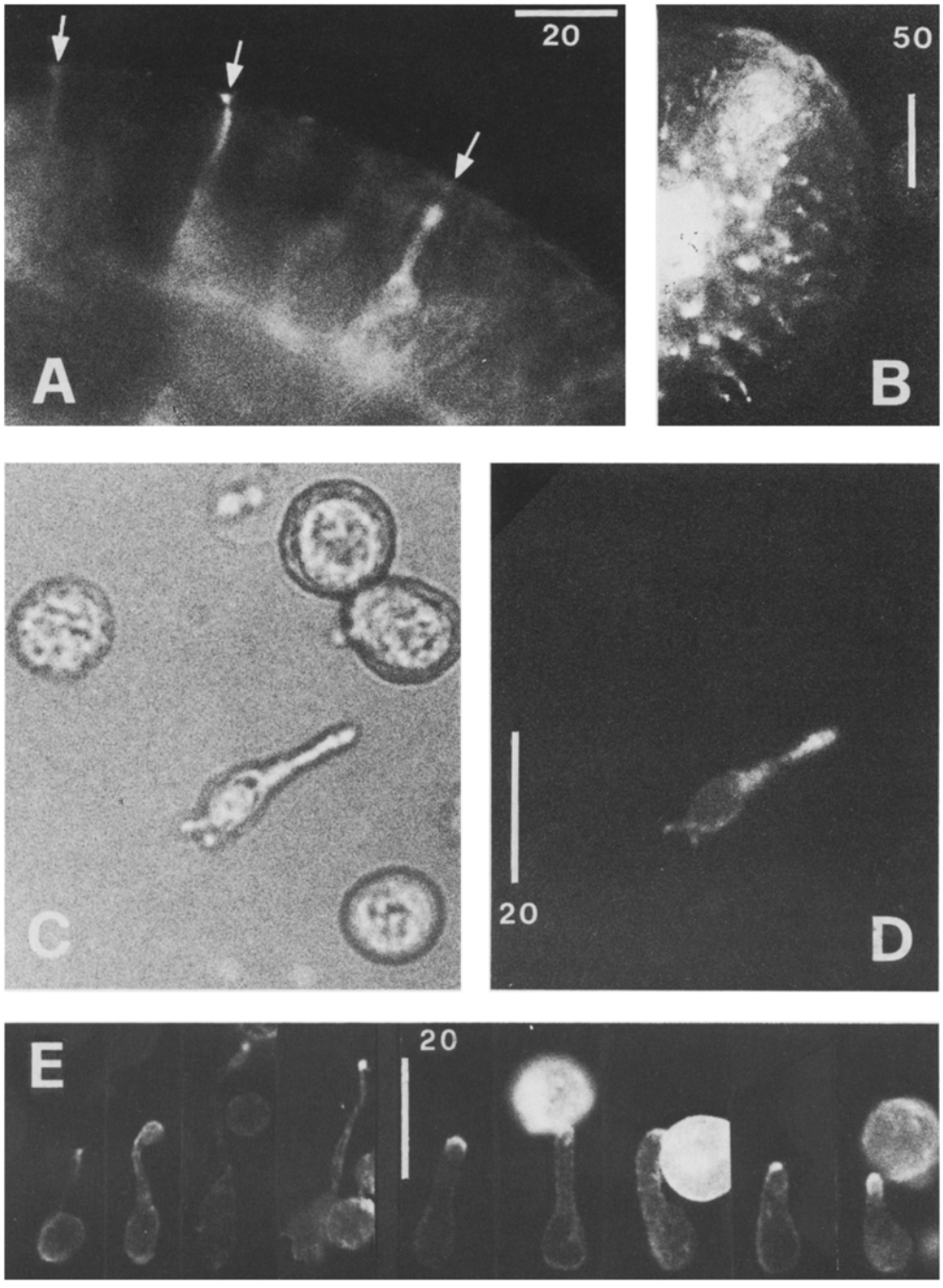

Fig. 2. (*A*) Vertical optical cross section of an intact taste disc stained with RH-414 (100 μg/ml of Na-Ringer's for 45 min). Three red-fluorescent "dendrites" of receptor cells could be recognized (arrows). When in focus (middle) they were seen to make contact with the mucosal surface of the disc. (*B*) Horizontal optical cross section of an intact taste disc stained with RH-414. In the lower part of the diagram the mucosal surface is in focus. Focussing through this preparation revealed numerous red-fluorescent dendrites which reached the mucosal surface. (*C*, *D*) Corresponding photographs of the same scene of isolated cells in transmitted light (*C*) and with green-excited fluorescence (*D*). Staining with RH-414 (*see* panels *A*, *B*) was done prior to tissue dissociation. The five globular cells (probably associated surface cells, the uppermost lysed) are not fluorescent. The spindle-shaped receptor cell showed maximal red fluorescence at the apical pole of the dendrite. (*E*) Four spindle-shaped (thin dendrites) and five flask-shaped TRCs (blunt dendrites), stained with Rh-phalloidin following fixation with glutardialdehyde and permeabilization with 1% Triton X-100. An intense red fluorescence of the apical cell poles was noted. Little F-actin was stained in more proximal parts of the cells

jor process of flask-shaped cells often measured somewhat shorter and blunter (Fig. 2*E*) than expected from TRCs. These cells may be in the process of rounding. Both flask- and spindle-shaped cells could be patch-clamped, but spindle-shaped cells were used preferentially. Occasional cells which appeared grossly shrunken or swollen (rounded) or which were blebbing, were not used.

When the intact disc was vital-stained with methylene blue (2.5 mM), the mucosal surface showed blue dots which were the apical poles of TRCs because on focussing down they were seen connected to spindle-shaped cells. The isolated flask- and spindle-shaped cells also stained with methylene blue, in contrast to the majority of rounded-off cells. The few rounded cells which stained may be damaged or, perhaps, sensory cells which have become globular. A similar pattern of staining was found with the fluorescent dyes merocyanin 540 and RH-414, as shown in Fig. 2(*A*) and (*B*). Particularly with RH-414, TRCs within the disc could be stained rather selectively and their shape followed by focussing down perpendicularly to the epithelial surface. With the taste bud resting on its side (Fig. 2*A*), the dendritic tips were often seen to protrude slightly from the surface. In ten isolated discs we counted in the mean 60 ± 10 (SD) RH-414 fluorescent dendrites.

When discs prestained with RH-414 were dissociated, most isolated flask- and spindle-shaped cells were fluorescent while the majority of rounded cells was not fluorescent (Fig. 2*C* and *D*), suggesting that the TRCs were the major component among the isolated nonglobular cells. Very few of these cells were not fluorescent. These may be "basal cells" whose processes do not radiate to the mucosal surface [17].

We attempted to stain specifically the characteristic bundle of microfilaments which provides for the rod-shape of the apical pole of the chemo-receptor cells [15, 21]. Discs and isolated cells were fixed with buffered 2% formaldehyde, permeabilized with 1% Triton X-100 and incubated for 60 min in a solution containing Rh-phalloidin. Phalloidin is known to bind specifically to F-actin (e.g., ref. 65). In both cases cells of elongated shape stained predominantly at the tip of their apical process which appeared as a brightly red-fluorescent dot. (With this method about 700 receptor terminals per taste disc were counted; Richter, Avenet, Mestres and Lindemann, *in preparation*). It appears, therefore, that the microvillus of chemo-receptor cells contains a high concentration of F-actin, which is retained by isolated TRCs (Fig. 2*E*). However, hours after dissociation the stained apical structures of some cells became more distributed, indicating that the cytoskeleton associated with the receptor area reacted to the cell isolation.

Whole-Cell Recording

After breaking the patch and establishing whole-cell recording, TRCs had a membrane slope-resistance of 150 to 750 MΩ, a capacitance of 10 to 20 pF and a zero-current voltage of $V_0 = -65.1 \pm 9.5$ (SD) mV (22 cells) when Na Ringer's was used in the bath and the pipette filling solution contained 110 mM KCl. When the K concentration of the bath was reduced from 3.5 to 1 mM, V_0 was -70.8 ± 7 mV (17 cells). When with 1 mM K in the bath the pipette solution contained 5 mM ATP, V_0 was initially in the order of -65 to -70 mV, but depolarized spontaneously with about 1 mV/min and stabilized at -48.6 ± 7.2 mV (12 cells). During patch-clamping, an increase in cell volume was sometimes observed without a noticeable change in membrane properties.

At a pipette holding voltage of -80 mV the subtraction of membrane capacitive current transients was adjusted, square command pulses applied to the pipette and the resulting whole-cell currents recorded. With the solutions specified above and depolarizing voltage pulses, we obtained the current time-courses shown in Fig. 3 (*A*, *B*). A fast transient inward current (plotted downwards) was followed by a pronounced outward current which developed more slowly and inactivated partially at positive voltages. Replacement of Cl ions in the pipette (by glutamate) or in the bath (by sulfate) left the currents essentially unchanged.

The peak and steady-state current values, depicted as a function of pulse voltage in Fig. 3(*C*), suggest that the recorded currents resulted from the superposition of a fast, transient Na inward current and K outward currents of slower dynamic response.

Na Currents

When the K content of the pipette filling solution was replaced by Cs, the outward currents shown in Fig. 3 were much smaller (Fig. 4*A*). The remaining outward currents were blockable by Ba added to the bath (Figs. 5*C* and 8*C*). The transient inward current could then be studied in more detail. It was abolished by replacement of the external Na by N-methyl-D-glucamine or Ba or Ca ions. Furthermore,

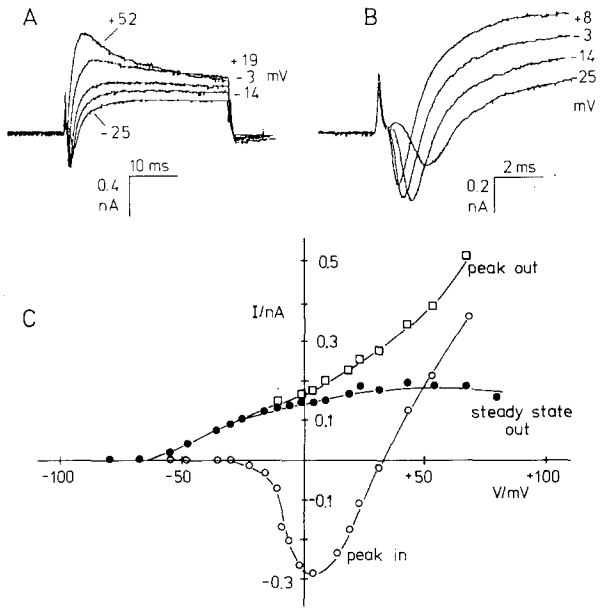

Fig. 3. (*A*) Time-course of whole-cell currents (spindle-shaped TRC) in response to depolarizing voltage pulses, from a holding voltage of −80 mV to the values indicated. Current from taste receptor cell to bath (outward current) is plotted upwards. Bath: NaCl-Ringer's with 3.5 mM K. Pipette: standard filling solution with 110 mM KCl, no ATP. Pipette resistance not compensated. (*B*) Records from same TRC at higher time and current resolution. From left to right: Current at holding voltage, capacitive outward current spike (with cell-capacitive current cancellation), transient inward current, superimposed more slowly developing outward current. (*C*) Peak-inward, peak-outward and steady-state-outward currents as a function of command pulse voltage. Data from a different cell to that in panels (*A*) and (*B*)

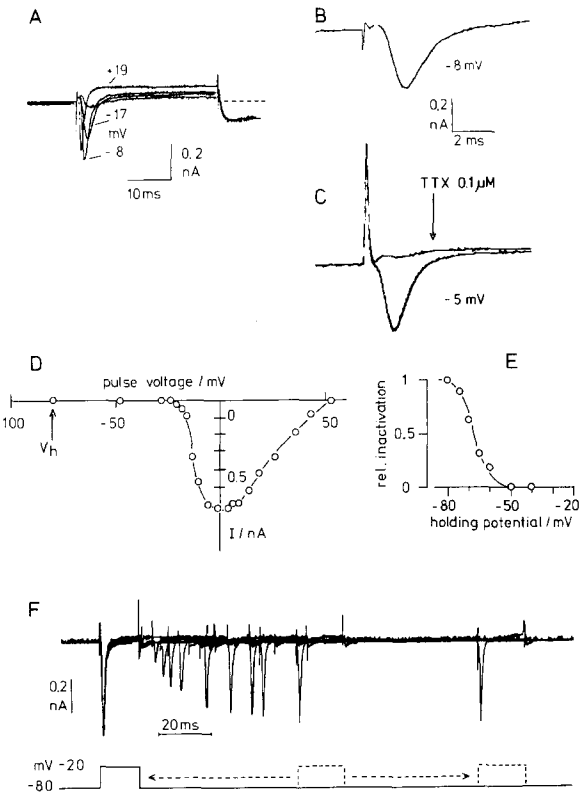

Fig. 4. (*A–C*) Transient inward currents recorded from a spindle-shaped TRC as in Fig. 3(*A*, *B*), but with a pipette solution containing 110 mM CsCl replacing KCl. Thereby most but not all of the outward current was suppressed. (*C*) The remaining transient inward current was suppressed by 0.1 μM TTX present in the bath. (*D*) Peak inward currents, obtained under conditions of panels (*A*) and (*B*) as a function of pulse voltage. Holding voltage V_h = −80 mV. The left branch of this curve is taken as an estimate of the voltage dependence of Na current activation. (*E*) Na current steady-state inactivation-voltage relationship. Peak inward current-voltage curves, like that of panel (*D*), were obtained for holding voltages between −80 and −40 mV. The most negative currents of these curves (maximal peak currents) were plotted against the holding voltage. The currents were normalized with respect to that obtained with V_h = −80 mV. (*F*) Recovery at −80 mV from Na current inactivation (*see* text). For this voltage the time constant of inactivation was estimated to 15.6 msec

it was completely blocked by 0.1 μM tetrodotoxin present in the bath (Fig. 4*C*). It seems safe to conclude, therefore, that the transient inward current is a Na current flowing through TTX-blockable channels which are voltage-gated. Amiloride (80 μM) had no effect but quinine blocked the Na currents (*see below* and Fig. 9*C*). In some patched TRCs the Na currents were, for unknown reasons, small or almost absent.

Panel (*B*) of Fig. 4 shows that activation of the Na current sometimes occurred with a delay which was particularly long at small depolarizations (*see also* Fig. 3*B*). The delay was pronounced when the patch was not completely open (then it could be removed by applying gentle suction to the pipette), or when the pipette was connected to the cell through a thin membrane evagination which sometimes developed when the cell moved away from the pipette. Thus the delay may be attributed to nonideal space-clamp conditions.

With good space-clamp conditions, the activation threshold was at −30 to −25 mV. Maximal peak amplitudes were reached with pulses close to 0 mV and ranged from −0.1 to −1 nA/cell (Fig. 4*D*). A steady-state inactivation curve was obtained by plotting the maximal peak currents (e.g., −0.63 nA in Fig. 4*D*), obtained with pulses from different holding voltages, against the holding voltage (panel *E*). Thus inactivation was found to be half-maximal at −67 mV and complete at −50 mV. An inactivation threshold more negative than the activation threshold is commonly found with excitable cells [39]. It implies that inactivation is considerably slower than activation, and that at depolarizations

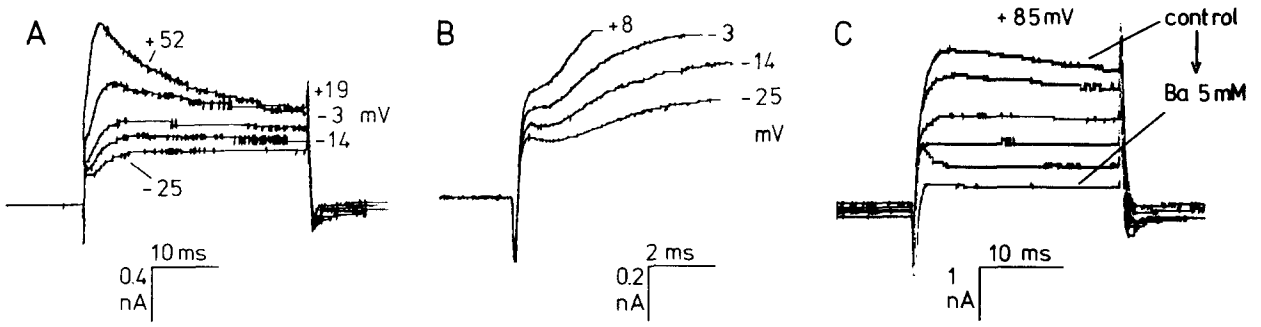

Fig. 5. (*A*, *B*) K outward currents recorded in response to depolarizing voltage pulses from the spindle-shaped TRC of Fig. 3(*A*, *B*) following replacement of all extracellular Na with NMDG. Holding potential −80 mV. Pipette: standard KCl filling solution, no ATP. (*C*) Time evolution of blockage of K outward current observed during 3 min of slow superfusion of a TRC with NMDG-Ringer's containing 5 mM $BaCl_2$ (K_o 1 mM). V_H −80 mV, pulse voltages +85 mV. Note the TRC of panels (*A*) and (*B*); note the difference in rate of K current inactivation between cells. Due to Ba exposure, the slope-resistance at −80 mV increased from 133 to 300 MΩ. The inward tail current was also blocked. Pipette: standard KCl filling solution, no ATP

beyond −50 mV the Na current will eventually turn off completely. Thus the TTX-blockable Na conductance cannot contribute to stationary membrane potentials.

Figure 4(*F*) shows recovery of Na currents from inactivation with a two-pulse protocol. The first pulse moved the voltage from −80 to −20 mV, causing activation followed by full inactivation of the Na channels. A second pulse, of the same amplitude, was applied after a variable delay spent at −80 mV. At this voltage recovery from inactivation had a time-constant of 15.6 msec.

K Currents

With K-containing pipettes and depolarizing pulses pure outward currents could be recorded when the extracellular Na was replaced by N-methyl-D-glucamine (Fig. 5*A* and *B*). These currents were diminished when Cs replaced the K in the pipette solution and were blocked by 5 to 60 mM Ba in the bath (Fig. 5*C*). They will be designated K currents. With depolarization to 0 mV the currents activated within 10 msec but did not show a clear subsequent inactivation within 100 msec. However, during depolarizations beyond 0 mV, inactivation became noticeable in that peak currents, secondary current decline and subsequent steady-state currents could now be distinguished. The ratio of peak to steady-state currents varied between cells. K currents were not blocked by 20 mM TEA applied from the pipette, 7.5 mM TEA applied externally or 1 mM 4-aminopyridine or 2 μM D600 or 5 mM Co applied externally. Quinine, cAMP, forskolin, IBMX and ATP caused blockage as described below. The K currents were only slightly increased when the pipette contained 1 mM Ca in the absence of EGTA, as described below.

K Tail Currents

On termination of depolarizing voltage pulses a transient inward current was seen (Fig. 4*A*). It took about 2 msec to reach its peak (due to imperfect capacitance compensation), then decayed in about 100 msec at −80 mV, and was of larger amplitude when the pipette K was replaced by Cs. Tail currents remained unchanged when the external NaCl was replaced by 60 mM $CaCl_2$, or when the external Ca was lowered to 0.1 mM. They were not blockable by TTX or amiloride.

Tail amplitudes increased with the length of the preceding depolarizing pulse, as shown in Fig. 6(*A*) and (*B*), and with the external K concentration (Fig. 6*C*). Furthermore, tail currents were blocked by 5 mM Ba in the external solution, suggesting that they are due to K flow through inactivating K channels. Indeed, the reversal potential of tail currents was identical with that of steady-state K currents, in the order of −20 mV with cellular K replaced by Cs from the pipette and an extracellular K concentration of 10 mM (Fig. 6*D*). Identity of reversal potentials was also found with 110 mM K in the pipette and 20 mM K outside (Fig. 6*E*).

These results indicate that the tail currents were mediated by Ba-blockable channels which were activated by the preceding depolarization and passed predominantly K and to a smaller extent Cs ions.

Screening by Divalent Cations

In a search for Ca-activated K channels, 10 μM Ca was added to the KCl containing pipette solution

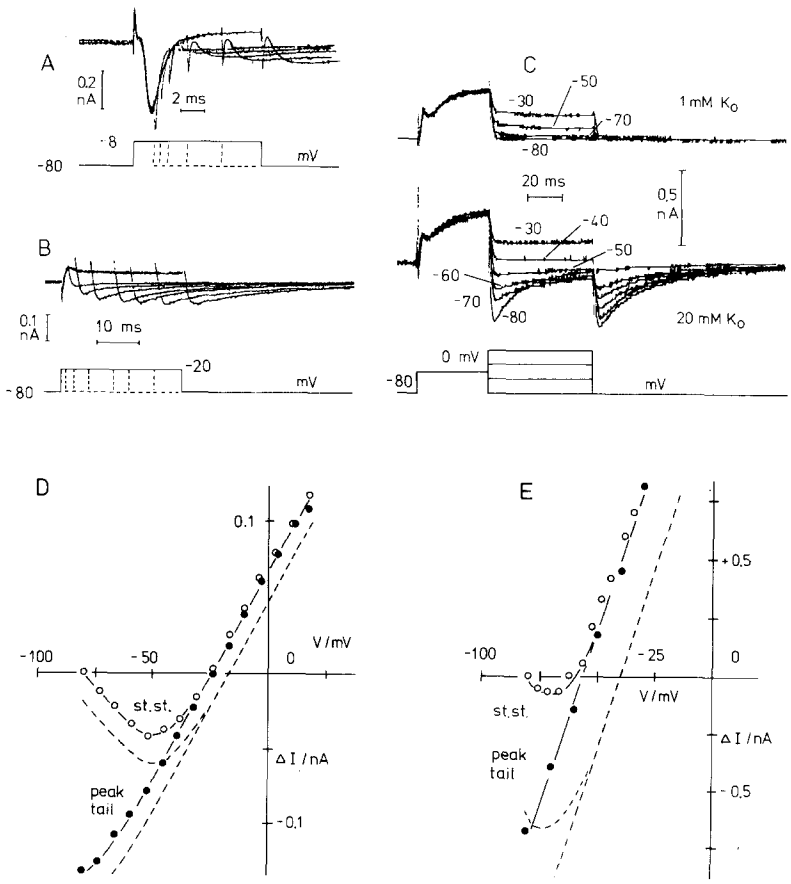

Fig. 6. Analysis of inward-tail currents seen after termination of depolarizing voltage pulses. Spindle-shaped TRC. (*A*, *B*) The peak-tail current increased with the duration of the preceding depolarizing voltage pulse. Bath: Na-Ringer's (*A*) and NMDG-Ringer's (*B*) containing 3.5 mM K_o. Pipette: standard filling solution with K replaced by 110 mM Cs; no ATP. (*C*) Dependence of tail currents on the external K concentration. Bath: NMDG-Ringer's; upper trace 1 mM K_o, below 20 mM K_o (90 mM NMDG). Pipette: standard KCl filling solution, no ATP. (*D*, *E*) $\Delta I(V)$-curves of steady-state K currents (read at end of second pulse, open circles) and peak-tail currents, which had similar reversal potentials. Tail currents were read 2 msec after repolarization if peaks were not apparent. The data points depict differences to the stationary current observed at V_h = −80 mV. Dashed lines: same data without subtraction of the stationary current. Pulse protocol of panel (*C*). (*D*) Prepulse −21 mV. Stationary current at −80 mV: −23 pA. Bath: Ringer's with NaCl replaced by 60 mM $CaCl_2$, containing 10 mM K_o. Pipette: standard filling solution with K replaced by 110 mM Cs; no ATP. (*E*) Prepulse −24 mV. Stationary current at −80 mV was −0.56 nA. Bath: Ringer's with 110 mM Na replaced by 90 mM NMDG, containing 20 mM K_o. Pipette: standard KCl filling solution, containing 5 mM ATP

and EGTA omitted. Then the K currents appeared to be slightly larger and seemed to activate at smaller depolarizations, but this was not certain because the protocol did not allow for low-Ca control recordings from the same cell.

We therefore removed Ca and EGTA from the pipette solution, recorded Na and K currents with Na-Ringer's solution as the extracellular medium and then added 1 μM of the Ca ionophore A23187 [41] to the bath by superfusion. After 10 min an increase of both peak and steady-state K currents became noticeable. The dynamics of K current activation and inactivation did not change significantly, but the K current-voltage curve was shifted to more negative voltages by about 15 mV (Fig. 7*A*). Furthermore, peak Na currents (activation) were shifted by 25 mV to more negative voltages in response to A23187 (Fig. 7*A*), and the steady-state Na inactivation curve was shifted by 8 to 10 mV in the same direction (Fig. 7*B*).

In many cells an increase of cytosolic Ca, as achieved with A23187, opens Ca-activated K channels (e.g., ref. 53). However, while the TRCs showed the expected increase in outward current, this was accounted for by a shift of the steady-state K current-voltage curve to more negative voltages, and was accompanied by a similar shift of Na current parameters. These effects may be due to screening of fixed charges at the inner surface of the membrane by Ca. Similar shifts by about 10 mV, but to positive voltages, were effected by 5 mM $NiSO_4$ added to the outer solution (Fig. 7*C*). This may be explained by screening of negative fixed charges at the outer membrane surface.

Ca Currents

Pipettes were filled with 110 mM Cs containing solution and patched onto TRCs bathed in Na-Ringer's. The 110 mM NaCl of the bath was then replaced with 60 mM $CaCl_2$ by slow superfusion in order to depress Na-inward currents and increase putative Ca inward currents. The transient-inward Na current gradually disappeared (Fig. 8*A*) and the membrane responded to depolarization from −30 to +30 mV merely with a small outward current. At elevated external K concentration (10 mM) this current

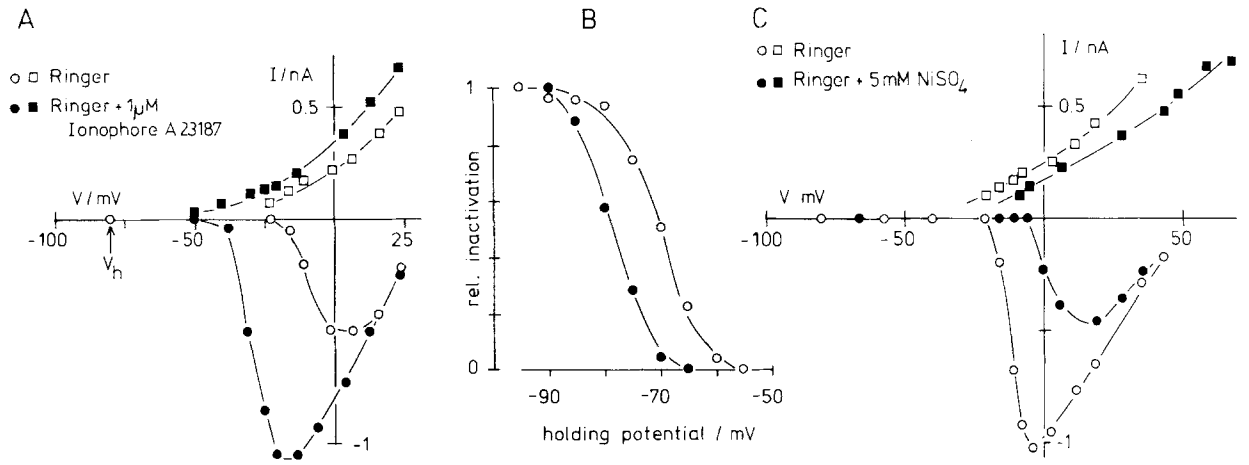

Fig. 7. Shifting effect of divalent cations on peak-K-outward and peak-Na-inward currents of spindle-shaped TRCs, and on the steady-state Na-inactivation curve. Recording conditions as in Fig. 3. Pipette: KCl filling solution without Ca, EGTA and ATP. (*A*, *B*) Data obtained before (open symbols) and during presence (filled symbols) of 1 μM of the Ca ionophore A23187 in the bath (Na-Ringer's with 3.5 mM K). (*C*) Data obtained before (open symbols) and during presence (filled symbols) of 5 mM $NiSO_4$ in the bath

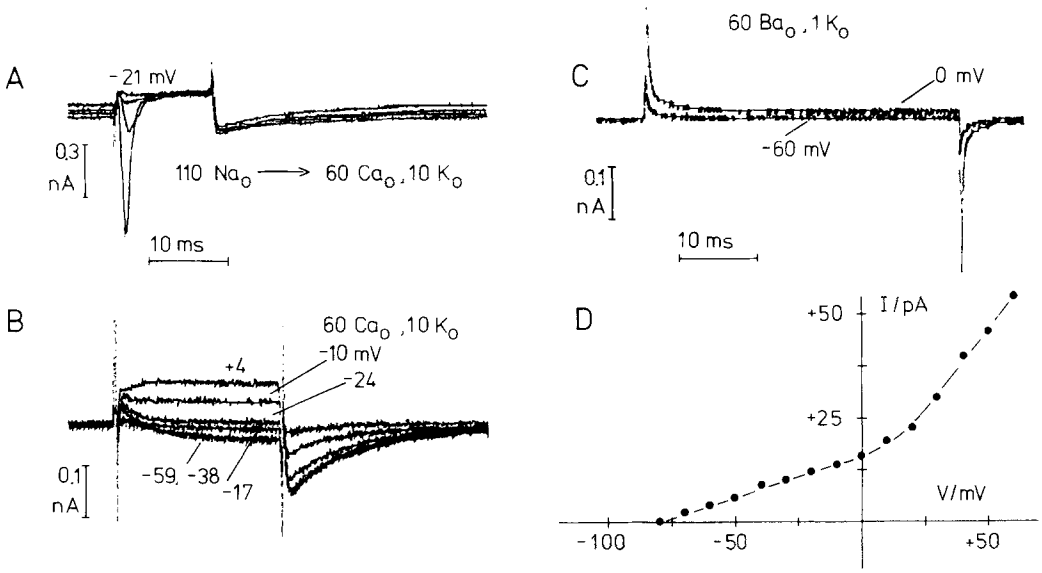

Fig. 8. Search for Ca currents with 110 mM Cs replacing K in the pipette. Spindle-shaped TRCs. Holding potential −80 mV, depolarizing voltage pulse amplitudes as indicated. (*A*) On replacing the 110 mM NaCl in the bath with 60 mM $CaCl_2$ by slow superfusion, the transient inward current disappeared within 1 min. A Ca-inward current did not develop at −21 mV. In spite of a concomitant elevation of K_o from 3.5 to 10 mM, the peak-tail current and the steady-state-inward current recorded at −80 mV became less negative. This may have been due to the increased Ca concentration shifting the K-current activation curve towards positive voltages, as previously observed with Ni (Fig. 7*C*). (*B*) Same TRC, K_o = 10 mM. In the voltage range −20 to −60 mV slowly activating inward currents developed. However, these currents were not Ca but K currents, because (*C*) when 60 mM Ca is replaced by Ba, which passes Ca channels but blocks K channels, no inward current was recorded at 0 mV. A fresh TRC was used for this experiment to avoid rundown. Records completed 4 min after patching. K_o = 1 mM. (*D*) Under these conditions the slope resistance increased to 5 GΩ at −50 mV and to 1.3 GΩ at +50 mV, with a transition at +15 mV

showed a slowly activating inward component at −59 mV (Fig. 8*B*) which was, however, blocked by 5 mM Ba. On replacing the extracellular Ca with 60 mM Ba (Fig. 8*C*), which blocks K channels but passes Ca channels, depolarization to +50 mV did not elicit transient inward currents. Outward current at this voltage was decreased to 50 pA (Fig. 8*D*). The same negative result was obtained during

Table. Gustatory stimuli reported to elicit receptor potentials of TRCs, or glossopharyngeal nerve activity in the frog

Stimulus		Concentration range	Reference
Distilled water		56 M	1, 26, 33, 42, 48, 57, 70
Salty	NaCl	0.1–1 M	1, 24, 26, 30, 33, 42, 57
	KCl	0.1–1 M	1, 24, 30, 33
	NH_4Cl	0.1 M	33
	$CaCl_2$	1–50 mM	1, 22, 24, 26, 30, 33, 42, 48, 70
	$MgCl_2$	1–100 mM	24, 26, 30, 33
Sweet	Sucrose	0.25–1 M	1, 30, 33
	D-galactose	0.8–1 M	42
	Saccharin	0.4 mM	33
Amino acids	Glycine	5–50 mM	67
	L-alanine		67
	L-serine		67
	L-threonine		42, 67
	L-glutamate		18
Acid	Acetic acid	1–15 mM	24, 30, 33
	HCl	0.1–1 mM	1, 42
Bitter	Quinine	0.1–10 mM	1, 18, 30, 33, 70
	Strychnine	0.3–3 mM	30, 70
	Theophylline	2 mM	42
Narcotics	Ethanol	400 mM	26, 42
Electrical current		0.7 mA	26

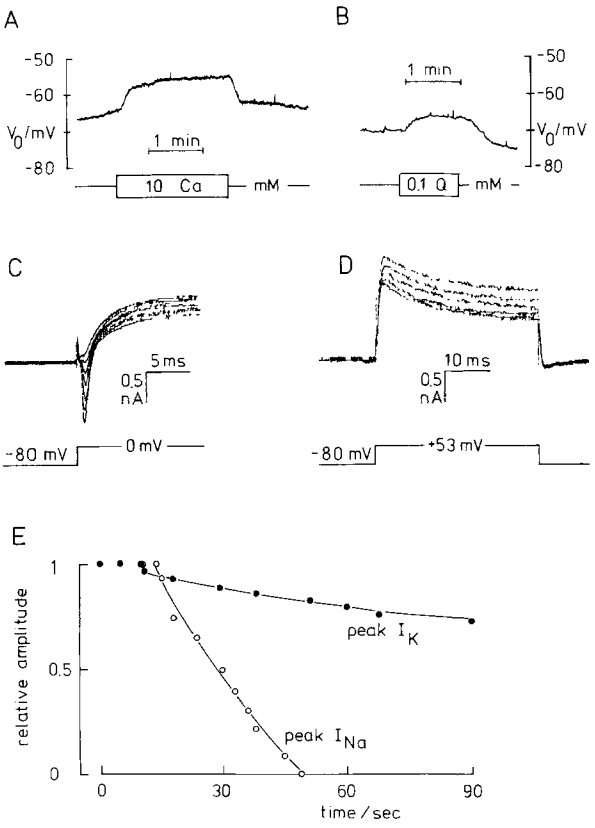

Fig. 9. (*A*) In the current-clamp mode, superfusion of a spindle-shaped TRC with Na Ringer's containing 10 mM Ca caused a small reversible depolarization (which did not increase on repetition and did not elicit action potentials). The standard KCl-pipette solution without ATP was used. (*B*) Slow superfusion with 0.1 mM quinine in Na Ringer's caused a small reversible depolarization without eliciting action potentials. (*C*, *D*) Time evolution of blockage by quinine. In the voltage-clamp mode, the depolarization by 0.1 mM quinine was accompanied by a block of transient Na-inward currents, complete in 45 sec, and an incomplete block of K-outward currents. (*E*) Time-course of decrease in peak-Na and peak-K currents during slow superfusion with 0.1 mM quinine

additional exposure to 5 mM cAMP, which has a strong effect on K currents (*see below*). We conclude, therefore, that voltage-activated Ca channels conducting Ca or Ba ions were not active in the TRCs investigated.

Taste Stimulants

Salts and chemicals known to stimulate the frog's gustatory system are listed in the Table. It will be appreciated that many of these agents are effective only at high, osmotically relevant concentrations. Applied in the bathing solution of isolated TRCs they must be expected to cause rather unspecific effects like cellular shrinkage and regulatory volume increase (e.g. sucrose, NaCl), depolarization (KCl), charge screening ($CaCl_2$), cellular pH changes (NH_4Cl, acids), channel blockage (acids) and others. We therefore concentrated on the stimulants quinine, saccharin, amino acids and $CaCl_2$, which are effective at small or still manageable concentrations.

TRCs were bathed in NaCl-Ringer's and patched with pipettes containing the 110 mM KCl solution with or without 5 mM ATP. After control recordings of the whole-cell current, current-clamp conditions were established and the zero-current potential V_0 monitored (about −65 mV) while the cells were now superfused with a solution containing the stimulant. Thereby a stimulant-induced voltage change in the way of a generator potential and/or an action potential should have become noticeable. L-threonine, L-serine, L-leucine and L-glutamate (all 10 mM), saccharin (5 mM), glucose (5 mM) and sucrose (50 mM) had no noticeable effect. A depolarization of 5 to 10 mV was found with 10 mM $CaCl_2$ (e.g. Fig. 9*A*), while KCl (30 mM added to Na-Ringer's or replacing NaCl) caused depolarization by 50 mV. With 5 mM Ba, V_0 depolarized down to −10 to −20 mV and the voltage noise increased significantly (Fig. 10*A*). All depolarizations were revers-

ible. None of the depolarizing agents elicited action potentials.

Chemoreception mediated by adenylate cyclase requires cellular GTP, which is, however, a common contaminant of commercial ATP [49]. Addition of 0.2 or 1 mM GTP to a pipette solution containing 5 mM ATP did not improve the negative results obtained with amino acids and sweeteners. It is noteworthy that ATP in millimolar concentrations was reported to block cAMP-phosphodiesterase [12]. Therefore the pipette filling solution used was particularly suitable to detect a sensory response mediated by cAMP.

Quinine (0.1 mM) caused a small reversible depolarization (Fig. 9*B*). When this effect was investigated in the voltage-clamp mode, it became apparent that the drug inhibited the transient Na current completely (Fig. 9*C*). This reversible change was not due to a shifting of the Na channel activation curve. In addition quinine caused a partial block of K currents (Fig. 9*D*), which, at the concentration used, developed more slowly than the blockage of Na current (Fig. 9*E*), and was more slowly reversible. It explains the depolarization of V_0 caused by quinine. Addition of 1 mM EGTA or 1 μM $CaCl_2$ to the pipette solution had no effect on the response to quinine, indicating that intracellular Ca release did not mediate the response.

Depolarization by quinine did not elicit action potentials, even when the resting voltage was increased by lowering the external K concentration to 1 mM. Blockage of Na and K conductance by quinine or quinidine is well known (e.g. ref. 66). Ozeki [51] correctly predicted that quinine decreases the K conductance of TRCs. The membrane-permeant bitter taste stimuli quinine and strychnine, like methylxanthines, were reported to affect the activity of the cAMP-phosphodiesterase of TRCs [31, 34, 54]. It seemed interesting, therefore, to search for membrane effects elicited by IBMX and cAMP.

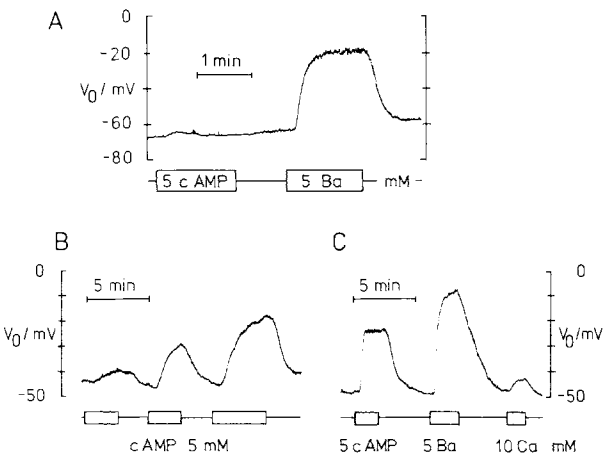

Fig. 10. Time-course of reversible depolarizations of spindle-shaped TRCs observed in the current-clamp mode during slow superfusion with various agents dissolved in Na Ringer's buffered with HEPES-Tris and containing only 1 mM K. The standard pipette solution containing 110 mM KCl was used. (*A*) Without ATP added to the pipette solution, superfusion with 5 mM cAMP had almost no effect (repeated four times) while superfusion at the same flow rate with 5 mM $BaCl_2$ caused a reversible depolarization and an increase of voltage noise. (*B*) On adding 5 mM ATP to the pipette solution, repeated superfusion with 5 mM cAMP caused reversible depolarizations of increasing amplitude. (*C*) The fourth superfusion with cAMP caused a rapid depolarization of this TRC, the rate being comparable to that obtained with 5 mM Ba. The depolarization achieved with 10 mM Ca was much smaller, showing that the blocking effect of Ba cannot be explained by shifting of the K activation curve by divalent cations (as in Fig. 7*B*). The depolarizations did not elicit action potentials

Cyclic Adenosinemonophosphate

When 5 mM ATP was included in the pipette solution, addition of 5 mM cAMP to the superfundate caused depolarization of TRCs in the current-clamp mode (Fig. 10*B*, *C*; Fig. 11), and a small increase in voltage noise. (cAMP is poorly permeant. In order to raise its cellular concentration into the μM range and elicit an intracellular response, millimolar concentrations are needed in the bathing solution [64].) Without ATP in the pipette the depolarization was negligible (Fig. 10*A*). Voltage-clamp recordings showed the depolarization to be caused by blockage of K currents (Fig. 11, insets). K-tail currents were also blocked; Na currents were not affected. The rate of depolarization was sometimes slower but often comparable to that observed with 5 mM Ba with the same cell (Fig. 10*C*). Repetitive exposure to 5 mM cAMP often showed a tendency for the second depolarization to be larger than the first, even though the first wash-out reestablished the initial membrane potential (Fig. 10*B*).

Depolarizations of similar amplitude were obtained when 0.3 to 1 μM *forskolin* was added to the superfundate (Fig. 11*A*), showing that the TRCs contained a competent adenylate cyclase and that the effect obtained with cAMP was most likely due to an intracellular action of this agent. The depolarization obtained with forskolin was less easily reversible than that obtained with cAMP. The depolarizations did not elicit action potentials at 3.5 or 1 mM of external K.

When 0.5 μM cAMP was included in the pipette solution together with 5 mM ATP, breakage of the patch was followed by a spontaneous depolarization which was at least twice as fast as that observed with ATP alone (*see below*), and which was

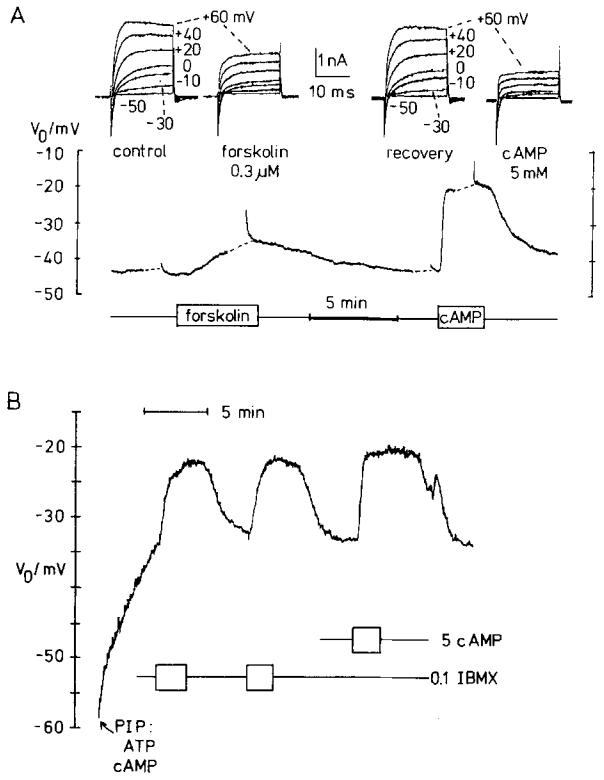

Fig. 11. (*A*) Time-course (bottom trace) of reversible depolarizations observed in the current-clamp mode during slow superfusion of a spindle-shaped TRC with forskolin and cAMP dissolved in Na Ringer's buffered with HEPES-Tris and containing only 1 mM K. The standard pipette solution containing 110 mM KCl and in addition 5 mM ATP was used. The first superfusion with 300 nM forskolin (vehicle 2.4 μl ethanol/10 ml) caused a slow reversible depolarization, the subsequent exposure to 5 mM cAMP at the same flow rate a faster and larger depolarization (second exposure of this cell to 5 mM cAMP). During four periods indicated by dashing of the time record, currents were recorded in the voltage-clamp mode (insets), demonstrating that K-outward currents and tail currents were partially blocked during exposure to forskolin or cAMP, while Na-inward currents were not affected. cAMP blocked 60% of the K outward current observed prior to its addition. (*B*) Depolarizing effect of 0.5 μM cAMP added with 5 mM ATP to the standard pipette solution. Bath as in panel (*A*). When the patch was broken (where the record begins on the left) V_0 depolarized rapidly. Superfusion with 0.1 mM IBMX (expected to inhibit cAMP breakdown due to the phosphodiesterase) caused further depolarization. The same voltage range of about −20 mV was reached when additional cAMP was provided by superfusion (5 mM). Thus 0.5 μM of intracellular cAMP must be close to the saturating value with respect to the depolarization effect

more extensive (Fig. 11*B*). The mean of voltages reached in four experiments was −35 mV. Further depolarization could be achieved by adding 5 mM cAMP to the bath.

Inhibition of Phosphodiesterase

Addition of 0.1 mM IBMX, a permeant inhibitor of cAMP-phosphodiesterase, to the superfundate caused reversible depolarization of varying magnitude, indicating that the rate of spontaneous generation of cAMP differed among the isolated TRCs. When less than 1 μM cAMP was present in the pipette, superfusion with IBMX always caused a further depolarization (Fig. 11*B*). When TRCs were exposed to external cAMP during continued exposure to IBMX, further depolarization down to about −30 mV was recorded. Repolarization during washout of cAMP was significantly delayed and slowed down by the presence of IBMX (Fig. 12*A*). These results may be explained by the presence of a methylxanthine-inhibitable cAMP-phosphodiesterase in the TRCs. They show that 5 mM cAMP had an intracellular effect which was saturating at this concentration, and that inhibition of the enzyme [12] by ATP supplied from the pipette was not a serious problem. The blockage of K conductance exerted by 5 mM external cAMP comprised about 60% of the initial conductance. The remainder was blockable by external Ba.

Blocking Effect of ATP

When, directly after perforating the patch, membrane voltage was observed at zero pipette current, it usually increased slightly and stabilized at about −65 mV (with 3.5 mM K_o) or −70 mV (with 1 mM K_o). This increase was also seen when the pipette contained 5 rather than 0.1 mM EGTA. However, when the pipette contained 5 mM ATP, V_0 *decreased* at a rate of about 1 mV/min to values near −50 mV (with 1 mM K_o). It will be recalled that addition of cAMP blocked K outward currents only when the cell was loaded with ATP from the pipette. Combined, these observations are compatible with blockage of K channels by phosphorylation, effected by the cAMP-activated protein kinase A which requires ATP as substrate.

To test this hypothesis 2 mM ATP-γ-S was added to the pipette solution instead of ATP. The thiophosphate analog of ATP is a substrate for protein kinase A, but the resulting protein–P = S is more resistant to dephosphorylation by the corresponding phosphatases than the normal phosphorylation product Pr − P = O (e.g. refs. 23, 69). Thus in the presence of ATP-γ-S the equilibrium between conducting and blocked (phosphorylated) K channels should be shifted to the blocked fraction. The rate of spontaneous initial depolarization then ob-

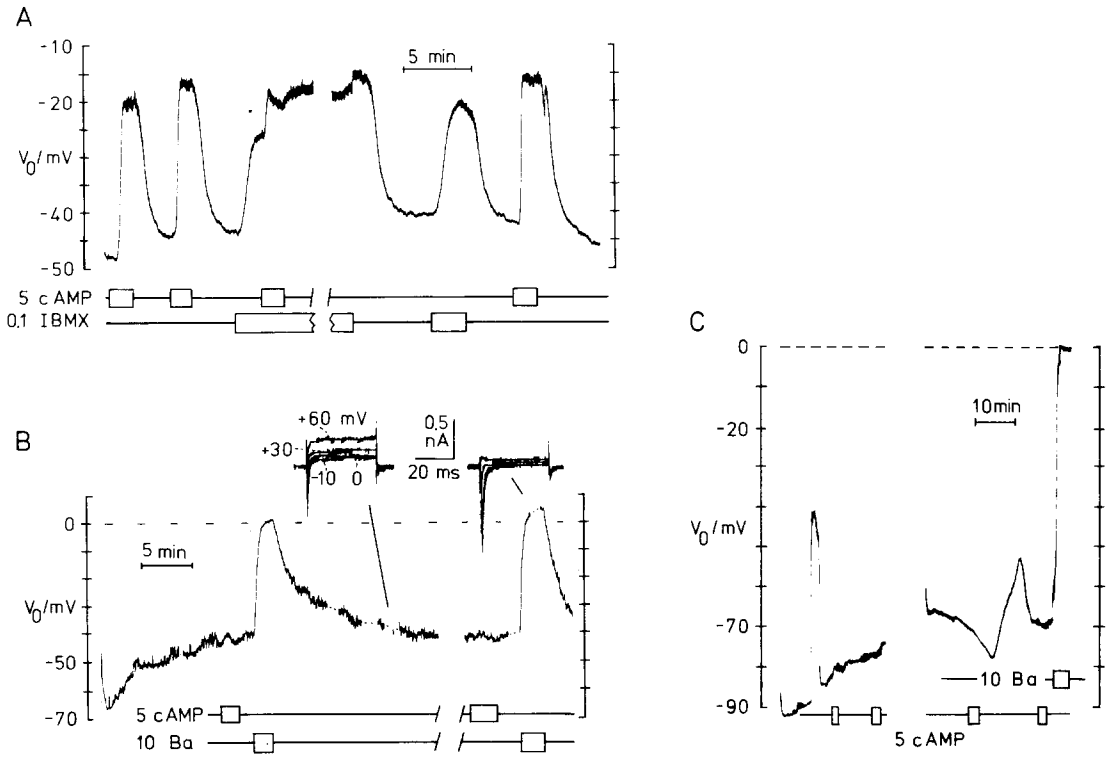

Fig. 12. (*A*) Reversible depolarization by 5 mM cAMP and by blockage of cAMP-phosphodiesterase with 0.1 mM IBMX. Whole-cell recording from this spindle-shaped TRC lasted 85 min. Where the trace is interrupted (for 15 min), voltage-clamp records were taken. Bath: Na-Ringer's buffered with HEPES-Tris and containing only 1 mM K. Pipette: standard KCl filling solution with 5 mM ATP added. (*B*) Depolarizing effect of ATP-γ-S (2 mM) which replaced ATP in the pipette solution. (With this thiophosphate analog of ATP, sealing of the pipette to TRCs was more difficult.) Otherwise solutions as in panel (*A*). The patch was broken where this record begins on the left. Note the spontaneous initial depolarization (slower than in Fig. 11*C*) and the lack of effect of cAMP. Where the trace is interrupted, voltage-clamp records were taken. The same voltage protocol was used for the insets. The left inset shows that with ATP-γ-S the K conductance is smaller than with ATP (*compare* Fig. 11). (*C*) V_0 time-course of two spindle-shaped TRCs. Pipette: standard filling solution with 5 mM AMP-PNP instead of ATP. On breaking the patch (at the start of these records) a slow and moderate depolarization or hyperpolarization was seen. The spontaneous transients are unexplained. Superfusion with 5 mM cAMP was without effect

served with ATP-γ-S was about as seen with ATP, but the depolarization was more extensive, stabilizing at −36 mV (mean of four observations with 2 mM ATP-γ-S) or −28 mV (mean of three observations with 5 mM ATP-γ-S). The membrane resistance increased. The subsequent superfusion with 5 mM cAMP did not cause a further depolarization, but 10 mM Ba decreased V_0 reversibly to values near 0 mV (Fig. 12*B*).

When ATP was replaced by 5 mM AMP-PNP in the pipette, an analog which is not a good substrate for kinase reactions [69], there was no spontaneous depolarization upon breakage of the patch. In four experiments V_0 remained in the range −70 to −80 mV except for some spontaneous transients (Fig. 12*C*). The depolarizing response to cAMP was abolished in the presence of AMP-PNP.

The results with ATP-γ-S and AMP-PNP are compatible with inactivation of K channels of TRCs by cAMP-dependent phosphorylation. However, a noticeable fraction of the K channels was not controlled by this reaction.

Action Potentials

The failure of the depolarizing taste stimulants Ca and quinine, and the other depolarizing agents K, Ba, cAMP, forskolin and IBMX to elicit action potentials in the current-clamp mode raised the question whether the patched TRCs are able to generate action potentials at all, even though their expression of voltage-gated Na and K channels suggests that they would. When TRCs were patched under standard conditions, current-clamp established and depolarizing current pulses applied to the pipette, action potentials were seldomly demonstrated.

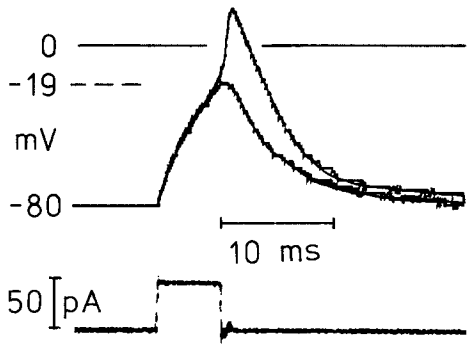

Fig. 13. In the current-clamp mode this spindle-shaped TRC (following a long experiment with exposures to forskolin and cAMP) had a low zero-current membrane potential of only −30 mV at K_o = 1 mM. The potential was driven to −80 mV by decreasing K_o to 1 mM and passing a constant inward current of −0.17 nA. On lowering the current to −0.12 nA for 5.6 msec (pulse on lower trace) the voltage responded with a quasi-exponential depolarization. On repetition, a slight increase of the depolarizing current pulse elicited an action potential (threshold −19 mV, maximal rate of rise 47 V/sec, overshoot +18 mV, duration at threshold 4.2 msec). Bath: Na-Ringer's buffered with HEPES-Tris, 1 mM K_o. Pipette: standard KCl filling solution with 5 mM ATP added

However, when the prestimulation potential was increased to about −80 mV by passing inward current, action potentials were seen more regularly (Fig. 13). The threshold voltage was in the order of −20 mV, a value somewhat smaller than the Na current activation threshold of −30 to −25 mV (Fig. 4*D*). Dynamic voltage changes were also seen on termination of hyperpolarizing current pulses.

Discussion

The gustatory receptor cells of the tongue are sensory epithelial cells whose transduction mechanism still awaits clarification. While chemoreceptor membrane material can be studied in vitro (e.g. refs. 38, 61), the functional response of apical receptor proteins and the cellular events leading to secretion of transmitter at the basolateral synapse are speculative. The discovery that the Na transport blocker amiloride blocks the taste of salt and sweet in mammals (e.g. refs. 10, 20, 58) has added a new challenge to this field. We have developed an isolation procedure for taste receptor cells (TRCs) which allows patch-clamping of the basolateral membrane. Ionic currents through this membrane, which are expected to participate in the sensory signal chain, could thus be characterized.

The conventional isolation procedure [2, 9] involved exposure to collagenase and mechanical agitation for cellular detachment from the basement membrane, and low extracellular Ca concentration for opening of tight junctions. Each of these measures may have led to cell damage, and it is not surprising, therefore, that some obviously permeabilized cells were found in the final suspension. While the suspension contained many flask- and spindle-shaped TRCs without gross damage (Figs. 1 and 2), we cannot exclude the possibility that these cells were altered in a more subtle way by the isolation procedure. Yet, the membrane resistance of 150 to 750 MΩ, the resting potential of about −65 mV and the absence of anion-conductive pathways suggests that many membrane functions were preserved even though, as will be discussed, some appeared to be missing.

The identification of TRCs in the cell suspension was not difficult since most of them maintained their characteristic shape. Furthermore, fluorescence labeling of the apical sensory processes with RH-414 prior to isolation showed that most of the spindle- and flask-shaped cells of the suspension are those, which in the intact tissue make contact with the mucosal medium through one or two slim "dendritic" processes. At their tips these processes bear a rod-shaped microvillus (e.g. refs. 15, 21) which contains a cylindrical bundle of F-actin stainable with phalloidin, as is typical for the gustatory cells (Richter, Avenet, Mestres and Lindemann, *in preparation*). The dendritic tips of most isolated flask- and spindle-shaped cells were still stainable with phalloidin-TRITC.

No exceptional difficulty was met in patch-clamping the isolated taste cells. In cases where sealing proved difficult, a transient increase in extracellular Ca usually solved the problem. Our whole-cell recordings did not indicate anion-conductive pathways, but we did find a voltage-gated TTX-blockable Na current and voltage-gated K currents which were blocked by Ba. Therefore the TRCs should be capable of firing action potentials, which were indeed observed during passage of depolarizing currents under current-clamp conditions. Action potentials and anode-break responses were also observed in microelectrode recordings from taste cells in the intact mucosa [7, 25, 29, 55]. Some other epithelial cells, like those of the adeno-hypophysis (e.g. ref. 50) and β-cells of Langerhans islets [56] are also able to generate action potentials which trigger Ca inflow and thereby hormone release.

It may be concluded, therefore, that action potentials do not only serve for propagation of electrical excitation over long distances, but may in "short" epithelial cells have another function. In sensory epithelial cells this may perhaps be related to coding of different stimuli. In the tongue mucosa,

taste cells will be subject to some mechanical deformation which may result in slow transient changes of the membrane potential. It is conceivable that such slow depolarizations are barred from causing emission of transmitter, which rather requires the characteristic fast depolarization of regenerative responses. This would imply that action potentials are not elicited by slow depolarization, and that occupation of taste receptors leads either to fast depolarizations or to generation of action potentials by other means. Therefore *slow* superfusion resulting in a slow increase in concentration of a taste agent may not lead to action potentials. This problem awaits further experimentation.

The isolated taste cells did not show voltage-gated Ca currents or currents blockable by D-600 or $CoCl_2$. This negative result was obtained with control cells and also when ATP and/or GTP was replenished through the pipette or when the cells were stimulated with cAMP. It is at variance with the conclusion by Kashiwayanagi et al. [25] and Roper [55] that the basolateral membrane of taste cells contains voltage-gated Ca channels. It is also at variance with our own finding that one fraction of *Necturus* taste cells *in situ* fires slow, low-amplitude action potentials which strongly resemble Ca-spikes [7]. In ongoing experiments we attempt to establish the conditions for activation of Ca channels in the isolated TRCs. Furthermore, within the limitation of the protocols used, we did not find Ca-activated K channels.

Of the gustatory stimulants which were applied by slow superfusion, none elicited action potentials. While $CaCl_2$ and quinine caused a consistent reversible depolarization, trial experiments with sweet and amino-acid stimuli remained inconclusive. It may be that the cell isolation procedure has affected the sensory response to these agents. Particularly a tryptic split of the apical receptor protein (*compare* ref. 16) must be considered. This possibility is presently under investigation. The patch pipette may have dialyzed second messenger or another essential component from the cytosol of these small cells (*compare* ref. 37). However, this putative component was not GTP, or not GTP alone, since 0.2 of 1 mM GTP in the pipette solution was without effect. Recordings without breakthrough, i.e. in the cell-attached mode, also did not show the generation of action potentials in response to gustatory stimuli. There is a remote possibility that the apparent lack of sensory function is related to the absence of contact to nerve fibers; taste buds contain vesiculated nerve endings and, furthermore, are well known to depend on neurotrophic maintenance supported by axonal transport (e.g. refs. 8, 63).

Electrophysiological and biochemical experiments have implied cAMP- and cGMP-dependent processes in the signal chain of gustatory cells (e.g. refs. 32, 35, 40, 41, 68). However, the significance of cyclic nucleotide effects remained controversial. Adenylate cyclase, guanylate cyclase and cAMP-phosphodiesterase were histochemically localized at the apex of taste buds [45], and, in fact, on the apical microvillus membrane of type I (dark) receptor cells [3, 46], but more recently these results have become doubtful. A sensitivity to gustatory chemicals, as expected of a nucleotide cyclase, could not be demonstrated, even though a substrate of this enzyme, AMP-PNP, was utilized in the reaction [4]. Functionally, cyclic nucleotides could as yet not be established as second messengers in taste cells. They were merely recognized as "modifiers" of the chemosensory process (e.g. refs. 11, 40). The effect of membrane-permeant bitter substances on the cAMP-phosphodiesterase is also controversial (e.g. refs. 31, 34, 45, 57, 54).

The isolated taste cells responded to extracellular cAMP with a consistent, reversible depolarization (Figs. 10-12) based on blockage of about half of the K-conductance. It is likely that cAMP exerted this effect after diffusing into the cell, since the same reversible blocking response was found with forskolin, a diterpene which activates the catalytic subunit of the adenylate cyclase directly (e.g. refs. 59, 60). The cellular level of cAMP is thus increased without the need for receptor-protein or GTP-binding protein. Furthermore, blockage of cAMP-phosphodiesterase affected the response to exogenous cAMP as expected for an intracellular action of this messenger, and small concentrations of cAMP applied intracellularly through the pipette had comparable effects to large concentrations of extracellular cAMP.

The depolarizing blockage of K currents initiated by cAMP and forskolin, and the enhancing effect of IBMX, suggest that cAMP may, in analogy to olfactory transduction [43, 52], be one of the second messengers in taste transduction. The recent report by Kinnamon and Roper that acid gustatory stimuli depolarize *Necturus* taste cells *in situ* by decreasing the K conductance [27], is in a general way consistent with our results if one postulates that the acid stimulus leads to an increase of the cAMP-level in intact taste cells.

The depolarizing effect of cAMP was consistently absent when ATP was not present in the pipette solution. AMP-PNP did not substitute for ATP in this respect. Furthermore, the open-circuit membrane potentials were consistently larger when ATP was left out of the pipette solution or replaced by AMP-PNP. The results with AMP-PNP argue against allosteric blockage of K channels by binding of ATP, i.e. blockage without protein phosphorylation (e.g. refs. 13, 44). Rather, the effect of cAMP

and its clear ATP requirement point to a phosphorylation reaction. It appears possible, therefore, that the cAMP-activated protein kinase A phosphorylated one set of K channels to a nonconductive conformation, and that this process went on to a limited degree even when exogenous cAMP was not provided.

The hypothesis was tested by replacing ATP with ATP-γ-S in the pipette solution. This ATP analog leads to thiophosphorylated proteins which are more resistant to dephosphorylation than the normal phosphorylation product [23, 69]. Therefore the majority of K channels susceptible to phosphorylation should become blocked in the presence of ATP-γ-S. Indeed, in these experiments a slow depolarization due to blockage of the K conductance was found, and subsequently cAMP was without effect (Fig. 12*B*). Possibly phosphorylation was sufficiently stimulated by endogenous cAMP to block all K channels which were susceptible to this control, while the remaining fraction of conducting K channels was not subjected to this reaction. Ongoing experiments with purified protein kinase A and natural inhibitors of protein kinase A also point to an involvement of this enzyme in the depolarizing reaction to cAMP [5].

While it is known from several invertebrate cells that a cAMP-dependent control reaction can lead to phosphorylation and thereby closure of K channels (e.g. ref. 62; for a review *see* ref. 36), this mechanism was not previously established for vertebrates. A significance for taste transduction, which is the only known task of TRCs, may be suspected, but a demonstration of cAMP generation initiated by nonbitter taste agents is still wanting.

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Note Added in Proof

While this paper was in press, our attention was drawn to Striem, Pace, Zehawi, Naim & Lancet, 1986, *Chem. Senses* **11:**669. The abstract reports that sucrose and other sweeteners increase the rate of cAMP generation in homogenates of tongue epithelia (rat) containing taste buds. *See* the last sentence of our Discussion.