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Odorant Response of Isolated Olfactory Receptor Cells is Blocked by Amiloride

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Summary. Olfactory receptor cells were isolated from the nasal mucosa of *Rana esculenta* and patch clamped. Best results were obtained with free-floating cells showing ciliary movement. 1) *On-cell mode:* Current records were obtained for up to 50 min. Under control conditions they showed only occasional action potentials. The odorants cineole, amyl acetate and isobutyl methoxypyrazine were applied in saline by prolonged superfusion. At 500 nanomolar they elicited periodic bursts of current transients arising from cellular action potentials. The response was rapidly, fully and reversibly blocked by 50 μM amiloride added to the odorant solution. With 10 μM amiloride, the response to odorants was only partially abolished. 2) *Whole-cell mode:* Following breakage of the patch, the odorant response was lost within 5 to 15 min. Prior to this, odorants evoked a series of slow transient depolarizations (0.1/sec, 45 mV peak to peak) which reached threshold and thus elicited the periodic discharge of action potentials. These slow depolarizing waves were reversibly blocked by amiloride, which stabilized the membrane voltage between −80 and −90 mV. We conclude that amiloride inhibits chemosensory transduction of olfactory receptor cells, probably by blocking inward current pathways which open in response to odorants.

Key Words chemoreception · olfaction · periodic discharge · cyclic AMP · amiloride

Introduction

The nasal mucosa responds to odorants with an inwardly directed short-circuit current which depends on the presence of Na ions in the mucosal solution [65]. Recently it was shown that part of the "chemosensory" inward current is blocked by amiloride, an inhibitor of several Na-transport systems. Based on these transepithelial measurements, it was suggested that the first electrogenic response of the receptor cells to odorants may be the opening of amiloride-blockable Na channels at the apical cell pole [56].

We report initial progress with patch-clamped, free-floating olfactory receptor cells isolated from the nasal mucosa of the frog by a special procedure. Our protocol appears to allow a more reliable response to odorants than was obtained previously [3, 20, 47, 66]. We found that amiloride does indeed block the odorant-induced depolarization of single receptor cells.

Materials and Methods

Isolation of Olfactory Receptor Cells

Frogs of the species *R. ridibunda/esculenta* were maintained at about 4°C in tap water. Animals were sacrificed by decapitation and pithing. The upper jaw was removed from the forehead and two frontal sections were cut through the head, isolating the major parts of the nasal cavities. The nasal cavities were opened and the mucosa constituting the dorsal and ventral lining was scraped free, tearing the axons of the primary olfactory neurons.

The scrapings were collected into a hypertonic Ringer's solution (solution RS of Table 1, modified from ref. 20), where they remained for 10 min at room temperature. Then they were cut into pieces with an area of about 1 mm^2 which were placed into the Ca complexing medium of pH 10.3 (solution DS, modified from ref. 38) for 45 min at room temperature. This solution was freshly prepared directly before use [38]. At the end of this period, tissue dissociation was not yet noticeable. The pieces of mucosa were then portioned into 6 parts which were briefly washed with solution RS. Each part was stored in 1 ml of RS, to which 1% albumin was added, at 4°C for up to 6 hr.

For experimental use, a portion was decanted and covered with 1 ml of solution RS (albumin-free, 4°C), in which it was triturated gently 3–5 times with a fire-polished Pasteur pipette. Thereby, the pieces of epithelium released large amounts of single cells. Enzymes were not needed for cell dissociation and DNase was not needed for cleaning cell surfaces. The cell suspension obtained from one stored portion was partitioned into three experimental chambers and maintained at 4°C for up to 120 min.

Chambers, Microscopy and Change of Solution

For experimental chambers, standard glass slides, onto which a cured silicon ring of 1 mm thickness and 10 mm inner diameter

Table 1. Solutions used for cell isolation and for recording (in mM)

	RS (Ringer's)	DS (dissociation solution)	NaP ("high Na" pipette-sol)	KP ("high K" pipette-sol)
NaCl	120	100	115	5
NaOH	4	4	—	—
KCl	3	2.5	—	115
KOH	—	—	4	5
$CaCl_2$	1	1	1	—
$MgCl_2$	2	—	3	3
Na_2CO_3	—	10	—	—
$NaHCO_3$	—	10	—	—
Na_3-citrate	—	10	—	—
HEPES	10	—	10	10
EGTA	—	—	—	1
Albumin %	—	1	—	—
Glucose	5	—	—	—
Na-pyruvate	5	—	—	—
pH	7.4	10.3	7.4	7.2
ATP	—	—	—	5
mOsm	250	254	229	224

was pressed, were used [6]. For patch clamping, a chamber containing the cell suspension was placed on the stage of an inverted microscope (Olympus IMT2-F), where it warmed to room temperature. The preparation was viewed in transmitted light at a total magnification of 400×. Numerous rounded cells with long or short cilia settled onto the bottom of the chamber, among them olfactory receptor cells, recognized by their bipolar shape. As described by Anderson and Hamilton [3], a thin process of variable length (the torn axon) was usually seen at one end of the olfactory cells, and a longer and thicker process (the dendrite) with apical cilia, which exceeded 20 μm in length, at the other end.

Many receptor cells attached to the glass bottom of the chamber and, typically, did not show ciliary movement. Others were propelled through the solution by uncoordinate, slow ciliary movement and, apparently, were thus kept from attaching to the glass. Cells were patched in solution RS. After sealing the patch pipette to a cell membrane, the cell was superfused with RS, using gravity feed and a suction pump (inverted aquarium pump), with a rate of about 1 ml/min at room temperature. The suction outlet was placed such that the chamber volume was reduced to about 100 μl. The resulting exchange time was 10–20 sec.

Electrical Recording

The procedure for patch clamping of cell membranes closely followed the description by Hamill et al. [31]. Patch pipettes were pulled from thick-walled borosilicate glass capillaries (Jencons Scientific Ltd., Bedfordshire, UK; outer diameter 2 mm, inner diameter 1–1.25 mm) on a two-stage puller designed in the laboratory. When filled with solutions NaP or KP (Table 1), the tips had resistances of about 7 MOhm which were doubled by fire-polishing.

The pipettes were mounted at an angle of 35° to the horizontal on a hydraulic 3-D manipulator (Narishige MO-103-R). Patch-clamp signals were recorded with an amplifier of our own design. The current-to-voltage converter was AD 515 with a feedback resistor of 1 GΩ, frequency response flat to 2 kHz. The specified input-bias current of this operational amplifier is 0.3 pA. The pipette resistance was not compensated. Current and voltage signals were digitized at 40 kHz (16 bits) with an audio-PCM processor (Sony 501 ES, modified to pass zero frequency) and stored on a video recorder. Leakage current was not subtracted. In the figures, cell membrane depolarizations (whole-cell) and patch membrane depolarizations (on-cell) are plotted upwards. Currents out of the cell (out of pipette for whole-cell and into pipette for on-cell) are also plotted upwards.

Solutions and Reagents

The solutions for tissue dissociation, superfusion of cells and filling of patch pipettes are specified in Table 1. Osmolarities were measured with a vapor pressure osmometer (Wescor 5100C). The odorants used were those which Persaud et al. [56] reported to stimulate the chemosensory inward current of the frog olfactory mucosa, i.e. 1,8-cineole (eucalyptol (minty)), *n*-amyl acetate (fruity) and 2-isobutyl-3-methoxypyrazine (bell pepper). A 1 mM stock solution was prepared with methanol. We used either 1,8-cineole alone or the three odorants together at a final concentration of 500 nM in solution RS. This concentration gave good responses even though it was somewhat smaller than the cineole threshold (1 μM) of ref. [56]. The final methanol concentration was 0.05%. Odorants, ATP and GTP were obtained from Sigma. Amiloride (3,5-diamino-6-chloro-N(diaminomethylene)pyrazine carboxamide) was a gift from Sharp & Dhome GmbH, FRG.

Molar water/air partition coefficients of odorants were roughly estimated as the ratio of saturation-concentrations [70] in water and gas phase, disregarding properties of nonideal solutions and binding to molecules in the mucus. The equilibrium vapor pressure of cineole is 1.5 mm Hg at 20°C [64, 70], which corresponds to a saturation concentration of 82 μM in the gas phase. The solubility in water is about 18 mM, the water/air partition coefficient (18/0.082), therefore, near 200 at equilibrium. The measured value is near 280 [2]. For *n*-amyl acetate

Table 2. Properties of isolated receptor cells

Type	I	II	III	IV	V
Ciliary movement	yes	yes	yes	yes	no (attached)
Resting pot. [mV]	−90	−90	not stable	−90 or not stable	−40 to −90
Spont. transient depolarization	small irreg. bumps	large regular waves	irregular fluctuations −60 to −40	occasional −90 to −40	no
Spont. action pot.	yes	yes	yes	yes	no
Response to odorants	yes	no	no	no	no
Response to amiloride	yes	no	no	no	?

(vapor pressure 2.95 mm Hg at 20°C (i.e. 160 μM), solubility 14 mM [58, 68]) the water/air partition coefficient is in the order of 80 [37]. The coefficients 200 (cineole) and 80 (amyl acetate) were used to convert gas concentrations to those in water (Table 3).

Results

Selection of Competent Receptor Cells

Of the isolated olfactory cells, many attached to the glass bottom of the chamber. It was not difficult to seal pipettes to the membrane of these cells [3, 20, 47]. On breaking the patch, the resting membrane voltage was found to be in the range −40 to −90 mV, and the cell resistance of the order of 2 GΩ. However, an attached cell either had no apical cilia or lacked ciliary movement, and did not show spontaneous transient depolarizations. While action potentials could be elicited by passing depolarizing current pulses through the cell, there was typically no response to odorants (cell type V of Table 2).

Receptor cells with spontaneously beating cilia were usually found to move slowly through the chamber. Initially it was difficult to patch onto these cells, but with practice the success rate reached 90%. The membrane surrounding the soma was usually patched. Seal resistances were of the order of 20 to 40 GΩ. Uncoordinate ciliary movement continued during on-cell recording and caused a slow, irregular motion of the cell body but did not usually break the seal. Following breakage of the patch, which sometimes occurred spontaneously, in other cases by application of a brief pulse of reduced pressure to the pipette, membrane voltage was close to −90 mV if stable, and cell resistance 5–6 GΩ. Auto-mobile cells were grouped according to the type of potential change which spontaneously occurred under whole-cell current-clamp conditions (*see* Table 2 and Fig. 1). Since only cells of type I responded to the odorants used, we focussed on the properties of these cells. Further discussion will be limited to this type. Records were obtained from 98 receptor cells in total.

On-Cell Channel Activity

On sealing pipettes to the surface of receptor cells, stable recording conditions could be maintained for 30 to 50 min. Single-channel currents were often observed. Most often these were due to K channels of 60 and 30 pS slope conductance. Occasionally a K channel of 21 pS slope conductance was found. In addition, fast biphasic current transients were noted to pass the patch at irregular intervals (Fig. 2). Their initial cell-outward current component lasted about 4 msec, followed by a somewhat slower inward transient. There was an increase in high-frequency noise which began with the rising phase of the outward current and lasted for most of the inward current (Fig. 2*A*). When the transient occurred while a channel was open in the patch, the initial transient current component had an amplitude of 6–10 pA, otherwise it was smaller. As observed by others, such on-cell transients are due to cellular action potentials [16, 17, 47, 59]. Figure 2 shows that the transients were also biphasic when the pipette contained only 5 mM Na or when the voltage across the patch was apparently close to the K equilibrium potential. The late, inwardly directed component of these current transients cannot be due to mere capacitive and conductive properties of the patch. Fenwick et al. attributed it to Ca-activated channels in the patch membrane [17].

Under special conditions, cellular action potentials could be triggered through the patch: with a

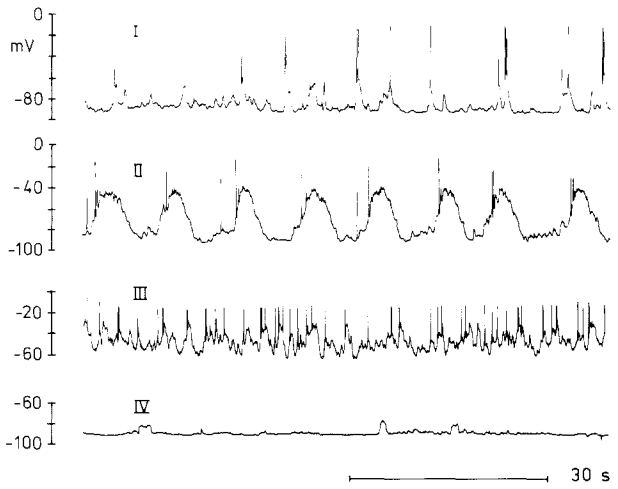

Fig. 1. Whole-cell voltage records from isolated olfactory receptor cells showing the different kinds of spontaneous depolarization which characterize the cell types I to IV of Table 1. Pipette solution: KP. Bath: Ringer's (RS) without added odorants. The cells were ciliated, *moving* until patched and fired action potentials in response to injected current. Therefore, type I to IV will belong to the second class of cells (mature receptor cells) as defined by Masukawa et al. [44]. The records shown were obtained in the first 5 min after patch breakthrough. They represent steady states of basal activity observed for 10 to 25 min. Action potentials appear as upwardly directed spikes. The mean spike rates are I: 0.16; II: 0.12; III: 0.68; IV: 0.0/sec. The periodic depolarizations of type II (0.1/sec) resembled those of type I in the odorant-stimulated state (*see* Figs. 4 and 5). Thus in type II-cells the generator potential mechanism may be *uncoupled* from the receptor mechanism. Amiloride had no effect on the "slow waves" of type-II cells

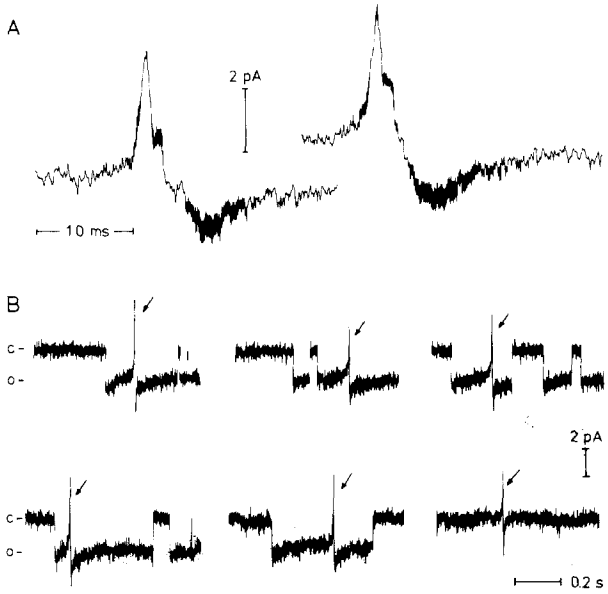

Fig. 2. On-cell current records. (*A*) Biphasic transients of patch current due to spontaneous cellular action potentials. Current from cell to pipette is plotted upwards. Pipette solution NaP, pipette potential clamped to 0 mV, bath solution NaR. During the current transient an increase in high-frequency noise, beginning with the upstroke, was seen with and without odorants added to the bath solution. (*B*) Pipette solution KP, pipette voltage +80 mV, bath solution RS without added odorants. Opening of a K channel (21 pS slope conductance) caused current flow from pipette to cell (downward deflection by 2.2 pA). Thereby the cell was depolarized and fired an action potential which drove a fast transient current (arrows) through the patch. The last record shows a biphasic transient recorded while the K channel was closed

high K solution (KP) in the pipette and the pipette voltage clamped to +80 mV, the opening of one K channel caused enough current flow into the cell (2.2 pA in Fig. 2) to depolarize the cell membrane to threshold. The phenomenon was observed previously [47, 66] and requires a slope resistance of the resting cell membrane in the 5–10 GΩ range. A similar sensitivity to injected current was reported by others [16, 20, 33, 47].

Gradual Loss of Response to Odorants

Shortly after breaking the patch, type I cells responded to superfusion of odorants with slow periodic depolarizations and by firing action potentials at an increased rate (typically in bursts), as will be described below. This response reversed when odorants were washed out. However, the responsiveness to odorants was typically lost 5 to 15 min after breaking the patch. Ciliary beating stopped at about the same time and the cell seemed to convert from type I to type IV. This decline in physiological function was possibly due to diffusional loss ("wash-out") of cytosolic solutes into the pipette compartment [1, 19, 47, 66]. Inclusion of ATP (5 mM) and GTP (1 mM) in the pipette solution did not improve the situation. Therefore the response to odorants was first studied without opening the cellular compartment, i.e. in the on-cell mode.

On-Cell Current Response to Odorants

Patch pipettes filled with the high-Na solution (NaP of Table 1) were sealed onto moving receptor cells. The pipette potential was clamped to zero and the patch current recorded. It will be appreciated that under these conditions, and with cell potentials close to −90 mV, any current through K channels present in the patch was close to zero. Indeed, the resulting current trace did not show any open/close events. However, transient patch currents due to cellular action potentials were noted at irregular intervals.

With unstimulated cells, spontaneous action

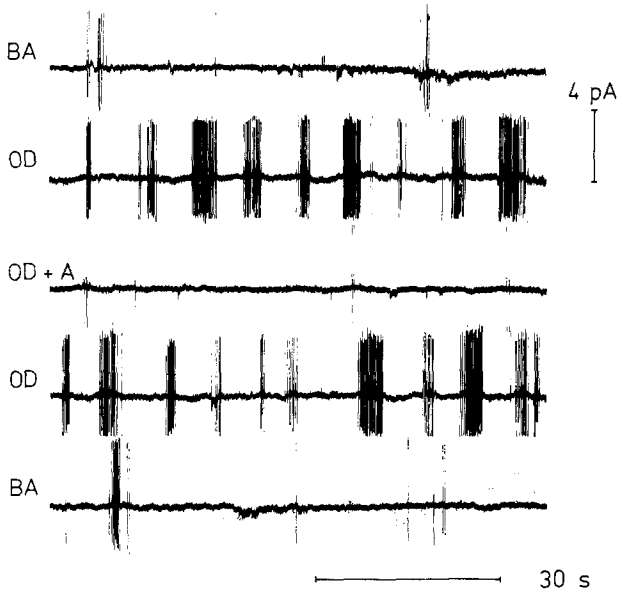

Fig. 3. Response of one isolated, free-floating receptor cell to odorants and amiloride. On-cell current record. A mixture of the three odorants (*OD*, each 500 nM in RS) and, where indicated, amiloride (*A*, 50 μM) was applied to the cell by superfusion. Pipette solution NaP, pipette potential clamped to zero. The uppermost and lowest trace show basal activity (*BA*) in the absence of added odorants. Mean spike rate 0.24 and 0.16/sec. *OD:* Odorant-evoked periodic discharge of action potentials began 1 min after starting the slow superfusion. Mean spike rate 1.5, burst repetition 0.12, mean intra-burst spike rate 5.6/sec, mean burst duration 2.3 sec. This was observed for 6 min, then the amiloride solution reached the cell. The inhibition by amiloride was observed for 4 min, the subsequent reactivation following washout of amiloride for 3 min. Odorant-washout took 1 min and, after 6 min, the whole experiment could be repeated (*not shown*)

potentials occurred in groups of 2 to 5. Their mean rate was less than 0.3/sec (basal activity). While superfusing a cell with odorants, the burst duration and the burst repetition frequency increased (Fig. 3). The mean spike rate increased to 1.5/sec. This response was observed with a mixture of the three odorants (each at 500 nM) and also with 500 nM of cineole alone. On changing to a superfundate which in addition to the odorants contained 50 μM amiloride, the spike rate decreased rapidly to 0.1/sec. With 10 μM amiloride the inhibition was smaller but still noticeable (*not shown*). The blocking effect of amiloride and the stimulating effect of odorants were fully reversible (Fig. 3).

On-Cell Voltage Response to Odorants

We attempted to record changes in cell voltage, without disrupting the continuity of the cell membrane, by the following procedure: Pipettes were filled with the high K solution (KP of Table 1), which, following eventual breakthrough of the patch, allows the establishment of membrane voltage in the presence of the standard gradients of Na and K. After sealing the tip to the surface of a free-floating receptor cell, the pipette voltage, recorded in the current-clamp mode, was in the range -80 to -90 mV. It showed slow waves of transient depolarizations as expected (from the previous whole-cell recordings, *see* Fig. 1) of type I cells. Apparently, the membrane patch was leaky enough to transmit not only fast current transients arising from cellular action potentials (*see above*), but also a signal resembling the low-frequency profile of intracellular voltage to the pipette. Similar conductive properties of the patch were reported for other cells [29]. They depend on a surprising leakiness of the patch membrane which can be much larger than expected from a phospholipid bilayer [21]. In our experiments the absolute values of the on-cell voltage signal contained an offset of about -10 mV, as shown by subsequent whole-cell recordings. The offset may in part have been due to the input-bias current (0.3 pA) of the front-end current-to-voltage converter connected to a patch resistance of more than 20 GΩ. This, however, was not a serious problem because a better estimate of the membrane voltage could later be obtained by breaking the patch.

In the presence of odorants added to the superfundate (Fig. 4, OD), the slow depolarizing waves became larger and more sinusoidal and now resembled the pattern of type II cells. The duration of one depolarizing halfcycle was about 6 sec, the period close to 0.11/sec. The patch current showed periodic discharges of fast transients which coincided with the depolarizing phases of the voltage waves. On adding 50 μM amiloride to the odorant solution, voltage waves and periodic discharges were abolished (Fig. 4, center). The periodic electrical activity reappeared at 0.11/sec when amiloride was washed out. In the case of Fig. 4, removal of amiloride left the cell more active than it was prior to amiloride. This, however, was not a typical observation. Adaptation to the stimulus or run-down of the odorant response was not seen during on-cell recordings.

Whole-Cell Recordings

In the experiment of Fig. 4 basal electrical activity (type I) resumed when odorants were washed out 13 min after establishing the seal. The patch was then broken and basal activity observed in the whole-cell configuration (Fig. 5, BA). It consisted of slow "triangular" depolarizations (0.08/sec) which elicited action potentials near -60 mV. Superfusion with

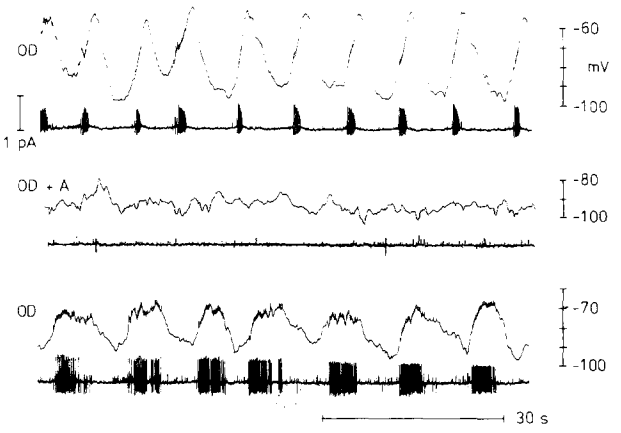

Fig. 4. On-cell record from an isolated receptor cell (Type 1, which was moving prior to being patched) with a pipette filled with a solution of high K concentration (KP). With the mean pipette-current clamped to zero pA (current-clamp mode), the current (but not the voltage) record shows fast transients due to cellular action potentials. In the presence of odorants they occur in bursts. Mean spike rate 1.8, burst repetition 0.12, intra-burst spike rate 10.1/sec, mean burst duration 1.5 sec. The simultaneous voltage record (above) shows the much slower changes of the cellular membrane potential, as recorded through the patch. When the cell was superfused with 50 μM amiloride (*A*), added to the odorant mixture (*OD*, 500 nM), the regular waves of spontaneous depolarization were abolished. Thus membrane voltage remained more negative than threshold and the firing of action potentials stopped. On washing out amiloride, periodic discharging resumed and now was stronger than prior to inhibition by amiloride: the slow waves were broader, increasing the burst duration threefold. Mean spike rate 3.1, burst repetition 0.1, intra-burst spike rate 7.6/sec, burst duration 4.4 sec. Each of the traces shown represents a steady state of at least 3 min. Eleven minutes after start of the record, odorants were washed out, the patch broken and whole-cell recording conditions established (*see* Fig. 5)

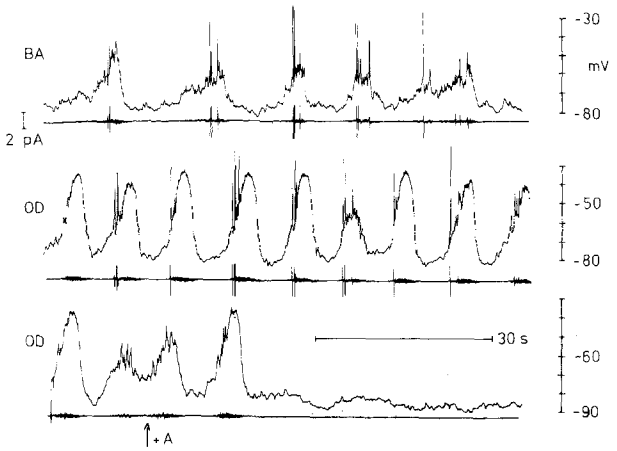

Fig. 5. Whole-cell record of the cell from which Fig. 4 was first obtained. Current-clamp mode. Action potentials are seen as fast transients on the voltage trace and (due to frequency limitations of the clamp circuit) also on the current trace (*shown below*). *BA:* basal activity in the absence of added odorants (recorded for 3 min); mean spike rate 0.18/sec. *OD:* odorants (mixture in RS, 500 nM each; recorded for 3 min). The repetition rate of bursts (0.11/sec) is comparable to that of Fig. 4, but the burst duration is shorter (run-down). Mean spike rate 0.15/sec. The lower two records are continuous, the bottom record showing the time course of inhibition by 50 μM amiloride (*A*) in the superfundate (added to the odorant solution)

odorants changed the pattern to more sinusoidal waves (0.11/sec, 45 mV peak to peak). Action potentials were fired during the depolarizing phase, but the burst duration was less than during on-cell recording. Addition of 50 μM amiloride to the superfundate containing odorants arrested the membrane voltage near −85 mV, inhibiting the periodic activity.

In Fig. 5, not only the response to odorants, but also the basal activity, was abolished by amiloride. This, however, was only observed when the basal activity was relatively high. (A high basal activity could be due to the presence of odorants in the "odorant-free" solutions, or to partial "uncoupling" of receptor mechanisms from the depolarizing channel activity, resulting, for instance, from an inwardly directed leak current.) Several cells had negligible basal activity but responded to added odorants. In these cases, amiloride had no effect without added odorants.

During prolonged whole-cell recording, the response to odorants tended to be lost, as mentioned above. Before the loss became complete, the repetition rate of the slow depolarizing waves decreased while the amplitude of these waves was unchanged (Fig. 6). Amiloride was still effective in this state.

Discussion

Odorant Detection

Odorants dissolve in the nasal mucus, where they are expected to interact chemically with binding sites at the apical cilia of receptor cells (reviewed in 26, 40). The binding may either open ion-conducting channels *directly* [39], or cause *indirectly* through G-proteins [4] and adenylate cyclase [54, 63] the generation of cyclic nucleotides [48, 49] which, in turn, open ciliary channels [50]. It is interesting that the odorant isobutyl methoxypyrazine was found to evoke both the direct and indirect response, of which the first appeared to be the more sensitive one [39, 63]. The induced inward currents passing ciliary channels and perhaps also somal channels will cause depolarization ("receptor potential") and thus evoke action potentials which are conducted to the olfactory bulb. The study of odorant reception

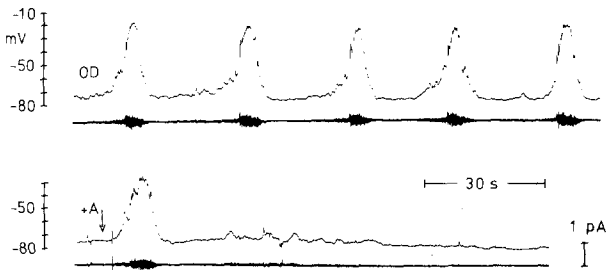

Fig. 6. Whole-cell record obtained in the current-clamp mode 15 min after breaking the patch. The type I-cell was superfused with RS containing 500 nM cineole as odorant (OD). Pipette filled with KP. The run-down phenomenon had decreased burst duration and the repetition rate of spontaneous depolarizations, but the amplitude of the spontaneous depolarizations remained in the order of 55 mV. Action potentials are upward-directed spikes reaching −15 mV. During depolarizations the current noise increased due to voltage-dependent activation of K channels. Cell-to-bath current is plotted upwards. Action currents were clipped

at the level of single cells is hampered by the fact that after isolation the receptor cells tend to have a very poor response to odorants.

Cell Preparation

The features which we consider important in the isolation of olfactory receptor cells are slight hypertonicity [12, 20], avoidance of EGTA by Ca complexing with 10 mM citrate at pH 10.3 for 45 min only [38], use of hypertonicity with 1% albumin for storage prior to trituration as well as cell dissociation by trituration no longer than 2 hr before use [3, 38]. While none of these features is novel, we found that their combination is quite effective. In addition, as pointed out by Trotier [66], the recording from free-floating cells having moving cilia is possible. When combined with the procedure detailed above, it gives a good yield of odor-competent receptor cells. For chemical stimulation, it is helpful to avoid attachment of cilia to the chamber, to concentrate on cells with a type I voltage pattern and to observe the "washout" time.

Odorant Concentration

The odorants which we used are known to elicit on-responses of olfactory cells in many species of vertebrates, and to activate the adenylate cyclase of olfactory cilia. Part of the information available is summarized in Table 3. The supra-threshold concentrations of odorants used in this study (500 nM in saline) are seen to be larger than the threshold concentrations specified for the intact olfactory system, but somewhat smaller than the threshold concentrations for activation of the adenylate cyclase preparations and for stimulation of the transmucosal short-circuit current. This is not surprising because the enzyme assay and the short-circuit current across the mucosa will average properties of highly responsive and poorly responsive cells, while single-cell recordings allow to determine the threshold of sensitive cells *per se*. Probably for a similar reason the threshold concentrations determined from the integrated olfactory nerve response of the tortoise [68] are about 100-fold larger than those of the most sensitive single olfactory units [45]. For the latter, values of about 1 nM (in water) were specified (e.g. 24) or can be calculated from the air thresholds [45]. While the threshold concentrations for stimulation of single neuroblastoma cells, *Nitella* and melanocytes [35, 36, 41, 69] are considerably larger than those for stimulation of primary olfactory neurons, only nanomolar concentrations are needed to directly activate single odorant-sensitive channels from olfactory cilia [39].

Previous patch-clamp studies, to avoid receptor cell adaptation, used "puffs" of odorants in solution; the resulting odorant concentrations in the saline were not easily specified [3, 47, 66].

Voltage Response to Odorants

The low odorant specificity of olfactory cells (e.g. 45, 57) may explain, why quite a number of isolated receptor cells responded to our cocktail of three odorants, or even to cineole alone. The response to a maintained exposure to odorants was an increase in the rate of action potentials. At the odorant concentration used, the action potentials were typically grouped into bursts (on-cell recordings). Except for bursting, there was no indication of adaptation. Occasional bursts of 3 or more spikes were also seen by Maue and Dionne in their work with isolated cells [46, 47], but results obtained from the intact mucosa (reviewed in ref. 25) indicate this phenomenon rather infrequently. Getchell and Shepherd [27, 28] report only rare cases of "waning and then recovery of impulse frequency" during a pulse of odorants. Gesteland et al. apparently observed synchronized bursting in response to cigarette smoke [23], and some published spike sequences show bursting (e.g. Fig. 2*B* of ref. 52, Fig. 4 of ref. 57, Fig. 2*A* of ref. 61). In few cases was bursting discussed (e.g. 14, 57).

The repetitive bursting with a constant stimulus became understandable from simultaneous voltage recordings (on-cell and whole-cell): the response to odorants was not a maintained depolarization but

Table 3. Concentration dependence of responses to some odorants

Preparation	Odorant	Delivery	Concentr. in air	Concentr. in water	Reference
Tortoise					
int. nerve	AMA	puff up to 1 min	—	14 μM–14 mM	68
int. nerve	AMA	"	0.16–1.6 μM th 1.6 nM	13–130 μM th 130 nM	68
s. neur. act.	CIN	puff	0.03–0.8 μM	6–160 μM	
. .	AMA	"	0.05–1.6 μM th 16 pM	4–130 μM th 1.3 nM	45
R. ridibun.					
s. neur. act.	CIN	puff	4–40 μM	0.8–8 mM	57, 62
isol. receptor cell	CIN	cont.	—	500 nM	this study
R. catesbei.		puff of			
olf. bulb	AMA	minutes	—	50–1000 μM	35
transmucosal SCC	CIN	cont.	—	1–500 μM	
	AMA	"	—	10–100 μM	
	IBMP	"	—	20–1000 μM	56
R. ridibun.					
adenyl.-cyclase	CIN	cont.	—	th 2.5 μM	
	AMA	"	—	"	54
R. catesbei.	CIN	cont.	—	th 1 μM	
adenyl.-cyclase	IAMA	"	—	"	
	IBMP	"	—	"	63
Neuroblastoma	CIN	cont.	—	th 120 μM	36
(N-18)	AMA	"	—	th 300 μM	35
Nitella	IAMA	cont.	—	th 1 mM	69

int. nerve: integrated nerve response; s. neur. act.: single neuron activity; SCC: short-circuit current; CIN: 1,8-cineole; AMA: *n*-amyl acetate; IAMA; iso-amyl acetate; IBMP: isobutyl methoxypyrazine; odorants were delivered as a puff (in air or in solution) or continuously (in solution). For comparison, air concentrations were converted (*see* Materials and Methods) into equilibrium concentrations in water (not in mucus). However, air-mucus equilibrium may not always have been reached when short puffs were used. Where concentrations in air are not given, odorants were delivered in solution; th: estimated concentration threshold (where several thresholds were observed with different cells, the lowest is quoted).

the appearance of slow cyclic voltage changes which gave rise to the bursts of action potentials during the later part of their depolarizing phase. The slow oscillations may be related to the slowly fluctuating components ("waxing and waning") occurring in the electro-olfactogram at high stimulus concentrations (reviewed in 25 and 53).

In olfactory receptor cells, such *repetitive* waves were not previously observed, probably because odorants were mostly applied in brief "puffs" of unknown time course. Only a single depolarizing transient was then seen per "puff" [3, 66], and it may have been easy to overlook the possibility that the hyperpolarizing phase of the transient was due to a mechanism other than the decline of the odorant concentration. The nonrepetitive depolarizing transients observed by Anderson and Hamilton required the passage of hyperpolarizing inward current to obtain odorant-evoked action potentials [3].

Bursting, based on slow voltage waves, is a well-known feature of pacemaker neurons [10, 15, 30, 32], but is also shown by many other cells, like cold-receptors of the skin [11], some types of smooth muscle cell [9] and insulin-secreting β-cells [5, 59]. Furthermore, fibroblasts show slow voltage oscillations [71] and cochlear hair-cells fast voltage oscillations [19], whose origin seems related to that of pacemaker potentials.

In many of these cases the depolarization itself, or the action potentials which it evokes, triggers inflow of Ca through voltage-activated Ca channels. The resulting rise in cytosolic Ca activates Ca-dependent K channels, thus inducing the delayed repolarization which terminates Ca inflow. Then the cytosolic Ca concentration decreases again, and the concomitant closure of K channels permits the onset of the next depolarizing phase.

Both Ca inward currents and Ca-activated K

outward currents have been observed in olfactory cells in patch-clamp experiments [20, 47, 66]. They may play a role in the repolarizing phase of slow waves [66]. While equilibration of the cytosol with the pipette and diffusion of EGTA into the cell will have limited excursions of cytosolic Ca transients [1, 18, 59], the slow waves were nevertheless observed for minutes in whole-cell experiments. However, we used small-tipped pipettes and patched at a location of the elongated cells which may be remote from the sites of Ca inflow. Yet, blockage of putative Ca pathways by exposure to 2 mM Co (*compare* 66) had no effect on the slow waves. Thus the role of Ca transport in the generation of slow waves requires further investigation.

Bursting and oscillations in the systems mentioned above differ in the way in which the depolarization is brought about. It is likely that in the case of olfactory cells the depolarizing phase of the slow waves and the depolarizing phase of the "receptor potential," which is recorded as a single transient depolarization following a short pulse of odorants [3, 67], have the same origin. Since the depolarization is based on an increase in conductance [47, 67], it will be due to the activation of one or more types of *odor-induced inward currents* (even though it may be accompanied by a decrease in Ca-activated K outward currents).

Amiloride

The *classical Na channel* of apical epithelial membranes, which in the toad urinary bladder is about 1000-fold more permeable to Na than to K [55], is blocked by amiloride with a (microscopic) inhibition constant ≤ 0.4 μM [42, 43, 60]. The *Na/H-exchanger* is blocked by amiloride with an inhibition constant of 25 μM and by the ring-modified analog 5-N-ethylisopropylamiloride (EIA) with an inhibition constant of 30 nM [22]. Some other Na transport systems are inhibited by amiloride not at all or only with very low efficacy [22]. For instance, the *Na/Ca-exchanger* is blocked with an apparent inhibition constant of 1–2 mM [34]. However, the side-chain modified analog 3′,4′-dichlorobenzamil (DCPA) inhibits Na/Ca-exchange with an apparent inhibition constant in the range 15–50 μM [34, 51].

An amiloride-blockable cationic inward current was recently found for isolated *taste receptor cells* [7]. This current does not discriminate well between Na and K, such that its reversal potential is close to 0 mV. However, its amiloride sensitivity is much larger than that of the olfactory current, the apparent inhibition constant being as low as 0.3 μM. The dark-current of vertebrate *photoreceptor cells* (rod outer segments) is blocked by the amiloride analog DCPA (apparent inhibition constant 1 μM). However, this compound appears to block the cGMP-activated channels from the cytosolic side of the membrane [51]. It is not known whether the dark-current is affected by amiloride itself.

In *olfactory receptor cells,* 50 μM of amiloride caused a rapid, complete and reversible suppression of the odorant-evoked depolarizations, by locking the membrane potential to values near −80 mV. Amiloride had no effect on type I-cells in the absence of odorants. Similarly, amiloride affects the electro-olfactogram only in the presence of odorants [56]. This seems to indicate a blockage of an odorant-induced effect by amiloride. For instance, amiloride might bind to odorant receptors as a competitive antagonist. However, it is not obvious why amiloride, which is a pyrazine, should block the effects of such diverse compounds as cineole, amyl acetate and isobutyl methoxypyrazine (*see* 56). It seems more likely that amiloride blocks an odorant-induced inward current. It is noteworthy that the reversal potential reported for this current is near 0 mV for standard ionic gradients [3, 50, 66]. Thus, if the induced inward current is due to amiloride-blockable channels, the Na specificity of these channels and their sensitivity to amiloride is smaller than in the epithelial-type Na channel (e.g. 60).

In the olfactory mucosa, blockage of the transmucosal short-circuit current by mucosal amiloride suggests that the putative amiloride-blockable channels are located at the apical membrane. However, in isolated cells the channels will, perhaps, also be found on the somal membrane, since cases are known where the opening of tight junctions during cell isolation allows the spread of apical membrane proteins over the cell surface [8, 13, 72]. This may explain why odorant-induced inward currents were found in on-cell membrane patches of dendrite and soma [47] and cAMP-induced inward currents in excised somal patches of isolated cells [50]. (However, part of the cAMP-sensitive channel population could well serve a physiological function in the basolateral membrane.) Thus blockability by amiloride may allow easy recognition of the odorant-induced current in the future.

Conclusion

We observed a cyclic voltage response of isolated receptor cells to odorants. During *prolonged* exposure to 500 nM cineole, the depolarization lasted only 5–10 sec. It was terminated by an extensive

repolarization (possibly induced by Ca inflow). It appears that subsequent removal of the hyperpolarizing mechanism, in the face of continued inward current, allows initiation of the next depolarization/hyperpolarization cycle. The resulting "slow waves" of membrane potential generated a bursting pattern of action potentials. Bursting patterns are also known from some recordings of single unit activity in the intact olfactory mucosa; their significance is not clear.

Amiloride (50 μM) prevented the depolarization elicited by 500 nM of cineole, locking the membrane potential to high negative values. This inhibition is probably due to blockage of the odorant-induced inward current, but other mechanisms still are to be excluded. Future work also needs to address the nature of the induced inward current(s) and of the hyperpolarizing mechanism.

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