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Ion transport across the frog olfactory mucosa: the basal and odorant-stimulated states

Krishna C. Persaud [a], John A. DeSimone [a], Marilyn L. Getchell [b], Gerard L. Heck [a] and Thomas V. Getchell [b]

[a] *Department of Physiology, Virginia Commonwealth University, Box 551, MCV Station, Richmond, VA 23298 and* [b] *Department of Anatomy and Cell Biology, School of Medicine, Wayne State University, Detroit, MI 48201 (U.S.A.)*

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The Ussing method was adapted to study the basal electrolyte transfer as well as the events that occur upon odorant stimulation in frog olfactory mucosa. The unstimulated short-circuit current was due mainly to a furosemide-sensitive ion transport system on the apical side of the olfactory mucosa. This current was not amiloride sensitive. The current-voltage relationship of the unstimulated state was linear. That of the odorant-evoked current was non-linear and amiloride-sensitive. Ouabain caused collapse of both the unstimulated and odorant-stimulated short-circuit current. In this case, voltage-clamping the tissue to non-zero values restored the odorant-evoked current with polarity depending on that of the clamping voltage. This suggested that the direction of the current is determined by that of the sodium electrochemical potential difference. Our results indicate that the unstimulated short-circuit current occurs through an apical sodium cotransport system, while the odorant-evoked current is due to odorant-activated, passive sodium channels that are amiloride sensitive.

Introduction

The vertebrate olfactory mucosa has both sensory and secretory functions [1–3]. Both processes involve the transport of electrolytes, the respective mechanisms of which are not well understood. The sensory related ion transport mechanisms have been studied more extensively because of their presumed relationship to olfactory transduction. Takagi and co-workers [4–6] utilized isolated bullfrog olfactory mucosa to study the effects of ion replacement in the bathing medium on the odorant-induced voltage transient, the electro-olfactogram (EOG). They inferred that a sodium current plays a major role in transduction, although it was not measured directly. Odorants can also stimulate secretion from sustentacular cells [3,7–9], and can evoke changes in the intracellular potential of receptor [1,23,24] and sustentacular cells [10,23]. In addition, there is an active basal electrolyte transfer in the olfactory mucosa in the absence of odorants [11,12].

In this paper we have studied both states of the system under voltage-clamp in vitro utilizing a new adaptation of the Ussing method [14]. We show that odorant-evoked currents occur in parallel with and superimposed on the basal current. The ionic mechanisms associated with the unstimulated and odorant-stimulated currents across

Correspondence: J.A. DeSimone, Department of Physiology, Virginia Commonwealth University, Box 551, MCV Station, Richmond, VA 23298, U.S.A.

the olfactory mucosa are distinct and readily separable pharmacologically.

Materials and Methods

Tissue preparation, mounting, and odorant presentation

Adult bullfrogs (*Rana catesbeiana*) were anesthetised with 0.4% ethyl *m*-aminobenzoate (MS 222) for 30 min. The superficial skin, connective tissue, bone and cartilage overlying the dorsal olfactory mucosa were excised. The olfactory mucosa was then dissected as a single sheet of tissue. In a few cases the ventral olfactory mucosa was also removed. Tissues were placed on a sessile drop of oxygenated amphibian Ringer's solution, ciliated side up. The serosal side was supported by a nylon mesh backing (100 μm mesh) that was on a silicone rubber gasket. A lucite washer, lightly coated with a cyanoacrylate adhesive, was then fitted over the tissue so that after curing, the mucosa was sealed to the washer along its edges. The effective diameter of the tissue after mounting was 3.1 mm. The assembly was then placed between two Ussing chambers held against the washers by silicone rubber gaskets secured by a screw brace. The chamber volumes were 0.5 ml on the ciliated side and 1.5 ml on the submucosal side of the olfactory tissue. The tissue was maintained at room temperature (22° C) in oxygenated amphibian Ringer's solution. The solution on the ciliated side was replaced continuously at a flow rate of 0.2 ml/s via a constant pressure head flow system. Odorants were presented in aqueous solution to the ciliated side of the tissue by switching an odorant-charged loop of Ringer's solution into the flow system by means of a 2-gang-3-way valve. This method is analogous to that used in fast protein liquid chromatography for introducing samples. The stimulus profile was measured by placing a photodarlington transistor in the position where the tissue is normally seated and following the change in light absorbance when a blue dye (5,5'-indigosulfonic acid) was used instead of an odorant.

Electrical measurements

The transmucosal potential difference and short-circuit current were recorded using the methods described by Heck et al. [11]. Potentials were recorded with saturated calomel electrodes connected to each reservoir with 3% agar/0.15 M NaCl salt bridges. Small asymmetry potentials (< 0.5 mV) were nulled to zero and the solution resistance was compensated for automatically. Current was passed using Ag/AgCl electrodes connected to the reservoirs with NaCl/agar bridges. The tissue was voltage clamped using a Physiologic Instruments VCC 600 apparatus and the output was recorded with a strip chart recorder (Linseis 7045) equipped with a potentiometer backoff for the necessary scale expansion.

Chemicals and solutions

All salts were reagent grade. Amphibian Ringer's solution contained 100 mM NaCl, 5 mM $MgCl_2$, 2.5 mM KCl, 2.5 mM $CaCl_2$, 1.1 mM Na_2HPO_4, 0.4 mM NaH_2PO_4 and 10 mM glucose (pH 7.3 at 22° C). This was kept oxygenated using 100% O_2. *N*-Methyl-D-glucamine, furosemide, and ouabain were from Sigma Chemical Co., St. Louis, MO. Amiloride was a gift from Dr. E.G. Cragoe of Merck, Sharp and Dohme. When *N*-methyl-D-glucamine was used in ion substitution experiments, it was first titrated to neutrality with HCl forming the *N*-methyl-D-glucammonium ion. When a sodium free Ringer's solution was necessary the sodium phosphate salts were exchanged for the corresponding potassium salts, and the amount of KCl present was reduced to keep the total potassium concentration constant.

The odorants 1,8-cineole and amyl acetate were obtained from Aldrich Chemical Co., Milwaukee, WI. 2-Isobutyl-3-methoxypyrazine was obtained from Pyrazine Specialities, Inc., Atlanta, GA. This was stored in amber glass bottles at 4° C in the dark. Stock solutions of odorants (0.1 M) were made up in methanol. Working solutions were obtained by dilutions into amphibian Ringer's solution so that the concentration of methanol was typically 0.01% but never more than 0.1%. Changes in standing short-circuit current were not observed in control experiments when 0.1% methanol was delivered to the sensory epithelium. This is in agreement with the results of Menevse et al. [15] who used 0.5% methanol in Ringer's solution on the frog olfactory mucosa without affecting the electro-olfactogram.

Results

Steady-state parameters in amphibian Ringer's solution

After mounting the olfactory mucosa in the Ussing chamber, it was bathed symmetrically in oxygenated Ringer's solution and allowed to achieve a stable open-circuit potential usually within 20 min. This slowly declined over the next several hours, the short-circuit current decreasing about 10% per hour. Tissues that had been damaged during dissection and mounting generally showed a sharp decline in potential at this stage and were discarded. All preparations gave evidence of electrogenic active ion transport. The mean values ($\pm$S.D., $n = 18$) of the open-circuit potential, short-circuit current, and transmucosal resistance were respectively: -3.55 ± 0.43 mV (ciliated side electronegative), 53.0 ± 14.5 μA/cm^2, and 67 ± 26.5 ohm · cm^2. The current-voltage relation was linear for each tissue over the range $\pm$ 10 mV. Fig. 1 shows a representative current-voltage relation. These parameters and the linearity of the current-voltage relation are in agreement with our previous results [11,12]. Both dorsal and ventral olfactory mucosae had linear current-voltage relations with similar slopes.

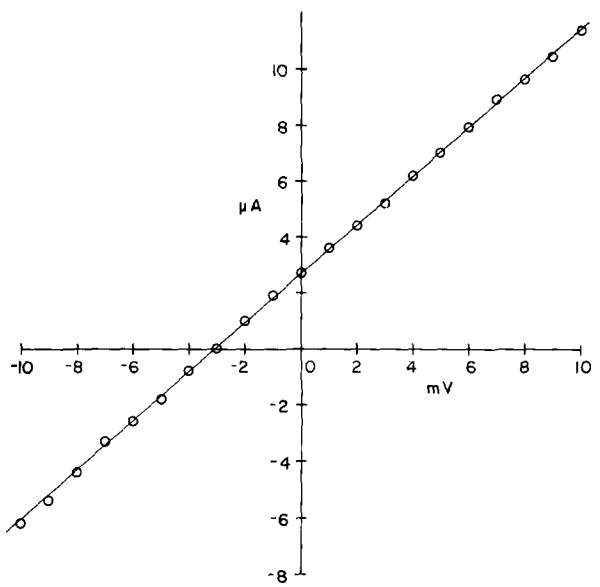

Fig. 1. Current-voltage relationship of the bullfrog olfactory mucosa. The olfactory tissue was mounted in the Ussing chamber and bathed symmetrically with oxygenated amphibian Ringer's solution. It was voltage-clamped and the transmucosal current was recorded. Voltage pulses between -10 and $+10$ mV were applied for 1 s and the resulting current transient measured. The figure shows a current-voltage relation representative of 18 tissue preparations. The effective area of tissue was 0.0755 cm^2. The open-circuit potential in this case was -3.2 mV (ciliated side electronegative), the short-circuit current 36 μA/cm^2, and the conductance was 11.6 mS/cm^2 giving a resistance of 86.1 ohm·cm^2.

Short-circuited response to odorants

Fig. 2a shows the time profile of the pulse of odorant solution introduced into the reservoir measured with the dye/phototransistor method described above. The stimulus concentration reached a peak within 1.2 seconds, was constant over a 9 s period and declined to zero in 30 s. There were no current changes when Ringer's solution or 0.1% methanol in Ringer's solution were delivered to either side of the tissue. Figs. 2b–e show representative responses to 10^{-4} M 1,8-cineole. The response in many cases was characterized by a steep rise in the inward positive short-circuit current, a sharp decline within 5 s (while the stimulus concentration was still at a constant magnitude), a plateau followed by a slow decline of the current towards baseline (Fig. 2b). When other odorants were presented at 10^{-4} M at 5-min intervals the responses were reproducible within 10% for at least 2 h, and lower amplitude responses could be observed even after 5 h. With an aging tissue preparation, the kinetics of the response became slower, and the amplitude decreased with each odorant stimulation. Repeated odorant presentations caused changes in the response profile (Fig. 2c). There was a gradual emergence of a pronounced slower second component. At first it was superimposed on the fast initial current response, but eventually overtook it and became greater in magnitude than the first component. This slow secondary response might take several minutes to return to baseline current after the stimulus had been washed out of the system. The response kinetics and amplitude typically varied in different tissue preparations. Responses of slower kinetics at the beginning of a recording period are shown in Fig. 2d and after repeated odorant stimulation in Fig. 2e. Such preparations, especially during the winter months, had a layer of

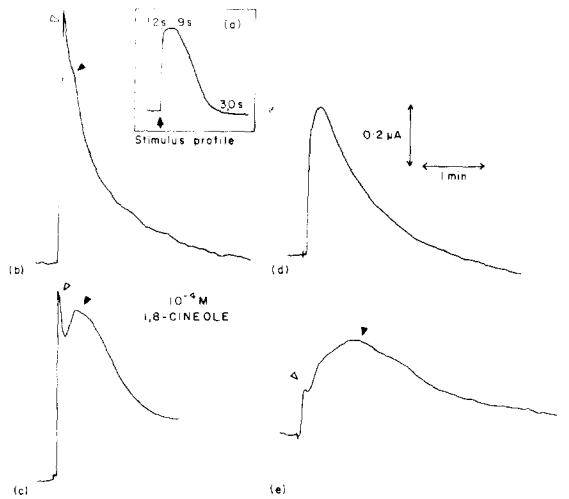

Fig. 2. Odorant-evoked current transients recorded to 10^{-4} M 1,8-cineole. (a) The inset shows the time profile of the pulse of odorant solution introduced into the reservoir facing the ciliated side of the mucosa. This was measured by replacing the mucosa with a photodarlington transistor and introducing a pulse of blue dye (5,5′-indigosulfonic acid) into the reservoir and measuring the current change across the phototransistor when the system was under constant illumination. The arrow shows the onset of the pulse. The peak amplitude was reached after 1.2 s, this peak was maintained for 9 s and about 30 s were required for complete return to the baseline.
(b) The trace shows the odorant-evoked current transient recorded from the olfactory mucosa when a pulse of 10^{-4} M 1,8-cineole was presented. The response shows a steep rise in inward positive short-circuit current (open arrowhead), a sharp decline towards a plateau while the stimulus is still present at constant magnitude (closed arrowhead), and a slow decline of the current towards baseline.
(c) The profile of the odorant-evoked current after repeated odorant presentation in the same tissue preparation is shown. The trace shows a fast component (open arrowhead) and the emergence of a pronounced slower second component (closed arrowhead).
(d) The trace shows the odorant-evoked current from a different tissue preparation where the response kinetics are slower than that of (b).
(e) The trace shows the initial fast component (open arrowhead) followed by emergence of an even slower second component (closed arrowhead) after repeated odorant stimulation of the tissue preparation shown in (d).

viscous mucus on the ciliated surface. This may have increased odorant diffusion times, giving rise to slower responses.

We recorded short-circuit current responses from the olfactory mucosa when compounds that have markedly different odours and chemical properties (1,8-cineole (eucalyptus), amyl acetate (fruity), 2-isobutyl-3-methoxypyrazine (green peppers)) were each delivered as a defined pulse. Fig. 3 shows the concentration-response functions of these compounds for the initial rapid component of the odorant-induced current recorded from different tissue preparations. For each odorant, the tissue exhibited a different dose-response function. The tissue had a higher sensitivity to 1,8-cineole than to the other odorants tested. Both amyl acetate and 2-isobutyl-3-methoxypyrazine gave dose-response relations which were shifted to

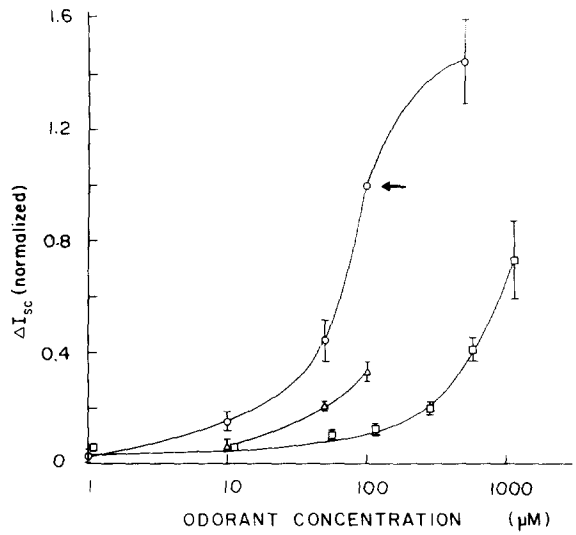

Fig. 3. Concentration response for 1,8-cineole, amyl acetate and 2-isobutyl-3-methoxypyrazine. The voltage-clamped olfactory mucosa was presented with pulses of increasing concentrations of odorants and the odorant-evoked current transient recorded. Prior to each presentation of a particular odorant concentration, the response to a pulse of 10^{-4} M cineole was recorded. This was used as an internal control to adjust for the gradual decrease in response over the time course of the experiment as well as to normalize the amplitudes of the responses to other odorants so that different preparations might be directly compared. The amplitude of the initial fast component of the current transient was obtained for a given concentration of odorant. For each preparation the current was normalized by dividing by the current evoked by 10^{-4} M cineole. This normalized current (ΔI_{sc}) is plotted as a function of the odorant concentration. Each point is a mean value ($\pm$S.D.) representing three preparations for cineole and amyl acetate and five preparations for 2-isobutyl-3-methoxypyrazine. The arrow indicates the reference concentration of 1,8-cineole for which the normalized current is unity. ○, 1,8-cineole; △, amyl acetate; □, 2-isobutyl-3-methoxypyrazine.

the right along the concentration axis relative to the curve for cineole. In all cases the response was an increasing function of concentration over the entire range. We did not attempt to define the thresholds, which are related to the signal-to-noise characteristics of the preparation and apparatus. Saturation was demonstrated for 1,8-cineole but not for the other odorants. This may reflect the fact that high concentrations of some odorants (> 1 mM) elicited initially a large response but subsequently were unable to stimulate the tissue. Under these conditions the responses may also be associated with nonspecific increases in membrane permeability.

Effect of an externally imposed transmucosal potential

The olfactory mucosa was voltage clamped between -20 mV and $+40$ mV (relative to the submucosal side). The unstimulated transmucosal current was proportional to the voltage. The mean conductance of the preparation shown in Fig. 4a was 10.1 mS/cm^2 $\pm$ 0.2 (S.D.) In all cases the values of the open-circuit potential, short-circuit current, and the resistance were consistent with the mean values ($\pm$S.D.) quoted above, and with the *I-V* relation using pulsed voltage excursions (Fig. 1). This suggests that the transmucosal resistance is due mainly to paracellular shunts or low resistance pathways through sustentacular cells [10] but probably not through the higher resistance receptor neurons [16]. In contrast, the *I-V* relationship of the odorant-evoked current was nonlinear (Fig. 4b). The odorant-stimulated inward current transient increased in amplitude as the potential became more negative. This showed a linear relationship with applied voltage (Fig. 4b, left branch) with a conductance of 0.075 mS/cm^2 $\pm$ 0.015 (S.D.). When voltage was clamped in the reverse direction so that the ciliated side was positive with respect to the submucosal side, the odorant response decreased in amplitude and underwent a transition when the external clamping voltage was about 15 mV (arrowhead, Fig. 4b, right branch). The current transients were of opposite polarity. They were linearly proportional to the applied voltage but with the larger mean conductance of 0.19 mS/cm^2 $\pm$ 0.07 (S.D.). The volt-

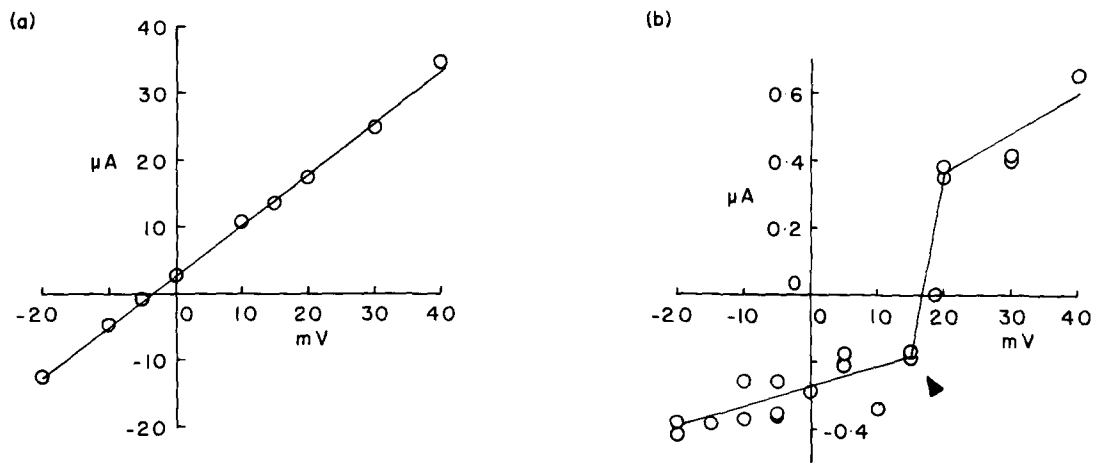

Fig. 4. Effect of an externally imposed transmucosal potential. (a) *I–V* relationship of the unstimulated current under a stable clamping voltage. The olfactory mucosa (0.0755 cm^2) was clamped between -20 mV and $+40$ mV and the resulting transmucosal current recorded. The mean conductance of the tissue preparation shown was 10.1 mS/cm^2 $\pm$0.2 (S.D.).
(b) *I–V* relationship of the odorant-evoked current response. The olfactory mucosa was voltage clamped to potentials between -20 mV and $+40$ mV and the amplitudes of the current transient evoked were measured for each clamping voltage when a pulse of 10^{-4} M 1,8-cineole was applied to the ciliated side. The data plotted are the pooled results from five tissue preparations. The amplitude of the odorant-evoked current transient at short-circuit for each preparation was normalised to that of an arbitrarily chosen preparation and the data points for each preparation scaled to this value. The figure shows that the *I–V* relationship is nonlinear and undergoes an abrupt transition at a clamping voltage of 15 mV (closed arrowhead). The conductance of the linear portion (left) was 0.075 mS/cm^2 $\pm$0.015 (S.D.), while that on the other portion (right) was 0.19 mS/cm^2 $\pm$0.07 (S.D.).

age dependence of the odorant-evoked current and its reversal suggest that a passive channel is opened upon stimulation and that the direction of the response is determined by the direction of the driving force for passive electrodiffusion. This is discussed further below.

Effect of ouabain

When 10^{-4} M ouabain was introduced into the submucosal bath of an olfactory tissue preparation, the standing short circuit current decreased exponentially to zero with a rate constant of 0.027 $\pm$ 0.006 min^{-1} ($n = 3$). Accordingly after one hour the standing short-circuit current decreased more than 80%. This was significantly greater than the spontaneous loss of standing current, which as noted previously, declined approximately 10% per hour. Ouabain was effective only from the submucosal side. It is well established that the standing short-circuit current is a direct measure of transmucosal active ion transport under symmetrical bath conditions. Inhibition by ouabain indicates that the active part of the ion transport process is sustained by a metabolically driven sodium pump that depends on a $(Na^+ + K^+)$-ATPase located on the submucosal side of the tissue. Ion fluxes across the apical membranes are probably passive, driven by forces that depend either directly or indirectly on the maintenance of a trans-apical membrane Na^+ ion electrochemical potential gradient.

Although the transmucosal voltage was held at zero, the voltages across the apical and basolateral membranes of all cell types were, of course, unclamped. Intracellular recordings from receptor cells [16,23,24] and sustentacular cells [10,16,23] under open circuit indicate that cell interiors are electronegative. This is also likely the case under tissue short circuit. In addition the sodium pump maintains the intracellular sodium ion concentration below that of extracellular fluids. A trans-apical membrane sodium ion electrochemical potential difference is therefore maintained even when the tissue as a whole is short-circuited. The energy in the sodium gradient may support a variety of trans-apical membrane passive ion transport mechanisms. If sodium channels are available, both the chemical and electrical gradients will drive sodium into the cells. The electrical gradient will drive anions out of the cells via appropriate channels. The sodium chemical potential gradient can also drive a variety of sodium-coupled electroneutral ion transport processes, given the appropriate transporter.

It is notable that the amplitude of the odorant response also decreased during the time that ouabain was decreasing the standing current. Figs. 5a and 5b show the responses to 10^{-4} M 1,8-cineole before and 45 min after ouabain treatment, respectively. The loss of response to odorant may reflect the collapse of both the sodium chemical potential difference and the electrical potential difference across the apical membranes of the olfactory cells. This would occur as a consequence of ouabain inactivation of the sodium pumps on the submucosal sides of the receptor cells. The disappearance of an odorant response would be expected if odorant-evoked currents depend on an increase in conductance of a passive ion channel located in the apical membranes of receptor cells.

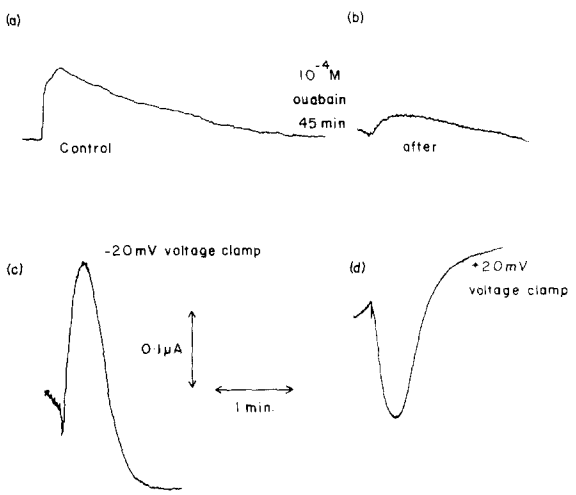

Fig. 5. Effect of ouabain. 10^{-4} M Ouabain was introduced into the submucosal reservoir. The standing short-circuit current dropped exponentially to zero with a rate constant of 0.027 ± 0.006 min^{-1} ($n = 3$). The figure shows (a) the response to cineole prior to the introduction of ouabain, and (b) the response 45 min later. At this point the tissue was voltage clamped to -20 mV and the odorant-evoked current transient recorded (c); the tissue was then clamped to $+20$ mV which gave the odorant-evoked transient shown in (d). The results show that an inward positive current response occurred when clamped to -20 mV (c) that was reversed when the tissue was clamped to $+20$ mV (d).

Following sodium pump inhibition, the electrochemical potential difference for sodium ion across the apical membrane will approach zero asymptotically. If a nonequilibrium state can be reestablished, the odorant response should be restored even in the presence of ouabain. This was achieved by fixing a biasing potential across the olfactory mucosa that would perturb electrochemical equilibrium. Furthermore, the direction of the odorant-evoked current should now depend on the direction of the perturbation from equilibrium. To test these hypotheses we measured the odorant response in the ouabain treated tissue when clamped to −20 mV and +20 mV. With the standing short-circuit current at zero after treatment with ouabain, the tissue was voltage clamped so that the ciliated side was either −20 mV (Fig. 5c) or +20 mV (Fig. 5d) with respect to the submucosal side. The tissue was then stimulated with 10^{-4} M 1,8-cineole. Figs. 5c and 5d show an inward current response with −20 mV bias and an outward current response with +20 mV bias, respectively. This supports the hypothesis that the odorant-activated current pathway is a passive channel (probably for sodium) through which the direction of ion transport depends solely on the ion electrochemical gradient.

Effect of amiloride and furosemide on the unstimulated short-circuit current

Fig. 6 shows the effects of 10^{-4} M amiloride and furosemide on the unstimulated standing short-circuit current. Amiloride bathing either the ciliated or submucosal sides resulted in negligible change after 10 min of exposure. Furosemide added to the ciliated side reduced the current by 66% in 5 min. It was not effective on the submucosal side. The effects of furosemide were reversible; when washed with Ringer's solution, the short-circuit current returned to normal within 40 min. These results show that the unstimulated current is dominated by a furosemide-sensitive apical ion transport pathway.

Effect of amiloride on the odorant induced current transient

In contrast to the unstimulated current, the current evoked by an odorant was inhibited rapidly and reversibly by amiloride. Amiloride (10^{-4} M)

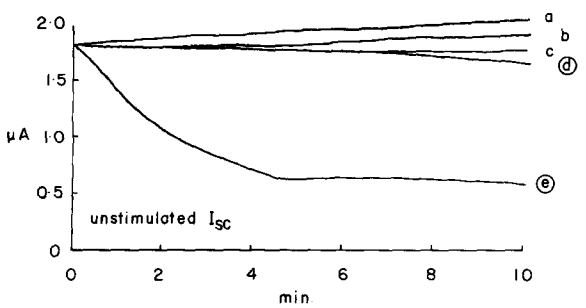

Fig. 6. Effect of amiloride and furosemide on the standing short-circuit current. The figure shows the short-circuit current over a 10 min period. Drugs were added at time zero. (a) untreated, (b) 10^{-4} M amiloride introduced into the submucosal reservoir, (c) 10^{-4} M amiloride added on the ciliated side, (d), 10^{-4} M furosemide added on the submucosal side, (e), 10^{-4} M furosemide added on the ciliated side. Furosemide on the ciliated side reduced the short-circuit current by 66%; when the tissue was then bathed symmetrically with Ringer's solution the standing current slowly recovered within the next 40 min. Each trace shown is representative of three preparations.

elicited no current transient when presented as a pulse to the ciliated surface (cf. Fig. 6c). When a mixture of 10^{-4} M amiloride and 10^{-4} M 1,8-cineole in Ringer's solution was presented, the response amplitude decreased progressively with repeated stimulations (Figs. 7b, c, d) to less than 25% of the control within 15 min. This effect was fully reversible within 15 min. Amiloride concentrations of 10^{-5} M and less also partially inhibited the odorant-evoked response. This is the first direct evidence that the odorant-activated ion channel associated with olfactory transduction is an amiloride-sensitive channel. The immediate action of amiloride at concentrations of 10^{-4}M and less and its complete reversibility are consistent with its well characterized action as a specific blocker of apical membrane sodium channels in epithelial tissues [17]. What is unique in this instance is that these channels are ligand activated, i.e., they are only in evidence when an odorant is introduced on the apical side of the mucosa.

Effect of furosemide on the odorant-induced current transient

Furosemide (10^{-4} M) caused a slow decrease in the standing short-circuit current when presented to the ciliated side as a pulse (Fig. 8e) using the odorant delivery system. Following a fur-

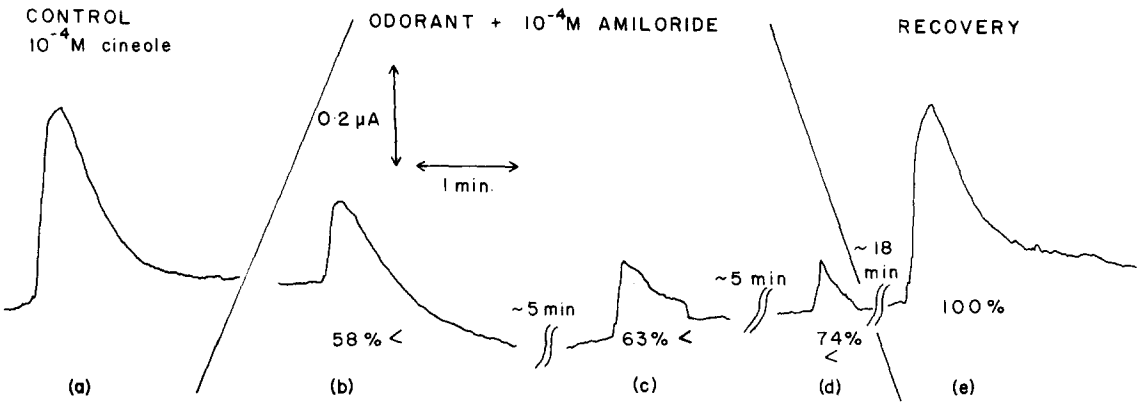

Fig. 7. Effect of amiloride on the odorant-evoked current transient. The traces show (a) control response to 10^{-4} M 1,8-cineole, (b–d), responses to repetitive pulses of the odorant $+10^{-4}$ M amiloride at 5 min intervals showing decreases in amplitude of 58%, 63% and 74% with successive pulses, and (e) recovery of the response when a pulse of the odorant alone was presented after 18 min, all in the same tissue preparation. The same effect was observed in five tissue preparations.

osemide pulse, the current returned to its orginal value within 15 min. The decline in baseline current due to furosemide occurred without significant latency. As shown in Fig. 8b, furosemide (10^{-4}M) and 1,8-cineole (10^{-4}M) presented together produced a response which preserved the early fast component of the current, but markedly truncated the second component. When 1,8-cineole was again presented, there was partial recovery of the original appearance of the response (Fig. 8c) and of the baseline current. As noted in Fig. 2, it was often possible to distinguish two components in the odorant-evoked current transient. It appears that the first of these is furosemide-insensitive while the second or slower component is strongly suppressed by furosemide. The results suggest that the early and late parts of the response involve different ion transport mechanisms.

Sodium ion replacement with N-methyl-D-glucammonium

(a) Unstimulated short-circuit current. Unlike earlier preparations of the isolated olfactory mucosa [4–6], the present system permits unilateral changes in the reservoir composition on each side of the mucosa. The presence of an electropositive submucosal potential under conditions of symmetrical ion composition and the unilateral effect of furosemide suggest that the standing current is at least in part due to inward sodium ion transport. To investigate this further we replaced sodium on the ciliated side with the large cation *N*-methyl-D-glucammonium, while keeping the submucosal side in normal amphibian Ringer's solution. In this case the unstimulated inward positive short-circuit current decreased and eventually went negative as the concentration of Na^+ in the external bathing medium was lowered (Fig. 9a). The current was restored when the ciliated side was again bathed in Ringer's solution, suggesting that Na^+ absorption is an important contributor to the total ion flux across the olfactory mucosa. In support of this, when the medium bathing both sides of the mucosa was replaced with sodium free Ringer's solution, the unstimulated short circuit current was zero. *N*-methyl-D-glucammonium appeared to be biologically inert to the preparation since the effect of Na^+ replacement was reversible. This was not the case when choline or tetraethylammonium cations were used. These caused degradation of both the standing short-circuit current and the odorant-evoked response.

(b) Odorant-stimulated short-circuit current in sodium-depleted medium. Fig. 9b shows that progressive replacement of Na^+ in the medium bathing the ciliated side of the mucosa had no immediate effect on the odorant-stimulated short-cir-

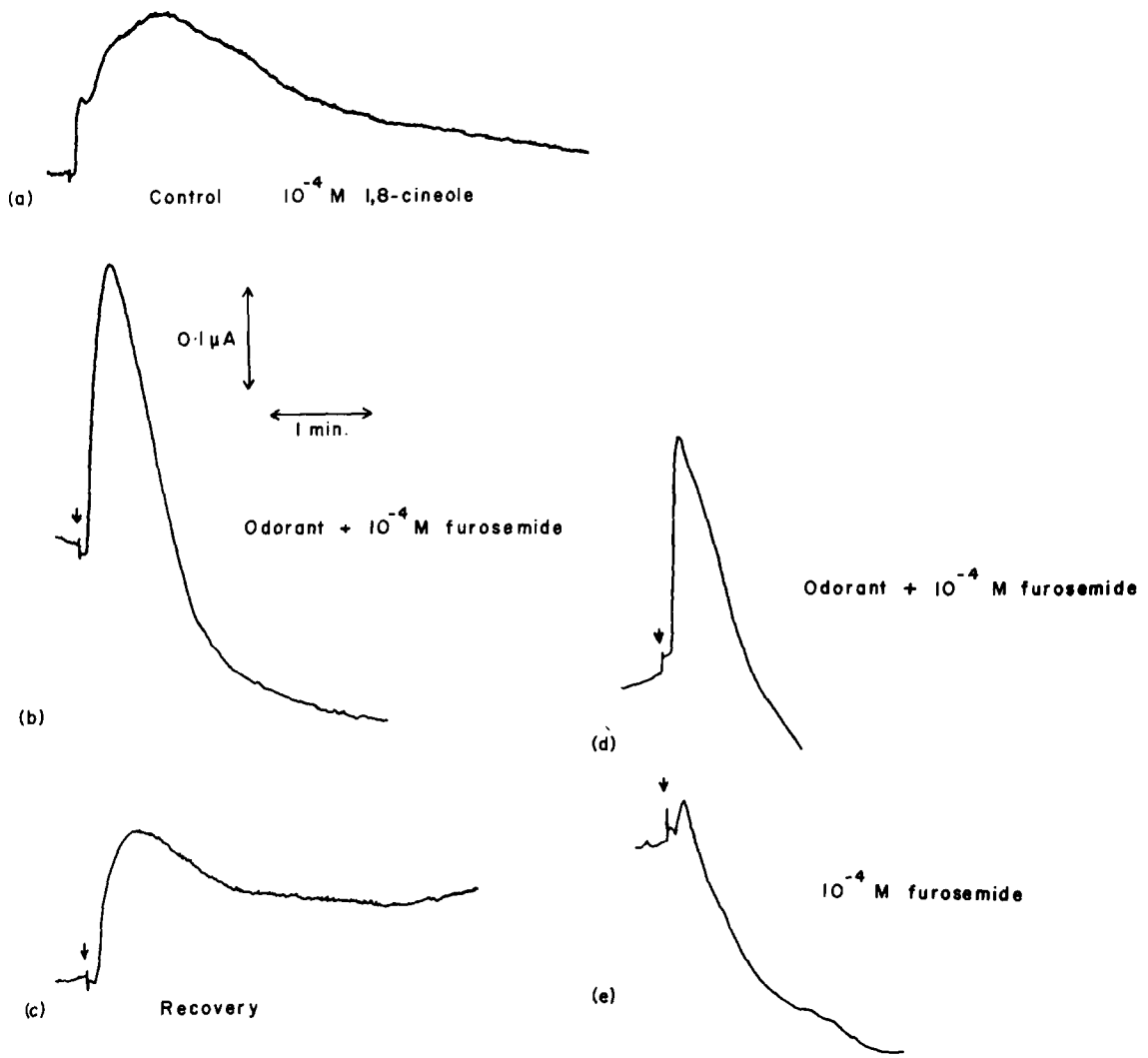

Fig. 8. Effect of furosemide on the odorant-evoked current transient. The traces show: (a) control response to 10^{-4} M 1,8-cineole; (b) response when the mixture of odorant and 10^{-4} M furosemide were presented showing truncation of the current transient and decrease in baseline short-circuit current; (c) partial recovery of the response and baseline after 15 min when odorant alone was presented; (d) in a different tissue preparation the effect of a mixture of odorant and furosemide on the response; this compares with the trace shown in (b); (e) the effect of a pulse of 10^{-4} M furosemide, showing an immediate decrease in the basal current. The traces shown are representative of four tissue preparations.

cuit current. At 75% replacement the amplitude of the response increased by 20% but after total replacement the odorant response was comparable to the controls prior to ion substitution. We had shown earlier that the odorant-evoked current transient was mediated by an amiloride-blockable mechanism, probably a sodium channel (Fig. 7). Thus, it would be anticipated that the response would diminish if Na^+ ion were removed from the cilated side. It was found that the odorant response after 100% sodium substitution was still reversibly blocked by amiloride, suggesting the continued presence of a sodium current (Figs. 9b and 10). Amiloride sensitivity would indicate that Na^+ ions are still present in the microenvironment of the ciliated side of the epithelium probably

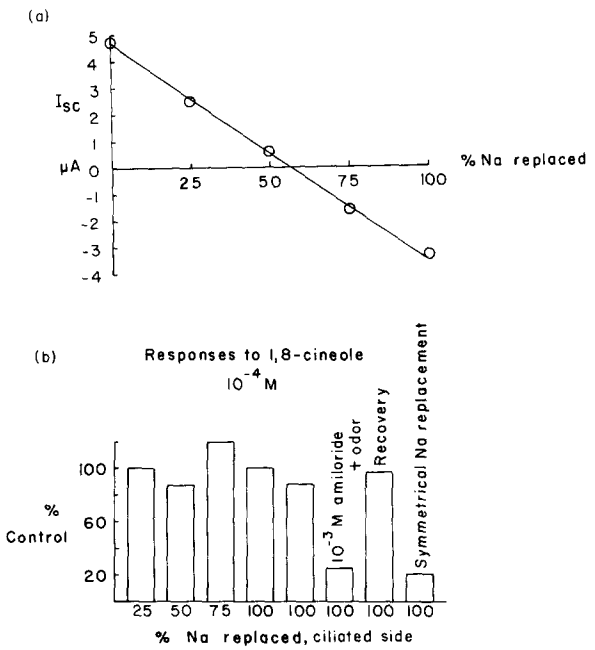

Fig. 9. Sodium ion replacement with *N*-methyl-D-glucammonium. (a) The figure shows the unstimulated short-circuit current (I_{sc}) plotted as a function of % sodium concentration replaced by *N*-methyl-D-glucammonium on the ciliated side, showing a decrease in the inward positive current as a function of lower sodium concentrations. The polarity of the current was reversed at about 45 mM Na^+ in the bathing solution. (b) The figure shows the responses to 10^{-4} M 1,8-cineole plotted relative to the control response with Ringer's solution on both sides of the mucosa, after each sodium replacement. There was little change in the amplitudes of the responses. After 100% sodium replacement on the ciliated side, a mixture of odorant and 10^{-3} M amiloride was presented that decreased the response to 25% of control, and the effects of which were completely reversible as shown. When 100% sodium replacement was carried out on both sides of the mucosa, the response was initially 21% of the control, which decreased with further stimulation and was partially recoverable when the tissue was again bathed in symmetrical Ringer's solution. Similar effects were observed in four tissue preparations.

supplied by the submucosal reservoir. It should be noted that at 100% nominal sodium replacement there was no evidence of a second or slow component in the response. Fig. 9a shows that at 100% nominal sodium replacement the standing short-circuit current was reversed. Under these conditions short-circuit was maintained by passing an inward (mucosa to submucosa) current. The possibility that a sodium flux associated with the fast component might still be inward, although sodium replacement in the apical bath was nominally complete, can be explained by the inward direc-

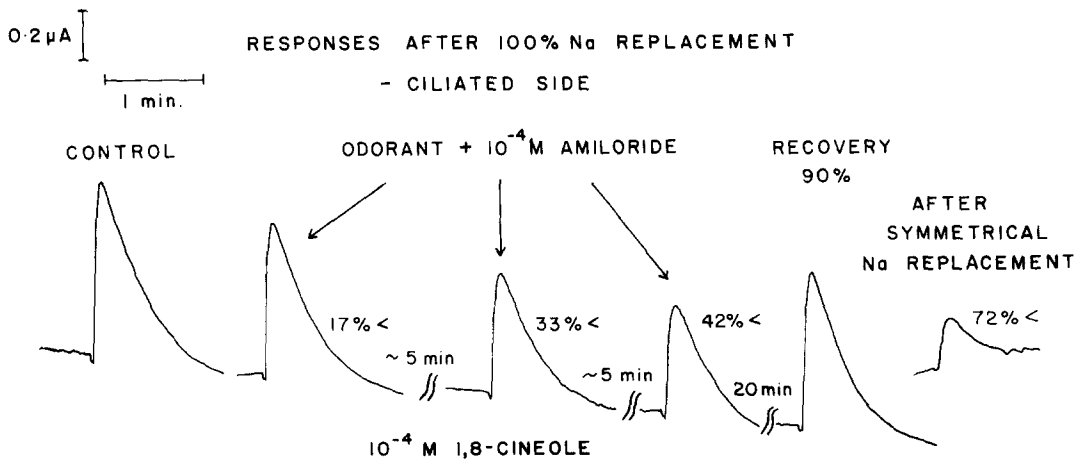

Fig. 10. Effect of amiloride on the odorant-evoked current in sodium free medium on the ciliated side of the mucosa. The ciliated side of the mucosa was bathed in Ringer's solution in which all sodium ions had been replaced by *N*-methyl-D-glucammonium. The response to 10^{-4} M 1,8-cineole after 100% sodium replacement is the control. Responses to repeated presentations of a mixture of odorant and 10^{-4} M amiloride at 5-min intervals showed decreases in amplitude of 17%, 33% and 42% with each subsequent stimulation. 90% Recovery of the response was achieved when odorant alone was presented after 20 min. A 72% decrease in the response was observed when sodium was replaced on both sides of the mucosa.

tion of the clamping current. This applied current would then serve to drive sodium inward in the event that an odorant increased sodium channel conductance. That sodium ion was still a major factor in the odorant-evoked current was proved by bathing both sides of the mucosa in sodium free Ringer's solution. In this case, the odorant-evoked current was significantly diminished and eventually disappeared, and could be recovered only when the system was replenished with sodium. This result shows that the local sodium concentration on the apical side is significantly sustained by passive sodium movement from submucosal sources. The mechanism of transport of Na^+ ions is probably diffusion through the paracellular shunts and/or secretion from glands or sustentacular cells [3,7–9].

Replacement of Na^+ with K^+

The medium bathing the ciliated side of the mucosa was progressively replaced with Ringer's solution containing K^+ substituted for Na^+. In contrast to sodium replacement with *N*-methyl-D-glucammonium, the unstimulated inward positive short-circuit current increased by about 25% when all the sodium had been replaced. This suggests that unlike *N*-methyl-D-glucammonium, which is apparently impermeable, there are potassium transport pathways across the olfactory mucosa. Recent work by Trotier and MacLeod [10], showed that the sustentacular cells are depolarized by high apical K^+ ion concentrations, suggesting K^+ channels in sustentacular cells. Path-clamp studies by Maue and Dionne [18] also showed that olfactory receptor neurons have Ca^{2+}-activated K^+ channels. Thus it is likely that with elevated K^+ concentrations, the transmucosal K^+ pathways in both olfactory receptor and sustentacular cells are operative. It can also be seen in Fig. 11 that odorant-evoked current transients were markedly reduced when the apical K^+ concentration was elevated. This is also consistent with the earlier experiments that indicated that transduction involves the activation of sodium channels. High K^+ levels evidently depolarize both receptor and sustentacular cells, further reducing the driving force for inward sodium movement. The precise role of the apical K^+ channels in olfactory transduction is unknown. We observed partial recovery of both standing short-circuit current and odorant response when the ciliated side was again bathed in normal amphibian Ringer's solution. Replacement of Na^+ with K^+ on both sides of the mucosa caused loss of both standing current and odorant-induced current.

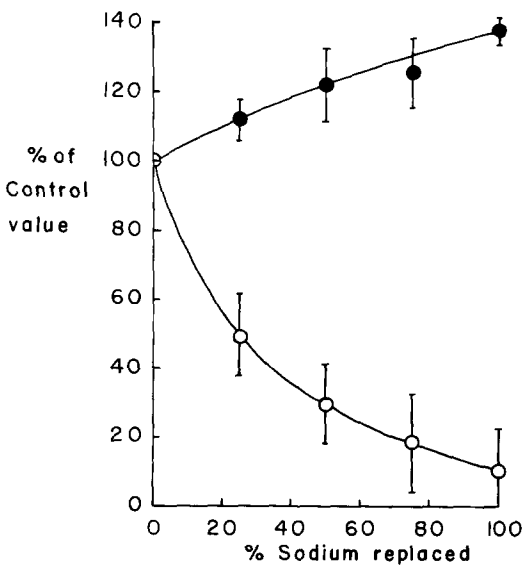

Fig. 11. Replacement of Na^+ with K^+. The sodium ions in the medium bathing the ciliated side of the mucosa were replaced with potassium. At each concentration the standing short-circuit current and the odorant-stimulated current transient were measured. The figure shows that the standing short-circuit current (●) increased as a function of K^+ concentration, while there was a corresponding decrease in the amplitude of the odorant-evoked current (○). The data are plotted as the percentage change relative to control values at normal Na^+ and K^+ concentrations. The data show the means and standard deviations from four tissue preparations.

Discussion

(a) The unstimulated electrical characteristics of the bullfrog olfactory mucosa

When bathed symmetrically with amphibian Ringer's solution, a potential difference existed across the bullfrog olfactory mucosa; the ciliated side was electronegative by 3.6 ± 0.3 mV. When voltage-clamped, there was a standing short-circuit current, which decayed to zero when the submucosal side was incubated with ouabain or when the tissue was oxygen-deprived. The fact that furosemide blocked the standing current preferentially from the ciliated side suggests that a

Na^+/Cl^- or a $K^+/Na^+/Cl^-$ cotransport site is located there [19,20]. Thus, sodium may enter across the apical membranes of epithelial cells via the cotransport pathway and be pumped out to the submucosal side via the basolateral Na^+/K^+ pump. The apical specificity for furosemide and the collapse of the short-circuit current after ouabain indicates a role for transmucosal sodium transport as a current source. Unilateral Na^+ ion replacement with *N*-methyl-D-glucammonium on the ciliated side resulted in a reversal of the short-circuit current, and bilateral replacement caused the current to approach zero. These results suggest an inward transcellular sodium transport pathway, which includes active or metabolically driven components, and a parallel passive outward sodium flux that is probably paracellular. Under symmetrical and open-circuit conditions, the latter is driven only by the potential generated by the inward active component, but when the apical Na^+ concentration is reduced below that of the submucosal bath, a passive concentration driving force for Na^+ ions also exists. Fig. 9 shows that the short-circuit current reversed direction when the apical sodium ion concentration was reduced to 45 mM. This means that the active transport system for sodium can move sodium inward against a 2.2-fold sodium concentration ratio.

The reversal of the short-circuit current when sodium on the ciliated side was replaced with *N*-methyl-D-glucammonium reflects the fact that the tissue permeability to the latter cation is low, and that net secretion of sodium dominates over absorption. However, when the ion replacing sodium is potassium, the standing current increased (Fig. 11). This indicates that potassium ion pathways are available on the apical side, and as discussed earlier, these may be channels in both the receptor neurons and the sustentacular cells [10,16,21]. Thus, it is likely that both cell types are depolarized readily by an elevated apical K^+ concentration.

The unstimulated olfactory mucosa exhibited a linear *I-V* relationship with either an external voltage applied as a pulse (Fig. 1) or as a clamping voltage (Fig. 4a). This, together with the low tissue resistance of 67 ohm $\cdot$ cm^2 and low open-circuit voltage indicates that the olfactory mucosa is a 'leaky' tissue in physiological terms, much like oral-cavity and respiratory epithelia [11,22]. This suggests that the passive transmucosal resistance is determined primarily by low impedance pathways such as the paracellular shunts. It should be noted, however, that the active surface area of the mucosa is greater than the geometric area of the exposed tissue since both olfactory cilia and sustentacular microvilli are present, though it is not known what contribution they make to the unstimulated short-circuit current. Therefore, the current density may be overestimated, and the specific resistance may be underestimated.

(b) The odorant-stimulated short-circuit current

The short-circuited bullfrog olfactory mucosa responded to a pulse of odorant solution with an inward positive current transient, the amplitude and shape of the response varying among preparations. The shapes of these transients can be divided broadly into the three classes shown in Figs. 2b, 2c and 2d. Some preparations clearly showed two distinct current transients, an initial fast component followed by a slower component that was often of greater amplitude and that returned to baseline several minutes after the odor stimulus was washed out of the system. In addition, there was often a progressive decline in the rate of onset and amplitude of the early component during an experiment, while the slower component remained relatively stable. The two current components were affected quite differently when the tissue was stimulated with mixtures of odorant and furosemide (cf., Fig. 8). This pharmacological dissection of the current components suggests that they are associated with different ion transport mechanisms. It is likely that these mechanisms reside in different cell types.

Odorant-evoked secretion of simple anions such as Cl^- or polyelectrolyte anions such as mucopolysaccharides could also be sources of inward current. Cytological evidence for odorant-evoked secretion from the sustentacular cells has been obtained in the salamander by M.L. Getchell et al. [3,7,8] and in the bullfrog by Okano and Takagi [9]. Mobilization of secretory activity occurs over several minutes during exposure of the mucosa. This would be consistent with the fact that repeated stimulation of the olfactory mucosa in our experiments caused a significant increase in the

size of the slower component. The selective suppression of the second current component by furosemide suggests that it is associated with a cotransport pathway which is non-conductive rather than with a conductive ion channel. This is supported by the electrophysiological experiments of Trotier and MacLeod [10] on the salamander. They show that the odorant responses of sustentacular cells do not occur with measurable changes in the cell conductance. These results along with histochemical data suggest that at least part of the second current component arises in the sustentacular cells.

The second current component was also suppressed by substituting *N*-methyl-D-glucammonium for sodium on the ciliated side. The remaining fast component was amiloride sensitive, as seen in Fig. 9b. This and the voltage dependence of the amiloride-sensitive component suggests that in addition to the nonconductive sodium pathway, there are odorant-activated conductive apical membrane sodium channels. It seems unlikely that both sodium transport systems would be present in the same cell type. The Na^+/Cl^- cotransport system in other tissues is associated with fluid and electrolyte absorption and secretion [19,20]; it is reasonable to assume that it serves the same function in olfactory mucosa.

Amiloride - sensitive sodium channels are rare in non-innervated leaky epithelia [17]. The fact that these are present in the olfactory mucosa, but are exclusively odorant-activated suggests that they reside uniquely in the apical parts of the receptor neurons. The tissue resistance, and current under unstimulated conditions, are unaffected by amiloride, indicating that it does not block the paracellular shunts. The voltage-dependence of the olfactory response makes it unlikely that the odorant-evoked current is due to a nonconductive Na^+/H^+ cotransporter (affected by amiloride in some leaky epithelia). In addition the odorant-evoked response is inhibited by amiloride concentrations below 10 μM. These results suggest that the amiloride-sensitive transport system is a sodium channel. Sodium ions are presumably supplied to the ciliated side of the mucosa through glandular secretion. The leakiness of the paracellular shunts, however, indicates that should the apical surface of the mucosa become Na^+ depleted, passive sodium gradients would replenish the mucosa via passive diffusion from the submucosal compartment.

The odorant-activated sodium channels appear to be passive. In support of this, ouabain placed on the submucosal side inhibited the odorant-evoked response at short-circuit. As ouabain acts only from the submucosal side, its effects must be indirect, i.e. through degradation of the sodium gradient. If the sodium electrochemical gradient across the receptor apical membranes is responsible for the odorant-evoked current, restoring the gradient through applied potentials should also restore the current. Fig. 5 shows that this is the case. The direction of the current may be inward or outward as the polarity of the electrochemical potential gradient dictates.

The initial fast component of the odorant-evoked current increased as a function of increasing concentrations of odorant. It is apparent from Fig. 3 that the sensitivities for the compounds tested differed. These compounds evoke widely different odorant descriptors in humans (eucalyptus, fruity, and green peppers, respectively). Since the olfactory epithelium has receptor populations of differing specificities [1,2], the differences in sensitivity probably reflect the responses of a varying population of receptor neurons. The detection limits of the odorants were not investigated since these were mainly restricted by the signal to noise ratio of the preparation. However, it is clear that the detection limits of the tissue must be lower than 10^{-6}M with a response range of at least three orders of magnitude. The summed odorant-evoked currents are only observable when a significant fraction of the cellular current generators are functioning synchronously. Since single-unit recordings from olfactory receptor neurons show that different units have different thresholds of stimulation by a given odorant [1,23,24], it is expected that more and more receptor neurons are recruited as the concentration of an odorant is increased. The receptor neuron density of the frog olfactory mucosa is $(5–8) \cdot 10^6/cm^2$ [25]. Thus the current passed by an individual receptor neuron when stimulated by 10^{-4} M 1,8-cineole for example would be 1.3–2.1 pA/cell assuming that all the available neurons are stimulated. This compares well with the recent results of Firestein and

Werblin [21] who carried out single cell patch-clamping of olfactory receptor neurons. Odorants eliciting a generator current of 6 pA caused depolarization of the cell and initiation of spike activity. It is clear that while the odorant-evoked current in our experiments represents less than 10% of the standing short-circuit current, its magnitude is consistent with that expected for stimulation of the receptor neurons.

(c) The odorant-stimulated current-voltage relation

By measuring the odorant response over clamping voltages ranging from −20 mV to +40 mV, the *I-V* relationship of the odorant-evoked current for a fixed concentration of odorant was determined (Fig. 4b). In contrast to the *I-V* relation of the unstimulated current under voltage clamp (Fig. 4a), that for the odorant-evoked current was nonlinear. The current-voltage relation showed a transition between two states characterized by different conductances. Accordingly, the amplitude of the odorant-evoked current showed marked rectification with respect to the direction of the clamping voltage. These results may be interpreted in two ways: (a) there may be voltage-gated sodium channels that can be activated during stimulation by an odorant in addition to passive odorant-activated sodium channels, or (b) the efflux of potassium via K^+ channels may account for the increased conductance when the driving force for ion flow is outward. If the former is true, a voltage-dependent change in Na^+ permeability of the type suggested by these results would enhance the rate of sodium entry and hence the rate of depolarization. This would increase the sensitivity of the system. Independent evidence for the presence of voltage-gated sodium channels has been obtained from single cell patch clamp experiments on olfactory receptor neurons [18,21]. This is supported by preliminary reports of single channel properties of isolated olfactory receptor neurons obtained using patch clamp methods that show that, in addition to potassium channels, voltage-gated sodium channels are also detectable [18,21].

Conclusion

Gesteland [27], Getchell [28], Juge et al. [29], and Heck et al. [11] have presented models of olfactory transduction that propose that transductory currents are at the apical processes of receptor neurons. The importance of return and shunt pathways were also considered. From the present work we can assign the specific transport mechanisms to various elements of the mucosa. These are summarized in Fig. 12. The standing short-circuit current is in large part generated through a furosemide-sensitive apical membrane transport system. From the established specificity of furosemide as a blocker of electroneutral Na^+/Cl^- or $Na^+/K^+/Cl^-$ cotransport in other systems, we propose that the standing short-circuit current depends on the electroneutral Na^+/Cl^- cotransporter located on the apical membranes of sustentacular cells [19,20]. This system probably maintains fluid and electrolyte balance as in other non-excitable tissues. Part of the odorant induced current is also furosemide-sensitive, which would suggest the same electroneutral ion transport sys-

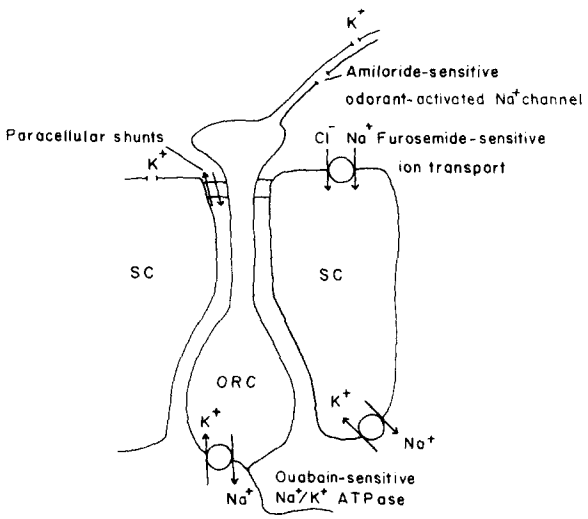

Fig. 12. Tentative assignment of specific transport mechanisms to various elements of the mucosa. The standing short-circuit current is in large part generated through a furosemide-sensitive apical membrane transport system. This is probably a Na^+/Cl^- electroneutral cotransporter located in the sustentacular cells (SC). The tranductory generator current is mediated through amiloride-sensitive, odorant-activated sodium channels thought to be located in the ciliary membranes of the olfactory receptor neurons (ORC). The paracellular shunts are leaky to NaCl. Both receptor cells and sustentacular cells have ouabain-sensitive ($Na^+ + K^+$)-ATPase linked to sodium pumps in the basolateral membranes. There are likely to be passive K^+ channels on both cell types.

tem is also stimulated by odorants. This suggests that this system may also have a role in odorant clearance.

The transductory current is mediated through odorant-activated amiloride sensitive sodium channels located in the ciliary membranes [1,2]. These channels may also be modulated through a second messenger system involving cyclic nucleotides [13,30–32]. The nonlinearity of the odorant-evoked current as a function of clamping voltage suggests that voltage-sensitive channels may also be activated subsequent to the initial events of transduction in accord with current models of olfactory neuron stimulation [1,18,21,26]. Both receptor neurons and sustentacular cells have a ouabain-sensitive ($Na^+ + K^+$)-ATPase linked to sodium pumps in their basolateral membranes. Our results suggest that potassium channels are present on the apical and basolateral membranes, probably of both major cell types. The paracellular shunts would appear to act as passive pathways for NaCl flux from submucosal to the mucosal side of the tissue. The driving force for this flow would be the potential generated by the active ion inward current.

Acknowledgements

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