Gated currents in isolated olfactory receptor neurons of the larval tiger salamander

(olfaction/electrophysiology/patch clamp)

STUART FIRESTEIN AND FRANK S. WERBLIN*

Graduate Group in Neurobiology, Zoology Department, University of California, Berkeley, CA 94720

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ABSTRACT The electrical properties of enzymatically isolated olfactory receptor cells were studied with whole-cell patch clamp. Voltage-dependent currents could be separated into three ionic components: a transient inward sodium current, a sustained inward calcium current, and an outward potassium current. Three components of the outward current could be identified by their gating and kinetics: a calcium-dependent potassium current [$I_K(\text{Ca})$], a voltage-dependent potassium current [$I_K(\text{V})$], and a transient potassium current (I_a). Typical resting potentials were near -54 mV, and typical input resistance was $3\text{--}6$ G Ω . Thus, only 3 pA of injected current was required to depolarize the cell to spike threshold near -45 mV. The response to a current step consisted of either a single spike regardless of stimulus strength, or a train of <8 spikes, decrementing in amplitude and frequency over ≈ 250 msec. Thus, the receptor response cannot be finely graded with stimulus intensity.

Recent studies of vertebrate olfactory receptor neurons have yielded conflicting results. Conventional intracellular recordings (1–3) show a cell with a resting level of -40 mV and an input resistance of several hundred M Ω . Patch-clamp studies in salamander (4, 5) show more negative resting potentials, much higher input resistances, and concomitantly greater membrane time and length constants.

Responses are reported to consist of spike trains with frequencies up to 40 Hz (2, 3), or a brief burst of 6–8 spikes in response to odorants or current injection (6).

Some of the membrane voltage-gated currents have also been measured. In mouse, single-channel recordings have revealed three outward potassium conductances (7, 8), and recently two additional potassium conductances have been reported (9). Whole-cell studies have variously described three (9), four (10, 11), and five (5, 6) gated currents.

We have attempted to resolve some of the discrepancies in the earlier work through a more thorough study of the electrical constants and gated membrane currents measured with whole-cell patch clamp.

MATERIALS AND METHODS

Preparation. Cells from the nasal epithelium were isolated using an enzyme treatment [papain type IV (Sigma), 0.75 mg/ml activated with *N*-acetyl cysteine (Sigma), 0.25 mg/ml for 25 min at room temperature] and gentle mechanical disruption (12). Aliquots (0.5 ml) of Ringer's solution with isolated cells were pipetted into a chamber on a glass slide and the cells were allowed to settle; no anchoring materials were used. The preparation and electrode were viewed with a Zeiss $\times 40$ water immersion objective.

Recording System. Patch electrodes of 5–7 M Ω resistance were made on a modified Kopf electrode puller (David Kopf, Waltham, MA) using borosilicate glass (outer diameter, 1.5 mm) (WPI Instruments, New Haven, CT; TW 150). We recorded with a Dagan patch clamp (Model 8900, Dagan Instruments, Minneapolis, MN) in the whole-cell configuration (13) using a 1 G Ω feedback resistor in the current-to-voltage converter. Data were digitized, stored, and processed using an IBM PC/XT with Data Translation (Marlboro, MA) analog interface board. Current injection was controlled by the IBM PC/XT with an A/D interface.

Solutions. Patch electrodes normally contained 116 mM KCl, 4 mM Hepes, 2 mM MgCl₂, 1.6 mM CaCl₂, and 2 mM EGTA to buffer Ca to 0.1 μM at pH 7.4. The extracellular medium contained 130 mM NaCl, 2.5 mM KCl, 4 mM CaCl₂, 0.4 mM MgCl₂, 10 mM Hepes, and 10 mM glucose at pH 7.5. This medium was hyperosmotic (298.2 mosM vs. 272 mosM) to prevent cells from swelling once isolated from the epithelium. Bath solutions were changed with a peristaltic pump at a rate of ≈ 0.5 ml/min; changes typically elicited their full effect within 2 min.

Cell Morphology. Isolated olfactory receptors resemble those reported in anatomical studies of *in situ* prepared cells (14). Cell bodies are elliptical with dimensions on the order of 20×12 μm . Most cells had both dendritic and axonal processes still attached; $\approx 25\%$ appeared to have only the dendrite. Axons were very fine (<1 μm) and generally no more than a 15- μm length remained attached to the cell. The dendritic process was much stouter (1–3 μm thick) and quite variable in length, ranging from 10 to >100 μm . A knob-like structure at the end of the dendrite was present in $>75\%$ of the cells, but cilia were seen in $<10\%$ of the cells viewed.

Only cells not granulated, with an axon segment, complete dendrite, and cilia were studied. Smaller cells with shorter dendrites were easier to space clamp and more likely to be mature receptors (15) with a full complement of conductances. More than 50 cells were looked at in the course of the experiments described in this report. In the following sections, the numbers of cells used to determine the values of the electrical constants are those for which specific and careful measurements were made. All cells were checked to determine whether they fit within these values before additional experiments were carried out.

Resting Potential. The resting potential was -54 mV $\pm$ 8.2 mV (mean $\pm$ SD; $n = 15$). This is considerably more positive than the calculated Nernst potential for K⁺ (-98 mV), suggesting that the resting membrane is permeable to other cations besides K⁺. It is, however, more negative than the values commonly reported by investigators using intracellular microelectrodes (1–3).

Input Resistance. The input resistance was determined by measuring the response to a step in command potential from -70 to -100 mV, a region where no gated currents are

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*To whom reprint requests should be addressed.

FIG. 1. Electrical constants. (A) Measurement of membrane capacitance. From the subthreshold voltage trajectory in current clamp, the membrane time constant was measured as the time required for the voltage to reach $1 - e^{-1}$ ($\approx 63\%$) of the plateau value. This gives a value of 104 msec. The slope of the straight line in a semilogarithmic plot of the response (not shown) gave a time constant of 98 msec. The input resistance measured as the potential change to a known input current during the plateau phase is 5 G Ω . Input resistance measured under voltage clamp conditions after the capacitive transient gives a similar value. The measured time constant and input resistance allows calculation of the cell capacitance. For this cell the value is 21 pF. (B) Determination of electrode series resistance. The capacitive current flowing in response to a 30 mV step (from -100 to -70 mV) decays with a time constant of 200 μ sec. Given the cell capacitance of 21 pF, the electrode series resistance is just under 10 M Ω . Electrode and system capacitance were compensated for electrically prior to patch rupture.

present. For 28 cells, the measured mean input resistance ($\pm$ SD) was 5.2 ± 1.3 G Ω . This is nearly an order of magnitude higher than that found in a recent study using intracellular electrodes (3).

Cell Capacitance. The cell capacitance was estimated from the trajectory of the voltage response to a subthreshold

current step (Fig. 1A). The time constant was typically ≈ 100 msec. With the input resistance of 5 G Ω , the estimated membrane capacitance was 20 pF (± 3 pF; $n = 12$).

Cell-Surface Area. Assuming that the specific membrane capacitance is $1 \mu\text{F}\cdot\text{cm}^{-2}$, the surface area of the cell would be $\approx 2000 \mu\text{m}^2$. An average cell was $20 \times 12 \mu\text{m}$ (with dendrite $10 \times 2 \mu\text{m}$). Approximating the cell as two cylinders, the surface area would be $\approx 1600 \mu\text{m}^2$.

Series Resistance. We estimated the series resistance from the current transient following a voltage step. The response in Fig. 1B has a time constant of 200 μ sec. With cell capacitance of 20 pF, this implies a series resistance of 10 M Ω or less. For a 500 pA current, this would cause a 5-mV error due to the voltage drop across the series resistance leading to only a minor error in the response kinetics.

RESULTS

Membrane Currents

Whole-Cell Currents. Fig. 2A shows the two major currents elicited by depolarizing steps of voltage from a holding potential of -80 mV. A transient inward current appeared at -40 mV and became larger, faster, and more transient with more depolarizing steps reaching up to 1 nA at -15 mV. With further depolarization to 0 mV, this current diminished but was followed in time by a more slowly developing sustained outward current. The current-voltage curves taken at the peak of the inward current and during the sustained portion of the outward current are shown in Fig. 2B.

Five Separate Ionic Components. These whole-cell currents could be decomposed into five distinct components: a transient inward current carried by Na^+ , a sustained inward current carried by Ca^{2+} , and three outward currents carried by K^+ . The three outward currents are distinct in their gating characteristics and their susceptibility to various pharmacological agents and inactivation by conditioning steps. The compositions of all bathing and electrode solutions and descriptions of procedures are given in the figure legends.

Transient Inward Current. Fig. 3A shows a rapid inactivating inward current elicited by depolarizing steps between -36 and -16 mV from a holding potential of -80 mV. At -15 mV, it reached a peak magnitude of almost 350 pA in <3 msec. In some cells, peak current was as large as 1 nA; the mean peak magnitude was 650 ± 100 pA ($n = 9$).

This current was insensitive to tetrodotoxin, even up to concentrations of 30 μM . Replacing Na^+ with either choline

FIG. 2. Whole-cell currents. (A) Responses to voltage steps. Step levels are shown at right of traces. Holding potential was -80 mV; step duration was 100 msec. (B) Current-voltage relations. Peak of the transient inward current ($\bullet$) and 50 msec after the onset of the voltage step during the sustained plateau of the outward current ($\times$). The transient inward current appears to reverse at $+40$ mV. This probably reflects a substantial activation of the outward currents at this potential. When the outward current is blocked, the transient inward current reverses near $+60$ mV.

FIG. 3. Inward currents. (A) Transient inward current. Transient inward current was elicited by depolarizing steps in the range -40 to -15 mV. Depolarizations beyond -15 mV elicited smaller currents. Outward currents were blocked by replacing K^+ with equimolar Cs^+ in the electrode. Co^{2+} (1 mM) in the bath blocked Ca^{2+} currents. Numbers near the current traces are the command potentials. Holding potential for all cells was -80 mV. (B) I - V plot for transient inward current. Note reversal near $+50$ mV. (C) Na^+ inactivation. Voltage protocol is as shown in lower traces. Conditioning pulse was delivered for 50 msec followed by test pulse to 0 mV. Numbers near current traces refer to conditioning pulse potential. Same cell as in B. (D) Na^+ inactivation curve. Normalized peak current (ordinate) vs. conditioning pulse potential. Note that at resting potential of -55 mV $<40\%$ of the current has been inactivated. (E) Sustained inward current. Outward currents were blocked by replacing K^+ with an equimolar combination of Cs^+ (90 mM) and tetraethylammonium (36 mM) in the electrode solution and substituting 30 mM tetraethylammonium for an equimolar amount of Na^+ in the bath medium. The transient inward current of A was abolished by replacing the remaining Na^+ in the bath with choline. This remaining current is completely blocked by the addition of 1 mM Co^{2+} to the bath. (F) I - V curve taken at the peak of the sustained inward current. (Bars: A and C, 100 pA and 10 msec; E, 25 pA and 10 msec.)

or Tris in the bathing solution abolished it completely. This and the reversal potential near $+50$ mV (see Fig. 3B) suggest that it is probably carried by Na^+ .

The current inactivated rapidly, approximately exponentially, with a time constant ranging from 12.5 msec at -60 mV to 2 msec at -20 mV. The inactivation curve of Fig. 3D shows that even at the measured resting potential of -55 mV one-third of the sodium current is inactivated. This suggests that the true resting level may be somewhat more negative (see Discussion).

Sustained Inward Current. Extreme pharmacological treatments were necessary (Fig. 3E and legend) to reveal the sustained inward current. It reached a maximal amplitude of <100 pA but was often smaller. It activated at -20 mV, was maximum at $+20$ mV, reversed near $+50$ mV (Fig. 3F), and was blocked by as little as 1 mM Co^{2+} in the bathing medium.

These results suggest that the sustained current is carried by calcium (16). When the transient inward Na^+ current was abolished by replacement with choline, the remaining Ca^{2+} current was not sufficient to generate a spike. In fact it does not even contribute to any substantial depolarization of the membrane since, as discussed below, it serves to activate an outward potassium current, which effectively opposes further depolarization to injected current.

Outward Current. Depolarizations beyond -20 mV elicited an outward current that increased dramatically beyond -10 mV, typically reaching 1 nA at $+40$ mV as shown in Figs. 2 and 4A. It was blocked by intracellular replacement of K^+ with Cs^+ or Cs^+ and tetraethylammonium, suggesting that it is carried by potassium.

This outward current is a composite of three separate potassium components distinguishable by activation and inactivation ranges, kinetics, and pharmacology. Their contribution to the net current varied from cell to cell: all three currents were not present in every cell.

Voltage-Dependent Current [$I_{K(V)}$]. Of the three components of the outward current, this was the least variable. Virtually all cells possessed this sustained voltage-dependent current. It was isolated by holding the membrane at -10 mV for 500 msec, thereby inactivating the other voltage-dependent outward currents (see below) and by blocking Ca^{2+} -dependent outward currents with Co^{2+} . The remaining current, $I_{K(V)}$ was elicited by steps beyond -15 mV. At $+30$ mV, it commonly reached 250 pA (Fig. 4B). It activated slowly and was sustained for up to 600 msec.

Tail currents showed a reversal near -65 mV, ≈ 30 mV positive to the calculated Nernst potential for K^+ . Tetraethylammonium blocked this current but also had nonspecific blocking actions on the total outward current. Therefore it could not be used to selectively characterize particular components of the net outward current.

Ca^{2+} -Dependent K^+ Current [$I_{K(Ca^{2+})}$]. We subtracted currents in the presence of 4 mM Co^{2+} in the bathing solution from normal records to reveal the calcium-activated potassium current (Fig. 4C). This current activated near -20 mV, reached a peak magnitude of 300 pA within 15 msec, and inactivated slowly with a time constant >1 sec. It was blocked by reduction of extracellular $[Ca^{2+}]$ to 10 nM or replacement of Ca^{2+} with equimolar Ba^{2+} or Cd^{2+} .

Inactivating K^+ Current [I_a]. We were able to isolate a portion of the potassium current in about one-half of the cells, which inactivated with a time constant of 45 msec. It shared many characteristics of the "A-current" described in molluscan neurons (17).

This current could be completely inactivated with a 500-msec conditioning step to -10 mV, and it was also blocked by the addition of 10 mM 4-aminopyridine to the bath. As shown in Fig. 4D, it reached a maximum amplitude of 350–400 pA at membrane potentials of $+20$ mV.

Voltage Responses to Injected Current

Spike Threshold. Receptor neurons fired one to several action potentials in response to current steps as small as 3 pA (Fig. 5A). Threshold for action potential generation in these cells was commonly between -45 and -50 mV. A variety of spiking patterns was seen (Fig. 5), ranging from cells that fired only a single action potential for any suprathreshold stimulus (Fig. 5B) to cells that generated brief trains of action potentials (Fig. 5D). Spike threshold was reduced and spike amplitude was increased when the membrane was held 15–20 mV more negative than the resting potential.

Spiking Patterns. Impulse trains typically consisted of up to 8 spikes. The first spike displayed a rapid rise (30 V/sec) to a peak potential of between 0 and $+20$ mV and an almost equally rapid repolarization. Some spikes were as large as 100

FIG. 4. Outward currents. (A) Total outward current. Response to a voltage step to +20 mV. Na^+ current was blocked by replacing Na^+ with choline extracellularly. Ca^{2+} current was not blocked because it gates a portion of the outward current. It does not seriously distort the measurement because of its small magnitude (<100 pA). (B) Voltage-dependent K^+ current [$\text{I}_{\text{K}}(\text{V})$]. Current remaining when 2 mM CO_2^{2+} and 10 mM 4-aminopyridine have been added to the bath Ringer's solution. It can also be revealed by treatment with 2 mM Co^{2+} and a voltage command protocol that inactivates the transient portion of the outward current (see below). (C) Ca^{2+} -dependent K^+ current [$\text{I}_{\text{K}}(\text{Ca})$]. Subtraction in the presence and absence of 2 mM Co^{2+} —i.e., the difference in outward current due to influx of Ca^{2+} . The inward transient at the beginning of the record is an artifact of the subtraction procedure. Note very slow inactivation. (D) Transient outward current [I_{a}]. A current measured by subtraction of two responses to a voltage step to +20 mV with and without a 500-msec conditioning pulse at -10 mV. A virtually identical current was blocked by 10 mM 4-aminopyridine (not shown). Bath contained 2 mM Co^{2+} and choline replaced Na^+ .

mV. In single spiking cells the membrane potential returned to a plateau level near -40 mV.

Subsequent spikes were slower in both the rising and falling phase (3.1 and 4 msec, respectively) and were smaller both at the peak (-10 to 0 mV) and during the afterhyperpolarization. In addition, the interspike intervals increased by as much as 5 times, from 30 msec to >150 msec.

With longer current steps, from 0.5 to 2 sec (Fig. 5D), the spikes degenerated into oscillations around a plateau membrane potential commonly between -35 and 0 mV. This rapid damping of the response would not have been seen at shorter times (compare, for example, traces in Fig. 5C and D).

DISCUSSION

Our results can be correlated with those of earlier studies to resolve some of the discrepancies that seem to exist in the values of electrical constants and identities of gated currents

in olfactory receptors. The resolution to some of the main issues follows.

Resting Potential. In contrast with both intracellular studies (1–3) and other whole-cell patch studies (4, 9–11), which reported resting potentials of about -40 mV, we have found the mean resting level to be -55 mV. The range was -35 to -70 mV. The less-negative resting potentials are problematic since at -40 mV $>80\%$ of the sodium current would be inactivated (see Fig. 3D), and it is difficult to see how the cell could generate a spike.

The discrepancy in measurements could be due to several factors. Immature receptor cells are reported to have more depolarized resting potentials (18) and it is possible that some studies have unintentionally selected for these cells. In intracellular experiments, the shunt caused by electrode penetration could cause a depolarization that would be just matched by the activation of the potassium current near -40

FIG. 5. Voltage responses. (A) Threshold response. Voltage response to a current step of 3 pA. Resting level was -70 mV and threshold was near -45 mV. Step duration is 2 sec. (B) Single spike response. Elicited by a 10 pA stimulus current maintained for 1.5 sec. All current steps from 5 to 50 pA elicited only a single spike. (C) Early response phase. Voltage response during the first 200 msec (note expanded time scale). Current step was 8 pA, and resting potential was -70 mV. (D) Extended response. Voltage response to a 2-sec-long step of 8 pA showing characteristic firing pattern. The initial spike is 80 mV; subsequent spikes are smaller, degenerating into oscillations within 500 msec. Resting potential was -70 mV.

mV. Entrance of Ca^{2+} through the damaged membrane would also serve to activate a potassium current. In patch-clamp studies, the resting potential is set in large degree by the ionic concentration gradient, which is determined by the experimenter.

The substantial inactivation of the sodium current, which occurs at membrane potentials more positive than -60 mV, and the increase in the spike response when the membrane is maintained at -60 to -70 mV strongly suggest that the more-negative resting levels are likely to be correct.

Input Resistance. Intracellular studies report input resistances between 0.2 and 0.6 G Ω (2, 3), whereas patch-clamp recordings place the input resistance near 3 – 6 G Ω . The lower values obtained with intracellular electrodes probably result from a depolarization and shunt due to the penetrating electrode, coupled with a hyperpolarization and shunt due to increased calcium-activated potassium current (19).

The average area of an olfactory cell is ≈ 2000 μm^2 , not including the membrane infolding. For an input resistance of 5 G Ω , the specific membrane resistance would be 10^5 , a high but not unreasonable value.

Capacitance and Time Constant. Under current clamp we measured a membrane time constant of 100 msec (Fig. 1A). The membrane capacitance would be 20 pF given an input resistance of 5 G Ω . With a membrane area of 2000 μm^2 , the specific capacitance would be near the standard 1 $\mu\text{F}\cdot\text{cm}^{-2}$. Trotier (9) calculated a membrane time constant of 30 msec using a capacitance derived from the time constant of the capacitive transient of the voltage-clamp response and assuming a series resistance. We found this measure to be unreliable because of the uncertainty in the measurement of the electrode series resistance.

Voltage Response and Spike Activity. Because of the high input resistance of these cells, only 3 pA of injected current was required to reach spike threshold, and maximum spiking output was achieved with only ≈ 10 pA. Cells responded to depolarizing currents either with a single spike or a brief burst of 2 – 8 spikes, which were only crudely graded with the intensity of the stimulating current. It was impossible to characterize a spike frequency because the interval between the few spikes in the response increases with each subsequent spike. Intracellular studies have reported firing frequencies of up to 40 Hz based on the interval between the 1st and 2nd spikes, but this may be misleading since a burst contains only ≈ 8 spikes and the interval between the last 2 spikes can be >5 times greater than that between the first 2 spikes.

Gated Currents. We have separated the voltage-gated currents into five components: a transient inward sodium current, a sustained inward calcium current, and three outward potassium currents. With the exception of ref. 9, other voltage clamp studies have described only two currents: a transient inward sodium current and a delayed rectifier outward current (10, 11).

Sodium current. We measured a transient inward current with peak magnitude between 0.2 and 1.0 nA. Although this current is surprisingly tetrodotoxin resistant (5, 11), it is eliminated when sodium is replaced by choline in the bath. Trotier (9) measured a similar transient current but believed it to have a significant calcium component since it was eliminated by 10 mM cobalt in the bath. We found that as little

as 5 – 6 mM cobalt often blocked all gated currents and that 2 – 4 mM cobalt was sufficient to completely block the calcium current (see below) while leaving the transient inward current intact.

Calcium current. We measured a small (<200 pA) sustained inward current that was blocked by 2 mM cobalt and other divalent cations but was unaffected by removal of sodium from the bath. Curiously, Trotier (9) measured a sustained inward current of less than half the magnitude, but only after increasing the calcium concentration in the bath to 10 mM.

Potassium currents. The net outward current can be dissected into three components: a voltage-dependent potassium current that was found in all cells; a calcium-dependent component found in only half the cells; and a transient current with properties similar to those of the A current described by Connor and Stevens (17), also found in half the cells. The relative contribution of these three components varied from cell to cell. Trotier (9) described an anomalous rectifying conductance based on single-channel recordings, but we have never seen its macroscopic counterpart in our whole-cell recordings.

Thus, olfactory receptor neurons appear to be homogeneous in their passive electrical characteristics being electrotonically compact and possessing a low spike threshold. They are quite heterogeneous however in regard to their spike responses and the gated currents underlying those responses.

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