Amiloride-sensitive sodium signals and salt appetite: multiple gustatory pathways

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Rats that are depleted of Na⁺ will avidly ingest Na⁺-containing solutions, even at concentrations that they would normally reject, a behavior known as Na⁺ appetite (5, 21). Consumption of Na⁺ solutions during Na⁺ appetite is enhanced further when inhibitory, postigestive signals are minimized by allowing ingested fluid to pass through an open gastric fistula (sham drinking) (10, 19, 22). Under Na⁺-depleted conditions, rats are highly motivated to consume and will selectively target Na⁺-containing solutions when non-Na⁺ salt solutions are also presented (5). The selectivity of the behavior necessitates that the rat be equipped with sensory machinery for detecting the Na⁺ and distinguishing it from others. Two potential players in the taste coding of Na⁺ have emerged from the literature: the chorda tympani (CT), which is a branch of the greater superficial petrosal nerve (GSP) nerves] have been reported to lack amiloride sensitivity (Refs. 8 and 11, respectively), it could be suspected that amiloride-sensitive Na⁺ signals are conveyed exclusively by the CT. However, recent evidence suggests otherwise. Spector et al. (30) have observed greater disruption of NaCl-KCl discrimination with amiloride treatment than with CT transection, suggesting that amiloride-sensitive taste receptors other than those in the CT-innervated anterior tongue mediate taste-guided behavior toward Na⁺. This idea was strongly supported by recent electrophysiological evidence of amiloride sensitivity of another primary gustatory nerve, the GSP (27).

The present studies further examine the hypothesis that amiloride-sensitive Na⁺ signals are critical for taste-guided behavior toward Na⁺ and that these signals are not conveyed exclusively by the CT.
GENERAL METHODS

Subjects
Male Long-Evans rats (University of Washington breeding colony), weighing between 325 and 375 g at the start of experiments, were housed singly in stainless steel wire cages on a 12:12-h light-dark schedule. Teklad Rodent Chow (Madison, WI) and water were available ad libitum except where otherwise noted.

Presurgery Habituation

For all experiments, rats were habituated to the testing apparatus before surgery. All solutions used during habituation and testing were made with reagent-grade chemicals in distilled water. Rats were weighed each morning and then placed individually in a cylindrical Plexiglas chamber with a raised, grated floor. Approximately 18 h before the first habituation session, animals were deprived of food and water, and a graduated drinking tube filled with an 8% sucrose solution was made available to the animals for 1 h. The sucrose solution was used during habituation because of its palatability and its ability to promote solution sampling. After the habituation session the animals were returned to their home cages and given ad libitum access to food and water. Ad libitum food and fluid access was allowed on all subsequent habituation days. Daily access to the sucrose solution in the libitum food and fluid access was allowed on all subsequent home cages and given ad libitum access to food and water. Ad libitum food and fluid access was allowed on all subsequent habituation days. Daily access to the sucrose solution in the testing apparatus continued for 6 days. On day 7 of habituation, all animals were depleted of Na⁺ (see Sodium Depletion) and received 1-h access to a 0.1 M NaCl solution 24 h post-Na⁺ depletion. Intakes were measured at 1 h, and subjects were matched on their NaCl intake when assigned to experimental conditions.

Sodium Depletion

The diuretic furosemide (Astra USA, Westborough, MA) was used to induce acute Na⁺ depletion in a procedure based on Wolf (35) and discussed previously (22). Briefly, rats were accustomed to Na⁺-deficient chow (ICN Biochemicals, Cleveland, OH) ~12 h before the diuretic treatment. Just before depletion, food and water were removed from all cages, and the animals were weighed. Depletion was accomplished by administering a total of 10 mg/kg of furosemide in two subcutaneous injections 1 h apart and was confirmed by comparing weights taken 3 h after the first injection with preinjection weights. The criterion for weight loss was ≥15 g. On average, animals lost ~25 g, and no animals had to be excluded. After animals were weighed, they were given ad libitum access to Na⁺-deficient chow and distilled water.

Surgery

Animals were fitted with gastric cannulas by means of techniques described previously (24). Rats were food deprived 18 h before surgery and were anesthetized with a mixture of ketamine (100 mg/ml; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (20 mg/ml; Phoenix Scientific, St. Joseph, MO) in a ratio of 2:1 (ketamine/xylazine; 0.12 ml/100 g body wt ip). The peritoneal cavity was opened, and the stomach was exposed. The cannula was inserted into the lumen of the stomach through a puncture wound made along the greater curvature of the stomach and secured by purse-string sutures. It was then exteriorized through the muscle and skin layers and sutured in place. The cannula was closed with a stainless steel screw that was removed for sham-drinking sessions. Animals were permitted 7 days to recover from surgery before the cannula was opened again for rinsing of the stomach.

Postsurgery Habituation

Animals recovered from surgery for 3 days. On the fourth day animals had 1-h access to an 8% sucrose solution as before surgery. This continued until day 7 after surgery. For all subsequent days of habituation, animals had their food and water removed 1 h before access to the sucrose solution. Then each animal had its stomach rinsed with ~60 ml of distilled water (37°C) until the rinse became clear. One hour later, animals had access to the sucrose solution for 1 h. This procedure continued for 4 days, at which time testing began.

Sham Drinking Test

All studies used the sham-drinking method to focus on orosensory signals generated by the salt solutions and to minimize their postdigestive effects. This approach also prevents any diuretic and natriuretic effects of the amiloride solution. Na⁺ appetite was induced in CTX and sham-operated rats, and the rats were then allowed to sham drink NaCl solutions with or without amiloride. In each experiment, just before the start of the test session, gastric fistulas were fitted with a stainless steel insert attached to a lightweight polyethylene tube. The tube was led out of the experimental apparatus and into a collection vial. Collected fluid was measured at the end of the test session and compared with the fluid intake of the rats. In all cases, collected fluid closely matched intake values (>85% of fluid ingested was recovered).

Statistical Analysis

To statistically confirm group differences, both parametric (ANOVA, t-tests) and nonparametric (Kruskal-Wallis, Mann-Whitney U tests) statistical analyses were performed. The rationale for the addition of nonparametric analyses was to confirm results obtained with parametric analyses on tests with small sample sizes and nonhomogeneous variances (see EXPERIMENT 2, Results). In all cases of null hypothesis rejection, with one exception (see EXPERIMENT 2, Results), parametric and nonparametric analyses yielded identical statistical outcomes. Thus only parametric analyses will be presented except when statistical outcomes differed.

EXPERIMENT 1

Method

We tested the effect of blocking amiloride-sensitive Na⁺ channels on the sham drinking of a 0.1 M NaCl solution in rats with Na⁺ appetite and compared this intake with that of rats sham drinking a non-Na⁺ salt solution (0.15 M KCl). Na⁺-depleted rats will avidly consume hypertonic (0.3 M NaCl) as well as hypotonic (0.1 M NaCl) solutions. The lower concentration was used here to increase the likelihood that the 100 μM amiloride solution would be effective in blocking Na⁺ at the taste bud membrane and yield unambiguous results. It should also be noted that, although considered palatable, 0.1 M NaCl is not consumed in significant amounts by Na⁺-replete rats in short-term drinking tests. Rats were randomly assigned to one of three groups that differed only in what they would receive to sham drink: a 0.1 M NaCl solution (n = 9), a 0.1 M NaCl
in 100 μM amiloride solution (n = 10), or a 0.15 M KCl solution (n = 10). KCl was chosen as a control solution because other laboratories have shown deficits in NaCl-KCl discrimination after CTX or amiloride application (3, 30). We selected 0.15 M KCl because it is a concentration intermediate to that used by Breslin et al. (3). These groups were matched for similar intake during their first Na⁺ appetite session (see Presurgery Habituation). Twenty-four hours before the start of the test session, rats had their stomachs rinsed, and Na⁺-deficient food and water were removed from their home cages for the 1-h interval. Just before the start of the intake test session, gastric cannulas were opened, and a tube was inserted to carry fluid leaving the stomach into a collection vial. Rats were then placed in the testing chamber and allowed to sham drink for 2 h, with intake measured every 15 min for the first hour and every 30 min thereafter.

Results

Substantial drinking was observed in rats consuming the 0.1 M NaCl solution, and the response was markedly attenuated in animals drinking either 0.1 M NaCl mixed with amiloride or 0.15 M KCl (see Fig. 1). Using a one-way ANOVA, we observed a significant main effect of type of solution on 2-h sham drinking [F(2,24) = 12.2, P < 0.01]. This was a result of a significant difference between the group receiving 0.1 M NaCl and that receiving 0.1 M NaCl in 100 μM amiloride [41.9 ± 9.0 vs. 6.9 ± 3.7 ml, respectively; t(17) = 3.37, P < 0.01], as well as a significant difference between the group receiving 0.1 M NaCl and that receiving 0.15 M KCl [41.9 ± 9.0 vs. 8.8 ± 2.3 ml, respectively; t(17) = 3.46, P < 0.01]. There was no statistically reliable difference between the group receiving 0.1M NaCl in 100 μM amiloride and that receiving 0.15 M KCl.

Discussion

Na⁺-depleted rats displayed strong avidity for a 0.1 M NaCl solution, especially compared with the low intake of non-depleted rats (1.46 ± 0.34 ml; n = 10). On the other hand, Na⁺-depleted rats were not driven to sham drink a substantial quantity of a non-Na⁺ salt solution. Rats sham drinking the same NaCl solution but with added amiloride showed significantly less avidity for the solution, with intakes similar to those seen with KCl. These results are consistent with results obtained using different methods (2, 17). Although it is possible that rats drink so little of the amiloride-NaCl solution because amiloride rendered the NaCl solution tasteless, we believe this is unlikely for the following reasons. There is a significant, although reduced, CT response to an amiloride-0.1 M NaCl solution (7). Behaviorally, 0.05–0.2 M NaCl solutions, when mixed with amiloride, appear to be more difficult for rats to discriminate from KCl (30). The present results are consistent with the idea that signals generated by the passage of Na⁺ through amiloride-sensitive Na⁺ channels on the tongue are critical for the detection of Na⁺ and therefore of the normal, robust expression of Na⁺ appetite. Experiment 2 used a similar methodology to determine if amiloride-sensitive Na⁺ signals, which drive Na⁺ appetite, are conveyed exclusively by the CT. If the CT is the sole carrier of amiloride-sensitive Na⁺ signals that drive Na⁺ appetite, then amiloride should have no additional suppressive effect on intake in animals with bilateral CT transection.

**EXPERIMENT 2**

Method

A new group of rats was habituated as described in **EXPERIMENT 1**. During the gastric cannula implantation surgery, half of the rats received bilateral CT transection, and the other half received sham surgeries (see CT transection). Animals were randomly assigned to surgery condition, and the groups were matched for NaCl intake during their initial Na⁺ appetite test. On the test day rats were further divided into four groups: sham operated-0.1 M NaCl (n = 4), sham operated-0.1 M NaCl in 100 μM amiloride (n = 4), CTX-0.1 M NaCl (n = 3), and CTX-0.1 M NaCl in 100 μM amiloride (n = 4). Postsurgery habituation was carried out as described in **EXPERIMENT 1**. Twenty-four hours before the start of the test session, all rats were Na⁺ depleted as described above. One hour before the start of the test session, the rats’ stomachs were rinsed, and food and water were removed from the home cages for the interval. Just before the start of the intake test session, gastric cannulas were opened, and a tube was inserted to carry fluid leaving the stomach into a collection vial. Rats were then placed in the testing chamber and allowed to sham drink for 2 h, and intake was measured every 15 min for the first hour and every 30 min thereafter.
CT transection. CT transection was performed according to a previously published procedure (31). Briefly, the animal was turned on its side and, with the aid of a dissecting microscope, the tympanic membrane and the auditory bones behind it were visualized. The membrane was punctured with no. 5 microforceps, and a large portion of it was removed. This constituted the sham operation. For nerve transection, a small caudal portion of the bony meatus was removed, and the CT was visualized passing behind the malleus. The nerve was sectioned with microscissors, and the auditory ossicles were removed. Rats then followed the postsurgery habituation regimen described in EXPERIMENT 1.

Histology. To confirm nerve transection, at the end of behavioral testing rats were deeply anesthetized (100 mg/kg pentobarbital sodium) and perfused intracardially with isotonic PBS, and tongues were removed and postfixed in vials filled with 4% paraformaldehyde in 0.1 M phosphate buffer. After 2 wk of postfixing, tongues were removed and rinsed in distilled water. Under a light microscope, fungiform papillae were identified and counted. In the intact rat, each fungiform papilla contains a single taste bud with a pore. If no pore exists, then the taste bud is believed to have degenerated, which indicates that the innervation of the taste bud has been eliminated (18). The percentage of papillae with a pore served as a measure of denervation. A criterion of <15% of papillae with pores was set as an indication of a successful transection, based on previous work (10, 32). An observer blind to experimental categories counted papillae and pores. Every CTX rat met criterion and none had to be excluded from the study.

Results

As in experiment 1, intact rats displayed substantial sham drinking of a 0.1 M NaCl solution, and intake was dramatically suppressed in intact rats by adding amiloride to the solution. Consistent with previous reports (10), CTX rats displayed a blunted Na+ appetite when drinking a 0.1 M NaCl solution, but intake was even further reduced in CTX rats drinking NaCl with amiloride added (see Fig. 2). To determine the significance of these effects, we performed a two-way ANOVA. The presence of amiloride in the NaCl solution significantly reduced sham drinking in rats with Na+ appetite [F(1, 14) = 6.47, P < 0.05; see Fig. 2]. There was no significant main effect of CT transection, but there was a trend toward a significant interaction between CT status and solution [F(1, 14) = 3.66, P = 0.082]. This trend suggested that the effect of CT transection mattered only when no amiloride was present. Whereas a parametric test of significance (t-test) could not confirm a statistical difference, a nonparametric test of significance (Mann-Whitney U), which is less affected by small sample sizes and unequal variances (present here), revealed a significant difference between sham-operated and CTX rats drinking 0.1 M NaCl (28.0 vs. 11.0 ml median, respectively; U = 0.5, P = 0.05) but no statistically significant difference between sham-operated and CTX rats drinking 0.1 M NaCl in 100 µM amiloride.

The significant main effect of amiloride in solution suggested that amiloride treatment reduced intake regardless of whether the CT was intact or not. Results from experiment 1 showed a significant reduction in intake with amiloride in the NaCl solution. In this study, we replicated that effect and also found a significant effect of amiloride in CTX rats [8.7 ± 3.4 vs. 0.5 ± 0.3 ml, CTX-0.1 M NaCl vs. CTX-0.1 M NaCl in 100 µM amiloride; t(5) = 2.86, P < 0.05]. There was no significant difference between sham-operated and CTX rats when amiloride was in solution.

Discussion

Amiloride treatment and CT transection each appeared to reduce the NaCl intake of sham drinking rats. These results suggest that signals that are conveyed via the CT are important for the full expression of Na+ appetite. A significant effect of CT transection on sham drinking of NaCl has been demonstrated previously, although NaCl concentrations were higher and group sizes were larger (10). The overall effect size, however, was similar in magnitude. Interestingly, amiloride treatment had a greater effect than did surgical transection of the CT. If all behaviorally relevant, amiloride-sensitive Na+ signals were conveyed by the CT, then disruption of amiloride-sensitive taste receptors or CT transection alone should result in similar effects. That this was not the case, combined with the fact that amiloride treatment reduced NaCl intake in CTX and not just sham-operated rats, strongly suggests that at least one other primary gustatory pathway carries behaviorally relevant, amiloride-sensitive Na+ signals.

In both experiments 1 and 2, the addition of amiloride to NaCl dramatically decreased intake. The proposed explanation of this effect is that, by blocking amiloride-sensitive Na+ signals, the ionic content of the solution is disguised and therefore fails to trigger a full Na+ appetite. A less interesting explanation is that amilo-
ride tastes bad or its addition to NaCl leads to an unpalatable taste. It is unlikely that amiloride in solution, by itself, tastes bad, inasmuch as previous research suggests an inability of rats to taste amiloride (15). Experiment 3 investigated this issue further by assessing whether the amiloride-NaCl solution has an inherently unpalatable taste. If the amiloride-NaCl mixture is genuinely unpalatable, then thirsty rats should avoid it.

EXPERIMENT 3

Method

Intact rats from experiment 2 were water deprived for ~20 h and then offered either 0.1 M NaCl or 0.1 M NaCl in 100 µM amiloride to sham drink for 2 h.

Results

Intake did not differ between thirsty rats drinking 0.1 M NaCl with or without amiloride in solution [35.5 ± 10.7 vs. 47.2 ± 13.2 ml, 0.1 M NaCl vs. 0.1 M NaCl in 100 µM amiloride; t(6) = −0.69, P = 0.52; see Fig. 3].

Discussion

These results suggest that the amiloride-adulterated NaCl solution is not inherently unpalatable because thirsty rats sham drank similar amounts of a 0.1 M NaCl solution irrespective of whether the solution contained amiloride. Therefore, these results favor the explanation that Na⁺-depleted rats sham drank less 0.1 M NaCl with amiloride in the previous experiments because of a specific disruption of Na⁺ detection rather than a nonspecific aversive quality of the solution. These findings are in agreement with previous work suggesting that rats are unable to taste amiloride itself (15).

GENERAL DISCUSSION

Sodium appetite is a motivated behavior triggered by Na⁺ deficit. The appetite is highly specific for Na⁺-containing substances and is apparently unlearned (5). The specificity and selectivity of this behavior require appropriate gustatory coding to support it. To date there has been evidence that gustatory signals conveyed by the CT nerve play an important role in the identification of Na⁺-containing solutions (3, 16, 29, 32). Recordings of the activity of the whole CT nerve indicate a robust response to stimulation of the tongue by Na⁺ salts. Recordings of single-fiber activity indicate a population of fibers within that nerve that are highly selective for Na⁺ salts (9, 13, 20). Transection of the CT nerve interferes with Na⁺ appetite, reducing intake (10) and impairing the ionic specificity of the response (3). Another seemingly important component to Na⁺ coding is the mechanism for transduction of Na⁺ signals within taste buds. This function is believed to involve the passage of Na⁺ across the taste receptor cell apical membrane through amiloride-sensitive Na⁺ channels (for review, see Ref. 34). Until recently only the CT nerve has been proposed as a pathway by which amiloride-sensitive Na⁺ signals are conveyed to the central nervous system (CNS). It has been reported that the glossofaryngeal (8) and GSP (11) nerves do not convey amiloride-sensitive Na⁺ signals. Therefore, it could be concluded that amiloride-sensitive Na⁺ signals guide taste-mediated behavior toward Na⁺ via the CT innervation of the anterior tongue.

However, more recent studies suggest that the CT innervation of the anterior tongue is not the sole pathway by which amiloride-sensitive Na⁺ signals reach the CNS. St. John et al. (32) have examined the role of the CT and amiloride-sensitive Na⁺ channels in NaCl discrimination tests. They found that removal of the CT reduces performance on NaCI-KCl discrimination tasks, but that rats still perform above chance levels. However, adulteration of the salt solutions with amiloride reduced performance to chance levels, suggesting that in the CTX rat some amiloride-sensitive information is still present and contributing to discrimination performance (30). The residual amiloride-sensitive information must arise from a different taste receptor field. Recently, Sollars and Hill (27) have convincingly shown that the taste receptor field innervated by the GSP nerve contains amiloride-sensitive taste receptors, inasmuch as whole nerve responses of the GSP to Na⁺ stimulation are dramatically reduced by lingual application of amiloride. The present set of experiments extends the conclusions reached by these experiments by providing the first direct evidence that amiloride-sensitive Na⁺ receptors in taste fields not innervated by the CT contribute to taste-guided behavior toward Na⁺.

In sham-drinking rats, which have been depleted of Na⁺, gustatory signals are the primary signals that can direct and promote intake. The present studies provide clear evidence that amiloride-sensitive Na⁺ signals are essential for the display of Na⁺ appetite. The addition of amiloride to an NaCl solution dramatically reduced the sham intake of rats expressing Na⁺ appetite. That intake of amiloride-NaCl was not greater than that of KCl suggests that when amiloride-sensitive Na⁺ channels are blocked, identification of Na⁺ in solution does not occur. This general conclusion is consistent with prior studies (2, 17).
As previously reported (10), we observed that CT transection also reduced the sham intake of rats expressing Na⁺ appetite. However, the reduction in sham drinking was greater for animals offered amiloride-adulterated NaCl. The more dramatic effect of amiloride compared with CT transection is consistent with the work from Spector et al. (30). This difference in the magnitude of the effect is not consistent with the hypothesis that behaviorally relevant, amiloride-sensitive Na⁺ signals are conveyed through only the CT nerve. Also inconsistent with this hypothesis is the clear evidence that amiloride suppresses intake even in animals with bilateral transection of the CT nerve. Instead, these observations strongly suggest that, although the CT nerve may be an important pathway for amiloride-sensitive Na⁺ signals, it is not the only pathway that transduces amiloride-sensitive Na⁺ signals in the service of guiding behavior toward Na⁺.

In conclusion, the present studies provide strong support for the idea that amiloride-sensitive Na⁺ signals are critical to identification of Na⁺ during expression of Na⁺ appetite. These studies also support recent electrophysiological evidence that amiloride-sensitive Na⁺ signals travel to the CNS not only through the CT nerve but via at least one additional gustatory pathway. Finally, the results implicate this additional pathway as playing a role in taste-guided behavior toward Na⁺.

Perspectives

Ideas regarding physiological mechanisms underlying behavior gain significant power when results from physiological and behavioral experimentation support each other. Recording from the CT nerve, Formaker and Hill (7) reached the conclusion that “the membrane component sensitive to amiloride is the exclusive pathway in the generation of sodium taste responses.” Specter and colleagues (30) later showed that amiloride exposure reduces NaCl-KCl discrimination to chance levels, strongly supporting the conclusions reached from the physiological studies. Recently, Sollars and Hill (27) identified a second gustatory afferent nerve as having responses to Na⁺ that are suppressed by amiloride. Our findings strongly support this observation, showing that amiloride reduces Na⁺-directed behavior even when CT signals have been eliminated. Together, these data support the original statement by Formaker and Hill (8) but provide evidence that amiloride-sensitive signals critical to the behavioral response to the taste of Na⁺ are conveyed by at least two pathways: the CT and the GSP. Unlike effects on the expression of Na⁺ appetite and NaCl-KCl discrimination, amiloride exposure and CT transection only minimally affect behavior toward NaCl solutions in the absence of need (4, 25). Therefore, we support a rather specific, adaptive role for amiloride-sensitive Na⁺ signals in the gustatory identification of Na⁺. This specific detection ability is necessary during fine sensory discriminations and during the expression of Na⁺ appetite. In contrast, this ability does not normally play a role in NaCl preference in the absence of need.

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