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OLFACTORY SENSITIVITY TO CHANGES IN ENVIRONMENTAL [Ca^{2+}] IN THE MARINE TELEOST *SPARUS AURATA*

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Summary

Estuarine and/or migratory teleosts may experience large and rapid changes in external [Ca^{2+}]. Previous studies have largely centred on the physiological mechanisms that maintain a constant plasma [Ca^{2+}] in the face of such external fluctuations, but little work has been directed to examining how these changes may originally be detected. We present evidence that the olfactory system of the gilthead seabream (*Sparus aurata*) is highly sensitive to reductions in environmental [Ca^{2+}] and suggest a possible mechanism by which this may be mediated. Multi-unit extracellular recordings were made from the olfactory nerve of *Sparus aurata* while the [Ca^{2+}] of artificial sea water flowing over the olfactory epithelium was varied from 10 to 0 mmol l^{-1}. Reductions in [Ca^{2+}] caused a large, non-accommodating increase in the firing rate of the olfactory nerve (apparent IC_{50}=1.67±0.26 mmol l^{-1}, apparent Hill coefficient=–1.22±0.14; means ± S.E.M., *N*=6). This response was not due to the concomitant reduction in osmolality and was specific for Ca^{2+}. During continuous exposure of the olfactory epithelium to Ca^{2+}-free sea water, the apparent IC_{50} and Hill coefficient in response to increases in [Ca^{2+}] were 0.48±0.14 mmol l^{-1} and –0.76±0.16 (means ± S.E.M., *N*=6), respectively, suggesting an adaptation of the Ca^{2+}-sensing system to low-[Ca^{2+}] environments. Ca^{2+} is intimately involved in signal transduction in the olfactory receptor neurones, but our data support a true olfactory response, rather than a non-specific effect to lowering of external [Ca^{2+}]. The absence of Ca^{2+} from sea water only partially and temporarily blunted the olfactory response to the odorant L-serine; the response amplitude recovered to control levels within 20 min. This suggests that the olfactory system in general is able to adapt to low-[Ca^{2+}] environments. We suggest that the Ca^{2+} sensitivity is mediated by an extracellular Ca^{2+}-sensing receptor similar to the recently characterized mammalian Ca^{2+}-sensing receptor.

Key words: Ca^{2+}, olfaction, Ca^{2+}-sensing receptor, marine, teleost, gilthead seabream, *Sparus aurata*.

Introduction

Marine animals live in an environment rich in many inorganic ions including the divalent cation Ca^{2+} which, in sea water, is present at approximately 10 mmol l^{-1} (Bentley, 1998; Withers, 1996). Ca^{2+} is an extremely important ion, and both intracellular and extracellular Ca^{2+} are involved in a multitude of physiological processes, including muscular contraction, neurotransmitter release and cellular signalling. Marine teleosts have evolved a plethora of mechanisms by which extracellular [Ca^{2+}] is held constant and is maintained at a level substantially lower than that of the surrounding sea water (total plasma [Ca^{2+}] of fish is 2.0–3.0 mmol l^{-1}; Bentley, 1998; Withers, 1996). Much work has been directed to determining how these systems maintain this differential and what the endocrine factors responsible for the regulation of these mechanisms may be (for reviews, see Flik and Verbost, 1993, 1996; Bentley, 1998).

Surprisingly, much less work has focused on how teleosts may detect changes in environmental [Ca^{2+}] directly (Bodznick, 1978) or changes in the circulating plasma [Ca^{2+}] that may occur as a result. In general, the [Ca^{2+}] of sea water remains fairly constant, both over time and from sea to sea. However, coastal and estuarine or migratory species may experience large and rapid fluctuations in environmental [Ca^{2+}] from nearly zero (fresh water) to 10 mmol l^{-1} (full sea water). Therefore, it would be highly advantageous for these species to detect changes in environmental [Ca^{2+}] and to activate the appropriate physiological and/or behavioural responses before large and possibly lethal changes in circulating [Ca^{2+}] occur. The most obvious site for such a potential [Ca^{2+}]-sensing mechanism would be the olfactory system.

The gilthead seabream (*Sparus aurata*, Sparidae) is a coastal species of European, Mediterranean and North African seas, often thriving in estuarine or brackish waters of substantially reduced salinity (Wheeler, 1978). However, the sea bream is

unable to withstand full fresh water (below a salinity of less than 5‰), where death occurs after a matter of hours. Therefore, such a fish would be a prime candidate to possess a system that detects fluctuations in environmental [Ca^{2+}] so that appropriate physiological and/or behavioural responses can be activated. The present study was undertaken to examine whether the olfactory system of *Sparus aurata* is sensitive to changes in environmental [Ca^{2+}] and to explore the mechanism(s) by which this sensitivity, if present, is mediated.

Materials and methods

Experimental animals

Adult (300–450 g, length 25–28 cm) gilthead seabream (*Sparus aurata* L.) were obtained from a local fish farm (TiMar-Culturas em Água-Lda, Fuzeta, Portugal) and maintained in 400 l tanks with recirculating filtered sea water under natural day-length and temperature (18–20 °C) conditions at the Universidade do Algarve and fed once a day with commercial fish food.

Electrophysiological recording of olfactory responses

Fish were anaesthetized by immersion in sea water containing 50 mg l^{-1} MS222 (3-aminobenzoic acid ethyl ester; SigmaAldrich Co., Madrid, Spain) and immobilized by intramuscular injection of 3 mg kg^{-1} of the neuromuscular blocker gallamine triethiodide (SigmaAldrich) in 0.9 % saline. The fish were then maintained with recirculating aerated sea water containing 50 mg l^{-1} MS222 flowing over the gills at a rate of approximately 100 ml min^{-1} 100 g^{-1} whilst surgery to expose the olfactory nerves was performed. The olfactory nerves were exposed by removal of the bone of the skull between the snout (from immediately dorsal and posterior to the nares to the posterior edge of the orbits) and clearing the connective tissue and fat between the eyes. The fish was then placed in a Perspex V-clamp, and the gills were irrigated with aerated sea water at a flow rate of approximately 80 ml min^{-1} 100 g^{-1} body mass. During the experiment, the wound was anaesthetized with the local anaesthetic tetracaine [4-(butylamino)benzoic acid 2-(dimethylamino)ethyl ester; 3 % in 0.9 % saline, SigmaAldrich] applied with a small paint brush. The condition of the fish was monitored by observing the opercular rhythm (40–50 cycles min^{-1}).

Extracellular recording was carried out using two parylene-coated tungsten electrodes (resistance 0.8–1.2 MΩ, World Precision Instruments, Stevenage, UK) inserted into one of the olfactory nerves and connected to an a.c. preamplifier (Neurolog NL104, Digitimer Ltd, Welwyn Garden City, UK). The signal was then filtered (low-pass 3000 Hz, high pass 200 Hz; Neurolog NL125, Digitimer Ltd) and integrated (time constant 1 s, Neurolog NL703, Digitimer Ltd). Both the direct and integrated signals were digitized (Digidata 1200, Axon Instruments, Inc., Foster City, California, USA) and displayed on a computer running Axoscope 1.1 software (Axon Instruments, Inc.).

Stimuli were delivered to the olfactory epithelium *via* a glass tube held in position in the inhalant olfactory opening; this species has clearly defined inhalant (small, circular, anterior) and exhalant (large, slit-like, posterior) nares. The glass tube was connected to a gravity-fed tubing system, and stimulus solutions were fed into the tubing *via* a computer-operated three-way valve. The flow rate of the stimulus solutions was approximately 6 ml min^{-1}. The optimal positions for both stimulus delivery tube and electrodes were ascertained using 10^{-3} mol l^{-1} L-serine in sea water as a stimulus. Using this experimental approach, stable and reproducible recording could be obtained up to 8 h after the initial surgery on the fish.

Stimulus solutions

Artificial sea water (ASW) was made up using deionised water with the following composition: 460 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 25 mmol l^{-1} $MgSO_4$ and 25 mmol l^{-1} $MgCl_2$, pH adjusted to 8.0–8.2 using 0.1 mol l^{-1} NaOH (osmolality 991–1004 mosmol kg^{-1} H_2O). Ca^{2+}-free artificial sea water (Ca^{2+}-free ASW) was made up using deionised water with the following composition: 460 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 25 mmol l^{-1} $MgSO_4$, 25 mmol l^{-1} $MgCl_2$ and 20 mmol l^{-1} choline chloride, pH adjusted to 8.0–8.2 with 0.1 mol l^{-1} NaOH (final osmolality 1000–1013 mosmol kg^{-1} H_2O). Thus, the only difference in ionic composition between ASW and Ca^{2+}-free ASW is that Ca^{2+} was replaced by choline in Ca^{2+}-free ASW. Control experiments using ASW plus 20 mmol l^{-1} choline chloride as a stimulus were also carried out. The range of Ca^{2+} concentrations was made up by appropriate dilution of ASW with Ca^{2+}-free ASW. Mg^{2+}-free ASW and SO_4^{2-}-free ASW were made up as follows: 410 mmol l^{-1} NaCl, 25 mmol l^{-1} Na_2SO_4, 10 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 50 mmol l^{-1} choline chloride (pH adjusted to 8.0–8.2 with 0.1 mol l^{-1} NaOH; osmolality 950 mosmol kg^{-1} H_2O) and 460 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 10 mmol l^{-1} $CaCl_2$, 50 mmol l^{-1} $MgCl_2$ (pH adjusted to 8.0–8.2 with 0.1 mol l^{-1} NaOH; osmolality 1024 mosmol kg^{-1} H_2O) respectively. Serine solutions (10^{-3} mol l^{-1}) were made up in normal, charcoal-filtered sea water (the same water perfusing the olfactory epithelium between experiments) in ASW or in Ca^{2+}-free ASW as appropriate. A period of at least 1 min was allowed between successive stimuli to allow for washout from the olfactory epithelium.

Treatment of data

The olfactory epithelium was continuously superfused with ASW and stimulated with Ca^{2+}-free ASW. The amplitude of the integrated response (in arbitrary units) was taken to represent the maximal response of the Ca^{2+}-sensing system (i.e. equal to 1). All subsequent responses to intermediate Ca^{2+} concentrations were normalized to this response. The inverse experiment was then performed; the olfactory epithelium was continuously superfused with Ca^{2+}-free ASW and stimulated with ASW, and the decrease in integrated response was taken

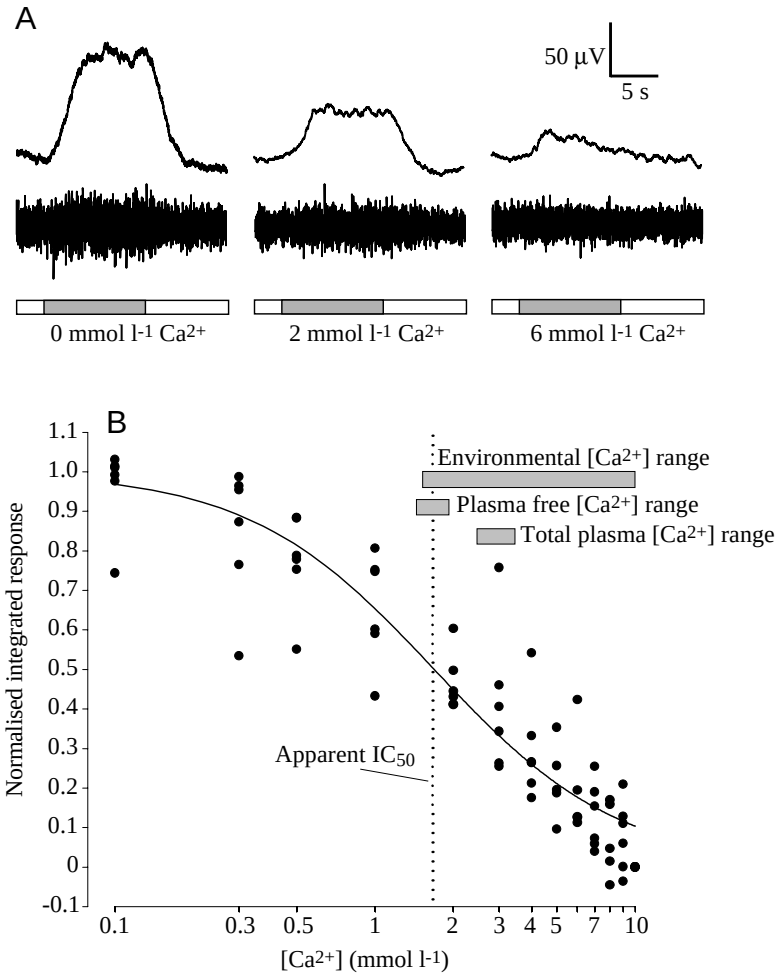


Fig. 1. Olfactory responses of *Sparus aurata* to changes in environmental [Ca^{2+}]. (A) Typical extracellular recordings from the olfactory nerve (lower traces) and their integrated activity (upper traces) in response to changes in Ca^{2+} concentration from 10 mmol l^{-1} (open horizontal bars) to 0, 2 and 6 mmol l^{-1} (shaded horizontal bars) flowing over the olfactory epithelium. All three traces were recorded from the same fish. The vertical scale bar refers to the nerve traces only, but the horizontal scale bar refers to both nerve and integrated traces; all integrated traces are shown at the same gain. Note that the amplitude of the integrated response is large and shows no accommodation within the time scale of the stimulus (10 s). (B) Semi-logarithmic plot of pooled data (N=6) showing the concentration dependency of the olfactory response to changes in [Ca^{2+}] with an apparent IC_{50} value of 1.67±0.26 mmol l^{-1} Ca^{2+} (mean ± S.E.M.). The range of total plasma [Ca^{2+}], free plasma [Ca^{2+}] and environmental [Ca^{2+}] that *Sparus aurata* is likely to encounter are also marked. The data have been normalized to the response to a change in [Ca^{2+}] from 10 to 0 mmol l^{-1}. Note the coincidence of the plasma free [Ca^{2+}] with the IC_{50} value and also the lower end of the range of environmental [Ca^{2+}].

to be maximal (i.e. equal to −1). Subsequent responses to increasing concentrations of Ca^{2+} were normalized to this response in a similar manner. No further response was seen to increases in Ca^{2+} concentration above 10 mmol l^{-1}. The data were then fitted to a conventional three-parameter Hill equation ($A=A_{max}[Ca^{2+}]^n/(IC_{50}^n+[Ca^{2+}]^n)$, where A is the normalized amplitude of the integrated response, A_{max} is the maximum value of the integrated response (i.e. 1 or −1), n is the Hill coefficient and [Ca^{2+}] and IC_{50} have their usual meanings, using the least-squares method of SigmaPlot 2000 (SPSS Science).

Statistical analyses

The data from each individual fish fitted the logistic equation given above acceptably (r^2=0.89–0.98 in ASW; r^2=0.77–0.94 in Ca^{2+}-free ASW). Thus, IC_{50} and Hill coefficients were calculated for each fish. These data were then compared using Student's t-test for paired data. For the adaptation experiment, the data were analysed by repeated-measures analysis of variance (ANOVA) followed by Dunnett's test for comparisons between groups and control (SigmaStat 2000, SPSS Science). P<0.05 was taken to represent statistical significance.

Results

Olfactory responses to decreases in environmental [Ca^{2+}]

The olfactory system of *Sparus aurata* responded to a reduction of [Ca^{2+}] in artificial sea water with a large increase in both firing frequency and amplitude in the olfactory nerve (Fig. 1A). This effect was concentration-dependent and allowed us to construct a concentration/response curve (Fig. 1B). The apparent IC_{50} value was 1.67±0.26 mmol l^{-1} Ca^{2+} (mean ± S.E.M., N=6), a value below the lower limit for the serum levels of [Ca^{2+}] in *Sparus aurata* (2.5–3.0 mmol l^{-1}; Mancera et al., 1994). However, if it is considered that approximately half the serum Ca^{2+} is protein-bound (chiefly to albumin, as in mammals), then this corresponds to a free [Ca^{2+}] of 1.5 mmol l^{-1}, very close to the IC_{50} of the olfactory response. The apparent Hill coefficient for this response was −1.22±0.14. Conversely, whilst the olfactory epithelium was superfused continuously with Ca^{2+}-free ASW, exposure to increasing concentrations of Ca^{2+} caused a decrease in both frequency and amplitude of activity within the nerve (Fig. 2A). The lack of any marked accommodation to the Ca^{2+}-free ASW allowed us to construct a similar concentration/response curve (Fig. 2B). The apparent IC_{50} for this response was 0.48±0.14 mmol l^{-1} Ca^{2+} and the apparent Hill coefficient was −0.76±0.16 (means

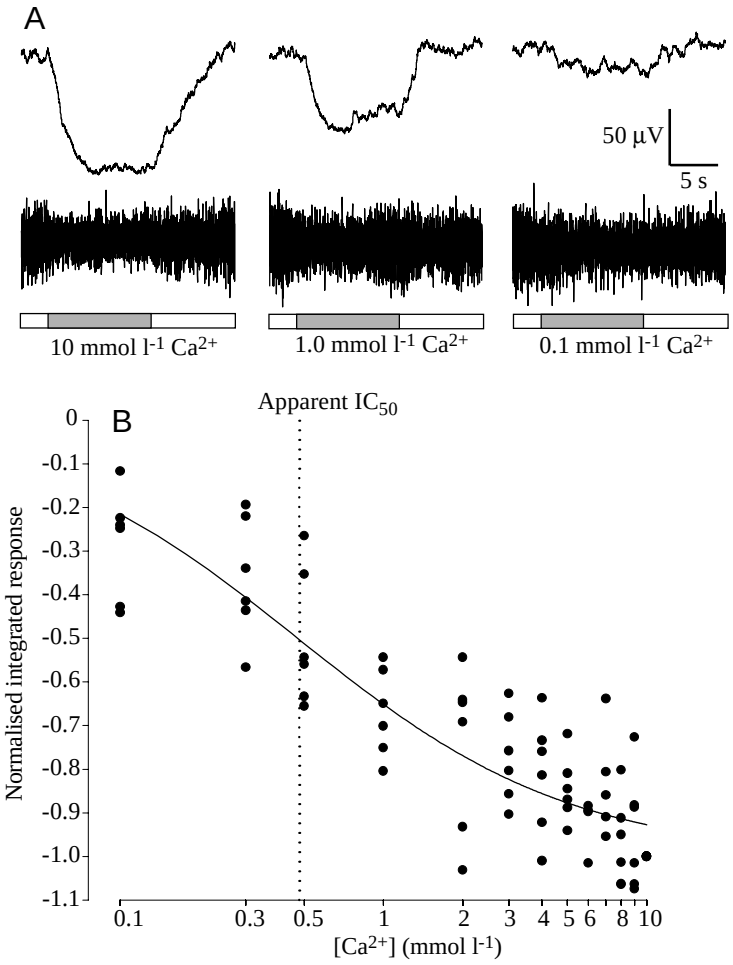


Fig. 2. Olfactory responses of *Sparus aurata* to changes in environmental [Ca^{2+}] in Ca^{2+}-free artificial sea water. (A) Typical extracellular recordings from the olfactory nerve (lower traces) and their integrated activity (upper traces) in response to changes in Ca^{2+} concentration from 0 (open horizontal bars) to 10, 1 and 0.1 mmol l^{-1} (shaded horizontal bars) in Ca^{2+}-free artificial sea water (Ca^{2+}-free ASW) flowing over the olfactory epithelium. All three traces were recorded from the same fish. The vertical scale bar refers to the nerve traces only, but the horizontal scale bar refers to both nerve and integrated traces; all integrated traces are shown at the same gain. Note how the response is large and shows little or no accommodation within the time scale of the stimulus (10 s). (B) Semi-logarithmic plot of pooled data (N=6) showing the concentration dependency of the olfactory response to changes in [Ca^{2+}] with an apparent IC_{50} of 0.48±0.14 mmol l^{-1} Ca^{2+} (mean ± S.E.M.). Data have been normalized to the response to a change in [Ca^{2+}] from 0 to 10 mmol l^{-1}. Note the shift in apparent IC_{50} compared with Fig. 1 (t=3.872, d.f.=5, P<0.05).

± S.E.M., N=6), both values significantly different from their respective values in ASW.

Specificity of the response to Ca^{2+}

The olfactory response of *Sparus aurata* to changes in [Ca^{2+}] is not merely due to a change in osmolality because the olfactory stimulus was delivered against a background osmolality of approximately 1000 mosmol kg^{-1} H_2O (ASW) and would therefore represent a very small change in the overall osmolality, and this was minimised by the replacement of Ca^{2+} by choline in Ca^{2+}-free ASW. The addition of 20 mmol l^{-1} choline chloride to ASW failed to provoke any olfactory response (Fig. 3), suggesting that not only is the olfactory epithelium insensitive to small changes in osmolality but also that it is insensitive to choline. Furthermore, neither Mg^{2+}-free ASW ([Mg^{2+}] of ASW=50 mmol l^{-1}) nor SO_4^{2-}-free ASW ([SO_4^{2-}] of ASW=25 mmol l^{-1}) had any observable effect. Thus, larger changes in concentration of other divalent ions present in sea water failed to evoke a similar response to changes in [Ca^{2+}] (Fig. 3).

Comparison of the response to Ca^{2+} with the response to a 'conventional' odorant

Comparison of the olfactory response of *Sparus aurata* to changes in environmental [Ca^{2+}] with the response to a general odorant, 10^{-3} mol l^{-1} L-serine, revealed a number of important points (Fig. 4). First, the amplitude of the integrated response to Ca^{2+}-free ASW was comparable with that to 10^{-3} mol l^{-1} L-serine, at this concentration an extremely potent olfactory stimulus (e.g. Hara, 1992). This may belie the underlying biological importance of the response to Ca^{2+}. Second, the response to Ca^{2+} was slower to reach its peak amplitude (time to peak=4.22±0.75 s, mean ± S.E.M., N=6) than the response to serine (10^{-3} mol l^{-1} serine in ASW time to peak=2.38±0.15 s, N=6, P<0.05; Student's t-test for paired data). Conversely, it was faster to return to baseline after the termination of the stimulus (time to baseline=4.43±0.42 s, mean ± S.E.M., N=6) than the response to serine (9.43±1.31 s, N=6, P<0.01; Student's t-test for paired data). Third, the response to Ca^{2+} does not show the typical biphasic time course (no sharp initial peak, followed by a rapid reduction in response amplitude). Fourth, the response to Ca^{2+} showed little accommodation with time (amplitude of integrated response after 100 s of exposure to stimulus 84±7.4 % of initial amplitude, mean ± S.E.M., N=3), whereas the response to serine showed marked accommodation with time (amplitude of integrated response after 100 s of exposure to stimulus 46.6±4.8 % of initial amplitude, mean ± S.E.M., N=5).

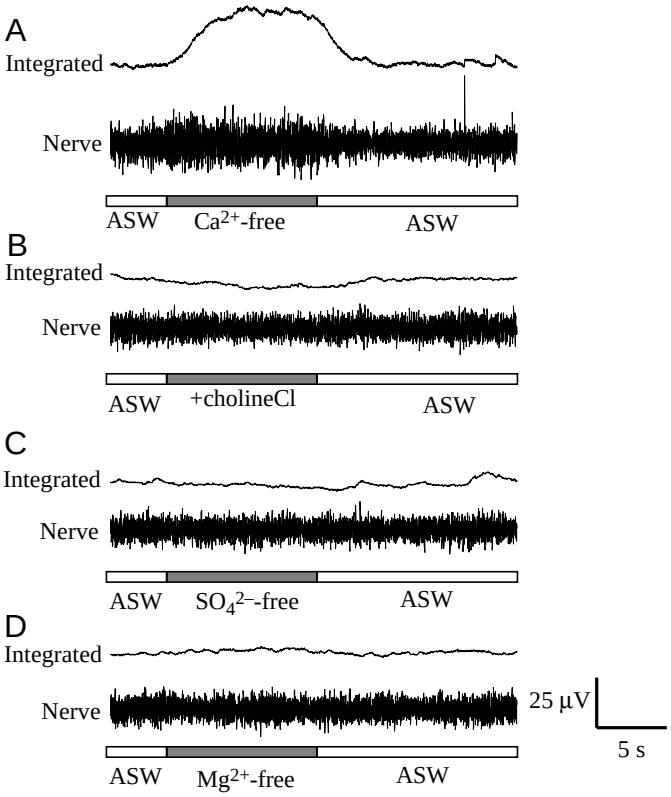


Fig. 3. Specificity of olfactory response in *Sparus aurata* to changes in environmental [Ca^{2+}]. (A) Typical response recorded extracellularly from the olfactory nerve (lower trace) and its integrated activity (upper trace) to a change in [Ca^{2+}] from 10 mmol l^{-1} (open horizontal bars) to 0 mmol l^{-1} (shaded horizontal bar) in artificial sea water (ASW) flowing over the olfactory epithelium. (B) The lack of response to a change in choline chloride (cholineCl) concentration (the physiologically inert cation used to substitute for Ca^{2+} in Ca^{2+}-free ASW) from 0 mmol l^{-1} (open horizontal bars) to 20 mmol l^{-1} (shaded horizontal bar). (C,D) The lack of response to the removal of sulphate and magnesium (shaded horizontal bars), respectively, from ASW (open horizontal bars). All traces were obtained from the same fish and are representative of three independent experiments. The vertical scale bar refers to the nerve trace alone, but the horizontal scale bar refers to both nerve and integrated traces. All integrated traces are shown at the same gain.

Adaptation to Ca^{2+}-free ASW

Ca^{2+} is intimately involved with signal transduction in vertebrate olfactory receptor neurones (Schild and Restrepo, 1998; Menini, 1999). Therefore, we wanted to examine the effect of removal of Ca^{2+} from sea water on the ability of *Sparus aurata* to detect the odorant L-serine. Superfusion of the olfactory rosette with Ca^{2+}-free ASW caused an initial attenuation (5–10 min after exposure to Ca^{2+}-free ASW) of the integrated response to 10^{-3} mol l^{-1} L-serine, which was especially evident in the tonic phase of the response (Fig. 5). The extent of this attenuation was highly variable (10–100 %), but the response was always blunted compared with that in ASW. However, after a period of adaptation of 20 min, the response always regained the same amplitude as in the presence of 10 mmol l^{-1} Ca^{2+}, and no further change was seen (up to 30 min).

Discussion

To the authors' knowledge, this is the first study to demonstrate an olfactory sensitivity to reductions in environmental Ca^{2+} concentrations in a marine animal. Bodznick (1978), recording extracellularly from the olfactory bulb (olfactory electro-encephalograph, EEG) of freshwater-reared sockeye salmon (*Onchorhynchus nerka*), demonstrated that this species shows olfactory sensitivity to increases in environmental Ca^{2+} concentration. He proposed that this contributed to their ability to identify their natal rivers and thereby aid in the homing migration that this species demonstrates. We would not argue against this interpretation, but would add that there are other, perhaps more physiologically pertinent, reasons for a fish to be able to monitor the environmental Ca^{2+} concentration – namely the maintenance of a constant extracellular [Ca^{2+}] or to forewarn the fish that it is reaching the limit of its salinity tolerance. In this respect, it is pertinent to note that *Sparus aurata*, although predominantly marine, can survive in brackish water as low as 5 ‰, and thus can osmoregulate in a hypo-osmotic environment (5 ‰ sea water has a NaCl concentration of approximately 70 mmol l^{-1} and an osmolality of approximately 140 mosmol kg^{-1} H_2O). However, the Ca^{2+} concentration of this dilute sea water is approximately 1.3 mmol l^{-1}, very close to the plasma free Ca^{2+} concentration of this species and the IC_{50} of the olfactory response to Ca^{2+}. It seems possible, therefore, that it is not the low osmolality of diluted sea water *per se* that limits the penetration of this species into freshwater environments, but its inability to maintain its plasma Ca^{2+} concentration in a low-[Ca^{2+}] medium. Ionic imbalance, especially involving Ca^{2+}, has been implicated as a source of toxicity (Douglas et al., 1996). It would be advantageous, therefore, for this species to have a robust and sensitive 'advance warning system' for [Ca^{2+}] to activate the appropriate endocrinological/physiological mechanisms (to maintain plasma [Ca^{2+}]) and/or manifest the appropriate behavioural response (e.g. avoidance of low-[Ca^{2+}] water). It is therefore not surprising that the response of the olfactory epithelium of a marine teleost should differ in direction (i.e. respond strongly to decreases in [Ca^{2+}]) from that of a freshwater-adapted euryhaline teleost (i.e. respond strongly to increases in [Ca^{2+}] from normal fresh water). It would appear that our original hypothesis, that the most likely site for such a Ca^{2+}-sensing system would be within the olfactory epithelium, is well supported by the present study. Furthermore, the apparent leftward shift of the concentration/response curve whilst the olfactory epithelium is superfused with Ca^{2+}-free ASW suggests that the Ca^{2+}-sensing mechanism is able to adapt to changes in external [Ca^{2+}], becoming more sensitive as external [Ca^{2+}] falls.

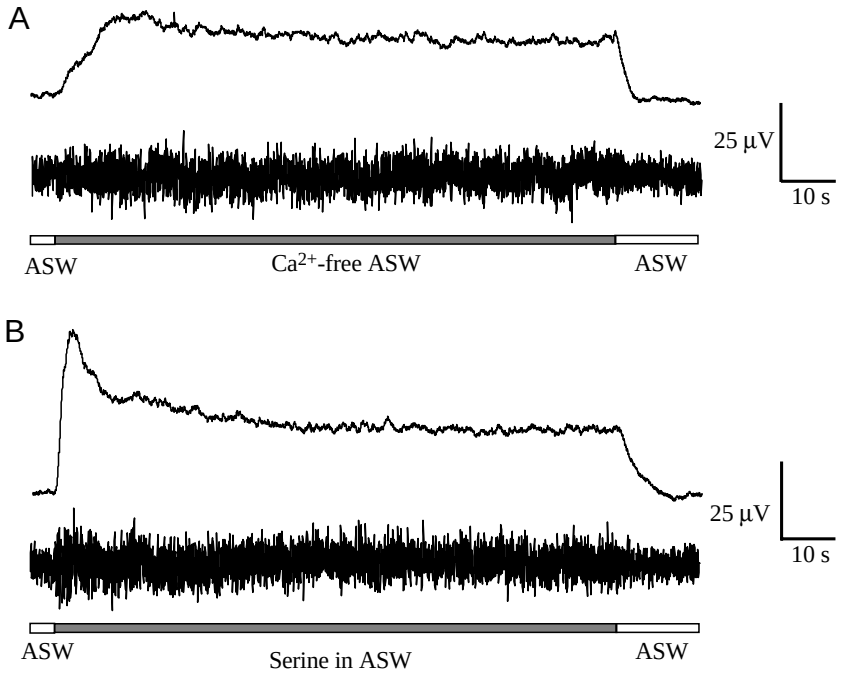
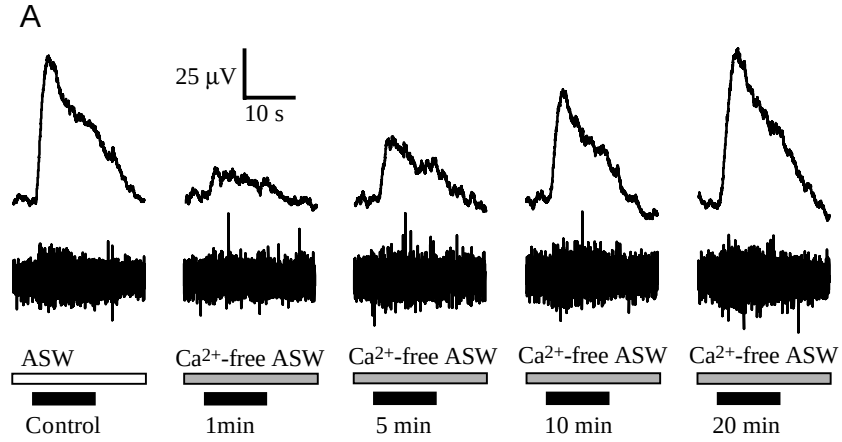


Fig. 4. Comparison of olfactory responses of *Sparus aurata* to changes in [Ca^{2+}] and to L-serine. (A) Effect of long-term (100 s; shaded horizontal bar) exposure of the olfactory epithelium to 0 mol l^{-1} Ca^{2+} on the response recorded extracellularly from the olfactory nerve (lower trace) and its integrated activity (upper trace), and (B) the subsequent trace of a similar long-term exposure to the conventional odorant L-serine (10^{-3} mol l^{-1} in ASW; shaded horizontal bar). Note that the tonic response to Ca^{2+} is of a similar amplitude to that of the L-serine response (in olfactory terms, 10^{-3} mol l^{-1} serine is a very potent stimulus) but that the sharp phasic response shown to to serine is absent. Furthermore, the time to peak of the Ca^{2+} response is slower than that of the serine response, whilst the return to baseline is quicker. Traces are representative of three independent experiments.



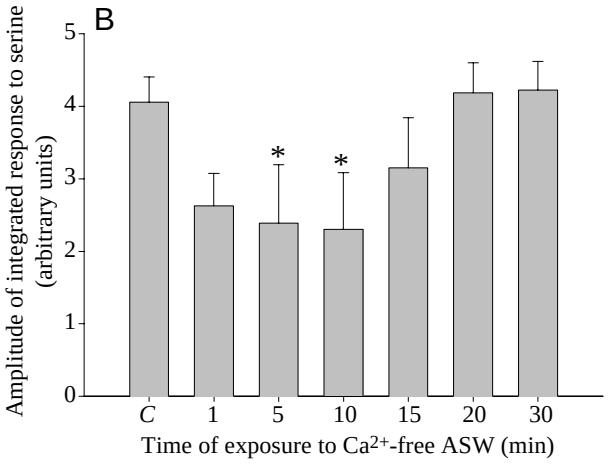


Fig. 5. Effect of the absence of external Ca^{2+} on olfactory transduction in *Sparus aurata*. (A) Consecutive extracellular nerve recordings (lower traces) and integrated activity (upper traces) from the olfactory nerve in response to stimulation of the olfactory epithelium by 10^{-3} mol l^{-1} L-serine (black horizontal bars; 10 s duration) in artificial sea water (ASW; open horizontal bar; first trace) or in Ca^{2+}-free artificial sea water (Ca^{2+}-free ASW; shaded horizontal bars; all subsequent traces). After 1 min of continuous exposure to Ca^{2+}-free ASW, the olfactory response to L-serine is still clearly manifest, but it is of a markedly lower amplitude than in normal ASW (control). However, the fish adapts to the absence of external Ca^{2+} within 20 min so that the response to 10^{-3} mol l^{-1} serine regains the same amplitude as the control. (B) A histogram of the pooled data (means + S.E.M.) from six similar independent experiments. An asterisk indicates a significant difference (P<0.05) compared with the control (*C*), using repeated-measures ANOVA followed by Dunnett's test.

Mechanism of Ca^{2+} sensitivity

An obvious candidate for the olfactory receptor for Ca^{2+} would be the mammalian Ca^{2+}-sensing receptor that was first isolated and cloned from bovine parathyroid tissue (Brown et al., 1993). Its presence has subsequently been demonstrated in kidney (Riccardi et al., 1995) and gut (Cheng et al., 1999), both tissues intimately involved with Ca^{2+} homeostasis in tetrapods. This receptor has seven transmembrane domains and is a G-protein-linked receptor related to the metabotropic glutamate receptors (for a review, see Chattopadhyay et al., 1997) that acts *via* the phospholipase C/inositol trisphosphate intracellular transduction pathway. Ingleton et al. (1999) have recently demonstrated, using both immunocytochemistry and *in situ* hybridisation, the presence of a similar Ca^{2+}-sensing receptor in several tissues from two species of fish: the puffer fish (*Fugu rubripes*) and the flounder (*Platichthys flesus*). The similarity between the apparent IC_{50} value of the observed olfactory response of sea bream and that of the mammalian Ca^{2+}-sensing receptor suggests that it could be mediating this sensitivity (the IC_{50} for plasma free $[Ca^{2+}]$ inhibition of parathyroid hormone release from parathyroid glands *in vivo* is 1.2 mmol l^{-1}; Brown et al., 1993).

However, the mammalian Ca^{2+}-sensing receptor also responds to changes in plasma $[Mg^{2+}]$ with slightly reduced sensitivity compared with Ca^{2+} (Brown et al., 1993); the data in the present study suggest that the Ca^{2+}-sensing receptor of the olfactory system of *Sparus aurata* is much less sensitive to alterations in the Mg^{2+} concentration. This may be related to the fact that the concentration of Mg^{2+} is much higher in sea water than that of Ca^{2+} ($[Mg^{2+}]$=50 mmol l^{-1}, $[Ca^{2+}]$=10 mmol l^{-1}), and thus Mg^{2+} availability may not be physiologically limiting until very dilute sea water is encountered. The study of Brodznick (1978) similarly showed that the olfactory system of the sockeye salmon was relatively insensitive to changes in environmental $[Mg^{2+}]$, compared with $[Ca^{2+}]$, even though the concentrations of environmental Mg^{2+} are much lower in fresh water than in sea water. Thus, if this mechanism is mediated by a piscine Ca^{2+}-sensing receptor (Ingleton et al., 1999), then it would appear to be functionally slightly different from the mammalian Ca^{2+}-sensing receptor in that it is more specific for Ca^{2+} and relatively insensitive to Mg^{2+}. A series of pilot experiments in our laboratory have shown that the olfactory response to reductions in $[Ca^{2+}]$ was blunted by Mg^{2+} only when present at a concentration of 100 mmol l^{-1}. Mg^{2+} concentrations of 50 mmol l^{-1} or less, the range that *Sparus aurata* is likely to encounter in its natural environment, had no effect on the Ca^{2+} response.

Alternatively, the observed increase in olfactory nerve activity could be due to reduced external $[Ca^{2+}]$ leading to a hyperexcitability of the plasma membrane (Hille, 1992) of the cilia (and/or microvilli) of the olfactory receptor neurones. This may explain the enhancing effect of citrate on the olfaction and gustation of amino acids in freshwater fish (Ogawa and Caprio, 1999; Parker et al., 2000); the citrate chelates Ca^{2+}, and this leads to an increase in the amplitude of response to a given concentration of amino acid. However, we believe that this phenomenon is unlikely to explain the response to Ca^{2+} in *Sparus aurata* (i) because the olfactory response to reductions in external $[Ca^{2+}]$ was manifest at concentrations above that of extracellular fluid (Fig. 1) and (ii) because there was no such enhancement of the amplitude of response to the amino acid L-serine in the presence of Ca^{2+}-free ASW (Fig. 5). In fact, the amplitude of the response was initially attenuated, followed by a period of adaptation during which the amplitude returned to control levels. Furthermore, a non-specific effect of a reduction in external $[Ca^{2+}]$ could not easily explain the shift in sensitivity of the concentration/response curve during continuous exposure to Ca^{2+}-free ASW (Fig. 2).

Conversely, a hyperexcitability of the olfactory neurones as a result of the removal of external Ca^{2+} (Hille, 1992) could possibly explain the bluntening of the integrated response to serine within the first 10 min of exposure to Ca^{2+}-free ASW (Fig. 5). A sudden shift in external $[Ca^{2+}]$ could lead to a hyperexcitability of serine-sensing neurones (and presumably other odorant neurones as well) causing a large increase in basal activity and rendering them unable to respond normally to the presence of odorant. Assuming that fish are able to regulate, at least to some extent, the concentrations of various inorganic ions in the mucous layer surrounding the sensory cilia and microvilli (see below), these neurones would then be stabilised, returning the membrane potential to normal levels, and then be able to respond to serine stimulation as if they were in normal sea water (after approximately 20 min; Fig. 5). The continuous high level of activity observed, even after the return of the serine response to normal, may then be due to the activity of Ca^{2+}-specific receptor neurones.

Involvement of Ca^{2+} in olfactory transduction

Ca^{2+} is intimately involved in the olfactory transduction process of vertebrates (for reviews, see Schild and Restrepo, 1998; Menini, 1999). The current, widely accepted, model for terrestrial, air-breathing tetrapods is that odorants bind to G-protein-linked receptors on the ciliary membranes of the olfactory receptor neurones, which then activate the adenylate cyclase cascade. The resultant increase in intracellular cyclic AMP concentration then opens cyclic-nucleotide-gated channels that allow an influx of extracellular Ca^{2+} which subsequently open Ca^{2+}-dependent Cl^- channels, and the consequent efflux of Cl^- depolarizes the neurone and generates action potentials (see Schild and Restrepo, 1998; Menini, 1999; and references therein). Recent evidence suggests that a similar mechanism may also be operating in the olfactory receptor neurones of fish (Sato and Suzuki, 2000). Thus, a reduction in the Ca^{2+} concentration of the medium surrounding the cilia may affect olfaction in a non-specific manner.

However, two main lines of evidence suggest that a non-specific response due to the absence of external Ca^{2+} cannot account for the responses observed in the present study. First, the olfactory system of *Sparus aurata* responds to reductions

in external Ca^{2+} concentration with an increase in firing rate of (presumably) the receptor neurones. If this were a non-specific effect, the opposite effect would be expected (i.e. a decrease in activity of the olfactory neurones). Second, the olfactory response is manifest at environmental concentrations of Ca^{2+} that are in excess of the free plasma Ca^{2+} concentration (as previously stated, the apparent IC_{50} value is similar to plasma free [Ca^{2+}]), and therefore, presumably, to the [Ca^{2+}] of the mucous layer. A complicating factor is that the olfactory cilia do not penetrate directly into the medium, but into a mucous layer, the ionic composition and regulation of which have received little attention (Schild and Restrepo, 1998). This may have profound effects on the olfactory process because both the odorant receptors and cyclic-nucleotide-gated ion channels are believed to be found predominantly on the ciliary membrane (Nakamura and Gold, 1987).

In air-breathing tetrapods, the ionic composition of the mucous can be tightly regulated by secretory epithelial cells without significant loss to, or gain from, the surrounding medium. This may not be the case, however, for aquatic water-breathing vertebrates, especially those exposed to widely fluctuating ionic environments, such as euryhaline fish. Shoji et al. (1994) found no difference in the olfactory response of chum salmon (*Oncorhynchus keta*) to 1 mmol l^{-1} L-alanine or L-methionine when the olfactory epithelium was superfused with artificial sea water ([Ca^{2+}]=10.7 mmol l^{-1}) or artificial fresh water ([Ca^{2+}]=0.4 mmol l^{-1}), and proposed that external Ca^{2+} (or other ions) played little or no part in the olfactory transduction process. Freshwater teleosts, which are perpetually in low-[Ca^{2+}] environments, still have highly sensitive olfactory systems. This may be due to the phospholipase C/inositol trisphosphate intracellular pathway being utilized by teleost olfactory receptor neurones (Restrepo et al., 1993; Speca et al., 1999), rather than the adenylate cyclase/cyclic AMP pathway (as in air-breathing tetrapods), and consequent mobilization of intracellular stores of Ca^{2+}, rather than reliance on influxes of extracellular, or even external, Ca^{2+}. It is pertinent to add that the Ca^{2+}-sensing receptor in mammals acts *via* the phospholipase C/inositol trisphosphate intracellular pathway (Brown et al., 1993). Thus, the transduction mechanism of this receptor is not dependent on extracellular [Ca^{2+}] (i.e. the very parameter that it is monitoring) to evoke a cellular response. How the olfactory epithelium of fish retains sensitivity to 'conventional' odorants in the face of very large fluctuations in environmental ionic composition is not clear (Shoji et al., 1994, 1996). Is the ionic composition of the olfactory mucous layer kept constant in the face of such changes? If so, how can the olfactory epithelium be sensitive to changes in concentration of ions such as Ca^{2+}? Are Ca^{2+}-sensing neurones exposed directly to the environment? Is the transduction mechanism of fish olfactory neurones independent of external inorganic ions and, if so, how? These are all questions that need to be addressed by further research.

Concluding remarks

In conclusion, we have shown that the olfactory system of a marine teleost is highly sensitive to changes in environmental [Ca^{2+}] within the range that this species is likely to encounter in their natural habitat. Furthermore, we suggest that this may be mediated by a Ca^{2+}-sensing receptor similar to that recently identified in the parathyroid glands and other tissues involved in Ca^{2+} homeostasis in mammals. The physiological and behavioural responses to such olfactory information remain to be explored.

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