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Electrophysiological Properties of Frog Olfactory Supporting Cells

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Abstract

Cells, identified as supporting cells by Lucifer Yellow injection, were recorded from slices of frog olfactory epithelium using patch-clamp recordings. Cell-attached single-channel recordings indicated that the intracellular potential (IP) was –68 ± 7 mV (n = 22) with 4 mM K^+ in the bath ($[K^+]_o$). IP was –67 ± 4 mV (n = 32) in whole-cell conditions with 100 mM KCl inside the cell, suggesting a low membrane permeability for Cl^-. IP depended on $[K^+]_o$ in a manner described by the Goldman–Hodgkin–Katz equation with a permeability ratio $p_{K+}:p_{Na+}$ of 40. The input resistance was 32 ± 14 MΩ (n = 15), indicating a high membrane conductance at rest. Odorant stimulations evoked passive membrane depolarizations, probably reflecting an increase in $[K^+]_o$ due to the neuronal activation. Whole-cell recordings with 100 mM CsCl instead of KCl in the pipette, together with the block of gap-junctions with octanol, indicated the existence of an electrical coupling between supporting cells. The electrical coupling between these glial-like cells could facilitate the clearance of K^+ ions released by olfactory receptor neurons during odorant stimulation.

Introduction

Supporting cells isolate and protect receptor neurons in the olfactory epithelium. In the frog, the cell body, in direct contact with the mucus layer at the surface of the epithelium, is surrounded by a single layer of 6–8 dendrites of receptor cells and 6–7 other supporting cell bodies (Graziadei, 1975). A basal process runs deep into the epithelium to make contacts with receptor cell bodies, capillaries and superficial Bowman's glands (Rafols and Getchell, 1983). Intracellular studies *in vivo* (Getchell, 1977; Trotier and MacLeod, 1986) and *in vitro* (Masukawa *et al.*, 1985a) indicate that amphibian olfactory supporting cells share common properties with glial cells. Odorant stimulations induce an increase of $[K^+]_o$ in the olfactory epithelium (Khayari *et al.*, 1991) and supporting cells are depolarized. The aim of the present study was to better characterize the properties of these cells using patch-clamp recordings.

Materials and methods

Slice preparation

Frogs (*Rana esculenta*) were anesthetized by cooling, doubly pithed and decapitated. The dorsal olfactory mucosa was dissected and placed on a filter paper soaked with Ringer. It was cut (200–300 µm thickness) under optical control using a razor blade, as previously described (Firestein and Werblin, 1989; Trotier, 1990a,b). The slice was secured, with two drops of paraffin, on a horizontal 14-mm-diameter glass coverslip. Another coverslip, placed 1.5 mm above, delimited an experimental chamber of ~70 µl which was constantly perfused using a Gilson minipulse peristaltic pump at a rate of 100 µl/min. As shown in Figure 1A, the slice (e) was placed with its luminal surface vertical and the recording electrode was moved horizontally through the layer of the olfactory cilia (c) to reach the apical membrane (am) of a supporting cell. The slice was observed under Normarsky illumination using an upright Zeiss Axioskop microscope (magnification ×400). In most cases the apical membrane was discernible. Recordings were made from cells at a distance from Bowman gland ducts, which were easily distinguished.

Recordings

The tip of the pipette (~1 µm in diameter after heat polishing; 6–10 MΩ) was gently pressed against the apical membrane of a supporting cell. The initial positive pressure applied to the pipette was suppressed when the electrical resistance of the pipette increased to ~20 MΩ, indicating a close contact with the membrane. A seal resistance >1 GΩ appeared within seconds either spontaneously or after the application of a mild negative pressure (2–10 cm of water) to the pipette. The rate of success of the sealing process was ~70%. The pipette potential was then kept at –100 mV for a few minutes to get a higher value of the seal resistance (8–80 GΩ). In a typical experiment the cell-attached configuration was used to estimate the intracellular potential from single-channel events and then the sealed membrane was

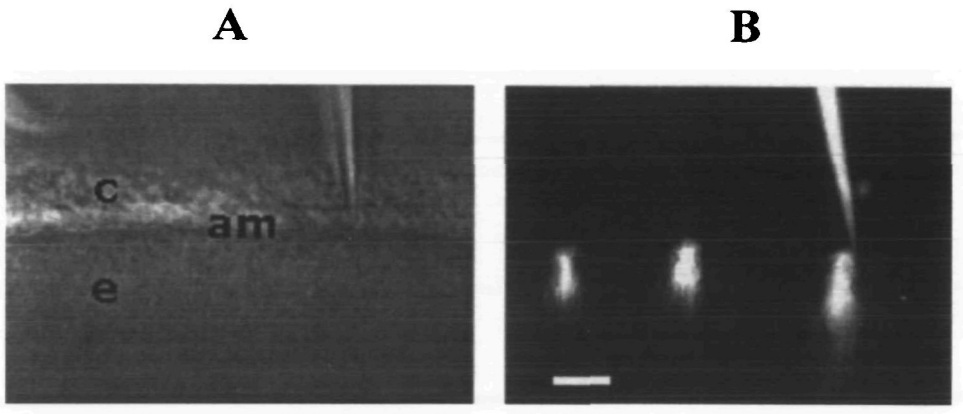

Figure 1 **(A)** Phase–contrast photomicrograph of the recording condition. The slice of epithelium (e) was placed with the apical (luminal) membrane (am) vertical and the electrode tip was moved horizontally through the cilia (c) of olfactory receptor cells. The electrode tip was sealed on the apical membrane of supporting cells. **(B)** The same slice was observed under UV illumination, without changing the field of observation, after 10 min of whole-cell recording with a pipette containing Lucifer Yellow. The recorded cell had the typical morphology of a supporting cell with a large cell body located at the luminal pole of the epithelium. Two other supporting cells, previously recorded with two different electrodes, can be seen on the left. Note the absence of spread of Lucifer Yellow from the recorded cells to other cells.

ruptured by suction to get the whole-cell configuration. Only whole-cell recordings which were followed by the excision of an outside-out patch membrane at the end of the experiment were considered.

Recordings were made with a patch-clamp amplifier (RK 300 with a 10 GΩ resistor; Biologic, France). Data were low-pass filtered (usually dc to 440 Hz; –3 dB; 5 poles Tchebicheff), digitized and stored on a computer for analysis with Biopatch software (Biologic, France). Results are given as mean ± 1 SD.

Solutions

All concentrations are given in mM. The pH of all solutions was 7.1 ± 0.1. The temperature in the experimental chamber was 21 ± 1°C. The bath contained NaCl 110, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10 and glucose 11 (Ringer's solution). The solution in the pipette was either KCl 100, $MgCl_2$ 1, HEPES 10, EGTA-Na_2 1.25 and $CaCl_2$ 1 (KCl internal solution) or CsCl 100, KCl 4, $MgCl_2$ 1, HEPES 10, EGTA-Na_2 1.25 and $CaCl_2$ 1 (CsCl internal solution). To measure the intracellular potential with various $[K^+]_o$s the slice was successively exposed to Ringer's solutions containing 2.5, 4, 8.5, 16, 30, 57 and 100 mM K^+, NaCl being replaced by KCl. The intracellular potential was measured after 7–12 min in the new solution. To block gap-junctions 1 mM octanol was added to the bathing Ringer's solution through the perfusion system. The slice was changed after each octanol treatment.

Lucifer Yellow injection

Lucifer Yellow CH (Lithium salt, Sigma ref. L-0259, 4 mg/ml in KCl or CsCl solution with 0.5 instead of 1 mM $CaCl_2$) was allowed to diffuse from the pipette into the recorded cell for 10 min in the whole-cell configuration. The slice was observed under UV illumination after ~30 min.

Results

Identification of recorded cells

Lucifer Yellow injections (12 cells from eight slices) indicated that recorded cells had the typical morphology of olfactory supporting cells, with an elongated cell body located near the surface of the epithelium (Figure 1B). Sometimes a long and thin process extending towards the depth of the epithelium was distinguishable. The diffusion of the dye from the recorded cell to the neighboring ones was not observed, except in two recordings where a very slight fluorescence was detectable in a few adjacent cells (not shown).

Single channel recordings

Cell-attached single-channel recordings were used to estimate the intracellular potential without any modification of the cell content. The pipette, filled with the KCl-solution, was sealed on the apical membrane and the patched membrane was depolarized by a ramp of potential to activate ionic channels (Figure 2A). These channels were characterized, after excision of the membrane patch and recording in the inside-out configuration (not shown), as calcium-dependent potassium channels of large conductance (BK channels; Marty and Neher, 1985). The conductance of these channels (253 pS in Figure 2A) was large enough to allow the estimation of the intracellular potential by extrapolating the current amplitude flowing through the open channel to the zero current level (dashed line in Figure 2A). The pipette potential at which the single-channel current was null was taken as the intracellular potential (–71 ± 4 mV for the cell recorded in Figure 2A). The mean value of 22 cells was –68 ± 7 mV with 4 mM K^+ in the bath, with a range from –58 to –82 mV.

Cell-attached single-channel recordings were also used to

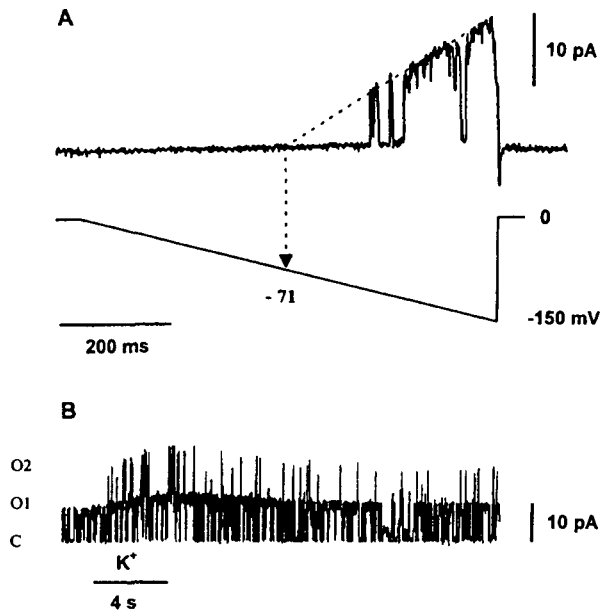

Figure 2 Cell-attached recording of BK channels. **(A)** Estimation of the intracellular potential. The patched membrane was depolarized by applying a ramp of potential (from 0 to −150 mV) to the recording pipette. The membrane depolarization induced the activation of a BK channel. The amplitude of the outward current flowing through the open channel was extrapolated to the zero-current level (dashed line). The pipette potential (−71 mV) at which the channel current was 0 pA corresponded to the intracellular potential. The pipette contained the KCl solution and the bath contained Ringer's solution. The leak current was substracted from the recording. **(B)** Membrane depolarization induced by increasing $[K^+]_o$ from 2.5 to 8 mM. The pipette potential was held at −110 mV to depolarize the membrane and activate BK channels (C: close state; O1 and O2: open states). $[K^+]_o$ was increased for the time indicated by the horizontal bar. As a result there was an increase of the amplitude of the outward current flowing through the open channels, indicating the depolarization of the membrane potential. The recording pipette contained the KCl solution.

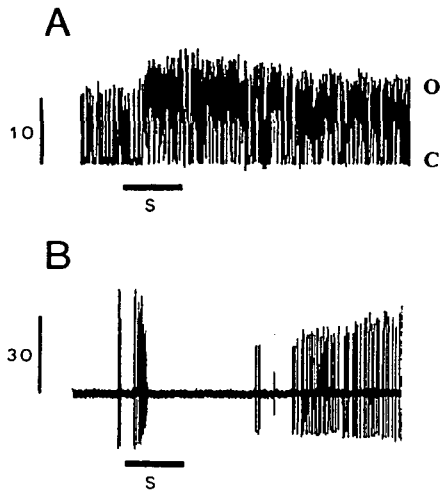

Figure 3 Responses to odorant stimulation. **(A)** Cell-attached recording of a BK channel. The stimulation (S) of the epithelium with butanol (1 mM in the stimulating pipette) increased the amplitude of the current flowing through the open channel (O), indicating cell depolarization. The pipette containing the KCl solution was held at −100 mV; the bath contained Ringer's solution. The level C corresponds to the closed channel. **(B)** Simultaneously with the record shown in (A), the extracellular impulse activity was recorded from an olfactory receptor cell using another recording pipette filled with Ringer's solution. The recording was band-pass filtered (0.1–1 KHz). Note that the odorant stimulation (S) evoked a rapid burst of action potentials of decreasing amplitude, followed by a silent period and a sustained impulse activity. Vertical scales are in pA; the stimulation (S) lasted for 5 s.

demonstrate that increasing $[K^+]_o$ evoked depolarization of supporting cells. In the example shown in Figure 2B the pipette potential was held at −110 mV and the single-channel current was 8.2 ± 0.1 pA with 2.5 mM $[K^+]_o$. The resting intracellular potential, measured as previously indicated, was −76 ± 3 mV. For the time indicated by the bar, $[K^+]_o$ was increased to 8 mM. Consequently, the amplitude of the single-channel current increased to 11.6 pA. The conductance of this channel was 242 pS. Therefore, the observed change of the channel current (3.4 pA) was calculated as being due to a 14 mV depolarization of the cell potential. The effect was reversible upon washing. Similar results were observed on 12 cells.

Odorant stimulations also evoked the depolarization of the membrane. In the example shown in Figure 3A butanol (1 mM in the stimulating pipette) was applied for 5 s and induced an increase of the single-channel current from 9.6 to 14.4 pA at a pipette potential of −100 mV. The channel conductance was 274 pS and the intracellular potential was −65 mV. Therefore, the change (4.8 pA) of the single-channel current observed during odorant stimulation was due to a depolarization of 17.5 mV of the intracellular potential. In this experiment the extracellular impulse activity of a receptor neuron at ~80 µm was simultaneously recorded (Figure 3B). The response consisted in a burst of spikes of decreasing amplitude, followed by a silent period and then by a long-lasting period of sustained firing activity. This kind of response is typical of a very large depolarization of olfactory receptor cells (e.g. Trotier and MacLeod, 1983). The comparison of the time course of both responses (Figure 3A,B) clearly indicates that supporting cells are depolarized during activation of olfactory receptor neurons by odorants. According to the relationship shown in Figure 4B (see below), the 17.5 mV depolarization of the supporting cell is representative of an increase in $[K^+]_o$ of ~9 mM.

Whole-cell recordings with KCl solution in the pipette

A typical experiment is shown Figure 4A. The upper trace represents the current and the lower trace the potential. The pipette was voltage-clamped at −75 mV and 10 mV depolarizing voltage pulses were repeatedly applied. At the onset of the whole-cell configuration (indicated by the arrow in Figure 4A) the amplitude of the current pulses elicited by the imposed voltage pulses indicated an input resistance of 38 MΩ. During the period indicated by the

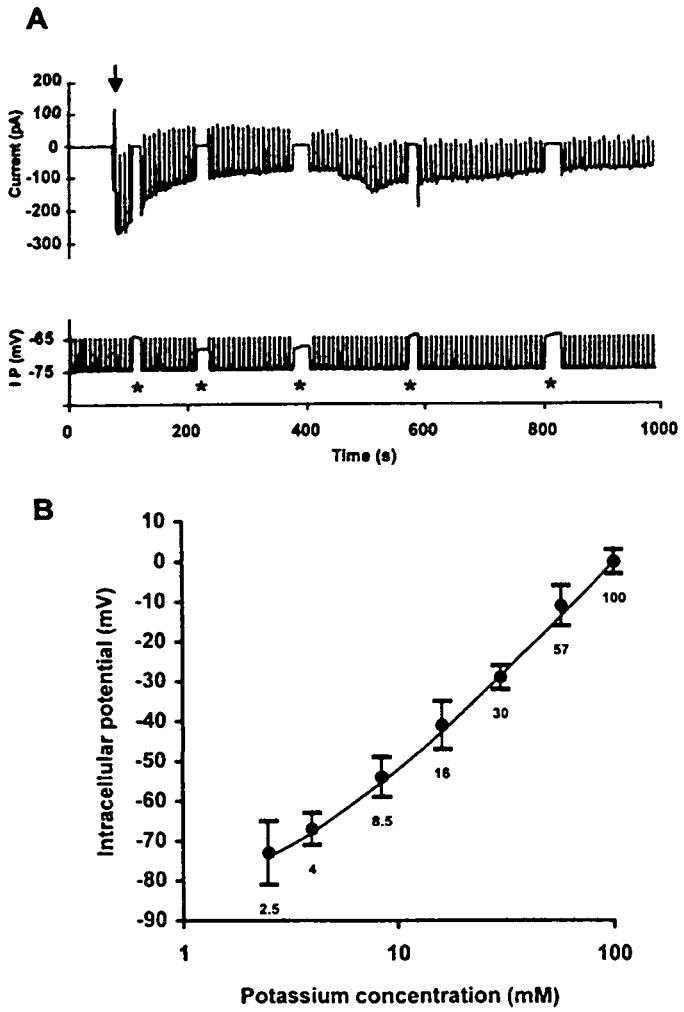

Figure 4 Measurement of the intracellular potential in whole-cell conditions. The pipette contained the KCl solution. **(A)** The pipette potential was held at –75 mV and 10 mV depolarizing voltage pulses were repeatedly applied. The arrow indicates the onset of the whole-cell recording. The input resistance was measured by dividing the imposed voltage pulse by the elicited current pulse. During the periods indicated by the stars the amplifier was shifted to the current-clamp mode at the zero current level to measure the intracellular potential (IP). The bath contained Ringer's solution. **(B)** Relationship between the intracellular potential and $[K^+]_o$. The mean potential from 12 cells (± 1 SD) is indicated for each $[K^+]_o$ value. Data were fitted (Pearson's $r > 0.97$) with the Goldman–Hodgkin–Katz equation with a ratio of permeabilities ($R = p_{K+}:p_{Na+}$) of 40. The pipette contained KCl solution.

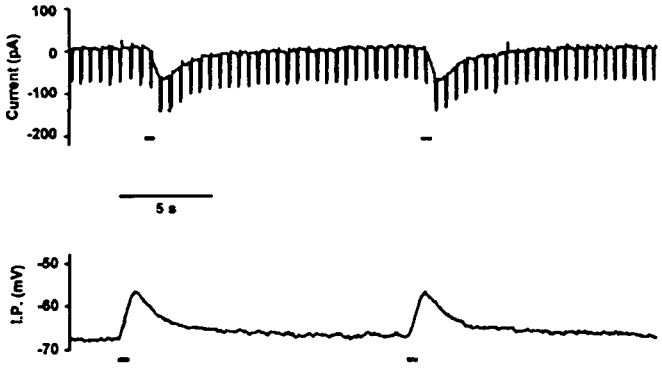

Figure 5 Responses to odorant stimulation in whole-cell conditions. **(A)** Voltage-clamp recording at –65 mV. Two successive stimulations with butanol, indicated by the underlying bars, evoked two inward current waves. No change of the input resistance was observed, as indicated by the constant amplitude of the current pulses elicited by voltage pulses of 10 mV. **(B)** Recording of the same cell in the current-clamp condition at the zero current level. Odorant stimulations were repeated and evoked depolarizations of ~10 mV. Odorant stimulus: butanol at 1 mM in the stimulating pipette. The recording pipette contained KCl solution, the bath Ringer's solution.

first underlying star (lower trace) the amplifier was shifted to the current-clamp mode at the zero current level and the intracellular potential was measured (IP = –64 mV). This measurement, repeated during other periods indicated by the stars, denoted the stability of the intracellular potential within ~5 mV. The mean IP measured in this condition, from 32 cells bathed in Ringer's solution containing 4 mM $[K^+]_o$, was –67 ± 4 mV. This value was very close to the value previously observed in cell-attached single-channel recordings (–68 ± 7 mV), indicating that the presence of a high Cl^- concentration inside the cell had little effect upon the resting potential value.

In the recording shown in Figure 4A, the input resistance gradually increased to reach 113 MΩ within ~10 min. The same observation was made on 14 other cells. At the beginning of the whole-cell recording the mean input resistance was 32 ± 14 MΩ ($n = 15$), with a range from 16 to 68 MΩ. After 8–12 min of recording it reached 164 ± 32 MΩ ($n = 15$), with a range of 68 to 212 MΩ.

The effect of changing $[K^+]_o$ in the bathing Ringer's solution was measured on 12 cells (Figure 4B). Decreasing the $[K^+]_o$ to 2.5 mM increased the IP to –73 ± 8 mV whereas increasing the $[K^+]_o$ to 8.5 mM decreased the IP to –54 ± 5 mV. Experimental points were fitted by the Goldman–Hodgkin–Katz equation ($IP = 58\log((R[K^+]_o + [Na^+]_o)/(R[K^+]_i + [Na^+]_i))$. A good fit (Pearson's $r > 0.97$) was obtained with $R = 40$, the $p_{K+}:p_{Na+}$ membrane permeability ratio. Therefore the membrane of olfactory supporting cells at rest appears to be highly and selectively permeable to K^+ ions.

Responses to odorant stimulations were recorded by applying butanol (1 mM in the stimulating pipette) near the site of recording. In the example presented in Figure 5 (upper trace), the stimulation evoked an inward current of ~70 pA in voltage-clamp conditions at –65 mV. During these responses there was no significant change in the input resistance ($n = 8$ cells) measured by applying repetitive 10 mV depolarizing voltage pulses. In current-clamp conditions (Figure 5, lower trace) the stimulation of the same cell evoked depolarizations of ~10 mV. Using the relationship indicated in Figure 4B, this depolarization was representative of a shift of $[K^+]_o$ from 4 to ~8.5 mM. Similar

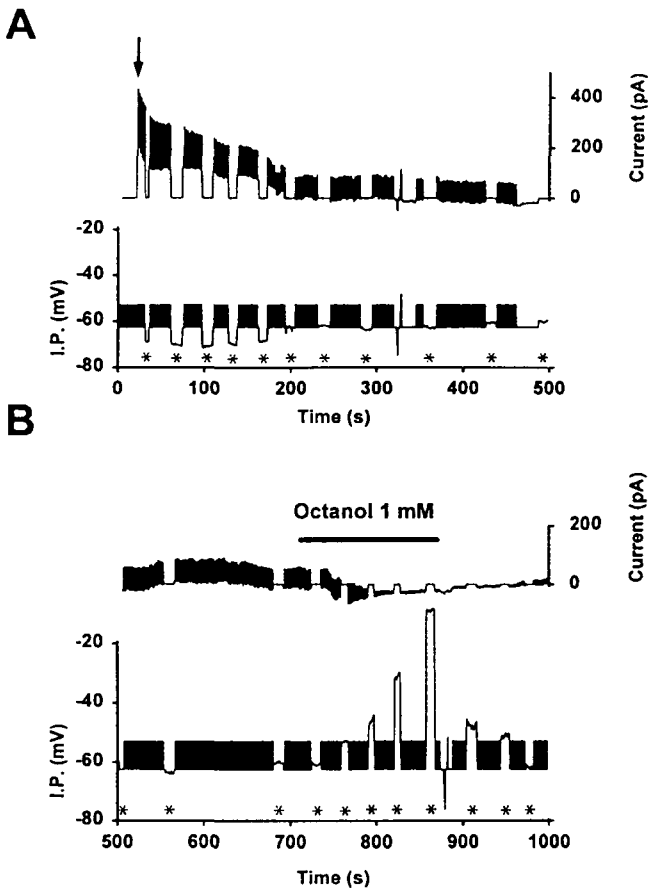

Figure 6 Whole-cell recording with CsCl solution in the recording pipette. **(A)** The pipette potential was held at –62 mV and 10 mV depolarizing voltage pulses were repeatedly applied. The arrow indicates the onset of the whole-cell recording. The intracellular potential (IP, lower trace) was measured as the zero current potential in the current-clamp mode during the periods indicated by stars. Notice that the intracellular potential remained around –60 mV. **(B)** Continuation of the record. After 11 min of recording the IP octanol (1 mM) was added to the Ringer's solution in the bath to block gap-junctions. As a result there was a large increase in the input resistance, indicated (upper trace) by the decrease in the current shifts evoked by the 10 mV depolarizing pulses, together with the decrease in the intracellular potential towards 0 mV (lower trace). At the end of the perfusion with octanol the membrane potential gradually returned to its original value and the input resistance decreased.

odorant-evoked responses were recorded from six other cells.

Whole-cell intracellular potential with CsCl solution in the pipette

As the intracellular potential of these cells depends on the ionic gradient of K^+ ions through the membrane, a rapid depolarization was expected by using CsCl instead of KCl in the whole-cell recording pipette. On the contrary, the intracellular potential remained negative (–60 mV in the example shown in Figure 6A, lower trace), even after >10 min. The mean potential measured on 14 cells was –48 ± 8 mV with 4 mM K^+ in the bath. Odorant-evoked depolarizations similar to those recorded using the internal KCl solution were recorded with the CsCl internal solution (not shown; n = 5). There was a gradual increase of the input resistance, which reached 123 ± 43 MΩ (n = 14) after 8–12 min of recording, a value close to that observed with the internal KCl solution.

Closure of gap-junctions with octanol

The results of the experiments with internal CsCl made it plausible that the recorded intracellular potential did not correspond simply to the membrane potential of the cell connected to the recording electrode but rather to the intracellular potential of an ensemble of electrically coupled supporting cells. To test this hypothesis 1 mM octanol was added to the bath because octanol is known to block transcellular communications through gap-junctions (e.g. Spray *et al.*, 1997). Octanol induced a large increase of the input resistance (upper trace in Figure 6B) together with a nearly complete membrane depolarization of the recorded cell loaded with CsCl (Figure 6B, lower trace). In 10 cells octanol increased the input resistance to 485 MΩ–1.1 GΩ and decreased the intracellular potential to –8 ± 7 mV. The effect of octanol was partially reversible after extensive washing, as shown at the end of the recording presented in Figure 6B. When octanol was applied on cells (n = 3) recorded with the KCl internal solution a depolarization in the range of 15–22 mV was recorded but the large increase of the input resistance observed with Cs^+-loaded cells was never observed.

Discussion

Electrical properties of frog olfactory supporting cells

The present study demonstrates that olfactory supporting cells are amenable to patch-clamp studies *in situ* from slices of frog olfactory epithelium. The mean intracellular potential was –68 mV with 4 mM K^+ in the bath, a concentration close to the physiological K^+ concentration that we measured *in vivo* in the mucus layer using K^+-sensitive microelectrodes (Khayari *et al.*, 1991). There was no significant difference between the mean intracellular potential recorded in the cell-attached configuration and in the whole-cell mode with a high intracellular Cl^- concentration, suggesting a negligible effect of a high internal Cl^- concentration. In the present study the intracellular potentials were less negative than those observed in the salamander using intracellular electrodes (near –95 mV: Masukawa *et al.*, 1985a; Trotier and MacLeod, 1986). This difference could reflect either variations between species or a side effect of the recording conditions.

The dependence of the intracellular potential upon $[K^+]_o$ indicates that the membrane of olfactory supporting cells is ~40 times more permeable to K^+ than to Na^+. Similar $p_{K+}:p_{Na+}$ permeability ratios have been measured from

Müller cells, the principal glial elements of the vertebrate retina (Conner *et al.*, 1985). In the present *in vitro* experiments supporting cells were depolarized during odorant stimulations. These depolarizations were similar, in time course and amplitude (up to ~15 mV), to those recorded previously *in vivo* with intracellular electrodes (Trotier and MacLeod, 1986), but were not associated with a conspicuous change in membrane resistance. As the membrane potential of these cells is very sensitive to variations in $[K^+]_o$, it is very likely that the odorant-evoked depolarizations were induced by an increase of $[K^+]_o$ following neuronal activation. We previously showed (Khayari *et al.*, 1991) that the resting $[K^+]_o$ is ~3.7 mM in the mucus layer of the frog olfactory epithelium and increases to ~8 mM during stimulations with odorants. According to the relationship shown in Figure 4B, such an increase depolarizes supporting cells by ~13 mV.

Electrical coupling between olfactory supporting cells

Perfusing the recorded cell with CsCl instead of KCl decreased the intracellular potential somewhat but never abolished it even after >10 min of recording. To test the hypothesis of electrical coupling between the recorded cell and other supporting cells octanol was used to block gap-junctions. The uncoupling induced a large increase of the input resistance and a complete depolarization of the recorded cell loaded with CsCl. The effect of octanol was reversible during washing and the opening of the gap-junctions fully restored the intracellular potential.

It should be emphasized that the uncoupling effect of octanol could be recorded only when the high resting K^+ permeability of the cells was blocked by internal Cs^+. When the cells were recorded with the KCl internal solution, octanol induced a depolarization of no more than 22 mV. As octanol is a potent odorant this depolarization probably resulted from a strong excitation of olfactory receptor neurons. Nevertheless the block of gap-junctions by octanol did not lead to a large increase of the input resistance of K^+-loaded cells because, in that case, the high resting K^+ membrane conductance dominates.

There is no indication of gap-junctions between frog olfactory supporting cells in the literature. However, gap-junctions between supporting cells have been identified in other species during the development of the olfactory epithelium (Kerjaschki and Hörandner, 1976; Miragall *et al.*, 1992) and also in adult animals (Usukura and Yamada, 1978). These gap-junctions are mostly present in corners where two tight-junctional ridges fuse (Kerjaschki and Hörandner, 1976). In adult mice (Miragall *et al.*, 1994) gap-junctions are observed between supporting cells, associated with the apical tight-junctional strands or in deeper regions of the epithelia. Immunocytochemistry using specific polyclonal antibodies indicates the presence of connexin in the apical third of the olfactory neuro-epithelium and in association with the apical junctional complex (Miragall *et al.*, 1994). Cx43 is the major constituent of gap-junctions in astrocytes (Paul, 1995). In the present study, it is proposed that these gap-junctions allow electrical communication between supporting cells.

There are several instances in which a glial syncytium is more efficient than a population of independent cells. For example, astrocytic gap-junctions play an essential role in dissipation and homeostasis of K^+ ions, supply of nutrient metabolites to neurons, and regulation of the cell volume and intercellular Ca^{2+} signaling between astrocytes, and between astrocytes and neurons (Giaume and McCarthy, 1996). In the retina K^+ is transported via Müller-cell spatial-buffering currents (Newman and Reichenbach, 1996) and amphibian Müller cells are coupled together by gap-junctions (Mobbs *et al.*, 1988). Gap-junctional coupling of Müller cells increases by 60% the 'spatial buffering' that these cells can carry out to reduce localized rises in extracellular potassium concentration during neuronal activation. It is highly probable that supporting cells play a similar role in the olfactory epithelium and maintain the neuronal microenvironment. The existence of a functional syncytium of supporting cells around olfactory sensory neurons would provide a homogeneous environment for the process of olfactory reception (Miragall *et al.*, 1994).

In the present study the input resistance at the site of recording was 32 ± 14 MΩ. Similar low values, indicative of a high membrane conductance at rest, have been observed in salamander olfactory supporting cells using intracellular electrodes (Masukawa *et al.*, 1985a; Trotier and MacLeod, 1986). The input resistance of some supporting cells was higher after olfactory nerve transection and receptor cells degeneration (Masukawa *et al.*, 1985b).

Additional experiments are needed to estimate the contribution of gap-junction conductance to the recorded input resistance and to establish the space constant of the electrical coupling.

A slow increase in the input resistance, reaching ~160 MΩ within a few minutes of whole-cell recording, was observed. Applying several pulses of suction to the pipette interior did not reverse this increase. Therefore, it probably did not result from an increase in the access resistance at the tip of the pipette. The origin of this systematic change remains to be determined. It is important to consider that transcellular connections through gap-junctions are not static but dynamic. For example, the junctional conductance through gap-junctions is modulated by pH and internal Ca^{2+} concentration (Spray *et al.*, 1982), or by activation of membrane receptor and activation of transduction pathways (Giaume and McCarthy, 1996). Therefore, recording in the whole-cell configuration, which rapidly modifies the intracellular content, could have interfered with this regulation.

The diffusion of the dye Lucifer Yellow CH was restricted to the recorded cell. The same observation was made using intracellular recordings (Masukawa *et al.*, 1985a). Nevertheless, this does not imply the absence of electrical

intercellular communications because electrical coupling without dye coupling has been observed in many preparations: mammalian astrocytes and oligodendrocytes in cell culture (Ransom and Kettenmann, 1990), neurons (Powel and Westerfield, 1984) and pancreatic beta cells (Perez-Armendariz *et al.*, 1991).

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