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Whole-cell Response Characteristics of Ciliated and Microvillous Olfactory Receptor Neurons to Amino Acids, Pheromone Candidates and Urine in Rainbow Trout

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Abstract

Olfactory lamellae of teleosts contain two morphologically different types of olfactory receptor neurons (ORNs): ciliated ORNs (cORNs) and microvillous ORNs (mORNs). However, little is known about the functional difference between these two types of ORNs in fish olfaction. We isolated cORNs and mORNs using a Ca^{2+}-free solution method from olfactory organs of the rainbow trout and examined their response characteristics to various odorants including fish pheromone candidates by whole-cell voltage-clamp techniques. Quadruple mixture of amino acids, single amino acids, steroids (analogues of DHP; 17α,20β-dihydroxy-4-pregnen-3-one and ECG; etiocholan-3α-ol-17-one glucuronide), prostaglandins (PGFs) and urine samples collected from immature and mature female fish were applied focally to olfactory cilia or microvilli using a multi-barreled stimulation pipette with a pressure ejection system. Inward current responses to odorants were recorded from both cORNs and mORNs at a holding potential of –60 mV. cORNs responded to the amino acid mixture, single amino acids, urine samples and ECG, whereas mORNs responded specifically either to the amino acid mixture or single amino acids. The response profiles of both cORNs and mORNs to various odorants varied widely. None of cORNs and mORNs responded to fish pheromone candidates, PGFs and DHPs. Androgen treatment of immature fish did not influence olfactory sensitivity of both cORNs and mORNs to the amino acid mixture and both urine samples. Amino acid and bile acid analyses by HPLC showed that both urine samples contained 35 amino acids (1–40 mM) and trace amounts of taurocholic acid and glycoursodeoxycholic acid. Our results suggest that cORNs are 'generalists' that respond to a wide variety of odorants, including pheromones, whereas mORNs are 'specialists', specific to amino acids, and also suggest that PGFs and DHPs are not pheromones for the rainbow trout.

Introduction

In amphibians, reptiles and mammals, there are two types of olfactory organs: the main olfactory organ and vomeronasal organ. The main olfactory organ contains ciliated olfactory receptor neurons (cORNs) and detects a variety of general odorants, whereas the vomeronasal organ has microvillous olfactory receptor neurons (mORNs) and detects pheromones that play important roles in the territorial defense, reproductive behavior and synchronism of sexual maturation (Farbman, 2000; Johnston, 2000). In teleosts, which do not have vomeronasal organs, both cORNs and mORNs are distributed in the olfactory epithelium (Yamamoto and Ueda, 1977; Zielinski and Hara, 1988; Eisthen, 1992; Zeiske *et al*., 1992). Cloning of fish odorant receptor genes has been performed in the catfish (Ngai *et al*., 1993), zebrafish (Barth *et al*., 1996) and goldfish (Cao *et al*., 1998), and showed that the gene families of odorant receptors in these teleosts consisted of 100–300 G-protein-coupled receptor genes. In the goldfish, functional expression of odorant receptor genes has been reported by Speca *et al*. (Speca *et al*., 1999). However, the distinct distribution of these odorant receptors between cORNs and mORNs was not investigated. Moreover, patch-clamp studies of isolated ORNs of channel catfish and rainbow trout (Restrepo *et al*., 1990; Miyamoto *et al*., 1992; Ivanova and Caprio, 1993; Sato and Suzuki, 2000) have recorded the responses to amino acids only from cORNs. Another patch-clamp study on the responses of salmon ORNs to imprinted odorants (Nevitt *et al*., 1994) also did not distinguish between two types of ORNs in respect of their functional differences.

The urine of mature females is a potent odorant in the Atlantic salmon (*Salmo salar*) (Moore and Scott, 1992), and the major source of primer and releaser pheromones for

the rainbow trout (*Oncorhynchus mykiss*), which regulate male plasma levels of gonadotrophin II (Scott *et al*., 1994) and play important roles in male attraction (Yambe, 2001). However, the pheromones present in the urine of mature female rainbow trout have not yet been identified. F-type prostaglandins (PGFs) and several steroids such as 17α,20β-dihydroxy-4-pregnen-3-one (DHP) and DHP 20-sulphate (DHP-s) have been identified as pheromones in several species of fishes, which regulate mate attraction and spawning synchrony (Stacey *et al*., 1994; Sorensen and Caprio, 1998). The detection of PGFs and DHPs by the olfactory organ of the goldfish (*Carassius auratus*) (Sorensen *et al*., 1988, 1991) leads to the production of sperm (Dulka *et al*., 1987) and sexual behavior (Sorensen *et al*., 1988, 1989). The sensitivity of olfactory organ to pheromones is enhanced during the fish spawning season. In the tinfoil barb (*Puntius schwanenfeldi)*, androgen treatment increases the sensitivity of ORNs to 15-keto prostaglandin $F_{2\alpha}$ (15KPGF), as measured both by electro-olfactograms (EOGs) and the frequency of courtship behavior (Cardwell *et al*., 1995). In Atlantic salmon, the sensitivity of ORNs to PGFs as measured by EOG responses and the level of expressible milt increase as the reproductive season progresses (Moore and Waring, 1996). PGFs are also potent odorants for Arctic char (*Salvelinus alpinus*), lake trout (*Salvelinus namaycush*), brown trout (*Salmo trutta*) and lake whitefish (*Coregonus clupeaformis*) (Hara and Zhang, 1998; Sveinsson and Hara, 2000). Similar hormonal influence on the sensitivity of isolated ORNs to odorants by a direct application of adrenaline has been noted in the newt (Kawai *et al*., 1999).

In the present study, we isolated cORNs and mORNs from the olfactory organ of rainbow trout with a Ca^{2+}-free solution method and examined for the first time their response characteristics to the quadruple mixture of amino acids, single amino acids, pheromone candidates such as PGFs and steroids, and urine samples collected from immature and ovulated female fish, using whole-cell voltage-clamp techniques. We also studied the effect of androgen treatment on the sensitivity of ORNs to the amino acid mixture and urine samples. Amino acid and bile acid analyses of urine samples were also performed by HPLC. Our results showed that cORNs responded to the amino acid mixture, single amino acids, urine samples and etiocholan-3α-ol-17-one glucuronide (ECG), whereas mORNs responded specifically either to the amino acid mixture or single amino acids.

Materials and methods

Fish and androgen treatment

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

To examine the effect of androgen treatment on the current responses of ORNs, anaesthetized (0.02% 2-phenoxyethanol) rainbow trout were injected i.p. with 17α-methyltestosterone (MT) (231-02, Nacarai Tesque, Kyoto) or 11-keto androstendione (11KA) (28, 499-8, Aldrich Chemicals, Milwaukee, WI). These steroids were dissolved in 50% ethanol–standard Ringer's solution [in mM: NaCl (100), KCl (3), $CaCl_2$ (2), $MgCl_2$ (1), D-glucose (10), HEPES (5) and NaOH (2.2), pH 7.4], and were injected into the test fish at a dose of 5.0 μg/g body wt. Three days after the first injection, an additional injection of 1.0 μg/g body wt was administered. Two days after the second injection, body color of androgen-treated fish became obviously darker than before and persisted for another 13–15 days. The test fish were not fed during treatment and were used for experiments 3–21 days after the second injection.

Procedure for isolating ORNs and whole-cell voltage-clamp recording techniques

Cell isolation procedure with Ca^{2+}-free solution method and whole-cell voltage-clamp recordings were performed as follows. The fish were killed by quick decapitation with a knife. Olfactory rosettes were first dissected out from both olfactory organs and stored in standard Ringer's solution on ice. For one series of electrophysiological experiments, olfactory epithelial tissues were collected from olfactory lamellae isolated from one rosette and incubated in Ca^{2+}-free Ringer's solution [in mM: NaCl (100), KCl (3), $MgCl_2$ (1), 10 D-glucose, HEPES (5) and NaOH (2.2), pH 7.4] on ice for 1 h. After incubation, olfactory epithelial cells were dissociated by sucking them in and out of a fire-polished Pasteur pipette with a tip opening of 0.2 mm. The epithelial cell suspension was filtered using a nylon mesh (40 μm mesh size) and 0.5–0.7 ml of final cell suspension was obtained. The cell suspension was plated onto a concanavalin A (C2010 Type IV; Sigma, St Louis, MO) coated coverglass that was inserted into a perfusion chamber attached to the stage of an inverted phase-contrast microscope (Diaphot TMD, Nikon, Tokyo), and was allowed to stand for 20 min until the cells became attached to the surface of the coverglass. The standard Ringer's solution was then flown at a rate of 1.0 ml/min through the test chamber and the cells were examined microscopically before the experiment to distinguish two types of ORNs in the electrophysiological preparation. In the present study, only cORNs bearing more than four cilia, each >3 μm long and mORNs bearing clearly identifiable microvilli were selected for electrical recording. Isolated ORNs were also separately examined with trypan blue staining. A small amount of the final epithelial cell suspension (25–50 μl) was transferred to a glass tube and mixed with the same volume of 0.1% trypan blue dissolved in standard Ringer's solution. After 5 min incubation at room temperature, trypan blue-

stained cell suspension was transferred to a hemocytometer and examined under a Nomarski differential interference contrast microscope (BX50WI, Olympus, Tokyo).

A standard whole-cell voltage-clamp technique (Hamill *et al.*, 1981) was used to record the current responses of cORNs and mORNs. For fabrication of recording pipettes, thick-walled borosilicate glass tubes (1.5 mm o.d., 1.2 mm i.d.; G-1, Narishige, Tokyo) were pulled using a micropipette puller (PD-5, Narishige, Tokyo). The pipette resistance was 8–12 MΩ, when filled with K^+-internal solution [in mM: KCl (93), EGTA-2K (5), HEPES (5), ATP-2Na (1.0), GTP-Na (0.1) and KOH (2.26), pH 7.4]. The recording pipette was connected via an Ag–AgCl wire to the headstage of a patch-clamp amplifier (CEZ-2200, Nihon Kohden, Tokyo). The reference electrode was an Ag–AgCl plate immersed in the bath solution. Tight seals (>1 GΩ) were established by applying a small negative pressure to the recording pipette after contact with the cell soma surface. Rupture of the membrane for whole-cell mode recordings was achieved either by application of negative pressure or zapping voltage pulses (±1.0 V, 1–10 ms) to the recording pipette. Whole-cell recordings from ORNs usually lasted 10–20 min. The recording was discontinued when a high seal resistance could not be maintained properly or deterioration of the ORN responses to odorants became evident. At each end of the recordings, voltage-gated channel currents to stepping voltage pulses (up to ±140 mV, 25 ms) were obtained to confirm the proper whole-cell recordings. Current signals were low-pass-filtered at 3 kHz and stored on the magnetic tape of a PCM data recorder (PCM-501ES, Sony, Tokyo; DC-13 kHz bandwidth) for later off-line analysis. Current and voltage data were digitized at 1 kHz sampling speed and analyzed using PowerLab (AD Instruments, Mountain View, CA) on a Power Macintosh computer and DataSponge (WPI, Sarasota, FL) on an IBM PC-AT compatible computer. The current data were further processed for presentation using graphics software, Canvas 6.0 (Deneba, Miami, FL).

Application of odorants by multi-barreled stimulation pipette

Odorants were applied focally to olfactory cilia or microvilli of an ORN using a three-, four- or seven-barreled stimulation glass pipette (each tip opening diameter 1.0 μm). A barrel of three, four or seven glass capillaries (G-1.2-filament; Narishige, Tokyo) fixed on both ends with heat-shrinkable polyolefin tube (FP-301; MMM, Austin, TX) was pulled with a programmable pipette puller (PMP-100; WPI). Each of the barreled stimulation pipettes filled with different odorants was connected via a silicon rubber tube to a custom-built pressure ejection system (Sato and Suzuki, 2000). The tip of the barreled pipettes was always positioned 20 μm away from the cilia or microvilli of ORNs using a hydraulic micromanipulator (MB-PP2; Narishige). In all experiments, the stimulus was delivered to ORNs at an ejection pressure of 1.0 kgf/cm^2 and a pressure pulse duration of 25 ms. To examine the whole-cell responses of cORNs and mORNs to various odorants, experiments were performed as in the following procedure. After the transition to the whole-cell recording configuration, a quadruple amino acid mixture [1.0 mM: L-Glu, L-Arg, L-Ala and L-Nva; these four amino acids are representative amino acids for the four independent amino acid receptor sites of ORNs in the channel catfish (Caprio and Byrd, 1984)] was applied first to an ORN, to determine the presence of any response. Then, 2–6 different odorants were successively applied to the ORN in a random order at 30–60 s intervals. The amino acid mixture was frequently applied to check the deterioration of current response during the period of odorant response recording. If the deterioration became obvious, the recorded data were excluded from analysis.

Odorants

Eight amino acids—L-Ala, L-Arg, L-Glu, L-Nva, L-Thr, L-Met, Gly and taurine (Nacarai Tesque)—were used for odorants. The first four of these amino acids were used as a quadruple mixture and dissolved in standard Ringer's solution (1 mM, pH 7.6). Other amino acids dissolved in standard Ringer's solution (1 mM, pH 7.3–7.4) were used as single amino acid odorants. The quadruple amino acid mixture was also used at higher concentrations (10 mM, pH 7.6) to determine the dose-dependency, current–voltage relationship and reversal potential for responses of mORNs. The following steroids were used for odorants as pheromone candidates: DHP (P6285, Sigma), DHP 20-acetate (DHP-a) (P1664, Sigma), DHP-s (a gift from Dr Peter W. Sorensen, University of Minnesota) and ECG (E 8000, Sigma). PGF and its analogs: PGF (163-10831; Wako, Osaka), 15KPGF (K 0127; Sigma), dPGF (39746-23-1; Cayman Chem, Ann Arbor, MI) and U-46619 (56985-40-1, Cayman Chem) were also used as pheromone candidates. Steroids and PGFs were dissolved in 0.5% ethanol–standard Ringer's solution (U-46619 was dissolved in 0.35% methylacetate–standard Ringer's solution) to a final concentration of 0.1 mM. Urine of immature rainbow trout was collected in February 2000 from immature fish (GSI: 0.56 ± 0.30) without distinction of sex. A plastic pipette tip (200 μl; Gilson, Villers, France) was directly inserted into the bladder of each fish to collect the urine. The fish were autopsied to check the correct placement of the pipette tip within the bladder. Urine of mature female fish (a gift from Dr Hidenobu Yambe, Hokkaido University) was collected in May 1999 from ovulated female rainbow trout. Urine samples of immature and mature female fish were diluted to 20 times with standard Ringer's solution, to a final millimolar-concentration range for their major amino acid components. Ten-times concentrated stock solutions of amino acids and other odorants were prepared before experiments and stored at 4C or –80°C. The stock solutions were diluted with standard Ringer's solution to the final concentration just

prior to the experiments. Once diluted, test solutions were stored at 4°C and subsequently used within 3 days. The stimulus concentration at the target was estimated, by measuring liquid junction potentials, to be diluted by at least a factor of 10 compared with the concentration in the stimulating pipette liquid. Therefore, the actual concentrations for amino acid stimuli at ORNs, when stimulated with 1 mM solution in a stimulation pipette, were of the order of 0.1 mM, which was slightly higher than the concentration ranges for amino acids to yield the half-maximal olfactory responses in the rainbow trout (Hara, 1982; Evans and Hara, 1985). Depending on the number of barreled stimulation pipettes used in different experiments, three, four, or seven odorants were tested in each ORN. Odorants except ECG, dPGF and U-46619 were tested for ORNs isolated from non-treated fish. The amino acid mixture, urine samples and PGFs were tested for ORNs isolated from MT-treated rainbow trout. The amino acid mixture, L-Met, dPGF, U-46619, ECG and urine samples were tested for ORNs isolated from 11KA-treated fish.

Analyses of urine samples for amino acids and bile acids

Urine samples collected from the rainbow trout were subjected to free-form amino acid and bile acid analyses. For amino acid analysis, a 150 µl of urine sample was directly loaded on an amino acid analyzer (L-8500; Hitachi, Tokyo) without pretreatment for hydrolysis and protein removal. Amino acid analysis was performed at the Center for Instrumental Analysis, Hokkaido University. For bile acid analysis, bile acids were first extracted from 700 µl of urine samples in the same volume of methanol with Bilepak II cartridge (JASCO, Tokyo). The extraction was loaded on a bile acid analysis system (LCSS-905, JASCO). Bile acid analysis was performed at the Analysis Center of JASCO Engineering Co. Ltd.

Statistical analysis

All data are expressed as mean ± SD. Differences between groups were examined for statistical significance using analysis of variance (ANOVA), two-way factorial ANOVA and Student's *t*-test. A P value <0.01 denoted the presence of a statistically significant difference.

Results

Morphology of cORNs and mORNs and the ratio in which they are found

Microscopic examination of isolated olfactory epithelial cell preparation for electrophysiological study confirmed that there were two types of morphologically distinct ORNs, which had either olfactory cilia or microvilli on the olfactory knob (Figure 1). Morphologically, both types of isolated ORNs were bipolar neurons with a slender dendrite (cORN, 19.0 ± 8.50 µm long, n = 20; mORN, 16.6 ± 4.24 µm long, n = 20), a cell soma (cORN, 12.5 ± 2.44 µm major axis, 7.03 ± 1.39 µm minor axis, n = 20; mORN, 13.3 ± 2.93 µm major axis, 6.44 ± 1.56 µm minor axis, n = 20) and often, an initial segment of axon (<1 µm diameter, 3–20 µm long). There were no significant differences in these dimensions between cORNs and mORNs (two-way factorial ANOVA: $P > 0.1$). cORNs had 2–10 cilia (3.0–10 µm long) on their knob, whereas mORNs had numerous microvilli (1.0–2.0 µm long) on their knob. mORNs were clearly distinguishable by their characteristic lump structure of short and thin microvilli from deciliated cORNs, which had short broken cilia. The trypan blue exclusion test showed that 617 000 ± 20 100 (n = 16) epithelial cells were isolated from one rosette. Of these epithelial cells, 45 000 ± 26 600 ORNs were distinguished (7.3% of total epithelial cells). Of total ORNs, the number of cORNs was 11 000 ± 4300 (24.4%; viability 97.2%), that of mORNs was 1000 ± 269 (2.2%; viability 100%), and that of unidentified ORNs was 33 000 ± 22 600 (73.4%; viability 46.6%). Thus, the ratio in which mORNs to cORNs were found in the preparations was 1:11. The majority of isolated ORNs in electrophysiological preparations became round within 3 h of continuous flow of bath solution and the morphological characteristics of the olfactory knob structures of the two types of ORNs became indistinguishable.

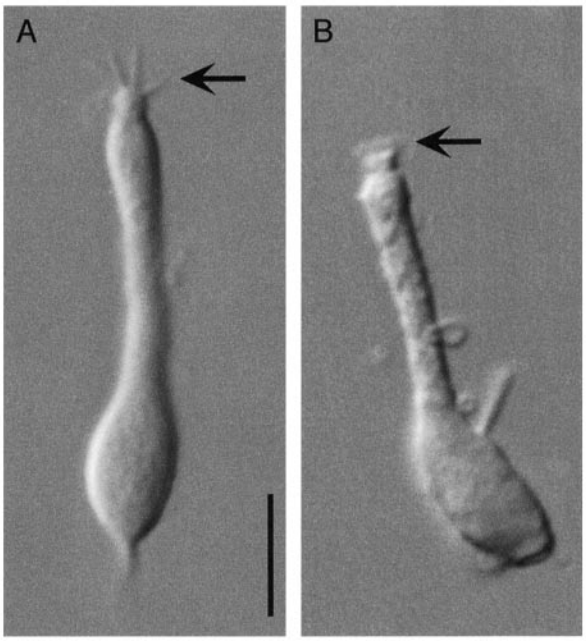

Figure 1 Nomarski differential interference contrast micrographs showing a major morphological difference between cORN **(A)** and mORN **(B)** isolated from olfactory epithelium of the rainbow trout. The olfactory knob of the cORN supported five cilia (arrow) that were 3–5 µm long, whereas that of the mORN bore numerous microvilli (arrow) 1.0–2.0 µm long. Bar = 10 µm.

Whole-cell current responses and reversal potential of mORNs to the amino acid mixture

The inward current responses to the amino acid mixture

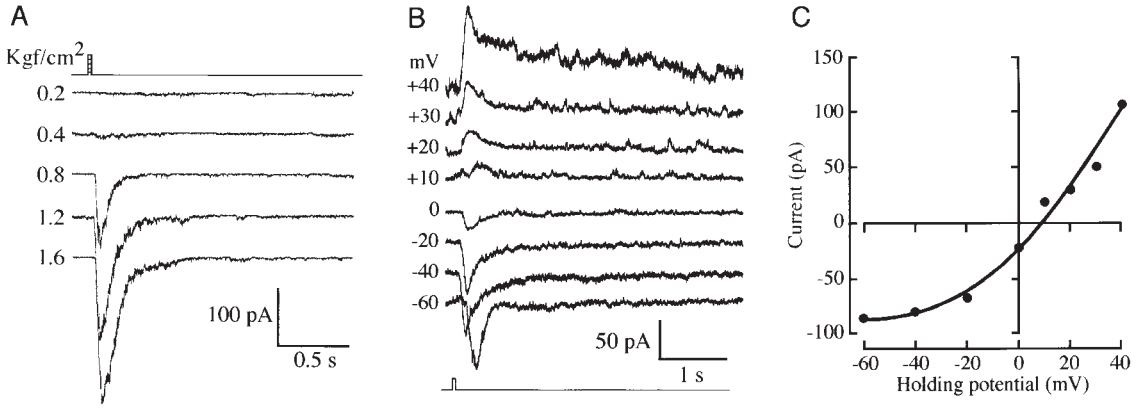

Figure 2 Whole-cell current responses of representative mORNs to the amino acid mixture. **(A)** Whole-cell inward current responses of mORN to the amino acid mixture in different stimulus ejection pressures (0.2–1.6 kgf/cm^2; duration 25 ms). Stimulus ejection pressure (kgf/cm^2) is shown on each trace. Top trace shows the timing of ejection pressure pulses. **(B)** Current traces at different holding potentials recorded from another mORN shown in (A). Holding potential (mV) is indicated on the left of each trace. Current offsets due to changes in holding potential are not shown for convenient trace illustration. Bottom trace shows the timing of ejection pressure pulse. The amino acid mixture was, on a 60 s interval, applied focally to microvilli at different holding potentials. Data in (A) and (B) were sampled at 1 kHz and displayed at a compression factor of 10 by DataSponge. **(C)** Current–voltage relationship. The peak current of each response shown in (B) was plotted against voltage, and the curve was drawn manually. The reversal potential determined from the curve was +8.0 mV.

occurred in mORNs at a holding potential of –60 mV. The magnitude of the peak inward current response to the amino acid mixture increased with higher stimulus ejection pressure (Figure 2A). We determined the current peak–voltage relationship (*I–V* relationship) for stimulation with the amino acid mixture by varying the holding potential between –60 and +40 mV (Figure 2B). 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 this condition (Figure 2C) showed a marked outward rectification. The reversal potential determined for this and other mORNs in the same condition was +6.0 ± 1.73 mV (n = 3).

Response profiles of cORNs and mORNs to amino acids, steroids, PGFs and urine samples

Whole-cell current responses of cORNs held at –60 mV to any one of odorants tested were obtained from 44 of 175 (25.1%) cORNs (Figures 3A and 4A–D). Although the response profiles varied markedly from one cORN to another, cORNs could be roughly divided into three groups based on their response characteristics to the amino acid mixture, single amino acids and urine samples: cORNs that responded only to amino acids, to either the amino mixture or single amino acids, and to either or both of them (group I in Figure 3A; Figure 4A); cORNs that responded to either or both urine samples (group II in Figure 3A; Figure 4B,C); and cORNs that responded to either or both of the amino acid mixture and single amino acid and either or both urine samples (group III in Figure 3A; Figure 4D). Since cORNs of Figure 3A, no. 1–12 were not tested with urine samples, these cORNs were excluded from this classification. The percentages of groups I–III were 21.9, 31.3 and 46.9% (n = 32) respectively. None of the cORNs responded to DHP (85/175 cORNs), DHP-a (80/175 cORNs), DHP-s (29/175 cORNs), PGF (42/175 cORNs), 15KPGF (30/175 cORNs) and taurine (27/175 cORNs).

Whole-cell current responses of mORNs held at –60 mV were obtained from 20/70 (28.6%) mORNs (Figure 3B). Figure 4E,F show typical inward current responses of two mORNs. These mORNs responded only to the amino acid mixture or a single amino acid. None of mORNs responded to both urine samples (54/70 mORNs), DHP (33/70 mORNs), DHP-a (33/70 mORNs), DHP-s (11/70 mORNs), PGF (10/70 mORNs), 15KPGF (8/70 mORNs) and taurine (9/70 mORNs).

All current responses recorded from both types of ORNs were phasic inward currents (current peak levels: 1.4–187.0 pA) at a holding potential of –60 mV. Outward current responses were not observed in the present experiments. There was no significant difference between the mean peak inward current responses of cORNs (43.2 ± 40.7 pA, n = 32) and mORNs (48.1 ± 55.8 pA, n = 16) to the amino acid mixture at a holding potential of –60 mV (t-test: $P > 0.1$).

Response profiles of cORNs and mORNs from androgen-treated fish

Whole-cell current responses of cORNs isolated from 11KA-treated fish to any of odorants tested were obtained from 18/33 (54.5%) cORNs (Figures 3C and 5A,B). The response profiles of cORNs isolated from 11KA-treated fish varied widely, as observed in those of cORNs isolated from the control fish. However, they could be roughly divided into three groups by their response characteristics to the amino acid mixture, a single amino acid and urine samples, as

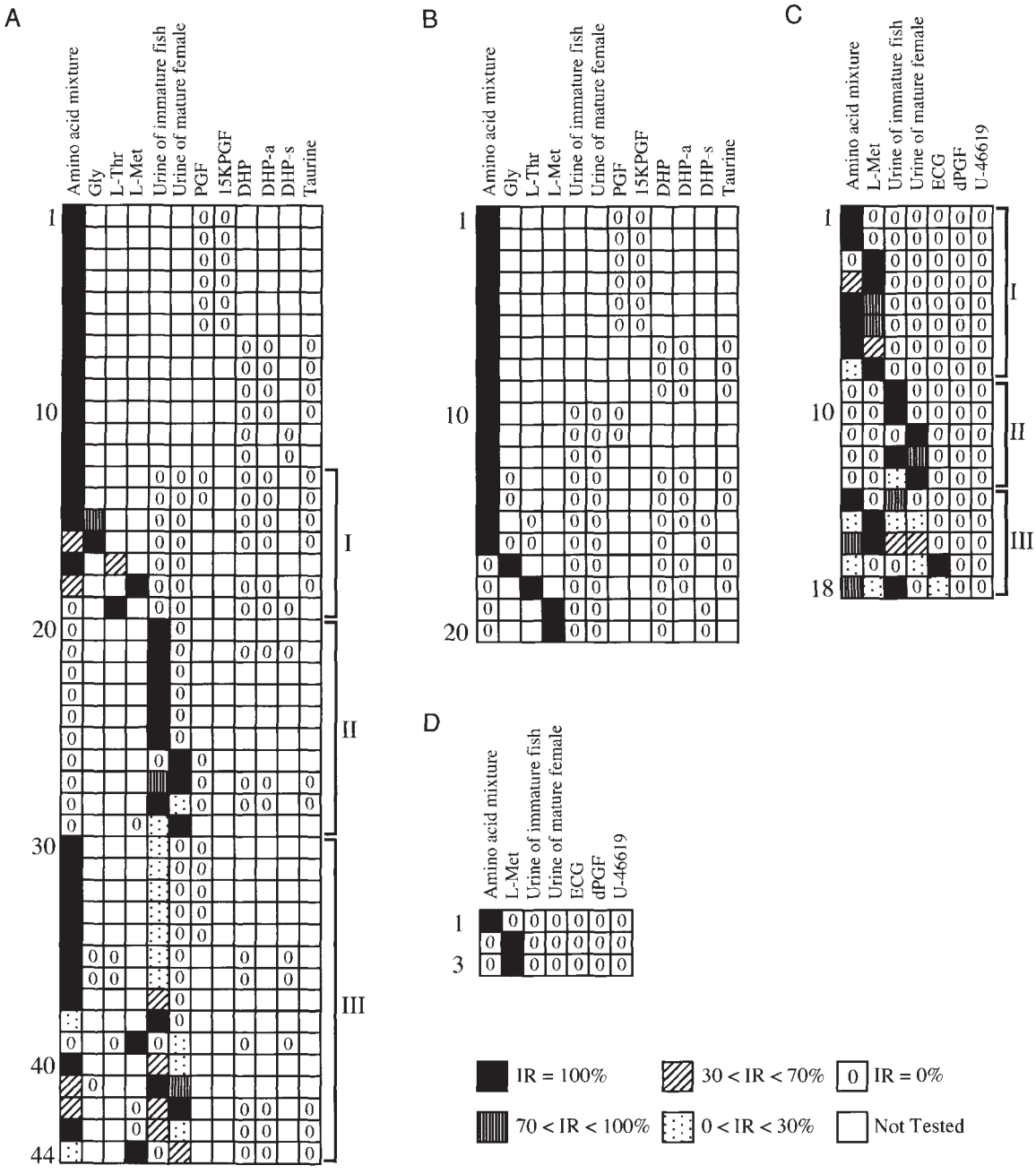

Figure 3 Olfactory response profiles of cONRs and mORNs isolated from rainbow trout. The magnitude of the inward current response is expressed as a percentage (IR; box drawing) of the maximum response for each ORN. Blank squares indicate untested odorants. All current responses were recorded at a holding potential of –60 mV and were phasic inward currents. In cORNs **(A,C)**, olfactory response profiles were divided into three groups (I, II and III) based on their response characteristics. (A) Response profiles of cORNs isolated from control rainbow trout. (B) Response profiles of mORNs isolated from control rainbow trout. (C) Response profiles of cORNs isolated from 11KA-treated rainbow trout. (D) Response profiles of mORNs isolated from 11KA-treated rainbow trout.

classified in cORNs isolated from the control fish: cORNs that responded to either the amino mixture or a single amino acid, and or both of them (group I in Figure 3C), cORNs that responded to either or both urine samples (group II in Figure 3C) and cORNs that responded to either the amino acid mixture or a single amino acid and one or both urine samples (group III in Figure 3C). The percentages of groups I, II and III were 44.4, 27.8 and 27.8% (n = 18) respectively. None of cORNs (n = 33) isolated from 11KA-treated fish responded to dPGF or U-46619 (Figure 3C). Figure 5 shows examples of current responses of cORNs of group III to ECG. One cORN responded strongly to ECG and weakly to the amino acid mixture and urine of mature female (Figure 5A). Another

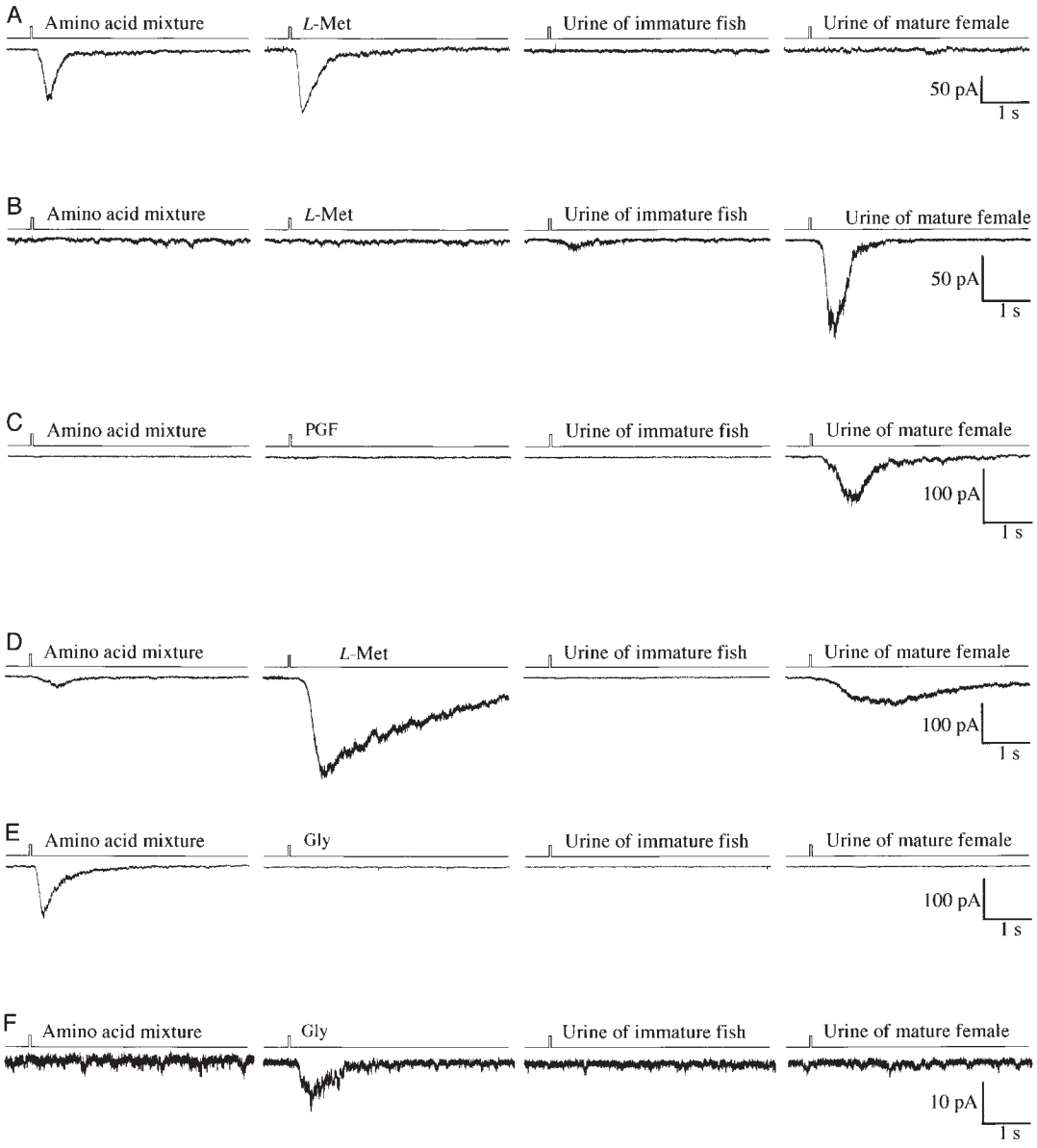

Figure 4 Typical inward current responses of four cORNs **(A–D)** and two mORNs **(E,F)** to the amino acid mixture, single amino acids, PGF and urine samples diluted 20-fold by standard Ringer's solution. ORNs were chosen from cells shown in Figure 3. Upper and lower traces in (A)–(F) indicate the timing of odorant application and whole-cell current recordings respectively. Holding potential was –60 mV. Data were sampled at 1 kHz and displayed by PowerLab. (A) cORN (Figure 3A, no. 18) that responded to the amino acid mixture and L-Met. (B) cORN (Figure 3A, no. 29) that responded to both urine samples. (C) cORN (Figure 3A, no. 26) that responded specifically to urine of mature female fish. (D) cORN (Figure 3A, no. 44) that responded to the amino acid mixture, L-Met and urine of mature female fish. (E) mORN (Figure 3B, no. 13) that responded specifically to the amino acid mixture. (F) mORN (Figure 3B, no. 17) that responded specifically to Gly.

cORN responded strongly to the amino acid mixture and urine of immature fish but weakly to ECG (Figure 5B).

Whole-cell current responses of mORNs isolated from 11KA-treated fish to the amino acid mixture or L-Met were obtained from 3/5 (60%) mORNs (Figure 3D). One mORN responded specifically to the amino acid mixture and two mORNs responded specifically to L-Met. None of these mORNs (n = 5) responded to dPGF, U-46619, ECG and both urine samples.

Whole-cell current responses of cORNs isolated from MT-treated fish to any of the odorants—amino acid mixture and urine samples—were obtained from 16/40 (40%) cORNs. Only the amino acid mixture elicited inward current responses in 6/22 (27.2%) mORNs (data not shown). None of cORNs and mORNs isolated from MT-treated fish responded to PGF and 15KPGF. In androgen-treated fish, cORNs responded to multiple odorants, whereas mORNs responded specifically either to the amino acid mixture or to a single amino acid (L-Met), as observed in the control fish.

All current responses of ORNs isolated from androgen-

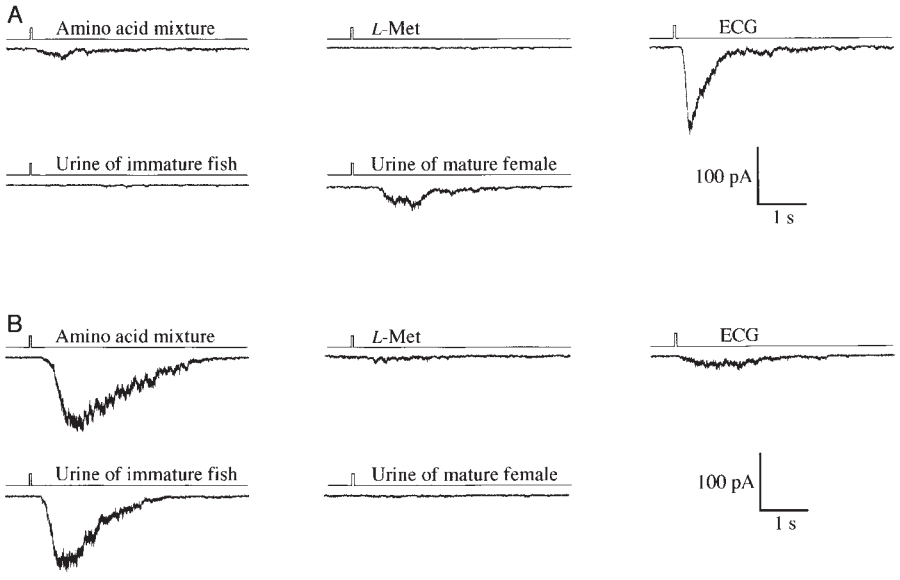

Figure 5 Typical inward current responses of cORNs isolated from 11KA-treated rainbow trout to ECG, amino acids and urine samples diluted 20 fold with standard Ringer's solution. Upper and lower traces in **(A)** and **(B)** indicate the timing of odorant application and whole-cell current recordings respectively. Holding potential was –60 mV. Data were sampled at 1 kHz and displayed by PowerLab. (A) cORN (Figure 3C, no. 17) showing a strong response to ECG. The cell also responded to the amino acid mixture and urine of mature female. (B) cORN (Figure 3C, no. 18) that responded to the amino acid mixture, L-Met, urine of immature fish and ECG.

treated fish were inward currents (current peak levels: 3.3–192.5 pA for ORNs from 11KA-treated fish, 2.2–106.2 pA for ORNs from MT-treated fish) at a holding potential of –60 mV. No outward current responses were observed.

Figure 6 compares the magnitudes of inward current responses of cORNs isolated from the control fish and androgen-treated fish to the amino acid mixture and both urine samples at a holding potential of –60 mV. Androgen treatment had no significant effect on whole-cell current responses of cORNs to the amino acid mixture and both urine samples.

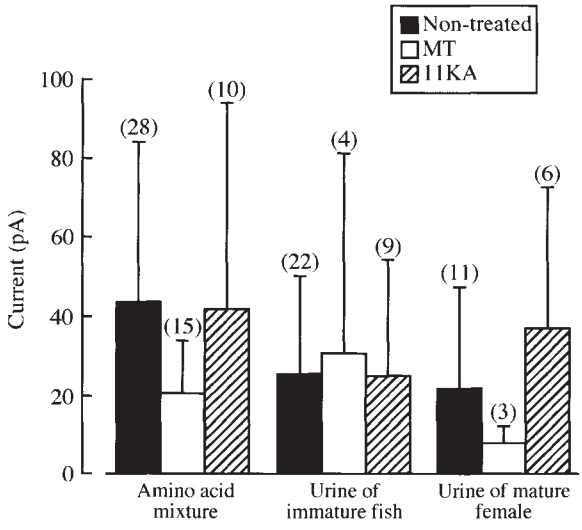

Figure 6 Effects of androgen treatment on whole-cell current responses of cORNs. Magnitudes of peak inward current responses of cORNs to the amino acid mixture and urine samples are indicated. Holding potential was –60 mV for all cORNs tested. Values are mean ± SD. Numbers of measurement are shown in parentheses. Androgens did not affect the olfactory responses of cORNs to the amino acid mixture and both urine samples (ANOVA; $P > 0.1$).

Amino acids and bile acids in urine samples

Table 1 shows the concentrations of amino acids, amino group substances and bile acids found in urine samples collected from immature and mature female fish. The composition of urinary amino acids varied between immature and mature female fish. Both urine samples contained mainly urea, taurine and ammonia (>100 mM) together with 35 amino acids (1–40 mM). Phosphoethanolamine and carnosine were not detected in urine of immature fish but in urine of mature females. The number of amino acids and their concentrations in urine samples of mature female fish tended to be higher than those of immature fish. The results of bile acid analysis indicated that both urine samples contained a small amount of glycoursodeoxycholic acid (GUDCA) and taurocholic acid (TCA). Other bile acids (tauroursodeoxycholic acid, ursodeoxycholic acid, glycocholic acid, cholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, glycodeoxycholic acid, taurodeoxycholic acid, chenodeoxycholic acid, deoxycholic acid, glycolithocholic acid, taurolithocholic acid and lithocenocholic acid) were not detected.

Table 1 Amino acids, amino group substances (mM) and bile acids (μM) in urine samples

	Urine of immature fish	Urine of mature female
Amino acids		
α-Aminoadipic acid	1.28	1.46
α-Aminobutyric acid	0.68	0
β-Alanine	7.20	20.2
β-Amino-isobutyric acid	15.0	4.04
γ-aminobutyric acid	3.88	0.74
Carnosine	0	4.12
Ethanolamine	12.2	37.1
Glycine	16.8	22.5
Hydroxylysine	1.66	3.30
Isoaspartic acid	4.18	16.4
Isoglutamine	3.38	5.58
L-Alanine	8.78	18.8
L-Anserine	10.9	0
L-Arginine	24.8	2.02
L-Aspartic acid	3.26	0
L-Cytosine	2.46	0.96
L-Glutamic acid	12.8	3.02
L-Histidine	1.90	2.00
L-Isoleucine	2.42	9.00
L-Leucine	6.46	18.1
L-Lysine	4.72	9.80
L-Methionine	1.78	3.70
L-1-Methylhistidine	41.2	1.76
L-3-Methylhistidine	1.70	0.80
L-Ornithine	2.60	2.72
L-Phenylalanine	4.78	11.1
L-Proline	3.40	0.16
L-Serine	11.2	4.08
L-Threonine	23.3	25.6
L-Triptophan	1.66	1.98
L-Tyrosine	2.88	3.10
L-Valine	7.14	21.7
O-Phosphoserine	4.74	7.38
Phosphoethanolamine	0	21.6
Sarcosine	2.42	3.34
Taurine	476	644
Amino group substances		
Ammonia	85.5	389
Urea	215	809
Bile acids		
GUDCA	0.096	0.016
TCA	0.626	0.788

Discussion

Functional differences between cORNs and mORNs

In the present study, we isolated two different types of ORNs—cORNs and mORNs—from the olfactory lamellae of rainbow trout and examined for the first time their response properties to various odorants. The previous SEM study of the surface structure of olfactory lamellae of several salmonid species, including rainbow trout (Thommesen, 1983), showed that the density of cORNs increased towards the peripheral margin of each lamella, whereas that of mORNs increased towards the central margin and the distribution ratio of mORNs to cORNs was 1:1.2–4.0. This distribution ratio is 2–10 times larger than the ratio of mORNs to cORNs found in our study in epithelial cell preparations for electrophysiology. The lower ratio of mORNs to cORNs in our study is probably due to underestimation of the number of isolated mORNs; these olfactory knob structures might be more easily damaged than cORNs during Ca^{2+}-free solution cell isolation procedure, resulting in underestimation of the number of mORNs by microscopic examination. The present patch-clamp recordings showed that the response characteristics to the amino acid mixture for mORNs such as the dose–response dependence and the reversal potential of *I–V* relationship were essentially similar to those of cORNs, as demonstrated in our previous study (Figures 1 and 2 in Sato and Suzuki, 2000). Comparison between peak values of inward current responses of cORNs and mORNs to the amino acid mixture also showed that there was no significant difference in responsiveness between these two types of ORNs at least to the four amino acids, L-Glu, L-Arg, L-Ala and L-Nva in the mixture. However, cORNs responded to several different species of odorants—amino acid mixture, other single amino acids, urine samples and ECG—whereas mORNs responded specifically to either the amino acid mixture or other single amino acids. This specific response of mORNs to amino acids in the present study is consistent with the previous finding of the higher amplitude of EOG (electro-olfactogram) response to amino acids in the central margin of the lamella, where mORN distribution density is higher than in the peripheral margin (Thommesen, 1983). Similar specific responses of mORNs to amino acids have been noted in the functional expression study of goldfish odorant receptors (Speca *et al.*, 1999) and in the labeling study of zebrafish ORNs with activity-dependent ion-channel-permeant probes (Lipschitz and Michel, 1999). The small population (25–29%) of ORNs responded to any one odorant tested in the present study. Since the trypan blue exclusion test showed that most of isolated ORNs were viable (viability 97–100%), the rest of the population of ORNs (71–75%), which did not respond to any of odorants we tested, might have responded to other odorants we did not test. Thus, the basic difference of response characteristics between cORNs and mORNs was the response selectivity to different species of odorants. Therefore, cORNs of the rainbow trout might be termed 'generalists', which respond to various species of odorants, whereas mORNs might be called 'specialists', which respond specifically to amino acids.

Natural olfactory stimuli for fish, such as odorants from

river water and urine, are themselves odorant cocktails. Masu salmon can discriminate the composition difference of amino acids dissolved in different river waters (Shoji *et al*., 2000). Analyses of amino acids and bile acids of urine samples of immature and mature female rainbow trout in the present study showed that these samples contained many amino acids together with trace amounts of two bile acids. Some steroids and their metabolites have also been identified in the urine of mature female rainbow trout (Scott and Liley, 1994). Urine from ovulated female Atlantic salmon also contained large quantities (18 ng/ml) of immunoreactive PGFs (Moore and Waring, 1996). Thus, the wide variety of cORNs and mORNs which have not only different responsiveness to different species of odorants but also many different types of amino acid receptor sites (see following Discussion) may help fish to detect a particular odorant of biological significance among many natural odorants at the peripheral level of the olfactory system.

Amino acid receptor sites and odorant receptors

Amino acid receptor sites in channel catfish ORNs have been classified into four independent receptor sites: the sites for acidic amino acids, basic amino acids, short-chained neutral amino acids and long-chained neutral amino acids (Caprio and Bryd, 1984). The amino acid mixture used in the present study contained L-Glu, L-Arg, L-Ala and L-Nva, each of which is the representative for these four independent receptor sites. In our study, we identified various types of cORNs and mORNs with regard to amino acid receptor sites. One type of cORN (Figure 3A, no. 39), for example, responded to L-Met, one of the long-chained neutral amino acids, but not to an amino acid mixture containing L-Nva for this site. Another cORN (Figure 3A, no. 19) responded to L-Thr, one of the short-chained neutral amino acids, but not to the amino acid mixture that contained L-Ala for this site. Other cORNs (Figure 3A, nos. 15–18) responded not only to the amino acid mixture but also to other single amino acids common for the four amino acid receptor sites. On the other hand, some mORNs (Figure 3B, nos. 13–16) responded only to the amino acid mixture for the four amino acid receptor sites. Other mORNs responded to a single amino acid, Gly (Figure 3B, no. 17), L-Thr (Figure 3B, no. 18) and L-Met (Figure 3B, no. 19-20), but not to the amino acid mixture. Thus, the response profiles of cORNs and mORNs to different amino acids for four independent amino acid receptor sites varied greatly from one ORN to another. Although amino acid receptor sites for different groups of amino acids are not always rigidly determined when stimulated by rather high concentrations of amino acid stimuli (Hara, 1976; Hara, 1982), it seems that individual ORNs of the rainbow trout may have much more complex combination of multiple amino acid receptor sites than those of the channel catfish (Kang and Caprio, 1995).

Gene cloning studies of G-protein-coupled odorant receptor have shown that individual ORNs express only one of a family of 100–300 genes in the channel catfish, zebrafish and goldfish (Ngai *et al*., 1993; Barth *et al*., 1996; Cao *et al*., 1998) and 1000 genes in the rat (Buck and Axel, 1991). Furthermore, recent studies of functional expression of mouse odorant receptor (Malnic *et al*., 1999; Touhara *et al*, 1999) have shown that one odorant receptor detects multiple species of odorants, and, conversely, one odorant is detected by multiple odorant receptors. The present results showed that individual cORNs responded to different species of odorants, amino acids, ECG and urine samples, and that individual cORNs and mORNs responded to different amino acids for different amino acid receptor sites. Therefore, one odorant receptor of an individual ORN in the rainbow trout could detect multiple odorants of different species as in the rat and mouse. In addition, since electrophysiological properties, such as *I–V* relationship and reversal potential for the amino acid mixture, were in this study essentially similar both in cORNs and mORNs, different types of odorant receptors might be equipped within the transduction machinery of individual cORNs and mORNs, which have similar efferent ion channels for the transduction machinery.

Responses of ORNs to pheromone candidates and urine samples

PGFs and DHPs have been identified as pheromones for several fish species (Stacey *et al*., 1994). However, neither cORNs nor mORNs of rainbow trout responded to these pheromone candidates. U-46619 and dPGF which elicited the bulbar responses of Arctic char (Sveinsson and Hara, 2000) were also ineffective on the rainbow trout. In rainbow trout (Scott *et al*., 1994) and Atlantic salmon (Waring *et al*., 1996), DHP-s had only a small priming effect on their steroid level. An EOG study of rainbow trout has shown that the olfactory epithelium does not respond to PGFs (Hara and Zhang, 1998). Together with these previous results, it could be concluded that PGFs and DHPs are not pheromones for the rainbow trout. In the African catfish (*Clarias gariepinus*), ECG was identified as a pheromone by behavioral observation and recordings of EOG and olfactory tract activities (Resink *et al*., 1989). Small EOG responses to ECG have also been recorded in several salmonid species (Hara and Zhang, 1998). In the present study, ECG induced inward current responses in two cORNs. However, this substance may not be a pheromone for the rainbow trout, because cORNs responsive to ECG did not show a specific response to this substance; ECG-responsive cORNs also responded to the amino acid mixture and both urine samples. These cORNs did not show a specific response similar to that of mORNs in the mouse vomeronasal organ, which showed specific responses to putative pheromones (Leinders-Zufall *et al*., 2000). Moreover, putative pheromones induced responses in only a particular subset of mORNs (0.2–3% of total mORNs) in

the epithelium of vomeronasal organ (Leinders-Zufall, 2000). If there is a similar situation for the specific responses of a small population of ORNs, the number of ORNs recorded in our study might not be sufficient to detect the responses of many ORNs to pheromones. On the other hand, we recorded specific current responses to urine of mature female, which should contain unidentified substances as pheromones (Scott *et al*., 1994; Yambe 2001), from two cORNs (Figures 3A, no. 26; 3C, no. 11; 4C). As mentioned above, urine from mature females contained many amino acids and trace amounts of two bile acids. Since we diluted urine samples 20-fold for stimulation, the concentration ranges of bile acids in urine samples should be close to the rainbow trout's olfactory threshold (Døving *et al*., 1980), whereas those of amino acids in urine samples were similar to those of amino acid mixture and single amino acids tested in our study. Therefore, amino acids in urine samples should be the main contributors to inward current responses of cORNs to urine samples. However, these cORNs responded to only urine of mature female. The specific components for urine of mature female, phosphoethanolamine and carnosine, might also contribute to the specific responses of cORNs to urine of mature female. In any case, the present results suggest that cORNs may serve as pheromone reception neurons in the rainbow trout, unlike in the rat and mouse, where mORNs serve as pheromone reception neurons.

Effect of androgen treatment on ORN sensitivity

The magnitudes of EOG responses to PGFs increase as the male reproductive season progresses in *S. salar* (Moore and Waring, 1996) and this phenomenon was reproduced by androgen treatment in *P. schwanenfeldi* (Cardwell *et al*., 1995). In the present study, however, PGFs did not induce any current responses in ORNs and androgen treatment did not influence inward current responses of cORNs to the amino acid mixture and urine samples. In our previous study (Sato *et al*., 2000), olfactory nerve responses of lacustrine sockeye salmon (*Oncorhynchus nerka*) to river water did not change significantly with different degrees of maturation. PGFs did not induce responses in olfactory nerves of sockeye salmon and masu salmon (*Oncorhynchus masou*) (Sato and Shoji, unpublished data). Therefore, olfactory response properties of ORNs of *Oncorhynchus* salmonids to different odorants may be different from those of ORNs of *Salmo* salmonids during maturation and also in pheromone detection. Many fish species of non-ostariophysi, including salmonids, do not show any EOG responses to PGFs and DHPs (Stacey *et al*., 1994).

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