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Intrinsic and extrinsic neural and neurohumoral control of the decapod heart

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Abstract. The intra-cardiac nervous system of the decapod heart is composed of large and small ganglionic cells (LGCs and SGCs) and axons of extrinsic cardio-acceleratory and -inhibitory neurons (CAs and CIs). Candidate neurotransmitters for the neurons have been determined by pharmacological, cytochemical and immunocytochemical tests. SGCs may be cholinergic, LGCs and CAs are probably dopaminergic, and CIs are GABAergic. Serotonin and octopamine were cardio-excitatory neuromodulators of the heart. Proctolin, crustacean cardio-active peptide (CCAP), red pigment concentrating hormone (RPCH), and FMRFamide also had modulatory actions on the heart. Proctolin was the most potent peptide, which acted primary on the cardiac ganglion. Insect adipokinetic hormones had little effect on the heart.
Key words. Decapod heart; cardiac ganglion; cardio-acceleratory neuron; cardio-inhibitory neuron; neurotransmitters; neurohormones; neuropeptide.

Introduction

Since Carlson [5] reviewed studies on invertebrate hearts, a considerable number of papers on the neuroanatomy, physiology and pharmacology of crustacean hearts have been published. Those include studies of various cardio-active substances. Cooke [7], however, concluded in his review that the neurotransmitters are not established. The neuronal constituents of the intra-cardiac nervous system are small and large ganglionic cells (SGCs and LGCs, respectively) composing the cardiac ganglion itself, and axons of cardio-acceleratory and -inhibitory neurons (CAs and CIs, respectively) running from the central nervous system to make synaptic contact with the ganglion and myocardium. Activities of the neural constituents and the myocardium are modulated by neurohormones which are liberated by the neuro-secretory tissue, the pericardial organ, into the blood of pericardial sinus. Serotonin, octopamine and proctolin are major neurohormones found in the pericardial organ (see Cooke and Sullivan [9] for review).

In this report, we will describe effects of a variety of putative neurotransmitters and humoral substances on the heart of hermit crabs, and propose the most likely candidates for natural neurotransmitters of extrinsic inhibitory and acceleratory axons and of intrinsic LGCs and SGCs. Preliminary studies have appeared elsewhere [63, 64].

Materials and methods

Giant marine hermit crabs (*Aniculus aniculus* and *Dardanus crassimanus*) were treated as reported before [59, 64]. As far as the present report is concerned, there was no significant difference between the two species.

Electrophysiological and pharmacological methods were the same as those described previously [59]. Filtered natural seawater and artificial seawater (NaCl 526, KCl 11, $CaCl_2$ 18, $MgCl_2$ 24 [in mM], and Tris-buffer 5 mM at pH 7.4) were used as the perfusion medium for preparations.

The following chemicals were used: acetylcholine, atropine, scopolamine, pilocarpine, hexamethonium, nicotine, picrotoxin, serotonin, dopamine, l-noradrenaline, yohimbin (Wako); arecoline, carbamylcholine, muscarine, muscimol, octopamine, ergonovine, chlorpromazine, apomorphine (Sigma); methacholine (Nakarai); d-tubocurarine (Tokyo Kasei); phentolamine (Ciba Geigy); haloperidol (Dai-Nippon); fluphenazine (Yoshitomi, gift); methysergide (Sandoz); crustacean cardio-active peptide (CCAP) (KosmoBio-Bachem AG); red pigment concentrating hormone (RPCH) (Funakoshi-PLI); adipokinetic hormone II of *Schistocerca gregaria* (AKH-II), adipokinetic hormone I of the cockroach (AKH-I), FMRFamide, proctolin (Sigma).

General neuroanatomy and physiology of the cardiac nervous system in hermit crabs

Figure 1 is a schematic drawing of the cardiac nervous system. Neurons governing the heart beat in decapods are four small ganglionic cells (SGCs) and five large ganglionic cells (LGCs) in the cardiac ganglion, and one pair of CIs and two pairs of CAs in extrinsic nerves. SGCs, the so-called pacemaker cells, innervate LGCs but do not innervate myocardial cells[59]. LGCs receive synaptic inputs from SGCs and induce excitatory junction potentials (EJPs) in myocardial cells[61]. LGCs possess spontaneity even if they are surgically severed from SGCs as shown in other decapods[29, 51, 52] (and unpublished observations). CAs innervate both LGCs[59] and SGCs[63], and also the myocardium[60]. CIs innervate both LGCs[59] and SGCs[63], but not the myocardium[59]. The heart is under the modulatory influences of neurohormones released from the pericardial organ (see Cooke and Sullivan[9]).

Spontaneous activities of nerve and muscle of the heart of the hermit crab are shown in figure 2. Figure 2A shows periodic bursts of impulses recorded from the main trunk of the cardiac ganglion (ER 1 site shown in fig. 1). In the trace, the small and large spikes show discharges of SGCs and LGCs respectively. In the upper record of figure 2B, two successive LGC bursts were intracellularly recorded simultaneously with extracellular LGC impulses at the site of the circular trunk of the cardiac ganglion (ER 2 site in fig. 1). The trains of SGC-evoked excitatory postsynaptic potentials (EPSPs), recorded in a LGC, were followed by bursting discharges of LGC spikes. The LGC bursts and corresponding mus-

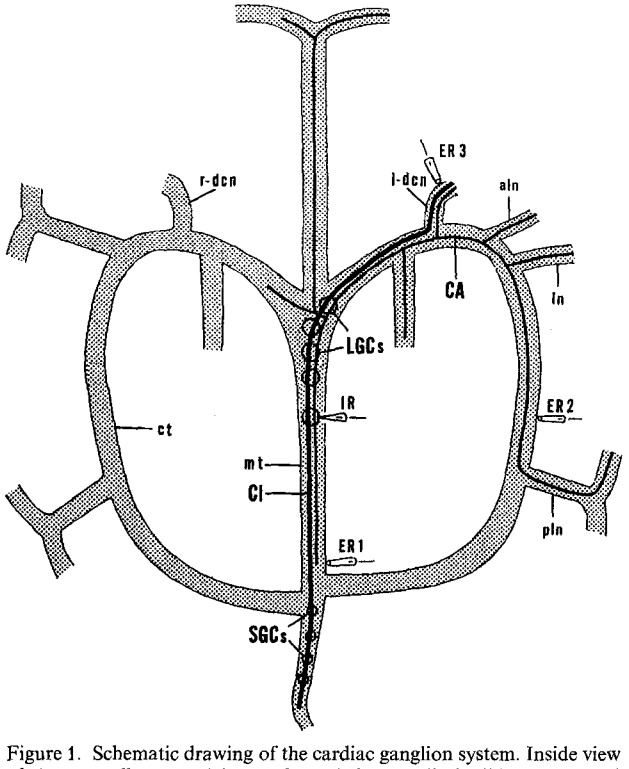

Figure 1. Schematic drawing of the cardiac ganglion system. Inside view of the ventrally opened heart. CI and CA, cardio-inhibitor axon and cardio-accelerator axons (illustrated only on left side); r- and l-dcn: right and left dorsal cardiac nerves; mt and ct, main trunk and circular trunk of the cardiac ganglion; aln, ln and pln: anterior lateral, lateral and posterior lateral nerves; LGCs and SGCs: large and small ganglionic cells; ER 1 - ER 3: sites on the ganglion system where nerve impulses were recorded extracellularly; IR: intracellular recording from large ganglionic neurons.

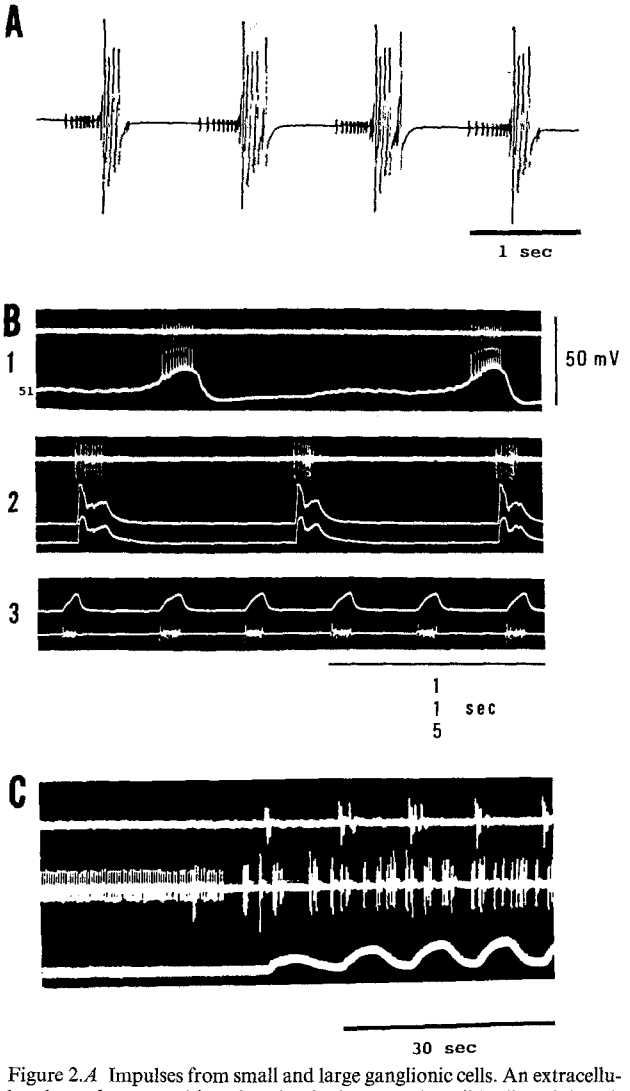

Figure 2. *A* Impulses from small and large ganglionic cells. An extracellular electrode was positioned at the site between the cell bodies of the 5th LGC and 1st SGC (the site ER 1 in fig. 1). Small and large impulses are respectively from SGCs and LGCs.

B1 Simultaneous recordings of large ganglionic cells activity from the circular trunk of the cardiac ganglion at the site ER 2 in figure 1 (upper trace) and a LGC soma (lower trace). Small deflections in the lower trace show EPSPs from SGCs, which appeared prior to the burst of LGC spikes. The most hyperpolarized potential of the LGC was 51 mV.

B2 Simultaneous recording of LGC impulses from the circular trunk of the cardiac ganglion (upper trace) and corresponding compound EJPs from two muscle cells. *B3* Simultaneous recording of force of the heart (upper trace) and LGC impulses from the circular trunk of the cardiac ganglion at the site ER 2 in figure 1.

C Simultaneous recording of electrocardiogram (upper trace), spontaneous impulse discharge of the dorsal cardiac nerve (dcn, at the site ER 3 in fig. 1) (middle trace), and force of the heart beat. In the dcn trace, CI impulses are smallest ones, and impulses of two CAs are larger ones. CI impulses caused cardiac arrest in the left part of the record.

cle EJPs are recorded simultaneously from two myocardial cells (fig. 2B, middle). The LGC bursts also corresponded to heart beats (fig. 2B, lower traces). Figure 2C shows simultaneous records of electrocardiogram (ECG), activities of a dorsal cardiac nerve (dcn, ER 3 site in fig .1) and heart beat. When CI impulses in dorsal cardiac nerve discharged at a high frequency, ECG and heart beat stopped completely (at the left in fig. 2C). When CI became inactive and instead CAs impulses appeared, ECG and heart beat reappeared.

In the following sections, we will describe the actions of the major classical neurotransmitters and peptide hormones.

Acetylcholine

Acetylcholine (ACh) has been shown to enhance the neurogenic heart beat in many decapod species. The cardiac ganglion of crustaceans has been considered to be a cholinergic system[30]. Florey and Rathmayer[17] showed that the cardiac ganglion of *Astacus* and *Eriphia* has a muscarinic nature. On the other hand, ACh has long been postulated to be the transmitter of cardio-accelerators[7, 54]. Actually, two recent reports on *Homarus*[18, 48] showed muscarinic cholinergic responses of LGCs. Thus, ACh has become 'a front-runner of the moment' as the candidate transmitter of the cardio-acceleratory nerves[7]. We observed, however, that cholinergic blockers (curare, hexamethonium, and atropine) did not antagonize effects of the accelerator nerve on the cardiac ganglion in the hermit crabs[61]. Since CAs innervate myocardial cells[60] as well as LGCs[59], we applied ACh to myocardial cells. ACh did not change the membrane potential of myocardial cells (data not shown). In *Homarus*, it has also been reported that cardiac muscle cells did not respond to ACh[17]. Therefore, it is likely that CAs may not be cholinergic.

In the hermit crab heart, we observed that ACh depolarized LGC membrane. The response was accompanied by a decrease of membrane resistance[63]. LGCs responded to cholinergic agonists (arecoline, carbamylcholine, methacholine, muscarine, nicotine, pilocarpine, and tetramethylammonium) though there were differences in potency between the agonists. Thus, we considered the possibility that ACh is a neurotransmitter of some other neuronal constituent in the heart. We eventually observed that synaptic transmission from SGCs to LGCs was susceptible to cholinergic blockers such as atropine and scopolamine. Figure 3A shows that SGCs-evoked EPSPs in a LGC were suppressed by atropine. Therefore, it is most likely that SGCs are cholinergic[64]. ACh directly enhanced SGCs as well as LGCs[63]. Thus, SGCs may synapse chemically with other SGCs, since SGCs are known to connect with each other in *Panulirus interruptus*[19] and in *Panulirus japonicus*[49].

Our conclusion is supported by certain points of circumstantial evidence. That SGCs do not directly innervate

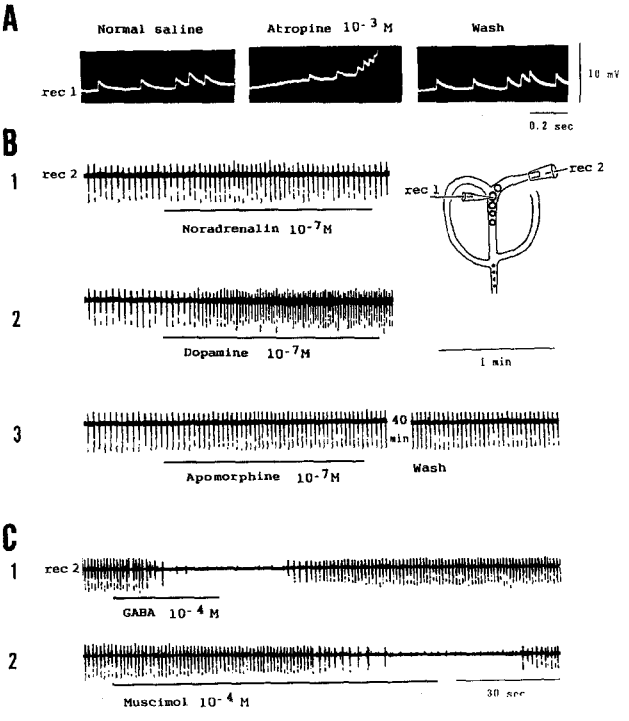

Figure 3.*A* SGC-induced EPSPs recorded from LGC (rec 1 in insertion). Left, in normal saline; middle, in atropine at 10^{-3} M; right, wash in normal saline. *B* Effects of noradrenaline (*B1*), dopamine (*B2*) and apomorphine (*B3*) at a concentration of 10^{-7} M on the cardiac ganglion activity of the circular trunk (rec 2 in insertion). *C* Effects of GABA and muscimol at a concentration of 10^{-4} M on the cardiac ganglion activity. The recording site was the same as in B but in a different preparation.

myocardial cells accords with the fact that muscle cells did not respond to ACh. Cholinesterase was present around the cell body of LGCs in certain lobsters, *Panulirus argus* and *Homarus americanus*[32]. Anticholinesterase excited the decapod heart and increased the action of applied ACh in *Cancer*, *Astacus*, *Panulirus*, *Homarus* and *Carcinus*[24]. Furthermore, Sullivan and Miller[48] demonstrated ACh synthesis in the lobster cardiac ganglion.

In *Homarus americanus*, when ACh was applied to isolated LGC, the second messenger system was activated[46]. Therefore, cholinergic cardiac ganglion receptors are probably similar to the muscarinic ACh receptor in sympathetic ganglionic neurons[7]. We obtained evidence that forskolin at 10^{-4} M activated the cardiac ganglion of the hermit crab heart, although we could not specify the site of the action of forskolin (data not shown). Since it is known that, in *Homarus americanus* heart, cAMP levels are elevated by both octopamine and serotonin[1] and by peptide neurohormone[27], further investigations are necessary to determine the involvement of cAMP in any synaptic action in decapod heart.

Biogenic amines

Monoamines have excitatory effects on the heart in many decapod species[25, 34, 56] (see also Cooke and Sullivan[9]

for review). Krijgsman[24] suggested in his review that motoneurons (LGCs) may have adrenergic characteristics, even though there had been no unequivocal physiological and pharmacological evidence.

We, therefore, reexamined effects of monoamines on the hermit crab hearts. Dopamine, noradrenaline, adrenaline, octopamine and serotonin (5HT) have cardio-excitatory effects, on the hearts presently studied, in a dose dependent manner[62]. Effects of dopamine, octopamine and 5HT were more potent than those of adrenaline and noradrenaline. Figure 3B1 and 3B2 shows the potency difference between noradrenaline and dopamine in the effects on the cardiac ganglion. An adrenergic agonist, clonidine, did not have significant effects on muscle cells and on the cardiac ganglion; nor did the beta-agonist, isoproterenol. A dopaminergic agonist, apomorphine, showed excitatory effects on the cardiac ganglion (fig. 3B3).

Adrenergic, serotonergic and cholinergic antagonists (phentolamine, phenoxybenzamine and yohimbin; methysergide; curare, hexamethonium and atropine, respectively) failed to block myocardial EJPs evoked by LGCs[62]. However, several blockers such as ergonovine, chlorpromazine, fluphenazine and haloperidol blocked EJPs[62]. Furthermore, excitatory effects of dopamine were antagonized by the dopaminergic blockers, chlorpromazine and haloperidol (unpublished observation). The results suggest that LGCs may be dopaminergic[64]. This postulate is in agreement with histochemical observations of glyoxylic acid-induced fluorescence observed in LGCs of the lobster[37] and of *Aniculus*[61]. Immunoreactivity to an anti-serotonin antiserum was not observed in any neuronal processes inside the heart of *Aniculus*[63]. The cardiac ganglion of the lobster was revealed as a dopamine-containing tissue by HPLC analysis and high voltage paper chromatography[37]. The presence of dopamine in the cardiac ganglion of *Aniculus*, was confirmed by HPLC analysis (unpublished observation).

On the other hand, in the studies on CAs, we reached a different conclusion from that of Sullivan and Miller[48] who pointed out the possibility that CAs are cholinergic. (Sullivan and Miller also pointed out the other possibility that intrinsic neurons are cholinergic). Our results strongly suggest that CAs are dopaminergic because dopaminergic blockers (haloperidol and chlorpromazine) but not adrenergic and serotonergic blockers (phentolamine and methysergide) eliminated excitatory effects of CA 1 and CA 2 stimulation on the cardiac ganglion[62]. This notion agrees with the histochemical experiments on the cardiac nervous system of the hermit crabs: 1) two CA axons in the dorsal cardiac nerve were revealed as amine-containing fibers that showed glyoxylic acid-induced fluorescence; and 2) serotonin immunoreactivity was never found in any axons in the dorsal cardiac nerve[61,63].

It is possible that two neurotransmitters are present in one neuron[2,10]. However, the most prominent characteristic which CAs and LGNs showed in our results was dopaminergic.

Neuroleptics, chlorpromazine and haloperidol, may block the conduction of nerve impulses[39,57]. Since we monitored pre-impulses, we could exclude the possibility that the observed synaptic blockade by the drugs was brought about by a conduction block of the pre-impulses.

Biogenic amines are ubiquitous in the crustacean brain, and of these octopamine is a particularly dominating amine[26]. Although octopamine has strong effects on the hermit crab heart, we have no evidence that octopamine mediates neurotransmission inside the crustacean heart. For example, adrenergic (octopaminergic) blockers did not antagonize any neuro-neuronal or -muscular transmission in the heart[62]. Octopamine probably does not have a neurotransmitter function in the neurogenic heart[20]. Octopamine is one of the neurohormones released from the pericardial organ of the decapod[7,45] and from neurosecretory cells, the root cells, of the lobster[14,28]. In the present specimens, cells similar to the root cells stained with neutral red (see Evans et al.[14]), were observed (unpublished observation).

Noradrenaline was not detected in any of the nervous structures of the lobster by Sullivan et al.[45]. On the other hand, the presence of noradrenaline has been shown in the cardiac ganglion of *Homarus*[37]. In the hermit crab heart, noradrenaline and adrenaline were less potent than other monoamines (see fig. 4B), and none of the adrenergic antagonists and agonists used were effective. Therefore, adrenergic innervation seems less likely in the hearts of hermit crabs.

Serotonin is another cardio-excitatory neurohormone released from the pericardial organ of decapods[6,8,45]. In the hermit crabs, serotonin immunoreactivity was found in the pericardial organ but not in the heart[63]. It has been argued that neither 5HT nor octopamine is a transmitter of cardio-acceleratory nerves[7,16]. We agree with this conclusion.

Amino acids

Although glutamate has effects on the myocardium of the lobster and crab[3,21], it (at least up to 10^{-4} M) showed little effect either on the cardiac ganglion or on muscle cells in the present hermit crab hearts (unpublished observation). In isopods, glutamate is a candidate for the transmitter of motoneurons of the heart[22,65].

Histamine, glycine, beta-alanine, and taurine did not show any effect on the heart of hermit crabs at concentrations at least up to 10^{-4} M (unpublished observation). In the lobster, Maynard[31] showed that effects of CI on cardiac ganglion activity were mimicked by gamma-aminobutyric acid (GABA). In the hermit crab heart, GABA and muscimol inhibit cardiac ganglion activity (fig. 4C). CI axon in the dorsal cardiac nerve showed anti-GABA immunoreactivity[63]. It has been reported

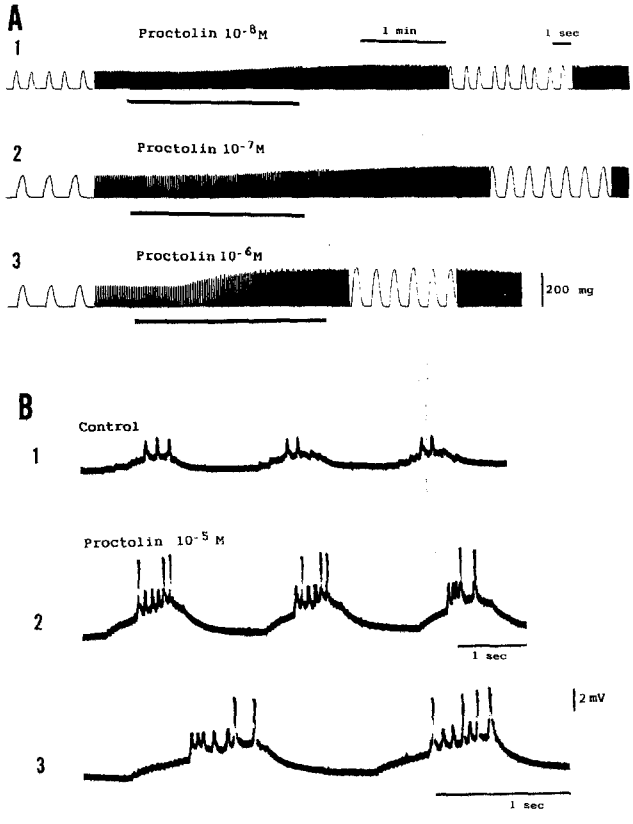

Figure 4. *A* Effects of proctolin on the force of heartbeat. Proctolin at 10^{-8} M (*A1*), 10^{-7} M (*A2*), and 10^{-6} M (*A3*) were perfused into the heart during bars. *B* Effects of proctolin on the cardiac ganglion activity. Intracellular recording from a LGC. *B1* Control activity. *B2* Maximum effects of proctolin (10^{-5} M) are shown. *B3* Faster recording right after *B2*. Note SGC-induced EPSPs appearing in a higher frequency, a larger amplitude of burst, and a longer duration of burst than in the control. At an initial phase of this high dosage application, frequency of LGC burst transiently increased (not shown) and then settled as in *B2*.

that picrotoxin blocked the effects of inhibitory nerves on the heart in the lobster[15,41], in isopods[11,50,58] and in *Squilla*[53]. GABAergic cardiac inhibition seems common in crustacean hearts.

Peptides

Proctolin has been found in the crab pericardial organ[43,44]. In the lobster, proctolin immunoreactivity was found throughout the nervous system, but the bulk is in the pericardial organ[38,42]. Excitatory actions of proctolin on the heart have been reported in the lobster[4,25,35,47] and in the crab[55]. Proctolin had positive chronotropic and inotropic effects in a dose dependent manner (fig. 4A). The excitatory actions were much greater than those of other peptides tested. The threshold concentration for proctolin was about 10^{-10} M.

Effects of high dosage of proctolin at 10^{-5} M on the cardiac ganglion neurons are shown in figure 4B. During an initial phase of this perfusion, only burst rate was increased (not shown). At a later period (fig. 4B), increases in the number of LGC spikes per burst and intensification of duration and amplitude of bursts were observed. Proctolin also strongly affected SGCs. This can be noticed in denser SGC-induced EPSPs prior to each LGC burst (fig. 4B3). The major effect of proctolin on the cardiac ganglion was the enhancement of SGC and LGC bursting discharges which resulted in argumentation of contraction force of the heart. Proctolin did not significantly change the amplitude of unitary EJPs. In the present heart preparations, it seems that proctolin acted prominently on the cardiac ganglion rather than on muscle cells. A similar ganglionic activation by proctolin has also been reported in the lobster[47]. It is likely that the enhancement of contraction force may have resulted from increases of amplitude and duration of compound EJPs caused by an increase of the cardiac ganglion impulse rate.

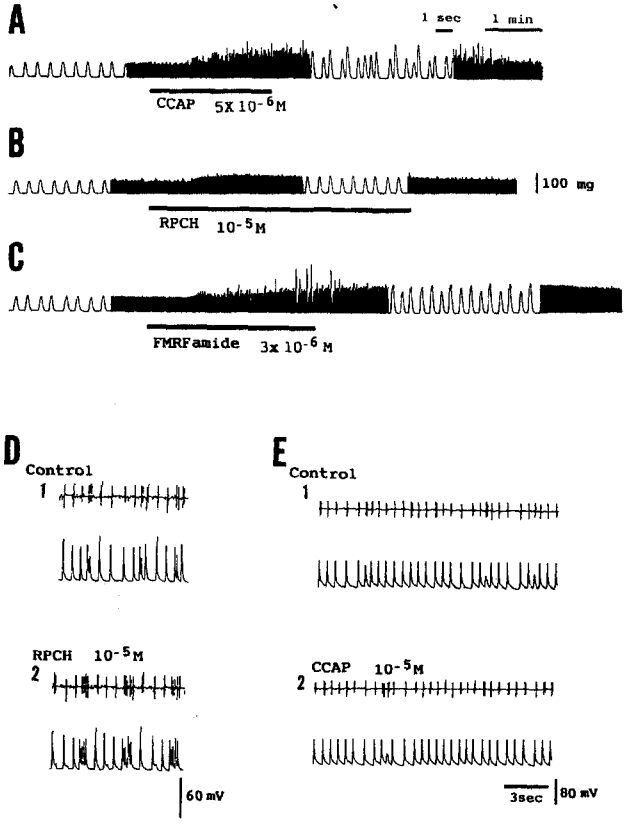

Figure 5. *A*, *B* and *C* Excitatory effects of CCAP, RPCH and FMRFamide on the force of the isolated heart. Chronotropic and tonotropic effects are seen at concentrations above threshold. Irregular heartbeats were frequently observable when using isolated heart but less observable when dorsal carapace and the internal skeleton around the heart were left intact, so as not to cut ligaments suspending the heart. *D* and *E* Effects of RPCH and CCAP on the same specimen. Simultaneous recordings of extracellular impulses from the main trunk of the cardiac ganglion (upper trace) and intracellular muscle EJPs. *D1* and *E1* Control records in normal artificial seawater. *D2* and *E2* Test records respectively in RPCH and CCAP at 10^{-5} M. RPCH (*D*) and CCAP (*E*) increased the rate of discharge of LGC impulses, but the effects in *E2* cannot be seen in this slow record. SGC impulse rate also was enhanced by RPCH and CCAP (not noticeable in these slow records). Amplitude of EJPs was little affected by the peptides tested.

Red pigment concentrating hormone (RPCH) modulated activities of the crayfish swimmeret[40], the lobster cardiac sac[12] and the crab gastric mill[36]. FMRFamide-like immunoreactivity has been found in the pericardial organ of the lobster and crayfish[23,33]. Processes immunoreactive to crustacean cardio-active peptide (CCAP) were found in the pericardial organ of the shore crab[13]. We found that these three neuropeptides were much less potent than proctolin in their actions on the heart, although we could observe positive chronotropic and inotropic effects. Threshold concentrations for CCAP, RPCH and FMRFamide were from 10^{-6} M to 10^{-5} M (fig. 5A, B and C).

As shown in figure 5D and E, the number of the cardiac ganglion spikes was increased by CCAP and RPCH at 10^{-5} M, although the amplitude of compound EJPs was not significantly changed by them. In figure 5E, CCAP produced many double or triple spikes though it is hard to see the effects in the panel in figure 5E2, recorded by a pen-writer, where shortened vertical swings in the ganglion trace indicate double or triple spikes.

RPCH-family peptides, a locust adipokinetic hormone (AKH-II) and a cockroach AKH-I-like peptide did not significantly affect heart beat. Neither the cardiac ganglion nor myocardial cells showed any perceptible modulation from AKHs at a concentration of 10^{-5} M (data not shown).

It is characteristic, in general, that recovery from effects of peptides was slower than from neurotransmitters.

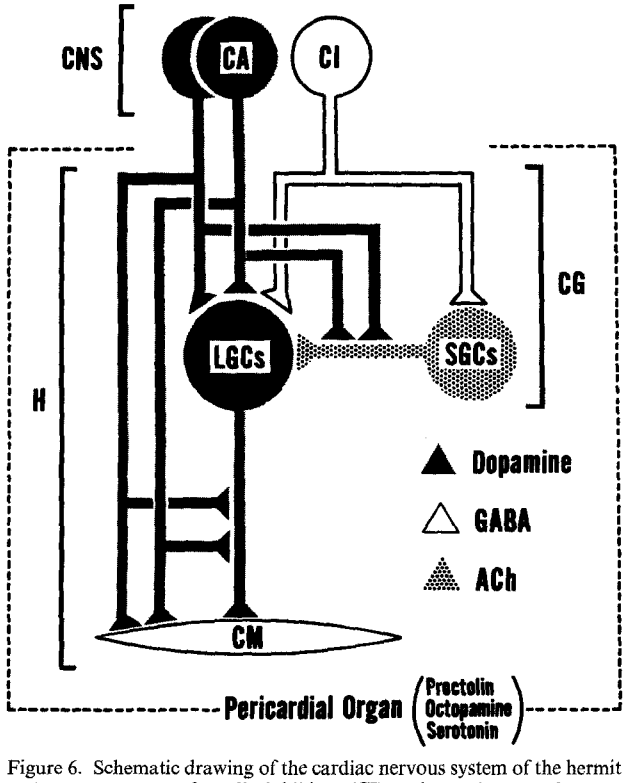

Figure 6. Schematic drawing of the cardiac nervous system of the hermit crab. The neurons of cardio-inhibitor (CI) and -accelerator (CA) are located in the central nervous system (CNS). A pair of CI axons innervates the cardiac ganglion (CG) making synaptic contact with both small and large ganglionic cells (respectively SGCs and LGCs). Two pairs of CAs innervate both the cardiac ganglion (both SGCs and LGCs) and the myocardium (CM). SGCs innervate LGCs. LGCs innervate the myocardium. Hormonal substances are liberated from the pericardial organ. H: heart. Transmitter candidates are dopamine for CAs and LGCs, GABA for CI, and ACh for SGCs. Major cardio-excitatory hormones are proctolin, octopamine and serotonin.

Conclusion

In the hermit crab heart, neurotransmitters of small ganglionic cells, large ganglionic cells, cardio-accelerators and cardio-inhibitors can be, respectively, acetylcholine (ACh), dopamine, dopamine and gamma-aminobutyric acid (GABA). Proctolin, octopamine and serotonin (5HT) may be major cardio-excitatory hormones.

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