The Journal of Experimental Biology 202, 997–1003 (1999)
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JEB1922

INHIBITORY AND EXCITATORY RESPONSES OF OLFACTORY RECEPTOR NEURONS OF *XENOPUS LAEVIS* TADPOLES TO STIMULATION WITH AMINO ACIDS

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Accepted 1 February; published on WWW 22 March 1999

Summary

Recordings were made from olfactory receptor neurons of *Xenopus laevis* tadpoles using the patch-clamp technique to investigate the responses of these cells to odorants. Four amino acids (glutamate, methionine, arginine and alanine) both individually and as a mixture were used as stimuli. Of the 156 olfactory neurons tested, 43 showed a response to at least one of the stimuli. Of the cells tested, 19 % responded to glutamate, 16 % to methionine, 12 % to arginine and 10 % to alanine. Each amino acid was able to induce both excitatory and inhibitory responses, although these occurred in different cells. Each amino acid produced approximately equal numbers of inhibitory and excitatory responses. Inhibitory responses could best be observed in the perforated-patch configuration using gramicidin as an ionophore and a recording configuration that is a current-clamp for fast signals and a voltage-clamp for slow signals. The diversity of the odorant responses, in particular the existence of excitatory and inhibitory responses, is not consistent with a single transduction pathway in olfactory neurons of *Xenopus laevis* tadpoles.

Key words: olfaction, olfactory neuron, inhibition, tadpole, *Xenopus laevis*.

Introduction

The standard model of olfactory transduction consists of odorant receptors with seven transmembrane domains, the G-protein G_{olf}, an adenylyl cyclase, a cAMP-activated channel which is highly permeable to Ca^{2+}, and a Ca^{2+}-activated Cl^- conductance (for reviews, see Schild and Restrepo, 1998; Dionne and Dubin, 1994). The Ca^{2+}-activated Cl^- current is depolarizing because the Nernst potential of these channels is less negative than the resting membrane potential (Dubin and Dionne, 1994; Kurahashi and Yau, 1993; Zhainazarov and Ache, 1995). This model of olfactory transduction explains depolarizing receptor potentials and an increase in firing rate. Inhibitory responses, i.e. hyperpolarizing receptor potentials and a decrease in firing rate, cannot be explained using this model.

Inhibitory responses have, however, been reported in insects (Kaissling, 1986; Boeckh, 1967), crustaceans (Michel et al., 1991; Derby and Harpaz, 1988; McClintock and Ache, 1989; Derby et al., 1988; Seelinger, 1983), fish (Kang and Caprio, 1995a; Suzuki, 1977, 1982) and amphibians (Gesteland et al., 1965; Duchamp et al., 1974; Revial et al., 1978; Dionne, 1990, 1992; Morales et al., 1994). The presence of inhibitory responses in amphibian olfactory receptor neurons is somewhat controversial, and it has been suggested that they may be partly attributable to the effect of local ion depletion (Gesteland and Adamek, 1987; for a review, see Getchell, 1986). Recent evidence (for reviews, see Morales and Bacigalupo, 1996; Bacigalupo et al., 1997) strongly suggests the involvement of a Ca^{2+}-activated K^+ conductance in the inhibitory responses of the olfactory receptor cells of the Chilean toad. Interestingly, excitatory and inhibitory signalling pathways appear to coexist within the same olfactory receptor neuron (lobster: Michel and Ache, 1994; Ache, 1994; toad: Morales et al., 1994; fish: Kang and Caprio, 1995b).

We have reinvestigated the question of inhibitory responses in amphibians using tadpoles of *Xenopus laevis* as an experimental model because tadpole olfactory receptor neurons appear to be sensitive to amino acids. This sensitivity offers experimental advantages such as the possibility of applying a limited number of water-soluble stimuli. Furthermore, two classes of olfactory receptor are found in tadpoles; one is similar to the receptors found in air-breathing higher vertebrates, and the other to the receptors found in fish (Freitag et al., 1995). The latter class of receptor appears to bind amino acids (Kang and Caprio, 1995b). For these reasons, we have chosen to record odorant responses to amino acids in tadpoles.

Recording odorant responses can easily fail for a number of reasons. For instance, in the whole-cell configuration of the patch-clamp technique, constituents of an intracellular signalling pathway may diffuse into the patch pipette. The

concomitant decrease in the intracellular concentrations of these molecules is sometimes termed 'wash-out'. Furthermore, the pipette solution used may not be adequate in some respects; for example, it could influence the buffering of Ca^{2+} and thus the intracellular concentration of Ca^{2+}. We have, therefore, used the perforated-patch configuration (Horn and Marty, 1988) rather than the standard whole-cell configuration in this study.

Obviously, an odorant-dependent inhibitory effect upon the firing rate of a neuron can only be detected if the firing rate is above zero. However, many olfactory receptor neurons of *X. laevis* were silent or had a fairly low spontaneous activity. We therefore modified an EPC7 patch-clamp amplifier so that 'slow voltage-clamp' recordings could be performed. In this mode, the instrument works as a normal current clamp for fast events such as action potentials and as a voltage clamp for slow events. In this way, we were able to hold the membrane potential at a pre-established depolarized value. This induced an increase in spiking rate so that odorant-induced inhibition of the neuron and the corresponding decrease in spiking rate could be observed clearly.

Materials and methods

Preparation and tissue dissociation

Olfactory receptor neurons were prepared from the main olfactory mucosa of tadpoles (stages 52–55; Nieuwkoop and Faber, 1956) of *Xenopus laevis*. The animals were bred in the laboratory. After immobilization in a mixture of water and ice, the animals were killed. The tissue above the mucosae was removed under a dissection microscope, and the mucosae were extirpated and placed into the cell dissociation solution consisting of 109 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 10 mmol l^{-1} glucose and 10 mmol l^{-1} Hepes (pH 7.8, 230 mosmol l^{-1}) which contained papain (12–15 units ml^{-1}, 27 °C) and 2 mmol l^{-1} EDTA. After 3 min in this solution, the mucosae were transferred into approximately 600 µl of cell dissociation solution without papain and EDTA and were mechanically macerated using two pairs of fine forceps. The resulting pieces of mucosae were then triturated with a plastic pipette (2 mm inner diameter), and 100 µl of Ringer's solution 1 (in mmol l^{-1}: NaCl, 98; KCl, 2; $CaCl_2$, 3; $MgCl_2$, 2; glucose, 10; Hepes, 10; pH 7.8, 230 mosmol l^{-1}) was added. Samples (100–150 µl) of the cell suspension were stored at 5 °C on glass coverslips coated with Concanavalin A (1 mg ml^{-1}). Some experiments were carried out using uncoated coverslips, and no obvious difference in results was observed. The cells were used within 6 h in a recording chamber mounted on the stage of an inverted microscope (Axiovert 135, Carl Zeiss, Jena, Germany). During the experiments, the cells were constantly superfused (at a rate of approximately 0.8 ml min^{-1}) with Ringer's solution 2 (in mmol l^{-1}: NaCl, 109; KCl, 2; $CaCl_2$, 0.3; $MgCl_2$, 0.5; glucose, 10; Hepes, 10; pH 7.8, 230 mosmol l^{-1}) unless otherwise noted.

Recording

Most details of the recording techniques were as described by Hamill et al. (1981). Patch electrodes of approximately 10 MΩ resistance and with a tip diameter of 1–2 µm were fabricated from borosilicate glass (1.8 mm outer diameter; Hilgenberg, Malsfeld, Germany) using a two-stage electrode puller (Narishige, Tokyo). The pipettes were fire-polished, which usually resulted in a slightly higher seal resistance.

Pulse protocol, data acquisition and evaluation programs were written in 'C'. Pulses were delivered from a microcontroller (Schild et al., 1996) to a D/A converter and then to the patch-clamp amplifier (EPC7; List, Darmstadt, Germany). Currents and voltages were recorded on video tape using a PCM unit (Instrutech, Elmont, NY, USA). The data were digitized off-line using an eight-pole Bessel filter, an A/D converter and a PC. Further data analysis was performed on a Sun Sparc Station.

Perforated-patch recordings

Gramicidin perforated-patch recordings (Horn and Marty, 1988; Abe et al., 1994) were performed as follows. A small section of tubing was placed in the pipette holder next to the Ag/AgCl electrode. This tubing was filled with the pipette solution (in mmol l^{-1}: NaCl, 5; KCl, 5; potassium gluconate, 71; $MgCl_2$, 5; glucose, 10; Hepes, 10; EGTA, 0.2; K-ATP, 1; cGMP, 0.2; pH 7.8, 230 mosmol l^{-1}) containing gramicidin (5–100 µmol l^{-1}) before putting the pipette onto the holder. The gramicidin solution was added to the pipette solution by gentle pressure ejection from the tubing after the formation of a gigaseal. To determine when the cell-attached configuration turned into a perforated patch, we developed a program written in 'C' which applied, *via* a microcontroller and a D/A card, voltage pulses to the cell and calculated the seal resistance, R_s, and the cell capacitance charging time constant, τ_{Rs}, from the transient current responses to the command pulses. The formation of gramicidin pores was accompanied by an increase in the capacitance C as seen by the amplifier and by a decrease in R_s. The formation of the perforated-patch configuration was thus best indicated by the ratio C/R_s (Fig. 1). We began recording when R_s had dropped to approximately 25–30 MΩ, corresponding to a value of C/R_s in the range 0.4–0.5 pF $M\Omega^{-1}$.

Slow voltage-clamp recordings

In neurons that have a cell resistance in the gigaohm range, the membrane potential (E_m) is easily short-circuited by the seal resistance between patch pipette and membrane. This effect of R_s upon E_m can be compensated for by injecting a negative current into the neuron. In particular, this can be performed automatically by a small control circuit added to the patch-clamp amplifier which injects a current proportional to the voltage difference ($E_{command}$–E_m) between a command voltage and the actual membrane potential. If the time constant τ_c of this control circuit is set at, for example, 2 ms, action potentials are little affected, because the circuitry takes more time for the voltage control than the duration of an action potential, i.e. the action potentials are recorded approximately as in the current-clamp mode, while slower potentials ($t \gg \tau_c$)

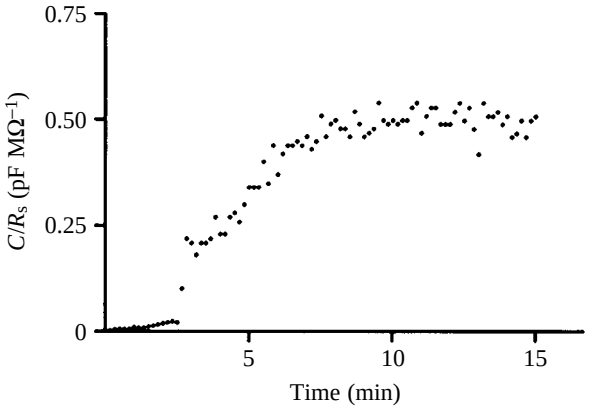

Fig. 1. Progress of the perforated-patch configuration. Cell capacitance C and series resistance R_s were evaluated every 10 s from current responses to a small, negative voltage-clamp pulse (no cell capacitance compensation). The ratio C/R_s was then plotted as a discrete function over time. In this recording, the sudden onset of the perforated-patch configuration was observed 3 min after the establishment of the on-cell configuration. The asymptotic value of C/R_s, which was reached only after approximately 10 min, reflects a series resistance of approximately 24 MΩ, given a cell capacitance of 12 pF.

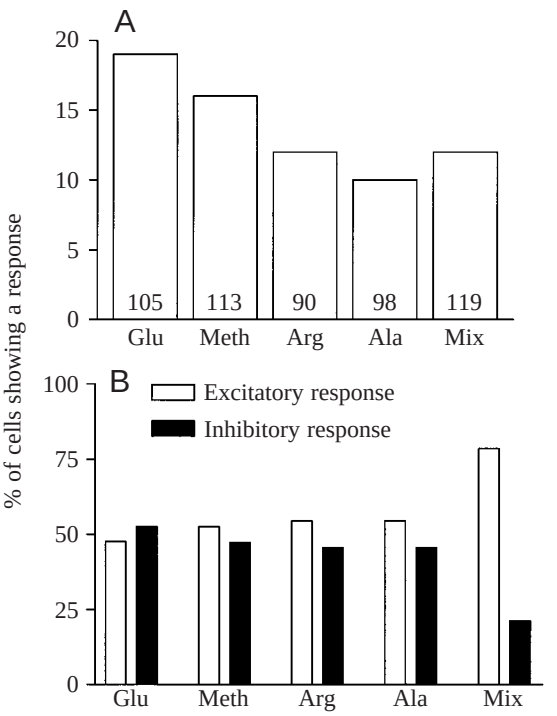

Fig. 2. Overview of the responses of *Xenopus laevis* tadpole olfactory neurons to the application of four amino acids. (A) Percentage of cells that responded to glutamate (Glu), methionine (Meth), arginine (Arg), alanine (Ala) and a mixture of the four amino acids (Mix). The concentration of each amino acid was 100 μmol l^{-1}. The number at the bottom of each bar indicates the number of olfactory neurons to which a particular stimulus was applied. (B) Using only those cells that responded to at least one of the stimuli, this histogram gives the relative frequency of excitatory and inhibitory responses for each stimulus. Each amino acid elicited either type of response with approximately the same frequency.

are voltage-clamped. We therefore call this clamp a slow voltage clamp, which is a voltage clamp for low frequencies and a current clamp for high frequencies. In this mode, receptor potentials can be recorded starting from membrane voltages predetermined by the experimenter.

Odorant application

The odorants used were dissolved in Ringer's solution 3 (in mmol l^{-1}: NaCl, 107; KCl, 2; $CaCl_2$, 2; $MgCl_2$, 0.5; glucose, 10; Hepes, 10; pH 7.8, 230 mosmol l^{-1}) and delivered by gravity feed from storage vials (1 ml, MoBiTec, Göttingen, Germany) to the bath. Each of the five stimulus tubes had a separate solenoid valve and a separate outlet (stainless-steel needle, internal diameter 0.2 mm). The five needles were placed in a linear arrangement approximately 20 μm from the cilia of the dissociated cells. The array of application outlets was micromanipulated in such a way that the odorant to be applied was next to the cilia. Continuous rinsing of the bath and tubing kept the leakage of odorants below a detectable minimum, as ascertained by applying a $KMnO_4$ solution instead of an odorant. As odorants, we used glutamate, methionine, arginine and alanine (each at 100 μmol l^{-1}) applied as single compounds or as a mixture of all four. Before applying a stimulus, the cells were rinsed for at least 2 min in Ringer's solution 3.

Results

We recorded from 156 olfactory receptor neurons of *X. laevis* tadpoles using the amino acids glutamate, methionine, arginine and alanine (100 μmol l^{-1} each), as well as a mixture of these four stimuli. Of the 156 cells tested, 43 responded to at least one of the stimuli, and 21 of the 43 responding cells showed a response to more than one stimulus: 19 % of the cells tested responded to glutamate, 16 % to methionine, 12 % to arginine and 10 % to alanine (Fig. 2A). Each stimulus could be excitatory or inhibitory as assessed either by an increase or a decrease in spike rate or by depolarizing or hyperpolarizing receptor potentials. Interestingly, every type of stimulus produced approximately equal numbers of excitatory or inhibitory responses, albeit in different cells, while the net effect of the mixture of the four stimuli was mostly excitatory (Fig. 2B).

In the cell-attached configuration of the patch-clamp technique, individual action potentials are reflected by a biphasic, mixed capacitative/ohmic current associated with the time course of the membrane potential during an action potential (Lynch and Barry, 1989) (Fig. 3A, inset). Fig. 3 shows examples of an increase in firing rate upon application of glutamate (Fig. 3A) and a mixture of amino acids (Fig. 3B) and of a decrease in firing rate upon application of glutamate (Fig. 3C) and methionine (Fig. 3D). In most recordings, however, the firing rate of olfactory receptor

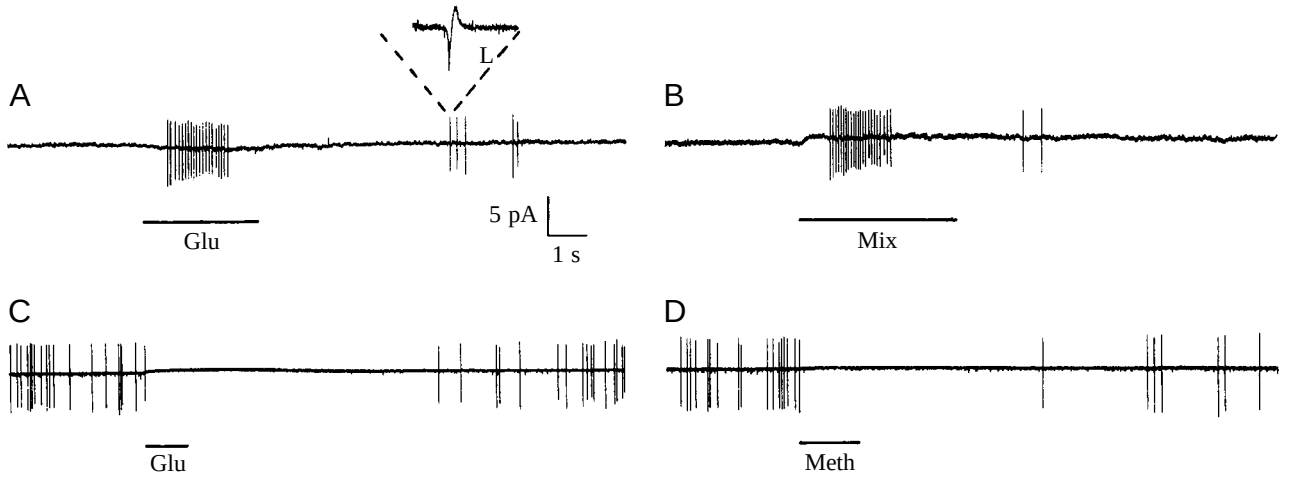

Fig. 3. On-cell recordings of olfactory receptor neurons stimulated by amino acid application. The inset in A shows a single spike-associated current at high time resolution; the calibration bars in the inset indicate 5 ms and 10 pA. The traces show an increase in spike rate upon application of both glutamate (Glu) (A) and the amino acid mixture (Mix) (B) and a decrease in spike rate upon application of glutamate (Glu) (C) and methionine (Meth) (D). Recovery from inhibition took between 5 and 15 s. Recordings are from four different cells.

neurons, as observed in the cell-attached configuration, was near zero, so that the presence of inhibitory responses could not be assessed.

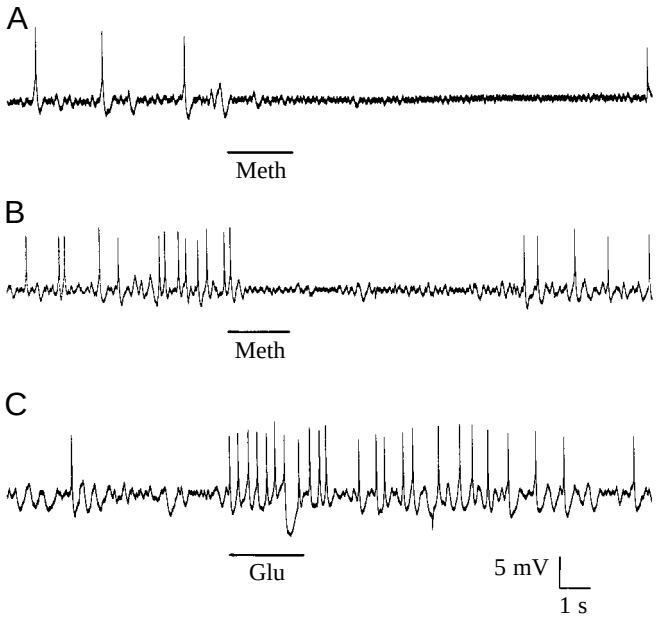

Fig. 4. Activity of olfactory receptor neurons in the current-clamp mode with electronic control of the holding potential on a slow time scale (slow voltage clamp). (A) Depression of spiking activity by methionine (Meth). Low-frequency clamp potential: −70 mV. (B) Recording of the odorant response in the same olfactory receptor neuron when the average pipette potential was held at −60 mV. The resulting average depolarizing effect induced a higher spiking rate and the inhibitory response to methionine became more obvious. (C) Recording of an excitatory odorant response in the slow voltage-clamp mode upon application of glutamate (Glu). The recording in C is from a different neuron from that shown in A and B.

To overcome this problem, we used the gramicidin perforated-patch method in conjunction with the 'slow voltage-clamp' (SVC) mode. This mode allowed easy checking for inhibitory responses, because a cell that had no spontaneous activity at a clamped potential of −80 mV typically exhibited moderate activity when clamped at −70 mV (Fig. 4A). Upon application of methionine, the spike activity decreased. When, in the same cell, the clamp voltage was increased to −60 mV, the activity increased and the methionine-induced decrease in the firing rate was more pronounced (Fig. 4B). In these recordings, the time constant τ_c of the clamp was 30 ms, so that action potentials were recorded because they were not voltage-clamped. As the time constant of the clamp control circuit was similar to the duration of the action potentials, their shape was distorted because the negative feedback action of the control circuit led to an artefactual hyperpolarization after every action potential. Fig. 4C shows an odorant-dependent increase in the firing rate in this recording mode.

When the time constant of the clamp circuit was set to much larger values, e.g. 10 s, receptor potentials could be recorded upon stimulus application. Fig. 5 shows examples of a hyperpolarizing and a depolarizing receptor potential elicited in two different cells by the application of alanine, again showing that the same stimulus can be coupled to different transduction pathways in different cells.

As the next step in our analysis, we studied the odorant-induced currents underlying the receptor potentials using conventional voltage-clamp in conjunction with the gramicidin perforated-patch method. Fig. 6 shows an example in which application of the stimulus mixture caused an inward current of approximately 300 pA to develop which reached its maximum amplitude approximately 5 s after the end of the stimulus. Command voltage ramps from −80 mV to +80 mV (160 ms) added to the command voltage led to current responses which appeared as spikes in Fig. 6A and which are

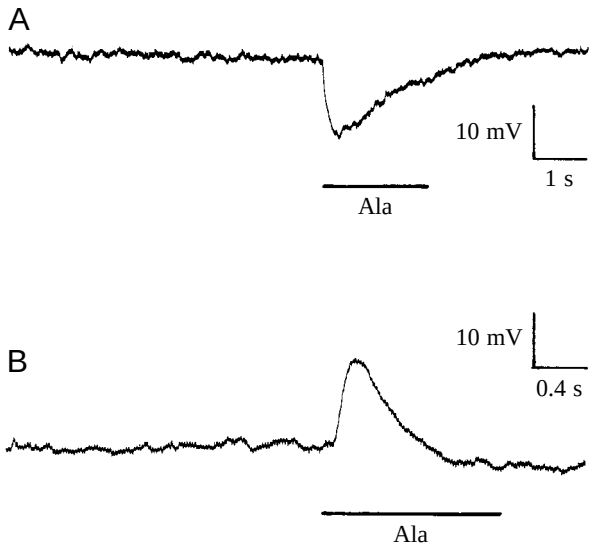

Fig. 5. Whole-cell standard current-clamp recordings of two different olfactory receptor neurons to stimulation with alanine (Ala). One cell shows a hyperpolarizing (A) and the other a depolarizing (B) receptor potential upon the application of alanine (100 μmol l^{-1}). The resting potentials were −63 mV (A) and −68 mV (B).

shown in more detail as single current responses to command voltage ramps (i.e. as current–voltage curves) in Fig. 6B. The generator currents reversed at approximately −10 mV, and the ramp response currents showed a clear inward rectification. These current responses to a mixture of odorants may be caused by a superposition of more than one conductance activated during stimulation.

Furthermore, using voltage-clamp ramps, we observed odorant-induced increases and decreases in outward current with no change in the holding current at −80 mV (Fig. 7). These responses could arise from the activation or suppression of a K^+ conductance. A detailed analysis of the transduction pathways of these odorant responses was beyond the scope of this paper.

Taken together, the above results clearly suggest a diversity of transduction mechanisms present in olfactory receptor neurons of *Xenopus laevis* tadpoles.

Discussion

We investigated the responses of olfactory receptor neurons of larval *Xenopus laevis* to stimulation with the amino acids glutamate, methionine, arginine and alanine and to a mixture of these. The results show the following.

(i) The olfactory receptor neurons of *X. laevis* clearly react to amino acids. This does not, of course, exclude the possibility that there are other types of relevant stimuli.

(ii) The responses can be excitatory or inhibitory. In our experiments, each of the four amino acids used as stimuli elicited approximately equal numbers of excitatory or inhibitory responses. Although the relative frequencies of either type of response are not important in this context, there

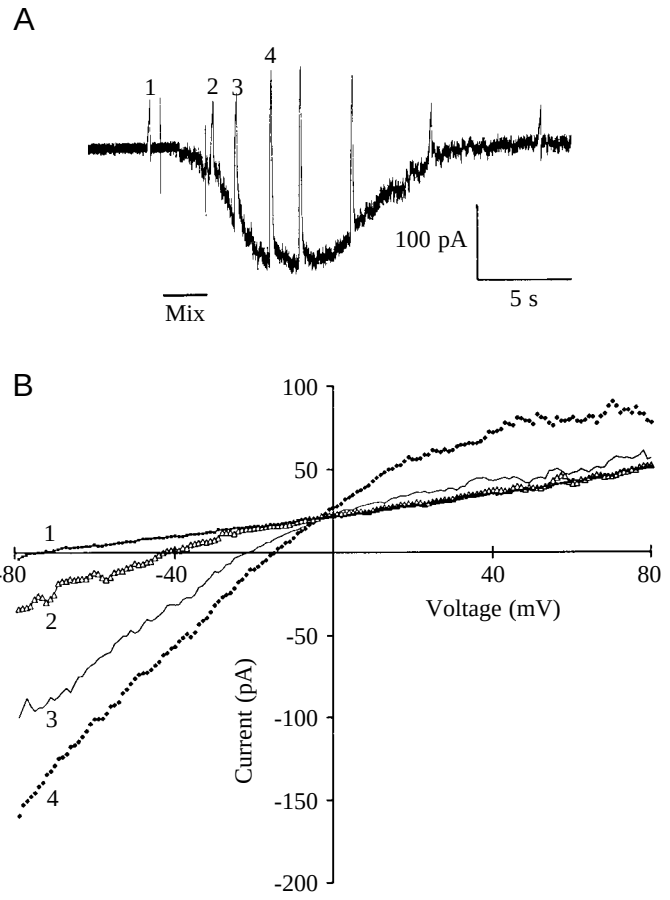

Fig. 6. Odorant response to application of the mixture (Mix) of amino acids in the whole-cell voltage-clamp configuration. The stimulus clearly induces an inward current at the holding potential (A). The current responses to the voltage ramps applied during the ongoing recording and labelled 1–4 indicate that the reversal potential of the stimulus-induced conductance is approximately −10 mV (B). The odorant-induced current shows a pronounced inward rectification for small currents, which became less marked with increasing current amplitudes.

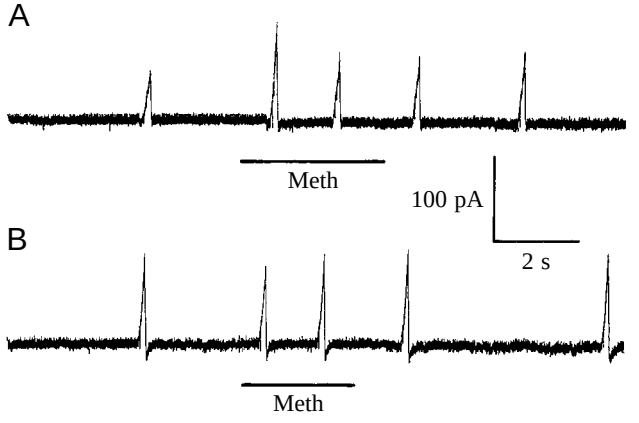

Fig. 7. Effects of odorant (methionine) application on voltage-clamp ramps. Measuring the current responses to voltage-clamp ramps before, during and after odorant application (Meth) revealed an increase (A) or decrease (B) in outward current, but the holding current was unaffected. Holding current −80 mV.

is no doubt that inhibitory responses do occur regularly and cannot be considered as exceptional events.

(iii) Owing to the low spontaneous spiking rate of many olfactory receptor neurons, there is a bias towards observing excitation. Inhibitory responses are masked in extracellular recordings because a decrease in the spiking rate is difficult to observe at low spontaneous spiking rates. We therefore injected current into the cell *via* gramicidin pores to depolarize the membrane potential and to elicit an increase in baseline firing rate. This current injection was performed in a controlled way, so that the potentials in the patch pipette and in the olfactory receptor neuron were clamped on a long time scale, but not on the time scale of action potentials or, depending on the time constant of the control circuit, receptor potentials. By this means, it was possible to increase the spiking rate to a detectable level and to measure odorant-induced decreases in the firing rate. The low spontaneous spiking rate of many olfactory receptor neurons implies that an inhibitory signal would not itself convey a signal to the olfactory bulb unless the neuron is excited by a different odorant *via* an excitatory pathway. In this case, the excitation would be short-circuited by the inhibitory effect. Such an interaction has been reported in the Chilean toad (Bacigalupo et al., 1997) and in the lobster (Michel and Ache, 1994).

(iv) As revealed by Freitag et al. (1995), larval *X. laevis* olfactory receptor neurons possess two types of receptor, one similar to those of mammals and the other to those of fish. Using amino acids as odorants, we presumably stimulated receptor neurons with receptors of the latter type. The responses we observed showed some similarity with those reported in the catfish by Kang and Caprio (1995b). These authors found that amino acids elicited inhibition as well as excitation and that many cells responded to more than one amino acid. In their experiments, inhibition occurred more frequently than excitation. The conductances underlying the responses of catfish olfactory receptor neurons have been investigated using the whole-cell configuration of the patch-clamp technique (Miyamoto et al., 1992a,b) as well as by imaging of $[Ca^{2+}]_i$ (Restrepo et al., 1990; Restrepo and Teeter, 1990). In these studies, it has been shown that catfish olfactory receptor neurons react to amino acids in the whole-cell mode and that there appear to be two different signal transduction cascades. However, an inhibitory pathway has not been described.

(v) Inhibitory responses are not consistent with the standard model of olfactory transduction, which assumes that stimuli eventually lead to an inward current and a depolarizing receptor potential. In *X. laevis* tadpoles, there appears to be at least one transduction mechanism that differs from the classical cAMP-activated cascade.

One working hypothesis would be that the cAMP-activated cascade is identical in all olfactory receptor neurons, except that E_{Cl} (the Nernst potential for chloride ions) may differ from cell to cell. At present, there are no measurements of the chloride concentration in the cytoplasm of olfactory receptor neurons and only very few of the concentration in the mucus (Minor et al., 1990, 1992; Chiu et al., 1988), and there is no evidence at all for E_{Cl} differences among different olfactory receptor neurons within one olfactory mucosa.

An additional finding from our experiments is preliminary evidence for an effect of amino acids on K^+ conductances. Odorant-modulated K^+ conductances have been previously described in the mudpuppy (Dubin and Dionne, 1994) and in the Chilean toad (Morales et al., 1994, 1995). The olfactory receptor neuron signal cascades targeting K^+ conductances thus appear to be more widespread than initially supposed and clearly need further investigation.

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