Citrate Enhances Olfactory Receptor Responses and Triggers Oscillatory Receptor Activity in the Channel Catfish

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Parker, James M., Qinhui Chang, and John Caprio. Citrate enhances olfactory receptor responses and triggers oscillatory receptor activity in the channel catfish, *Ictalurus punctatus*. Citrate, a normal constituent of cellular metabolism, in a binary mixture with an amino acid enhanced asynchronous olfactory receptor responses in the channel catfish, *Ictalurus punctatus*. In addition, high concentrations of either citrate (≥3 mM) alone or an amino acid (≥0.1 mM) in a binary mixture with citrate (≥1 mM) triggered synchronized voltage oscillations of olfactory receptor neurons (ORNs) known as “peripheral waves” (PWs). Binary mixtures containing lower concentrations of an amino acid also triggered PW activity if the concentration of citrate in the mixture was increased. Both the enhancement of asynchronous activity and the generation of PW activity were the result of citrate chelating calcium, which lowers the surface potential of ORNs making them hyperexcitable. These effects of citrate are replicated by EGTA. Inactivation of the chelating ability of citrate and EGTA with 1 mM calcium chloride, barium chloride, or strontium chloride abolished both the enhancement of asynchronous olfactory responses and PW activity, while not affecting olfactory receptor responses to the amino acids alone.

**INTRODUCTION**

In recent studies, citric acid (or trisodium citrate), a six-carbon tricarboxylic hydroxy acid (or salt), enhanced taste responses to amino acids in the largemouth bass (Ogawa and Caprio 1999), catfish (Davis and Caprio 1996), and rat (Gilbertson et al. 1997). Citric acid, which is an additive to human food, acts as a preservative, a pH buffer, an antioxidant, and a flavor enhancer (Gardner 1972). Citrate is also used as an additive to increase the palatability of a variety of animal foods, such as its combination with phosphoric acid to enhance the flavor of cat food (Kealy 1975). Increased daily food intake and the enhanced behavioral effects of citrate addition in feeding studies of fish and mammals, the present study tested whether the addition of citrate to an amino acid stimulus also generated synchronized voltage oscillations of olfactory receptor neurons (ORNs) known as “peripheral waves” (PWs). Binary mixtures containing lower concentrations of an amino acid also triggered PW activity if the concentration of citrate in the mixture was increased. Both the enhancement of asynchronous activity and the generation of PW activity were the result of citrate chelating calcium, which lowers the surface potential of ORNs making them hyperexcitable. These effects of citrate are replicated by EGTA. Inactivation of the chelating ability of citrate and EGTA with 1 mM calcium chloride, barium chloride, or strontium chloride abolished both the enhancement of asynchronous olfactory responses and PW activity, while not affecting olfactory receptor responses to the amino acids alone.

**METHODS**

**Experimental animals**

Channel catfish, *Ictalurus punctatus* (25–50 g), were obtained from three different sources. Most fish used in this study were raised at the Louisiana State University Aquaculture Center and were maintained in floating cages held in ponds at the facility. Additional fish were obtained from Sander’s Fish Farm, a local hatchery in Pride, Louisiana. A third source was the Louisiana State University School of Veterinary Medicine. All experimental fish were fed weekly with floating commercial fish chow. Each week catfish were transferred to an aerated, 250-l polyethylene aquarium filled with charcoal-filtered city tap water at the Louisiana State University Animal Care Facility and maintained on a 12:12 light/dark regime. The temperature was held above 27°C during the spring and summer and below 20°C during the fall and winter to help avoid bacterial (*Edwardsiella ictaluri*) infection (Morrison and Plumb 1994). The fish were used experimentally within 1 wk of their placement in the animal care facility and were not fed during this period.

**Experimental procedures**

The preparation of the animals was the same as that described by Kang and Caprio (1991). Each catfish was initially immobilized with an intramuscular injection of the neuromuscular blocking agent, Flaxedil (gallamine triethiodide, 0.03 mg/100 g). During the experiments, additional injections were applied as needed via a hypodermic needle embedded in the flank musculature. The immobilized fish was wrapped in a wet Kim-Wipe, placed into a Plexiglas container, and stabilized using a pair of orbital ridge clamps. The gills were irrigated using an orally inserted glass tube supplying a constant flow of aerated, charcoal-filtered city tap water containing the anesthetic, 0.005% (initial concentration) MS-222 (ethyl-m-amino-benzoate methane sulfonic acid). Access to the olfactory organ was achieved by removing the skin and connective tissue between the incurrent and excurrent nares, superficial to the olfactory organ.
Stimuli

Representatives of four different classes of amino acids: l-glutamic acid (acidic), l-arginine (basic), l-methionine [neutral with a long side-chain (LCN)] and l-alanine [neutral with a short side-chain (SCN)] were presented individually and in binary mixtures with citrate (trisodium), EGTA [ethylene glycol-bis(aminoethylether)-N,N,N′,N′-tetraacetic acid], distilled water, calcium chloride, barium chloride, and strontium chloride. All stimuli used in the study were prepared using charcoal-filtered city tap water (containing 0.1–0.5 mM Ca$^{2+}$), pH adjusted (8.5–9.0) to match the control water bathing the olfactory organ and presented at concentrations varying from 10$^{-6}$ M to 10$^{-2}$ M. Stock solutions at 10 mM were prepared weekly, except for citrate, EGTA, CaCl$_2$, BaCl$_2$, and SrCl$_2$, which were prepared daily. During the experiments, a series of four or five consecutive odorants were preceded and followed by the presentation of the standard (1 mM l-methionine). Data recorded in response to these stimulus series were included in the study only if the initial and final responses to the standard differed by more than a 10% change in response magnitude, the data were excluded from the analysis. Stimulus delivery was via a “gravity-feed” system employing a spring-loaded valve driven by a pneumatic actuator at 40 psi. Stimulus solutions and charcoal-filtered artesian water used to bathe the olfactory mucosa were delivered through separate Teflon tubes (0.8 mm diam) at a rate of 6–8 ml/min. The olfactory cavity was continuously perfused with charcoal-filtered tap water to facilitate stimulus delivery, protect the mucosa from desiccation, avoid the introduction of mechanical artifacts associated with stimulus presentation, and thoroughly rinse the olfactory cavity between stimuli (3–5 min interstimulus intervals).

![Graph](http://example.com/graph.png)

**FIG. 1.** Citrate enhances olfactory receptor responses to amino acids. Absence of response to control water (A1 and A2) and responses to 0.1 mM l-methionine (l-Met; B1 and B2), 0.1 mM citrate (C1 and C2) and enhanced response to the binary mixture 0.1 mM l-Met and 0.1 mM citrate (D1 and D2). Labels with the number 1 are raw data, and those with 2 are integrated (0.5 s). In this and succeeding figures, stimulus duration was 5 s, and arrows indicate stimulus onset.

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>0.1 mM Citrate</th>
<th>0.5 mM Citrate</th>
<th>1.0 mM Citrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>l-Ala</td>
<td>7 (19)</td>
<td>25 (0)</td>
<td>214 (49)</td>
</tr>
<tr>
<td>l-Met</td>
<td>27 (0)</td>
<td>PW</td>
<td>PW</td>
</tr>
<tr>
<td>l-Arg</td>
<td>47 (0)</td>
<td>66 (0)</td>
<td>PW</td>
</tr>
<tr>
<td>l-Glu</td>
<td>16 (0)</td>
<td>28 (29)</td>
<td>20 (2)</td>
</tr>
<tr>
<td>l-Met</td>
<td>0 (5)</td>
<td>56 (0)</td>
<td>PW</td>
</tr>
<tr>
<td>l-Arg</td>
<td>48 (0)</td>
<td>PW</td>
<td>PW</td>
</tr>
<tr>
<td>l-Glu</td>
<td>44 (0)</td>
<td>10 (PW)</td>
<td>PW</td>
</tr>
<tr>
<td>l-Met</td>
<td>22 (42)</td>
<td>26 (37)</td>
<td>64 (100)</td>
</tr>
<tr>
<td>l-Arg</td>
<td>100 (25)</td>
<td>68 (26)</td>
<td>42 (0)</td>
</tr>
<tr>
<td>l-Glu</td>
<td>68 (16)</td>
<td>68 (25)</td>
<td>96 (7)</td>
</tr>
<tr>
<td>l-Met</td>
<td>0 (0)</td>
<td>57 (0)</td>
<td>PW</td>
</tr>
<tr>
<td>l-Arg</td>
<td>13 (43)</td>
<td>59 (0)</td>
<td>PW</td>
</tr>
<tr>
<td>l-Glu</td>
<td>13 (0)</td>
<td>13 (0)</td>
<td>PW</td>
</tr>
<tr>
<td>l-Met</td>
<td>20 (39)</td>
<td>60 (0)</td>
<td>67 (5)</td>
</tr>
<tr>
<td>l-Arg</td>
<td>58 (0)</td>
<td>67 (28)</td>
<td>68 (20)</td>
</tr>
<tr>
<td>l-Glu</td>
<td>59 (0)</td>
<td>89 (0)</td>
<td>PW</td>
</tr>
<tr>
<td>l-Met</td>
<td>16 (16)</td>
<td>16 (16)</td>
<td>10 (PW)</td>
</tr>
<tr>
<td>l-Arg</td>
<td>42 (0)</td>
<td>42 (0)</td>
<td>PW</td>
</tr>
</tbody>
</table>

Number in parentheses indicates [(response to trisodium citrate)/(response to amino acid) × 100]. Fish preparation indicated by superscript number. $R_M$, response magnitude to binary mixture; $R_{am}$, response magnitude to amino acid; PW, occurrence of peripheral waves.

A foot switch connected to an electronic timer triggered the valve to introduce the odorants for a 5-s stimulus duration.

**Recording technique and data analysis**

In vivo recordings of multiunit olfactory neural activity were made using metal-filled glass capillary electrodes plated with platinum (~15 μm ball; impedance, 10–40 kΩ) placed against the sensory face of an olfactory lamella (Caprio 1995; Gesteland et al. 1959). The electrode was r.c.-coupled (220 pF capacitor, 20 MΩ resistor) to a high-impedance probe at one input with the other input grounded via a hypodermic needle embedded in the flank musculature of the fish. The receptor neural activity was amplified (band-pass 30–300 Hz), observed on an oscilloscope, integrated (0.5 s rise time), and displayed on a pen recorder. These amplified signals were stored on a hi-fi stereo VCR as an analogue signal via one of the audio inputs or as a digitized video signal. All recorded data were digitized at 32 kHz and analyzed off-line by Discovery software (Brainwave Systems Discovery package Version 5.0, DataWave Technologies, Longmont, CO) and printed.

**RESULTS**

**Citrate and EGTA enhance ORN activity and trigger PW activity**

Citrate (0.1 and 0.5 mM) in binary mixtures with an amino acid (0.1 mM) enhanced asynchronous olfactory receptor responses (Fig. 1, Table 1). In addition, single amino acids (≥0.1
and binary mixtures of citrate and an amino acid selected from any of four classes (acidic, basic, LCN and SCN) of amino acids generated PWs (Fig. 2). During the first 2 s of PW activity, the mean frequency displayed was 28 ± 5.6 Hz (mean ± SE, n = 25 fish, 283 trials); however, at stimulus concentrations just above PW threshold, some records show apparently lower frequency (e.g., Fig. 2, A and B), but interspersed with the large waves are smaller ones that increase in magnitude with increasing stimulus potency. Frequencies sampled from the responses of nine fish during the 5th s of PW activity in response to a 5-s stimulus decreased by 7 ± 3.7 Hz (n = 84) from those measured during the 1st s of synchronous activity. No specific frequency or range of frequencies was associated with a particular stimulus or fish preparation (Table 2). Components of binary mixtures that triggered PW activity included 0.5 mM amino acid and 0.1 mM citrate. A lower concentration of an amino acid (10 mM) resulted in PWs if the concentration of citrate was raised to 0.5 mM. Individual presentations of 0.1 mM EGTA or 3 mM trisodium citrate alone triggered PWs (Fig. 3). PW activity is stimulus driven, because intrinsic oscillatory activity was never observed.

**Divalent cations abolish both the enhancement of asynchronous activity and PWs**

The possibility that the enhancement of olfactory receptor neural activity was due to citrate’s ability to chelate calcium prompted the testing of EGTA, a classical calcium chelator, to see whether it also caused an increase in responsiveness.

EGTA (30 μM) caused a similar enhancement of asynchronous responses (Fig. 4) as was seen with citrate (0.1 mM). The addition of divalent cations (1 mM CaCl₂, BaCl₂, or SrCl₂) to binary mixtures of an amino acid and citrate (Fig. 5, A–C) or EGTA (Fig. 5D) eliminated the enhancement of asynchronous olfactory receptor activity to a response similar to that to the single amino acid alone. PWs triggered by binary mixtures of citrate and an amino acid were also abolished in 100% of 42 trials by the addition of CaCl₂, BaCl₂, or SrCl₂, but the magnitude of responses to single amino acids in the presence of divalent cations was not appreciably reduced (Fig. 6). PWs in response to calcium chelators (citrate and EGTA) alone were

### Table 2. PW frequencies (Hz)* to odorant stimuli

<table>
<thead>
<tr>
<th>Test Stimulus</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM L-Met + 1 mM citrate</td>
<td>22</td>
<td>22</td>
<td>26</td>
<td>30</td>
<td>24</td>
<td>21</td>
</tr>
<tr>
<td>1 mM L-Ala + 1 mM citrate</td>
<td>18</td>
<td>25</td>
<td>30</td>
<td>23</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>2 mM L-Arg + 1 mM citrate</td>
<td>33</td>
<td>28</td>
<td>24</td>
<td>30</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>1 mM L-Met</td>
<td>26</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mM L-Ala</td>
<td>34</td>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Response frequencies are listed in the order in which they were recorded from the preparation. * Measured during the initial second of activity in response to a 5-s stimulus.

**FIG. 2.** Peripheral waves (PWs) are generated in response to mixtures of citrate and a representative from each of 4 classes of amino acids. PW responses to binary mixtures of 1 mM trisodium citrate and 1 mM L-Met (neutral with long side chain [LCN]; A), 1 mM L-alanine (L-Ala; neutral with short side chain [SCN]; B), 1 mM L-arginine (L-Arg; basic; C), and 2 mM L-glutamic acid (L-Glu; acidic; D).

**FIG. 3.** Calcium chelators trigger PW activity. Trisodium citrate (2 mM; A) and 10 mM EGTA (C) were insufficient to trigger PWs; however, 3 mM citrate (B) and 0.1 mM EGTA (D) caused PW activity.
abolished in 100% of 16 trials by the addition of CaCl₂ or BaCl₂ (Fig. 7).

Effects of distilled water

Single amino acid stimuli presented in four fish preparations (5-s duration) at concentrations normally insufficient to produce PW activity generated PWs when prepared in distilled water. The addition of 1 mM CaCl₂, however, prevented PW activity (Fig. 8).

DISCUSSION

Prior studies reported enhancements of taste responses to binary mixtures of citrate and amino acids (Davis and Caprio 1996; Gilbertson et al. 1997; Ogawa and Caprio 1999). Because both olfactory and taste systems of channel catfish are highly sensitive to amino acids (Caprio 1978), it was hypothesized that a similar enhancement by citrate as seen in gustation might also occur in olfaction. This study shows that trisodium citrate also enhances olfactory receptor responses in the channel catfish to amino acids (Fig. 1) and can trigger voltage oscillations known as PWs (Fig. 2). Olfactory receptor response enhancement was observed to binary mixtures of citrate and a representative of each of four classes of amino acids: neutral amino acid with a long, linear side-chain (L-methionine), neutral amino acid with a short side-chain (L-alanine), acidic amino acid (L-glutamate), and basic amino acid (L-arginine).

The generation of PW activity depends on surpassing a threshold level of depolarization required to initiate a synchronization of ORN responses. The coordinated activity of multiple ORNs responding to an odorant generates large PWs, which have been observed in representatives of every class of vertebrate [fish (Sutterlin and Sutterlin 1971), amphibian (Otto et al. 1959; Takagi and Shibuya 1961), bird (Tucker 1975), and mammal (Adrian 1956)]. PWs are odorant-driven (i.e., do not occur intrinsically), and in most species studied have a frequency that is significantly higher than olfactory bulbar...
waves; 2) are generated within the olfactory sensory epithelium (Parker et al. 1997; Sutterlin and Sutterlin 1971; Takagi and Shibuya 1961); 3) are not derived from elements other than ORNs (Takagi and Shibuya 1961); 4) are not influenced by bulbar electroencephalographic (EEG) waves, because transection of the olfactory nerves have no effect on either their magnitude or frequency (Sutterlin and Sutterlin 1971); and 5) possibly function as a logic gate to facilitate the release of glutamate onto mitral/tufted cells possessing N-methyl-D-aspartate (NMDA) receptors (Ennis et al. 1996), resulting in long-term potentiation associated with learning and memory (Brunjes 2000; Parker et al. 1999).

The present report clearly indicates that response enhancement by citrate is due to its ability to chelate calcium. The addition of calcium, barium, or strontium chloride to the binary mixture of an amino acid and citrate abolished this enhancement by inactivating the calcium chelating ability of citrate, thus confirming that it is the reduction of divalent cations that is responsible for the hyperexcited state of the ORNs. Lowering the extracellular calcium concentration with a conventional calcium chelator (EGTA) in a binary mixture with an amino acid produced a similar enhancement to that seen with citrate. This enhancement was also eliminated by the addition of divalent cations (1 mM CaCl₂, BaCl₂, or SrCl₂) to the stimulus mixture, which did not affect olfactory responses to single amino acids (Fig. 5). PW activity triggered by the presence of a calcium chelator in a binary mixture with an amino acid was also abolished by the addition of divalent cations (Fig. 6). One millimolar calcium is a concentration similar to that reported in the mucus surrounding the cilia of ORNs in the frog (Joshi et al. 1987). However, due to the greater potency for calcium chelation by EGTA, lower concentrations of EGTA (30 μM) achieved a similar enhancement of asynchronous olfactory activity as that observed with citrate (0.1 mM). In addition, PWs were observed to amino acid stimuli that by themselves were not sufficiently potent to generate synchronized voltage oscillations, but did so dissolved in distilled water. Peripheral waves were abolished with the addition of calcium chloride or barium chloride, which resulted in a response similar to that to the amino acid component alone (Fig. 8).

A reduced extracellular calcium ion concentration can result theoretically in the increased excitability of vertebrate ORNs due either to 1) lessening the calcium block on olfactory receptor cyclic nucleotide-gated (CNG) channels (Frings et al. 1995; Kleene 1995; Kleene and Pun 1996; Zufall and Firestein 1993) and/or 2) by lowering of the surface potential of neurons, shifting ion channel activation to a more negative potential (Hille 1992). Although CNG channels are involved in the transduction of olfactory information in tetrapods (Belluscio et al. 1998; Brunet et al. 1996; Firestein et al. 1991; Kurahashi 1990; Nakamura and Gold 1987), and in situ hybridization studies demonstrated the extensive presence of CNG channels in the olfactory epithelium of the channel catfish (Ngai et al. 1993), there is no evidence linking this specific channel type to olfactory responses to amino acids in this species (Bruch and Teeter 1990). Current data clearly show that IP₃-gated ion channels mediate olfactory responses to amino acids in the channel catfish (Bruch 1996; Restrepo et al. 1993) and other teleosts (Speca et al. 1999). Thus the mechanism for the presently described enhancement of ORN activity is consistent with being an effect on the divalent field charge at the membrane of ORNs. Under conditions of a lowered divalent

![Figure 7](http://jn.physiology.org/lookup/fig/2680)

**FIG. 7.** Addition of calcium chloride abolished PW activity to calcium-chelating stimuli. PWs generated in response to 5 mM citrate (A) and 0.1 mM EGTA (C) were abolished by the addition of 1 mM calcium chloride (B and D). E: lack of response to a water control.

![Figure 8](http://jn.physiology.org/lookup/fig/2680)

**FIG. 8.** Amino acid dissolved in distilled water triggers PWs. PWs were generated by 1 mM l-Met in distilled water (A). The same amino acid dissolved in charcoal-filtered tap water did not initiate PWs (B). Calcium chloride (1 mM) abolished PW activity observed in A (C).
citation concentration as occurring in the presence of citrate and EGTA, sodium ions entering through ciliary and microvillous IP₃ channels are sufficient to activate the ORNs (Schild and Restrepo 1998).

Binary mixtures of citrate and amino acids are most likely similar to chemical stimuli the catfish would normally be exposed to during feeding in its natural environment. Amino acids are present in high (up to a few hundred millimoles/liter) concentration in all living tissue (Carr 1988; Carr et al. 1996) and are naturally released into the aqueous environment. Citrate is present in all living cells that undergo aerobic respiration via the citric acid cycle and is also released in high (>30 mM) concentrations by common food sources for the channel catfish (e.g., insects) (Wyatt 1961). The use in the present study of naturally occurring and physiologically relevant concentrations of stimuli suggests that PWs occur in the animal’s natural environment.

The enhancement of taste responses with citrate in rodents occurred only in response to stimuli that caused depolarization of the taste receptor cells (Gilbertson et al. 1997). This finding correlates with the proposed mechanism in the present experiments for the enhancement of olfactory receptor responses, i.e., the ability of citrate to chelate calcium. With respect to citrate’s flavor-enhancing ability in human food (Gardner 1972), citrate may not add only a sour component, but is likely to enhance the taste of gustatory stimuli whose normal action is to depolarize taste cells.

We thank S. Finckbeiner for computer graphics assistance. This research was supported by National Science Foundation Grant IBN-9221891 and National Institute on Deafness and Other Communication Disorders Grant DC-03792 to J. Caprio. Address reprint requests to J. Caprio.

Received 4 November 1999; accepted in final form 31 January 2000.

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