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## MODULATION OF OUTPUT FROM AN ISOLATED GASTROPOD HEART: EFFECTS OF ACETYLCHOLINE AND FMRFamide

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### SUMMARY

In this study, two cardioactive drugs, acetylcholine (ACh) and the tetrapeptide FMRFamide, are perfused through the isolated systemic heart of the gastropod, *Busycon canaliculatum*. Their effect is examined in terms of the regulation of output, and is then related to the *in vivo* regulation of stroke volume.

ACh decreases cardiac output by reducing both stroke volume and heart rate. End-diastolic volume and cardiac reserve increase with drug concentration. These effects are accompanied by a slowing in the rise time of the electromyogram prepotential and an increase in the duration of the plateau phase. Low concentrations of FMRFamide increase output by accelerating the heart rate. Stroke volume is only affected at higher concentrations ( $5 \times 10^{-7} \text{ mol l}^{-1}$ ), and then negatively. End-diastolic volume is reduced. Between  $10^{-9}$  and  $10^{-8} \text{ mol l}^{-1}$ , FMRFamide increases the rise time of the prepotential and the amplitude of the plateau; the duration of the plateau is markedly shortened. At  $5 \times 10^{-7} \text{ mol l}^{-1}$  and above, the plateau is extended and the cardiac reserve is reduced to zero. The two drugs have opposite effects on the characteristics of the aortic pressure pulse: ACh reduces the amplitude of the pulse, but increases its duration.

### INTRODUCTION

Factors modulating cardiac output are often examined separately, although *in vivo* they would act in concert. This is particularly apparent in the treatment of intrinsic and extrinsic modulation. Although this approach has obvious advantages, it can result in data which are difficult to relate to cardiac performance in the whole animal, a situation which has certainly occurred in studies on molluscan cardiovascular physiology. This paper, the third in a series (Smith, 1985a; Smith & Hill, 1986) uses the systemic heart of *Busycon canaliculatum* to examine some basic parameters of cardiac modulation *in vitro*, with the aim of extending and eventually testing the conclusions on an *in vivo* preparation.

The energetics and intrinsic modulation of the *Busycon* heart, under various preload and afterload perfusion pressures, have already been examined (Smith,

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1985a). The performance of the heart is predictable, with stroke volume varying in direct proportion to the preload value. In this respect, the *Busycon* heart conforms to Starling's law, as do the ventricles of two other molluscs examined, *Aplysia* and *Eledone* (Straub, 1901, 1904; Smith, 1981).

Intrinsic modulation will act *in vivo* in conjunction with such extrinsic effects as the action of cardioactive neurotransmitters. In *Busycon*, for example, 5-hydroxytryptamine (5-HT) alters heart rate in a dose-dependent manner, but has little or no effect on stroke volume (Smith & Hill, 1986). It does, however, have a profound effect on the manner in which the blood is ejected from the heart. Pressure pulse amplitude rises with 5-HT concentration at constant perfusion pressures, but the duration of the pulse drops in such a way that the stroke volume remains relatively constant.

The present study examines the action of two further putative cardioregulatory substances in the mollusc: the tetrapeptide FMRFamide (Phe-Met-Arg-Phe-NH<sub>2</sub>) and acetylcholine (for reviews see Jones, 1983; Painter & Greenberg, 1982). The emphasis is not on the pharmacological action of these two drugs, but on how they might contribute to the dynamic regulation of cardiac output, working in parallel with other cardioregulatory substances and the intrinsic properties of the myocardium. For this reason, attention is paid, not only to the effects on stroke volume and heart rate, but also to the manner of fluid ejection and the ventricular end-diastolic volume. These results are compared with changes in the electromyogram.

#### MATERIALS AND METHODS

The subject of this study was the prosobranch mollusc, *Busycon canaliculatum* (L.). The work was conducted at the Department of Zoology, University of Rhode Island, USA, during the months of March and April, 1985. Animals came from the Marine Biological Laboratory, Woods Hole and were maintained in a recirculating aquarium at a temperature of 20°C. This was also the experimental temperature.

The procedure for isolating and perfusing the heart of *B. canaliculatum* has already been described in detail in previous publications (Smith, 1985a; Smith & Hill, 1986). Briefly, the isolated systemic heart (atrium and ventricle), removed from the constraints of the pericardium, was perfused at controlled levels of preload (input perfusion pressure) and afterload (output back pressure). Cardiac responses to changes in the experimental conditions were assessed in terms of: stroke volume [mean measured output over a known number of beats (approximately 20)]; heart rate measured over a 2- to 3-min period; pressure pulse characteristics; electromyograms recorded with surface suction electrodes; and gross changes in ventricular end-diastolic volume. Except for changes in the ventricular volume, the methods of collecting and recording the data have been described in detail before (Smith, 1985a).

The cardiac volume was recorded crudely with a rotary motion transducer (Harvard, Model 356) equipped with a light wooden stick which was arranged so as to rest against the surface of the heart. As the volume of the heart changed, the stick

moved. The output signal was amplified using a Grass Low Level d.c. preamplifier (7P1B) coupled to the Grass Driver Amplifier (7DA) on a Grass Polygraph (76C). The results were unitless and serve only to monitor heart size during the *diastolic pause*.

Hearts were perfused both internally and externally with the test solutions. Two header-tanks connected to a single input reservoir allowed a rapid change-over from one perfusate to another, without interruption of either the pressure or flow of fluid to the heart (Smith & Hill, 1986). In contrast to the 5-HT study (Smith & Hill, 1986), the effects of acetylcholine iodide (ACh: Sigma) or FMRFamide (Cambridge Biochemicals) on cardiac performance were not examined over a range of preload values. Instead, the preload was fixed at 4 cmH<sub>2</sub>O (1 cmH<sub>2</sub>O = 98.1 Pa) with an afterload of 6 cmH<sub>2</sub>O. A fresh preparation was used for every experiment. The two drugs were never tested on the same heart.

The drugs were tested in ascending concentrations, a time-consuming procedure. To check against the deterioration of the preparation during a concentration series, the hearts were perfused with sea water for about 10 min between tests, and the performance was compared to the initial, control, run. No obvious deterioration was observed. All test solutions were made up either freshly in filtered sea water or, in the case of FMRFamide, from samples of 10<sup>-4</sup> mol l<sup>-1</sup> solutions stored at -70°C.

Fig. 1 shows the relationship between stroke volume and ventricular wet mass (measured as described by Smith, 1985a) for both sexes and at two different conditions of loading. The stroke volumes of hearts from the previous two studies are included. The relationship is non-linear. As cardiac volume might be expected to increase with the square of the mass, power curves, with the equations given in the figure, were fitted to the data. No ventricle heavier than of 0.7 g was used, so the behaviour of this relationship above that mass is not known. Stroke volume was corrected for ventricular size using the equation for a preload of 4 cmH<sub>2</sub>O and an afterload of 6 cmH<sub>2</sub>O and was expressed in ml 0.5 g<sup>-1</sup> ventricular wet mass. The mean heart masses were 0.46 g (±0.08 s.d.) and 0.36 g (±0.1 s.d.) for the ACh and FMRFamide experiments, respectively.

Since the performance of preparations varies considerably and independently of heart mass (Fig. 1), the response to drugs and pressure changes also varies, even after correction for mass. Therefore, quantitative comparisons between preparations, or between the *in vitro* and *in vivo* conditions, should be made cautiously. However, the qualitative responses to either pressure change (Smith, 1985a) or drug application (Smith & Hill, 1986; this study) are similar in all preparations and will therefore be emphasized in this report.

## RESULTS

### *Effects of acetylcholine (ACh)*

The response of the heart to ACh was tested over a concentration range of 10<sup>-14</sup> to 10<sup>-6</sup> mol l<sup>-1</sup>. Only the results from concentrations greater than 10<sup>-12</sup> mol l<sup>-1</sup> are shown in Figs 2-5. Threshold appeared to lie above 10<sup>-9</sup> mol l<sup>-1</sup>.

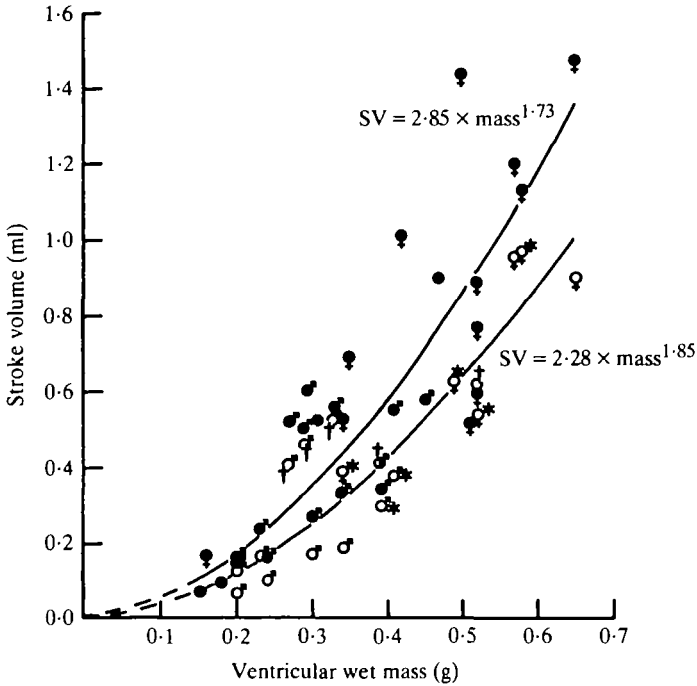


Fig. 1. The relationship between ventricular wet mass and stroke volume (SV) at two different, but constant, perfusion levels. (●) Preload 6 cmH<sub>2</sub>O, afterload 8 cmH<sub>2</sub>O; upper curve. (○) Preload 4 cmH<sub>2</sub>O, afterload 6 cmH<sub>2</sub>O; lower curve. The sex of four animals was not noted. Preparations used to measure the effects of acetylcholine (●) and FMRFamide (†) on stroke volume and heart rate are marked.

ACh acts upon the heart to reduce cardiac output (stroke volume  $\times$  heart rate). This is achieved by a reduction in both the stroke volume (Fig. 2A) and the heart rate (Fig. 2B). The response is dose-dependent. Stroke volume began to show a clear decline at  $5 \times 10^{-8} \text{ mol l}^{-1}$ , whereas heart rate was reduced at  $10^{-8} \text{ mol l}^{-1}$ . At these concentrations, returning the heart to sea water after perfusion with the drug caused a return to control values. As the concentration was increased, however, there was an after-effect which did not wash out during an intermediate period of sea water perfusion. This effect, an increase of both the stroke volume and the heart rate, was dependent on the preceding ACh concentration. At  $10^{-6} \text{ mol l}^{-1}$ , ACh arrested the heart.

Increasing the concentration of ACh also altered the characteristics of the aortic pressure pulse. The perfusate was ejected from the heart over a longer period (Fig. 3A) but at a lower pressure (Fig. 3B). On return to normal sea water, however, there was no appreciable change in the pressure pulse duration compared to the control value, but there was a clear increase of the amplitude. Thus the change in stroke volume on wash-out was accommodated purely by an amplitude increase acting against a fixed resistance (the afterload). The wash-out increase in heart rate was not the result of an accelerated systolic pulse, as this period was unaltered (Fig. 3A).

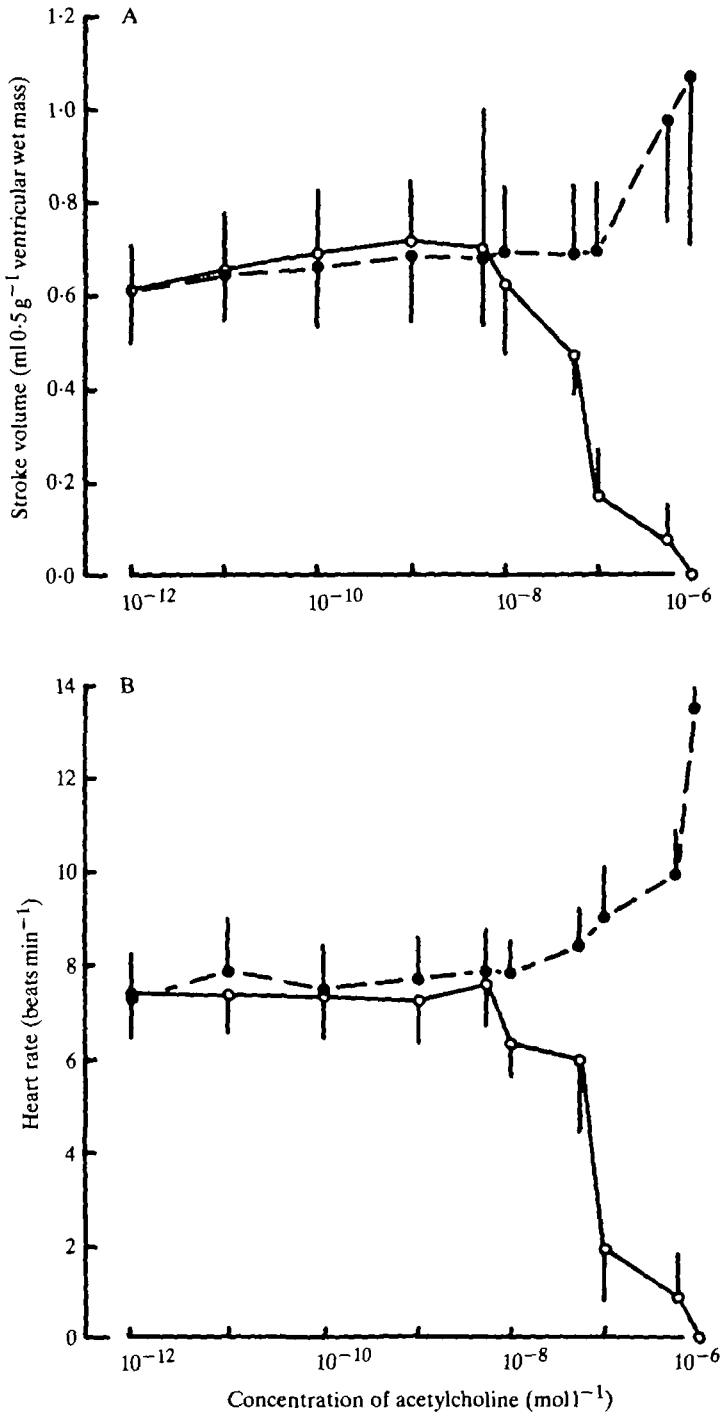


Fig. 2. Effect of acetylcholine (ACh, O) on the stroke volume (A) and heart rate (B). Before perfusion with a new concentration of ACh the hearts were washed out with sea water (●). At about  $10^{-8} \text{ mol l}^{-1}$  ACh reduces both stroke volume and the rate of contraction. On washing out, however, these values increase ( $N = 6$ ; S.E. is shown on one side of each mean value).

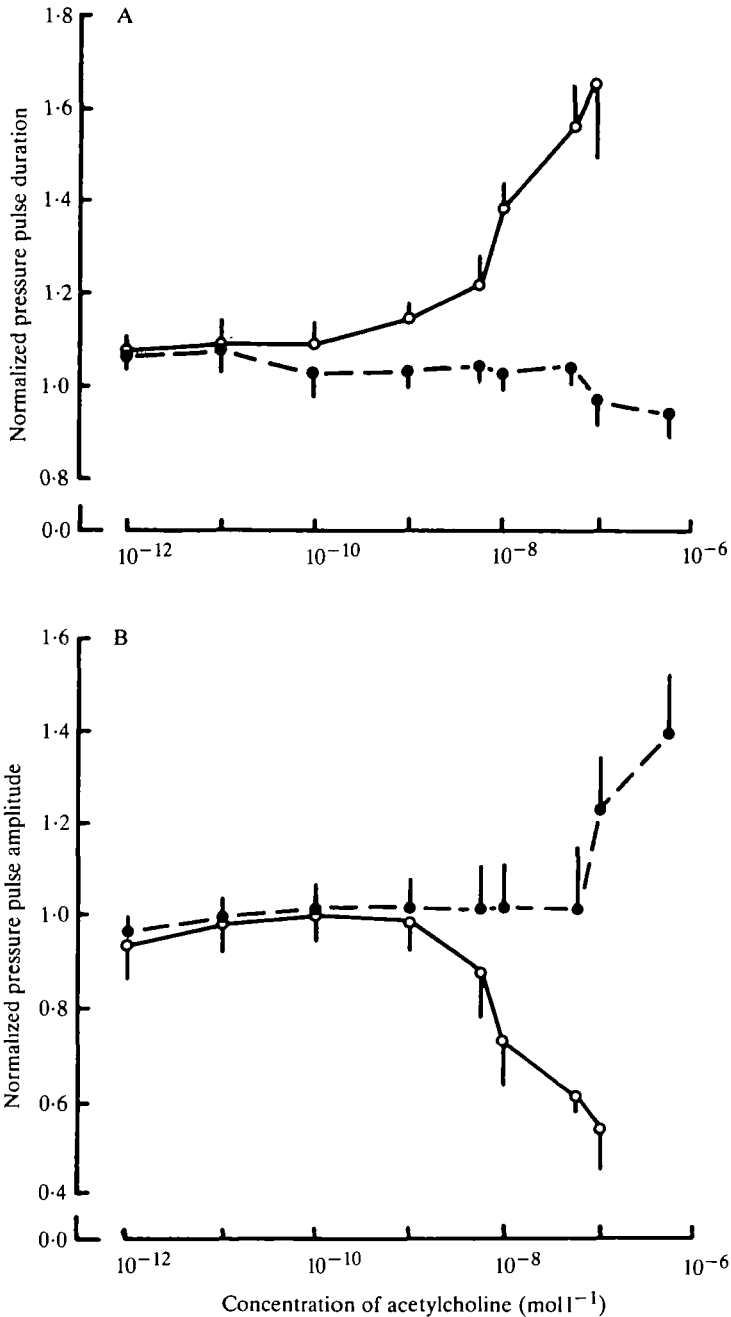


Fig. 3. Effect of acetylcholine (ACh, ○) on the aortic pressure pulse duration (A) and amplitude (B). The results have been normalized relative to the values recorded in sea water at the onset of the experiment ( $10^{-14} \text{ mol l}^{-1}$  ACh) and show a threshold around  $5 \times 10^{-9} \text{ mol l}^{-1}$ . The original characteristics of the pressure pulse were: mean amplitude,  $2.75 \text{ cmH}_2\text{O}$ ; duration,  $3.4 \text{ s}$ . Unlike the effect on rate and stroke volume, washout with sea water (●) returns the pulse duration to the control value but the amplitude remains elevated above  $10^{-7} \text{ mol l}^{-1}$  ( $N = 9$ ).

As with the physical performance of the heart, the electrical activity was first clearly affected by ACh between of  $10^{-8}$  and  $5 \times 10^{-8} \text{ mol l}^{-1}$  (Fig. 4). The effect was progressive, with increased concentrations causing an increased slowing of the prepotential and an increased prolongation of the plateau. The spike became less obvious as concentration increased and the plateau amplitude declined. Changes in the degree of suction will affect the electromyogram amplitudes, so this aspect of the results should be treated with caution. At  $10^{-6} \text{ mol l}^{-1}$  the heart was arrested, but reversibly (as shown in the bottom traces of Fig. 4).

The results in Fig. 4 all come from the same preparation and do not show an after-effect, as the heart was perfused with a high concentration of acetylcholine ( $10^{-6} \text{ mol l}^{-1}$ ) prior to recording the electromyogram. This gave a more stable electrical recording. Hearts perfused for the first time with acetylcholine ( $N = 4$ ) showed basically the same result but the suction, which held the electrode in place on the myocardium, invariably broke down during the recording period.

Fig. 5A,B shows examples of how ACh, at two concentrations, affects ventricular volume and aortic pressure pulse, the greater concentration causing arrest. In both cases it is clear that in addition to either greatly reducing or stopping the aortic pressure pulse, ACh also relaxed the myocardium. This resulted in a ventricular volume increase, although the preload level remained the same. The end-systolic volume, the cardiac reserve, also appeared to be increased. This would be inevitable if end-diastolic volume were increasing while stroke volume was decreasing with ACh concentration, as shown in Fig. 2A.

#### *Effects of FMRFamide*

FMRFamide, between  $10^{-8}$  and  $5 \times 10^{-7} \text{ mol l}^{-1}$ , increased cardiac output. This was achieved entirely by an increase in the heart rate, as no positive effect of FMRFamide on stroke volume was observed (Fig. 6A,B). Despite the variation around the mean values in Fig. 6A and 6B all the preparations showed exactly the same trend. Above  $10^{-7} \text{ mol l}^{-1}$  the stroke volume began to decline, as did the heart rate at  $5 \times 10^{-7} \text{ mol l}^{-1}$ . In the latter case, washing-out with sea water between concentrations returned the heart to control levels.

As with ACh, FMRFamide affected the characteristics of the aortic pressure pulse (Fig. 7A,B). It increased the pulse amplitude while decreasing its duration, an effect upon the ventricle opposite to that of ACh. The perfusate was therefore being ejected at a higher pressure, but over a shorter time. Flow-rate increased. Both responses showed a threshold at approximately  $10^{-9}$ – $5 \times 10^{-9} \text{ mol l}^{-1}$ , balancing one another out in such a way that there was no effect on the stroke volume until  $5 \times 10^{-8} \text{ mol l}^{-1}$ .

At lower concentrations ( $10^{-8}$ – $10^{-7} \text{ mol l}^{-1}$ ) FMRFamide acted on the electromyogram to reduce the duration of both the prepotential and the plateau; spike and plateau amplitude both increase (Fig. 8). This corresponds to an acceleration of beat frequency and rapid change in the pressure pulse characteristics (Figs 6B–8). At concentrations of  $5 \times 10^{-7}$ – $10^{-6} \text{ mol l}^{-1}$  the electromyogram form changed dramatically, with the plateau phase being greatly extended. Heart rate dropped at



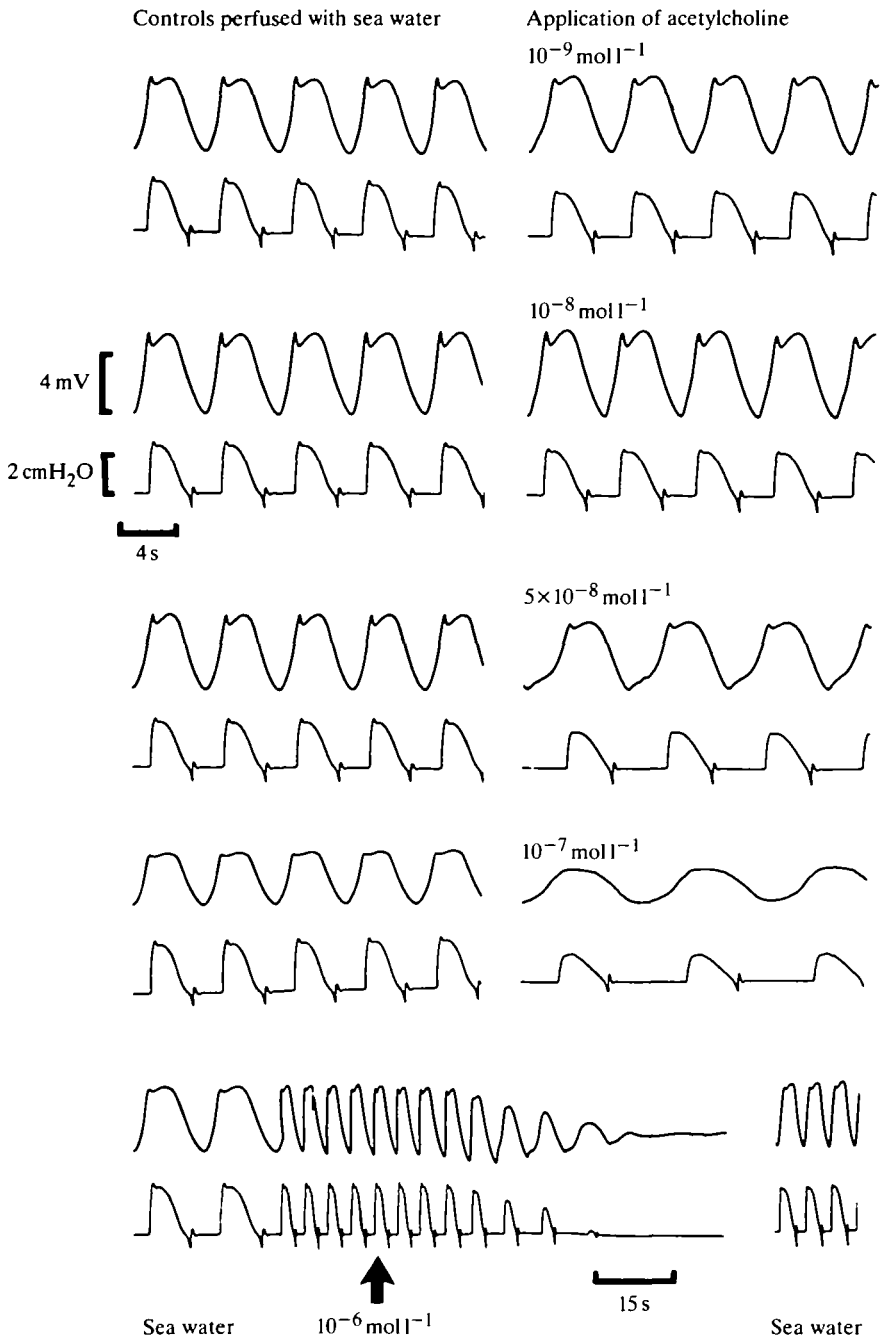


Fig. 4. Effect of acetylcholine (ACh) perfusion on the ventricular electromyogram and aortic pressure pulse. The left-hand column shows the activity in sea water prior to the application of ACh. Increasing concentrations decrease the amplitude of the pressure pulse (lower record) but increase the duration of both this and the electromyogram (upper record). Note the change in the time base in the last series of records.

these concentrations (Fig. 6B) as a result of the failure of the myocardium to repolarize in the normal time.

The effect of FMRFamide on ventricular volume is opposite to that of acetylcholine. End-diastolic volume decreased in a dose-dependent manner (Fig. 9). Visual observations also indicated that, at the higher concentration, the myocardium was rapidly contracted with no appreciable end-systolic reserve. This explains why there was such a rapid and short pressure pulse (Figs 7A, 8) while the plateau duration of the electromyogram was sustained (Fig. 8); all of the perfusate was ejected over the initial period of systolic contraction.

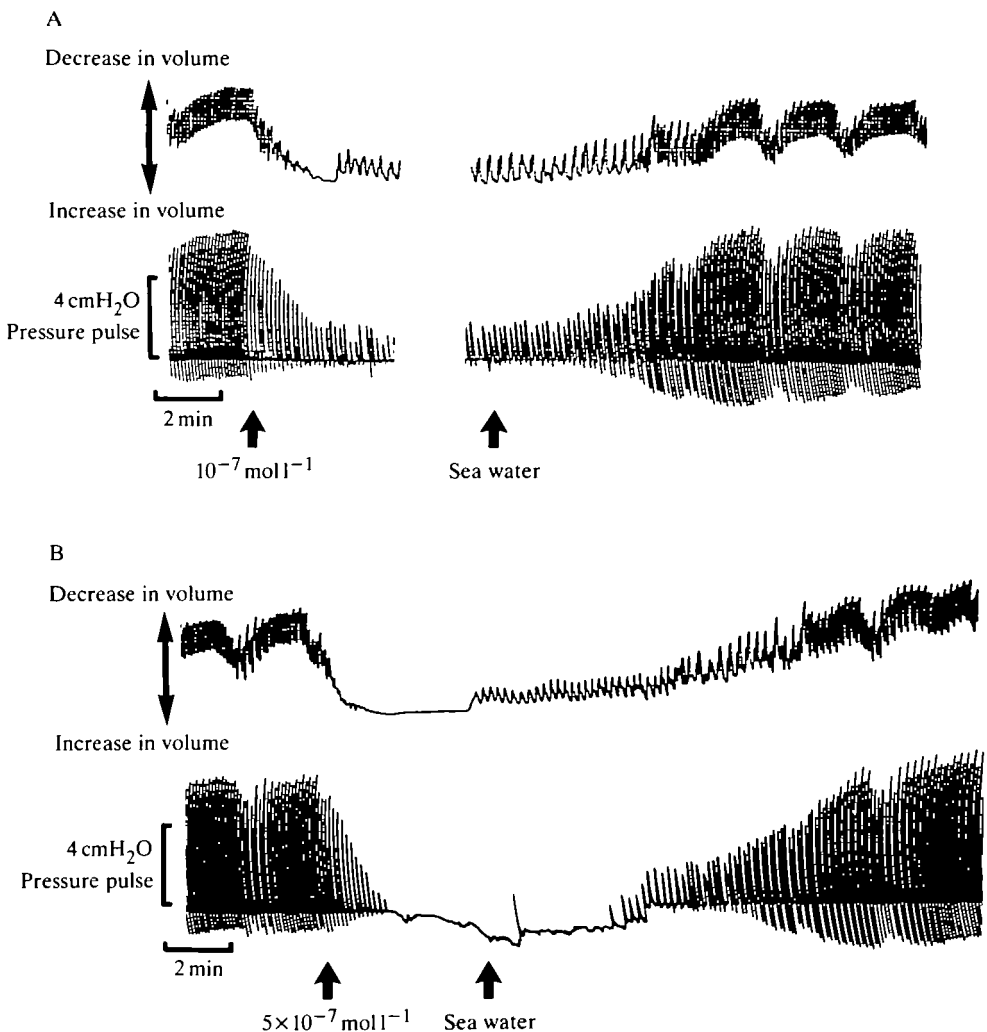


Fig. 5. The effect of acetylcholine (ACh) on the ventricular volume and pressure pulse at (A)  $10^{-7} \text{ mol l}^{-1}$  and (B)  $5 \times 10^{-7} \text{ mol l}^{-1}$ . The higher concentration arrests the heart. ACh relaxes the tone of the heart allowing a larger end-diastolic volume for the same preload value (in this case,  $4 \text{ cmH}_2\text{O}$ ).

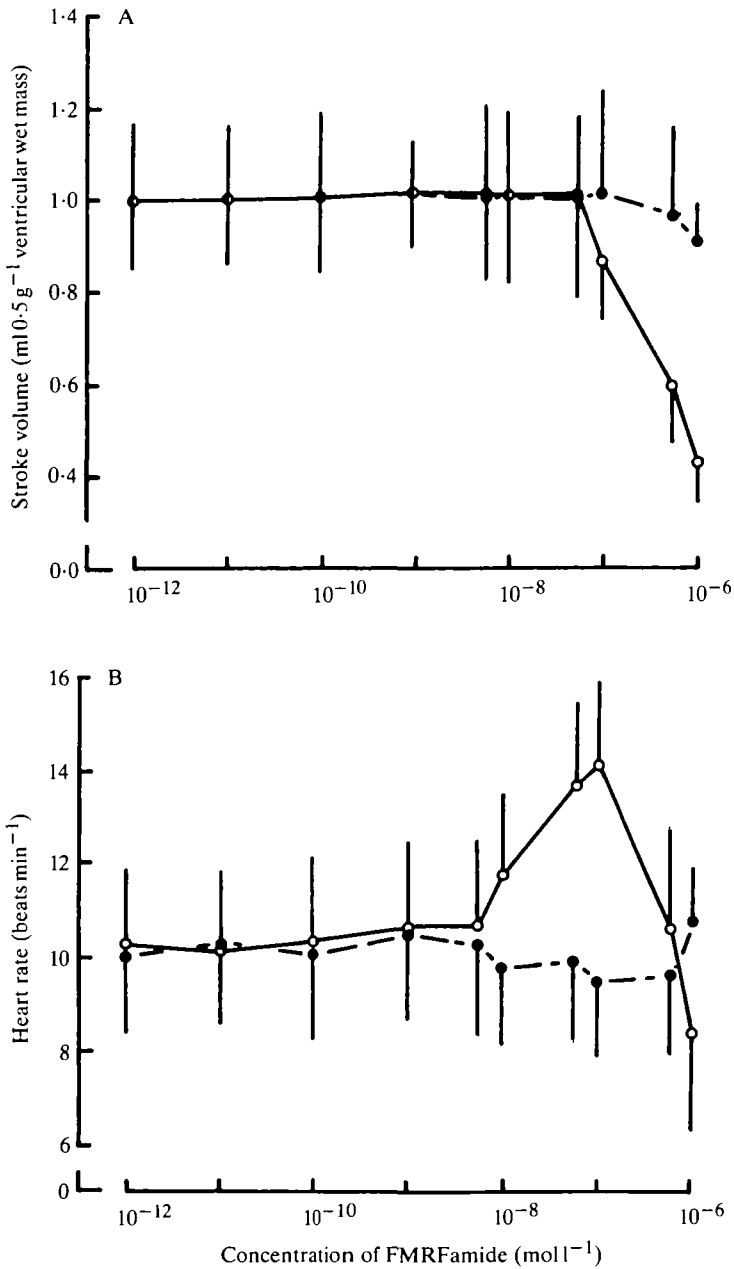


Fig. 6. Effect of FMRFamide on the stroke volume (A) and heart rate (B,  $\circ$ ). Stroke volume begins to decline at concentrations above  $5 \times 10^{-8} \text{ mol l}^{-1}$ . Rate is affected at concentrations above  $5 \times 10^{-9} \text{ mol l}^{-1}$ , but the action is reversed above  $10^{-7} \text{ mol l}^{-1}$ . Between doses of peptide the hearts were washed out with sea water ( $\bullet$ ) returning the performance to control values ( $N = 5$ ; s.e. is shown on one side of the mean value).

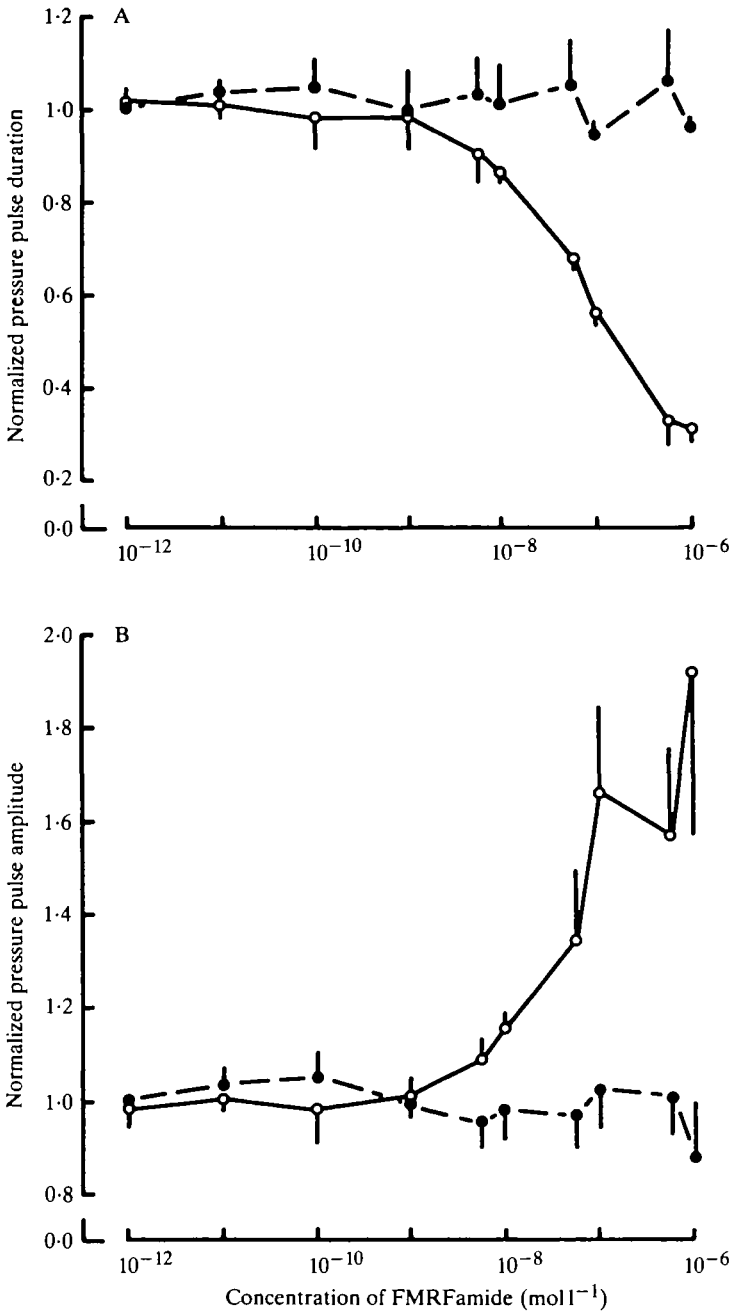


Fig. 7. Effect of FMRFamide on the aortic pressure pulse duration (A) and amplitude (B, ○). The data has been normalized relative to the initial values obtained during sea water perfusion. The original characteristics of the pressure pulse were: mean amplitude, 3.4  $\text{cmH}_2\text{O}$ ; duration, 4.0 s. Values in sea water prior to each perfusion with FMRFamide are shown by solid symbols. Both amplitude and duration are affected at concentrations above  $10^{-9} \text{ mol l}^{-1}$ . Single standard errors are shown on one side of the mean values ( $N = 6$ ).

## DISCUSSION

In the isolated *Busycon* heart, ACh reduces cardiac output (stroke volume  $\times$  heart rate) by its effect on both heart rate and stroke volume. The reduction in beat

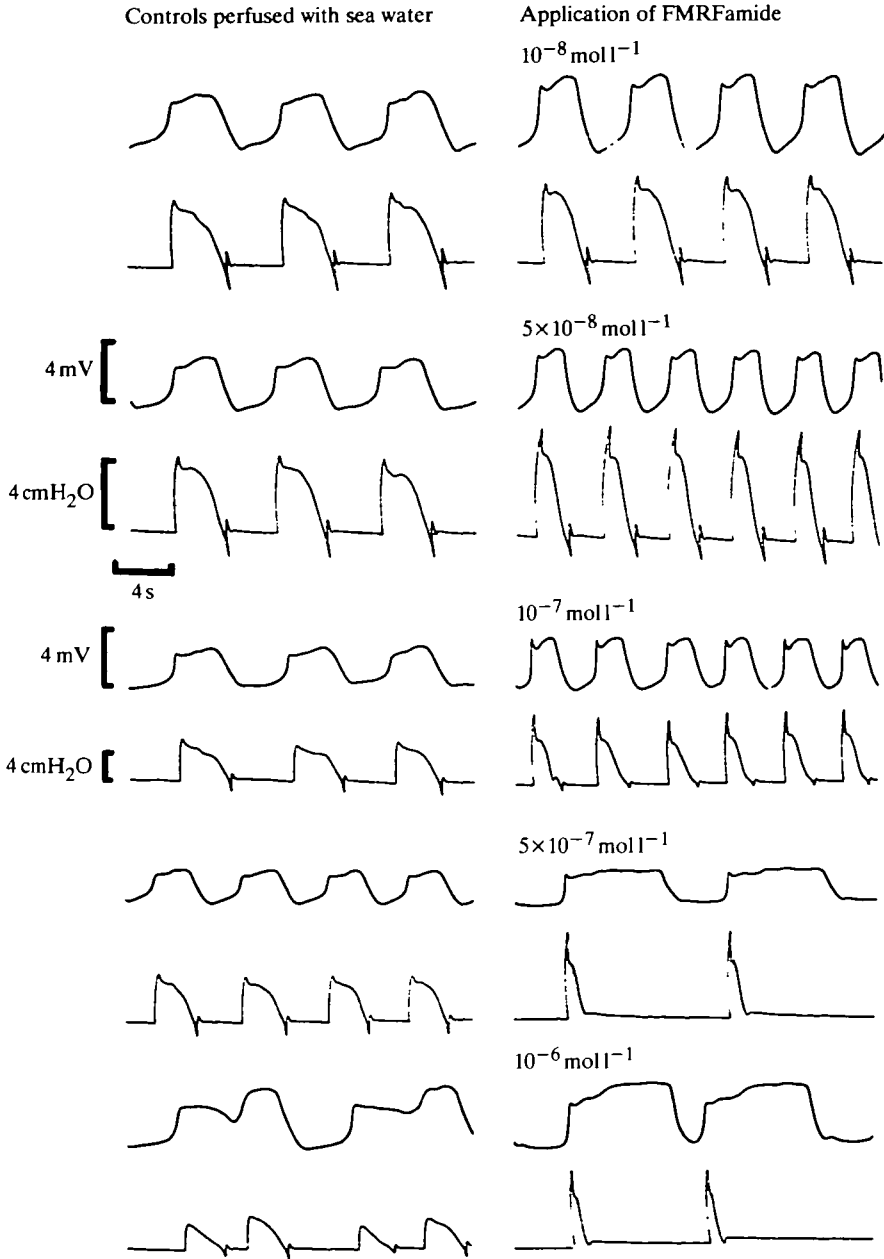


Fig. 8. Effect of FMRamide on the ventricular electromyogram and aortic pressure pulse. Initially there is a shortening of the plateau duration and an accelerated rise of the prepotential. At higher concentrations the plateau duration is greatly extended and the rate drops. Note that the scale of the pressure pulse amplitude changes at  $10^{-7} \text{ mol l}^{-1}$ .

frequency is reflected by a slowing in the rate of rise of the prepotential and an increase in the duration of the plateau. The latter corresponds to an increase in the duration of the aortic pressure pulse, but the amplitude of the pressure pulse, working against a constant pressure difference, decreases with ACh concentration. The end-diastolic volume increases with ACh concentration.

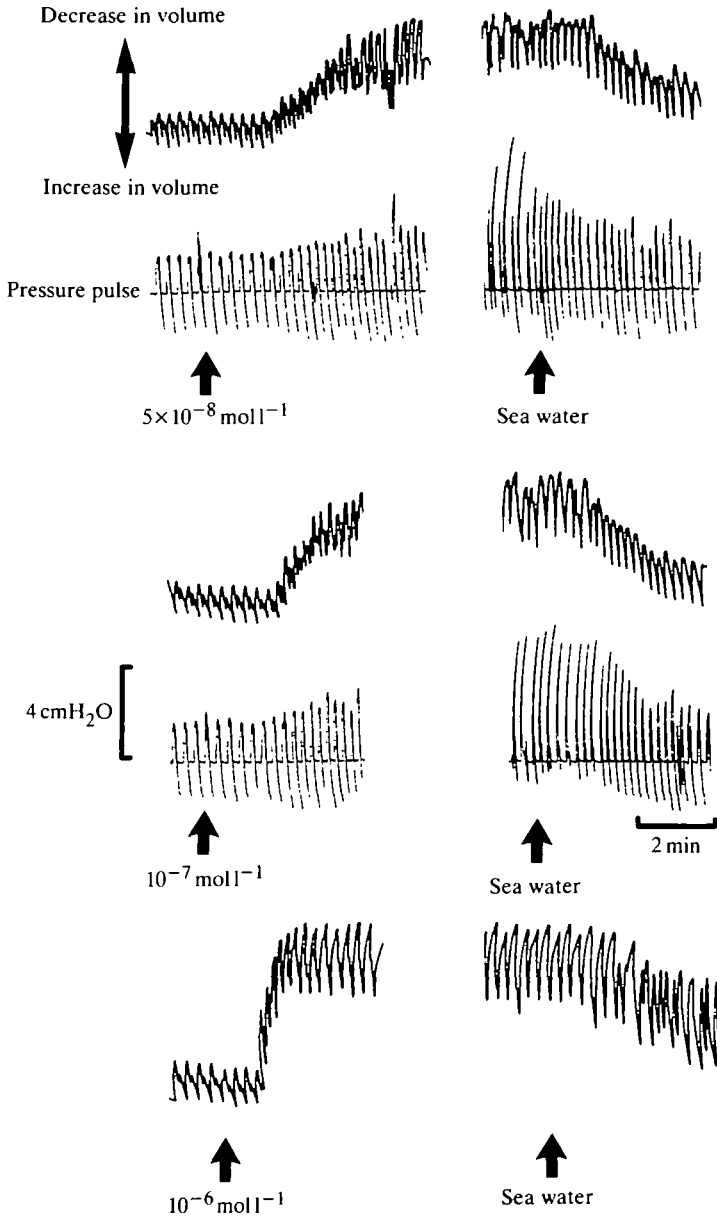


Fig. 9. The effect of FMRFamide on the ventricular volume and pressure pulse at three different concentrations. Here the ventricular volume is decreased at the end of diastole. The pulse pressure has been omitted from the last concentration.

At lower concentrations, FMRFamide acts in a manner opposite to that of ACh; cardiac output is increased. This is achieved solely by an increase in heart rate, as there is no indication that FMRFamide alters stroke volume. These responses correspond to a more rapid rise in the prepotential and a shortening of the plateau duration, making the action of FMRFamide, at concentrations below  $10^{-7}$  mol l<sup>-1</sup>, similar to those of 5-HT (Smith & Hill, 1986). Also, as with 5-HT, the pressure pulse amplitude is greatly increased while the duration decreases. Simultaneously, the duration of the electromyogram plateau is reduced while the amplitude increases. However, above  $10^{-7}$  mol l<sup>-1</sup> FMRFamide, both heart rate and stroke volume decline dramatically. This is not caused by the kind of cardiac inhibition produced by ACh, but by an extreme extension of the action potential plateau, perhaps as a result of the membrane failing to repolarize. [After washout of the FMRFamide the controls can show incomplete repolarization (Fig. 8, bottom left).] A similar plateau extension also occurs in calcium-free sea water (P. J. S. Smith, unpublished observation). Stroke volume declines as FMRFamide decreases the end-diastolic volume and the heart simply expels its entire content.

The results of this study seem to contradict the previously reported effects of ACh. In various molluscan species, *Crassostrea gigas* (Irisawa, Kobayashi & Matsubayashi, 1961), *Modiolus demissus granosissimus* (Wilkins & Greenberg, 1973) and *Dolabella auricularia* (Hill, 1974), the application of ACh leads to a reduction in the duration of the plateau. In the latter two studies this was correlated with a reduction in the force of contraction. However, the different species and experimental regimes used in the different studies complicate any comparison. In the present investigation, the heart, influenced by both intrinsic and extrinsic modulating factors which affect the form of the action potential (see also Smith, 1985a), works against an after-load while being perfused with the cardioactive substances. In addition it is being stretched internally during diastole by the preload levels. These experiments therefore reflect a complex interaction of regulatory mechanisms, making the results difficult to compare with the simpler force of contraction measured in earlier work. Despite these difficulties the perfused working heart is a better preparation for examining the regulation of output.

The reasons for the change in the action of FMRFamide at higher concentrations and the rebound effect after perfusion with acetylcholine cannot be answered from this study. These effects may reflect changes in the ionic permeabilities of the membrane or some persistent intracellular action.

Cardiac performance in the molluscs has a number of features in common with the lower vertebrates, most notably that changes in output may be primarily accommodated by altering stroke volume and not heart rate (for review see Smith, 1985b). This point is clearly seen in the cephalopod mollusc, *Octopus vulgaris*, where a five-fold increase in stroke work (stroke volume  $\times$  the pressure difference across the heart) accompanies exercise. Using indirect methods, Thompson, Livingston & de Zwaan (1980) showed similar changes in ventricular volume for the scallop. These authors have further shown that it is the end-diastolic volume which increases, proving that changes in stroke volume are not achieved solely by a

reduction in the end-systolic volume. Observations such as these, even if still indirect, do raise the interesting question of how stroke volume might be controlled in the molluscs.

The work on the isolated systemic heart of *Busycon* has shown that none of the three most obvious cardioactive substances can act alone to increase stroke volume. Regulation could, however, be achieved by the phasic action of the cholinergic innervation at the end of ventricular systole allowing the heart to achieve a greater volume for the same returning venous pressure. This could facilitate an increase in output either by an intrinsic reflex mechanism, perhaps similar to the rebound effect seen in this study, or by aminergic excitation of the myocardium at the onset of systole. Cholinergic innervation to the prosobranch heart has been shown (Kawakami & Kobayashi, 1984).

Cholinergic regulation of ventricular relaxation state, causing an increase in the end-diastolic volume, is already suggested by results from the opisthobranch *Aplysia*. Koester, Mayeri, Liebeswar & Kandel (1974) noticed that a pattern of cholinergic neuronal discharge to the heart occurs with the stereotyped repetitive behaviour during which fresh sea water flows over the gills; the gills contract, pumping an increased volume of oxygenated blood to the heart, which is then in a relaxed state as a result of the neuronal activity.

FMRFamide and 5-HT have several similar effects on cardiac performance in the isolated *Busycon* heart (this study; Smith & Hill, 1986). The action of both can be accompanied by increases in the level of cyclic AMP, although FMRFamide is not affected by the serotonergic antagonist methysergide (see Painter, 1982). However, with regards to the *in vivo* action of FMRFamide on cardiac performance, there are still clear problems in interpreting its role. Both FMRFamide and FMRFamide-like peptides, such as pQDPFLRFamide, are present in molluscan blood at concentrations around  $10^{-9} \text{ mol l}^{-1}$  (Nagle, 1982; Price *et al.* 1985). This concentration suggests that the peptides may be acting as circulating hormones with, as individual concentrations vary (Price *et al.* 1985), a modulating myotonal effect on their target organs. Unfortunately, it is not clear that FMRFamide acts on the heart *in vivo*, or, if it does, what aspect of cardiac function it might regulate (see Greenberg & Price, 1980).

In considering the actions of cardioactive peptides *in vivo*, it is worth remembering that none of the factors which regulate cardiac performance will act in isolation. This is clearly the case if these peptides circulate above threshold concentrations. Under these conditions, the cardiac tone will be set on a sliding scale, depending on the concentration present. Against this tone, other extrinsic or intrinsic factors will act. Thus, the activity of the cholinergic and aminergic innervation will be an integral part of an orchestrated response to regulate cardiac output at defined pressures. In this sense, to categorize cardioactive agents as either inhibitory or excitatory, with regards to cardiac output and performance, is too simplistic.



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