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THE CONTRIBUTION OF A Ca^{2+}-ACTIVATED Cl^- CONDUCTANCE TO AMINO-ACID-INDUCED INWARD CURRENT RESPONSES OF CILIATED OLFACTORY NEURONS OF THE RAINBOW TROUT

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Summary

To determine whether amino-acid-induced inward currents of ciliated olfactory receptor neurons (ORNs) in rainbow trout (*Oncorhynchus mykiss*) include a Ca^{2+}-activated Cl^- conductance, we first studied changes in reversal potential and the current/voltage relationships of the responses of ORNs to an amino acid mixture (L-alanine, L-arginine, L-glutamate and L-norvaline; all 10 mmol l^{-1}) with different concentrations of Na^+ and Cl^- in the perfusion and recording pipette solutions. We also examined the effects of six different Cl^- channel blockers on the responses of ORNs using a conventional whole-cell voltage-clamp technique. The amino acid mixture and one blocker were applied focally to the cilia of ORNs using a double-barrelled micropipette and a pressure ejection system. The expected shifts in reversal potential, indicating the contribution of the Ca^{2+}-activated Cl^- conductance, occurred in both positive and negative directions depending on the external and internal Na^+ and Cl^- concentrations. Niflumic acid, flufenamic acid, NPPB [5-nitro-2-(3-phenylpropylamino)-benzonate] and DCDPC (3′,5-dichlorodiphenylamine-2-carboxylate), at 0.5 mmol l^{-1}, reversibly blocked both the amino-acid-induced inward currents and the background activity in most ORNs. The effectiveness of these blocking agents varied from 77 to 91 % for ORNs perfused externally with standard Ringer's solution. SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonate), at 5.0 mmol l^{-1}, irreversibly inhibited the physiological response (100 % inhibition), whereas DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonate), at 5.0 mmol l^{-1}, had the smallest effect (45 %) of the inhibitors tested. The dose of niflumic acid inducing 50 % inhibition (IC_{50}), determined specifically for the current component of the Ca^{2+}-activated Cl^- channels, was 70 μmol l^{-1}. Our results suggest that these blockers are not specific for Ca^{2+}-activated Cl^- channels and that the density of these channels varies between individual ORNs. Our results also show that the Ca^{2+}-activated Cl^- conductance plays an important role in olfactory transduction and allows fishes to adapt to various ionic environments.

Key words: patch-clamp, olfaction, ciliated olfactory neuron, Ca^{2+}-activated Cl^- conductance, rainbow trout, *Oncorhynchus mykiss*.

Introduction

In vertebrates, it is widely accepted that odour transduction in olfactory receptor neurons (ORNs) is mediated by multiple second-messenger pathways: cyclic AMP and inositol 1,4,5-trisphosphate ($InsP_3$) pathways. Odorant binding to G-protein-coupled receptors (Buck and Axel, 1991) is the initial event of olfactory transduction, and this in turn results in the activation of adenylate cyclase or phospholipase C to produce the second messenger, cyclic AMP or $InsP_3$ (Lowe et al., 1989; Boekhoff et al., 1990). The resulting activation of cyclic-nucleotide-gated (CNG) channels or $InsP_3$-activated channels in the ciliary membrane of ORNs subsequently generates a depolarizing receptor potential (Nakamura and Gold, 1987; Zufall et al., 1994; Okada et al., 1994; Suzuki, 1994; Schild et al., 1995). It is controversial whether these two transduction pathways coexist in individual ORNs in the same species (Schild and Restrepo, 1998; Gold, 1999).

In the cyclic AMP pathway, the influx of Ca^{2+} through cyclic-AMP-activated CNG channels in the membrane of ORN cilia (Frings et al., 1995; Leinders-Zufall et al., 1997) plays an important role in further activation of Ca^{2+}-activated Cl^- channels (Kleene and Gesteland, 1991; Kleene, 1993) to amplify the inward receptor current (Lowe and Gold, 1993) and to trigger the feedback loop of the pathway for ORN adaptation (Kurahashi and Menini, 1997). The inward receptor current evoked by odorants consists of Na^+ and Ca^{2+} influx and Cl^- efflux components (Kurahashi and Yau, 1993; Kleene et al., 1994; Zhainazarov and Ache, 1995).

Salmonid fish are able to survive in fresh water and sea water. They hatch in fresh water, migrate to the ocean and

return to their natal river to reproduce. In general, fresh water contains approximately 0.5–1 mmol l^{-1} Na^+ and 0.1–2 mmol l^{-1} Ca^{2+} together with other cations and anions at low concentrations. In comparison, sea water contains approximately 500 mmol l^{-1} Na^+, 10 mmol l^{-1} K^+ and 10 mmol l^{-1} Ca^{2+} together with other cations and anions at higher concentrations than those in fresh water. The olfactory epithelium of fish is exposed directly to the external ionic environment. Its function is preserved during the migration of salmonid fish into sea water where the fish encounter an ionic environment completely different from that of fresh water. Experimental evidence for the persistence of olfactory function has been provided in rainbow trout (*Oncorhynchus mykiss*) and chum salmon (*Oncorhynchus keta*); the responses of the olfactory nerve response to amino acids are not affected by external Na^+ concentration (Shoji et al., 1994, 1996). In freshwater lamprey (*Lampetra fluviatilis*), the olfactory response to amino acids occurs even if Na^+ and K^+ are not present in water; only Ca^{2+} is necessary for the receptor responses to amino acids (Suzuki, 1978). In addition, the permeability of CNG channels for Ca^{2+} is high compared with the permeabilities for Na^+ and K^+ (Kurahashi and Shibuya, 1990), and Ca^{2+} influx triggered by amino acid stimuli has been demonstrated in the ORNs of channel catfish (*Ictalurus punctatus*) (Restrepo et al., 1990). Thus, if Ca^{2+}-activated Cl^- channels are present in the membrane of fish ORNs, they might enable olfactory adaptation to different ionic environments (Kurahashi and Yau, 1993; Reuter et al., 1998).

Although several studies have reported the responses of ORNs to amino acids in channel catfish (Miyamoto et al., 1992; Ivanova and Caprio, 1993) and coho salmon (*Oncorhynchus kisutch*) (Nevitt et al., 1994), the isolation of functional ORNs has remained a major problem. In the present study, we describe the development of new techniques for the isolation and stimulation of ORNs that allow the recording of robust ORN responses to various amino acids. We examined changes in the reversal potential and current/voltage (*I*/*V*) relationships of the responses of ciliated ORNs to a mixture of amino acids at different Na^+ and Cl^- concentrations. The effects of Cl^- channel blockers on the responses of ciliated ORNs were also investigated. Our results show that a Ca^{2+}-activated Cl^- conductance plays an important role in olfactory transduction and allows fishes to adapt to various ionic environments.

Materials and methods

Procedure for isolating cells without using proteolytic enzymes

Rainbow trout *Oncorhynchus mykiss* (Walbaum) (fork length 17–20 cm, mass 60–90 g) were obtained from a local fishery and maintained on a 12 h:12 h light:dark cycle in an Aqualex water circulating tank (AR18-300-10, NK System, Osaka, Japan) at approximately 15 °C. The fish were fed daily with a goldfish food pellets and were used for experiments less than 1 month after transportation from the local fishery.

To avoid ORN dysfunction caused by the narcotic MS-222 (Lewis et al., 1985), the fish were killed by quick decapitation with a knife. The olfactory rosettes were dissected out from both olfactory organs and kept in standard Ringer's solution (in mmol l^{-1}): 100 NaCl, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, 5 Hepes and 2.2 NaOH, pH 7.4, on ice until use. Individual olfactory lamellae were isolated from one rosette, using a microsurgical blade under a dissecting microscope, and transferred to Ca^{2+}-free Ringer's solution (in mmol l^{-1}): 100 NaCl, 3 KCl, 1 $MgCl_2$, 10 D-glucose, 5 Hepes and 2.2 NaOH, pH 7.4, on ice. Olfactory epithelial tissues were stripped from individual lamellae on a cutting board using two pairs of fine tweezers. The olfactory epithelial tissue samples collected in this way were then incubated in Ca^{2+}-free Ringer's solution on ice for 1 h. These incubation conditions provided the best environment for subsequent isolation of ORNs and for the preservation of their morphological and functional properties. After incubation, the epithelial tissues were collected using a Pasteur pipette to a volume of 1.0 ml in Ca^{2+}-free Ringer's solution in a small watchmaker's glass. Olfactory epithelial cells were then dissociated carefully by sucking them into and out of a fire-polished Pasteur pipette with a tip diameter of 0.2 mm under a dissecting microscope. The epithelial cell suspension was filtered through nylon mesh (40 μm pore size), and 0.5–0.7 ml of the final cell suspension was obtained. The cell suspension was plated onto a coverslip coated with concanavalin A (C2010, type IV, Sigma, St Louis, MO, USA) that was inserted into a perfusion chamber attached to the stage of a Nikon Diaphot TMD inverted phase-contrast microscope, and was allowed to stand for 20 min until the cells became attached to the surface of the coverslip. The bath solution was maintained at 16–18 °C and was circulated, at a rate of 1.0 ml min^{-1}, by a peristaltic pump (Minipuls 3, Gilson, Villiers, France) through the test chamber. Microscopic examination of the cell preparations revealed ciliated and microvillous ORNs (Zeiske et al., 1992). The olfactory knob of ciliated ORNs supports 2–10 cilia that are 3.0–10 μm long, whereas that of microvillous ORNs bears numerous microvilli 1.0–2.0 μm long. The ratio of microvillous to ciliated ORNs was 1:20–30. In the present study, only ciliated ORNs containing more than four cilia and with cilia more than 3 μm in length were selected for electrical recordings to maximize the possiblity of recording robust amino acid responses.

Whole-cell voltage-clamp recording technique

A standard whole-cell voltage-clamp technique (Hamill et al., 1981) was used to record the current responses of ciliated ORNs. To fabricate recording pipettes, thick-walled borosilicate glass tubes (1.5 mm o.d., 1.2 mm i.d.; G-1, Narishige, Tokyo, Japan) were pulled using a micropipette puller (PD-5, Narishige, Tokyo, Japan). The pipettes were coated with silicone (TE-106, Shinetsu Chemicals, Tokyo, Japan) and fire-polished using a microforge (MF-79, Narishige, Japan). The pipette resistance was 8–12 MΩ. Unless indicated otherwise, the recording pipette was filled with K^+-internal solution (in mmol l^{-1}): 93 KCl, 5 potassium EGTA, 5 Hepes, 1.0 ATP, 0.1 GTP and 2.26 KOH,

pH 7.4. The recording pipette was connected *via* a Ag/AgCl wire to the headstage of a patch-clamp amplifier (CEZ-2200, Nihon Kohden, Tokyo, Japan). The reference electrode was a Ag/AgCl plate immersed in the bath solution. For whole-cell recording, the pipette was first placed onto the cell soma of an ORN using a mechanical micromanipulator (MX-2, Narishige, Japan). Tight seals (>1 GΩ) were established by applying a small negative pressure to the recording pipette after contact with the cell soma surface. Gigaohm seal formation and subsequent rupture of the underlying membrane were achieved either by application of negative pressure or using zapping voltage pulses (±1.0 V, 1–10 ms) to the recording pipette. The seals were monitored by measuring the capacitative current transients triggered by 0.1 mV test pulses. Whole-cell recordings from ORNs usually lasted 15 min and could be maintained for up to 30 min in some ORNs. Preliminary experiments showed deterioration of the response, probably due to 'wash-out' of intracellular molecules by the whole-cell pipette solution, particularly during long-term recordings. Recording was therefore discontinued when a high seal resistance could not be maintained or when deterioration of the ORN responses to amino acids became evident. Current signals were low-pass-filtered at 3 kHz and stored on magnetic tape on a PCM data recorder (PCM-501ES, Sony, Tokyo, Japan) with a bandwidth of direct current to 13 kHz for later off-line analysis. During off-line analysis, the reproduced current signals were digitized at 500 Hz or 1 kHz sampling speed and analyzed using DataSponge software (WPI, Sarasota, FL, USA) installed on an IBM-PC-AT-compatible computer. The current data were further processed for presentation using the graphics software Canvas 5.0 (Deneba, Miami, FL, USA).

Application of amino acid stimuli and Cl^- channel blockers

A mixture of four amino acids, L-alanine, L-arginine, L-glutamate and L-norvaline (Nakarai Tesque, Kyoto, Japan), each at 10 mmol l^{-1}, was used as an olfactory stimulus in the present study to maximize the probability of recording ORN responses. These amino acids bind to four independent amino acid receptor site groups in the channel catfish (Caprio and Byrd, 1984). To avoid possible superposition of liquid junctional currents, due to stimulus application, on the true current responses of ORNs, the amino acids were dissolved in external solution in each test. Six different Cl^- channel blockers were used to examine the inhibitory effects on amino acid responses of ORNs: niflumic acid (N0630, Sigma), flufenamic acid (F9005, Sigma), SITS (4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonate) (001-30-Flu, Nakarai Tesque, Kyoto, Japan), DIDS (4,4′-diisothiocyanatostilbene-2,2′-disulphonate) (D3514, Sigma), NPPB [5-nitro-2-(3-phenylpropylamino)-benzonate] and DCDPC (3′,5-dichlorodiphenylamine-2-carboxylate). The last two agents were kindly provided by Dr H.-J. Lang of Höchst Aktiengesellschaft (Frankfurt am Main, Germany). The channel blockers were dissolved in external solution at concentrations that effectively blocked the Ca^{2+}-activated Cl^- conductance (Kleene and Gesteland, 1991; Kleene, 1993; Kurahashi and Yau, 1993; Lowe and Gold, 1993).

The mixture of amino acids was applied using a single glass pipette (tip diameter 1.0 μm) fabricated using a PD-5 Narishige puller. The mixture of amino acids and Cl^- channel blockers was also applied focally to the cilia of ORNs using a double-barrelled glass pipette (tip diameters 1.0 μm) fabricated using a programmable pipette puller (PMP-l00, WPI) from double-barrelled glass tubing (G-1.2-filament, Narishige). The double-barrelled pipette was filled with the amino acid mixture and a Cl^- channel blocker. The tips of the single and double-barrelled pipettes were always positioned 20 μm away from the cilia of the ORNs using a hydraulic micromanipulator (NHW-194 and MB-PP2, Narishige). The pipettes were connected *via* a silicone rubber tube to each of two independent channels of a custom-built pressure ejection system constructed as described by Ito et al. (1994). Using this pressure ejection system, we were able accurately to control the pressure level (0–19.6 N cm^{-2}), pressure pulse duration (25 ms to 10 s) and timing of application of two independent channels, using TTL pulse generators. The pressure ejection system was calibrated by measuring liquid junction currents induced by diluted standard Ringer's solution under conditions similar to those used during actual stimulus application. This calibration confirmed that the concentration of the stimulus solution applied at the target correlated linearly with the ejection pressure applied (Ito et al., 1995). The stimulus concentration at the target when applied at 0.98 N cm^{-2} and 25 ms pulse duration was estimated to be diluted by at least a factor of 10 compared with the concentration in the pipette fluid.

To investigate the effects of Cl^- channel blockers on whole-cell current responses to the amino acid mixture, experiments were performed using the following procedure. After the transition to the whole-cell recording configuration, the amino acid mixture was applied two or three times to the olfactory cilia at 30 s intervals. A Cl^- channel blocker was then applied for 10 s. To examine the blocking effect and the recovery from blockade, the amino acid mixture was then applied at 30 s intervals starting 4 s after the end of the application of the channel blocker. The inhibitory effect of blockers was expressed as an inhibition index (%): [1–(peak current 4 s after the end of blocker application)/(peak current before the blocker application)]×100.

Test solutions

To examine the contribution of the Ca^{2+}-activated Cl^- current to the whole-cell current during the response to the amino acid mixture, we recorded shifts in reversal potential in response to changes in equilibrium potential for specific ions in different external and internal solutions in which Na^+ and Cl^- were replaced by ions that do not permeate the cell membrane. Na^+ was substituted with choline, and Cl^- was substituted with either gluconate or acetate (Kurahashi, 1989; Hallani et al., 1998). The following external (perfusate) and internal (pipette) solutions were used (all concentrations in mmol l^{-1}): Na^+-free external solution, 105 choline chloride, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, 5 Hepes and 2.2 NaOH, pH 7.4; low-Cl^- external solution, 30 NaCl, 80 sodium gluconate, 3 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 D-glucose, 5 Hepes

and 2.2 NaOH, pH 7.4; low-Cl^- internal solution, 10 KCl, 83 potassium acetate, 5 EGTA, 5 Hepes, 1 ATP-2Na, 0.1 GTP-Na and 2.6 KOH, pH 7.4; and Cs^+-internal solution, 93 CsCl, 5 EGTA-2Cs, 5 Hepes, 1 ATP-2Na, 0.1 GTP-Na and 2.0 CsOH, pH 7.4. The free Ca^{2+} concentration of all internal solutions was calculated to be 10^{-8} to 10^{-9} mol l^{-1} using CALCIUM.BAS software (Suzaki, 1987).

Statistical analyses

All values are expressed as means ± S.D. Differences between groups were examined for statistical significance using analysis of variance (ANOVA) and a Fisher's *post-hoc* test. A P value less than 0.01 was accepted as a statistically significant difference.

Results

General characteristics of ORN responses to amino acid mixture

Initially, we recorded whole-cell currents triggered by the amino acid mixture dissolved in the Ringer's solution using K^+-internal solution in the recording pipette. The inward current responses to the amino acid mixture (1.96–19.6 N cm^{-2} ejection pressure; 25 ms ejection duration) occurred in 107 of 312 ORNs (34 %). However, no outward current responses were observed in our experiments. Fig. 1 shows a typical example of the current responses of an ORN to changes in stimulus ejection pressure. Inward current responses to the amino acid mixture were monotonic and showed a fast rise followed by a slow decline, on which deflections due to the opening and closure of ion channels were superimposed. The mean values of the response amplitude and latency measured in selected ORNs at the same stimulus ejection pressure (9.8 N cm^{-2}, 25 ms) were 86.0±63.7 pA (mean ± S.D., N=53) and 49.1±25.6 ms (N=40), respectively. The amplitude of the inward current peak of the response to the amino acid mixture increased with increasing stimulus ejection pressure, reaching a plateau at approximately 9.8 N cm^{-2}. To obtain a saturated current response, the standard stimulus was delivered to ORNs at an ejection pressure of 9.8 N cm^{-2} and for a duration of 25 ms in the following experiments.

We then determined the peak current/voltage relationship (*I*/*V* relationships) for stimulation with the amino acid mixture by changing the holding potential between −60 mV and +60 mV in the standard external and internal solutions (Fig. 2A). The time course of the rising phase of the responses did not change at different voltages. However, a significant prolongation of the response decay time was noted at positive voltages. The *I*/*V* curve obtained under these conditions (Fig. 2B) showed a marked outward rectification. The reversal potential determined for this and other ORNs in the same condition was +4.3±0.58 mV (N=3).

Shifts of reversal potential in different external and internal solutions

We first examined the shifts in reversal potential in *I*/*V* relationships in Na^+-free external solution. When Na^+-free solution was used as the perfusate and K^+-internal solution was used for filling the recording pipette, inward current responses to the standard stimuli were recorded in 48 of 173 (28 %) ORNs held at −60 mV. The response characteristics of the rise and decay phases were similar to those of ORNs examined in the standard combination of external and internal solutions (Fig. 3A). The *I*/*V* relationship examined in three ORNs showed strong rectification, as observed in the standard combination of external and internal solutions (Fig. 3B). However, the reversal potential shifted in the negative direction, as expected from the change in equilibrium potential for Na^+ (E_{Na}) across the ORN membrane, and was −10.7±1.16 mV (N=3).

To determine whether the current component contributed by Cl^- channels is involved in inward current responses to the amino acid mixture, we examined the shifts in reversal potential caused by changes in external and internal Cl^- concentrations. If Cl^- channels are involved in the generation of the inward current responses of ORNs, a positive shift in reversal potential would be expected to occur with a decrease in external Cl^- concentration. Using a combination of low-Cl^- external solution and K^+- or Cs^+-internal solution, responses to the amino acid mixture were obtained from 15 of 46 (32 %) ORNs held at −60 mV. The *I*/*V* relationship examined in four ORNs showed that the reversal potential shifted in the positive direction, as

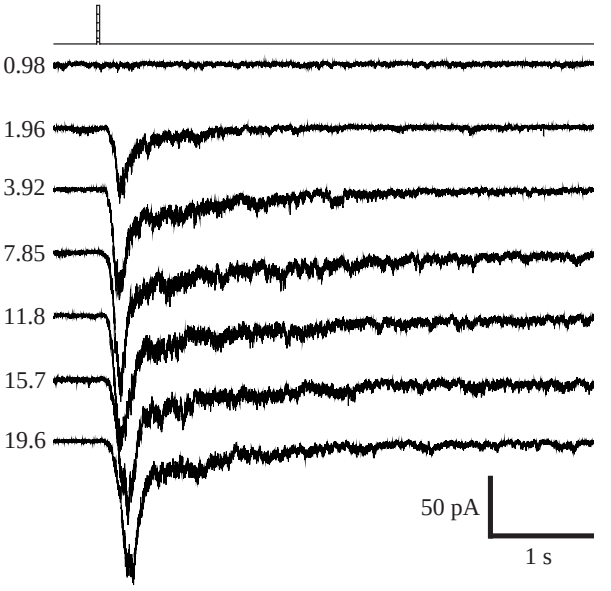

Fig. 1. Whole-cell inward current responses of a representative olfactory receptor neuron (ORN) to the amino acid mixture (10 mmol l^{-1}: L-alanine, L-arginine, L-glutamate and L-norvaline) at different stimulus ejection pressures (0.98–19.6 N cm^{-2}; duration, 25 ms). Stimulus ejection pressure (N cm^{-2}) is shown beside each trace. The top trace shows the timing of the ejection pressure pulses. A single stimulating pipette was positioned 20 µm from the cilia of the ORN. Standard Ringer's solution was perfused externally at 1.0 ml min^{-1}. K^+-internal solution (see Materials and methods) filled the recording pipette. Data were sampled at 1 kHz and displayed using a compression factor of 10.

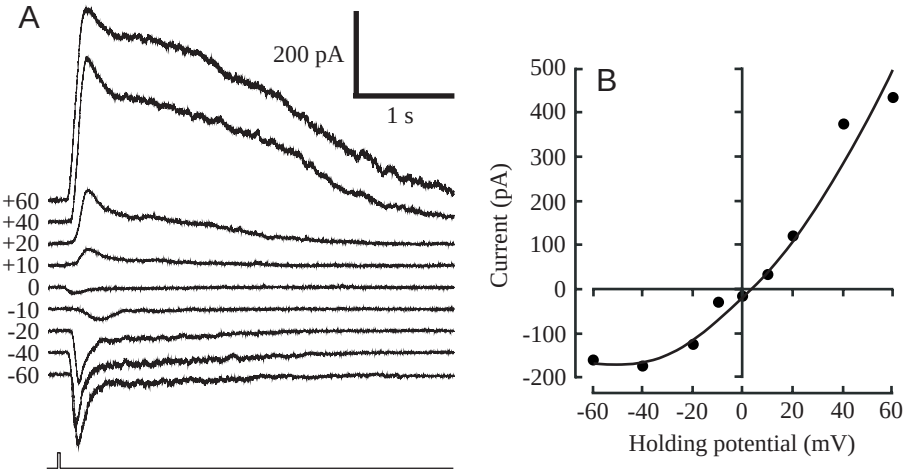

Fig. 2. Whole-cell inward current responses of a representative olfactory receptor neuron (ORN) to the amino acid mixture at different holding potentials. Standard Ringer's solution was perfused externally. K^+-internal solution (see Materials and methods) filled the recording pipette. (A) Current traces at different holding potentials. The holding potential (mV) is indicated beside each trace. Current offsets due to changes in holding potential in this and the following figures (Figs 3–5) are not shown for clarity. The duration of application of the amino acid mixture was 25 ms. Stimulus ejection pressure was 9.8 N cm^{-2}. The bottom trace shows the timing of the ejection pressure pulse. Data in this and the following figures (Figs 3-5) were sampled at 1 kHz and displayed using a compression factor of 10. (B) Current–voltage relationship. The peak current of each response shown in A was plotted. The curve was drawn manually. The reversal potential determined from this curve was +4.0 mV.

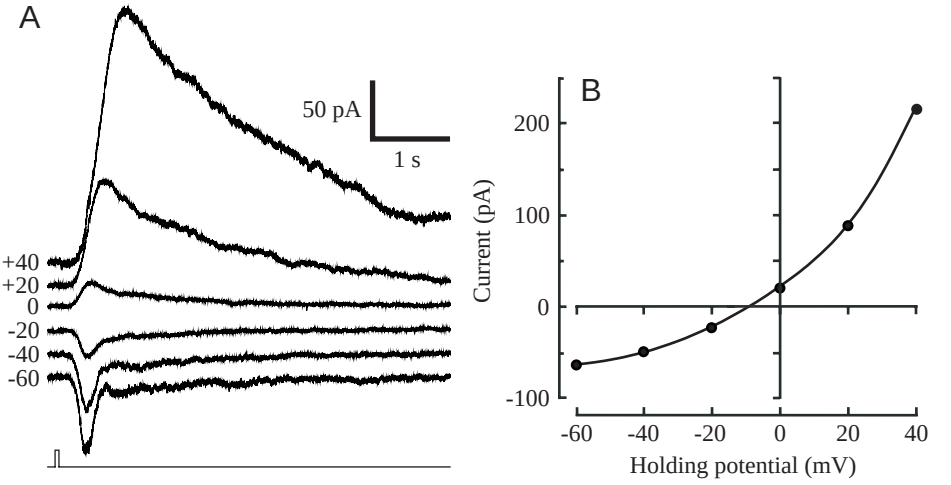

Fig. 3. Whole-cell current responses of a representative olfactory receptor neuron (ORN) to the amino acid mixture (9.8 N cm^{-2}; 25 ms). Na^+-free external solution (see Materials and methods) was perfused and Cs^+-internal solution filled the recording pipette. (A) Current traces at different holding potentials. The holding potential (mV) is indicated beside each trace. The bottom trace shows the timing of the ejection pressure pulse. (B) Current–voltage relationship. The peak current of each response shown in A was plotted. The curve was drawn manually. The reversal potential determined from this curve was –10 mV.

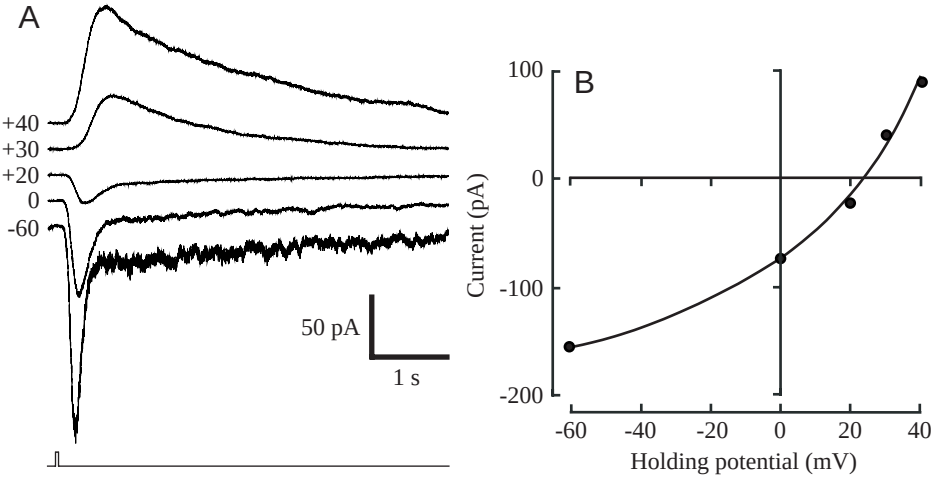

Fig. 4. Whole-cell current responses of a representative olfactory receptor neuron (ORN) to the amino acid mixture (9.8 N cm^{-2}; 25 ms). Low-Cl^- external solution (see Materials and methods) was perfused and Cs^+-internal solution filled the recording pipette. (A) Current traces at different holding potentials. The holding potential (mV) is indicated beside each trace. The bottom trace shows the timing of the ejection pressure pulse. (B) Current–voltage relationship. The peak current of each response shown in A was plotted. The curve was drawn manually. The reversal potential determined from this curve was +23 mV.

expected from the change in E_{Cl}, and was +17.8±6.3 mV (N=4; Fig. 4). Using a combination of low-Cl^- internal solution and standard Ringer's solution, inward current responses to the amino acid mixture were recorded in 29 of 90 (32 %) ORNs held at –60 mV. The I/V relationship examined in three ORNs showed slight outward rectification under these conditions, but the reversal potential shifted in the negative direction, as expected from the change in E_{Cl}, and was –0.67±0.67 mV (N=3;

Fig. 5). Taken together, these results indicated that the inward current responses to the amino acid mixture contained not only Na^+ and Ca^{2+} current components through CNG channels, but also Cl^- current components through Cl^- channels.

The magnitude of the inward current responses of ORNs to the amino acid mixture with different combinations of external and internal solutions

Fig. 6 compares the magnitude of inward current responses of ORNs to the amino acid mixture for the standard stimulus (9.8 N cm^{-2}; 25 ms) with different external and internal solutions. In all recording conditions, the peak values of initial inward current response of all ORNs held at −60 mV were taken in order to exclude the effect of response run-down. The magnitude of inward current responses in the combination of low-Cl^- external and Cs^+-internal solution was the largest for all combinations of external and internal solutions (P<0.01). The result was consistent with the hypothesis that the inward current responses to the amino acid mixture contain current components through both CNG channels and Cl^- channels.

Inhibition of inward current responses of ORNs to amino acid mixture by Cl^- channel blockers

In the next series of experiments, we examined the inhibitory effects of six Cl^- channel blockers on the inward current responses to the amino acid mixture at a holding potential of −60 mV using the standard combination of external and internal solutions. Fig. 7 shows the inward current responses of ORNs, held at −60 mV, 4 s after the termination of the 10 s application of blocker. At the concentrations indicated in Fig. 7, each blocker exhibited an inhibitory effect on inward current responses. Niflumic acid was the most potent blocker among the six. Most of the inhibitory effect of each blocker on inward current responses was reversible, although, at 5.0 mmol l^{-1}, SITS caused irreversible inhibitory effects on the responses of most of the ORNs tested (data not shown). In addition to the inhibition of the inward current response, these blockers also inhibited the spontaneous activity of ORNs (Fig. 7D,F). Therefore, these Cl^- channel blockers were not specific for Ca^{2+}-activated Cl^- channels, but were rather non-specific for other conductances. The inhibitory effects and dose-dependence of the six Cl^- channel blockers are summarized in Table 1. The inhibitory indices for the blockers at different concentrations varied greatly among ORNs.

To examine the specific inhibitory effects of niflumic acid on Cl^- channel conductance, the inward current responses were measured in Na^+-free external solution. Under these conditions, the CNG channel current was abolished (Fig. 8). In

Table 1. *Inhibitory effects of Cl^- channel blockers on whole-cell current responses of olfactory receptor neurons to the amino acid mixture*

Inhibitor	Concentration (mmol l^{-1})	Inhibition index (%)	Number of tested cells: Total (reversible, irreversible)
SITS	5.0	99.5±1.88	12 (1,11)
	2.0	35.2±43.0	16 (15,1)
	1.0	22.3±33.3	4 (4,0)
DIDS	5.0	45.3±48.7	6 (4,2)
Flufenamic acid	0.5	77.1±34.8	5 (5,0)
Niflumic acid	0.5	90.5±13.3	5 (3,2)
NPPB	0.5	81.0±22.4	4 (3,1)
DCDPC	0.5	82.6±29.0	9 (6,3)

Values are means ± S.D.
SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonate.
DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulphonate.
NPPB, 5-nitro-2-(3-phenylpropylamino)-benzonate.
DCDPC, 3′,5-dichlorodiphenylamine-2-carboxylate.
Reversible denotes that the current responses recovered to a level greater than 50 % of the initial control value.
Standard Ringer's solution was perfused during recording of the response at a holding potential of −60 mV. K^+-internal solution was used for filling the recording pipette (see Materials and methods).

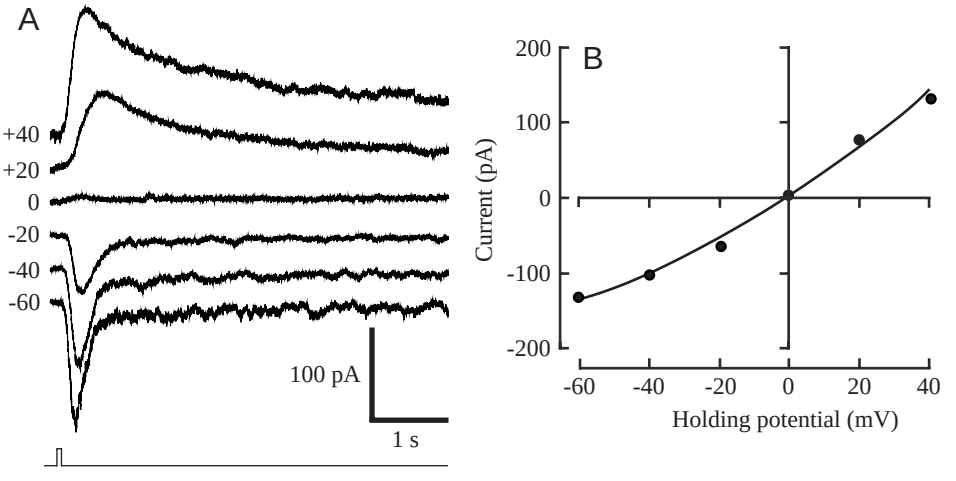

Fig. 5. Whole-cell current responses of a representative olfactory receptor neuron (ORN) to the amino acid mixture (9.8 N cm^{-2}; 25 ms). Standard Ringer's solution was perfused externally and low-Cl^- internal solution (see Materials and methods) filled the recording pipette. (A) Current traces at different holding potentials. The holding potential (mV) is indicated beside each trace. The bottom trace shows the timing of the ejection pressure pulse. (B) Current–voltage relationship. The peak current of each response shown in A was plotted. The curve was drawn manually. The reversal potential determined from this curve was −2.0 mV.

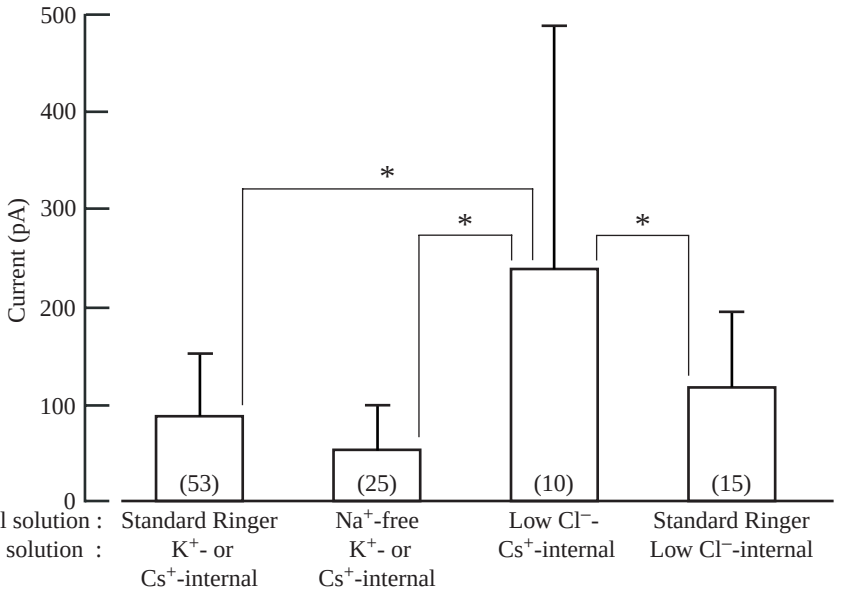

Fig. 6. Comparison between the magnitudes of the peak inward current responses of olfactory receptor neurons (ORNs) to the amino acid mixture (9.8 N cm^{-2}; 25 ms) using different combinations of external and internal solutions. The peak response of each ORN was obtained from the first response to the amino acid mixture to exclude the effects of ORN response run-down. Combinations of external and internal solutions (see Materials and methods for details) are indicated on the horizontal axis. The holding potential was −60 mV for all ORNs tested. Values are means + S.D. The number of measurements is shown in parentheses. An asterisk indicates a significant difference (P<0.01) between current responses in different combinations of external and internal solutions.

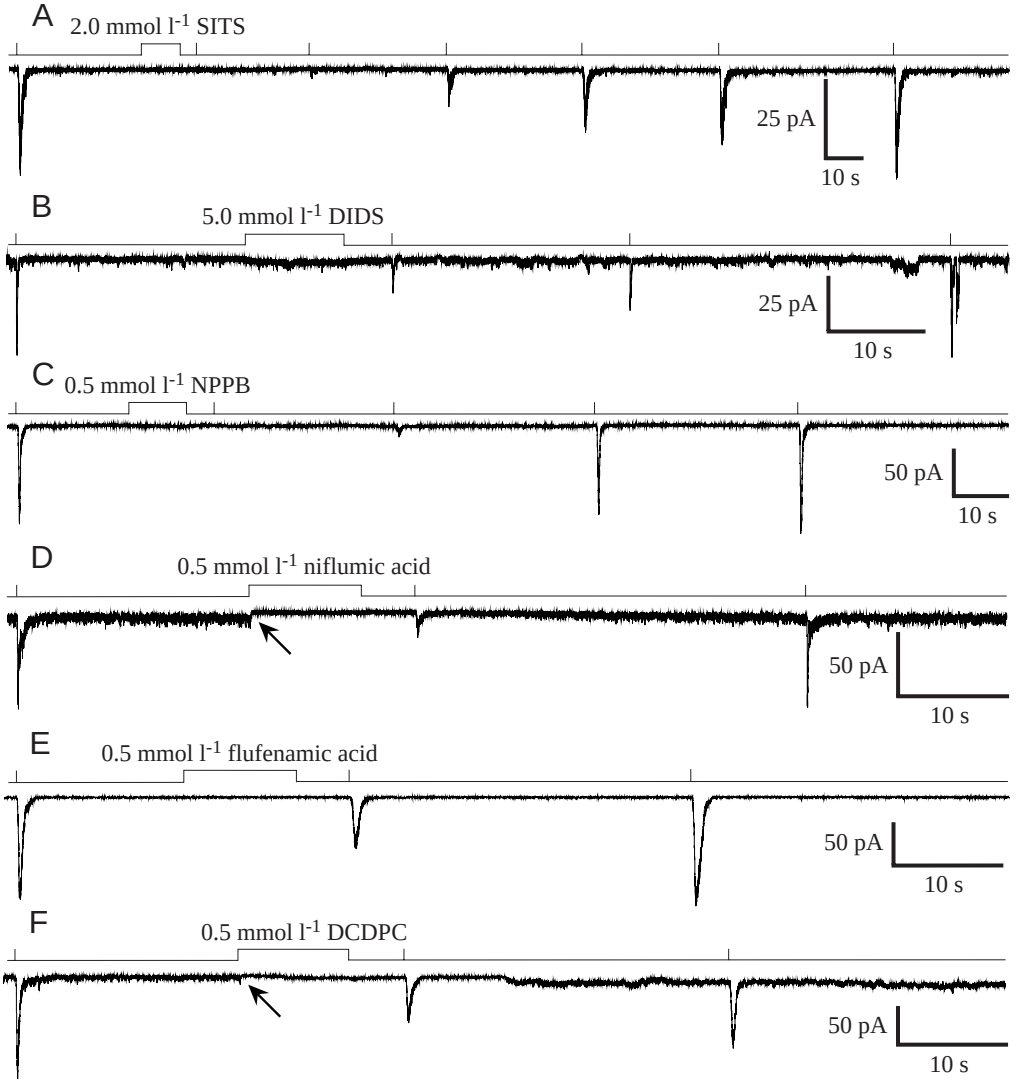

Fig. 7. Effects of six different Cl⁻ channel blockers on whole-cell current responses of olfactory receptor neurons (ORNs) to the amino acid mixture. Different Cl⁻ channel blockers (A–F) were applied for 10 s at 9.8 N cm^{-2} through one of the double-barrelled stimulating pipettes, which was positioned 20 µm away from the olfactory cilia. Current responses to the amino acid mixture (9.8 N cm^{-2}, 25 ms), applied through the second barrel of the double-barrelled stimulating pipette, were monitored at the times indicated in the upper traces in A–F. The lower traces show whole-cell current responses. In D and F, note that Cl⁻ channel blockers inhibited not only amino-acid-induced currents but also the background activity of ORNs (arrows). Traces were recorded from different ORNs in different experimental sessions. Standard Ringer's solution was perfused during recording at a holding potential of −60 mV. K⁺-internal solution (see Materials and methods) filled the recording pipette. Data were sampled at 500 Hz and displayed using a compression factor of 10.

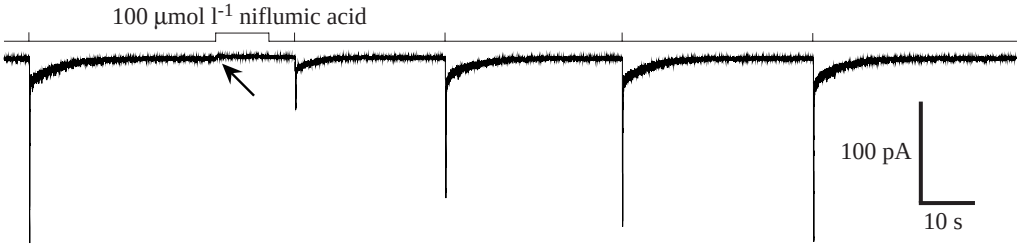

Fig. 8. Effects of niflumic acid on whole-cell current responses of a representative olfactory receptor neuron (ORN) to the amino acid mixture. Na^+-free external solution (see Materials and methods) was perfused and K^+-internal solution filled the recording pipette. The arrow indicates inhibition of Ca^{2+}-activated Cl^- channel activity by niflumic acid during the adaptation stage of the ORN response. The ORN was held at a holding potential of −60 mV during the recording. The amino acid mixture and niflumic acid solution were applied using a double-barrelled stimulating pipette as explained in Fig. 7. Data were sampled at 500 Hz and displayed using a compression factor of 10.

these experiments, the inhibitory effects of niflumic acid on the residual activity of ORNs evoked by the preceding stimulus were sometimes observed at the beginning of the application of this blocker, indicating that the activity of the Cl^- channel during the adaptation stage of the ORN response was also inhibited by niflumic acid. The dose-dependence of the inhibitory effect is shown in Fig. 9. The concentration of niflumic acid that resulted in 50 % inhibition (IC_{50}) determined from the curve was 70 μmol l^{-1}.

Discussion

Previous patch-clamp studies of ORNs of channel catfish (Miyamoto et al., 1992), coho salmon (Nevitt and Moody, 1992) and zebrafish (*Danio rerio*) (Corotto et al., 1996) have characterized the properties of the CNG channels and voltage-gated channels of ORNs. In these studies, proteolytic enzymes were used to isolate ORNs. In our preliminary studies on rainbow trout ORNs, we also used various proteolytic enzymes such as papain, collagenase and collagenase/dispase in combination with DNase to isolate ORNs. However, we were unable to record robust amino acid responses in the enzyme-treated ORNs that retained their shape. The method of ORN isolation involving incubation with Ca^{2+}-free solution used in the present study was far superior to the enzyme methods. Characteristic morphological features of the olfactory knob and cilia were well preserved. Voltage-gated current responses evoked by depolarizing voltage steps were recorded in all ORNs. However, only 28–34 % of ORNs tested in the different external and internal solutions responded to the amino acid mixture. In many ORNs, the cell isolation procedures using the Ca^{2+}-free solution damaged the transduction mechanisms and left the voltage-gated channels functional. The internal solutions used in the pipettes contained 5 mmol l^{-1} EGTA to maintain a free Ca^{2+} concentration of between 10^{-8} and 10^{-9} mol l^{-1}. The internal solution may either have induced a rapid wash-out of molecules needed in the transduction pathway from the cell into the pipette or stabilized the change in Ca^{2+} concentration required for activation of Ca^{2+}-activated Cl^- channels. The Ca^{2+} concentration giving 50 % activation ($K_{1/2}$) for the Ca^{2+}-activated Cl^- channels was estimated to be 4.8 μmol l^{-1} in the cilia of frog ORNs (Kleene and Gesteland, 1991).

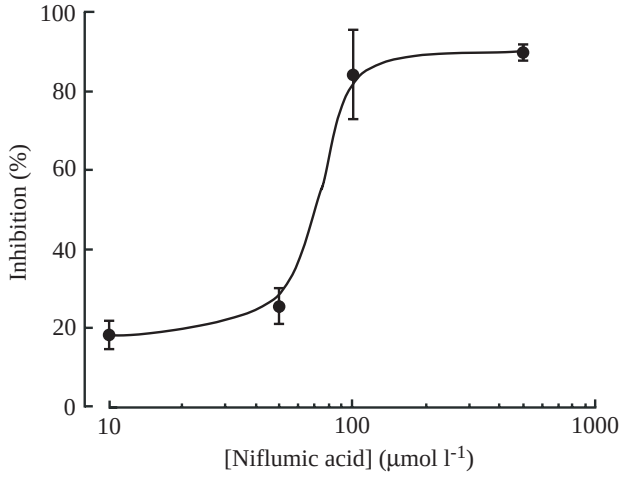

Fig. 9. Dose-dependence of niflumic acid inhibition on the responses of olfactory receptor neurons (ORNs) to the amino acid mixture when Na^+-free external solution was perfused and K^+-internal solution filled the recording pipette (see Materials and methods). Data were obtained from 2–4 different ORNs for each concentration tested. Values are means ± S.D. The curve was drawn manually. IC_{50} determined from the curve was 70 μmol l^{-1}.

Previous studies on *in vivo* ORNs in lamprey (Suzuki, 1977, 1982) and channel catfish (Kang and Caprio, 1995) showed that some ORNs responded to a single amino acid, such as glutamate, alanine and methionine, with either excitation or suppression of spontaneous activity. Recent experiments on ORNs isolated from tadpoles of *Xenopus laevis* showed that 50 % of responses to a single amino acid (glutamate, alanine, methionine or arginine) were inhibitory. A small percentage of isolated ORNs responded with inhibitory responses even to a mixture of these same amino acids (Vogler and Schild, 1999). In the present study, however, the responses of 199 ORNs held at −60 mV to a mixture of four amino acids (alanine, arginine, glutamate and norvaline) in different combinations of external and internal solutions were inward currents that resulted in depolarizing excitatory receptor potentials. No outward current response to the amino acid mixture yielding inhibitory and hyperpolarizing receptor potentials was observed. The reason for the discrepancy between the present results and those obtained in previous studies is not clear, but it is possible that outward current components, even if they were evoked by one

or more of the amino acids of the mixture used in the present study, were masked by simple arithmetic addition by larger inward current components evoked by other amino acids.

The shifts in the reversal potential in both negative and positive directions associated with external and internal Na^+ and Cl^- concentration changes, and the fact that the largest inward current responses to the amino acid mixture were evoked in low-Cl^- perfusate, indicated that Ca^{2+}-activated Cl^- channels contributed to the generation of amino acid-induced inward currents in ORNs. In lamprey, Ca^{2+} is the only ion required for an olfactory receptor response to amino acids to occur (Suzuki, 1978). An increased intracellular Ca^{2+} concentration caused by an influx of Ca^{2+} through CNG channels in the membranes of olfactory cilia has recently been confirmed by Ca^{2+} imaging analyses in various amphibian species (Leinders-Zufall et al., 1997, 1998). The resultant increase in Ca^{2+} concentration further activates Ca^{2+}-activated Cl^- channels (Kleene and Gesteland, 1991; Hallani et al., 1998) in ciliary membranes, leading to the efflux of the intracellular Cl^- that provides one component of the inward receptor current (Kurahashi and Yau, 1993; Lowe and Gold, 1993; Zhainazarov and Ache, 1995). Taken together with these previous results, we conclude that the inward current responses to the amino acid mixture in rainbow trout ORNs include inward current components caused not only by influxes of Na^+ and Ca^{2+} but also by efflux of Cl^- through Ca^{2+}-activated Cl^- channels.

In the present study, all six Cl^- channel blockers (niflumic acid, flufenamic acid, SITS, DIDS, NPPB and DCDPC) were effective in inhibiting the responses of ORNs to the amino acid mixture. The order of inhibitory effectiveness of these Cl^- channel blockers was essentially similar to that on Ca^{2+}-activated Cl^- channels in the frog (Kleene and Gesteland, 1991; Kleene, 1993). The similarity of the order of inhibitory effectiveness of Cl^- channel blockers on Ca^{2+}-activated Cl^- channels in different vertebrate species suggests that Ca^{2+}-activated Cl^- channels in these vertebrates may have a common molecular structure. However, the large variability in the inhibitory effectiveness of Cl^- channel blockers at the same dose among ORNs in the present study (see Table 1) suggests that there is a difference in the distribution of Ca^{2+}-activated Cl^- channels between individual ORNs.

Two of the Cl^- channel blockers used in this study (niflumic acid and DCDPC) inhibited not only the current responses to the amino acid mixture but also the background activity of ORNs, indicating that the blocking effects were not specific for Ca^{2+}-activated Cl^- channels. A similar non-specificity of Cl^- channel blockers for other ion channels has been reported for voltage-gated Cl^- channels (Dubin et al., 1994) and for Ca^{2+}-activated non-selective cation channels (Gögelein and Pfannmüller, 1989). In the present study, inhibition of background activity was probably due to other conductances such as the inwardly rectifying K^+ conductance that is slightly activated at −60 mV (Miyamoto et al., 1992).

There was no significant change in the magnitude of inward current responses to the amino acid mixture when ORNs were perfused with Na^+-free external solution, and no other cations apart from Ca^{2+} were required to generate inward current responses to the amino acid mixture. In fish, the olfactory epithelium is in direct contact with the ambient water. In fresh water, ionic concentrations over the receptive ciliary membrane of ORNs are extremely low. In such an environment, the olfactory receptor current components through CNG channels would be small since only a small influx of cations, including Ca^{2+}, occurs. However, increased intracellular Ca^{2+} concentration due to Ca^{2+} influx will further activate Ca^{2+}-activated Cl^- channels, resulting in the generation of a depolarizing receptor current sufficient for the sensory function of ORNs. In sea water, in contrast, the concentrations of Na^+, Ca^{2+} and other cations are high on the external receptive surface of ORNs, and the efflux of Cl^- noted in fresh water will be replaced by influxes of Na^+ and Ca^{2+} across the receptive membrane of ORNs to generate the receptor current. Similar results on olfactory function in fishes have been reported previously (Dubin et al., 1994; Zhainazarov and Ache, 1995; Kleene and Pun, 1996; Reuter et al., 1998).

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