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Physiologic Studies on the Heart of *Amblema peruviana* (Mollusca, Bivalvia)

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Physiologic Studies on the Heart of *Amblema peruviana*. The heart of the freshwater clam, *Amblema peruviana* was found to be relatively insensitive to changes in ionic concentrations, and could function in extremely dilute ionic environments. Pharmacologic studies showed a bimodal acetylcholine response, with inhibition at lower concentrations, and excitation at higher concentrations. Cl^- ion was found to potentiate the depressor acetylcholine response. The resting membrane potential was found to be more negative than that found in comparable marine species.

INDEX DESCRIPTORS: Mollusca, *Amblema peruviana*, clam heart, clam heart action potential, cardiac physiology, cardiac pharmacology.

The Phylum Mollusca, and even the Class Bivalvia, appear to exhibit a fairly wide and diverse response to pharmacologic substances and ionic concentrations (Hill and Welsh, 1966; Welsh and Twarog, 1960; Krijgsman and Divaris, 1955; Greenberg, 1960). Most of the literature on the physiology of the molluscan heart has dealt with marine species. There seems to be variation among species in response to pharmacologically active substances such as acetylcholine (ACh) (Pilgrim, 1954; Greenberg, 1962, 1965, 1969; Shigeto, 1970). The greatest differences are between marine and freshwater bivalves. Shigeto (1970) suggests that the variation in response could result from differences in membrane potentials or from differences in the normal membrane permeability to Na and Cl. Since the normal body concentrations of Na and Cl are vastly different in marine species and freshwater species (Pilgrim, 1953), this could possibly account for some of the variation in response.

ACh often has two separate modes of action (Greenberg and Windsor, 1962; Chiarandini and Gerchenfeld, 1967; Greenberg, 1965, 1969, 1970; Shigeto, 1970; Irisawa et al., 1973). Most bivalve hearts are inhibited by low, and excited by high, concentrations of ACh (Greenberg and Windsor, 1962; Greenberg, 1965). Wilkens and Greenberg (1973) suggest that the mechanism of the excitatory action is due to an increase in Na conductance, and Shigeto (1970) and Irisawa (1973) suggest that the inhibitory action is due to an increase in Cl conductance.

The scope of this project was to study some basic pharmacologic, ionic, and toxic effects on the isolated heart of the freshwater clam, *Amblema peruviana*.

MATERIALS AND METHODS

The freshwater clam, *A. peruviana* (three ridge), was taken from the Mississippi River near Lansing, Allamakee Co., Iowa. Collections were made in June and July, and the specimens were stored for up to four weeks in an indoor greenhouse pond. The heart was exposed, and ligatures were tied at both atrio-ventricular junctions. The ends of the intestine, which runs through the heart, were also ligatured. These served as a means to lift the heart out of the specimen. The intestinal ends were stretched, and tied to a glass microscope slide. One atrio-ventricular junction was tied to a light spring attached to the strain gauge transducer of a Grass polygraph. Stretching the heart from the intestinal ends proved to be an important aspect in keeping the hearts beating.

The Ringer's solutions used were taken from Potts and Perry (1964), and a Tris buffer was substituted for the sodium phosphate to prevent precipitation of calcium phosphate. The Ringer's consisted of 14.1 mM NaCl, 0.5 mM KCl, 5 mM $CaCl_2$, 1 mM glucose, and 10 ml Tris buffer per liter. The Tris buffer (Gomori, 1955) consisted of 50 ml 0.2 M Tris, and 38.4 ml of 0.2 M HCl with water added to make a volume of 200 ml. The final pH of the Ringer's was adjusted to 7.6 with NaOH.

The outside of the heart has a definite epicardium containing columnar and large mucous cells; no difinite endocardium has been found on the inside (Motley, 1933, 1934). Therefore, a hole was cut in the ventricle of many preparations to expose the inside of the heart to the test solutions. The cutting often resulted in stoppage of the heart, but it could usually be revived with 10^{-4}M 5-hydroxytryptamine (5-HT).

A gas mixture, 95% O_2 and 5% CO_2 was bubbled through the chamber at a slow rate, and the temperature was normally 23 to 26 degrees Centigrade. All concentrations of drugs are given in final molar concentrations in the chamber.

For studies of electrical phenomena the preparations were left in the half shell, totally immersed in a bowl of clam Ringer's, and aerated with a slow, steady stream of 95% O_2 and 5% CO_2. A dual beam oscilloscope, augmented by a high input impedance negative capacitance preamplifier, was used simultaneously with a Grass polygraph to record changes in membrane potential, as measured with glass microelectrodes of high resistance (30-120 megohms). Measurements were made on ten different hearts. Frog heart controls gave normal resting membrane and action potential values when measured with the same electrical system.

RESULTS

A relatively high acetylcholine (ACh) threshold of 10^{-4}M resulted in a reduction in rate and amplitude in most preparations and, occasionally, a decrease in tone or diastolic arrest. In some preparations, a combination depressor and excitor effect was observed in which rate and amplitude were depressed but tone increased. The threshold was lowered in preparations having a hole in the ventricle, which allowed easier access of solutions to the inside. These preparations were depressed in rate and amplitude by concentrations of 10^{-7}M to 10^{-6}M, and excited by concentrations of 10^{-4}M to 10^{-3}M, the excitor response being an increase in tone, followed by systolic arrest.

The anticholinesterase, eserine (10^{-4}M), was found to lower the threshold for the ACh depressor effect by a factor of 100; the threshold before treatment with eserine was 10^{-4}M and afterwards 10^{-6}M (Fig. 1). The ACh excitor effect was also found to be potentiated by eserine 10^{-4}M, the effect being an increase in tone at a given ACh concentration (Fig. 2).

Irisawa (1973) suggested that ACh causes hyperpolarization, and thus depression, by a mechanism which increases Cl^- conductance. This was investigated by doubling the Cl^- concentration in the external perfusate. Irisawa removed the external Cl^-, and thus blocked the effect due to ACh. We doubled the Cl^- concentration using choline chloride, and found that the doubled Cl^- concentration increased the sensitivity to ACh (Fig. 3). A control with doubled Cl^- alone had no effect.

The preparation seemed to be quite insensitive to changes in external ion concentrations. Sodium-free Ringer's were prepared by substitut-

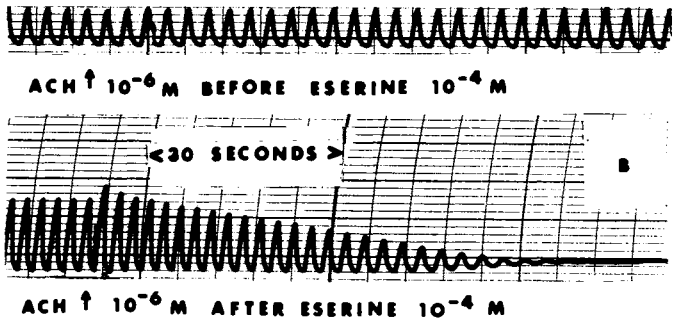

Figure 1. *Eserine potentiation of ACh depressor effect.*

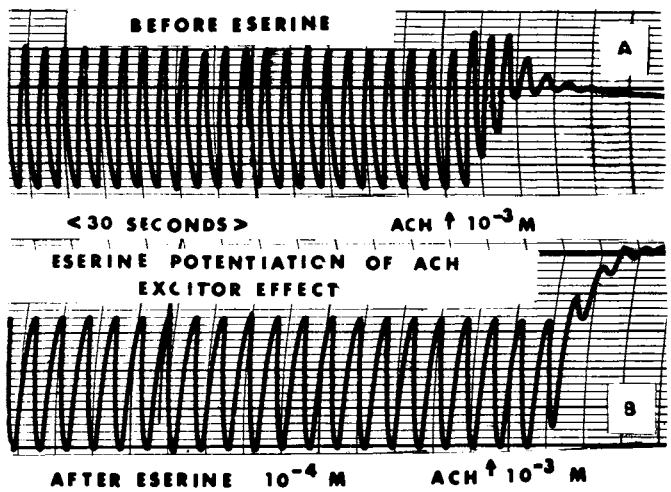

Figure 2. *Eserine potentiation of ACh excitor effect.*

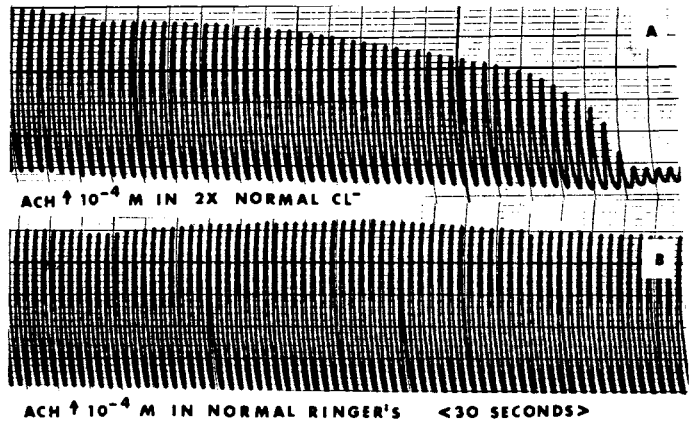

Figure 3. *Increased sensitivity to ACh in 2X normal Cl⁻ Ringer's.*

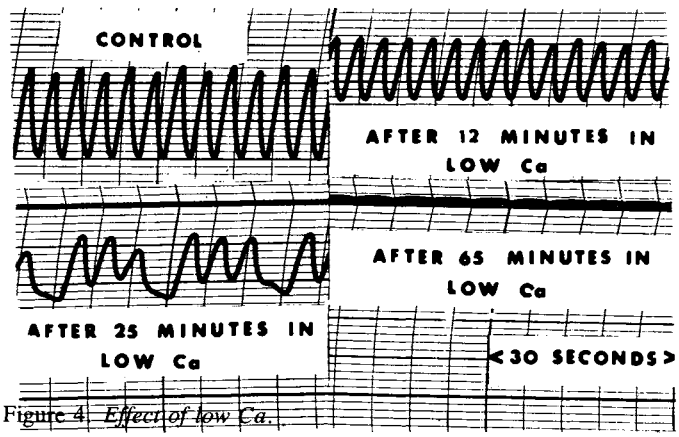

Figure 4. *Effect of low Ca.*

ing equimolar amounts of the large, nondiffusible choline ion (as choline chloride) for the NaCl, and by using triethylamine instead of the usual NaOH for pH adjustment. Such solutions had no effect on the heart, and one preparation was even maintained overnight in low-Na Ringer's. Holes were cut in most preparations to allow easy access to the inside, and to remove trapped fluid. To test the effect of blocking active Na transport, ouabain 10^{-3}M was added. This caused systolic arrest.

Calcium-free Ringer's were prepared using choline chloride as a substitute for the Ca, and most hearts continued to beat. The beating in some preparations became somewhat irregular after several minutes of exposure to low Ca and, after an hour, one preparation finally stopped in systolic arrest (Fig. 4). This preparation had been exposed to the Ca-free Ringer's for 13 minutes, after which fresh Ca-free Ringer's was added. This probably rinsed out some of the Ca that had been stored in the membrane.

Doubling the osmotic concentration with sucrose had no effect, but tripling it stopped the heart in diastolic arrest.

Three and four times normal KCl had no effect on the heart, but ten times normal KCl caused systolic arrest, presumably by decreasing the resting membrane potential, and thus allowing a state of continued depolarization (Fig. 5). The normal KCl concentration makes up such a small part of the total ion concentration that even a tenfold increase in KCl would have a negligible effect on the total osmotic concentration.

The heart was also unaffected by adrenaline (10^{-4}M), dopamine (10^{-8} to 10^{-4}M), and GABA (10^{-8} to 10^{-4}M). Serotonin (5-HT) caused excitatory responses at concentrations as low as 10^{-7} to 10^{-6} M in some preparations, and others required concentrations as high as 10^{-5} to 10^{-4}M for a noticeable effect. Acetyl-beta-methyl-choline chloride showed slightly inhibitory effects at 10^{-4}M, but was not nearly as effective as ACh.

Neither PCB Aroclor 1016, Aroclor 1254, nor atrazine, in concentrations as high as 400 ppm, showed any effects on the heart. Since these compounds are insoluble in water, alcohol was first used as a solvent, and then dimethylsulfoxide (DMSO). It was hoped that the DMSO would act as a carrier molecule, since it has high membrane permeability. Although no immediate toxic effects were observed under these conditions, the possibility still remains that the large atrazine and PCB molecules did not even reach the active sites of the tissue. However, some penetration may occur at a slower rate since unpublished data collected by several Luther College students at the EPA Lab in Duluth, Minnesota showed PCB concentrations of 1.4 and 0.5 ug/gram of tissue (wet weight analysis) in two different Mississippi River clams. They also found 177ug PCB and 42 ug PCB/gram of extracted lipid. Thus, the possibility of long term, toxic effects cannot be eliminated.

There are at least four different components of the clam cardiac action potential, as measured in *A. peruviana:* a pronounced prepotential, followed by a sharp spike, which is followed by a small plateau

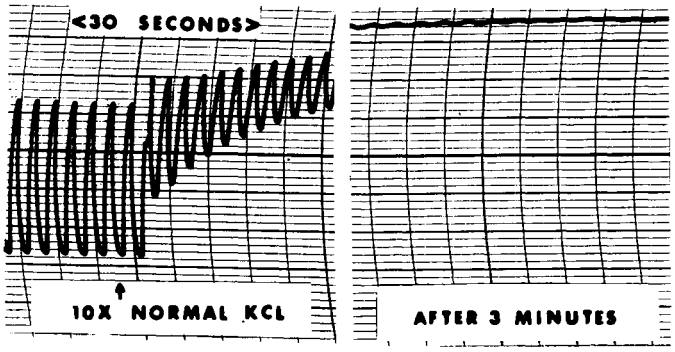

Figure 5. *Effect of 10X normal KCl.*

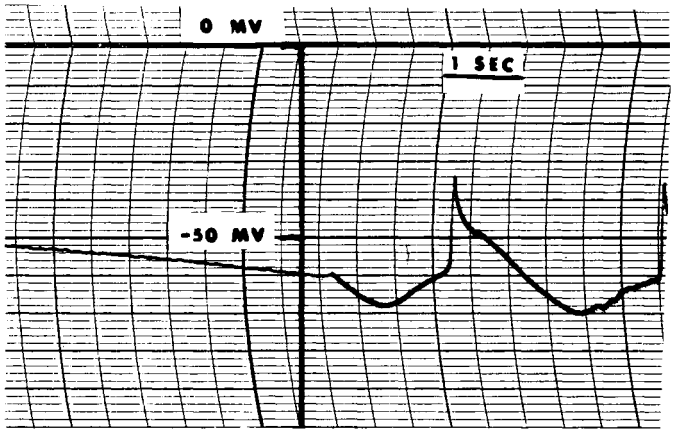

Figure 6. *Action potential as recorded with an intracellular microelectrode.*

which falls off rapidly at first and then more slowly to the resting membrane potential (Fig. 6). Data collected from ten separate hearts yielded an average resting membrane potential of -72 ± 10 mv (2SD); the average action potential peak was -41 ± 11 mv, making the average amplitude for the action potentials 31 ± 9 mv. Table 1 is a summary of our results.

Table 1. *Summary of pharmacologic effects of 5-HT and ACh on the* Amblema *heart.*

Chemical	Threshold	Effect
5-HT	10^{-7}M (2)[1]	Increased amplitude and rate
	10^{-5}M (1)	" " " "
	10^{-4}M (1)	" " " "
ACh	10^{-4}M (4)	Slowed rate and decreased amplitude (one arrested)
	10^{-7}M (2) Both had a hole in the ventricle	Slowed rate and decreased amplitude
		10^{-6}M resulted in inhibition, 10^{-5}M diastolic arrest, 10^{-4}M combination effect (arrest, but an increase in tone), and 10^{-3}M systolic arrest.

[1]Numbers in parentheses refer to the number of hearts giving this result.

DISCUSSION

Of the pharmacologically active substances tested, only ACh and 5-HT seemed to show major effects on the heart. The fact that the anticholinesterase, eserine, potentiates the action of ACh, leads to the suspicion that acetylcholinesterases are indeed present. Since the potentiation occurs for both the depressor and excitor effects, both are probably due to reactions at receptor sites. Since doubling the Cl^- concentration in the external medium caused a very marked increase in sensitivity to ACh, it would seem likely that ACh acts to increase membrane permeability to Cl^-. By removing the external Cl^-, Irisawa (1973) was able to abolish the ACh depressor effect. Together, these two observations offer a feasible explanation for the mechanism of the ACh depressor effect. Also, the reason that ACh thresholds are generally higher in freshwater species than in most marine species (Greenberg, 1965; Pilgrim, 1954) could be because freshwater clams have a much lower external concentration of Cl^- than marine species.

Wilkens (1972) has reported that the resting membrane potentials (RMPs) in the marine bivalve *Modiolus* ranged from -42 to -64 mv, while spike amplitude ranged from 26 mv to 56 mv. One possible explanation for the significantly more negative RMP found in the freshwater clam is the fact that the freshwater habitat provides a much more dilute environment; perhaps less availability of sodium and other positive extracellular ions has resulted in a more negative RMP. *Modiolus* has the capability of achieving spikes of greater amplitude, which may also be because the marine species is exposed to an environment of higher sodium ion concentration, with more sodium available to generate greater potential differences per influx of sodium.

The results presented earlier suggest that the heart can tolerate wide variations in the external ionic environment. Since most hearts continued to beat in extremely low Ca, it would seem that Ca is not necessary for the inward current (although it may be in the absence of Na). If Ca were necessary for the inward current, it would be difficult to account for the *systolic* arrest observed in one preparation that had previously been beating for over an hour in low Ca. This suggests that Ca may play a role in regulating membrane permeability to Na. Absence of Ca generally increases membrane permeability to Na (Prosser, 1973), and increased permeability to Na would be a likely explanation for the contracture effect. Blockage of the heart in contracture with ouabain suggests that Na is a normal inward current carrier. Ouabain blocks the Na pump (Prosser, 1973) and when inward Na currents depolarize the membrane, the Na cannot be pumped out to repolarize it, resulting in contracture.

The fact that the heart continues to beat in a low Na medium is more difficult to explain. Geduldig and Junge (1968) suggest that in the giant neuron of the mollusc *Aplysia,* Na carries the inward current in the absence of Ca, and Ca carries the inward current in the absence of Na. They felt that one can carry the current in the absence of the other, but that in normal Ringer's, Na plays a stronger role. In *A. peruviana,* the relative roles of Na and Ca under normal conditions remain to be seen, but the fact still remains that this heart can function under a wide variation of external ionic environments.

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