Discrimination of bile acids by the rainbow trout olfactory system: Evidence as potential pheromone

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ABSTRACT

Electro-olfactogram recording was used to determine whether the olfactory epithelium of adult rainbow trout is specifically sensitive to bile acids, some of which have been hypothesized to function as pheromones. Of 38 bile acids that had been pre-screened for olfactory activity, 6 were selected. The rainbow trout-specific bile acids, taurocholic acid (TCA), and tauroolithocholic acid 3-sulfate (TLS) were the most potent compounds tested. TLS had a distinctive dose-response curve. Cross-adaptation experiments demonstrated that sensitivity to bile acids is attributable to at least 3 independent classes of olfactory receptor sites. Our data suggest that bile acids are discriminated by olfaction in rainbow trout, supporting the possibility that these compounds function as pheromones.

Key terms: bile acids, chemical signals, electro-olfactogram, olfaction, pheromones, rainbow trout.

INTRODUCTION

Bile acids and amino acids, together with sex steroids and prostaglandins, comprise four major classes of chemicals that have been identified as specific olfactory stimulants for fishes and their stimulatory effectiveness characterized in over 30 species (Hara, 1994a; Hara, 1994b).

Extreme olfactory sensitivities to bile acids, coupled with their wide distribution and chemical variations have been implicated for their role in fish behavior (Dolving et al., 1980; Hara et al., 1984; Sola and Tosi, 1993; Zhang et al., 2001). Although the gustatory function of bile acids has yet to be ascertained, behavioral evidence suggests that one of their functions as olfactory stimulants may be to serve as pheromones for migratory anadromous fishes, some of which appear to recognize and select the odor of conspecifics when choosing spawning streams (Dolving et al., 1980; Stabell,

1992). Studies using electroencephalogram recording showed that bile acids are potent olfactory stimulants for the grayling and Arctic charr (Dolving et al., 1980). Taurocholic acid (TCA) is a potent olfactory stimulus to several salmonids (Hara et al, 1984; Quinn and Hara, 1986; Zhang and Hara, 1991; Hara and Zhang, 1996) and goldfish (Sorensen et al., 1987).

In teleosts, the principal biliary bile acids are (1) sulphated bile alcohol, mainly 5-cyprinol and 5-chimaerol; and (2) C₂₄ bile acids, mainly cholic acid (CA), chenodeoxycholic acid (CD), deoxycholic acid (DC), and haemulcholic acid (Haslewood, 1967; Goto et al., 1996). The C₂₄ bile acids are taurine amidated and/or sulphated. Also, cysteinolic acid-amidated bile acids were found in the bile of some marine species (Une et al, 1991; Goto et al, 1996). In the bile acid composition of rainbow trout, cholic acid was found to be the main component and constituted over 85% of total (Denton and Yousef, 1974).

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Chenodeoxycholic acid accounted for 14% and $3\alpha\pm$, $12\alpha\pm$ -7-keto and $7\alpha\pm$, $12\alpha\pm$ -3-keto- $5\beta\leq$ cholanoates for 1% or less of total. Based on these findings, we planned to investigate a possible pheromonal role of bile acids in rainbow trout, determining which of them would be detected by olfaction.

Despite growing interest in the role of bile acids in fish chemoreception (Zhang et al., 2001), little effort has been made to examine structure-activity relationships and receptor specificity for bile acids in the fish olfactory system. Analyses of this interaction will reveal the relative effectiveness of stimulants interacting with the putative receptors and, consequently, will provide information on whether a particular stimulus will be effectively detected by fish and which structural features are required for a stimulant to interact with the receptors.

To investigate a possible pheromonal role of bile acids in rainbow trout, our studies focused on: 1) which bile acids are detected by olfaction; and 2) how bile acids are detected by olfaction, using electro-olfactogram (EOG) recordings. The EOG, or transepithelial voltage transient, represents a summation of generator potential of olfactory neurons (Ottoson, 1971). EOG responses of fish are likely dependent upon activities of a large population of neurons over an entire area, if not the whole olfactory rosette, rather than the group of receptor cells on a single lamella in close proximity to the recording electrode. The high sensitivity and stability of EOG has proven advantageous for monitoring the olfactory sensitivity to odorants. The current study uses standard electrophysiological competition (cross-adaptation) procedures to determine whether bile salt odorants compete for shared odorant receptors.

MATERIAL AND METHODS

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) were obtained from a Manitoba provincial fish hatchery and maintained in the Freshwater

Institute, Department of Fisheries and Oceans, Winnipeg, Canada. Fishes were fed with commercial food pellets and supplied with flowing, aerated dechlorinated Winnipeg city water at 11.5-12.5°C under a 16L: 8D photoperiod. Experimental procedures employed in the studies complied with the guidelines issued by the Canadian Council for Animal Care.

Stimulus administration and EOG recording

Test fish were tranquilized with MS222 (1:8000), anaesthetized intraperitoneally with amobarbital (30 mg/Kg body weight), and immobilized with an intramuscular injection of Flaxedil (gallamine triethiodide, 3-4 mg/Kg body weight). The anaesthetized fish was wrapped with an absorbent tissue and secured in a holding apparatus. The right-side rosette of the olfactory sac was exposed by removing the dorsal aspect of the skin and the cartilage of the olfactory sac. The naris and the gills were perfused with dechlorinated plain water. To deliver plain water or stimulants to the naris, the method of Sveinsson and Hara (1990) was used. Briefly, a pneumatic activator switches plain water to/from the stimulant solution delivered by two identical polyethylene tubes. The pneumatic valve was controlled by a solenoid and an associated electronic timing device (Hara et al., 1973). The solution flowed through a glass capillary positioned over the rosette (10 ml/min). This system provides an approximate square stimulation at required concentration each time with no apparent interruption or disturbance of the flow to the naris. The stimulus duration used was 10 s.

EOG responses were recorded with a saline-gelatin (8%) filled capillary (Type diameter 100-150 μ m) bridged to an AgAgCl electrode (Type MEH-15, World Precision Instruments, Sarasota, FL, USA) filled with 3 M KCl and recorded on a polygraph (Model 79, Grass Instrument, West Warwick, USA). A reference electrode of the same type was placed lightly on the dorsal skin surface adjacent to the perfused olfactory cavity. The

recording electrode was positioned near the central ridge, posterior portion of the rosette, and slightly above the olfactory epithelium.

Cross-adaptation

Cross-adaptation experiments (Caprio and Byrd, 1984) were conducted to compare the EOG response to a test stimulus before and during adaptation to an adapting compound using a protocol by Sveinsson and Hara (1990). A standard test series measured a competitor odorant's effect on the responses to each test odorant. The 3 phases of a standard test series are: phase 1, measurement of the response to a test odorant when plain water bathes the olfactory epithelium; phase 2, measurement of the response to a test odorant when a competitor odorant bathes the olfactory epithelium (the olfactory epithelium was bathed by a competitor odorant for approximately 60 s prior to first application of the test odorant); and phase 3, the flow bathing the olfactory epithelium is returned to plain water, and the odorant is re-tested to confirm that adaptation is reversible.

Chemicals

Bile acids and amino acids were purchased from Steraloids (Newport, RI, USA) or Sigma Chemical (St. Louis, MO, USA). Trivial names for bile acids are used throughout the text. Names and

abbreviations of the most potent bile acids are listed in Table 1 (6 out 32 tested).

Stock solutions of the stimulants (10^{-3} M concentrations) were prepared with distilled water and stored in a refrigerator. Test solutions were formulated immediately before testing by diluting with plain water. To eliminate the effect of distilled water, aliquots of stock solutions were diluted at least 100 times with plain water to form test formulas.

Data analysis

A one-way ANOVA followed by a Dunnett post-hoc test upon significance ($P < 0.05$) was performed on the percentage of unadapted responses obtained in the cross-adaptation experiments. A significant response in the Dunnett test between the stimulation with the chemical used as adaptant and another stimulant was taken as evidence of the activation of distinct receptor sites.

All EOG responses were normalized as relative magnitudes of the standard reference stimulus 10^{-5} M L-Serine. All data are presented as means $\pm$ SEM. Differences between groups of means were determined using one-way analyses of variance. The t-test or a paired t-test, when appropriate, was used to determine differences between means from two groups. A p value < 0.05 was used to identify significant differences between groups.

TABLE I

Names and abbreviations for the bile acids tested

Abbreviation	Trivial name	Chemical name
CA	Cholic acid	$3\alpha\pm, 7\alpha\pm, 12\alpha\pm$ -Trihydroxy- $5\beta\pm$ -cholan-24-oic acid
CD	Chenodeoxycholic acid	$3\alpha\pm, 7\alpha\pm$ -Dihydroxy- $5\beta\pm$ -cholan-24-oic acid
DC	Deoxycholic acid	$3\alpha\pm, 12\alpha\pm$ -Dihydroxy- $5\beta\pm$ -cholan-24-oic acid
TCA	Taurocholic acid	$3\alpha\pm, 7\alpha\pm, 12\alpha\pm$ -Trihydroxy- $5\beta\pm$ -cholan-24-oic acid N-(2-sulfoethy)amide
TCD	Taurochenodeoxycholic acid	$3\alpha\pm, 7\alpha\pm$ -Dihydroxy- $5\beta\pm$ -cholan-24-oic acid N-(2-sulfoethy)amide
TLS	Taurolithocholic acid $3\alpha\pm$ -sulphate	$3\alpha\pm$ -Hydroxy- $5\beta\pm$ -cholan-24-oic acid N-(2-sulfoethy)amide

RESULTS

Characteristics of EOG responses

The EOG response to a bile acid was a rapid negative response reaching its peak amplitude 1-2 s after the stimulation switch was turned on. Then, the response declined exponentially to a maintained tonic level

throughout the 10-s stimulus period. Upon cessation of the stimulus, the potential returned quickly to the baseline. As the stimulant concentration increased, the time of latency phase became gradually shorter, whereas the time of recovery phase became longer. Figure 1 shows the tonic/phasic responses ratios for the six most representative bile acids tested.

Figure 1: (A) Example tracing of EOG. Superimposed EOG responses to 10^{-9} and 10^{-5} M L-cysteine, showing development of tonic and phasic components (scale bar: 10 s). (B) Tonic/phasic responses ratios for six representative bile acids at various concentrations. CA=cholic acid, TCA=taurocholic acid, TCD=taurochenodeoxycholic, CD=chenodeoxycholic, DC=acid deoxycholic acid, TLS=tauroolithocholic acid 3-sulphate. Responses are expressed as percentages of the response to the standard 10^{-5} M Serine (mean $\pm$ SEM). The sample size is 5.

Concentration-response relationships

Concentration-response (C-R) relationships of the six most potent bile acids tested were grouped according to the molecular structures (Fig. 2). Three groups were identified, and all compounds within each group had similar C-R relationships. The free bile acids deoxycholic (DC), chenodeoxycholic (CD), and cholic acid (CA) had similar, nearly parallel C-R curves with detection thresholds of 10^{-10} M, 10^{-11} M and 10^{-10} M, respectively (Fig. 2a). The bile acids conjugated with taurine, taurochenodeoxycholic acid (TCD), and taurocholic acid (TCA) had similar C-R curves, with relatively lower detection thresholds of 10^{-11} M and 10^{-10} M, respectively (Fig. 2b). Lastly, tauroolithocholic acid 3-sulphate (TLS), the only sulfated compound tested, had a C-R curve that was different from all other bile acids (Fig. 2c); it had a detection threshold of 10^{-11} M, with a nearly exponential increase between 10^{-10} and 10^{-8} M.

Cross-adaptation

When used as adapting stimuli (self-adaptation), all six bile acids eliminated EOG responsiveness to themselves. The results suggested that the interaction of bile acids with receptors is in a reversible and truly competitive manner and may be mediated by specific receptors (Fig. 3).

Adaptation to free bile acid DC inhibited responses to CA and itself (Fig. 3b), but adaptation to CD did not inhibit TCA responses completely (Fig. 3a), suggesting that separate receptors may exist for DC and CD and that DC and CA share the same receptors. None of the bile acids significantly inhibited TCD responses (other than itself), suggesting separate receptors for TCD. Although TLS responses were suppressed by DC, they were unaffected by others (Fig. 3c). ARG responses were unaffected by all bile acids.

DISCUSSION

The present study demonstrated that bile acids are a group of potent olfactory

stimulants for rainbow trout. This observation is consistent with EOG data in lake char (Zhang et al., 2001), zebrafish (Michel and Lubomudrov, 1995), sea lamprey (Li et al., 1995) and olfactory bulb recording in grayling and Arctic char (Doving et al., 1980).

Our cross-adaptation results indicated a competitive interaction of bile acids with their receptors which is fully reversible at the concentrations range tested. The results suggest that olfactory responses to bile acids may be mediated by specific receptors.

Cross-adaptation is useful in investigating the involvement of single or multiple receptors mediating an observed electrophysiological response. However, the concentration and potency of adapting compounds affect the degree of inhibition to test stimuli. Different experimental strategies and the complicated biological system in *in vivo* studies sometimes make it difficult to interpret cross-adaptation results (Mair, 1982; Zack-Strausfeld and Kaissling, 1986). For instance, it has been reported that two chemicals with similar concentrations or equipotencies may have different inhibitory power (Cain and Engen, 1969; Caprio and Byrd, 1984). In some cases, more potent compounds have higher adaptation effects on test stimuli (Sugimoto and Sato, 1981; Ohno et al., 1984). Inversely, Baylin and Moulton (1979) concluded that there is no correlation between a compound's potency and cross-adaptation power for olfactory responses. Moreover, Zhang et al. (2001) found that strong stimulants are, in general, better adapters for compounds of the same group. Following this hypothesis, in the present study, TCD should inhibit TCA because both are from the same group of amidated bile acids, however this was not evident in the data presented.

In general, the results of previous competing experiments have been interpreted as follows. If the response to an odorant is unaffected by a competitor odorant, it is assumed that the test and competitor odorants interact with distinct transduction pathways including different odorant receptors. Complete and reciprocal cross-adaptation between two odorants is

Figure 2: Concentration-response relationships of phasic responses for bile acids. Response magnitudes are normalized as percentages of response to 10^{-5} M L-serine. (a) free bile acids; (b) amidated bile acids; and (c) sulphated bile acids. Responses are expressed as percentages of the response to the standard 10^{-5} M Serine. Each point represents mean $\pm$ S.D. The sample size is 5.

Figure 3: Remained response magnitudes at 10^{-7} M during adaptation to (a) 10^{-7} M chenodeoxycholic acid (CD); (b) 10^{-7} M deoxycholic acid (DC); (c) 10^{-7} M tauroolithocholic acid 3-sulphate (TLS); (d) 10^{-7} M taurocholic acid (TCA) and (e) 10^{-7} M taurochenodeoxycholic (TCD). Sample size is 5 in a, b, c, e, f and 4 in d. Responses are expressed as percentages of the response to the standard 10^{-5} M Serine (mean $\pm$ SEM). Different letters above bars indicate a statistically significant difference when compared to the test of adapting stimulus.

interpreted as evidence that the two odorants compete for a common transduction pathway probably including a shared odor receptor.

Partial cross-adaptation, whether reciprocal or not, may be the result of competition for a shared odorant receptor or due to interactions at any step in a shared transduction cascade. Odors that partially, but never completely, cross-adapt each other

are common (e.g., CA and DC in this study) and are assumed to reflect interaction at some level of the receptive process.

Similarities and differences in the patterns of cross-adaptation of the test odorants in competitor odorant backgrounds provide information about the probable ligand binding sites of the odorant receptors (Caprio and Byrd, 1984; Ohno et al., 1984).

Presumably, structurally similar odorants have shared patterns of adaptation because their common structural features activate odorant receptors recognizing those features.

Odorants interacting with a common molecular receptor might be expected to bind to that receptor with different affinities. In some competition studies, the concentrations of the test odorants are adjusted until approximately equal response magnitudes are obtained in an attempt to correct for differences in binding affinity (Caprio and Byrd, 1984). Cross-adaptation between odorants presumed to interact with common receptor might not be expected to be reciprocal if their concentrations had not been so adjusted.

Our data showed that even when the adapting compound and the test stimuli were from the same group, there was no inhibition. This could mean that the inhibition (or lack of inhibition) of the compounds is group independent. For example, the free bile acids DC and CA were not even slightly inhibited when the adapting compound was another free bile acid (CD), whereas DC was totally inhibited when the adapting compound was TLS, a sulphated bile acid. This suggests that the adapting compound acts as a partial agonist that competes for receptors of other groups of bile acids. If there are multiple bile acid receptors, then it is expected that a bile acid would interact with more than one type of receptor because molecular structures among bile acids groups are similar.

Analyses of structure-activity relationship suggest that certain configurations are important for individual receptor recognition. For example, the position and orientation of hydroxyls presented on the ring structure affect the stimulus effectiveness. It appears that a-axial formed by hydroxyls of 3 a- and 7 a- is important in the stimulatory potency of the compound. These features suggest that bile acids interact with their receptor in pointedly positions rather than in collective effects.

Among bile acids, the most potent are CA, TCA, TCD, CD, DC, and TLS. At least three types of bile acid receptors are responsible for olfactory responses to these

most stimulatory bile acids. However, a bile acid may react with one or more bile acid receptors.

Li and Sorensen (1994) suggest that there are four types of bile acid receptors in the sea lamprey olfactory epithelium. Our results appear similar to those reported in sea lamprey. Thus, it seems that bile acid receptors exist in many fish species, but it is yet to be determined if similar receptors present in a wide range of species have significance in chemical signal detection.

Implication of bile acids as chemical signals

In salmonids, there is little degradation of bile acids in the intestine. It is thought that the extensive bile acid metabolism occurring in the mammalian intestine is caused by microorganisms (Gustafsson et al., 1968; Huijghebaert and Hofmann, 1986). In fish, especially in salmonides, the occurrence of fewer intestinal microorganisms probably accounts for the lack of metabolic transformation of bile acids (Trust and Sparrow, 1974; Trust, 1975).

Fish utilize bioactive byproducts as chemical signals for communication and the olfactory system plays a predominant role in perception of chemical signals (Hara, 1982; Hara, 1992). Feces and urine represent two sources for these bioactive byproducts in water. Although the ecological significance of bile acids in feces, urine and water remains to be established, spawning lake char are attracted to reefs treated with feces of juvenile lake char. Females approach and males contact the treated reefs, and successful spawning was observed at these sites. This suggests that chemical cues emanating from feces of juvenile lake char may play a role in the reproductive behavior of spawning adults. This is consistent with the evidence that chemical signals are often a mixture of related chemical (Sorensen et al., 1998). Presumably organisms have evolved to discern pheromonal mixtures because they contain more information and represent a more portent signal (Sorensen et al., 2003). Thus, a small portion of certain bile acid

components may be important in forming chemical cues. Experiments are presently underway to confirm the precise behavioral significance of these components.

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