

## AN ANALYSIS OF CONTROL OF THE VENTRICLE OF THE MOLLUSC *MERCENARIA MERCENARIA*

### I. THE IONIC BASIS OF AUTORHYTHMICITY

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#### Summary

During spontaneous beating (autorhythmicity) in the bivalve ventricle, the cardiac action potential (AP) was generated by calcium ( $\text{Ca}^{2+}$ ) and sodium ( $\text{Na}^+$ ) influx. The initial fast rising phase (the 'spike') of the cardiac AP was dependent on extracellular  $\text{Ca}^{2+}$  concentration, whereas the slow plateau phase was  $\text{Na}^+$ -dependent.

The initial fast rising phase of the cardiac AP was abolished by treatment with a  $\text{Ca}^{2+}$ -free saline or inorganic  $\text{Ca}^{2+}$  entry blockers, such as lanthanum chloride or cobalt. Conversely, this fast rising phase of the AP was potentiated by treatment with barium ions, the dihydropyridine-sensitive  $\text{Ca}^{2+}$  channel agonist Bay K 8644 or, unexpectedly, by the organic  $\text{Ca}^{2+}$  entry blocker diltiazem.

The force of systolic beating was directly proportional to the amplitude of the fast rising phase of the cardiac AP. The  $\text{Ca}^{2+}$ -dependent, fast rising phase of the AP was modulated by the level of extracellular  $\text{Na}^+$ . Both the amplitude of the fast rising phase of the AP and coupled systolic force were increased by progressive reduction of extracellular  $\text{Na}^+$  concentration.

The slow plateau phase was abolished by treatment with a  $\text{Na}^+$ -free saline and potentiated by the  $\text{Na}^+$  ionophore monensin. The size of the  $\text{Na}^+$ -dependent plateau was modulated by the level of extracellular  $\text{Ca}^{2+}$ . When extracellular  $\text{Ca}^{2+}$  was removed from the bathing saline, both the amplitude and duration of the plateau phase were increased. Conversely, restoring extracellular  $\text{Ca}^{2+}$  to physiological levels decreased the size of the  $\text{Na}^+$ -dependent plateau.

Autorhythmicity was dependent on the level of extracellular potassium. In the absence of  $\text{K}^+$ , neither a  $\text{Ca}^{2+}$ -dependent fast rising phase nor a  $\text{Na}^+$ -dependent plateau phase was recorded.

#### Introduction

Rhythmicity of bivalve hearts is directly regulated by inhibitory and excitatory neural inputs (Hill and Welsh, 1966; Jones, 1983). Prosser (1940) deduced that cardiac inhibition in ventricles from *Mercenaria mercenaria* is probably caused by release of

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acetylcholine (ACh) from cardiac nerves. Shigeto (1970) attributed the inhibitory action of ACh in bivalve hearts to changes in potassium or chloride conductances which decrease the excitability of the heart.

In contrast, 5-hydroxytryptamine (5-HT) causes cardioexcitation in *Mercenaria* (Greenberg, 1960a,b; Loveland, 1963), in certain other bivalves (Painter and Greenberg, 1982) and in some gastropods (in *Helix*, S.-Rosza and Perenyi, 1966; in *Aplysia*, Liebeswar *et al.* 1975). The pharmacology of the 5-HT receptor in the heart of *Mercenaria* has been described in detail (Greenberg, 1960a), but the ionic basis of the action of 5-HT on the bivalve heart has not been well studied. To understand the ionic mechanisms underlying excitation by 5-HT, the ions involved in autorhythmicity of the *Mercenaria* ventricle first need to be identified. An analysis of the ionic basis of autorhythmicity is described in the present paper. In the following paper (Devlin, 1993), experiments were conducted to determine the ionic mechanisms involved in excitation of the heart by 5-HT.

### Materials and methods

Fifty-four isolated ventricles of the northern quahog, *Mercenaria mercenaria* Linnaeus, were used in this study. The animals were obtained from a commercial supplier in Narragansett, Rhode Island, USA. All animals were housed in aerated filtered sea water at 21°C at the Department of Zoology, the University of Rhode Island, until use. The experiments were conducted in the fall and winter of 1990.

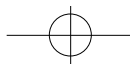
Intact animals ranged in mass from 136 to 237g (mean mass 164.8g, s.d. 26g). The wet mass of the isolated ventricles ranged from 203 to 391mg (314±67mg). The technique used for dissecting the ventricle from the intact clam has been described by Welsh and Taub (1948).

A portable sucrose-gap apparatus (Hill and Langton, 1988) was used to record compound cardiac action potentials (APs) and accompanying systolic contractions of the isolated ventricles simultaneously. The data were printed on chart paper using a Grass model 79C polygraph. Spontaneous activity in these experiments was measured by the following features: amplitude of cardiac APs (mV), amplitude of systolic force (N) and frequency of beats (beatsmin<sup>-1</sup>). Student's *t*-tests or analyses of variance (ANOVAs) were carried out using the statistics program Systat when appropriate.

Artificial sea water was made up according to the Marine Biological Laboratory formula (MBL, 1964). All ion-free (Ca<sup>2+</sup>-free, Mg<sup>2+</sup>-free and K<sup>+</sup>-free) and ion-modified salines were made up according to Wilkens (1972a,b), with the exception of the Na<sup>+</sup>-free and Cl<sup>-</sup>-free saline. See Table 1 for a description of the artificial sea water and all modified artificial salines used. Results are presented as mean ± s.d.

### Results

Cardiac APs generated by ventricles which were autorhythmic in normal sea water had two different waveforms (Fig. 1). The most common type of AP recorded was composed of a pre-potential followed by fast rising and rapid repolarizing phases (a 'spike'); a



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Table 1. Concentration of ions in artificial sea water and ion-free salines (mmol l<sup>-1</sup>)

Ion	ASW	Na <sup>+</sup> -free	Ca <sup>2+</sup> -free	K <sup>+</sup> -free	Mg <sup>2+</sup> -free
Na <sup>+</sup>	423.0	0	438.7	432.2	484.8
K <sup>+</sup>	9.7	9.7	9.7	0	9.7
Ca <sup>2+</sup>	9.9	9.9	0	9.9	9.9
Mg <sup>2+</sup>	51.2	51.2	51.2	51.2	0
Cl <sup>-</sup>	538.6	71.5	534.5	538.6	525.5
SO <sub>4</sub> <sup>2-</sup>	27.0	27.0	27.0	27.0	13.2
HCO <sub>3</sub> <sup>-</sup>	2.3	2.3	2.3	2.3	2.3

Modified from Wilkens (1972a).

The artificial sea water (ASW) was made according to the MBL formula (1964). Ca<sup>2+</sup>-free, Mg<sup>2+</sup>-free and K<sup>+</sup>-free salines were made up according to Wilkens (1972a,b). In making a Na<sup>+</sup>-free saline, 423mmol l<sup>-1</sup> sucrose was used as a substitute to maintain osmolarity when Na<sup>+</sup> was removed. A Cl<sup>-</sup>-free saline was made up with 423mmol l<sup>-1</sup> sodium isethionate, 9mmol l<sup>-1</sup> potassium isethionate, 9mmol l<sup>-1</sup> calcium sulphate and 51.2mmol l<sup>-1</sup> magnesium sulphate.

plateau phase was absent. Less frequently recorded were APs with a fast rising phase followed by a slow plateau phase, as is characteristic of APs in mammalian cardiac tissue.

The mean AP amplitude (both types pooled) was 4.8±4.4mV (*N*=9 ventricles) when recorded with the sucrose-gap method. This extracellular recording technique measures signals from a number of syncytial cells which may be in either an excitatory or a refractory state. For this reason, the signals are smaller than APs recorded using intracellular electrodes. Other factors that can attenuate cardiac APs measured using the sucrose-gap method are conductivity of the bathing saline, configuration of the muscle across the gap, resistance of the gap and capacitive coupling between recording sites.

The contractions accompanying the cardiac APs generated a force of 0.012±0.006N (*N*=9 ventricles). The frequency of beating was 5.6±2.0beatsmin<sup>-1</sup> (*N*=9) in normal sea water. The mean membrane potential recorded by the sucrose-gap technique was -47±14.2mV (*N*=9). This value is comparable with those recorded using the sucrose-gap technique from other molluscan cardiac and smooth muscles (Jones, 1983).

### The effect of extracellular calcium on autorhythmicity

Ca<sup>2+</sup>-modified salines (0–144mmol l<sup>-1</sup>) as well as a diverse group of pharmacological Ca<sup>2+</sup> probes were used to test the dependence of autorhythmicity on extracellular Ca<sup>2+</sup>. The pharmacological probes were inorganic (lanthanum or cobalt) and organic Ca<sup>2+</sup> entry blockers (diltiazem, nifedipine or verapamil). Ba<sup>2+</sup> and a Ca<sup>2+</sup> agonist, Bay K 8644, were tested as agents which may enhance Ca<sup>2+</sup> influx into the ventricular myocytes.

### The effect of Ca<sup>2+</sup>-modified salines

The first series of experiments used nine ventricles to determine the effects of Ca<sup>2+</sup>-modified salines on systolic activity of the ventricles, compared with normal activity recorded in the presence of 9mmol l<sup>-1</sup> calcium chloride (the concentration in natural sea water). The amplitude of the AP spike and the systolic force of the ventricles were both highly sensitive to changes in the concentration of extracellular Ca<sup>2+</sup>. Both variables

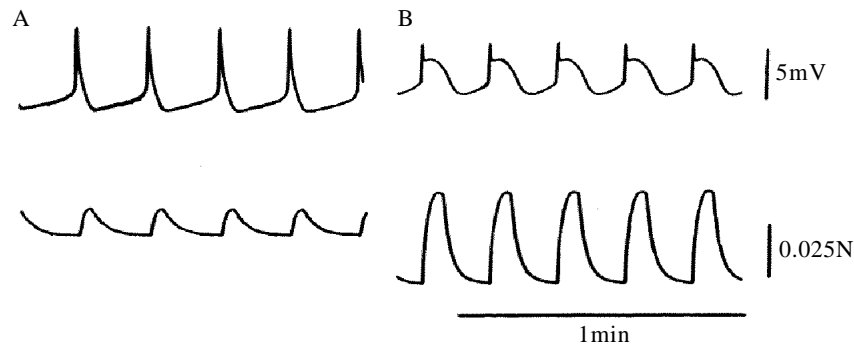
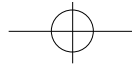


Fig. 1. Cardiac APs (upper traces) recorded from autorhythmic ventricles in normal sea water exhibited two waveforms. (A) The more typical type, with a pre-potential and fast rising and repolarizing phase; (B) a spike and plateau type. The APs were accompanied by rhythmic contractions of the hearts (lower traces).

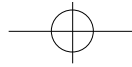
increased when external  $\text{Ca}^{2+}$  was raised from 0 to  $9\text{mmol l}^{-1}$ . Both the amplitude and frequency of beats were maximal at a  $\text{Ca}^{2+}$  concentration around  $9\text{mmol l}^{-1}$ . Concentrations of extracellular  $\text{Ca}^{2+}$  greater than  $18\text{mmol l}^{-1}$  inhibited beating and caused a slight membrane depolarization. The membrane became increasingly depolarized as the concentration of extracellular  $\text{Ca}^{2+}$  was raised from 18 to  $144\text{mmol l}^{-1}$ , with concurrent decreases in both the AP spike amplitude and systolic force. At relatively high concentrations of extracellular  $\text{Ca}^{2+}$  (36, 72,  $144\text{mmol l}^{-1}$   $\text{CaCl}_2$ ), the heart did not beat.

The dependence of the systolic activity of the nine ventricles on extracellular  $\text{Ca}^{2+}$  was further tested by removing  $\text{Ca}^{2+}$  from the bathing saline. Removal of  $\text{Ca}^{2+}$  had an effect on both the shape of the cardiac AP and force generation. Those APs initially characterized by a pre-potential followed by a fast rising phase were transformed into APs with a spike and plateau (Fig. 2). After prolonged  $\text{Ca}^{2+}$  deprivation (up to 30min), only a protracted plateau phase was recorded.

Ventricles which initially generated a fast rising phase and a plateau exhibited a short period of asynchronous beating lasting approximately 5min when perfused with  $\text{Ca}^{2+}$ -free saline. After 10min the initial fast rising phase and the systolic force were either reduced or abolished. A protracted plateau phase began to emerge so that, after 30–40min perfusion with  $\text{Ca}^{2+}$ -free saline, only APs with long plateau phases were recorded. At this time, the initial spike component was totally absent. Systolic beats were not coupled to APs consisting of only a plateau phase. Fig. 3 shows that the AP spike and systolic force immediately reappeared when  $9\text{mmol l}^{-1}$  calcium chloride was restored to the extracellular saline.

#### *The effect of inorganic $\text{Ca}^{2+}$ entry blockers*

Inhibition of the AP spike and systolic force was also recorded in the presence of  $\text{Ca}^{2+}$  entry blockers such as lanthanum ( $\text{La}^{3+}$ ) or cobalt ( $\text{Co}^{2+}$ ).  $\text{La}^{3+}$  inhibited cardiac APs and the coupled systolic force over a range of concentrations ( $10^{-7}$ – $10^{-3}\text{mol l}^{-1}$ ) in a dose-dependent manner in all six of the ventricles used during this experimental series (Fig. 4).



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Fig. 2. Treatment with  $\text{Ca}^{2+}$ -free saline reduced both the fast rising component of the AP (upper trace) and the systolic force (lower trace), and produced a protracted plateau phase. (A) The control: systolic activity in sea water. (B) APs recorded after 10min in  $\text{Ca}^{2+}$ -free saline. (C) APs recorded after 30min in  $\text{Ca}^{2+}$ -free saline.

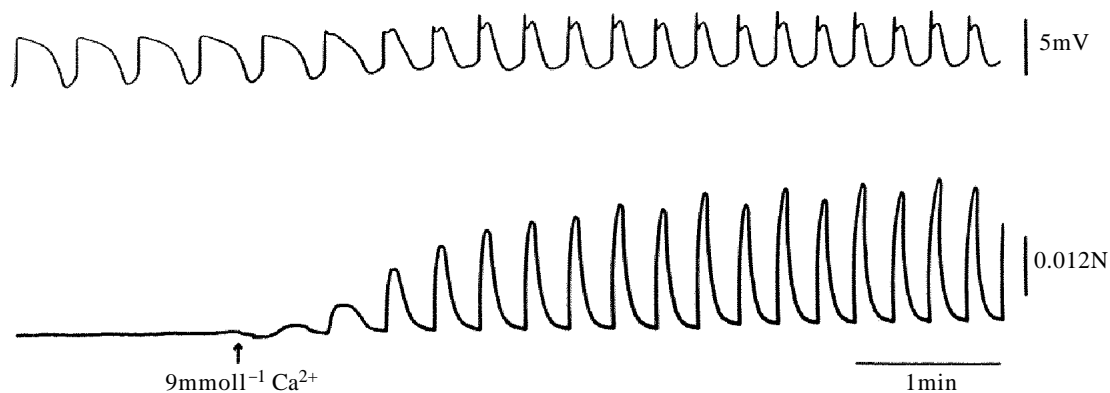


Fig. 3. APs with a protracted plateau recorded in  $\text{Ca}^{2+}$ -free saline were changed to APs with a spike and plateau by the addition of  $9\text{mmol l}^{-1} \text{Ca}^{2+}$  (top trace). A  $\text{Ca}^{2+}$ -dependent spike and forceful systolic contractions (bottom trace) were immediately restored by the addition of extracellular  $\text{Ca}^{2+}$ . The duration and amplitude of the plateau phase were also immediately reduced.

All rhythmicity stopped during treatment with  $10^{-3} \text{mol l}^{-1} \text{La}^{3+}$ .  $\text{Co}^{2+}$  also caused dose-dependent inhibition of autorhythmicity, but it could only stop systolic activity at a dose of  $10^{-2} \text{mol l}^{-1}$ . Both agents also selectively reduced the amplitude of the fast rising phase (Figs 4 and 5).

#### *The effect of organic $\text{Ca}^{2+}$ entry blockers*

Three organic  $\text{Ca}^{2+}$ -entry blockers, diltiazem, nifedipine and verapamil, were tested. Diltiazem ( $10^{-9}$ – $10^{-3} \text{mol l}^{-1}$ ) was tested on three ventricles. Between  $10^{-9}$  and  $10^{-5} \text{mol l}^{-1}$ , diltiazem had no significant effect on the shape or amplitude of the cardiac APs. However, at the higher doses of  $10^{-4} \text{mol l}^{-1}$  and  $10^{-3} \text{mol l}^{-1}$ , the drug greatly

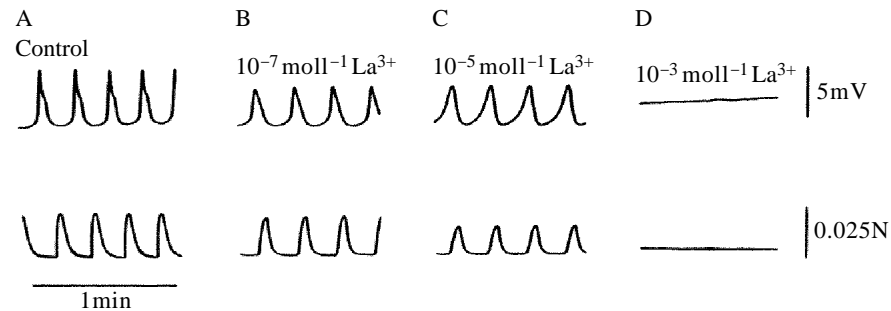
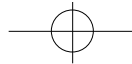


Fig. 4.  $\text{La}^{3+}$  inhibits the systolic activity of a ventricle in a dose-dependent manner. Upper traces show electrical activity, lower traces show systolic force. (A) The control: systolic activity in normal sea water. (B) The fast rising phase is eliminated by  $10^{-7} \text{ mol l}^{-1} \text{ La}^{3+}$  (upper trace). (C) Effects of  $10^{-5} \text{ mol l}^{-1} \text{ La}^{3+}$ . (D) Effects of  $10^{-3} \text{ mol l}^{-1} \text{ La}^{3+}$ .

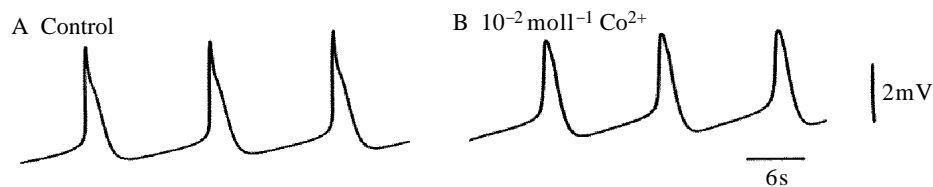


Fig. 5.  $\text{Co}^{2+}$  eliminates the fast rising phase of the AP. (A) The control: cardiac APs recorded in normal sea water. (B) The fast rising phase or 'spike' is reduced by treatment with  $10^{-2} \text{ mol l}^{-1} \text{ Co}^{2+}$ .

amplified and altered the shape of the cardiac APs (Fig. 6). Diltiazem caused an initial transient cessation in systolic activity that was immediately followed by an enhancement of the electrical activity of the ventricles. This excitatory response was sustained as long as diltiazem was present in the perfusion medium.

Diltiazem ( $10^{-3} \text{ mol l}^{-1}$ ) caused the membrane to depolarize (mean depolarization  $+7.5 \pm 2.5 \text{ mV}$ ,  $N=3$ ). Superimposed on the depolarization were APs composed of a fast rising phase followed by a plateau phase. This high dose of diltiazem caused APs with fast rising and repolarizing phases to be changed to APs with a spike and plateau waveform (Fig. 6). During treatment with diltiazem, the amplitudes of the cardiac APs recorded from the ventricle shown in Fig. 6 were increased to as much as 230% of control values.

There was no statistically significant change in the amplitude of systolic force generated over the range of diltiazem concentrations tested. This indicates that the drug had little or no effect on intracellular  $\text{Ca}^{2+}$  storage sites or release mechanisms that would have activated contractile proteins.

The effect of verapamil was tested on the autorhythmic beating of ten ventricles. Between  $10^{-10}$  and  $10^{-3} \text{ mol l}^{-1}$  verapamil had a non-significant effect on the AP amplitude and force produced, but it did have a significant excitatory effect on beat frequency.

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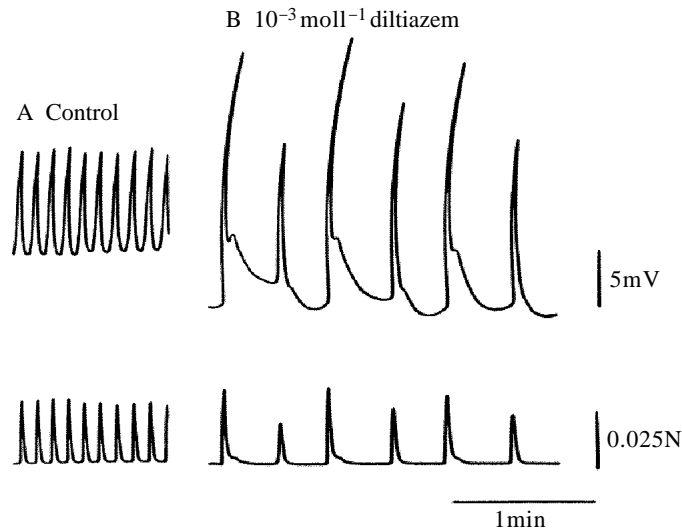


Fig. 6. The  $\text{Ca}^{2+}$  channel antagonist diltiazem had an excitatory effect on the cardiac AP, enhancing both the  $\text{Ca}^{2+}$ -dependent fast rising phase and the  $\text{Na}^{+}$ -dependent plateau phase. The upper traces show electrical activity, the lower traces show systolic force. (A) The control: systolic activity in normal sea water; (B) systolic activity during treatment with  $10^{-3} \text{ mol l}^{-1}$  diltiazem.

The effect of nifedipine was tested on five autorhythmic ventricles. Nifedipine slightly potentiated the amplitude of cardiac APs, systolic force and beat frequency over the concentration range tested ( $10^{-9}$ – $10^{-3} \text{ mol l}^{-1}$ ); however, the increases were not statistically significant.

*The effect of barium and Bay K 8644 on autorhythmicity*

$\text{Ba}^{2+}$  has been used to study  $\text{Ca}^{2+}$  channels in numerous muscle preparations because  $\text{Ba}^{2+}$  passes more readily through  $\text{Ca}^{2+}$  channels than does  $\text{Ca}^{2+}$ , so it creates a potentiated current. When  $\text{Ba}^{2+}$  replaced calcium chloride in the perfusate at concentrations ranging from 1 to  $9 \text{ mmol l}^{-1}$ , there was a large dose-dependent potentiation of both the AP spike and the coupled systolic force in all six of the ventricles tested. In  $9 \text{ mmol l}^{-1}$   $\text{Ba}^{2+}$ , the amplitude of the initial fast rising phase of the AP and the systolic force amplitude were increased to 242% and 213% of their respective controls (Fig. 7). Beat frequency declined as the  $\text{Ba}^{2+}$  concentration was increased.

A  $\text{Ca}^{2+}$  agonist, Bay K 8644, had a large potentiating effect on AP and force amplitude and on beat frequency over the range of concentrations tested ( $10^{-9}$ – $10^{-4} \text{ mol l}^{-1}$ ). AP amplitude, systolic force and beat frequency were increased to 413%, 555% and 188% of their respective controls during treatment with  $10^{-4} \text{ mol l}^{-1}$  Bay K 8644. This drug affected the cardiac AP by increasing the slope and amplitude of the fast rising phase.

*Regulation of the plateau phase of the AP*

In the absence of extracellular  $\text{Ca}^{2+}$ , the fast rising phase of the AP was absent and a large protracted plateau emerged. This suggested that the plateau phase was generated by

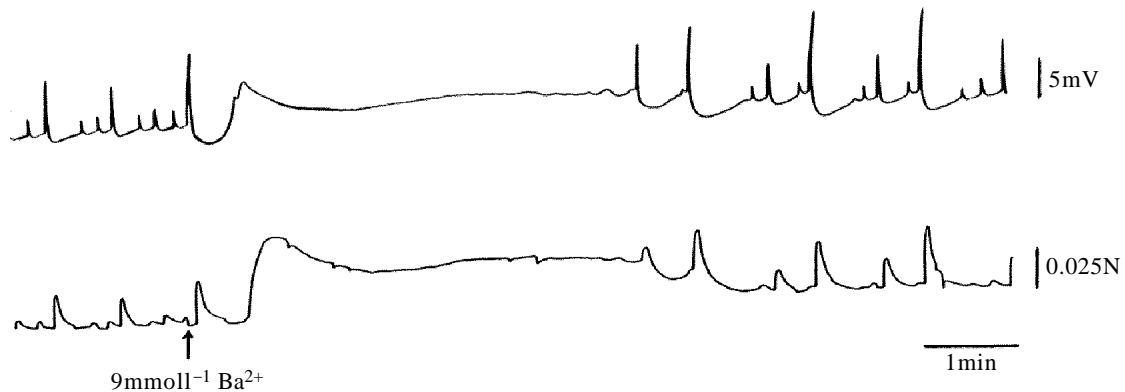
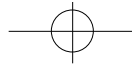


Fig. 7. Treatment with  $9\text{mmol l}^{-1} \text{Ba}^{2+}$  causes an increase in the amplitude of the cardiac APs (top trace) after a period when beating ceased (bottom trace).

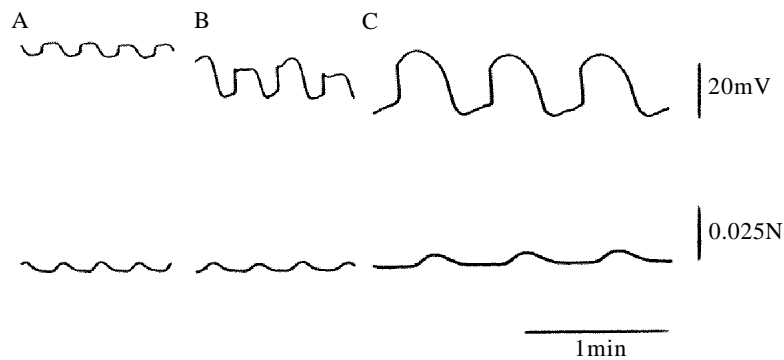


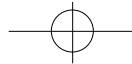
Fig. 8. Potentiation of amplitude and duration of the protracted plateau phase in  $\text{Ca}^{2+}$ -free saline by the  $\text{Na}^{+}$  ionophore monensin. The upper traces show electrical activity, the lower traces show systolic force. (A) APs with a large plateau recorded during perfusion with  $\text{Ca}^{2+}$ -free saline. (B) Monensin ( $10^{-3} \text{mol l}^{-1}$ ) prolonged the duration and increased the amplitude of APs after 15min. After 40min of treatment with the ionophore (C) the effects were more marked.

influx of an ion other than  $\text{Ca}^{2+}$ , possibly  $\text{Na}^{+}$ . Two series of experiments were performed to test this hypothesis. One series used monensin, a  $\text{Na}^{+}$  ionophore, while the other used  $\text{Na}^{+}$ -modified salines.

$10^{-3} \text{mol l}^{-1}$  monensin proved extremely effective at potentiating the amplitude and duration of the protracted plateau phase of the AP recorded in the presence of  $\text{Ca}^{2+}$ -free saline (Fig. 8). This response may have been due to an increase in intracellular  $\text{Na}^{+}$  concentration. Subsequent removal of monensin from the bathing saline caused the plateau to return to its original size and shape.

In contrast, the protracted plateau phase recorded during treatment with  $\text{Ca}^{2+}$ -free saline disappeared when the preparation was perfused with  $\text{Na}^{+}$ -free and  $\text{Ca}^{2+}$ -free saline (Fig. 9). Upon restoring  $\text{Na}^{+}$  to the bathing saline, the large plateau phase immediately





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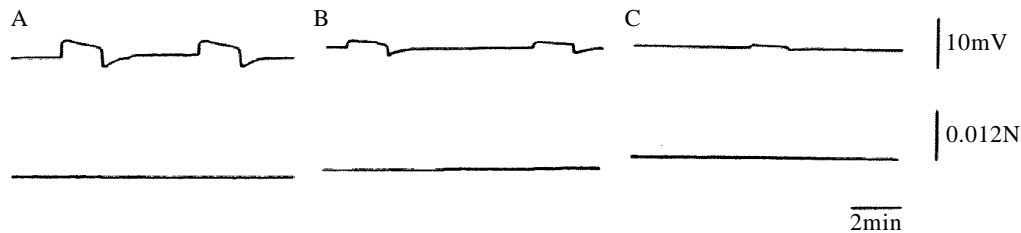


Fig. 9. The plateau phase of the cardiac AP was abolished by perfusion with a  $\text{Na}^+$ -free and  $\text{Ca}^{2+}$ -free saline. The upper traces show electrical activity, the lower traces show systolic force. (A) Control: APs recorded in  $\text{Ca}^{2+}$ -free saline. (B) 5min and (C) 15min after perfusion with  $\text{Na}^+$ -free and  $\text{Ca}^{2+}$ -free saline.

returned. The evidence from the experiments described above using monensin or  $\text{Na}^+$ -free saline suggests that the plateau was probably due to the influx of  $\text{Na}^+$  and/or to an increase in the concentration of intracellular  $\text{Na}^+$ .

$\text{Na}^+$  influx may be regulated by extracellular  $\text{Ca}^{2+}$  (cf. Fig. 3). When  $9\text{mmol l}^{-1}$  calcium chloride was restored to ventricles that had been treated with  $\text{Ca}^{2+}$ -free saline, the amplitude and duration of the  $\text{Na}^+$ -dependent plateau were immediately reduced. This may indicate that extracellular  $\text{Ca}^{2+}$  was blocking  $\text{Na}^+$  entry through  $\text{Na}^+$  channels and/or  $\text{Na}^+$  entry through  $\text{Ca}^{2+}$  channels.

#### *The effect of sodium ions on systolic activity*

Low- $\text{Na}^+$  salines were used to determine the  $\text{Na}^+$  dependency of the electrical and mechanical properties of nine bivalve ventricles. Artificial sea water containing 100%, 75%, 50%, 25% or 0% of the normal  $\text{NaCl}$  concentration of natural sea water ( $423\text{mmol l}^{-1}$ ) were tested on autorhythmic beating over 10min treatment periods.

As the extracellular  $\text{Na}^+$  concentration was serially decreased from 100% to 0%, the slope and amplitude of the AP were progressively increased (Fig. 10). Fig. 10 summarizes the effect of  $\text{Na}^+$  concentration on cardiac AP and systolic force amplitude. During treatment with  $\text{Na}^+$ -free saline, AP and force amplitude were respectively enhanced by 246% and 381% of control values recorded in normal sea water. Frequency of beating was slightly decreased over the same range of  $\text{Na}^+$  concentration, however, and fell to 88% of the control value during treatment with  $\text{Na}^+$ -free saline. APs recorded in the absence of  $\text{Na}^+$  consisted of a fast rising component and rapid repolarizing phase only; a plateau phase was never recorded.

#### *The effect of potassium ions on rhythmicity*

$\text{K}^+$ -free saline was used to study the effect of extracellular  $\text{K}^+$  on the autorhythmicity of nine ventricles. This saline caused a gradual decline in cardiac rhythmicity over the 10min treatment period in all ventricles tested. The slope of the AP pre-potential and spike gradually decreased, as did the spike amplitude. There was also a significant decrease in systolic force ( $P=0.038$ ,  $t$ -test). Spontaneous beating of four of the nine ventricles stopped completely within the 10min treatment period, whereas the remaining five ventricles beat very weakly. The AP amplitude recorded from the latter hearts was

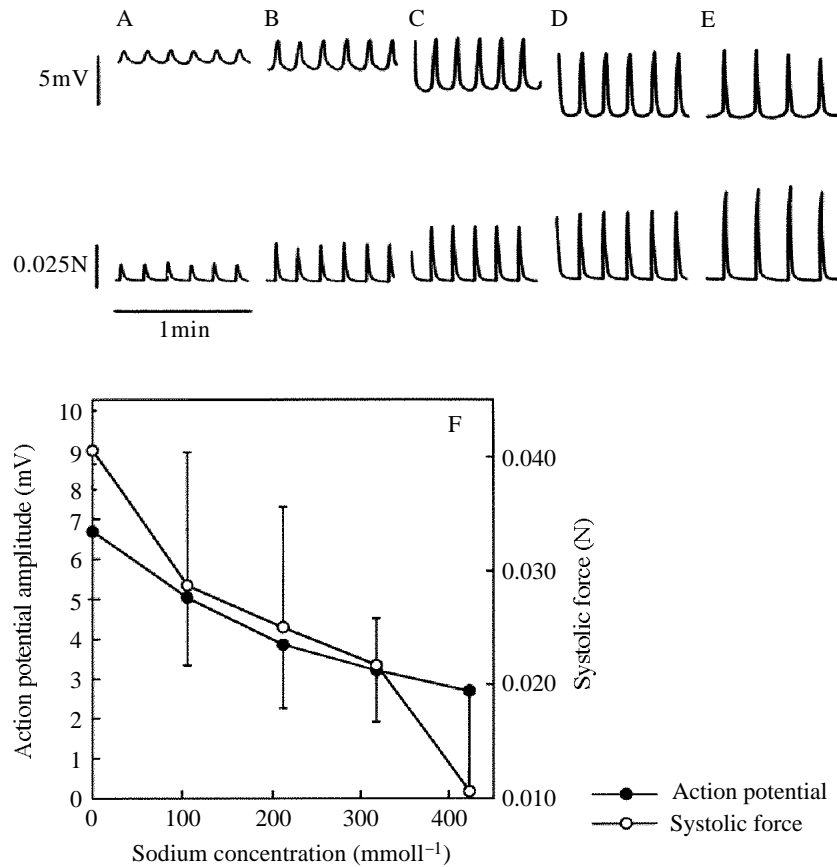
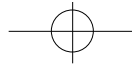
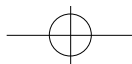


Fig. 10. The amplitudes of the fast rising phase (upper traces) and systolic force (lower traces) were progressively increased as the concentration of extracellular Na<sup>+</sup> was serially reduced from 100% to 0%. (A) Control: systolic activity in 100% Na<sup>+</sup> SW (423mmol<sup>-1</sup> Na<sup>+</sup>). (B-E) AP amplitude and systolic force measured after 10min in 75% Na<sup>+</sup> SW (B), 50% Na<sup>+</sup> SW (C), 25% Na<sup>+</sup> SW (D) and 0% Na<sup>+</sup> SW (E). (F) Na<sup>+</sup> had a concentration-dependent effect on systolic activity. Data points were averaged from six ventricles. Bars indicate S.D.

decreased to 30% and the systolic force to 29% of control levels. The frequency of beating was reduced to 58% of the control. Perfusion with K<sup>+</sup>-free saline had no effect on the resting potential or basal tone of the ventricles.

Ca<sup>2+</sup>-dependent spikes were not recorded in low-Na<sup>+</sup> saline when K<sup>+</sup> was absent. K<sup>+</sup> probably interfered with the pre-potential of the APs and thus increased the time required to reach the threshold for the opening of Ca<sup>2+</sup> channels. Na<sup>+</sup>-dependent plateaus were not recorded in K<sup>+</sup>-free saline.

At KCl concentrations of 7–18mmol<sup>-1</sup>, forceful systolic beating occurred. Optimal systolic activity was recorded during perfusion with 9mmol<sup>-1</sup> KCl (the concentration found in natural sea water). At a K<sup>+</sup> concentration greater than 25mmol<sup>-1</sup>, the membrane depolarized and this was coupled to a contracture. The degree of membrane depolarization and strength of contracture were directly correlated with the concentration



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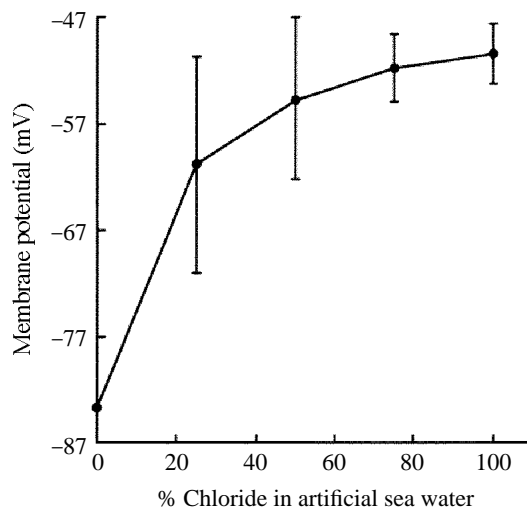


Fig. 11. Effect of  $\text{Cl}^-$  concentration on membrane potential. Removal of  $\text{Cl}^-$  had a marked hyperpolarizing effect on the membrane potential over the  $\text{Cl}^-$  concentration range tested (100% to 0%). Resting membrane potential was  $-47\text{mV}$ . Data points were averaged from three ventricles ( $\pm \text{S.D.}$ ).

of extracellular  $\text{K}^+$ , while the amplitude of systolic beating decreased as the membrane became increasingly depolarized. No systolic activity was generated during a sustained depolarization and contracture induced by  $\text{K}^+$  concentrations greater than  $31\text{mmol l}^{-1}$ . This effect was reminiscent of the response to high- $\text{Ca}^{2+}$  saline in which systolic activity was not generated when the membrane was depolarized.

#### *Effect of chloride ions on systolic activity*

The effects of 100%, 75%, 50%, 25% and 0% of the  $\text{Cl}^-$  concentration of natural sea water ( $538.6\text{mmol l}^{-1}$ ) were tested on three ventricles. Over this concentration range, autorhythmic beating was not significantly changed.

Treatment with low- $\text{Cl}^-$  salines (from 75% to 0%) had a large hyperpolarizing effect on the membrane potential (Fig. 11). In normal sea water the average resting membrane potential was  $-47\text{mV}$ ; however, in the presence of  $\text{Cl}^-$ -free artificial sea water, the membrane potential dropped to  $-84\text{mV}$ . This means that in  $\text{Cl}^-$ -free sea water the membrane was hyperpolarized to near the theoretical potassium equilibrium potential.

#### *Effect of magnesium ions on autorhythmicity*

The effect of extracellular  $\text{Mg}^{2+}$  (from 200% to 0% of the normal  $\text{Mg}^{2+}$  content of natural sea water) on autorhythmicity was tested. At 200%  $\text{Mg}^{2+}$  ( $102.4\text{mmol l}^{-1}$ ), the ions acted as an anaesthetic and completely blocked both electrical and mechanical activity of the ventricles. In 150%  $\text{Mg}^{2+}$ , some systolic activity returned, but the beating was very weak. Upon perfusion with normal sea water, both electrical and mechanical activity returned to control levels.

The ventricles responded in a complex manner as the extracellular concentration of  $\text{Mg}^{2+}$  was reduced from 100% to 0% of normal. Both the beat frequency and AP amplitude increased as the extracellular  $\text{Mg}^{2+}$  concentration was serially reduced. This response may have been due to  $\text{Ca}^{2+}$  channels being released from inhibition by removal of a competing divalent cation ( $\text{Mg}^{2+}$ ) at the membrane surface. This would increase  $\text{Ca}^{2+}$  influx and result in the increase in AP amplitude and frequency of beats.

The effect of  $\text{Mg}^{2+}$  on force generation was not statistically significant. In  $\text{Mg}^{2+}$ -free saline, systolic force was 71% of the control recorded in normal sea water. The ventricles continued to beat regularly and forcefully despite long treatments (greater than 20min) with  $\text{Mg}^{2+}$ -free saline. Only when  $\text{Ca}^{2+}$  was also removed did all activity of the ventricles stop. Activity was immediately restored when  $\text{Ca}^{2+}$  was again added to  $\text{Mg}^{2+}$ -free saline.

### Discussion

Jones (1983) described three types of cardiac APs that have been recorded from molluscan hearts: fast APs, slow APs, and spike and plateau APs. Two of the three types of cardiac APs were recorded from the *Mercenaria* ventricle: a fast AP (consisting of fast rising and repolarizing phases) and an AP with a spike and plateau. Fast APs, which were recorded most frequently in these hearts, could be transformed into those with a spike and plateau by treatment with low- $\text{Ca}^{2+}$  salines.

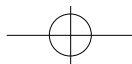
APs recorded from the *Mercenaria* ventricle using a sucrose-gap technique had a mean amplitude of 4.8mV. The sucrose-gap technique measures the compound AP from the entire syncytium of the heart muscle. Therefore, at any moment, some cardiac cells were firing while others were in a refractory state. This accounts for the relatively small amplitude of the APs recorded using the sucrose-gap technique as opposed to APs recorded with intracellular electrodes from single myocytes.

Resting potentials recorded from the *Mercenaria* ventricle with the sucrose-gap technique are similar to those recorded from other bivalve ventricles (Jones, 1983). Low resting potentials recorded from molluscan hearts are attributed primarily to a relatively low  $\text{K}^+$  equilibrium potential as well as large conductances to  $\text{Na}^+$  and  $\text{Cl}^-$  (Brezden and Gardner, 1984; Wilkens, 1972a).

In the present experiments, the cardiac APs from the ventricle of *Mercenaria* were found to have a  $\text{Ca}^{2+}$ -dependent fast rising phase (the spike) and, when present, a  $\text{Na}^+$ -dependent plateau. Other investigators have recorded this same ionic phenomenon in other molluscan species (Deaton and Greenberg, 1980; Hill and Yantorno, 1979; Irisawa *et al.* 1967, 1968; Wilkens, 1972a,b).

The  $\text{Ca}^{2+}$ -dependent fast rising phase was effectively reduced or abolished by treatment with  $\text{Ca}^{2+}$ -free saline or with inorganic  $\text{Ca}^{2+}$  blocking agents, such as  $\text{La}^{3+}$  or  $\text{Co}^{2+}$  (Devlin, 1991). Organic  $\text{Ca}^{2+}$  entry blockers such as diltiazem, verapamil and nifedipine, each of which is effective in blocking the slow inward  $\text{Ca}^{2+}$  current in vertebrate heart muscle (Fleckenstein and Fleckenstein-Grun, 1984), were ineffective in blocking the  $\text{Ca}^{2+}$ -dependent fast rising phase of the AP generated by these ventricles.

The  $\text{Ca}^{2+}$ -dependent fast rising phase could be potentiated by increasing the external  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_o$ ), by substituting  $\text{Ba}^{2+}$  for  $\text{Ca}^{2+}$  or by treatment with a



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dihydropyridine-sensitive  $\text{Ca}^{2+}$  agonist, Bay K 8644. Surprisingly, diltiazem, a  $\text{Ca}^{2+}$  antagonist, mimicked the effects of  $\text{Ba}^{2+}$  and Bay K 8644, and potentiated the amplitude of the fast rising component of the AP, thus acting as a  $\text{Ca}^{2+}$  agonist in this molluscan cardiac preparation.

Enhancement of the fast rising component by these agents was typically coupled to an increase in systolic force. In contrast, during block of the spike by  $\text{Ca}^{2+}$  blockers or with  $\text{Ca}^{2+}$ -free saline, systolic force was not detectable. The fact that force generation was highly dependent on  $[\text{Ca}^{2+}]_o$  lends support to Irisawa's argument that bivalve cardiac muscle may actually be 'smooth muscle' (Irisawa *et al.* 1973). When *Mercenaria* ventricles were perfused with  $\text{Ca}^{2+}$ -free saline, an AP with a protracted plateau was recorded. The amplitude of the protracted plateau was potentiated by monensin and abolished by perfusion with  $\text{Na}^+$ -free saline. This suggests that the plateau phase recorded from the ventricle was  $\text{Na}^+$ -dependent.

A protracted plateau phase appeared in the absence of extracellular  $\text{Ca}^{2+}$ , perhaps as a result of the passage of  $\text{Na}^+$  through  $\text{Na}^+$  channels and possibly by the passage of  $\text{Na}^+$  through  $\text{Ca}^{2+}$  channels. Hess *et al.* (1983) and Hess and Tsien (1984) found that the reduction or elimination of  $[\text{Ca}^{2+}]_o$  facilitates the passage of  $\text{Na}^+$  through  $\text{Ca}^{2+}$  channels of single cardiac myocytes. Restoration of  $9\text{mmol l}^{-1}$  calcium chloride to the saline immediately caused the reduction and/or elimination of the  $\text{Na}^+$ -dependent plateau. If the ion-ion interaction model described for vertebrate cardiac cells (Hess *et al.* 1983; Hess and Tsien, 1984) holds true for molluscan cardiac preparations, then  $\text{Ca}^{2+}$  would block the passage of  $\text{Na}^+$  through  $\text{Ca}^{2+}$  channels, which would cause the decrease in the amplitude and duration of the  $\text{Na}^+$ -dependent plateau.

The amplitude of the  $\text{Ca}^{2+}$ -dependent fast rising phase and systolic force were increased when  $\text{Na}^+$  was removed from the saline. Amphibian and mammalian cardiac cells also contract when treated with  $\text{Na}^+$ -free salines (Mullins, 1984). In this situation,  $\text{Ca}^{2+}$  may have passed through  $\text{Ca}^{2+}$  channels unimpeded without interference from  $\text{Na}^+$  at the surface of the  $\text{Ca}^{2+}$  channels. Perhaps systolic force was increased because  $\text{Ca}^{2+}$  was not pumped out of the cell in the absence of extracellular  $\text{Na}^+$  and, consequently, the intracellular  $\text{Ca}^{2+}$  concentration was elevated.

Systolic beating of the *Mercenaria* ventricle was dependent on extracellular  $\text{K}^+$  in the experiments reported here. Generation of the  $\text{Ca}^{2+}$ -dependent spike and  $\text{Na}^+$ -dependent plateau were absolutely dependent on extracellular  $\text{K}^+$ . It is probable that low extracellular  $[\text{K}^+]$  interfered with both the development of the AP pre-potential and the  $\text{Na}^+/\text{K}^+$  pump, though this was not investigated.

$\text{Mg}^{2+}$  causes a partial block of  $\text{Ca}^{2+}$  channels in barnacle muscle (Hagiwara and Takahashi, 1967).  $\text{Mg}^{2+}$  probably binds to the same membrane surface binding sites as  $\text{Ca}^{2+}$  and acts as a competitive inhibitor (Junge, 1981). The reduction in  $[\text{Mg}^{2+}]_o$  enhanced AP amplitude and beat frequency, possibly by reducing competition with  $\text{Ca}^{2+}$  for passage through the  $\text{Ca}^{2+}$  channel. When  $\text{Ca}^{2+}$  was omitted from  $\text{Mg}^{2+}$ -free saline, ventricles which were beating forcefully immediately stopped. This is also observed in the heart of the mussel, *Geukensia* (at the time of the work, referred to as *Modiolus demissus*) (Wilkens, 1972b). In contrast, when  $[\text{Mg}^{2+}]_o$  was increased to 1.5–2 times the

normal concentration found in natural sea water, all rhythmic activity of the ventricles immediately stopped, indicating that  $Mg^{2+}$  had blocked  $Ca^{2+}$  entry into the myocytes.

$Cl^{-}$  removal had little effect on the amplitude of systolic force, but it caused a large (37 mV) hyperpolarization of the *Mercenaria* ventricle to a value near the  $K^{+}$  equilibrium potential. Other investigators have found that  $Cl^{-}$ -free saline caused the membrane to depolarize by 6–22mV (Brezden and Gardner, 1984; Wilkens, 1972a), a predictable response based on the normal  $Cl^{-}$  distribution across the membrane. One obvious difference in the experimental regimes that may account for these large variations in the membrane response was the use of isethionate as the  $Cl^{-}$  substitute during the present experiments, while other investigators have used glutamate salts (Brezden and Gardner, 1984), propionate or sulphate (Wilkens, 1972a). H. Huddart (personal communication) found, however, that the *Busycon canaliculatum* heart hyperpolarized when he used sodium propionate as a  $Cl^{-}$  substitute. Wilkens (1972a) suggested that 15–30% of the total membrane potential of bivalve heart cells may be attributed to  $Cl^{-}$ . Brezden and Gardner (1984) also suggested that a  $Cl^{-}$  conductance was involved in the maintenance of the membrane potential in gastropod heart cells.

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### References

- BREZDEN, B. L. AND GARDNER, D. R. (1984). The ionic basis of the resting potential in a cross-striated muscle of the aquatic snail, *Lymnaea stagnalis*. *J. exp. Biol.* **108**, 305–314.
- DEATON, L. E. AND GREENBERG, M. J. (1980). The ionic dependence of the cardiac action potential in bivalve molluscs: systematic distribution. *Comp. Biochem. Physiol.* **67A**, 155–161.
- DEVLIN, C. L. (1991). An analysis of serotonergic control of the ventricle of a mollusc. PhD dissertation, University of Rhode Island, Kingston, Rhode Island, USA.
- DEVLIN, C. L. (1993). An analysis of control of the ventricle of the mollusc *Mercenaria mercenaria*. II. Ionic mechanisms involved in excitation by 5-hydroxytryptamine. *J. exp. Biol.* **179**, 63–75.
- FLECKENSTEIN, A. AND FLECKENSTEIN-GRUN, G. (1984). Effects of and the mechanism of action of calcium antagonists and other antianginal agents. In *Physiological and Pathophysiology of the Heart* (ed. N. Sperelakis), pp. 412–442. Boston: Martinus Nijhoff Publishing.
- GREENBERG, M. J. (1960a). Structure–activity relationship of tryptamine analogues on the heart of *Venus mercenaria*. *Br. J. Pharmac.* **15**, 375–388.
- GREENBERG, M. J. (1960b). The responses of the *Venus* heart to catecholamines and high concentrations of 5-hydroxytryptamine. *Br. J. Pharmac.* **15**, 365–375.
- HAGIWARA, S. AND TAKAHASHI, K. (1967). Surface density of calcium ion and calcium spikes in the barnacle muscle fiber membrane. *J. gen. Physiol.* **50**, 583–601.
- HESS, P., LEE, K. S. AND TSIEN, R. W. (1983). Ion–ion interactions in the calcium channel of single heart cells. *Biophys. J.* **41**, 293a.
- HESS, P. AND TSIEN, R. W. (1984). Mechanism of ion permeation through calcium channels. *Nature* **309**, 453–456.
- HILL, R. B. AND LANGTON, P. D. (1988). Use of a sucrose-gap apparatus to record electrical responses of gastropod radular protractor muscle to FMRFamide. *Comp. Biochem. Physiol.* **90C**, 207–214.
- HILL, R. B. AND WELSH, J. H. (1966). Heart, circulation and blood cells. In *Physiology of Mollusca*, vol. 2 (ed. K. M. Wilbur and C. M. Yonge), pp. 124–174. New York: Academic Press.
- HILL, R. B. AND YANTORNO, R. E. (1979). Inotropism and contracture of aplysiid ventricles as related to the action of neurohumors on resting and action potentials of molluscan hearts. *Am. Zool.* **19**, 145–162.

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- IRISAWA, H., IRISAWA, A. AND SHIGETO, N. (1973). Physiological and morphological correlation of the functional syncytium in the bivalve myocardium. In *Comparative Physiology of the Heart* (ed. F. V. McCann), pp. 176–191. Basel: Birkhauser.
- IRISAWA, H., NOMA, A. AND UEDA, R. (1968). Effect of calcium on the spontaneous activities of the oyster myocardium in sodium free solution. *Japan. J. Physiol.* **18**, 157–168.
- IRISAWA, H., SHIGETO, N. AND OTANI, M. (1967). Effect of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  on the excitation of the *Mytilus* (bivalve) heart muscle. *Comp. Biochem. Physiol.* **23**, 199–212.
- JONES, H. D. (1983). The circulatory systems of gastropods and bivalves. In *The Mollusca*, vol. 5 (ed. A. S. M. Saleuddin and K. M. Wilbur), pp. 189–238. New York: Academic Press.
- JUNGE, D. (1981). *Nerve and Muscle Excitation*. Sunderland, MA: Sinauer Associates, Inc.
- LIEBESWAR, G., GOLDMAN, J. E., KOESTER, J. AND MAYERI, E. (1975). Neural control of circulation in *Aplysia*. III. Neurotransmitters. *J. Neurophysiol.* **38**, 767–779.
- LOVELAND, R. E. (1963). 5-Hydroxytryptamine, the probable mediator of excitation in the heart of *Mercenaria (Venus) mercenaria*. *Comp. Biochem. Physiol.* **9**, 95–104.
- MULLINS, L. (1984). The role of Na–Ca exchange in heart. In *Physiology and Pathophysiology of the Heart* (ed. N. Sperelakis), pp. 199–214. Boston: Martinus Nijhoff Publishing.
- PAINTER, S. D. AND GREENBERG, M. J. (1982). A survey of the responses of bivalve hearts to the molluscan neuropeptide FMRFamide and to 5-hydroxytryptamine. *Biol. Bull. mar. biol. Lab., Woods Hole* **167**, 311–332.
- PROSSER, C. L. (1940). Acetylcholine and nervous inhibition in the heart of *Venus mercenaria*. *Biol. Bull. mar. biol. Lab., Woods Hole* **78**, 92–102.
- SHIGETO, N. (1970). Excitatory and inhibitory actions of acetylcholine on hearts of oyster and mussel. *Am. J. Physiol.* **218**, 1773–1779.
- S.-ROZSA, K. AND PERENYI, L. (1966). Chemical identification of the excitatory substance released in *Helix* heart during stimulation of the extracardial nerve. *Comp. Biochem. Physiol.* **19**, 105–113.
- WELSH, J. H. AND TAUB, R. (1948). The action of choline and related compounds on the heart of *Venus mercenaria*. *Biol. Bull. mar. biol. Lab., Woods Hole* **95**, 346–353.
- WILKENS, L. A. (1972a). Electrophysiological studies on the heart of the bivalve mollusc, *Modiolus demissus*. I. Ionic basis of the membrane potential. *J. exp. Biol.* **56**, 273–291.
- WILKENS, L. A. (1972b). Electrophysiological studies on the heart of the bivalve mollusc, *Modiolus demissus*. II. Ionic basis of the action potential. *J. exp. Biol.* **56**, 293–310.