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Expression and localization of amiloride-sensitive sodium channel indicate a role for non-taste cells in taste perception

(olfaction/potassium channel/cystic fibrosis/chloride channel/lung)

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ABSTRACT **Salty taste is blocked by the diuretic amiloride, which inhibits specific sodium channels. We have isolated an amiloride-sensitive sodium channel (ASSC) from taste tissues by polymerase chain reaction and screening of a cDNA library prepared from rat circumvallate papillae. Northern analysis reveals ASSC in taste and non-taste tissues with the highest level of expression of ASSC in the lung. *In situ* hybridization establishes ASSC localizations in the epithelia of lung and colon as well as tongue epithelial layers containing and lacking taste buds. These results support a model in which ASSC in non-taste cells regulates responses of taste cells to salt as well as other tastants.**

Unlike other central modalities, taste utilizes a diversity of signal transduction mechanisms. Sweet (1, 2) and bitter (3, 4) taste appear to involve G protein-linked receptors. Sour taste is mediated by a proton channel (5, 6) and possibly blockade of voltage-gated potassium channels (7). The salty taste of sodium requires a sodium channel (8, 9). The diuretic drug amiloride has clarified mechanisms of salty taste. Amiloride acts at specific receptor sites in the kidney to block sodium reabsorption (10). Amiloride also inhibits salty taste perception both in animals (8, 9, 11) and humans (11). Amiloride-sensitive channels may also modulate sour taste, which is diminished by amiloride (6).

The amiloride-sensitive sodium channel (ASSC) has been purified from kidney (12, 13) as a large protein complex, about 730 kDa, which comprises several subunits. Two different cDNAs for subunits of ASSC have been cloned, but the expressed proteins did not confer sodium channel activity (14, 15). Recently, Canessa *et al.* (16) and Lingueglia *et al.* (17) cloned a subunit of ASSC of about 70 kDa, which confers sodium channel activity and is sensitive to amiloride and various derivatives in proportion to their pharmacologic activity. Since electrophysiological studies suggest that the ASSC mediating salty taste resembles ASSC in kidney, we have constructed a cDNA library from rat taste buds and used it to clone ASSC. We report the isolation of ASSC from taste tissues. Using *in situ* hybridization we have localized ASSC to sites that imply a specific mechanism of salty taste perception.

MATERIALS AND METHODS

Construction of a Rat Circumvallate Papillae cDNA Library. Circumvallate papillae containing tongue epithelium and taste buds were dissected as described (4), and RNA was extracted from the circumvallate papillae of 500 rats. Poly(A)$^+$ RNA was prepared by passing RNA through an oligo(dT) column twice. About 5 μg of poly(A)$^+$ RNA was converted to cDNA for construction of the cDNA library, using the Lambda Zap vector cDNA library synthesis kit (Stratagene). The library consists of 1.5×10^6 independent clones with an average insert size about 1.2 kb.

Polymerase Chain Reaction (PCR) and Cloning. Ten micrograms of total RNA from various tissues was reverse transcribed using 1 μg of oligo(dT) as primer. One microliter of the reverse transcription mix was used in a 25-μl PCR reaction mixture containing 1.5 mM $MgCl_2$, 400 nM primers, 200 μM dNTP, and 0.5 unit of *Taq* polymerase (Boehringer Mannheim). The primers used for reverse transcriptase-PCR (RT-PCR) analysis of ASSC gene expression and for obtaining cDNA probes for screening taste tissue cDNA library are as follows: the forward Ena-S2, TTATGGATGATGGTGGCTTC, and the reverse Ena-A2, AGCACGGACGAGCCAAACCA, which correspond to the published ASSC nucleotide acid sequences 1162–1181 and 1752–1771, respectively (16). To identify homologues of ASSC, two degenerate primers were designed based on the published ASSC sequence. Ena-S1 [GGACAGAATTCGNGGNAA(T/C)TA(T/C)GGNGA(T/C)TG] is the forward primer, which corresponds to the amino acid sequence SLGGNYGDC (372–380); Ena-A1 [GATCCACTCGAGNGA(T/C)TTNACNGANGGCCA] is the reverse primer, which corresponds to ASSC amino acid sequence WPSVKSQ (520–526). PCR conditions were 35 cycles of 1 min at 95°C, 2 min at 45°C or 65°C, and 1 min at 72°C. The cDNA clone generated by PCR was labeled using a random primer kit and [^{32}P]dCTP and was used to screen a rat circumvallate papillae cDNA library under high stringency [50% formamide/5× SSC at 42°C for hybridization and 1× SSC at 55°C for a final wash (1× SSC = 0.15 M NaCl/15 mM sodium citrate)]. Positive clones were purified and cDNA was prepared for restriction mapping and sequence analysis.

Northern Analysis. Total RNA (30 μg) or poly(A)$^+$ RNA (5 μg) was prepared from various rat tissues or cultured cell lines (normal rat kidney epithelial-like cell line NRK-52E, human embryonic intestine cell line I-407, and human colon adenocarcinoma cell WIDR; all were obtained from the American Type Culture Collection), fractionated on 1% agarose/formaldehyde gels, and blotted onto a nitrocellulose membrane. The entire insert (2.1 kb) of the cDNA clone isolated from rat circumvallate papillae cDNA library was labeled with [^{32}P]dCTP. The blot was hybridized in 50% formamide/5× SSC hybridization buffer at 42°C and washed with 0.1× SSC at 65°C. The blot was then exposed to x-ray film for 5 days at −70°C.

***In Situ* Hybridization.** The cRNA probes were made for *in situ* hybridization on rat tongue tissue sections. The PCR clone in a Bluescript vector containing a 450-bp insert (between primers Ena-S1 and Ena-A1) was used to generate antisense RNA probes with [^{33}P]UTP labeling (NEN). Sense

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Abbreviations: DTT, dithiothreitol; ASSC, amiloride-sensitive sodium channel(s); RT, reverse transcriptase.

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RNA probes provided negative controls. Three antisense oligonucleotides (45-mer) based on the published ASSC sequence (16) were also labeled with [^{33}P]dATP by the terminal transferase reaction for *in situ* hybridization on rat lung and colon sections. The sequences of those oligonucleotides are ENA-A7, AAGTCATTCTGCTCTGTGCGCAGTGTCAGGGACAAACCATTGTTG (1053–1098); ENA-A9, CAGTTTATAATAGCAATAGCCCCAGGAGCTCTGCTTTCGGTAGTC (1416–1461); and Ena-A6, GCTGGGGAAGATGTGCTGAAGTGAGATATCCTCAGTTTTACAAGG (2134–2179). Tissue sections (16 μm) were fixed in 4% paraformaldehyde, rinsed in phosphate-buffered saline, and digested with 10 μg of proteinase K per ml at 37°C for 30 min. Sections were then rinsed in 0.1 M triethanolamine, acetylated in 0.25% acetic anhydride for 10 min, and dehydrated in a graded series of ethanol solutions. Hybridization was performed with 10^6 cpm/100 μl of probe in 50% formamide, 10% dextran sulfate, 0.3 M $MgCl_2$, 10 mM Tris (pH 8), 1× Denhardt's solution, 0.5 mg of tRNA per ml, and 10 mM dithiothreitol (DTT) overnight at 37°C for oligonucleotide probes and at 55°C for cRNA probes, respectively. Excess cRNA probe was removed by digestion with RNase A (20 μg/ml) for 30 min and washed at a final stringency of 0.1× SSC at 60°C for 30 min; excess oligonucleotide probe was removed by a final washing of 2× SSC at 55°C for 1 hr. Slides were hand-dipped in Kodak NTB2 emulsion, exposed for 1–4 weeks at 4°C, developed, and stained with Giemsa stain (Sigma).

RESULTS

Construction of a cDNA Library from Rat Circumvallate Papillae. RNA from the circumvallate papillae of 500 rats was poly(A)$^+$ selected and used to construct a directional cDNA library into the Lambda Zap vector. We obtained 1.5 million independent recombinants with an average insert size of 1.2 kb.

Tissue Distribution of ASSC mRNA. We examined for the presence of ASSC mRNA in taste bud-related tissues as well as other organs utilizing RT-PCR (Fig. 1). Utilizing primers based on the published ASSC sequence (16, 17), we conducted PCR under high stringency conditions (65°C annealing). We obtained PCR products of the expected size and confirm the presence of prominent bands in kidney and colon, with no mRNA evident in the brain and testis. The most prominent band is observed in the lung, with an intensity several times greater than that of colon and kidney. Bands of comparable intensity occur in epithelial tissue of the circumvallate papillae and in epithelium tissue of adjacent tongue sections that lack circumvallate papillae. Interestingly, olfactory epithelium displays a PCR band of similar intensity to bands in the tongue, while skin has a faint band. We subcloned and sequenced the PCR product from circumvallate papillae and observe a sequence that is identical to that previously reported (16).

Since PCR is not fully quantitative, we evaluated the distribution of ASSC by Northern blot analysis with a cDNA clone of ASSC obtained by high stringency screening of our circumvallate papillae cDNA library. From this library we obtained a 2.1-kb partial-length cDNA clone that was used for Northern blot analysis. Sequence analysis reveals that this partial clone is identical in sequence to the reported ASSC (16, 17).

Northern blot analysis confirms the results of PCR. The most prominent band by far is obtained in the lung. Bands in all tissues are about 3.5 kb, resembling previous reports (16). Substantial mRNA levels are evident in the kidney, while negligible levels occur in skin and in whole eye. Modest amounts are evident in olfactory epithelium. Interestingly, a continuous culture of rat kidney epithelium-like cells does not display ASSC mRNA, despite the high levels in kidney from intact rat. Similarly, no mRNA is observed in cell lines from human intestine or human colon cancer, rat spleen, rat muscle, or rat testes.

In the tongue substantial mRNA levels occur in epithelium containing circumvallate papillae as well as adjacent tissue lacking circumvallate papillae (Fig. 2). A strip of epithelium from the anterior region of the tongue also contains substantial ASSC mRNA. A poly(A)$^+$ preparation of epithelium from an area of tongue adjacent to the circumvallate papillae lacking taste cells displays higher levels of mRNA, presumably due to the poly(A)$^+$ enrichment.

Localization of ASSC mRNA by *in Situ* Hybridization. We conducted *in situ* hybridization utilizing a 450-bp PCR probe. In the tongue we compared areas enriched in taste buds, such

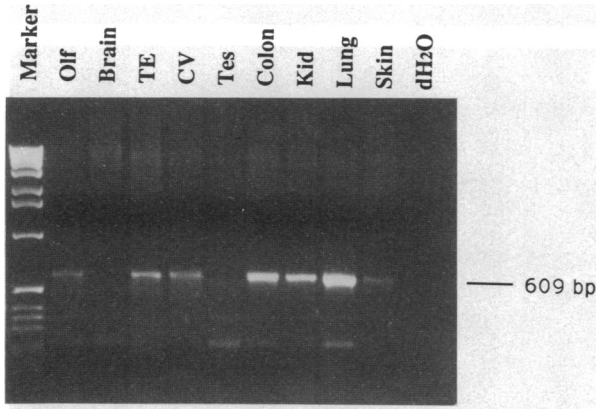

FIG. 1. RT-PCR analysis of ASSC expression in various tissues. Ethidium bromide-stained RT-PCR product from rat olfactory epithelial tissue (Olf), brain, tongue epithelium adjacent to circumvallate papillae (TE), circumvallate papillae (CV), testis (Tes), distal colon (Colon), kidney (Kid), lung, skin, and the negative control with water (dH_2O). A 1-kb DNA ladder was used as a marker. The expected size (609 bp) of PCR products using primers Ena-S2 and Ena-A2 is indicated.

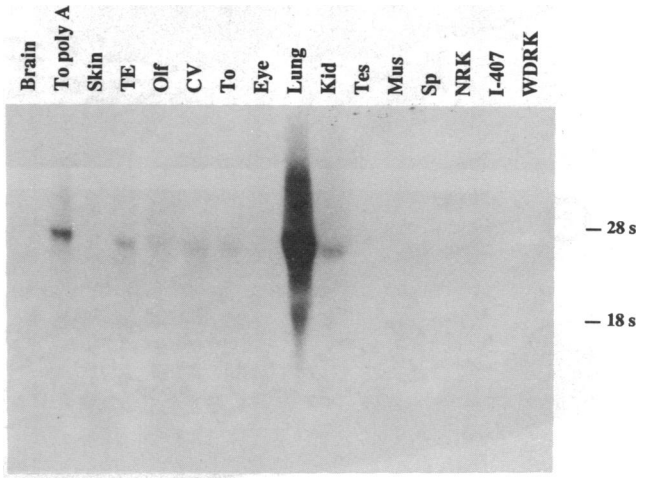

FIG. 2. Northern blot analysis of ASSC expression in various rat tissues and cell lines. A blot prepared from total RNA (30 μg) of rat brain, poly(A)$^+$ mRNA (5 μg) of rat tongue tissue adjacent to circumvallate papillae (To poly A), total RNA (30 μg) from rat skin, anterior tongue epithelium (TE), olfactory epithelial tissue (Olf), circumvallate papillae (CV), tongue tissue adjacent to circumvallate papillae (To), whole eye, lung, kidney (Kid), testis (Tes), skeletal muscle (Mus), spleen (Sp), rat normal kidney epithelia-like cell line (NRK), human embryonic intestine cell line (I-407), and human colon adenocarcinoma cell line (WDRK). The positions of 28S and 18S ribosomal RNA are indicated.

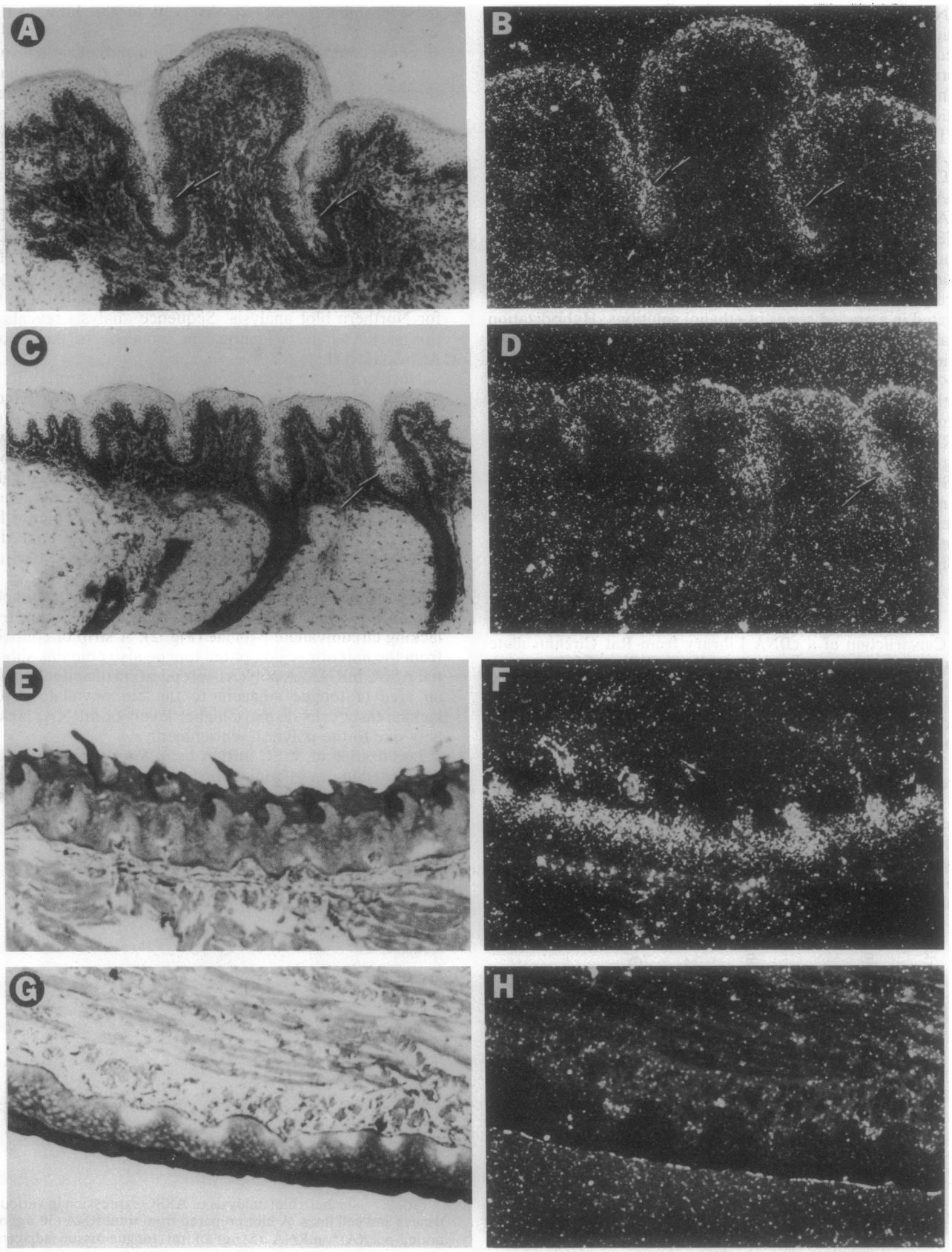

FIG. 3. Localization of ASSC mRNA in rat tongue epithelium. (*A*, *C*, *E*, and *G*) Bright-field views of cross sections of a rat circumvallate papillae (*A*), foliate papillae (*C*), anterior dorsal region (*E*), and anterior ventral region (*G*) of tongue epithelium, hybridized with a [^{33}P]cRNA probe. (×7.) (*B*, *D*, *F*, and *H*) Dark-field images of sections corresponding to the bright-field view. The taste buds are indicated by arrows.

as circumvallate and foliate papillae, with the anterior dorsal and ventral epithelia, which lack taste buds (Fig. 3). In all of these regions except ventral epithelium we observe high densities of silver grains associated with the epithelium with

negligible densities in the underlying muscle or glandular tissue. In circumvallate and foliate papillae grain density is the same in taste and non-taste epithelium. Grain density appears similar in epithelium from areas with or without taste cells. Experiments utilizing a sense probe reveal no hybridization.

Extremely high densities of ASSC grains occur in lung tissue localized to the epithelial layer of small bronchi and alveoli (Fig. 4). Low levels of silver grains are distributed evenly over the endothelial layer and smooth muscle of blood vessels. High densities of ASSC grains occur in the epithelium of the distal colon, but not in the lamina propria. Sense probes reveal a low background of grains in both lung and colon.

DISCUSSION

In the present study we have identified ASSC expression in various tissues. ASSC is expressed exclusively in epithelial layers of various organs. The extremely high levels in the lung, observed independently (18), might be relevant to pulmonary pathophysiology. The airway epithelium of patients with cystic fibrosis displays excessive active absorption of sodium (19, 20). Moreover, inhalation of amiloride diminishes pulmonary symptoms of cystic fibrosis patients (21).

One of our major findings is that ASSC occurs in epithelial layers of non-taste as well as taste cells. This finding might seem puzzling, since signal transduction for salty taste presumably occurs only in taste cells in an amiloride-sensitive fashion. However, various evidence suggests that the non-taste cells are of crucial importance for salty taste perception (Fig. 5). First, DeSimone and collaborators (8, 22) have shown that transepithelial sodium currents are sensitive both to amiloride and ouabain. Since the great majority of cells in these preparations are non-taste cells, this establishes that non-taste cells in the tongue possess amiloride-sensitive sodium channels, indicating that the ASSC that we have visualized in non-taste cells are functional. By analogy with the localization of ASSC in other organs (23), we infer that ASSC in lingual epithelia are located in the apical region of the cell. By contrast, the sodium–potassium transporters, which pump sodium outside the cell, are located in the basolateral surface of the cells. Accordingly, the movement of sodium is from the apical to the basal surface of cells. Thus, exposure to levels of NaCl that stimulate salty taste will cause a substantial augmentation in the sodium concentration in the interstitial fluid at the basolateral surface of taste and non-taste cells.

Interestingly, though ASSC are present in the dorsal epithelium of tongue, they are absent from the ventral epithelium. This is consistent with the finding that rat dorsal lingual epithelium, but not ventral epithelium, is permeable to NaCl (22). In taste cells the sodium current provides the depolarization that triggers firing of the sensory nerves. In non-taste cells the same sodium current provides elevated levels of sodium in the interstitial space that facilitate accumulation of chloride as a counteranion to sodium. The role of this transepithelial passage of chloride in taste is indicated by findings (24) that chorda tympani responses to sodium gluconate and sodium acetate are much less than to sodium chloride. All cells in the lingual epithelium are coupled by

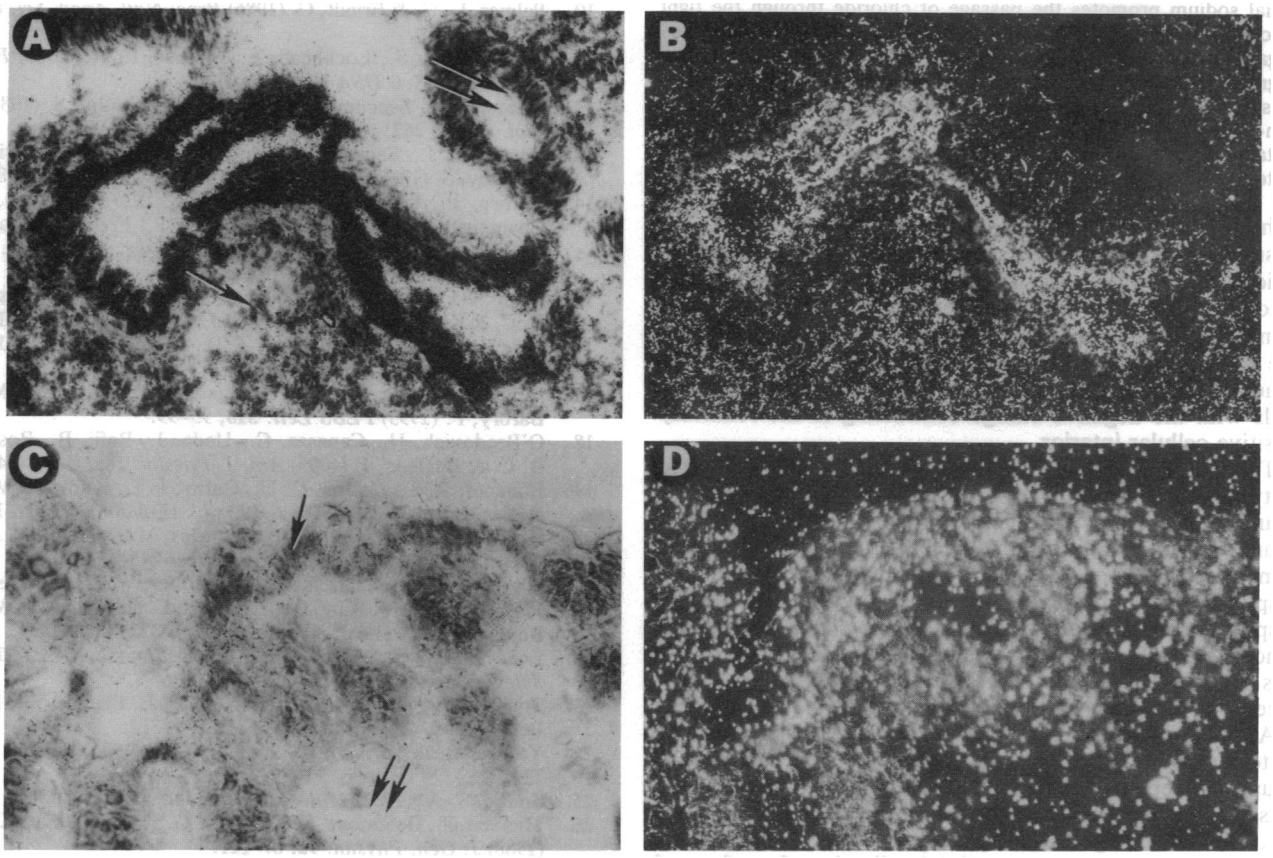

FIG. 4. *In situ* hybridization of ASSC mRNA in peripheral tissues. Three oligonucleotides (45-mer) labeled with ^{33}P were hybridized to adult rat lung (*A* and *B*) (×14) and distal colon (*C* and *D*) (×28) in light (*A* and *C*) and dark-field (*B* and *D*) microscopy. High densities of autoradiographic grains are over regions of the lung containing alveoli (arrows), alveolar epithelium, and the epithelium of small bronchi in *B* and epithelial cells of colon (arrows) in *D*. Note the absence of grain accumulation in the endothelial and smooth muscle lanes of the blood vessel in *B* and in lamina propria in *D* (double arrows).

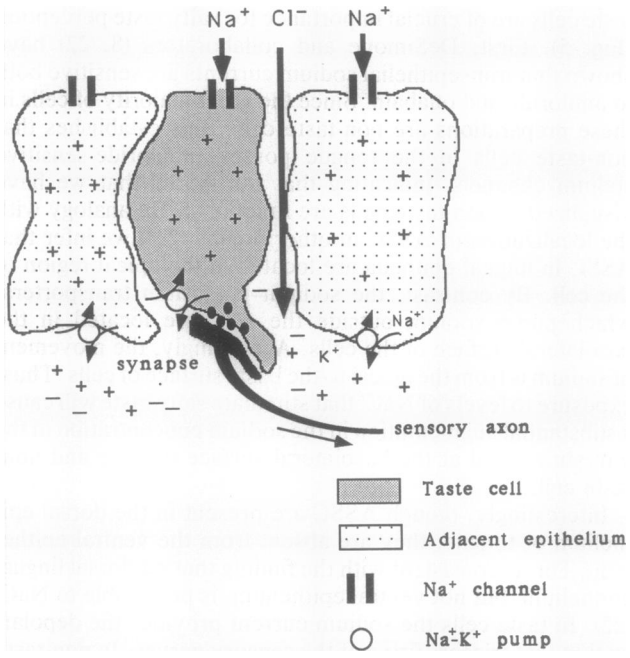

FIG. 5. Model of salty taste transduction mediated by taste cells and adjacent epithelial cells. Influx of sodium through apical membrane Na^+ channels of taste receptor cells depolarizes them and triggers the release of neurotransmitter to cause firing of the sensory axons. ASSC in the non-taste cells, along with the sodium–potassium transporter at the basolateral surface of these cells, causes a marked augmentation in the interstitial basolateral concentration of sodium when salty amounts of NaCl are applied to the tongue. This interstitial sodium promotes the passage of chloride through the tight junctions between cells to serve as a counteranion. The increased negative charges in the extracellular environment of the taste cell augment the depolarization of the taste cells. Since all taste modalities involve depolarization of the taste cell, activation of ASSC in non-taste cells by the increased sodium content of saliva when tastants are applied would be critical in maintaining the depolarized state of the taste cells.

tight junctions so that anion movement is restricted (24, 25). A small anion such as chloride may diffuse to the interstitial fluid, while the passage of gluconate, acetate, and other large anions is hindered. The requirement of chloride for salty taste transduction emphasizes the importance of anion passage for the transduction process. The chloride in the extracellular space presumably accentuates depolarization of the taste cells with the negative charge contrasting to the relatively positive cellular interior.

This model in which ASSC in non-taste cells are critical to salty taste may be relevant to other forms of taste (Fig. 5). Thus, sour taste involves the passage of protons through ion channels. The proton current depolarizes taste cells to initiate signal transduction. Cells that respond to sour stimuli also respond to sodium, albeit to a lesser degree, while NaCl-responsive cells are affected somewhat by sour tastants (6). Since sour taste in mammals is blocked by amiloride (5, 6), it is likely that some form of ASSC constitutes the sour taste receptor.

ASSC in non-taste cells may regulate yet other forms of taste perception. Sweet taste is thought to involve G-protein-coupled receptors linked to the stimulation of adenylyl cyclase (1, 2). Sweet taste perception is blocked by amiloride (11, 26). How might sweet perception be associated with ASSC activities in non-taste cells? Application of any form of tastant to the tongue stimulates the flow of saliva, and the stimulated saliva contains sodium concentrations of about 60 mM, more than 10 times the basal sodium concentration of saliva (27). This elevated sodium concentration should suffice to activate ASSC. The consequent increased interstitial chloride would accentuate the depolarization of the taste cells responding to sugar just as they enhance the response of taste cells responding to salt. In fact, application of low concentrations of salt to the tongue augments sweet taste responses (28). Since all taste transduction involves depolarization of taste cells, we suggest that ASSC in non-taste cells participates in signal transduction for all taste modalities, including umami taste, which is blocked by amiloride (29) and bitter taste.

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