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RE-EVALUATION OF THE STRETCH SENSITIVITY HYPOTHESIS OF CRUSTACEAN HEARTS: HYPOXIA, NOT LACK OF STRETCH, CAUSES REDUCTION IN HEART RATE OF ISOLATED HEARTS

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Summary

Decapod crustacean hearts are suspended by a three-dimensional array of alary ligaments. These ligaments are stretched during systole; diastolic filling *via* the ostia occurs as the ventricle is stretched by ligamental elastic recoil. There is no direct venous return to the hearts in these animals. In the present study, an isolated heart preparation with intact ligaments, hereafter called *in situ*, was used to evaluate the effects of artificially induced stretch on heart rate. Strongly beating *in situ* neurogenic hearts of the crab *Carcinus maenas* responded to direct perfusion of the ventricle with oxygenated saline and the attendant augmentation of natural stretch with a small increase in heart rate (f_H); however, f_H was well maintained for up to 15min after eliminating stretch by cutting the alary ligaments. In contrast to crabs, high rates of artificial perfusion usually depressed f_H in crayfish hearts. Crab heart rate falls during hypoxia and this is readily reversed by even low rates of perfusion with oxygenated saline. It is concluded that the gradual decline in f_H of totally isolated *in vitro* hearts arises from the deepening intraventricular hypoxia experienced by the cardiac ganglion.

Introduction

The striated muscle fibers of the decapod crustacean heart are not spontaneously active (Anderson and Cooke, 1971). Heart beat arises from the burst discharge of the cardiac ganglion (CG), which is located on the inner dorsal wall of the single ventricle (Alexandrowicz, 1932; Kuramoto and Yamagishi, 1990). Dendrites of the CG neurons ramify among the adjacent muscle fibers and the axons of the larger anterior motor neurons innervate the myocardium polyneuronally (Alexandrowicz, 1932; Kuramoto and Kuwasawa, 1980).

Since Carlson's early observations (1906), it has been assumed that the rhythmicity of these neurogenic hearts is responsive to and even dependent on stretch. In intact animals, the elastic recoil of the alary ligaments supplies the stretch that causes diastolic filling, while totally isolated hearts collapse and do not aspirate fluid during diastole. Isolated and

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unstretched hearts, even when held in oxygenated saline, are found to slow and eventually cease beating entirely, and direct ventricular perfusion has been used to maintain *in vitro* viability. Maynard (1960) reviewed earlier studies and presented a figure of his own (Maynard, 1960, Fig. 10) showing that artificial distension of isolated hearts caused increased heart rate (f_H). Both he and Cooke (1988) have concluded that stretch of the dendritic arborizations of the cardiac ganglion neurons is necessary to maintain bursting rhythmicity. Although not actually called the 'stretch hypothesis', it is generally accepted that heart rate in crustaceans is sensitive to stretch (Kuramoto and Ebara, 1984, 1985; Hill and Wyse, 1989).

Crustacean hearts lack a venous return and fill by a small negative pressure gradient which exists between the ventricle and the pericardial sinus (see reviews by McMahon and Wilkens, 1983; McMahon and Burnett, 1990). Thus, although the inflation of *in vitro* hearts by direct perfusion is unnatural, this procedure is thought to replace the stretch normally provided by the alary ligaments. The assumption that the CG requires some level of stretch in order to generate the rhythm of action potential bursts has not been rationalized with the many observations that isolated and unstretched CGs, pinned out in a dish, continue to generate a bursting rhythm (see review by Cooke, 1988). Of course, isolated ganglia are devoid of their dendrites, so the above stretch hypothesis cannot be directly tested. Even after reviewing studies on isolated ganglia in which pacemaker potentials were seen, Cooke (1988) speculated that leakage currents resulting from intracellular electrode penetration might account for bursting in isolated CG.

The semi-isolated hearts of crabs and crayfish, suspended by their alary ligaments, hereafter referred to as *in situ*, and maintained in oxygenated saline, beat strongly and regularly for many hours (Wilkens and McMahon, 1992; Wilkens and Walker, 1992). In the present investigation, the effect of direct perfusion of *in situ* and *in vitro* hearts on beating rate (f_H) is reported. Although perfusion-induced stretch does have a small effect on f_H, the work reported here supports a new hypothesis that hypoxia, rather than the absence of stretch of CG dendrites, is the primary cause of decreased f_H in decapod hearts *in vitro*.

Materials and methods

Experiments were conducted on shore crabs, *Carcinus maenas* L., and crayfish, *Procambarus bandingi* and *P. clarkii* Girard. Crabs were maintained in artificial sea water at 13°C and crayfish were held in fresh water at 20°C. Experiments were conducted at the same temperature as the animals were held. Salines formulated for *Carcinus* (Wilkens *et al.* 1989) and crayfish (Van Harreveld, 1936) were used for these experiments.

Observations were first made on *in situ* hearts removed from the animal, but left 'on the half-shell', and subsequently on many of the same hearts *in vitro*, i.e. after total isolation. The *in situ* preparation (Wilkens and McMahon, 1992) consisted of the heart suspended by its alary ligaments from the dorsal carapace, the thoracic walls and the pericardial septum. A cannula leading to an electromagnetic flow transducer (Biotronex BL-610 pulsed-logic flowmeter) having a resistance of $1.49\,\mathrm{N\,s\,m^{-5}}$ ($120\,\mathrm{Pa\,min\,ml^{-1}}$) was tied

into the sternal artery to measure arterial flow. Arterial pressure (Pa) was measured by a Gould P23Db Statham pressure transducer attached to a T-connector inserted into the arterial cannula. A second cannula, leading from a Hewlett Packard 311A amplifier and 267BC pressure transducer, was inserted into the ventricle through one of the hepatic arteries and its valve to measure ventricular pressure (Pv). All other arteries were tied shut. Cardiac output ($\dot{Q}$) was computed from the product of f_H and stroke volume, where stroke volume was obtained by integrating the area under arterial flow waveforms with a software program on a Nicolet 9094 digital oscilloscope.

A peristaltic pump delivered O_2- or N_2-equilibrated saline (P_{O_2}>67kPa or <0.7kPa, respectively) directly into the ventricular lumen *via* a 21 gauge syringe needle-tipped cannula inserted through the ventral wall of the ventricle. The insertion of this cannula had no effect on heart rate and pressures. Adding a small bolus of Methylene Blue solution to the perfusate was used to check for leaks and to confirm that all fluid was pumped out through the sternal artery. The perfusion cannula entered the heart at a shallow angle at a point midway between the front of the heart and the sternal artery. The perfusate was equilibrated to the bath temperature before it entered the heart. The actual P_{O_2} of the perfusate as it entered the heart was not measured, but that of the bath was measured with a Strathkelvin 781 O_2 electrode and amplifier. Following *in situ* observations, the effects of perfusion were retested after all alary ligaments and other connective tissue attachments had been cut. All cannulae remained in place during this surgery.

The signals from the flow and pressure transducers were stored on a Hewlett-Packard 2964A instrumentation tape recorder and displayed on a four-channel Gould 2400 polygraph. Heart rate (f_H) was measured with a Gould ECG amplifier.

Results

Crab hearts

Carefully prepared *in situ* crab and crayfish hearts pump strongly for hours when maintained in oxygenated saline. The direct perfusion of crab hearts with oxygenated saline had a small stimulatory effect on heart rate, while Pv, Pa and stroke volume were increased in proportion to perfusion rate (Figs 1A, 2). For the heart shown in Fig. 1A, the f_H and $\dot{Q}$ before perfusion were 40min^{-1} and $2.23\text{ml}\text{min}^{-1}$, respectively. Perfusion with oxygenated saline at rates above $2\text{ml}\text{min}^{-1}$ caused a moderate and progressive increase in f_H which reached 68min^{-1} at the highest perfusion rate. Fig. 2 summarizes the findings for 13 hearts under similar conditions. The mean percentage increase at the highest perfusion rate was 13.9%, although in three preparations f_H actually fell (mean decrease 13.4%) as perfusion rate was increased. The single point to the left of the perfusion curves in Fig. 2 shows that occluding arterial outflow by pinching the sternal artery shut did not affect f_H even though ventricular pressure more than doubled.

In four hearts the outflow resistance of the sternal arterial cannula (afterload) was increased from 1.49 to $6.44\ \text{N}\,\text{s}\,\text{m}^{-5}$ ($120–520\text{kPa}\,\text{min}\,\text{ml}^{-1}$). This restriction in outflow increased the perfusion-induced ventricular distension. The perfusion-induced changes in f_H at high afterload did not differ from those at the lower afterload. On one of these four

hearts, two flecks of carbon from a carbon rod were glued with cyanoacrylate adhesive to the ventral surface of the ventricle at a point where the heart was 8.29mm wide. Their spacing was measured with an ocular micrometer. In the absence of perfusion, the flecks were located 4.13mm apart during diastole and perpendicular to the long axis at the

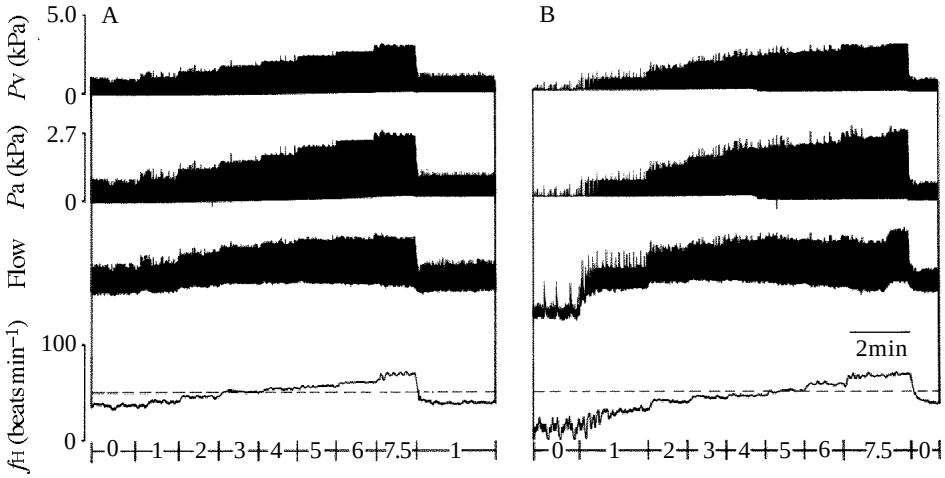

Fig. 1. Responses of an *in situ Carcinus* heart to stepwise increases in perfusion rate of oxygenated saline when the bath was (A) oxygen- (P_{O_2}>70kPa) and (B) nitrogen-equilibrated (P_{O_2}<0.7kPa). Ventricular (*P*v) and arterial (*P*a) pressures and stroke volume increased similarly in both cases. A calibration bar is not included for the flow traces because they were recorded in the pulse mode (see Materials and methods). The bottom line of each record shows the rate of perfusion in mlmin^{-1}.

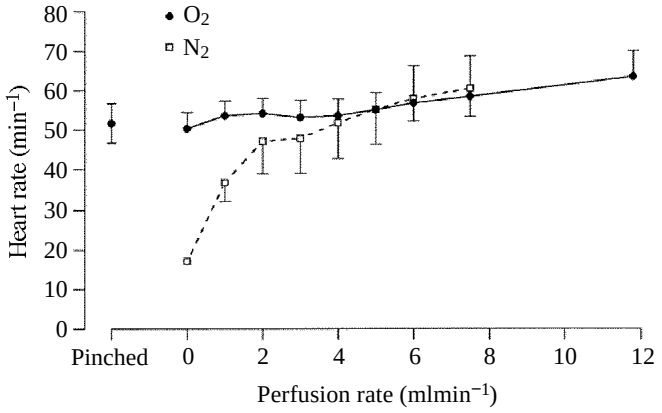

Fig. 2. Summary of the responses of 13 *Carcinus* hearts to artificial perfusion with oxygenated saline (solid line). When the bath was oxygenated, mean unperfused f_H was 51min^{-1} and this increased by 18% at the highest perfusion rate (solid line). For five of these hearts, the effect of pinching shut the sternal artery is shown as a single point to the left of the perfusion data. Also shown are the responses of five of these hearts held at a low bath P_{O_2} (<0.7kPa) to perfusion with oxygenated saline (dashed line). All of these hearts were tested at low afterload. Data presented as mean ± S.E.M.

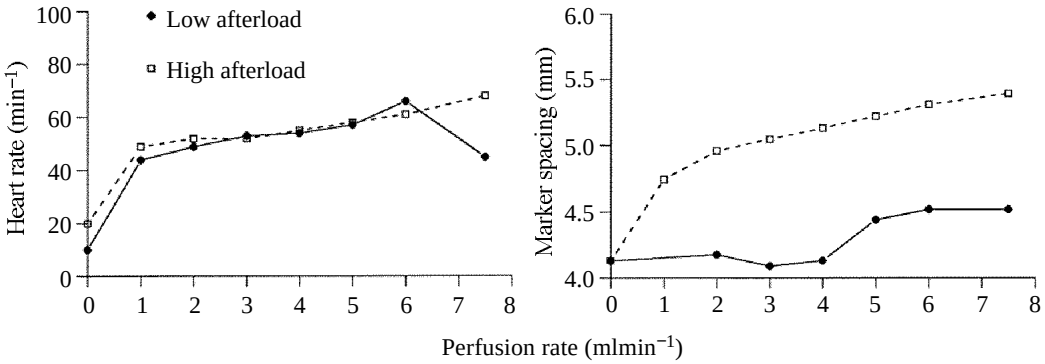

Fig. 3. The amount of perfusion-induced stretch of a single *Carcinus* heart was substantially increased when the afterload on the heart was increased fourfold. The increased stretch at the higher afterload did not change the f_H response to the perfusion.

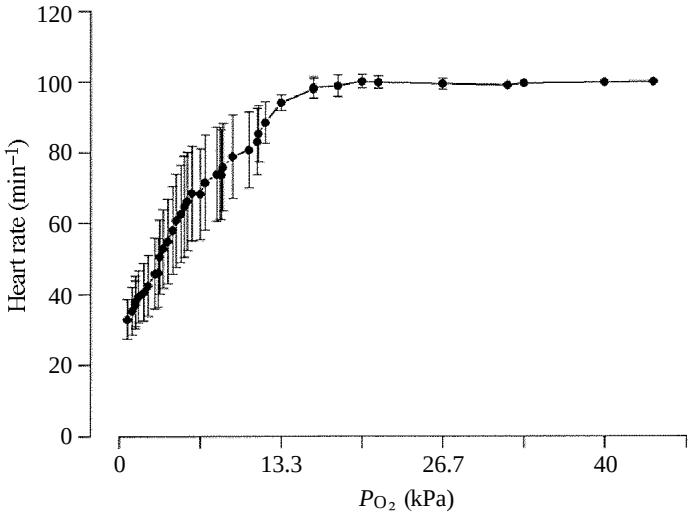

Fig. 4. The effect of decreasing bath P_{O_2} on f_H of *Carcinus*. The reductions in P_{O_2} required about 20min of rapid N_2 bubbling of the bath. Mean ± S.E.M., N=4.

middle of the heart. During perfusion, heart rate increased similarly under both afterload conditions even though the ventral wall, and presumably the dorsal wall with the CG, was stretched 43% more with the higher afterload (Fig. 3).

As the P_{O_2} of the bath was reduced by bubbling with N_2, f_H remained relatively stable until P_{O_2} fell below 13.3kPa. Below 13.3kPa, f_H fell linearly with further reductions in P_{O_2} (Fig. 4). In four hearts held at <0.7kPa for up to 5min f_H remained steady at 25–30min^{-1}; longer periods of hypoxia caused further reductions in rate. Figs 1B and 2 show the responses of hearts maintained at <0.7kPa to perfusion with O_2-equilibrated saline. In Fig. 1B the frequency of the unperfused hypoxic heart had fallen to 14min^{-1} at the point when perfusion was started; f_H increased to 66min^{-1} at the highest rate of perfusion with oxygenated saline. The increment in f_H in response to 1 and 2mlmin^{-1}

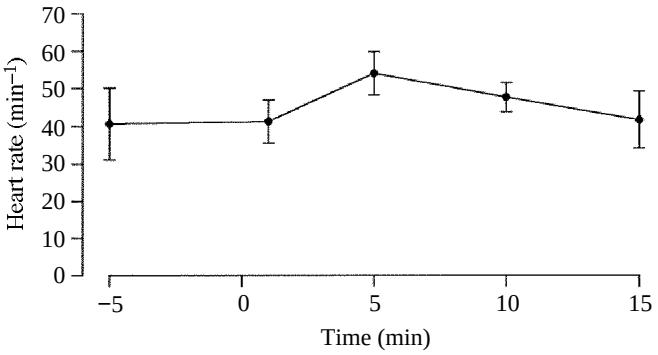

Fig. 5. Heart rate following ablation of the alary ligaments. All ligaments were cut just prior to the recording at time zero. When maintained in oxygenated saline, this operation had no effect on the f_H of crabs for up to 15min; however, heart rate gradually declined thereafter. Mean ± S.E.M., N=5.

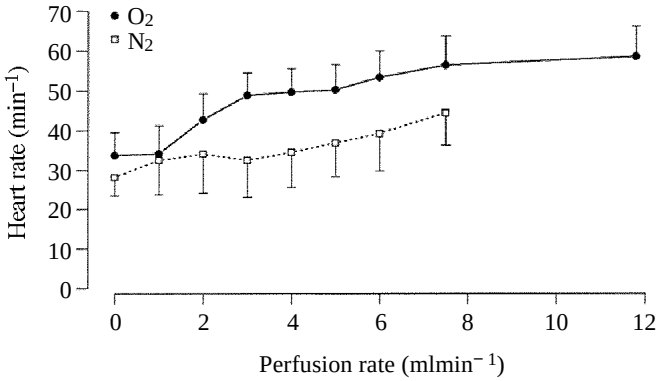

Fig. 6. Comparison of the effects of ventricular perfusion on f_H of *in vitro Carcinus* hearts perfused first with oxygenated saline and then with hypoxic saline. All hearts were held in oxygenated saline. Mean ± S.E.M., N=5.

perfusion was greater than that of all further increases. The maximum f_H attained by each heart approached that shown by the same heart when the bath was oxygenated.

Double systolic contractions were occasionally observed in crab and crayfish hearts, but usually only after a period of observation and manipulation and only in isolated hearts without ventricular perfusion. At this point, perfusion of crab hearts with oxygenated saline often abolished the double beats (not shown).

In several preparations, all ligamentous attachments were cut following the perfusion tests described above. Even without any form of stretch the rate of beating increased slightly after isolation and was well maintained for up to 15min (Fig. 5). Decapod hearts do not possess any elastic restoring properties, as do vertebrate hearts, and as a consequence they collapse and do not aspirate fluid during diastole. After isolation, sternal arterial flow stopped and ventricular pressure pulses were negligible. It is clear that diastolic expansion and an attendant stretch of CG dendrites is not required by the CG in order to maintain rhythmic bursting at the same rates as shown by *in situ* hearts.

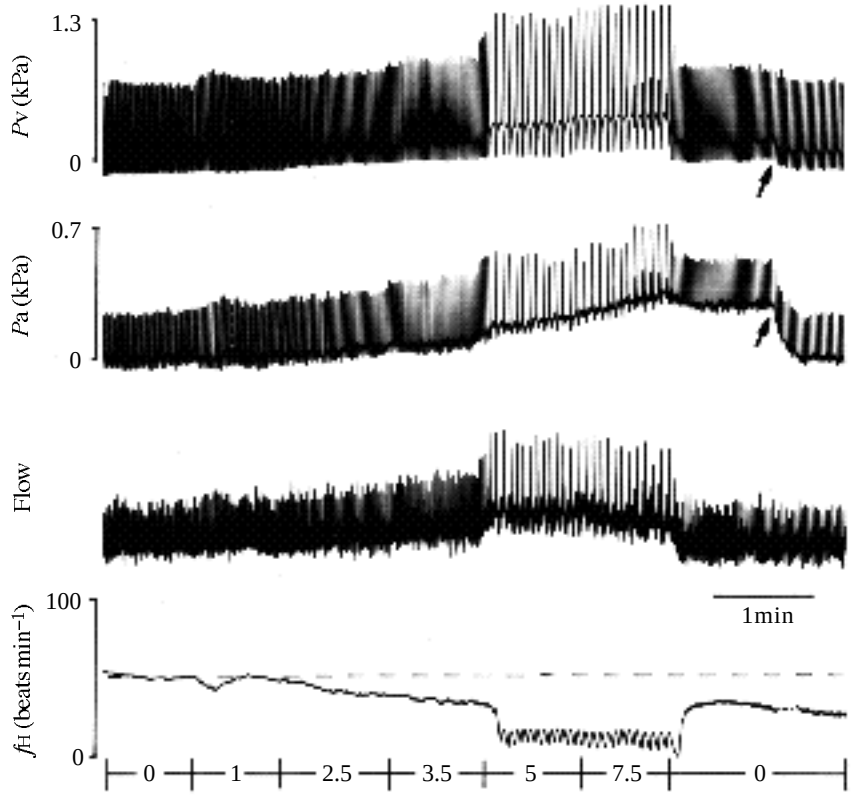

Fig. 7. An *in situ Procambarus clarkii* heart in which f_H declined steadily as perfusion rate increased. The bath and perfusate were oxygenated. The apparent increase in the baseline of diastolic ventricular and arterial pressures was an artifact caused by an increase in bath depth. This occurred because the bath-aspirating cannula had become blocked. At the arrows the original bath level was re-established.

After the *in vitro* f_H had declined to approximately $30 min^{-1}$, often 30 or more minutes after the ligaments had been cut, direct perfusion with oxygenated saline caused increases in f_H to levels slightly lower than the same hearts had shown prior to isolation (Fig. 6). Perfusion with N_2-equilibrated saline caused small increases in f_H, but of lower magnitude than the responses to O_2-equilibrated saline. During both types of trials the bath was oxygenated. Attempts were made to measure the responses of isolated hearts in anoxic media to oxygenated perfusate; however, these hearts failed rapidly when subjected to the double insults of isolation and anoxia. Such hearts showed only minimal recovery of function when perfused with oxygenated saline (data not shown).

Crayfish hearts

Six *in situ* crayfish hearts were directly perfused in order to test the generality of the crab findings. These hearts responded somewhat differently from those of crabs to perfusion-induced stretch. Heart rate was increased in one animal, showed no change in a second and decreased in the remaining four as perfusion rate was increased over the same

range as tested on crab hearts. In four of the six hearts, f_H decreased abruptly at a critical increase in perfusion rate; in the case of the heart illustrated in Fig. 7, the critical level occurred at the transition from 5 to 7.5mlmin^{-1}.

Discussion

It is worth noting again that the cardiac ganglion burst rate determines f_H of decapod crustacean neurogenic hearts and that any perturbations that affect f_H must do so by altering the pacemaking properties of these neurons. The f_H of oxygenated *in situ Carcinus* hearts can exceed 100min^{-1} during neurohormonal stimulation (Wilkens and McMahon, 1992) and *in situ* f_H in *Procambarus* can reach similar rates during cardio-accelerator nerve stimulation (Wilkens and Walker, 1992). The data presented here show that strongly beating *in situ* crab and crayfish hearts, removed from all nervous and neurohormonal input, are relatively insensitive to the stretch produced by artificial perfusion of the ventricle with oxygenated saline. Perfusion rates 4–5 times greater than $\dot{Q}$ of the unperfused hearts produced visible distention of the ventricle. In some cases, particularly in *Procambarus* hearts, perfusion actually caused decreases in f_H. Occluding all outflow of unperfused hearts by pinching the sternal artery shut does not in itself stretch the hearts, but causes them to contract isovolumetrically. P_V was doubled by such occlusion, but f_H was unaffected.

The amplitude of contractions, as represented by P_V, increased with ventricular perfusion. The minimum diastolic pressure in *Carcinus*, i.e. the baseline of the pressure traces, did not change as perfusion rates were increased, indicating that the ejection fraction also increased. The increased amplitude of contractions may have arisen from the stretch of the individual fibers of the myocardium to a more favorable Frank–Starling force/length relationship or from a change in orientation of the fibers relative to the circumference of the ventricle. The largely oblique fiber orientation in undistended hearts is complex (Maynard, 1960).

Heart rate and P_V were depressed when bath oxygen tension fell below 13.3kPa and perfusion of deeply hypoxic hearts with oxygenated saline dramatically increased f_H. The greatest increments of change occurred during the initial perfusion at 1–2mlmin^{-1}, perfusion rates which had virtually no effect on hearts bathed in oxygenated saline. In these cases, the delivery of oxygen was perfusion-limited and the major determinant of f_H was the availability of oxygen, not the perfusion pressure. The oxygenated saline is assumed to act directly on the cardiac ganglion neurons, which are located inside the dorsal wall of the ventricle.

When all ligamentous attachments are cut, the ventricle is not stretched and does not aspirate fluid during diastole because it has no intrinsic elastic restoring properties. In oxygenated bath conditions, the contraction rates of totally isolated hearts were maintained for up to 15min at the same rate or slightly above that immediately prior to the ablation. The eventual decline in f_H probably occurs as the intraventricular cardiac ganglion neurons become anoxic owing to an oxygen diffusion limitation. The maintenance of f_H for this period requires some explanation. These hearts had all pumped saline and had been perfused for more than an hour. Although not rigorously tested, I

have the strong impression that rapid removal of all traces of hemolymph is important for the continued function of the system. One difference in the *in vitro* hearts used here and those employed in other studies may be that these hearts had pumped out all traces of hemolymph.

Kuramoto and Ebara (1984, 1985, 1988, 1991) reported the occurrence of double contractions in isolated hearts of *Panulirus japonicus*. Double systolic contractions are occasionally observed in slowly beating crab and crayfish hearts. These were converted to single contractions as f_H increased during perfusion. Kuramoto and Ebara (1984, 1988, 1991) show a similar elimination of double beats by *in vitro Panulirus japonicus* hearts when perfusion pressure was increased. Another form of irregularity, namely the alternation of strong and weak beats seen in the first part of Fig. 1B, was also eliminated when perfusion pressure was increased. It is not possible to ascribe the elimination of these double and irregular beats during perfusion to the increased supply of oxygen or to stretch.

In the open circulatory system of crustaceans, the only natural stretch of the heart will come from the elastic recoil of the alary ligaments during diastole and possibly from ventral movements of the pericardial septum, which is connected to the ventral wall of the heart by numerous strands of connective tissue (Baumann, 1921). It is clear from the present study that such stretch has little effect on f_H. The dominant factor affecting f_H appears to be oxygen delivery to the cardiac ganglion.

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