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Ultrastructural Localization of Amiloride-sensitive Sodium Channels and Na^+,K^+-ATPase in the Rat's Olfactory Epithelial Surface

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Abstract

Several studies have indicated that olfactory responses are impeded by amiloride. Therefore, it was of interest to see whether, and if so which, olfactory epithelial cellular compartments have amiloride-sensitive structures. Using ultrastructural methods that involved rapid freezing, freeze-substitution and low temperature embedding of olfactory epithelia, this study shows that, in the rat, this tissue is immunoreactive to antibodies against amiloride sensitive Na^+-channels. However, microvilli of olfactory supporting cells, as opposed to receptor cilia, contained most of the immunoreactive sites. Apices from which the microvilli sprout and receptor cell dendritic knobs had much less if any of the amiloride–antibody binding sites. Using a direct ligand-binding cytochemical method, this study also confirms earlier ones that showed that olfactory receptor cell cilia have Na^+,K^+-ATPase. It is proposed that supporting cell microvilli and the receptor cilia themselves have mechanisms, different but likely complementary, that participate in regulating the salt concentration around the receptor cell cilia. In this way, both structures help to provide the ambient mucous environment for receptor cells to function properly. This regulation of the salt concentration of an ambient fluid environment is a function that the olfactory epithelium shares with cells of transporting epithelia, such as those of kidney.

Introduction

Two major epithelial cell types line the olfactory part of the nasal cavity, olfactory receptor cells and olfactory supporting cells. Although the main function of the receptor cells is to receive and transduce the odorous messages (Farbman, 1992; Buck, 1996; Menco, 1997), they are also involved in their own housekeeping, to maintain the ambient environment around their receptor cell cilia (see below). No such precise functions are known for the supporting cells, but all evidence points to the fact that these cells too have multiple roles, most of them relating to housekeeping (Farbman, 1992; Menco, 1992a; Getchell *et al.*, 1993; Lewis and Dahl, 1994).

For housekeeping, receptor and supporting cells, and also those of Bowman's glands (not further considered here), share functions with liver and kidney cells. Olfactory receptor cells share with those of the kidney the expression of Na^+,K^+-ATPase (Ernst, 1975; Kern *et al.*, 1991; this paper) and carbonic anhydrase (Brown *et al.*, 1984; Okamura *et al.*, 1996). With cells of the liver they share a presence of enzymes that regulate xenobiotic compounds, in particular glutathione metabolizing systems (Kirstein *et al.*, 1991; Krishna *et al.*, 1994; Starcevic and Zielinski, 1995).

Olfactory epithelial supporting cells share with liver cells the distinction that they are among the most active cell types in the body involved in detoxification of xenobiotic compounds, especially by means of various cytochrome P-450s (Adams *et al.*, 1991; Chen *et al.*, 1992; Lewis and Dahl, 1994; Miyawaki *et al.*, 1996). In addition, ultrastructural studies have shown that apices of rat olfactory supporting cells and of cells of kidney collecting tubules are remarkably alike in several aspects. For example, *Dolichus biflorus* agglutinin (DBA)-binding sites (Brown *et al.*, 1985; Weyer *et al.*, 1988) localized in both apical parts of principal cells of rat kidney collecting tubules and microvilli of olfactory supporting cells (Menco, 1992b). Furthermore, freeze-fracture studies in the rat demonstrated that membranes of supporting cell apices express a peculiar dumbbell-shaped particle (Hörandner *et al.*, 1974;

Kerjaschki and Hörandner, 1976; Menco, 1980, 1988, and discussion therein). This type of particle is also present in apical parts of renal collecting tubule intercalated cells (reviewed in Brown *et al.*, 1988). Circumstantial evidence suggests that these particles relate to a proton pump (Brown *et al.*, 1988; Brown, 1989). In the rat's olfactory system, the particles and DBA-binding sites are localized on two adjoining regions of the same olfactory supporting cell, apical regions and the microvilli sprouting from these. In the kidney particles and DBA-binding sites are localized on two neighboring cells.

Studies in rat kidney showed that antibodies to amiloride-sensitive but voltage-insensitive Na^+-channels (mol. wt ~ 730 kDa; Sorscher *et al.*, 1988; Kleyman *et al.*, 1991) bound to apical regions of principal cells of collecting tubules, the cells involved in Na^+-reabsorption (Brown *et al.*, 1989; Kleyman *et al.*, 1991). In a quest for other analogies between apices of rat olfactory supporting cells and cells of kidney collecting tubules, we applied the same antibodies to olfactory epithelia. Several physiological studies have suggested a role of amiloride-sensitive epithelial Na^+-channels in olfaction (Persaud *et al.*, 1987, 1988; Frings and Lindemann, 1988; Frings *et al.*, 1988; Delgado and Labarca, 1993; Lischka and Schild, 1993). The results of these studies implied that these channels are present on the receptor cells. However, this study and its preliminary reports (Menco and Benos, 1990; Menco, 1992a, 1994, 1995) indicate that most of these channels located on microvilli of olfactory supporting cells.

Materials and methods

Animals

Adult Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN). All procedures described below were performed in accordance with Federal and NIH animal use guidelines, using institution approved animal protocols.

Immunoblots: tissue preparation

Membrane preparations were prepared modified after Mishra (1986). Rat olfactory mucosal tissues were collected from rats deeply anesthetized with 0.85 mg/kg sodium pentobarbital i.p., fast-frozen in liquid nitrogen and stored at –80°C until homogenization. The samples were homogenized in 50 mM Tris (pH 7.4), 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM *p*-tosyl-L-arginine methyl ester (TAME), 1 mM dithiothreitol (DTT), 320 mM sucrose, 1 mM ethylenediamine tetra-acetic acid (EDTA) and 10 mM sodium metavanadate, using a Tekmar Tissumizer® (Cincinnati, OH) homogenizer. Following homogenization the samples were subjected to a 550 *g* centrifugation for 5 min at 4°C using a Beckman J2-21 Centrifuge (Beckman, Palo Alto, CA). The pellet was discarded and the supernatant was centrifuged in the homogenization buffer at 23 000 *g* for 15 min at 4°C. The resulting supernatant was discarded and the pellet was resuspended in the homogenization buffer, aliquoted and stored at –80°C. The protein content was determined using the Bradford (1976) method.

Light microscopy: tissue preparation

Adult rats were deeply anesthetized with 0.85 mg/kg sodium pentobarbital i.p. and transcardially perfused with 4% paraformaldehyde in 0.1 M Sorensen's phosphate buffer, pH 7.3. Nasal structures were dissected and left to fix in 4% paraformaldehyde for several hours or overnight. Fixation was followed by decalcification in RDO (Apex Engineering Corporation, Plainfield, IL) for 46 h. Washed tissues (Sorensen's buffer, overnight) were embedded in Tissue-Tek® OCT cryoembedding substance (Miles, Elkart, IN). Cryostat sections, 8–12 μm thick, cut with a LEO-Leica-Reichert Frigocut 2800N Cryostat (Heidelberg, Germany), were mounted on gelatin–alum-subbed slides.

Electron microscopy: tissue preparation

Adult rats were asphyxiated with CO_2. Nasal septa, containing olfactory and respiratory epithelia, were excised and rapidly frozen by dropping them on a liquid nitrogen-cooled copper block (Gentleman Jim Quick-Freeze System: Energy Beam Sciences, Agawam, MA; Phillips and Boyne, 1984). The otherwise unfixed specimens were freeze substituted in dry acetone/0.1% uranyl acetate (UAc). Freeze substitution, infiltration and low-temperature embedding were carried out in a CS Auto Cryo-Substitution System (LEO-Reichert Instruments, Vienna, Austria). Infiltrated specimens were embedded in the hydrophilic methracylate resin Lowicryl K11M while the temperature rose very slowly from –60°C to ambience (Phillips and Bridgman, 1991; Menco, 1995).

Antibodies

The preparation and specificity of the rabbit-raised polyclonal antibodies to purified epithelial Na^+-channels from bovine renal papilla has been described elsewhere (Sorscher *et al.*, 1988; Tousson *et al.*, 1989; Oh and Benos, 1993; Fuller *et al.*, 1995). The ultrastructural data presented in this paper are based on a limited (three repeats) but consistent set of experiments using antiserum bleeds from the same rabbit, Chan (Chan 29 and 32; Chan, Den and Hil indicate different rabbits, the numbers indicate the bleed number). Only relatively fresh antiserum of rabbit Chan worked well at an ultrastructural level; antibody reactivity deteriorated upon prolonged storage, even at –80°C. Moreover, bleeds from a different rabbit (Den 36 and 38) did not work well at an ultrastructural level (Menco, 1995). Light microscopy (LM) was only carried out with antiserum bleeds obtained from the latter animal. Bleeds from rabbit Hil were used for the Westerns blots. Intact Na^+-channel protein was used to generate the Chan and Hil antibodies

(see also below), while Den antibodies were made to denatured Na^+-channel protein.

Immunoblots

Resuspended supernatant (20 μg/ml), containing olfactory epithelial membranes, was separated over 8% SDS–PAGE gels according to the method of Laemmli (1970) under reducing conditions using 10 mM DTT. The bands were immunoblotted on Immobilon-P PVDF (polyvinylidene fluoride) microporous transfer membrane (Millipore, Bedford, MA) as previously described (Sorscher *et al.*, 1988). Lanes were probed either with polyclonal antibodies raised against purified bovine renal Na^+-channel (rabbit Hil), affinity purified over Protein A–Sepharose columns (Pierce, Rockford, IL) and controls, i.e. Protein A-purified pre-immune IgG from rabbit Hil and non-immune rabbit IgG (Jackson Immuno, West Grove, PA). All three were used at a concentration of 2.5 μg/ml. Blots were developed using donkey anti-rabbit secondary antibody conjugated to alkaline phosphatase at a dilution of 1:5000 in combination with the NBT/BCIP (nitro blue tetrazolium/5-bromo-4-chloro-3-inolyl phosphate) system from BioRad (Hercules, CA) according to the manufacturer's instructions. BioRad's Broadrange Standards served as molecular weight standards.

Light microscopy: post-embedding immunohistochemistry

Sections were preincubated in 0.1% SDS and 50% normal goat serum (Gibco, Grand Island, NY) in phosphate-buffered saline (PBS) at room temperature for 30 min. Next, they were incubated with primary antibodies in PBS (rabbit Den, dilution: 1:100) and 0.1% bovine serum albumin (BSA) for 1 h at 37°C. Immunoreactivity was visualized with secondary antibodies bound to biotin followed by an avidin–biotin–peroxidase reaction using rabbit Vectastain ABC (Vector, Burlingame, CA) with diaminobenzidine as chromophore (Carr *et al.*, 1989).

Electron microscopy: post-embedding immunocytochemistry

Sections of olfactory and nasal respiratory epithelia were collected on the rough side of uncoated 300 mesh nickel grids. Incubation with the primary antibody (rabbits Chan and Den) was done overnight at 4°C; all other procedures were carried out at room temperature. After blocking with 10% normal horse serum (Gibco, Grand Island, NY), 4% BSA (Sigma, St Louis, MO) or 0.1% acetylated BSA (AcBSA, Electron Microscopy Sciences, Fort Washington, PA; Leunissen, 1990) in Tris-buffered saline (TBS + 0.5 M NaCl, pH 8.0), sections were incubated with primary antibodies (dilution: 1:5 or 1:20) without intermediate washing and then, after washing, with protein G conjugated to 5 nm colloidal gold (Electron Microscopy Sciences; Bendayan and Garzon, 1988), with an optical density of 0.20 at 520 nm. Sections were washed with TBS–4% BSA or TBS–0.1% AcBSA and water, and air-dried. It was important to omit Tween 20 throughout the procedures, including the TBS–BSA or AcBSA washes, as this detergent was found to disrupt the labeling pattern for the antibodies used here (Menco *et al.*, 1994; Menco, 1995).

Dextran–ouabain–gold photoaffinity labeling

Instead of using an enzymatic assay to determine the localization of Na^+,K^+-ATPase in olfactory epithelia (Kern *et al.*, 1991) we elected for this purpose a method in which an inhibitor of this enzyme, ouabain, and the photoaffinity label sulfo-SANPAH [sulfo-succinimidyl-6(4′-azido-2′-nitrophenylamino)hexanoate] (Pierce) were covalently linked to a larger molecule. This molecule was dextran, which in turn coated 5–6 nm gold particles (prepared according to Slot and Geuze, 1985). Ouabain finds its target and the photoaffinity label, conjugated to the same gold particle, covalently anchors the dextran–ouabain–gold complex to the target, Na^+,K^+-ATPase. The ouabain retains its biological effect (Mrsny *et al.*, 1987). Incubation with the dextran–ouabain–gold photoaffinity conjugate was carried out for 3 h at 37°C. Next, a 100 W mercury lamp was used at a distance of ~20 cm of the specimens for 25 min in an otherwise dark cold room to covalently bind the complexes to the Na^+,K^+-ATPase (Mrsny *et al.*, 1987). The gold–dextran-conjugated ouabain photoaffinity label was used in 0.01 M phosphate buffer, 0.15 M NaCl, 0.01 M Na-azide, pH 7.4, and at an OD_{520} of ~1.0.

Imunocytochemical controls

Normal rabbit serum and secondary probes without primary probes served as negative controls for immuno-reactivity. Antibodies used in other studies, especially those to olfactory marker protein and to several olfactory signal transduction proteins, $G_{s\alpha}$, $G_{olf\alpha}$ and Type III adenylyl cyclase (Menco *et al.*, 1992, 1994; Menco, 1997), served as positive controls.

Electron microscopy

Following labeling the sections, 150–200 nm thick [thinner Lowicryl K11M sections fall apart on the water surfaces of the section-collecting boats (Menco, 1989)] mounted on 300 mesh nickel electron microscope grids, were stained with filtered 0.5% uranyl acetate dissolved in 50% methanol for 10–15 min, jet-washed with distilled water and air-dried. Next they were carbon-coated on one side to reinforce the sections. This procedure helps the sections to better resist exposure to the electron beam in the electron microscope. Coated, stained and labeled sections were examined at 120 kV in a JEOL 100 CX electron microscope.

Statistical analyses

Densities of gold grains overlaying and within surrounding areas of ~50 nm of supporting cell microvilli, olfactory cilia, and respiratory cilia and microvilli were determined on the

micrographs using a grid divided into 1 cm^2 squares. Densities were recalculated to grains/μm^2. The same was done for densities of gold grains overlaying olfactory supporting cell apices, dendritic receptor cell knobs and Lowicryl K11M resin devoid of tissue. ANOVAs were performed using the Statview 4.0 packet on a Macintosh microcomputer.

Results

Immunoblots

Figure 1 illustrates an immunoblot of a rat olfactory epithelial membrane fraction using the same antibodies as the ones used in the ultrastructural localization studies (albeit from a different rabbit). The antibody recognized a diffuse band migrating at 50–60 kDa. Neither pre-immune serum nor non-immune rabbit IgG recognized this protein complex. However, this rabbit polyclonal antibody, raised against a purified bovine renal Na^+-channel complex, recognized polypeptides of an identical molecular mass as antibodies raised against the α-subunits of rat renal Na^+-channels (Fuller *et al.*, 1995).

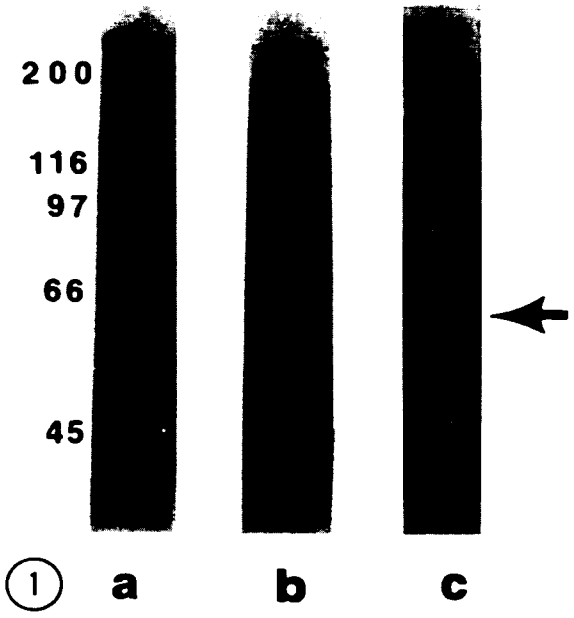

Figure 1 Western blot of rat olfactory epithelial membranes probed with a rabbit polyclonal antibody (rabbit Hil) to intact purified bovine renal Na^+-channel complex and controls. Lane a: non-immune rabbit IgG; Lane b: preimmune rabbit IgG; Lane c: anti-Na^+-channel IgG. Lane c shows a diffuse band migrating at between 50 and 60 kDa detected by the antiserum to the Na^+-channel, that was not detected by non-immune or pre-immune IgG.

Immunohistochemical labeling with antibodies to amiloride-sensitive Na^+-channels: light microscopy

At the level of the light microscope, olfactory epithelial surfaces (Figure 2) label more intensely with antibodies to amiloride-sensitive Na^+-channels than surfaces of adjacent non-sensory respiratory epithelia (Figure 3). LM observations were based on antisera from a different rabbit than EM observations, Den as opposed to Chan. Although the Den antisera did not work well at an ultrastructural level (Menco, 1995), LM results using this antiserum were consistent with the ultrastructural results based on antiserum from rabbit Chan.

Immunohistochemical labeling with antibodies to amiloride-sensitive Na^+-channels: electron microscopy

Nasal olfactory and respiratory epithelia have cells that bear cilia and microvilli, but these have different topographies and functions. While in the olfactory epithelium the cilia pertain to sensory cells and radiate like tentacles from a central dendritic knob (Figure 4), the cilia are aligned in parallel in the nasal respiratory epithelium (Figure 9). Also, respiratory cells bear both cilia and microvilli (Figure 9), whereas in the olfactory epithelium supporting cells surrounding the receptor cells bear most of the microvilli (Figures 4 and 5; see also Menco, 1980).

Supporting cell microvilli in particular bind the antibodies to the amiloride-sensitive Na^+-channels (Figures 4–6 and 8). Labeling of these structures is intense. Therefore,

Table 1 Densities of colloidal gold particles marking the ultrastructural localization of amiloride sensitive Na^+-channels and Na^+,K^+-ATPase

	Amiloride sensitive Na^+-channels (primary antibody + secondary antibody gold)		Na^+,K^+-ATPase (dextran–ouabain–gold photoaffinity conjugate)	
Olfactory cilia	108.6 ± 89.5 (n = 34, SE = 15.3)[a]	17%	160.1 ± 98.5 (n = 38, SE = 16.0)*	100%
Dendritic knob	103.6 ± 86.9 (n = 17, SE = 21.1)	16%	–	–
Supporting cell microvilli	640.1 ± 568.5 (n = 41, SE = 88.7)*	100%	28.1 ± 37.1 (n = 38, SE = 6.0)	18%
Supporting cell apex	73.5 ± 76.2 (n = 19, SE = 17.5)	11%	–	–
Respiratory cilia and microvilli	107.8 ± 95.5 (n = 13, SE = 26.5)	17%	16.1 ± 27.3 (n = 26, SE = 5.4)	10%
Resin and mucus background	77.3 ± 117.6 (n = 19, SE = 27.0)	12%	22.3 ± 49.5 (n = 21, SE = 10.9)	14%

[a]Densities of gold grains (grains per μm^2) overlaying assigned structures and within an area of ~50 nm surrounding these structures. Means ± SD; n = number of areas counted; SE = standard error; – = not enough counts for a reasonable estimate (the few values obtained were low, however). Values within columns two and four marked * differ significantly ($P < 0.0001$; significance level 5%) from other values in the same column. Values in the third and fifth columns are recalculated as percentage fraction of the most intensely labeled structure, supporting cell microvilli in the case of the amiloride-sensitive Na^+-channels and olfactory cilia in the case of Na^+,K^+-ATPase.

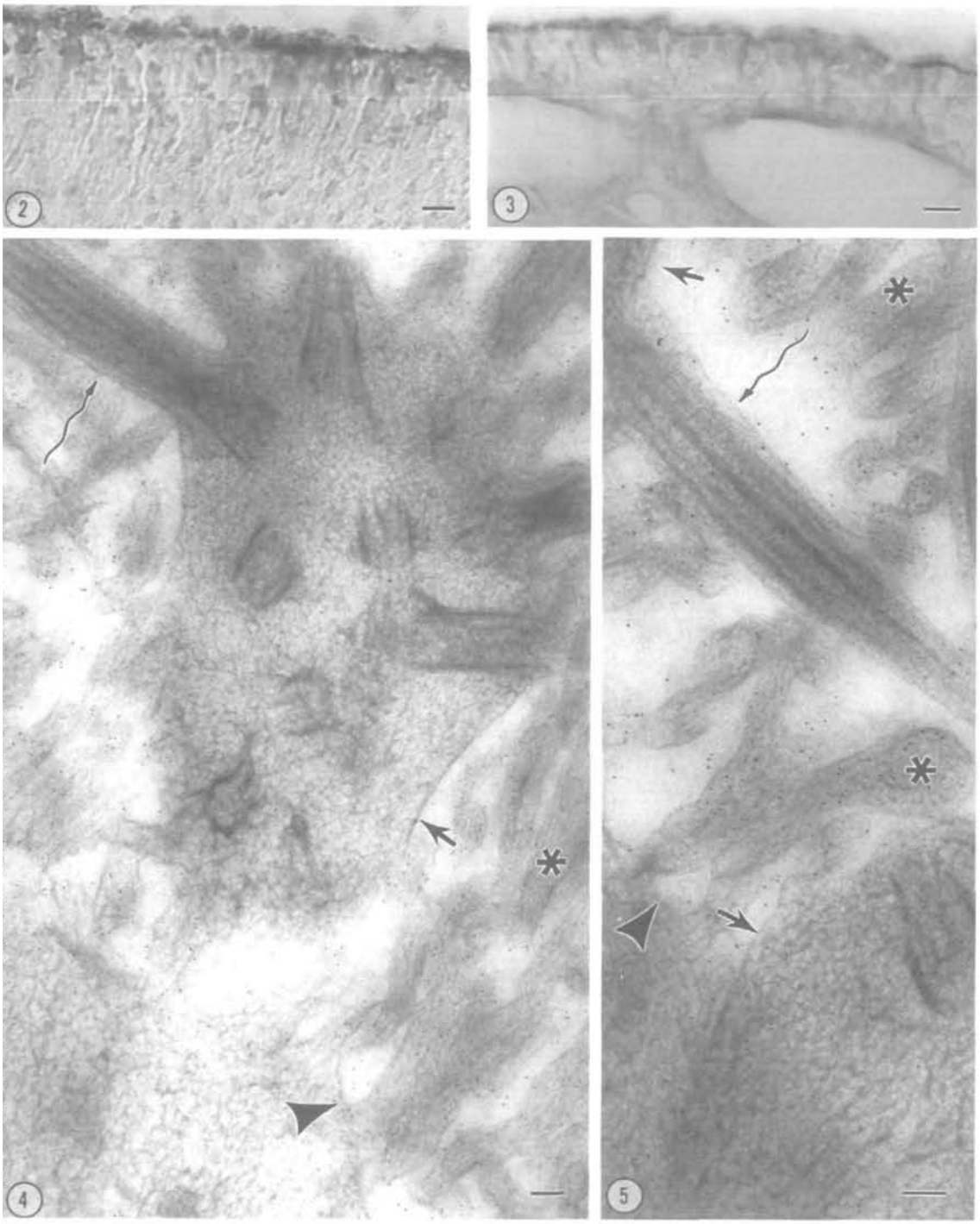

Figure 2 Light micrograph of rat olfactory epithelium immunolabeled with polyclonal antiserum Den 38 to amiloride-sensitive Na^+-channels. The olfactory epithelial surface is densely labeled. Scale bar: 10 μm.

Figure 3 Light micrograph of rat respiratory epithelium immunolabeled with polyclonal antiserum Den 38 to amiloride-sensitive Na^+-channels. Surface labeling is much less intense here than in the olfactory epithelium. Scale bar: 10 μm.

Figure 4 Olfactory supporting cell microvilli (asterisk) labeled with antiserum bleed Chan 29 against amiloride-sensitive Na^+-channels (dilution 1:5 in this and all subsequent micrographs). Labeling is considerably less intense in apical regions of the supporting cells from which the microvilli sprout (arrowhead), olfactory receptor cell dendritic knobs (straight arrow) and cilia (snake-shape arrow). Protein G conjugated to 5 nm colloidal gold was used as the secondary probe. Scale bar: 0.1 μm.

Figure 5 As Figure 4 with markers indicating the same structures, but a different area. Scale bar: 0.1 μm.

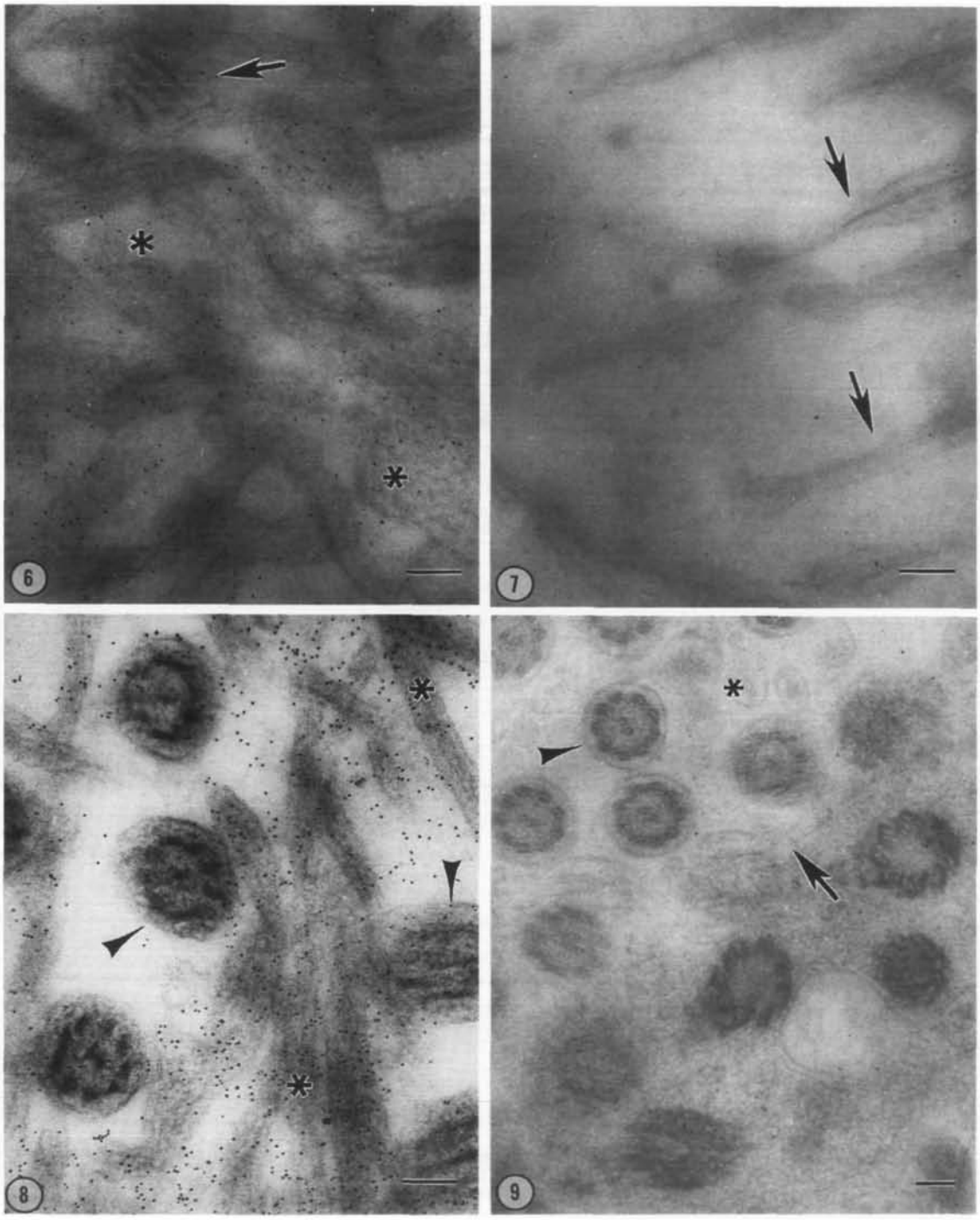

Figure 6 Supporting cell microvilli that clearly bound (asterisks) the antiserum Chan 29 to amiloride-sensitive Na^+-channels. The arrow marks a proximal part of an olfactory cilium that shows much less labeling. Scale bar: 0.1 μm.

Figure 7 Same section as that of the labeled supporting cell microvilli in Figure 6, but at the level of the tapering distal parts of the olfactory cilia (arrows), which show only a few gold grains. Scale bar: 0.1 μm.

Figure 8 *Proximal parts of olfactory cilia (arrowheads) showed much less immunoreactivity than surrounding supporting cell microvilli for* amiloride-sensitive Na^+-channels (Chan 32), also clearly visible in cross section (left hand cilia), Scale bar: 0.1 μm.

Figure 9 Respiratory cilia (arrowhead) and microvilli (asterisk) bound much less of the antibodies to amiloride-sensitive Na^+-channels (Chan 32, as used in Figure 8) than olfactory epithelial supporting cell microvilli. Arrow: apex ciliated cell. Scale bar: 0.1 μm.

is impossible to say for sure from micrographs like Figures 4 and 5 whether the much lower number of gold grains that overlay the apices of the supporting cells below the microvilli and the receptor cell structures, including cilia, reflect genuine labeling (Figures 4, 5 and 8). However, in certain areas the olfactory cilia are topographically quite remote from the supporting cell microvilli. When this was the case, it became clear that labeling of olfactory cilia is much lower, if present at all (Figures 6 and 7; see also Table 1). The same was true for microvilli of some minor populations of microvillous cells (Menco, 1992b; Pixley *et al.*, 1997) distinct from olfactory epithelial supporting cells (not shown). Labeling of respiratory cilia and microvilli was also low (Figure 9), as could even be seen with LM (Figure 3). The values presented in Table 1 show that gold grain densities in all areas mentioned above are within range of each other, apart from the value given for the supporting cell microvilli, which is much higher, roughly by a factor of 5–10. As this difference includes mucus and resin as well as cilia, this would suggest that labeling of olfactory cilia mainly reflects background.

Labeling with the dextran–ouabain–gold photoaffinity probe

We tested whether the antibody to amiloride-sensitive Na^+-channels bound to any other Na^+-transporting site. For this we elected Na^+,K^+-ATPase, which was shown to bind to olfactory cilia (Kern *et al.*, 1991). Employing a method using a photoaffinity-labeled dextran–ouabain–gold conjugate (Mrsny *et al.*, 1987) as opposed to cytochemical procedures (Kern *et al.*, 1991), we confirmed that olfactory cilia, rather than supporting cell microvilli (Figures 10–12; Table 1), possess Na^+,K^+-ATPase [because of freezing artifacts the basal region of the epithelium was not considered in this study (Phillips and Boyne, 1984; Menco, 1995)]. Labeling was also absent from microvilli of some minor populations of microvillous cells (Figure 12), and from respiratory cilia (Figure 13, Table 1) and microvilli (not shown). The quantitative evaluations presented in Table 1 further stress the distinctions presented here: density values of gold grains are 5–10 times higher in the olfactory cilia than in the other structures areas examined, including supporting cell microvilli, surrounding mucus and resin.

Immunocytochemical controls

In the olfactory epithelium the pattern of labeling was completely different from that of antibodies to olfactory signal transduction proteins, which all bound to olfactory cilia (Menco *et al.*, 1992, 1994; Menco, 1997). Omitting the primary antibody or replacement of primary antibody with normal rabbit serum gave scattered labeling or no labeling.

Discussion

Amiloride-sensitive Na^+-channels on supporting cell microvilli

This study demonstrated that antibodies to amiloride-sensitive voltage-insensitive Na^+-channels (Sorscher *et al.*, 1988; Fuller *et al.*, 1995) immunolabel the olfactory epithelial surface especially at the level of the supporting cells. Ultrastructurally these antibodies were predominantly seen bound to the microvilli of these supporting cells. Therefore, this study provides new insight into a possible function of olfactory epithelial supporting cell microvilli (see also Some technical points below).

There was accumulating evidence that (some) olfactory transduction might be mediated by an amiloride-sensitive but voltage-insensitive Na^+-channel (Frings and Lindemann, 1988; Persaud *et al.*, 1988; Lischka and Schild, 1993). The data of Frings *et al.* (1988) suggest that amiloride accesses the receptor cells via their basolateral membranes. The ultrastructural part of this study did not include observation of the basolateral parts of the supporting cells but LM did, and the antibodies used here did not label regions of basolateral membranes. Although we cannot completely exclude that structures of receptor cell apices genuinely bound some of the antibodies, albeit at a very low level, this study showed that, in areas devoid of supporting cell microvilli, cilium labeling was virtually absent and within the range of the background resin (Figure 7, Table 1). This suggests that apparent labeling in areas with microvilli is due to overlap caused by abundant microvillar labeling rather than genuine cilium labeling.

Functional implications of the present fimdings: receptor cells and supporting cells

Because of the above, the new data raise the question of whether physiological blocking of Na^+-channels by amiloride resulting in a reduced olfactory response (Frings and Lindemann, 1988; Persaud *et al.*, 1988; Lischka and Schild, 1993) might, at least in part, be caused by the action of amiloride on supporting cells instead of on receptor cells. Amiloride-sensitive channels are expressed in aldosterone-responsive cells (Rossier *et al.*, 1989; Duc *et al.*, 1994; Benos *et al.*, 1995; Garty and Palmer, 1997), taste bud cells being among these (Herness, 1992). Recent studies also support the presence of aldosterone receptors in the olfactory epithelium (Kern *et al.*, 1997). In combination with, among others, receptor cell cilium carbonic anhydrase (Brown *et al.*, 1984; Okamura *et al.*, 1996) and Na^+,K^+-ATPase (Kern *et al.*, 1991; this study), supporting cell microvilli could provide part of the buffering capacity to ensure that the surroundings of the receptor cells and their cilia are minimally affected by external variations. Therefore, the present study provides a first ultrastructural localization of an antigen with assigned function to supporting cell

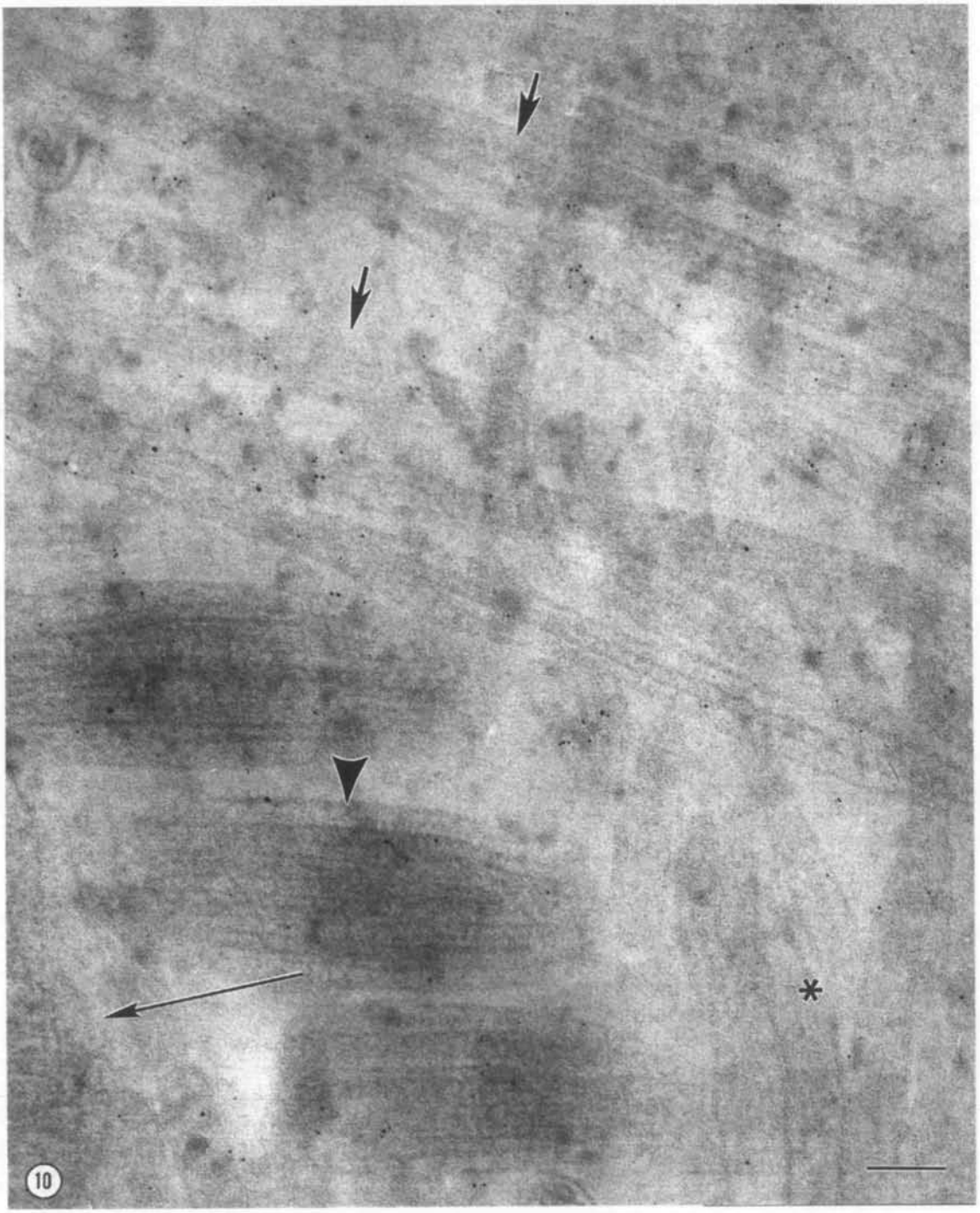

Figure 10 Proximal (arrowhead) and distal parts of olfactory cilia (small arrows) were found labeled with the dextran–ouabain–gold photoaffinity probe, localizing Na^+,K^+-ATPase; the dendritic knob (large arrow) and microvilli of surrounding supporting cells (asterisk) labeled much less, if at all. Scale bar: 0.1 μm.

microvilli. Blocking these supporting cell Na^+-channels conceivably affects receptor cell function.

The assumption that supporting cells help to balance the ionic environment around the receptor cells is reinforced by the fact that the same antibodies to amiloride-sensitive, but voltage-insensitive, Na^+-channels bound to apical regions of other cells where massive transport takes place, often also in combination with a nearby Na^+,K^+-ATPase, such as gastro-intestinal cells (Spicer *et al.*, 1989) and cells of collecting tubules in rat kidney (Ernst, 1975; Brown *et al.*, 1989; Kleyman *et al.*, 1991; for overview see Garty and Palmer, 1997). However, in contrast to olfactory epithelial

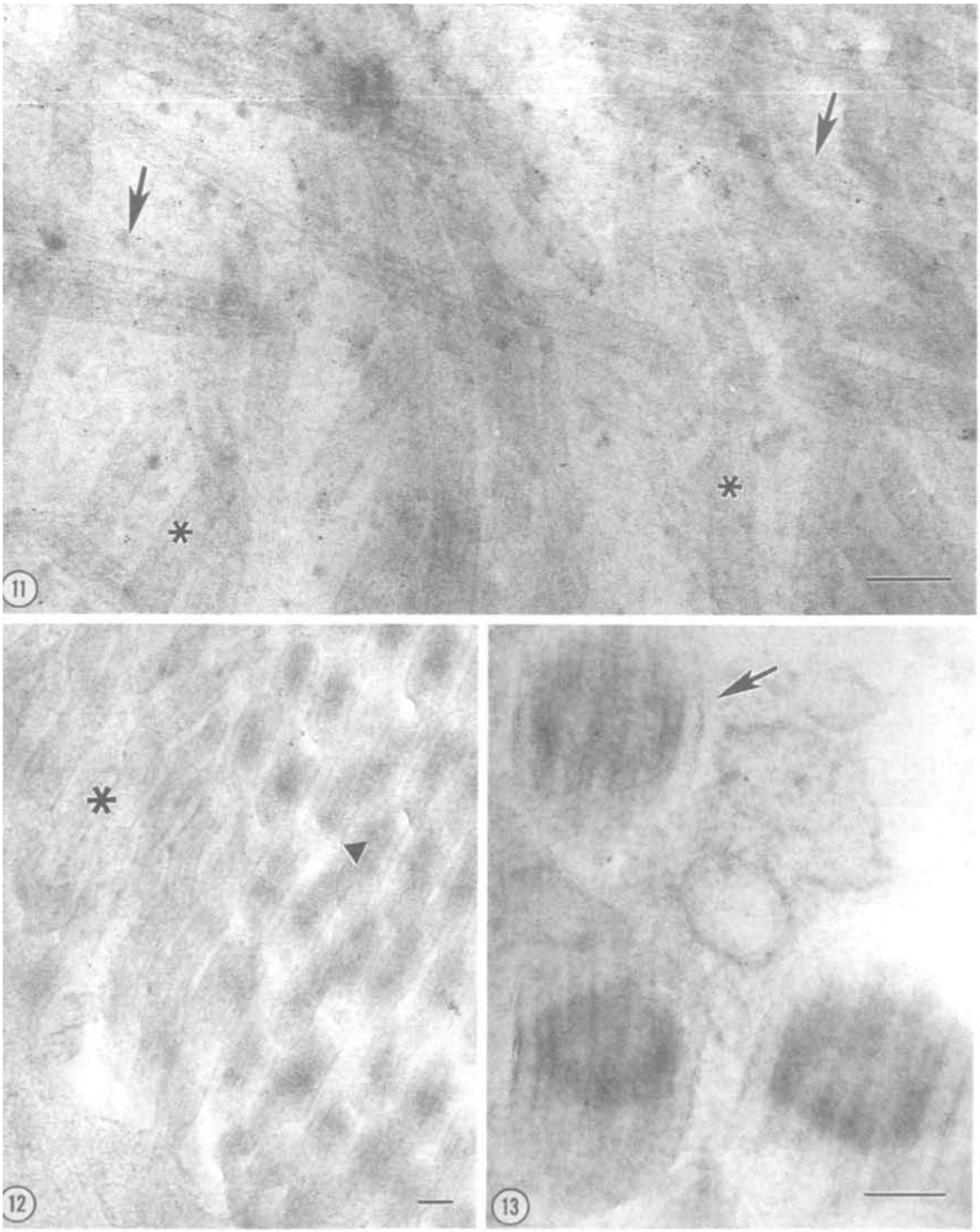

Figure 11 As Figure 10, but another example showing distal parts of olfactory cilia (arrows) that bound the dextran–ouabain–gold photoaffinity probe, whereas the supporting cell microvilli (asterisks) did not. Scale bar: 0.1 μm.

Figure 12 Like supporting cell microvilli (asterisk), brush cell microvilli (triangle) too did not bind the dextran–ouabain–gold photoaffinity probe. Scale bar: 0.1 μm.

Figure 13 Respiratory cilia (arrowhead) bound much less of the dextran–ouabain–gold photoaffinity probe complex than olfactory cilia (see Figures 10 and 11). Scale bar: 0.1 μm.

cells, Na^+,K^+-ATPase in these cells is found basolateral rather than apical.

Principal cells of collecting tubules in rat kidney have Na^+-reabsorption sites in their apical regions (Brown *et al.*, 1989). Moreover, from earlier studies (Menco, 1980, 1988) we know that dumbbell-shaped freeze-fracture particles in apices of mature supporting cells resemble such particles of apices of kidney collecting tubule intercalated cells (Brown, 1978: Brown *et al.*, 1978) and other transporting epithelia (see references in Menco, 1980). There was circumstantial proof that these particles relate to a proton pump, an H^+-ATPase, also present in apical regions of intercalated cells of collecting tubules in the kidney (Brown *et al.*, 1988; Brown, 1989; Brown and Stow, 1996). Though we were not able to show clearly the presence of this H^+-ATPase in vertebrate olfactory tissues (but see Menco, 1994), Klein and Zimmerman (1991) found this pump in apical regions of insect olfactory supporting cells. This suggests that an H^+-ATPase may also perform a function in olfaction, and could be important in mucus pH regulation and/or as an energy source for receptor potential generation (Klein and Zimmerman, 1991). Kern *et al.* (1991) suggested that Na^+,K^+-ATPase of olfactory cilia participates in the maintenance and restoration of the receptor's cell resting potential. Here, too, another function may be involvement in regulation of mucus ionic balance. Together, these findings give evidence for important roles of supporting cells in olfactory transduction: along with entities of olfactory receptor cell cilia they likely regulate the mucus ionic concentration and pH balance around receptor cell cilia.

From the literature it is known that the amiloride-sensitive Na^+-channels are present on the surfaces of trachea, bronchi and bronchioles, where they may help to hydrate airways (see review by Garty and Palmer, 1997). However, our findings suggest that immunolabeling of apical regions of the nasal respiratory epithelium was much lower than such labeling in the nasal olfactory epithelium.

A comparison with other sensory epithelia

Amiloride is known to have major effects on taste, particularly salt taste, and there are many studies to that effect (see reviews by Garty and Palmer, 1997; Lindemann, 1996). With LM, immunoreactivity to the same antibodies used here was seen all along the basolateral membranes of the taste bud cells, but also at the level of the taste bud microvilli (Simon *et al.*, 1993; Stewart *et al.*, 1995). Another study, using *in situ* hybridization techniques rather than immunocytochemistry, showed that these channels are also present in lingual non-taste-bud cells (Li *et al.*, 1994). On the basis of this finding the authors hypothesized these lingual cells as having a role in taste. All of these findings are in line with an involvement of an epithelial Na^+-channel on taste. More surprising was the localization of such channels, again using the same antibodies as the ones used here, on the modified microvilli or stereocilia of cochlear hair cells (Hackney *et al.*, 1991; Garty and Palmer, 1997). However, this is in line with the finding that such channels are present on supporting cell microvilli. Besides the speculated role of an involvement in mechanosensory transduction (see also Garty and Palmer, 1997), these channels may help to balance the mucus (endolymph) surrounding the hair cell stereocilia as proposed here for their role in olfaction.

A comparison with other supporting cell microvillous antigens

Several other antibody probes [1F4 (Pixley *et al.*, 1997) and S10/8A6 (Strotmann and Breer, 1991)] and also the lectin DBA bound rather specifically to supporting cell microvilli (Menco, 1992b), but no function of their binding sites is known. The pattern of binding of S10/8A6 is most similar to that of the antibodies to the amiloride-sensitive Na^+-channels used here (Menco, 1995), and the resemblance may suggest similarities between the various binding components. A main distribution difference between the patterns of labeling found here and those of the 1F4 antigen and also the DBA binding sites was that the latter were much more prominent on respiratory cilia and microvilli. However, like both probes used here—the antibody to amiloride-sensitive Na^+-channels and the ouabain probe to Na^+,K^+-ATPase—DBA, and the 1F4 and S10/8A6 antibodies only bound to supporting cell microvilli in the olfactory epithelium and not or much less so to microvilli of minor populations of microvillous cells distinct from supporting cells (Menco, 1992b, 1995; Pixley *et al.*, 1997).

Some technical points

Most gold particle labeling did not line membranes exactly. This lack of congruency is, in part, inherent to the techniques used here. Not all of the tissue is exposed to section surfaces in postembedding experiments, the plane of sectioning might not always have been optimal and some displacement may have occurred during experimental procedures (Menco *et al.*, 1992). Also, density values like those given in Table 1 are never absolute and depend on several factors, such as antibody affinity and steric hindrance (see e.g. Phillips and Bridgman, 1991; Menco, 1995).

As indicated in Materials and methods, the antisera caused some problems, apparently because of antibody instability. Best results were obtained in experiments where antigenic sites were least affected, making use of unfixed tissues that had been freeze-substituted. Even minor changes in the labeling method, such as inclusion of Tween 20, altered the immunolabeling pattern (see Materials and methods). Also, antibody activity deteriorated with prolonged storage and, at the level of the EM, antiserum bleeds from one rabbit (Chan) worked well whereas those of

another rabbit (Den) did not. In part, the latter may have been due to the fact that antibodies that worked well at an ultrastructural level were prepared against intact protein (rabbit Chan; antiserum obtained from rabbit Hil used for Westerns only, was also prepared in this way), whereas the ones that worked less well, at least at an ultrastructural level (rabbit Den), were prepared against denatured Na^+-channel protein. As Den antisera only were used for LM, this may also explain why labeling seemed somewhat more prominent in the respiratory epithelial surface with LM than with EM. Finally, molecular weights obtained in Western blots were somewhat lower than expected [50–60 kDa versus 75–80 kDa (Oh *et al.*, 1993; Fuller *et al.*, 1995)], suggesting that the olfactory epithelial Na^+-channel differs slightly from its renal analog, possibly in its glycosylation pattern.

Conclusion

Amiloride-sensitive but voltage-insensitive Na^+-channels are abundantly present on microvilli of olfactory epithelial supporting cells. This finding points to a major function of these microvilli in olfaction, specifically an involvement in the regulation of the salt balance surrounding the receptor cell cilia. The Na^+,K^+-ATPase of the olfactory cilia and the amiloride-sensitive Na^+-channels of the supporting cell microvilli may act jointly in this regulatory process. The salt-regulating entities noted here likely participate in maintaining a consistent mucus composition, enabling the olfactory receptor cells, including their cilia, to properly carry out their sensory functioning under various external environmental conditions. After all, the delicate cilia are uniquely exposed to the outside world, the chemical composition of which they have to perceive.

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