

OLFACTORY RESPONSES OF A EURYHALINE FISH, THE RAINBOW TROUT: ADAPTATION OF OLFACTORY RECEPTORS TO SEA WATER AND SALT- DEPENDENCE OF THEIR RESPONSES TO AMINO ACIDS

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Summary

Salmonid fishes are able to survive in both fresh water and sea water. Concentrations of NaCl in fresh water and sea water are 0.5 and 493 mmol l⁻¹, respectively, and, hence, salt concentrations in the medium at the olfactory epithelium are greatly changed when the fish migrate between fresh water and sea water. We used the rainbow trout, which is a salmonid fish, to examine the adaptation mechanisms of the olfactory receptors to high concentrations of salts in sea water. Application of sea water to the olfactory epithelium elicited only a very small response in the olfactory nerve, but 500 mmol l⁻¹ NaCl elicited a large response which did not adapt to a spontaneous level with time. It is considered that the olfactory nerve becomes fatigued when the olfactory epithelium is exposed to 500 mmol l⁻¹ NaCl for long periods. We found that the presence of 10 mmol l⁻¹ Ca²⁺ in sea water inhibited the response to 500 mmol l⁻¹ NaCl, suggesting that Ca²⁺ in sea water is essential for adaptation

of the olfactory receptors to sea water. In the second part of the study, we examined whether the olfactory nerve responses to amino acids, potent stimulants for fish, were altered between fresh water and sea water. The magnitudes of the responses to the six amino acids examined were similar in artificial pond water and artificial sea water, indicating that a large change in NaCl concentration between fresh and sea water does not affect the olfactory nerve responses to amino acids. We used fish reared in fresh water and fish acclimated to sea water and obtained similar results. It was concluded that the tolerance of the olfactory receptors for large changes in osmotic pressure is not acquired while fish are maintained in fresh or sea water, but that the receptors of these euryhaline fish naturally provide the tolerance.

Key words: rainbow trout, olfactory response, amino acids, sea water, *Oncorhynchus mykiss*.

Introduction

Salmonid fishes are able to survive in fresh water and sea water. They hatch in fresh water, migrate to their feeding grounds in the ocean and later return to their natal river for spawning after a marine existence of several years. In general, sea water contains approximately 500 mmol l⁻¹ Na⁺, 10 mmol l⁻¹ K⁺, 10 mmol l⁻¹ Ca²⁺ and 50 mmol l⁻¹ Mg²⁺ together with other cations and anions, while river or fresh water contains approximately 0.5–1 mmol l⁻¹ Na⁺ and 0.1–2 mmol l⁻¹ Ca²⁺. The olfactory epithelium of fish is always exposed to the external water and, hence, the Na⁺ concentration at the epithelium will change from 0.5 to 500 mmol l⁻¹ when the fish migrate from the river to the sea. In general, olfactory receptors respond directly to salts. For example, application of 500 mmol l⁻¹ NaCl to the olfactory receptors of a fish usually elicits a large response in the olfactory nerve, which does not then adapt to a spontaneous level. Hence, exposure of the olfactory epithelium to 500 mmol l⁻¹ NaCl solution for an extended period will fatigue the olfactory neurones because of continuous firing. How are the olfactory receptors adapted to

seawater salt concentrations when the fish migrate from the river to the sea?

In the present study, we used rainbow trout, which is a freshwater steelhead trout, reared in fresh water and trout reared in fresh water and acclimated to sea water, and examined the adaptation mechanism of the olfactory response. Application of sea water to the olfactory epithelium elicited only a very small response in both groups in spite of the presence of the high concentration of NaCl. We found that 10 mmol l⁻¹ Ca²⁺ in sea water caused the lack of response to this high concentration of NaCl.

Recent studies have suggested that second messengers such as cyclic AMP (Pace *et al.* 1985) and inositol trisphosphate (InsP₃) (Boekhoff *et al.* 1990; Reed, 1992) are involved in the olfactory transduction mechanism and that ionic permeabilities across channels gated by cyclic AMP (Pace *et al.* 1985; Nakamura and Gold, 1987; Goulding *et al.* 1992) and InsP₃ (Restrepo *et al.* 1990; Miyamoto *et al.* 1992; Suzuki, 1994) and Ca²⁺-activated Cl⁻ channels (Kleene, 1993; Kurahashi and

Yau, 1993) contribute to the transduction. In fish, the existence of cyclic-AMP-gated channels in the olfactory cells has been demonstrated by biochemical (Bruch and Teeter, 1989), electrophysiological (Bruch and Teeter, 1989, 1990; Goulding *et al.* 1992; Kolesnikov and Kosolapov, 1993) and histological (Goulding *et al.* 1992; Ngai *et al.* 1993) methods. InsP_3 (Bruch and Teeter, 1990; Huque and Bruch, 1986; Miyamoto *et al.* 1992; Restrepo *et al.* 1990, 1993) was also suggested to be involved in catfish olfactory transduction. Ivanova and Caprio (1993) suggested that catfish olfactory neurones possess a variety of odour receptors coupled to different types of transduction channels. These ion channels are generally considered to be located at the olfactory cilia and, hence, changes in salt concentrations in the medium in contact with the olfactory epithelium will directly affect the olfactory responses.

In order to examine the effects of the ionic environment on olfactory responses, it is desirable that the salt concentration in the medium in contact with the olfactory epithelium is changed over a wide range. In a land animal, salt concentrations in the medium in contact with the epithelium can be changed only over a limited range because the olfactory epithelium has no tolerance for high concentrations of salts. In freshwater and seawater fish, salt concentrations in the medium in contact with the olfactory epithelium cannot always be changed over a wide range; e.g. application of sea water to carp or distilled water to jacobopever, *Sebastes schlegeli* (Hilgendorf), caused irreversible damage to the olfactory receptors (K. Fujita, T. Shoji and K. Kurihara, unpublished data). However, the rainbow trout is useful for this purpose since the olfactory epithelium is tolerant to a large range of salt concentrations. We found that the magnitudes of the olfactory nerve responses to amino acids dissolved in artificial sea water (ASW) were similar to those to amino acids dissolved in artificial pond water (APW), indicating that the olfactory nerve responses to amino acids are independent of the salt concentration of the external medium.

Materials and methods

Rainbow trout *Oncorhynchus mykiss* (Walbaum), approximate mass 200–300 g, were obtained from a local fishery and maintained in running aerated fresh water. A second group of fish was maintained in sea water. Adaptation of these fish to sea water was carried out gradually; the fish were held in the diluted sea water (sea water: fresh water 1:2) for 1 week and then in sea water for at least 2 weeks.

The olfactory responses were recorded from the olfactory nerve as described by Sveinsson and Hara (1990). In brief, fish were immobilized using galamine triethiodide (3 mg kg⁻¹ body mass) and locally anaesthetized with lidocaine at the wound and head fixation points. The gills were perfused through the mouth with aerated solutions (well water or sea water) containing MS 222 (70 mg l⁻¹). Olfactory nerve responses were recorded from twin tungsten electrodes inserted into the olfactory nerve and integrated by a custom-built electric integrator (time constant 0.3 s).

Artificial pond water (APW) and artificial sea water (ASW) were used for perfusing the olfactory epithelium. The composition of APW was 0.5 mmol l⁻¹ NaCl, 0.05 mmol l⁻¹ KCl, 0.4 mmol l⁻¹ CaCl₂, 0.2 mmol l⁻¹ NaHCO₃, pH 6.9 at 18 °C. That of ASW was 493 mmol l⁻¹ NaCl, 11.0 mmol l⁻¹ KCl, 10.7 mmol l⁻¹ CaCl₂, 28.0 mmol l⁻¹ MgCl₂, 31.0 mmol l⁻¹ MgSO₄, pH 6.3 at 18 °C. The amino acids used (L-serine, L-alanine, L-methionine, L-glutamine, monosodium L-glutamate, L-arginine hydrochloride) were purchased from Wako Pure Chemical Industries, Tokyo. Amino acids were dissolved in APW or ASW. Before stimulation, the olfactory epithelium was perfused with APW or ASW for 10 min. Perfusion for 30 min with APW or ASW gave results similar to those obtained after a 10 min perfusion. Stimulating solutions were then applied to the olfactory epithelium at a flow rate of 30 ml min⁻¹. APW, ASW or distilled water was perfused for 5 min between each stimulus application. The stimulating solutions were 500 mmol l⁻¹ NaCl, 10 mmol l⁻¹ CaCl₂, 50 mmol l⁻¹ MgCl₂, sea water, ASW and amino acid solutions. Sea water used for stimulation was the same water that had been used for acclimating the fish. All the experiments were carried out at 18±2 °C.

Results

Fig. 1 shows the summed responses of the olfactory nerve to 500 mmol l⁻¹ NaCl, 10 mmol l⁻¹ CaCl₂ and 50 mmol l⁻¹ MgCl₂ in fish maintained in fresh water. The responses are composed of a phasic and a tonic component. In the present study, the initial height of the phasic response is taken as the magnitude of the response. Fig. 2 shows the relative magnitudes of the olfactory responses to NaCl, CaCl₂ and MgCl₂ of varying concentrations in fish maintained in fresh water; the magnitude of the response to 500 mmol l⁻¹ NaCl is taken as 1.0. The minimum concentrations required to induce a response (this concentration is referred to as threshold) were approximately 10⁻³ mol l⁻¹ for NaCl, 10⁻⁷ mol l⁻¹ for CaCl₂ and 3×10⁻³ mol l⁻¹ for MgCl₂. The maximum response to NaCl application is much larger than the responses to CaCl₂ and MgCl₂. The concentration–response relationships for the salts used in the fish acclimated to sea water were essentially similar to those shown in Fig. 2 (data not shown).

The tonic component of the response to 500 mmol l⁻¹ NaCl did not decrease to a spontaneous level even after long stimulation periods (Fig. 1D,H). It is likely that the olfactory neurones will become fatigued when the olfactory epithelium is exposed to 500 mmol l⁻¹ NaCl for long periods because firing of the neurones does not stop during exposure to the solution. Application of sea water or ASW induced only a very small response despite the fact that both contained high concentrations of NaCl (Fig. 1E,F). This suggests that other constituents in sea water and ASW suppress the expected response to the high concentration of NaCl. In the experiment shown in Fig. 1G, 10 mmol l⁻¹ CaCl₂ was applied to the epithelium and after the response to CaCl₂ had decreased to a spontaneous level, a mixture of 10 mmol l⁻¹ CaCl₂ and

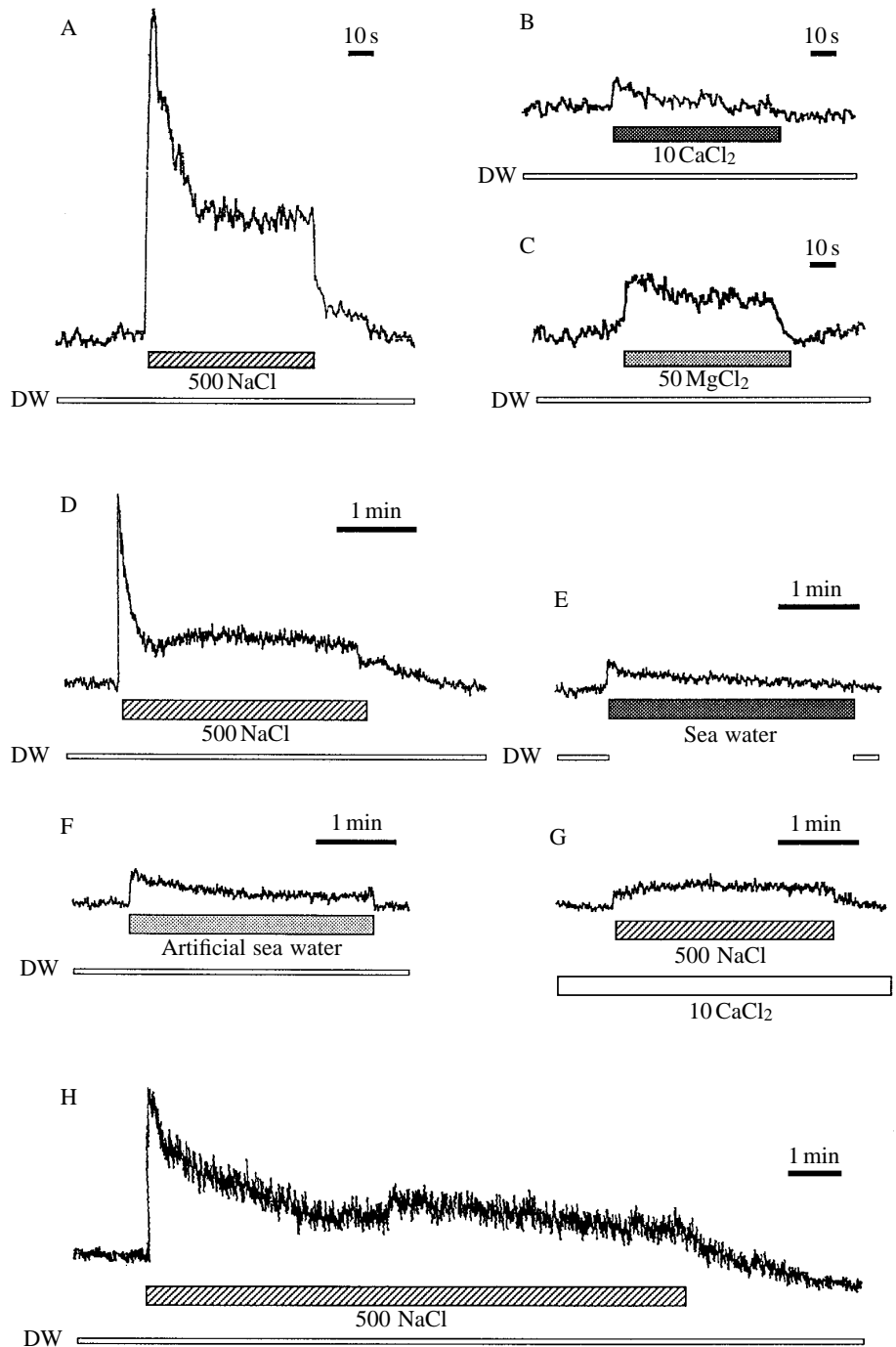


Fig. 1. Typical summed olfactory nerve responses of rainbow trout acclimated to fresh water and exposed to 500 mmol l^{-1} NaCl (A), 10 mmol l^{-1} CaCl_2 (B), 50 mmol l^{-1} MgCl_2 (C), 500 mmol l^{-1} NaCl (D), sea water (E), artificial sea water (F), a mixture of 500 mmol l^{-1} NaCl and 10 mmol l^{-1} CaCl_2 (G) and 500 mmol l^{-1} NaCl (H). The records A–C, D–G and H were measured from three different fish. The bar at the bottom of each record represents the duration of stimulation with the solution indicated. In G, 10 mmol l^{-1} CaCl_2 was first applied to the epithelium and, after the response had adapted to a spontaneous level, a mixture of 10 mmol l^{-1} CaCl_2 and 500 mmol l^{-1} NaCl was then applied. In H, the epithelium was stimulated for a long time (10 min). The epithelia were perfused with distilled water (DW) for 5 min between successive stimulations.

500 mmol l^{-1} NaCl was applied. This mixture induced only a very small response, suggesting that the presence of 10 mmol l^{-1} Ca^{2+} suppressed the expected response to 500 mmol l^{-1} NaCl.

Fig. 3 shows concentration–response curves for NaCl in the absence and presence of 0.1, 1 and 10 mmol l^{-1} CaCl_2 in fish acclimated to either fresh or sea water. The presence of CaCl_2 shifted the threshold for NaCl to a higher concentration. The curves are shifted increasingly to the right with an increase in CaCl_2 concentration and in proportion to it, suggesting that inhibition of the response to NaCl by Ca^{2+} is essentially of a

competitive type. However, the curves are distorted from a single isotherm, which suggests that a number of different factors may be involved. There was no essential difference between results for fish acclimated to fresh water or sea water.

Fig. 4 shows the effects of CaCl_2 and MgCl_2 of varying concentrations on the response to 500 mmol l^{-1} NaCl in fish acclimated to fresh or sea water. In both groups of fish, the response to 500 mmol l^{-1} NaCl was not significantly affected by MgCl_2 . The responses decreased with an increase in CaCl_2 concentration and reached a spontaneous level at about 10 mmol l^{-1} CaCl_2 . Sea water contains 10.7 mmol l^{-1} CaCl_2 ,

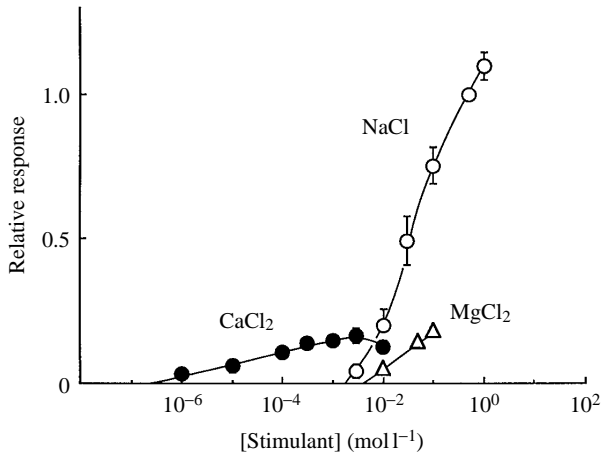


Fig. 2. Relative magnitudes of the olfactory nerve responses to NaCl, CaCl₂ and MgCl₂ of varying concentrations in rainbow trout maintained in fresh water. The magnitude of the response to 500 mmol l⁻¹ NaCl is taken as 1.0. Each point is the mean ± S.E.M. for data obtained from at least four preparations. Error bars for some points are within the symbol.

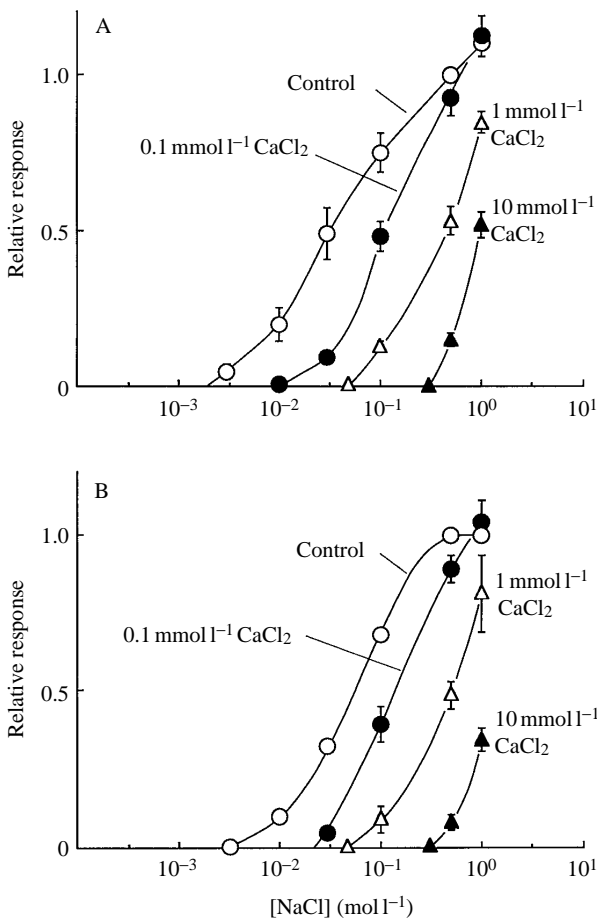


Fig. 3. Relative magnitudes of the olfactory nerve responses to NaCl of varying concentrations in the absence (control) and presence of 0.1, 1 and 10 mmol l⁻¹ CaCl₂. (A) Fish maintained in fresh water; (B) fish maintained in sea water. The magnitude of the response to 500 mmol l⁻¹ NaCl in the absence of CaCl₂ is taken as 1.0. Each point is the mean ± S.E.M. for data obtained from at least seven fish maintained in fresh water or three fish maintained in sea water.

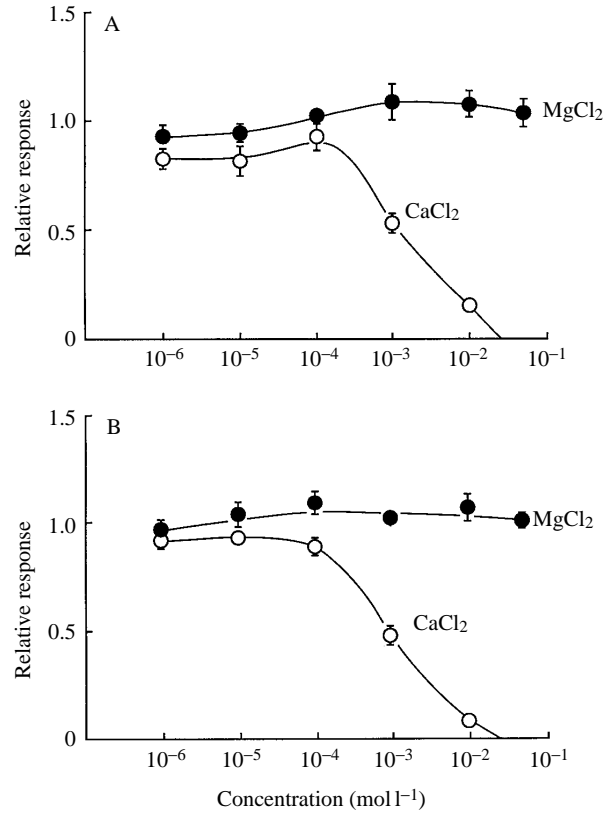


Fig. 4. Effects of CaCl₂ and MgCl₂ of varying concentrations on the relative olfactory nerve response to 500 mmol l⁻¹ NaCl in fish maintained in fresh water (A) and fish maintained in sea water (B). The magnitude of the response to 500 mmol l⁻¹ NaCl alone is taken as 1.0. Each point is the mean ± S.E.M. for data obtained from at least five preparations.

which implies that it contains the minimum concentration of Ca²⁺ required to suppress the expected response to the 493 mmol l⁻¹ NaCl present in sea water.

Fig. 5 shows the effects of different 1 mmol l⁻¹ salts containing various divalent cations on the response to 100 mmol l⁻¹ NaCl in fish reared in fresh water. To prevent any irreversible effect from the use of divalent cations, 100 mmol l⁻¹ NaCl instead of 500 mmol l⁻¹ NaCl was used, because the concentrations of divalent cations required to suppress the response to 100 mmol l⁻¹ NaCl will be much lower than those required to suppress the response to 500 mmol l⁻¹ NaCl. Like CaCl₂, SrCl₂ and BaCl₂ also have suppressing effects on the response to 100 mmol l⁻¹ NaCl, although their effects are smaller than that of CaCl₂. MgCl₂, CdCl₂ and CoCl₂ have no suppressive effect on the response.

Fig. 6 shows the summed olfactory nerve responses to 0.1 mmol l⁻¹ L-alanine and 0.1 mmol l⁻¹ L-methionine dissolved in APW or ASW. Fig. 6A represents the responses of the fish acclimated to fresh water to the amino acids dissolved in APW or ASW after the olfactory epithelium had been perfused with APW or ASW for 10 min. Fig. 6B

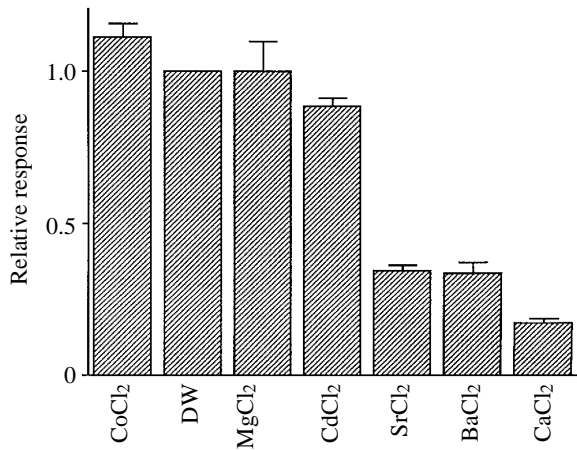


Fig. 5. Effects of 1 mmol l^{-1} salt solutions carrying various divalent cations on the relative olfactory nerve response to 100 mmol l^{-1} NaCl in rainbow trout maintained in fresh water. The ordinate represents the ratio of the magnitude of the response to 1 mmol l^{-1} salt solutions in the presence of 100 mmol l^{-1} NaCl to that in the presence of NaCl alone. Each value represents the mean ratio + S.E.M. obtained from four preparations. DW, distilled water.

represents the responses of the fish acclimated to sea water to the amino acids dissolved in ASW under the same conditions. The magnitude of the response to each amino acid dissolved in ASW is similar to that when the amino acid is dissolved in APW with both fish groups.

Fig. 7 shows the concentration–response curves for various amino acids in fish acclimated to sea water where the response to 0.1 mmol l^{-1} serine is taken as 1.0. The minimum concentrations of the amino acids required to induce a response are near $10^{-8} \text{ mol l}^{-1}$ and the responses do not saturate, even at 1 mmol l^{-1} . These concentration–response curves are similar to those reported by Hara (1982) for fish acclimated to fresh water.

Fig. 8 shows the average ratio of the magnitude of the response to an amino acid in ASW to that in APW. In both groups of fish, the ratios for L-alanine, L-methionine, L-glutamine, L-glutamic acid and L-arginine are close to 1.0, although the ratio for L-serine in the fish acclimated to sea water is a little larger than 1.0. Thus, the magnitudes of the responses to the amino acids are mostly equal between APW and ASW.

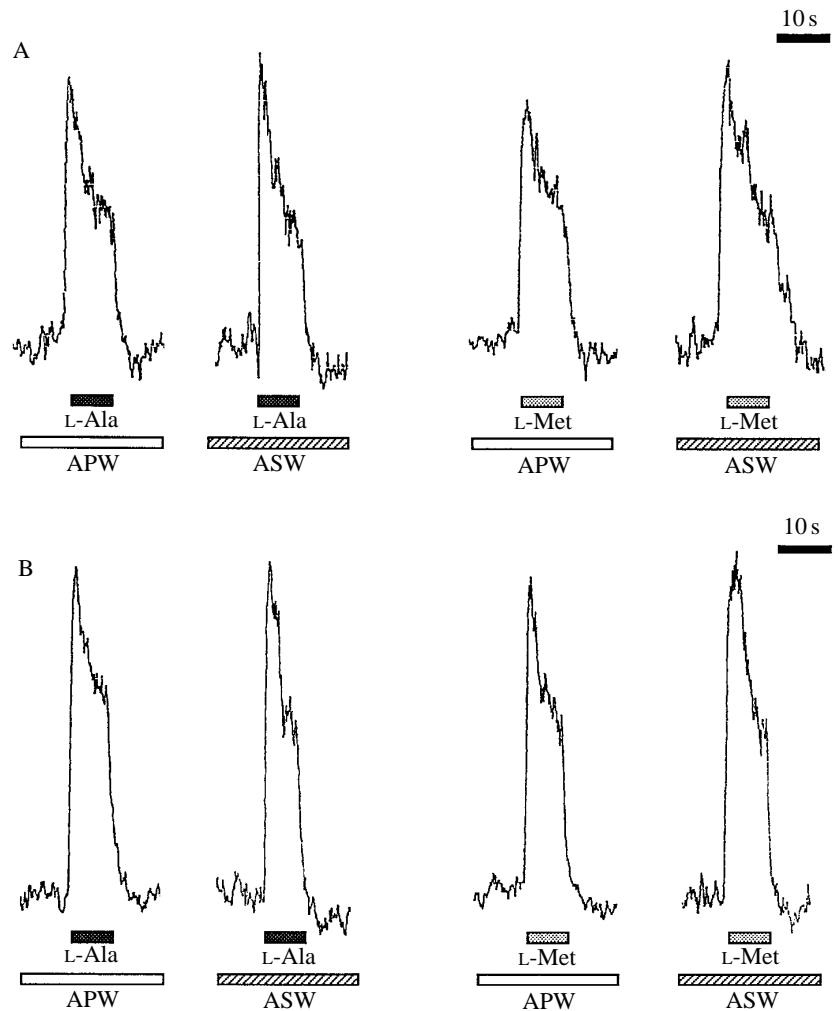


Fig. 6. Typical summed olfactory nerve responses to 0.1 mmol l^{-1} L-alanine (L-Ala) and 0.1 mmol l^{-1} L-methionine (L-Met). (A) Fish maintained in fresh water; (B) fish maintained in sea water. The bar at the bottom of each record represents the duration of application of the solutions indicated. Before stimulation with the amino acids, the olfactory epithelium was perfused with artificial pond water (APW) or artificial sea water (ASW) for 10 min; the amino acids dissolved in APW or ASW were then applied. The olfactory epithelium was perfused with APW or ASW between each stimulation.

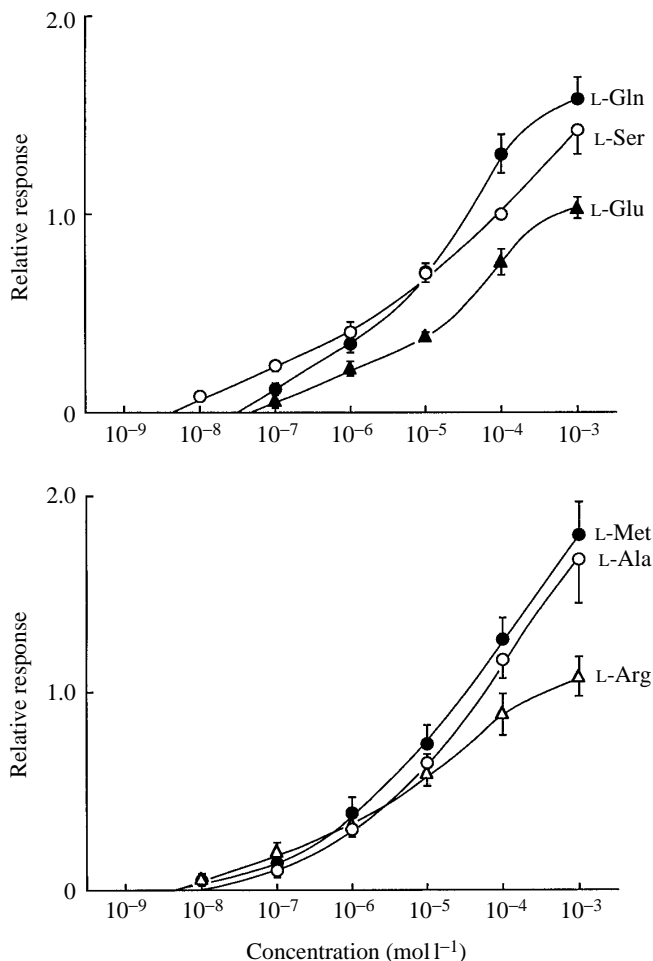


Fig. 7. Relative magnitudes of the olfactory nerve responses to amino acids of varying concentration in fish maintained in sea water. Amino acids were dissolved in ASW. The magnitude of the response to 0.1 mmol l⁻¹ serine is taken as 1.0. Each point is the mean \pm S.E.M. of data obtained from at least five preparations.

Discussion

Adaptation mechanism of olfactory receptors to sea water

Application of sea water to the olfactory epithelium of freshwater fish or application of fresh water to that of seawater fish usually brings about irreversible damage to the olfactory system. Hence, it is impossible to change the ionic environments of the olfactory epithelium of these fish over a wide concentration range. However, application of sea water to the olfactory epithelium of freshwater rainbow trout or of fresh water to rainbow trout acclimated to sea water does not cause irreversible effects on olfactory activity. This implies that the tolerance of the olfactory receptors for large changes in osmotic gradient is naturally present. Such tolerance of the olfactory receptors for large osmotic gradient changes has also been observed for chum salmon (Shoji *et al.* 1994).

The large response of the rainbow trout olfactory receptors to 500 mmol l⁻¹ NaCl was not reduced to spontaneous levels even after long stimulation periods. Hence, olfactory neurones are likely to become fatigued when the olfactory epithelium is

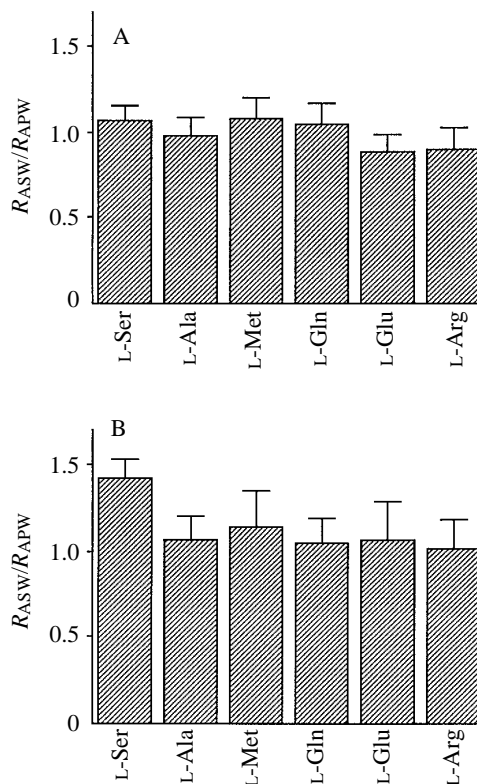


Fig. 8. Ratios of the magnitude of the olfactory nerve response to each amino acid in ASW (R_{ASW}) to that in APW (R_{APW}) in rainbow trout maintained in fresh water (A) or sea water (B). Amino acids (0.1 mmol l⁻¹) were dissolved in APW or ASW as appropriate. Each value represents the mean ratio \pm S.E.M. obtained from at least five preparations.

exposed to 500 mmol l⁻¹ NaCl for long periods. However, application of sea water elicited only a small olfactory response, which quickly adapted to a spontaneous level. Our experiments showed that the presence of 10.7 mmol l⁻¹ Ca²⁺ in sea water inhibits the olfactory response to the 493 mmol l⁻¹ NaCl present in sea water. An increase in Ca²⁺ concentration shifted the concentration-response curves for NaCl to the right, i.e. to a higher concentration, suggesting that the inhibition is essentially of a competitive type. It seems that Ca²⁺ binds to the receptor sites for Na⁺ or to Na⁺ channels on the receptor membranes and inhibits the response to NaCl. Sr²⁺ and Ba²⁺ also have suppressive effects, while Mg²⁺, Cd²⁺ and Co²⁺ have no such suppressive effect. This implies that the Ca²⁺ binding site accepts Sr²⁺ and Ba²⁺, but does not accept the other divalent cations. Our data for the inhibition of the response to NaCl by Ca²⁺ do not exactly match the theoretical curves for competitive inhibition; this implies that mechanisms other than competitive inhibition may also be involved in the inhibition observed.

Comparison of responses to amino acids in fresh and sea water

The present results show that the magnitudes of the olfactory

nerve responses to amino acids dissolved in ASW or APW are similar. This is important for these euryhaline fish, because amino acids are their most important chemical stimuli both in fresh water and in sea water. These results are not fully consistent with the olfactory transduction mechanism proposed recently. Recording of olfactory nerve activities is a more indirect method for elucidating the transduction mechanism than the use of intracellular recordings from isolated olfactory cells. It is, however, more difficult to change the ionic environments around isolated cells and, hence, isolated cells are not always suitable for determining whether ionic permeability changes occur at the apical membranes. In addition, even if the existence of ion channels at the apical membranes is proved using isolated cells, it is still unknown whether such channels work in olfactory reception *in vivo*. The present method gives information on ionic permeabilities at apical membranes during olfactory reception *in vivo*. The results are consistent with those obtained using lamprey *Lampetra japonica* (Suzuki, 1978), carp *Cyprinus carpio* (Yoshii and Kurihara, 1983), bullfrog *Rana catesbeiana* (Kashiwayanagi *et al.* 1991) and turtle *Geoclemys reevesii* (Shoji *et al.* 1991) olfactory systems, although the NaCl concentration in contact with the olfactory epithelium of these animals was changed only from 0 to 100 mmol l⁻¹.

It has been noted that cyclic-AMP-gated channels are blocked by the presence of divalent cations such as Ca²⁺ and Mg²⁺ in the external medium (Kolesnikov and Kosolapov, 1993; Kurahashi and Yau, 1993). When 3 mmol l⁻¹ Ca²⁺ was present in the external medium of olfactory receptors of the newt *Cynops pyrrhogaster*, the olfactory responses were almost independent of external Na⁺ concentration, and a Ca²⁺-activated Cl⁻ conductance contributes to the olfactory response in these conditions (Kurahashi and Yau, 1993). In our experiments, the ASW contained 10.7 mmol l⁻¹ Ca²⁺ and 59 mmol l⁻¹ Mg²⁺ and, hence, in the olfactory cells of the rainbow trout, cyclic-AMP-gated channels at the apical membranes, including ciliary membranes, may have been blocked by these divalent cations. The concentration of Cl⁻ in the ASW was 581.4 mmol l⁻¹. The intracellular Cl⁻ concentration of mammalian cells is 5–15 mmol l⁻¹ (Alberts *et al.* 1994), although that of fish olfactory cells is unknown. Even if the intracellular Cl⁻ concentration in fish olfactory cells is assumed to be 50 mmol l⁻¹, the equilibrium potential for Cl⁻ is -61.8 mV, which is close to the resting potential of olfactory cells. Hence, in the rainbow trout, a Ca²⁺-activated Cl⁻ conductance would not contribute to the olfactory response in ASW. The Ca²⁺ concentrations in APW and ASW were 0.4 and 10.7 mmol l⁻¹, respectively, but the magnitudes of the olfactory nerve responses to amino acids in APW were similar to those in ASW. This is not fully consistent with the hypothesis that an influx of Ca²⁺ through InsP₃-gated channels is involved in olfactory transduction (Miyamoto *et al.* 1992; Restrepo *et al.* 1990), but does not exclude the idea since the difference in Ca²⁺ concentration between APW and ASW is not large.

The present results are consistent with the proposal that

activation of ion channels in response to amino acids occurs at the cell membranes below tight junctions, although the density of the channels gated by cyclic AMP or InsP₃ is not high (Suzuki, 1994). Recently, it was pointed out that there may be new pathways in olfactory transduction other than the known pathways (Ivanova and Caprio, 1993; Kashiwayanagi *et al.* 1994). There is a possibility that some of these pathways are involved in the olfactory transduction of rainbow trout. Another possibility is that changes in the phase boundary potential at the apical membranes contribute to the generation of the receptor potential (Kurihara *et al.* 1986). Further study will be needed to clarify the transduction mechanism in the fish olfactory system.

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