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ORIGINAL PAPER

Daniel Weatherill · Ronald Chase

Modulation of heart activity during withdrawal reflexes in the snail *Helix aspersa*

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Abstract The beating activity of the molluscan heart is myogenic, but it is influenced by nervous signals of central origin. Previous studies have demonstrated changes in cardiac output during feeding and other behaviors. Here, we describe a short latency, transient cardiac response that accompanies withdrawal reflexes. When evoked by electrical stimulation of peripheral nerves, the response was detected within one or two heartbeats. Beat amplitudes increased on average 11.6%, and inter-beat intervals decreased on average 2.1%. The mean duration of the response was 28.1 s. A transient inhibitory phase often preceded the excitatory response. Results from testing various nerves and tissues show that the cardiac responses invariably occur whenever contractions of the tentacle retractor muscle are elicited. Even stimulation of the ovotestis and the kidney elicit responses despite their protected locations within the mantle cavity. Three excitatory cardioactive neurons are identified in the central nervous system of *Helix aspersa*, and their involvement in the reflex response is documented. The results suggest that the heart output is initially inhibited to relax the hydroskeleton and thereby aid withdrawal movements. A delayed increase in cardiac output then facilitates the re-inflation, hence eversion, of the withdrawn body parts.

Keywords Withdrawal reflex · Cardiac modulation · Heartbeat · Cardioactive neurons · Mechanoreception

Introduction

The tactile sense is extremely important for snails because they have no audition and have poor vision. Moreover, since they have no offensive weapons and no ability for fast locomotion, they can only respond to threats by withdrawal (Chase 2002). Weak tactile stimuli elicit withdrawal reactions limited to the stimulated region of the body, whereas stronger stimuli elicit withdrawals of remote regions as well, and with the strongest stimuli, the whole body is withdrawn into the shell. The central nervous system (CNS) is involved either minimally, or not at all, in local withdrawals, but it is progressively recruited with increasing stimulus strength (Prescott and Chase 1996).

The withdrawal behavior of snails is similar to the startle response of mammals in that the eliciting stimuli in both cases are brief, the response latencies are short and the reactions are defensive. The startle response varies from species to species, but it generally consists of a sequence of stereotypical motor acts such as, jumping, hunching or uncoordinated muscular contractions (Davis 1984). The overt expression of a startle response is accompanied by cardiovascular changes in rodents and humans (Casto et al. 1989; Holand et al. 1999). Both blood pressure and heart rate are transiently increased, with peak effects evident within 10 s following the stimulus. Some form of cardiac modulation similar to that seen during the mammalian startle response could be expected during the defensive withdrawal behavior of gastropod mollusks because of similarities in the behaviors, and also because the gastropod heart and the mammalian heart share several features in common. Both are chambered, myogenic and regulated by the CNS. In gastropods, various natural stimuli (e.g. temperature, food, and noxious stimuli) are known to affect the cardiac function (Skelton et al. 1992; Krontiris-Litowitz 1999; Chase 2002). However, most of the reported instances of cardiac regulation in gastropods involve modulation of activity lasting from minutes to hours. In the present study, we recorded reliable, but transient, modulations lasting only seconds.

As part of her study of sensitization in *Aplysia*, Krontiris-Litowitz (1999) briefly described some alterations of cardiac function following single pinches of the

D. Weatherill · R. Chase (✉)
Department of Biology, McGill University, 1205 Ave. Docteur Penfield, Montreal, QC, H3A 1B1, Canada
E-mail: ronald.chase@mcgill.ca
Tel.: +1-514-3986422
Fax: +1-514-3985069

tail or single electrical shocks applied to the tail. The diastolic pressure increased and the systolic pressure (beat amplitude) became more variable. These effects, recorded in the anterior aorta, lasted 10–30 s in vivo and 15–90 s in vitro. Since *Aplysia* has a hydrostatic skeleton, as do all gastropod mollusks, such acute responses could be caused by body wall contractions impacting on interstitial blood spaces. This could increase the resistance to blood circulation and change the cardiac output. Alternatively, they could be directly mediated by the activation of cardioactive CNS neurons. The issue was not resolved in the study of Krontiris-Litowitz (1999) because some trials were conducted in vivo and other trials were conducted in vitro but with an intact arterial flow.

Here, our investigation of cardiac modulation shows that it is reliably elicited by all stimuli that evoke withdrawal reflexes, it is quite constant in form, and it is mediated by CNS activity.

Materials and methods

Mature specimens of the brown garden snail *Helix aspersa*, 6–10 g, were obtained from natural populations in California. They were maintained under constant laboratory conditions (16:8 light:dark photocycle; 14–18°C).

Preparations

Animals were anesthetized by injecting 2–3 ml of isotonic $MgCl_2$, de-shelled, and dissected to produce a reduced preparation consisting of the posterior tentacles with associated retractor muscles, CNS, anterior aorta, albumen gland, seminal vesicle and ovotestis, as well as the lung, heart, kidney, and digestive gland. These structures were tightly pinned to the Sylgard bottom of a small dish filled with saline solution. Care was taken to ensure that the intestinal nerve and its pericardial and ovotestis branches remained intact. The inverted posterior tentacles were cut open to expose the olfactory epithelium (Prescott and Chase 1996). A cannula was inserted into one of the major pulmonary veins feeding into the auricle of the heart. The cannula was attached to a reservoir of saline solution positioned above the preparation, allowing gravity to drive perfusion of the heart. The flow rate of the perfusion was adjusted using a valve, until the heartbeat was constant in amplitude and frequency. The anterior aorta was cut close to the CNS, and a second cannula was inserted. This cannula was attached to a transducer (WPI, model BLPR) that monitored blood pressure and heart rate. Alternatively, in some experiments, heart activity was monitored using a CdS photocell positioned ~5 mm above the transilluminated auricle. Tentacle retraction was measured isometrically using a force transducer (Grass Instruments, FT.03C). These experiments were performed at room temperature (22–25°C), and all preparations were rested for at least 30 min before the experiments began.

Electrophysiology

For electrical stimulation, nerves were sucked into glass pipettes filled with saline. Nerves were stimulated with trains of 10 pulses, 1-ms duration and delivered at 10 Hz, except as noted. Tactile stimulation was done using a soft, hand-held artist's brush. For intracellular recording, the outer sheath of the subesophageal ganglia was removed, and the inner sheath was softened with a few milligrams of protease (Sigma, type XIV) for 30 s, followed by several washes. Glass microelectrodes were filled with 2 M potassium acetate and had resistances of 50–180 MΩ. Signals from the force and pressure transducers were amplified using AC/DC differential amplifiers (A-M Systems, model 3000). The force transducer recordings were low-pass filtered at 4 Hz. All electrophysiological signals were digitized (Axon Instruments, Digidata 1320A) and were recorded using AxoScope software (Axon Instruments).

Data analysis

The amplitude and timing of heartbeats were measured using Mini-analysis software (Synaptosoft) and Axoscope software (Axon Instruments). For Table 2, baseline values for inter-beat intervals and beat amplitudes were calculated as means using the ten heartbeats immediately preceding nerve stimulation. The durations of heart responses were measured from the time of the final nerve stimulus pulse to the time when the second consecutive beat amplitude fell to the level of the baseline amplitude. The mean heartbeat amplitude and the mean inter-beat interval were calculated over the full duration of each heart response, regardless of any transient inhibition. These means were then expressed as percentages of the corresponding baseline values. Means are reported as ± the standard error of the mean (SEM).

Results

Tactile stimulation of body organs and electrical stimulation of nerves were both effective in eliciting coordinated responses from the heart and the tentacle retractor muscle (Figs. 1 and 2). Weak electrical stimuli generally produced weak responses of both types (Fig. 1), but regardless of stimulus strength, the two types of response invariably occurred together (Table 1). The cardiac response was delayed for 2–4 s relative to the muscle response, corresponding to one or two heartbeats.

A surprising finding was that even some internal organs, namely the ovotestis and the kidney, are capable of generating defensive responses. Since both organs are

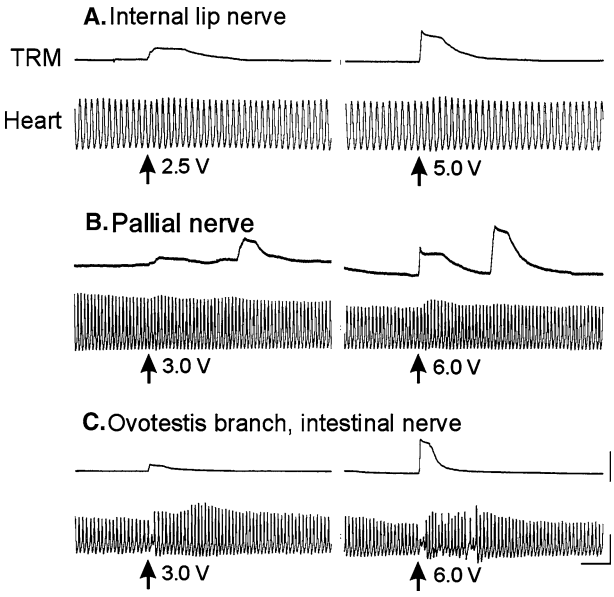

Fig. 1 Responses evoked by electrical stimulation of peripheral nerves. *Arrows* indicate the delivery of 10 pulses at 10 Hz. Threshold responses are shown on the *left*; responses to voltages 2× threshold are on the *right*. TRM, tentacle retractor muscle. Time calibrations: **a** 10 s, **b** and **c** 20 s. Force (TRM) calibrations: **a** 10 mN, **b** 5 mN ,**c** 16 mN. Pressure (heart) calibrations (measured as H_2O): **a** 50 mm **b** 55 mm; **c** 40 mm

located deep inside the mantle cavity, and hence, inside the animal's shell, they are not ordinarily subject to any type of intrusive mechanical stimulation. Nevertheless, tactile stimulation of either the ovotestis or the kidney (Fig. 2), or electrical stimulation of the ovotestis nerve branch (Fig. 1), was effective in eliciting coordinated responses of the tentacle retractor muscle and the heart. By contrast, tactile stimulation of other internal organs (seminal vesicle, albumen gland, bursa copulatrix, and digestive gland) was ineffective in producing defensive reactions (Table 1).

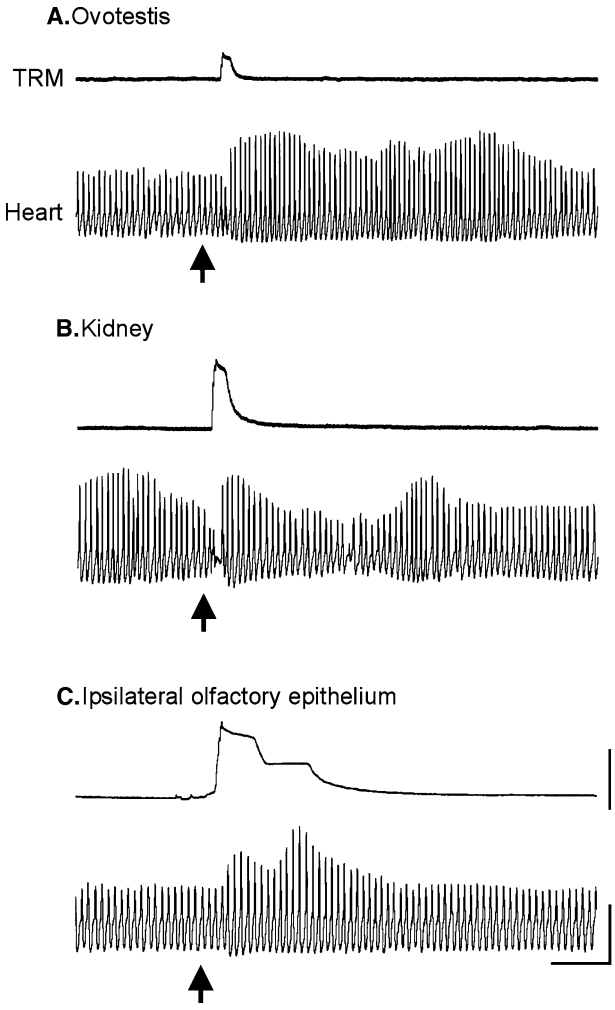

Fig. 2 Responses evoked by tactile stimulation. *Arrows* indicate timing of a light brush stroke over the entire exposed surface of the organ (1–2 s). TRM, tentacle retractor muscle. Calibrations: time, 10 s; force, **a** 10 mN, **b** 5 mN, and **c** 20 mN; pressure, **a** 35 mm, **b** 30 mm, and **c** 15 mm

Table 1 Reliability of responses observed in the heart and the tentacle retractor muscle following stimulation of various structures

Stimulus type	Stimulated structure	Number of preparations	Number of cardiac responses	Number of muscle responses
Electrical	Left tentacle nerve	4	4	4
	Right tentacle nerve	3	3	3
	Anal nerve	3	3	3
	Left pallial nerve	2	2	2
	Right pallial nerve	2	2	2
	Left internal lip nerve	1	1	1
	Ovotestis branch, intestinal nerve	1	1	1
Tactile	Ovotestis	3	3	3
	Kidney	3	3	3
	Left posterior olfactory epithelium	2	2	2
	Right posterior olfactory epithelium	1	1	1
	Seminal vesicle	2	0	0
	Albumen gland	2	0	0
	Bursa copulatrix	2	0	0
	Digestive gland	2	0	0
	Mantle cavity wall	1	0	0

Muscle responses were recorded as evoked contractions of the right posterior tentacle retractor muscle

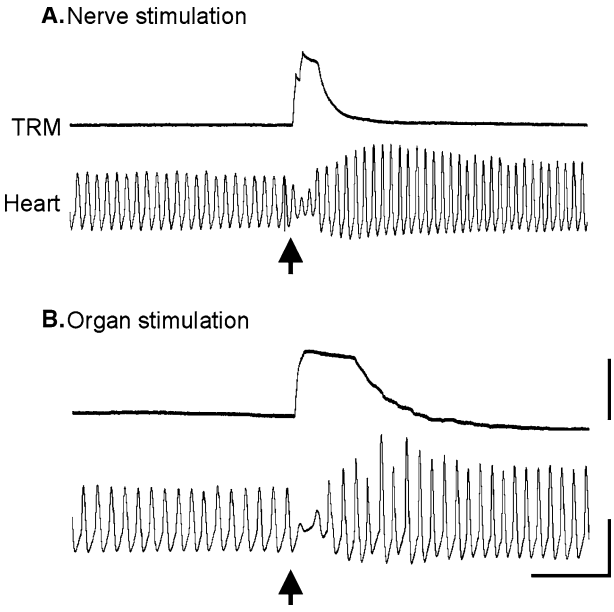

Fig. 3 Biphasic inhibitory-excitatory cardiac responses. **a** Electrical stimulation (*arrow*) of the ovotestis branch of the intestinal nerve. **b** Tactile stimulation (*arrow*) of the exposed surface of the ovotestis. **a** and **b** are from different preparations. Calibrations: time, 20 s; force, 8 mN; pressure, 40 mm H_20

Although the most prominent feature of the cardiac response is excitation, as seen in the increased beat amplitude, a transient inhibition often preceded the excitation. With the stimuli employed by us, the inhibitory phase comprised ≤ 3 heartbeats with amplitudes substantially smaller than baseline values (Figs. 1c, 2a, b, 3). Transient inhibition was elicited by electrical stimulation of five different nerves (tentacle, pallial, anal, lip, and intestinal) as well as by tactile stimulation of the ovotestis and the olfactory epithelium. Figure 3 illustrates the similar appearance of the biphasic cardiac response whether elicited by electrical stimulation or tactile stimulation. It is noteworthy that transient inhibition was not consistently observed, even within a given preparation. Overall, it was seen in 26 of 33 preparations (total of electrical stimulation and tactile stimulation). When nerve stimulation elicited inhibition in ≥1 trial in a given preparation, inhibition occurred, on average, in 70 ± 6.26% of repeated trials ($N = 25$). The variable occurrence of transient inhibition was not associated with differences in baseline heartbeat rate or with the phase of the heartbeat relative to the onset of stimulation.

Quantitative measures of the cardiac response are provided in Table 2. Various nerves were stimulated with a standard pulse train (see Materials and methods). In all cases, the change in beat amplitude (mean 111.6 ± 1.4% of base) was greater than the change in inter-beat interval (mean 97.9 ± 0.4% of base). Combining all trials, there was a weak negative correlation between changes in beat amplitude and changes in inter-beat interval (Pearson coefficient = 0.28), which is probably explained by intrinsic properties of the cardiac muscle. The mean duration of the response, measured from the change in beat amplitude, was 28.1 s.

To determine whether central neurons mediate the cardiac response, we identified cardioactive neurons in *H. aspersa*. In related gastropod species, heart excitatory motoneurons are located in the visceral and right parietal ganglia, especially along the common boundary, and they contain serotonin (Mayeri et al. 1974; Liebeswar et al. 1975; Rózsa 1987; Furukawa and Kobayashi 1987; Buckett et al. 1990). Hence, after treatment with 5,7-DHT, we systematically searched for labeled cells in the visceral and right parietal ganglia that have

Table 2 Cardiac responses to electrical stimulation of peripheral nerves

Nerve	Stimulus (V)	Response duration (s)	Beat amplitude (% of base)	Inter-beat interval (% of base)
Left tentacle[a]	2	15.4	109.6	96.9
Left tentacle[b]	2	31.7	113.3	99.9
Left tentacle[b]	2	17.6	111.7	99.6
Left tentacle[b]	3	29.0	116.3	100.1
Left tentacle[a]	4	19.3	107.8	98.7
Left tentacle[b]	4	38.6	118.2	95.7
Right tentacle[c]	10	18.2	107.6	97.5
Right tentacle[d]	14	18.9	107.2	96.7
Right pallial[d]	2	42.9	106.6	99.4
Right pallial[e]	8	27.8	109.8	98.5
Anal[d]	5	23.5	107.7	97.1
Anal[e]	5	46.7	126.4	94.4
Left internal lip[d]	5	32.5	108.5	97.5
Left internal lip[d]	5	37.1	105.7	97.4
Ovotestis[f]	4	18.4	109.6	98.0
Ovotestis[f]	5	31.8	119.5	99.2
Mean ± SEM		28.1 ± 2.46	111.6 ± 1.4	97.9 ± 0.4

Superscript letters designate different experimental preparations; some trials in some preparations were not suitable for inclusion here
Pearson correlation coefficient for changes in beat amplitude and inter-beat interval is 0.277 (not significant)
Stimulus trains contained 10 pulses of 1-ms duration delivered at 10 Hz

cardioactive properties. Three such neurons were identified. From the criteria of soma size, soma location and axonal projections into the intestinal nerve (determined by Lucifer Yellow injections), these cells correspond to cells E7, E17, and F89 in the scheme of Kerkut et al. (1975).

Direct driving of any one cardioactive neuron with intracellular current injection caused a pronounced increase in cardiac-beat amplitude and beat frequency, as shown in Fig. 4. Also, spontaneous bursts of activity in the same neurons were reliably associated with increases in beat amplitude (Fig. 4b). These increases were equivalent in shape and duration to the increases seen after inducing shocks in peripheral nerves (Fig. 1) or after touching organs (Fig. 2). Although our evidence is incomplete, it suggests, when considered together with information from related species, that the three cardioactive neurons identified by us in *H. aspersa* are motoneurons. The sensory pathway connecting the ovotestis and the identified cardioactive neurons was investigated by stimulating the ovotestis branch of the intestinal nerve with brief electrical pulses. The evoked slow potentials in cell E7 were identified as EPSPs from the progressive increase in initial slope with increasing cell hyperpolarization (Fig. 5). Given the reliability of the responses, and their constant latency, we conclude that they are probably monosynaptic.

To evaluate the role of neuron E7 in cardiac modulations evoked by tentacle nerve stimulation, we compared cardiac responses under conditions when firing in E7 was enhanced by direct depolarization versus when neuron E7 was prevented from firing by hyperpolarization. Depolarization trials alternated with hyperpolarization trials at 4-min intervals. As illustrated in Fig. 6, when E7 fired in response to nerve stimulation (left column), the associated changes in heartbeat amplitude were slightly greater than when E7 was prevented from firing (right column). Table 3 contains a quantitative analysis of these trials, together with three additional trials obtained after the stimulus voltage was increased (from 6 V to 8 V) and stimulus train duration was also increased (from 1.5 to 1.8 s). The amplitudes of five successive heartbeats (major contractions) were measured, peak-to-peak, immediately before and immediately after the stimulus. The results reveal that stimulus-evoked heartbeat amplitude changes were significantly greater when the cell was active than when it was suppressed (Mann–Whitney

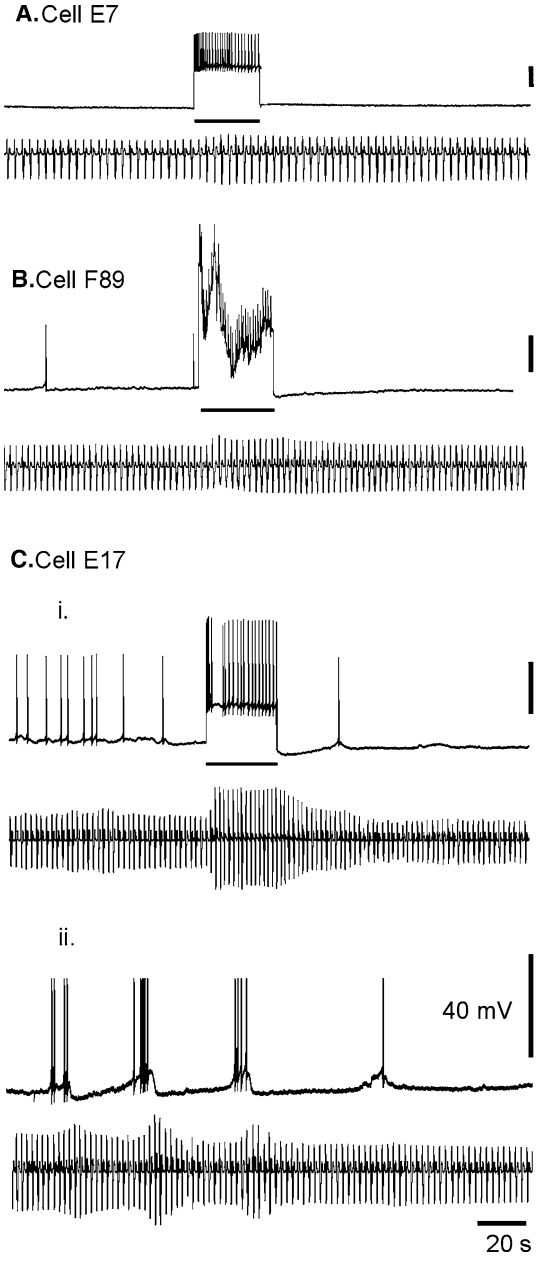

Fig. 4 Cardioexcitation elicited by three identified neurons. *Bars under the upper traces* (**a, b, c** *i*) indicate the injection of depolarizing currents (approximately 1 nA) to induce cell firing. **c** *ii* shows spontaneous bursts in cell E17 correlated with brief increases in heart beat amplitude. Cardiac activity was recorded with a photocell, hence uncalibrated

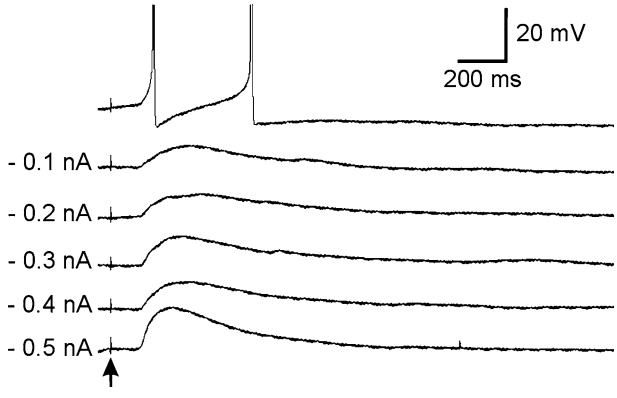

Fig. 5 Orthodromic responses elicited in the identified cardioactive neuron E7 by electrical stimulation (3 V, 1 ms) of the ovotestis branch of the intestinal nerve (*arrow*). The *top trace* shows the response at resting membrane potential; the action potentials are clipped. Other traces show responses elicited during injections of negative currents, as indicated

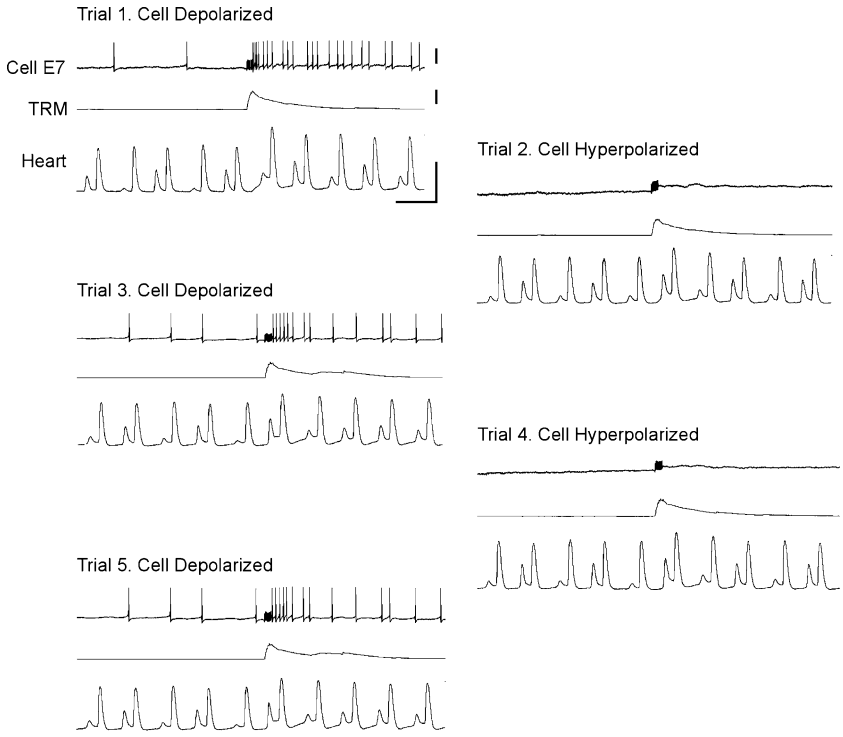

Fig. 6 Contribution of the identified neuron E7 to cardiac reflexes. In alternating trials the cell was either slightly depolarized (0.4 nA) to enhance spiking or slightly hyperpolarized (−0.5 nA) to prevent spiking. Reflex activity was evoked by electrical stimulation of the left tentacle nerve (10 pulses at 10 Hz) as indicated by artifacts in the intracellular trace. A quantitative analysis of the responses is given in Table 3. *TRM* Tentacle retractor muscle. Calibrations: time, 5 s; membrane potential, 40 mV; force, 5 mN; pressure, 30 mm H_20

Table 3 Prevention of firing in cell E7 consistently reduces heartbeat amplitude changes evoked by tentacle nerve stimulation but it does not affect either inter-beat intervals or muscle contractions

Trial No.	Cell treatment	No. action potentials	Muscle response (mN)	Beat amplitude response (% change)	Inter-beat interval response (% change)
1	Depolarized	23	7.99	+12.8	+0.108
2	Hyperpolarized	0	7.42	+2.04	−0.242
3	Depolarized	13	7.42	+4.82	+2.37
4	Hyperpolarized	0	7.36	+0.41	−0.573
5	Depolarized	13	7.02	+6.27	−1.18
6	Depolarized	26	8.40	+6.87	+1.2
7	Hyperpolarized	0	7.59	+1.88	−0.256
8	Depolarized	20	7.82	+3.42	+1.13
Mann–Whitney test (one-tailed)			NS	$p=0.025$	NS
Binominal test (one-tailed)			NS	$p=0.0156$	NS

Stimulus strength was increased between trials 5 and 6
Trials 1–5 are illustrated in Fig. 6
NS Not significant

test, $p=0.025$, one-tailed), whereas there was no significant effect of E7 activity on either stimulus-evoked changes in inter-beat frequencies or muscle contraction amplitudes ($p>0.05$). A binomial test was also calculated based on the six transitions from one current injection condition to another. It was predicted that cardiac and muscle responses would be *smaller* in hyperpolarization trials compared to the preceding depolarization trials, whereas responses would be *greater* in depolarization trials compared to the preceding hyperpolarization trials. This was always the case in respect to heartbeat amplitudes ($N=6$, $p=0.0156$, one-tailed), but not in respect to inter-beat intervals or muscle contraction amplitudes ($p>0.05$). These data support the conclusion that E7 is partly responsible for the stimulus-evoked increase in heartbeat amplitude.

Discussion

We have documented a new type of cardiovascular regulation in gastropod mollusks that is strongly associated with defensive reflexes. Before discussing its likely function, we need to rule out artifactual explanations. The experimental preparation itself eliminated sources of artifact that could have arisen from the obstruction of arterial flow, because the entire cardiac output (via the ventricle) was either blocked, by insertion of a transducer probe into the severed end of the anterior aorta, or

free-flowing due to severance of the remaining aortic branches. Also, the cardiac input (via the auricle) was maintained at a constant rate by perfusion. Because of these arrangements, it was not possible for the cardiac output to be affected secondarily by muscle contractions evoked anywhere in the preparation, contrary to what could occur in vivo or even in a minimally dissected in vitro preparation. Furthermore, and crucially, the same stimuli that elicited concurrent cardiac responses and withdrawal responses also provided strong excitation to cardioactive neurons, and the experimental removal of a single cardioactive neuron attenuated the evoked cardiac responses. Hence, it can be concluded that the cardiac responses were mediated by the activation of central nervous circuits.

Due to their brevity, the observed modulations of cardiac output are unlikely to have a significant impact on respiratory or metabolic functions. Rather, the significance of the response probably lies in its effect on the snail's hydrostatic skeleton. As the main component of the cardiac response that accompanies withdrawal behavior consists of increases in beat pressure and beat frequency, one can surmise that these effects on cardiac output contribute to the re-inflation, hence eversion, of the withdrawn body part(s), for example the tentacle(s). In many cases the increase in cardiac output was preceded by a transient inhibition of cardiac activity. A similar phenomenon was reported by Krontiris-Litowitz (1999). We hypothesize that this inhibitory component has an adaptive function in momentarily relaxing the hydroskeleton to facilitate the withdrawal of threatened body parts. Zhuravlev et al. (1991) described cardiac IPSPs as rather common in *Helix pomatia*, and they determined that the IPSPs are mediated by both central and peripheral motoneurons. Our results suggest at least one specific function for this inhibitory input. The variable occurrence of transient inhibition suggests that there is a small number of inhibitory motoneurons, perhaps as few as one. In an intact snail, the cardiac responses discussed above will likely interact with changes in arterial resistance and venous return, with consequences that are difficult to predict.

Our interpretation of the function of the cardiac response, as stated above, is consistent with the fact that its time course parallels the time course of the withdrawal response. Thus, the short latency, brief inhibition of cardiac activity corresponds to the rapid contraction of the retractor muscle. The longer latency, and more prolonged period of cardiac excitation corresponds to the time during which the withdrawn body part is re-inflated.

Interesting issues about the CNS organization are raised by the co-activation of tentacle withdrawal reactions and cardiac modulation. The neural circuitry responsible for tentacle withdrawal in *H. aspersa* has been well described (Prescott et al. 1997). Its central components comprise motoneurons (including the identified cell C3), and possibly interneurons, located in the cerebral ganglion. Whole body withdrawal responses are controlled by a small number of identified "command" interneurons located in the parietal and pleural ganglia (Balaban 1979). Also, here we have identified three neurons responsible for cardiac regulation, possibly homologous to motoneurons previously identified in related molluscan species (Rózsa 1987; Furukawa and Kobayashi 1987; Chase 2002).

The key question is whether the withdrawal control system and the cardiac control system are activated in parallel or in series. The slight delay of the cardiac response relative to the withdrawal response might suggest that the heart system is downstream of the withdrawal system, but more likely, this only reflects different mechanisms for regulating myogenic contractions (heart) and triggered contractions (tentacle). Furthermore, since withdrawal responses can be sensitized (Prescott and Chase 1996; Krontiris-Litowitz 1999), one would expect the cardiac response also to sensitize if the cardiac response were dependent on activity in cells commanding withdrawal. However, the cardiac response shows no short-term sensitization in *Aplysia* (Krontiris-Litowitz 1999), nor in *Helix* (our observations). The foregoing considerations argue against the idea that cardiac modulation is initiated secondarily to the initiation of withdrawal responses. On the other hand, if the two circuits are activated in parallel, the fact that one sensitizes while the other does not implies either that the excitatory signals originating in the snail's periphery diverge through interneurons, or that a common sensory pathway is subject to terminal-specific facilitation, as described in *Aplysia* (Clark and Kandel 1984; Martin et al. 1997). As many of the critical cellular mediators of withdrawal behavior and cardiac modulation are known and accessible, details of the relationships between these neurons is likely to be a fruitful area for future research.

Tentacle withdrawals and cardiac responses were elicited by stimulation of the kidney and the ovotestis, despite their protected locations inside the mantle cavity. This is a surprising finding with no obvious explanation. However, we believe that it may stem from the basic design feature of the gastropod nervous system. In gastropods, all peripheral nerves contain a mixture of sensory and motor fibers (Chase 2002). The kidney and the ovotestis are both heavily innervated, and a sensory function with respect to the ovotestis innervation was previously described (Antkowiak and Chase 2003). On the other hand, the internal organs from which no defensive responses could be elicited in the present study (seminal vesicle, albumen gland, bursa copulatrix, and digestive gland) are mostly not innervated (Schmalz 1914). The digestive gland is innervated, but only in a small portion of the left lobe. Primary mechanosensitive neurons, both peripheral and central are abundant in gastropods. Given that mechanoreception is probably a primitive feature in nervous systems (see Walters 1991), it may be that all innervated structures in gastropods are provided with mechanosensors. If so, the mechanosensor phenotype

might be sufficient to guarantee that such neurons have access to the defensive withdrawal circuitry of the CNS.

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