INTESTINAL \( \text{Na}^+ \) AND \( \text{Cl}^- \) LEVELS CONTROL DRINKING BEHAVIOR IN THE SEAWATER-ADAPTED EEL \textit{ANGUILLA JAPONICA}

MASAAKI ANDO AND KEI NAGASHIMA

\textit{Laboratory of Physiology, Faculty of Integrated Arts and Sciences, Hiroshima University, Higashi-Hiroshima, Hiroshima 739, Japan}

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Summary

To analyze drinking mechanisms in seawater teleosts, seawater-adapted eels were used as a model system. When the intestine of the eel was perfused with iso-osmotic mannitol, the eels drank sea water. However, when the perfusion medium was switched to iso-osmotic NaCl, seawater drinking was depressed. This depression was observed even after blocking NaCl absorption across the intestine by replacement of the perfusate with choline chloride or by treatment with furosemide, an inhibitor of NaCl and water absorption across the eel intestine. Furthermore, depression of drinking rate preceded an increase in urine flow by over 1 h. These results indicate that this depression is not due to a recovery of blood volume and suggest that intestinal \( \text{Cl}^- \) itself inhibits drinking. Direct action of luminal \( \text{Cl}^- \) on drinking behavior was further supported by the observation that perfusion with iso-osmotic NMDG–HCl, Tris–HCl, choline chloride and RbCl all inhibited seawater drinking. When NaCl in the perfusion medium was replaced with sodium acetate, sodium butyrate, sodium methylsulfate or NaSCN, the drinking rate was enhanced threefold, suggesting that \( \text{Na}^+ \) itself stimulates drinking in the absence of \( \text{Cl}^- \). In the present study, concentrations of \( \text{Na}^+ \) and \( \text{Cl}^- \) in the swallowed fluid were also measured simultaneously. As the drinking rate was enhanced, the \( \text{Na}^+ \) and \( \text{Cl}^- \) concentrations in the gastrointestinal fluid were increased. On the basis of these results, it seems possible that high concentrations of \( \text{Cl}^- \) in the intestine reduce the drinking rate, thus lowering esophageal \( \text{Cl}^- \) concentration due to desalination of the ingested sea water. When \( \text{Cl}^- \) concentration in the intestine falls below a certain level, \( \text{Na}^+ \) will stimulate seawater drinking again.

Key words: drinking behavior, mannitol perfusion, intestinal \( \text{Cl}^- \), intestinal \( \text{Na}^+ \), furosemide, esophagus, urine flow, seawater eel, \textit{Anguilla japonica}.

Introduction

Drinking behavior in most terrestrial vertebrates proceeds from ‘perception of thirst’ to ‘seeking for water’, to ‘ingestion into the mouth’ and finally to ‘swallowing’. In contrast, fishes hold water in their mouth constantly for respiration; thus, they can drink water by ‘swallowing’ alone immediately after perceiving thirst and may skip the second and third processes. This indicates that the mechanism regulating water intake in fishes may not be as complicated as that in terrestrial vertebrates, and fishes appear to be a simple model for analyzing drinking mechanisms.

However, little is known about drinking mechanisms in fishes. With respect to osmoregulation in fishes, it is known that seawater teleosts drink to compensate for osmotic water loss, while in fresh water they drink little water (Bentley, 1971; Evans, 1979). Water intake by eels is stimulated by removal of blood and inhibited by saline infusion into the venous system (Hirano, 1974). Similarly, hemorrhage stimulates drinking in two marine stenohaline fishes, winter flounder \textit{Pseudopleuronectes americanus} and longhorn sculpin \textit{Myxoceophilus octodecemspinosis}, but not in three freshwater fishes, goldfish \textit{Carassius auratus}, common shiner \textit{Natropis cornutus} and mottled sculpin \textit{Cottus bairdi} (Beasley et al. 1986).

These results suggest that the drinking behavior is controlled by a volume receptor, at least in some euryhaline and marine fishes. Although angiotensin II is well known as a dipsogen in mammals (see Bourque et al. 1994), it also induces drinking in some teleosts such as the eel \textit{Anguilla japonica}, the flounder \textit{Platychthys flesus} and the longhorn sculpin \textit{M. octodecemspinosis} (Takei et al. 1979; Carrick and Balment, 1983; Kobayashi et al. 1983; Hirano and Hasegawa, 1984; Beasley et al. 1986; Okawara et al. 1987). Therefore, some fishes may be suitable as a model system for analysis of the drinking mechanisms of vertebrates.

Among fishes, eels are a suitable subject for study because their drinking rate can be measured using esophagus cannulations as developed by Hirano (1974). However, the ingested water does not enter the intestine in the esophagus-cannulated preparations. Thus, most studies have used freshwater eels, since water absorption across the intestine is essential for body fluid homeostasis in seawater eels (Oide and Utida, 1968) and since dehydration becomes severe during the experiments in sea water.

By introducing a second cannula into the intestine of the seawater eels, various solutions were perfused into the lumen.
of the intestine at a constant rate in the present study. Using this system, it was demonstrated that perfusion with isotonic mannitol solution induced seawater drinking and perfusion with Cl−-depressed drinking.

Materials and methods

Japanese cultured eels Anguilla japonica Temminck & Schlegel weighing approximately 200 g, were kept in seawater aquaria at 20 °C for more than 1 week before use. Although they suffered an initial loss in mass, the body mass returned to the original level after 6 days in sea water (Oide and Utida, 1968). After anesthesia using 0.1% methane tricaine sulfonate (MS-222, Sigma) in sea water, an incision (2 cm long) was made longitudinally in the abdominal wall along the posterior half of the liver. A vinyl tube (o.d. 2.0 mm) was inserted into the esophagus at the entrance of the stomach and tied in place with silk threads as described by Hirano (1974). After fastening both the cardia and pylorus of the stomach with silk threads, another tube (o.d. 1.1 mm) was inserted into the gastrointestinal junction and tied. Through this cannula, various solutions were perfused using a peristaltic pump (RP-NE2, Furue Science, Tokyo) at a rate of 10 ml h−1. The incision was then closed using silk suture and the eel was transferred to a plastic trough of the same size as the eel. Well-aerated sea water was circulated continuously through the trough at room temperature (20–23 °C). In some preparations, a third cannula (o.d. 1.0 mm) was inserted into the urinary bladder following a procedure described by Kakuta et al. (1986) and urine flow was also measured simultaneously.

The cannulae from the esophagus and from the urinary bladder were connected to drop counters (SF-DCT, Advantic, Utunomiya, Japan) for continuous recording of drinking rate and urine flow, respectively. Each drop was recorded as a spike on a polygraphic chart recorder (EPR-121, TOA). One drop from the esophageal and urinary bladder cannulae corresponded to 27.8 and 17.9 μl, respectively.

After operation, the intestine was perfused with normal Ringer or iso-osmotic NaCl solution until drinking ceased, and then the perfused fluid was switched to mannitol to obtain a control measurement. After the experiments, the perfused fluid was returned to the NaCl solution at night. The next morning, mannitol perfusion started again. These treatments were repeated as necessary and all results were obtained within 1 week. Sensitivity to mannitol was reduced after 1 week. Sensitivity to mannitol perfusion started again. These treatments were repeated as necessary and all results were obtained within 1 week. Sensitivity to mannitol was reduced after 1 week.

To determine ionic concentrations in the swallowed fluid, drops were collected from the drop counter using a fraction collector (LKB 2112, Bromma, Sweden). Na+ concentration was measured using flame photometry (FPF-2A, Hiranuma, Mito, Japan) and Cl− concentration was measured using a chloridometer (CL-5M, Hiranuma, Japan). By inserting a cannula into the gastrointestinal junction, some fluids that passed through the stomach were also collected and Na+ and Cl− concentrations were determined as described above.

The perfusion fluids used in the present study were as follows: 155.2 mmol l−1 NaCl (279.4±1.5 mosmol kg−1, N=3), 271.3 mmol l−1 mannitol (279.3±1.3 mosmol kg−1), 155.2 mmol l−1 choline chloride (278.2±0.8 mosmol kg−1), 155.2 mmol l−1 Tris-HCl (281.5±0.8 mosmol kg−1), 155.2 mmol l−1 N-methyl-D-glucamine (NMDG)–HCl (280.5±0.9 mosmol kg−1), 155.2 mmol l−1 KCl (279.5±1.4 mosmol kg−1), 155.2 mmol l−1 NaBr (281.2±0.5 mosmol kg−1), 155.2 mmol l−1 NaNO3 (280.3±0.3 mosmol kg−1), 155.2 mmol l−1 NaSCN (279.4±1.3 mosmol kg−1), 155.2 mmol l−1 sodium methylsulfate (279.4±0.6 mosmol kg−1), 155.2 mmol l−1 sodium butyrate (279.1±1.8 mosmol kg−1), 155.2 mmol l−1 sodium acetate (280.9±0.4 mosmol kg−1). Normal Ringer’s solution contained, in mmol l−1: 141.3 NaCl, 5.7 KCl, 3.6 CaCl2, 1.4 KH2PO4, 1.4 MgSO4 (278.6±2.2 mosmol kg−1). Furosemide (0.01 mmol l−1) (Tokyokasei Co., Tokyo) was also applied to the lumen of the intestine.

Statistical analyses of results were performed using Student’s t-tests. Results are given as mean ± s.e.m. and are considered significant at P<0.05.

Results

Effect of intestinal perfusion

The esophagus-cannulated eels started drinking sea water immediately after recovery from anesthesia. However, when the intestine was perfused with normal Ringer’s solution, this copious drinking was depressed (Fig. 1). After stopping the perfusion (i.e. beginning dehydration), the eels started to drink again. Similar depression in the drinking behavior was also observed after perfusion with iso-osmotic NaCl. When iso-osmotic mannitol was perfused, the drinking rate was depressed initially but later increased gradually. In some preparations, drinking was finally induced by mannitol perfusion after 2 days. Water intake was also depressed by NaCl perfusion (Fig. 1). Since water absorption across the intestine of the seawater-adapted eel is due to a Na+/K+/Cl− cotransporter localized on the brush-border membrane of the intestinal epithelium (Ando and Utida, 1986; Ando and Subramanyam, 1990), it is likely that the luminal NaCl perfusion stimulates NaCl absorption, and consequently water absorption, hence increasing blood volume. Enhancement in the blood volume has been shown to inhibit drinking behavior in the eel (Hirano, 1974).

However, this possibility was ruled out by the following studies. In these experiments, the composition of the perfusion medium could be changed without changing the perfusion rate. Although sodium methylsulfate perfusion (no Cl− present) induced drinking, perfusion with choline chloride (no Na+ present) did not (Fig. 2). In addition, application of furosemide, an inhibitor of Na+/K+/Cl− cotransport in the seawater-adapted eel intestine (Ando and Utida, 1986), also did not induce drinking (Fig. 2).

Fig. 3 shows simultaneous recordings of the drinking rate and urine flow. During mannitol perfusion, the drinking rate was high and the urine flow was low. However, when the perfusion medium was replaced with iso-osmotic NaCl, the drinking rate was reduced after approximately 1 h and the urine flow increased.
Effects of intestinal Na⁺ and Cl⁻ levels on eel drinking behavior

After 2.5 h. Although urine collection was technically difficult, the mean time lag from a decreased drinking rate to an increased urine flow in six preparations was 111±21 min (mean ± S.E.M.).

Fig. 4 shows the time course of the drinking rate after replacement of mannitol with NaCl. During perfusion with iso-osmotic mannitol, a relatively constant drinking rate of 254.8±46.2 ml 10 min⁻¹ was maintained for more than 140 min. When iso-osmotic NaCl was substituted for mannitol, the drinking rate decreased, finally falling to near zero. Similar decreases in drinking rate were also observed after switching the perfusate to iso-osmotic NMDG–HCl, Tris–HCl, choline chloride or RbCl (Fig. 5). In contrast, perfusion with iso-osmotic KCl tended to increase the drinking rate, although its effect varied among individuals.

When mannitol was switched to iso-osmotic sodium acetate, sodium methylsulfate or NaSCN, the drinking rate was enhanced threefold (Fig. 6). Similar enhancement was also obtained after replacement with iso-osmotic sodium butyrate solution (data not shown). In contrast, NaNO₃ or NaBr substitution decreased the drinking rate, as in the case of NaCl (see Fig. 4).

**NaCl concentration in the fluid passed through the digestive tract**

The fluid that passed through the esophagus was also collected, and Na⁺ and Cl⁻ concentrations were determined. Fig. 7 shows the relationships between the drinking rate and Na⁺ or Cl⁻ concentrations in the fluids after ingestion. As the drinking rate increased from 0 to approximately 0.5 ml 10 min⁻¹, the concentrations of Na⁺ and Cl⁻ in this fluid also increased. However, a relatively constant concentration was maintained at drinking rates above 0.5 ml 10 min⁻¹. Even at the highest drinking rates, the Na⁺ and Cl⁻ concentrations were significantly lower than those in sea water (Table 1). Table 1 shows the mean concentrations of Na⁺ and Cl⁻ in sea water before and after it has passed through the esophagus or the stomach. In the latter case, a cannula was attached to the gastrointestinal junction, and all fluids passed through the stomach were collected. Fluids with these concentrations of Na⁺ and Cl⁻ presumably enter into the intestine in vivo.

**Discussion**

The esophagus-cannulated preparation appears to be a good system with which to measure drinking rate, because water absorption across the esophagus is negligible in seawater-adapted eels (Hirano and Mayer-Gostan, 1976; Nagashima and Normal Ringer Dehydration

Fig. 1. Effects of intestinal perfusion with NaCl or mannitol on the drinking rate of Anguilla japonica. The record is continuous from the top down. Perfusion with normal Ringer’s solution was started at the first arrow and stopped at the second arrow, at which point a period of dehydration begins. Iso-osmotic NaCl solution was perfused between the third and the fourth arrows. At the fifth and sixth arrows, iso-osmotic mannitol and NaCl solutions were perfused, respectively. Each spike represents 27.8 µl of swallowed water. The perfusion rate was 10 ml h⁻¹.

Fig. 2. Effect of inhibiting NaCl cotransport across the intestinal epithelium of Anguilla japonica. NaCl cotransport was inhibited by perfusing with media which did not contain Cl⁻ (upper panel) or Na⁺ (lower panel). Furosemide, an inhibitor of Na⁺/K⁺/Cl⁻ cotransport, was also applied to the lumen of the intestine (10⁻⁵ mol l⁻¹; lower panel). Each spike represents 27.8 µl of swallowed water. The perfusion rate was 10 ml h⁻¹ and the perfusate was switched from mannitol to NaCl at time zero.
Using the esophagus-cannulated eel, the present study demonstrates that intestinal perfusion with iso-osmotic mannitol induces drinking in seawater-adapted eels following an initial reduction in drinking rate. The initial reduction may be due to intestinal distention, since a similar distention of the stomach has been demonstrated to inhibit drinking rate in the seawater-adapted eel (Hirano, 1974). Enhanced water intake induced by intestinal perfusion with mannitol may be attributed to the lack of Cl$^-$ in the luminal fluid of the intestine, as discussed below.

After replacement of mannitol with iso-osmotic NaCl as the perfusate, the water intake was depressed (Figs 1, 4). Since an enhancement in blood volume inhibits drinking in the seawater-adapted eel (Hirano, 1974), this inhibition may be due to an increase in water absorption across the intestine. In an in vitro system, we have also demonstrated that intestinal water absorption depends on luminal Na$^+$, K$^+$ and Cl$^-$ concentrations (Ando, 1983; Ando and Utida, 1986). The present study also

**Fig. 3.** Simultaneous recordings of drinking rate and urine flow. At time zero, the perfusate was switched from mannitol to NaCl. The upper record shows the drinking rate (each spike represents 27.8 μl) and the lower record shows the urine flow (each spike represents 17.9 μl). Perfusion rate was 10 ml h$^{-1}$.

**Fig. 4.** Time course of the effect of NaCl perfusion. After perfusion with iso-osmotic mannitol solution (M), the perfusion medium was replaced with iso-osmotic NaCl solution at time zero (●). As a control (○), mannitol solution was continuously perfused until 120 min. Each point represents the mean ± S.E.M. Numbers of eels are indicated in parentheses. Perfusion rate was 10 ml h$^{-1}$.

**Fig. 5.** Effect of intestinal perfusates containing Cl$^-$ on drinking rate. The drinking rate is expressed as a percentage of the mean value during mannitol (M) perfusion (−20 to 0 min). At time zero, the perfusion medium was switched from mannitol to iso-osmotic KCl (△, N=5), NMDG–HCl (○, N=5), RbCl (▲, N=5), Tris–HCl (□, N=5) or choline chloride (●, N=8). Each point represents the mean value, error bars have been omitted for clarity. Perfusion rate was 10 ml h$^{-1}$.
demonstrates that luminal substitution of Cl⁻ by CH₃SO₄⁻ induces drinking (Figs 2, 6), which might possibly be explained by inhibition of intestinal water absorption.

Table 1. Na⁺ and Cl⁻ concentrations along the digestive tract of seawater-adapted eels Anguilla japonica

<table>
<thead>
<tr>
<th>Region</th>
<th>N</th>
<th>[Na⁺] (mequiv l⁻¹)</th>
<th>[Cl⁻] (mequiv l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>External medium (sea water)</td>
<td>10</td>
<td>413.3±12.5</td>
<td>482.3±5.8</td>
</tr>
<tr>
<td>Esophagus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>High drinking rate (&gt;0.5 ml 10 min⁻¹)</td>
<td>80</td>
<td>310.5±2.8*</td>
<td>407.5±4.8*</td>
</tr>
<tr>
<td>Low drinking rate (&lt;0.3 ml 10 min⁻¹)</td>
<td>30</td>
<td>213.5±4.8*†</td>
<td>285.0±8.8*†</td>
</tr>
<tr>
<td>Stomach</td>
<td>24</td>
<td>177.5±4.5*</td>
<td>228.8±4.3*</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M.  
*P<0.001 compared with sea water (Student’s t-test).  
†P<0.001 compared with high rate of drinking (Student’s t-test).

Fig. 6. Effect of intestinal perfusates containing Na⁺ on drinking rate. The drinking rate is expressed as a percentage of the mean value during mannitol (M) perfusion (−20 to 0 min). At time zero, the perfusion fluid was switched from mannitol to iso-osmotic sodium acetate (○, N=5), sodium methylsulfate (●, N=5), NaSCN (△, N=4), NaNO₃ (■, N=5) or NaBr (▲, N=5). Each point represents the mean value; error bars have been omitted for clarity as in Fig. 5. Perfusion rate was 10 ml h⁻¹.

Fig. 7. Relationship between esophageal desalination and drinking rate. (A) Relationship between esophageal Na⁺ concentration and drinking rate (DR); (B) Relationship between esophageal Cl⁻ concentration and drinking rate. Each point represents an individual value obtained in the experiments shown in Figs 4–6. Open circles and dashed lines indicate the Na⁺ and Cl⁻ level in the external environment (sea water). Solid lines show regression lines ([Na⁺]=140DR+183, r=0.5075, P<0.005; [Cl⁻]=187DR+270, r=0.4850, P<0.005).

However, this explanation is not appropriate, because replacement of Na⁺ with choline does not induce drinking (Figs 2, 5). Although Na⁺ might diffuse out from the blood and hence increase the luminal Na⁺ concentration, because the eel intestine is more permeable to Na⁺ than to Cl⁻ (Ando et al. 1975), this possibility was eliminated in the present study. Even in the presence of luminal furosemide, an inhibitor of the Na⁺/K⁺/Cl⁻ cotransporter and consequently of water absorption, choline chloride perfusion did not induce drinking (Fig. 2). Since NaCl and water absorption are blocked by furosemide or bumetanide in the seawater-adapted eel intestine (Ando and Utida, 1986; Ando, 1987; Ando and Subramanyam, 1990), these results suggest that intestinal Cl⁻ itself inhibits drinking of sea water.

The inhibition of drinking preceded by 1–2 h the increase in urine flow after NaCl perfusion (Fig. 3). This suggests that it
takes much longer for a sufficient increase in blood volume to trigger increased urine flow than to inhibit drinking. However, the time lag seems to be too long to accept this explanation. The most simple explanation that is compatible with our other observations is that the inhibition of drinking precedes the increase in blood volume.

The inhibition of drinking seems to be specific to Cl\(^-\) perfusion, since perfusion with NMDG–HCl, Tris–HCl, choline chloride and RbCl all inhibit seawater drinking (Fig. 5). Although KCl also contains Cl\(^-\), an increase in drinking followed its perfusion; however, high K\(^+\) levels may depolarize many cells, including neurons along the intestinal tract, and thus may result in stimulation of drinking.

When Cl\(^-\) is omitted from the perfusion medium, Na\(^+\) appears to stimulate drinking, since sodium acetate, sodium butyrate, sodium methylsulfate and NaSCN perfusions all enhanced water intake (Fig. 6). Although NaNO\(_3\) and NaBr have inhibitory actions, this may be explained by similar actions of NO\(_3\)^-, Br\(^-\) and Cl\(^-\). From these results, it seems likely that intestinal Na\(^+\) and Cl\(^-\) produce opposite actions on seawater drinking behavior but that the action of Cl\(^-\) is dominant, since simultaneous application of Na\(^+\) and Cl\(^-\) inhibits drinking (Fig. 4). Under natural conditions, the concentrations of Na\(^+\) and Cl\(^-\) in the ingested fluid vary as shown in Table 1, but do not vary independently of each other as in the present study. However, when intestinal NaCl concentration ([NaCl]) is reduced below a certain level, Na\(^+\)-induced drinking may accelerate the water intake, if the threshold concentration of Cl\(^-\) required for inhibition of drinking is higher than that of Na\(^+\).

The results from the present study suggest a role for [NaCl] in the seawater drinking of eels. If a high concentration of NaCl enters the lumen of the intestine, the drinking rate will be lowered; thus, the fluid passing through the esophagus will be slowed down. This process will result in a reduction in the NaCl concentration of the fluid in the esophagus, as shown in Fig. 7, and hence in [NaCl]. Intestinal absorption of NaCl further accelerates the decrease in [NaCl]. When [NaCl] is lowered below a critical point, at which Cl\(^-\) inhibition becomes ineffective, Na\(^+\) may stimulate seawater drinking. The absence of Cl\(^-\) in the luminal fluid may therefore have contributed to the mannitol-induced drinking observed in this study. However, mannitol-induced drinking was also observed in the absence of Na\(^+\), although after a long latent period which differed among preparations. Since the esophagus of the seawater-adapted eel is able to remove NaCl from the ingested sea water (Hirano and Mayer-Gostan, 1976; Nagashima and Ando, 1994; Table 1) and since the efficacy of this desalinization is higher at high concentrations of Na\(^+\) and Cl\(^-\) (Nagashima and Ando, 1994), the Na\(^+\) and Cl\(^-\) concentrations in the esophagus may be kept at a relatively constant level at higher drinking rates (Fig. 7). The processes by which Na\(^+\) and Cl\(^-\) in the intestine actually control drinking behavior still remain to be explored.

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