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BEHAVIORAL RESPONSES OF THE CRAYFISH *PROCAMBARUS CLARKII* TO SINGLE CHEMOSENSORY STIMULI

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ABSTRACT

In some crustaceans, compounds that weakly stimulate peripheral chemoreceptor cells elicit disproportionately large behavioral responses. Here, we investigated whether this is the case in the crayfish *Procambarus clarkii*. Resting animals were exposed to either a blank or ammonium, glucose, glutamate, glycine, maltose, or trehalose at predicted final concentrations of 200 μM to 2 mM. Glycine significantly increased the time spent walking. Maltose increased the time spent walking and the number of clasps of the small claws (dactyl clasps). Trehalose triggered leg probing/waving and dactyl clasping. Ammonium and glutamate failed to elicit responses. These results are consistent with the varied efficacies of those compound in stimulating leg chemoreceptor cells as determined previously with physiological methods. Glucose, however, elicited all three behaviors that we quantified - a result inconsistent with the earlier finding that glucose fails to elicit action potentials in the leg's nerve. To determine whether glucose-sensitive chemoreceptor cells are present in the legs, 150 μl of glucose or trehalose, at concentrations of 10 μM or 100 μM, was applied focally to crayfish legs and dactyl-clasp frequency was determined. At a concentration of 100 μM, glucose elicited a significantly higher dactyl-clasp frequency than at 10 μM. Trehalose elicited high dactyl-clasp frequencies at both concentrations. Crayfish legs are, therefore, sensitive to glucose but they are more sensitive to trehalose. Overall, behavioral responses to single compounds largely paralleled the relative abilities of those compounds to stimulate leg chemoreceptor cells.

INTRODUCTION

A key question in chemical ecology is that of the identity of the chemical signals that an animal can sense. For crustaceans, the range of stimulatory compounds has been investigated with a variety of behavioral and physiological methods. In physiological nerve recordings, a compound's effectiveness as a stimulus can be quantified as the number of chemoreceptor cells that respond to that compound, the average number of action potentials elicited in those cells, the total number of spikes summed across all identified single cells, or the total amount of activity that the compound elicits in axon bundles (Case, 1964; Shepheard, 1974; Johnson and Ache, 1978; Bauer and Hatt, 1980; Johnson et al., 1984; Derby and Harpaz, 1988; Voigt and Atema, 1992; Corotto and O'Brien, 2002). A compound's effectiveness can also be quantified in terms of the magnitudes of the behavioral responses elicited by that compound (Hartman and Hartman, 1977; Robertson et al., 1981; Trott and Robertson, 1984; Borroni et al., 1986; Johnson and Atema, 1986; Harpaz et al., 1987; Tierney and Atema, 1988).

If one ranks chemical stimuli by their effectiveness, there are a number of reasons why rankings may differ depending on whether the data were obtained from the study of primary chemosensory neurons or from behavioral responses. Physiological studies generally focus on one appendage while behavioral studies often target the whole animal, and therefore other appendages with potentially different sensitivities. Physiological properties of chemoreceptor cells are known to vary from appendage to appendage in crustaceans (Voigt and Atema, 1992; Garm et al., 2005). Another possibility is that behavioral thresholds may be higher than physiological ones so that, at the right concentration, a chemical stimulus that elicits a physiological response may not trigger a behavior. This issue is complicated by the fact that physiological thresholds change through adaptation (Borroni and Atema, 1988), and behavioral thresholds change with an animal's activity state (Zimmer-Faust et al., 1996). Another reason why the relative efficacies of physiological stimuli may not parallel their abilities to evoke behavioral responses would be if chemoreceptor cells tuned to a particular compound, i.e., sensitive to that compound, exert a greater effect upon the CNS than cells tuned to other stimuli. In that case, an animal would be rendered especially sensitive to a key compound not through high sensitivity of its peripheral chemosensory system but by sensitivity of its CNS to sensory neurons that are responsive to that compound.

To see if an animal's CNS is particularly sensitive to afferent neurons tuned to key chemical stimuli, one would first compare the rank order of effectiveness of chemical stimuli obtained through study of peripheral physiology with the relative abilities of those stimuli to evoke behaviors. Studies have shown varied degrees to which behavioral and physiological efficacies parallel each other. In the shrimp *Macrobrachium rosenbergii* De Man, 1879, taurine evokes behavioral responses at lower concentrations than betaine and L-arginine (Harpaz et al., 1987), but all three compounds generate similar physiological response magnitudes when they are applied at 10 mM (Derby and Harpaz, 1988). In other cases, there is a greater disparity between rankings of stimulatory effectiveness obtained through behavioral and physiological methods. Clasping of the small claws (dactyl clasping) in the lobster *Homarus americanus* Milne Edwards, 1837, is evoked by ammonium but not glutamate (Borroni et al., 1986), even though more primary chemoreceptor cells respond to glutamate than ammonium and the glutamate-sensitive cells fire at a higher frequency (Johnson et al., 1984). Also, in the spiny lobster *Panulirus argus* Latreille, 1804, a variety of

amino acids and nucleotides stimulate antennular chemoreceptor cells (Johnson and Ache, 1978; Derby et al., 1984; Steullet and Derby, 1997), but glutamate is far more effective than the other compounds in eliciting antennular grooming behavior (Barbato and Daniel, 1997).

A similar mismatch between behavioral and physiological sensitivities may be present in crayfish. Sugars elicit feeding behavior in *Orconectes rusticus* Girard, 1852 (Tierney and Atema, 1988) but do not stimulate leg chemoreceptor cells in another crayfish, *Austropotamobius torrentium* Schrank, 1803 (Bauer and Hatt, 1980). While this could result from the presence of a very small number of sugar-sensitive chemo-receptor cells that carry a disproportionate influence on the CNS, the issue is confounded by the species difference and the fact that whole animals were exposed to stimuli in the behavioral study but not in the physiological study. To determine if there is a mismatch between physiological and behavioral sensitivity in crayfish, and to begin to assess whether some primary chemoreceptor cells have more influence on the CNS than others, both the physiological and behavioral studies must be done with the same animal. In addition, the behavioral work should involve focal stimulation of the same appendage as studied physiologically. Here, we analyzed behavioral responses of the crayfish *Procambarus clarkii* Girard, 1852, to compounds studied in a prior physiological study of the same species. The behavioral work involved exposing whole animals and single appendages to chemical stimuli.

Materials and Methods

Female and form I male red swamp crayfish (*Procambarus clarkii*) with carapace lengths of 29-48 mm were obtained from a local supplier and from Carolina Biological Supply Co. (Burlington, NC). They were maintained at room temperature in aerated, aged tap water on a 14:10 light:dark cycle and fed chicken, carrots, and spinach. Two bioassays were performed to investigate behavioral responses to chemical stimuli. In one, whole animals were exposed to potentially stimulatory compounds (the whole-animal bioassay). In the other, compounds were applied focally to crayfish legs (the focal-application bioassay). Animals used in the whole-animal bioassay were fed every other day. For the focal-application bioassay, animals were fed every third day and were always tested three days after having last been fed. All animals used had tapered, i.e., not truncated, first and second antennae and at least three of their second through fifth pereiopods (walking legs) intact on each side.

Whole-Animal Bioassay

Chemical stimuli were chosen to allow a comparison of their effectiveness in eliciting behavioral responses with their effectiveness in evoking action potentials in the pereiopod nerve as established by Corotto and O'Brien (2002). The following compounds were investigated in the whole-animal bioassay: trehalose, which is the most effective physiological stimulus found for *P. clarkii* leg chemoreceptor cells; L-glycine, which is a moderately effective stimulus for *P. clarkii* leg chemoreceptor cells; maltose and ammonium chloride, which are weak physiological stimuli; and D-glucose and L-glutamate, both of which failed to elicit unambiguous physiological responses from *P. clarkii* leg chemoreceptor cells. Stimuli were dissolved at a final concentration of 10 mM in dechlorinated tap water and frozen until use. Aliquots of dechlorinated tap water were also frozen to serve as blank controls. All stimuli, including the blanks, contained 0.05% fluorescein dye to allow us to determine the exact time when the stimulus contacted a crayfish. Initial experiments showed that crayfish do not respond to fluorescein dye alone.

For testing, crayfish were first placed individually into test chambers with flow-through dechlorinated water (Fig. 1A) and allowed to acclimate for at least two hours. Test chambers with crayfish were subsequently moved to the testing area, which was also equipped with flow-through dechlorinated water. Animals were tested no less than 15 minutes later. A crayfish was tested only when it was stationary in its shelter, the only allowable movements being antennule flicking (Schmidtt and Ache, 1979) and leg raking (Atema and Cobb, 1980). Chemical stimuli were introduced by injecting a 10-ml aliquot of 10 mM stimulus into the carrier flow of dechlorinated water. Based on conductivity measurements, we predict that crayfish were exposed to stimulus concentrations of at least 200 μM but not more than 2 mM (Fig. 1B). The high value of 2 mM would only be experienced for an instant and only by an animal directly in front of the stimulus inflow. Responses were filmed from below at 30 frames per second and analyzed with the use of iMovie (Apple Computer, Inc. Cupertino, CA). Each crayfish was tested once.

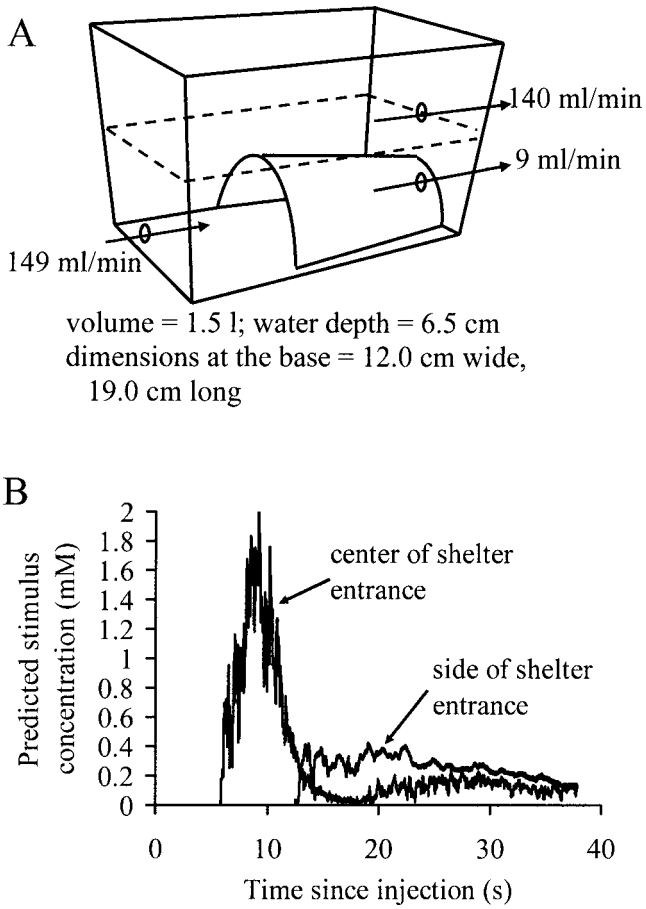

Fig. 1. A, Testing chamber for the whole-animal bioassay. Half of a 470 ml plastic cup served as the crayfish shelter. Flow rates refer to the carrier-flow of dechlorinated water into which a 10-ml volume of stimulus was introduced. Observations of injected fluorescein dye indicated that the stimulus entered the shelter along its midline, swirled within the shelter, and emerged at the sides of the shelter's opening. Crayfish within the shelter were presumably exposed to test stimuli that were intermediate in concentration between that at the center of the shelter's opening, where the stimulus entered, and that at the side, where the stimulus emerged. B, Stimulus dilution profile. To predict the concentration of the stimulus where it first entered the shelter and where it exited along the shelter opening's sides, conductivity was measured at those two positions while 10 ml of 50 mM NaCl was injected into a carrier flow of dH_2O. Chemical stimuli would presumably be diluted to the same degree as the NaCl. Ordinate values correspond to predicted concentrations of injected chemical stimuli (initially at 10 mM) not NaCl (initially at 50 mM). The conductivity probe consisted of two Ag/AgCl wires that were 6 mm long and 2 mm apart. The probe was positioned vertically with the tips of the wires 16 mm from the bottom of the shelter. To measure conductivity, the probe was placed in series with a 46 kΩ resistor. Voltage was measured across the resistor while 100 mV pulses were applied to the circuit. Knowing the voltage applied to the circuit, and the voltage falling across the resistor allowed us to calculate the current flowing through the circuit, the voltage across the probe, and the conductivity of the probe.

Observers that were unaware of the identity of the stimulus quantified the number of dactyl clasps within 60 seconds, the time spent probing/waving within 50 seconds, and the time spent walking within 50 seconds of the stimulus contacting the crayfish. Use of 50 seconds rather than 60 seconds for the latter two behaviors allowed the inclusion of experiments that were terminated prematurely thus increasing the sample size. To count dactyl clasps, the most clearly visible claw on a second or third pereiopod (the large claws are considered the first pereiopods) was observed and the number of times the dactyl moved towards the opposing surface of the propodus was recorded. Results, therefore, correspond to the number of times a single small claw clasped in 1 min; crayfish have four small claws. Probing/waving was considered to be any movement of a pereiopod other than a large claw provided that the movement was unrelated to movement of the animal as a whole. Walking was any movement of the whole animal. Dactyl clasping and probing/waving represent attempted food acquisition. Walking can result from attempts at food acquisition but can also represent escape behavior.

One-way analysis of variance (ANOVA) was used to determine if any of the three behaviors were significantly affected by the stimulus. The mean number of dactyl clasps was proportional to the variance, so dactyl-clasp data were subjected to the square root transformation before analysis (Zar, 1999). The time spent probing/waving and the time spent walking were expressed as proportions of 50 seconds. Since proportions fit a binomial distribution, these data were treated to the arcsine transformation to achieve normal distributions (Zar, 1999). Following significant ANOVA results, Fisher's Protected Least Significant Difference test was employed to compare responses to the individual stimuli. Because not all crayfish provided results for all three dependent variables, use of multivariate analysis of variance was not feasible.

Focal-Application Bioassay

For the focal-application bioassay, trehalose and D-glucose were dissolved at final concentrations of 10 μM and 100 μM in dechlorinated tap water. Stimuli included 0.001% fluorescein dye to allow the location of the stimulus to be followed visually.

One day prior to being tested with the focal-application method, animals were anesthetized in ice water and then blinded with a coat of nail polish over their eyes. For testing, animals were individually placed in aquaria measuring 30 cm long by 18 cm wide and filled with aged tap water to a depth of 5.5 cm. An air stone provided both oxygen and a background level of mechanical vibration. Constant vibration made it easier for us to approach the crayfish with a hand-held pipette without alerting them. After allowing at least 1 hour for acclimation, stationary crayfish were tested by gently pipetting 150 μl of a test solution onto the small claw of a second or third pereiopod. The number of dactyl clasps was noted as was the length of time between stimulus application and when the stimulus reached another part of the crayfish (2.0-7.2 seconds), at which point counting of dactyl clasps stopped. Results were expressed as dactyl-clasp frequency. Each crayfish was tested once. Square root transformed dactyl-clasp frequencies were analyzed via two-way ANOVA, the factors being stimulus (glucose or trehalose) and concentration (10 μM or 100 μM). Analysis of simple effects (Keppel, 1991) was performed following the finding of a significant interaction.

Results

In the whole-animal bioassay, different chemical stimuli elicited significantly different numbers of dactyl clasps ($F_{6,54} = 4.835$, $P < 0.001$), probing/waving times ($F_{6,80} = 7.459$, $P < 0.001$), and walking times ($F_{6,100} = 3.155$, $P = 0.007$). For the number of dactyl clasps, glucose, maltose, and trehalose were significantly more effective than the blank (Fig. 2A; Fisher's PLSD tests: $P = 0.008$ for glucose, $P = 0.009$ for maltose, and $P = 0.002$ for trehalose). Probing/waving was elicited by glucose and trehalose (Fig. 2B; $P < 0.001$ for glucose, $P = 0.018$ for trehalose) while walking was promoted by glucose, glycine, and maltose (Fig. 2C; $P = 0.029$, $P = 0.002$, and $P = 0.002$). Glutamate and ammonium failed to elicit responses.

In the focal-application bioassay, there was a significant difference in mean dactyl-clasp frequency between the two concentrations, but that difference depended on which stimulus was applied (Fig. 3; interaction of stimulus and concentration: $F_{1,43} = 7.827$, $P = 0.0077$). According to analysis of simple effects, the two concentrations of glucose elicited significantly different dactyl-clasp frequencies ($F_{1,20} = 10.783$, $P = 0.0037$), but that was not the case for trehalose ($F_{1,23} = 0.661$, $P = 0.4247$).

Discussion

Most of the results of the whole-animal assay are consistent with what one would expect given prior physiological data obtained from crayfish legs. Trehalose, the most effective physiological stimulus identified (Corotto and O'Brien, 2002), elicited significant dactyl clasping and probing/waving (Fig. 2A, B). Glycine, which is a moderately effective physiological stimulus (Corotto and O'Brien, 2002), elicited significant walking behavior (Fig. 2C). Neither ammonium nor glutamate elicited significant dactyl clasping, probing/waving, or walking (Fig. 2). Ammonium elicits relatively few action potentials in the pereiopod nerve while glutamate is ineffective at the 100 μM concentration employed by Corotto and O'Brien (2002). In contrast, ammonium is the only single compound identified that elicits dactyl clasping in the lobster *Homarus americanus*, although only across a certain range of concentrations (Borroni et al., 1986). While we applied test stimuli at a single initial concentration in this study (diluted in the chamber to 200 μM to 2 mM), our own unpublished data suggest that ammonium fails to elicit dactyl clasping across a broader range of concentrations in *P. clarkii*. It appears, therefore, that the one single compound shown to elicit dactyl clasping in *H. americanus* is ineffective at doing so in *P. clarkii*.

Glucose elicited all three of the behaviors we quantified in the whole-animal bioassay so, in that sense, it was the most effective chemical stimulus for *P. clarkii* when applied to the entire animal. Also, the magnitudes of the responses to glucose were similar to those evoked by maltose and trehalose (Fig. 2). This contrasts with the relative effectiveness of these carbohydrates as determined through extracellular recordings from pereiopod nerves; at a concentration of 100 μM, trehalose elicits the largest number of action potentials, maltose elicits relatively few spikes, and glucose elicits none (Corotto and O'Brien, 2002). If one considers only the whole-animal bioassay results, and not the results of the focal-application bioassay, the effectiveness of glucose as a behavioral stimulus could be explained in two ways: glucose-sensitive receptor cells may be absent in pereiopods but present elsewhere or receptor cells for glucose may be present in pereiopods but may generate too few action potentials in the nerve to be detected with Corotto and O'Brien's (2002) methods. Results of the focal-application bioassay show that the latter is the case. When applied only to the legs, 100 μM glucose elicited a significantly greater frequency of dactyl clasps than when applied at 10 μM (Fig. 3). Had the clasping behavior been elicited only by the mechanical stimulus associated with pipetting the solution onto the legs, one would expect no difference in clasp frequency between the two concentrations. This shows that receptor cells that are responsive to glucose are present in *P. clarkii* pereiopods. They presumably generate few spikes and thus went undetected by Corotto and O'Brien (2002).

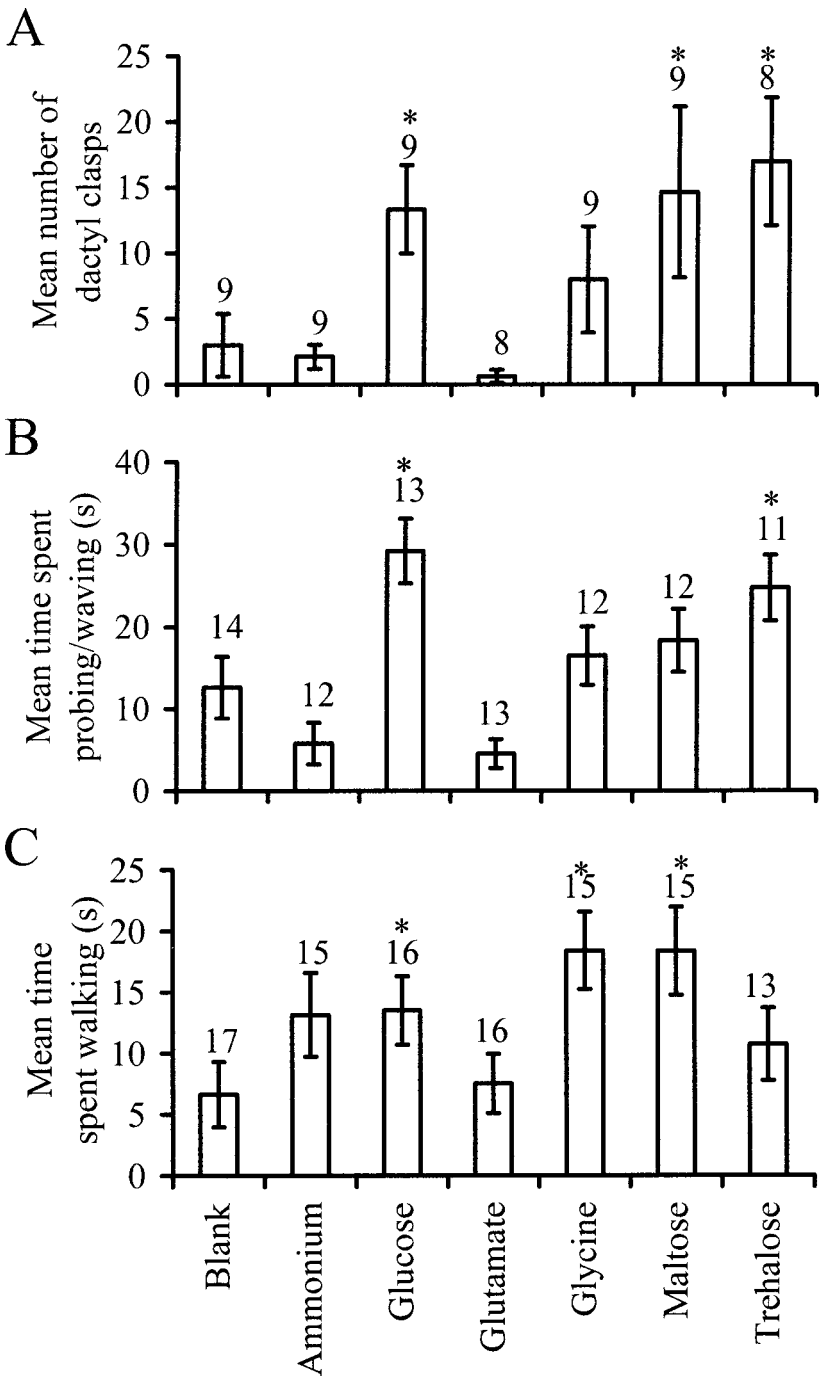

Fig. 2. A, Mean number of dactyl clasps counted over a 60 second time period starting when the stimulus first contacted the crayfish. Data are shown prior to the square root transformation. B and C, Mean time spent probing/waving (B) and walking (C) determined over a 50 second time period starting when the stimulus first contacted the crayfish. Data are shown prior to the arcsine transformation. Asterisks indicate mean responses that were significantly different from that elicited by the control blank, as determined by Fisher's Protected Least Significant Difference test. Numbers indicate sample size. Bars show standard error of the mean.

Knowing that glucose-sensitive receptor cells are present in the pereiopods, and that they generate few spikes in the pereiopod nerve, what accounts for the roughly equivalent response magnitudes elicited by trehalose and glucose in the whole-animal bioassay? One possibility is that glucose-sensitive cells are more abundant on other appendages. Another possibility is that the concentration of glucose employed in the whole-animal bioassay (200 μM-2 mM) was so high that it evoked the maximum behavioral response, in spite of low sensitivity of the animal to glucose. It is also possible that glucose-sensitive cells in the pereiopods have a greater influence upon the CNS than do other, more abundant, receptor cells. If the latter were the case, then high sensitivity to glucose would be achieved through sensitivity of the CNS to

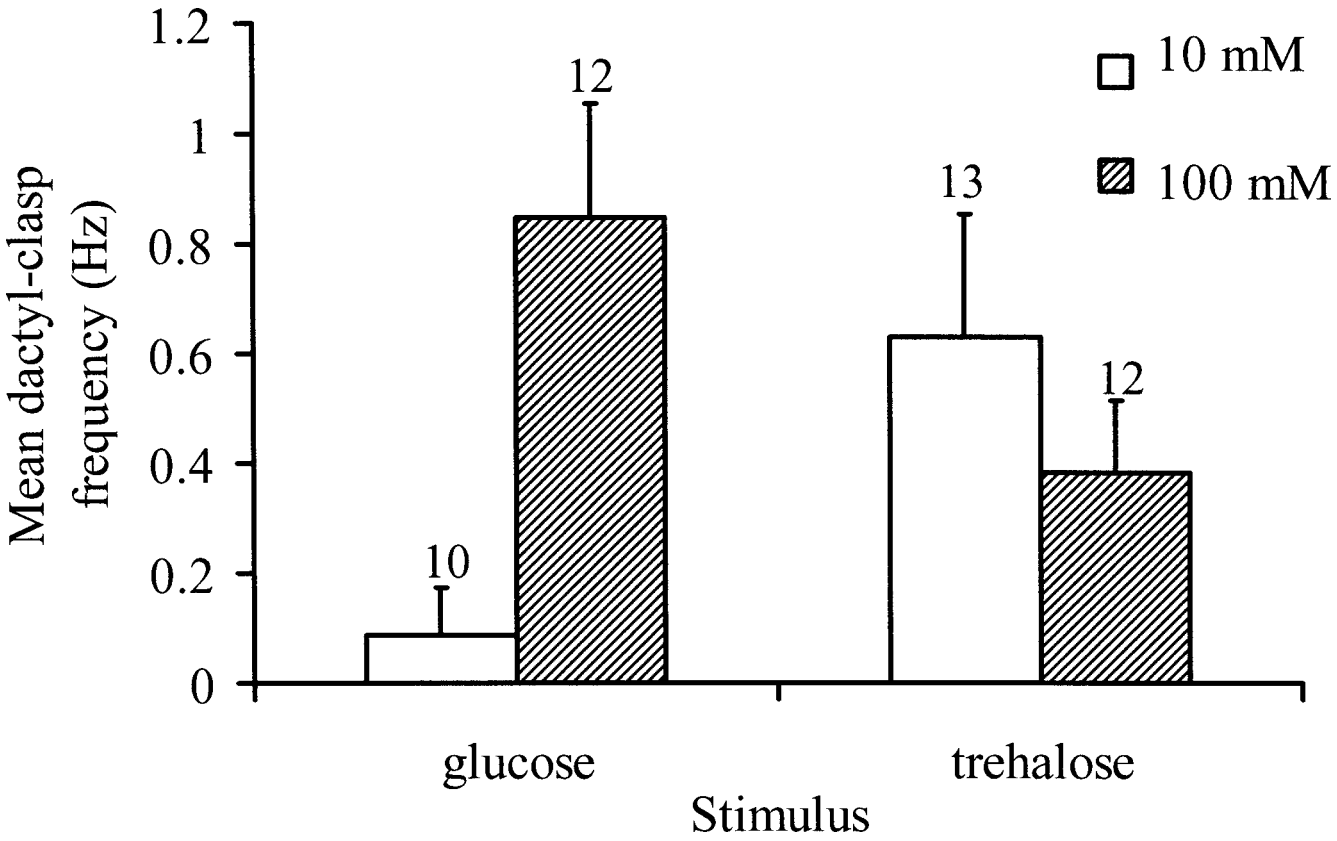

Fig. 3. Mean dactyl-clasp frequency following focal application of chemical stimuli. Data are shown prior to the square root transformation. Numbers indicate sample size. Bars show standard error of the mean.

cells responsive to that compound, rather than through having large numbers of those cells or high firing rates within them. Results of the focal-application bioassay fail to support this possibility. At a concentration of 10 μM, glucose evoked a lower dactyl-clasp frequency than at 100 μM (Fig. 3). For trehalose, both concentrations evoked high clasp frequencies that did not differ from one another statistically. This suggests that 10 μM trehalose evokes the maximum behavioral response but for glucose that concentration is too low to do the same. Crayfish legs are, therefore, more sensitive to trehalose than glucose, a finding consistent with trehalose's greater physiological effectiveness.

Results for the whole-animal and focal-application bioassays combined are consistent with what one would expect based on prior physiological data. Glycine, maltose, and trehalose, which are the stronger physiological stimuli, all triggered behavioral responses. The remaining stimuli were chosen because they either failed to elicit responses in the pereiopod nerve (glucose and glutamate) or evoked few spikes (ammonium). Of these weak physiological stimuli, only glucose produced behavioral responses in the whole-animal bioassay and, in the focal-application bioassay, it was less effective than trehalose. Overall, the behavioral responses of *P. clarkii* to single chemical stimuli largely paralleled the ability of those stimuli to elicit responses in the pereiopod nerve. We find no evidence that peripheral chemoreceptor cells tuned to a particular stimulus exert a greater influence on the CNS than cells sensitive to other stimuli.

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