PHYSIOLOGICAL AND PHARMACOLOGICAL STUDIES OF CARDIOREGULATION IN THE HEART OF THE HORSESHOE CRAB LIMULUS POLYPHEMUS (LINNAEUS)

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ABSTRACT

PHYSIOLOGICAL AND PHARMACOLOGICAL STUDIES OF CARDIOREGULATION IN THE HEART OF THE HORSESHOE CRAB LIMULUS POLYPHEMUS (LINNAEUS)

By

Vincent James Palese, Jr.

There is a large amount of information concerning the effects of stimulation of the cardioregulator nerves and application of exogenous drugs on the frequency and strength of contraction of the muscle of isolated spontaneously beating arthropod hearts. Only a few studies have been concerned with the effects of stimulation of the cardioregulator nerves and application of drugs on the intracellular electrical activity from cells located in the cardiac ganglion. Furthermore, with the exception of the crayfish stretch receptor neuron, few pharmacological studies have been performed on peripheral nerve cells. For these reasons electrophysiological and pharmacological experiments were performed on the large unipolar cells of the cardiac ganglion of limulus.

Abdominal cardiac nerves 10 through 12, the accelerator nerves, were stimulated individually near their junctions with the right pericardial nerves for one min periods at 5V, 8 and 20 Hz, and 3 msec duration. Increases in bursting

frequency were seen in the 20 cells examined with microelectrodes. Before stimulation of the abdominal nerves the mean bursting frequency was 16.6 bursts/min. One minute after onset of stimulation the average bursting rate was 20.8 bursts/min. The greatest increase in rate was 9.9 bursts/min. Initial decreases in bursting frequency ranging from 0.5 to 1.0 bursts/min were seen in 46% of the cases in which nerve 11 was stimulated. No significant changes in other parameters of the electrical activity from the unipolar cells were apparent. No postsynaptic potentials appeared in these cells and there were no changes in membrane resistance. These results suggest that the increases in burst frequency are due to an indirect effect through the pacemakers.

Segmental nerves 7 and 8, the inhibitory nerves, were stimulated as they entered the cardiac ganglion for 15 to 30 sec at 5V, 2 to 200 Hz, and 3 msec duration. There were no marked changes in the amplitudes of the initial and recovery phases or in the number of spikes occurring on the initial phase. The durations of the initial and recovery phases were increased by inhibitory nerve stimulation. The number of spikes on the recovery phase was decreased by 4 to 6 at stimulus frequencies above 2 Hz. All cells showed decreases in bursting frequency of 20 to 46%, when the inhibitors were stimulated at frequencies from 2 to 60 Hz. These results

indicate that the inhibitory nerves have direct inputs to the pacemaker cells. Stimulation of the inhibitors at 200 Hz failed to affect the electrical activity from the unipolar cells.

At stimulus frequencies of 2 to 5 Hz small inhibitory postsynaptic potentials (ipsp's) of 2 mV amplitude were apparent. These potentials reversed at -72 mV. These results suggest that chemical synaptic transmission is present between the inhibitory nerves and the unipolar cells.

Picrotoxin and strychnine were perfused over the ganglion. In each of the seven hearts examined picrotoxin completely blocked the ipsp's and partially blocked the decreases in bursting frequency produced by stimulation of the inhibitory nerves. Strychnine had no effect.

L-glutamic acid, dopamine, 5-hydroxytryptamine (5-HT) and gamma-aminobutyric acid (GABA) were applied to the unipolar cells in 1 μl amounts with a pipette and perfused over the entire ganglion. L-glutamic acid (10^{-3} M) had no effect when applied with a pipette. When perfused, l-glutamic acid (5×10^{-4} M) decreased the durations of the initial and recovery phases and the number of spikes on the recovery phase. The bursting frequency was increased from 42 to 319%.

Dopamine (10^{-3} M) decreased the number of spikes in each burst and the amplitude of the recovery phase when pipetted

onto the soma. When perfused over the ganglion, 5 x 10^{-4} M dopamine increased the duration of the recovery phase and decreased the number of spikes. The bursting frequency initially decreased by 26%, but within five min the frequency increased to 34% greater than the control rate.

When pipetted onto the soma, 10^{-3} M 5-HT decreased the amplitude of the recovery phase, the number of spikes and the bursting frequency. When perfused, 5 x 10^{-5} M 5-HT decreased all parameters of activity in 4 cells and completely blocked bursting activity in 5 cells.

GABA decreased all parameters of electrical activity when pipetted (10^{-3} M) onto cell bodies. In contrast to the other drugs examined, GABA decreased the membrane resistance by an average of 28%, and showed a reversal potential of -50 mV (range -39 to -65 mV). When perfused over the ganglion, 5 x 10^{-5} M GABA blocked all electrical activity for 2 to 10 min.

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By

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With love to my devoted wife, Joyce

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INTRODUCTION

Among the arthropods the heartbeats of the horseshoe crab, Limulus polyphemus, of decapod and stomatopod crustaceans and of some arachnids have been shown to be neurogenic (Brown, 1964a, 1964b; Carlson, 1905a, 1909; Sherman and Pax, 1968; Welsh and Maynard, 1951). The heartbeat in these arthropods is initiated by nerve cells which are located in a cardiac ganglion which lies on the dorsal aspect of the heart. The activity of these cardiac ganglion cells and ultimately the frequency and strength of contraction of the heart muscle can be modified by nerves from the brain and ventral nerve cord (Carlson, 1905b; Florey, 1960; Heinbecker, 1936; Pax, 1969; Pax and Sanborn, 1964; Maynard, 1953, 1960, 1961; Smith, 1947; Wiersma and Novitski, 1942).

It is thought that the nerves from the central nervous system (cardioregulator nerves) regulate the heartbeat through chemical mediation. A number of pharmacological experiments have been performed in an attempt to determine the chemical nature of the mediator. For the most part, these experiments have been concerned with an examination of the effects of various drugs on the mechanical contraction of the heart muscle. The effects produced by the drugs are then compared

with the changes in mechanical contraction produced by stimulation of the cardioaccelerator and cardioinhibitor nerves. These comparisons serve as the basis for the acceptance or rejection of a drug as the possible biological mediator.

However, such methods have several disadvantages, the principal shortcoming being the lack of knowledge as to the site of action of the applied drug in comparison with the site of action of the actual biological mediator. In some cases it is possible to eliminate a site of drug action. For example, when a drug changes the frequency of a heartbeat, it is possible to eliminate the cardiac muscle as the site of drug action. The conclusion can then be drawn that the drug is probably affecting the pacemaker cells located in the cardiac ganglion.

In other cases the site of action is not so easily determined. If a drug should affect the strength of contraction of the heart muscle, there are several loci at which the drug could be acting--the heart muscle, the motor or pacemaker nerve cell terminals, or the spike-generating region of the pacemaker cell or motor neuron. Because of the large number of possibilities of action sites, it cannot easily be determined whether the site of action of the applied drug is the same as the site of action of the chemical mediator.

One method of overcoming the stated problems is by monitoring the intracellular electrical activity of cells in the

cardiac ganglion during stimulation of the cardioregulator nerves and during drug application. Such experiments would eliminate many of the possible sites of neurotransmitter and drug action and would make comparisons between applied drugs and natural mediators more valid.

In the Crustacea only three such experiments have been undertaken. Terzuolo and Bullock (1958) described the effects of stimulation of the cardioregulator nerves on the electrical activity of follower cells in the lobster cardiac ganglion. Accelerator fibers were found to have no effective endings on the follower cells. The acceleration in the rate of occurrence of the bursts of electrical activity observed in these cells is entirely due to an indirect effect through the pacemakers. The inhibitory fibers do have direct synaptic inputs into these cells (Otani and Bullock, 1959). Depending on the type of activity recorded from the follower cells, stimulation of the inhibitory nerve fibers caused either depolarizing or hyperpolarizing postsynaptic potentials in the follower cells. However, no drug experiments were performed in these studies.

Cooke (1966) recorded the intracellular electrical activity from cells in the cardiac ganglion of the crab, Cancer borealis. He found changes in burst frequency and burst duration in motor cells when the ganglion was perfused with 5-hydroxytryptamine (5-HT) or pericardial organ extract. Changes in electrical activity of the cells during stimulation of the cardioregulator nerves were not reported.

To obtain information about the mechanisms of cardioregulation and the effects produced by drugs on the electrical activity of cells in the cardiac ganglion, electrophysiological and pharmacological studies were performed on the isolated heart preparation of limulus. This animal was used because: (1) the effects of electrical stimulation of the cardioregulatory fibers on the mechanical contraction of the heart muscle have been examined extensively (Bursey and Pax, 1970a; Carlson, 1905b; Pax, 1969; Pax and Sanborn, 1964); (2) the cardioinhibitory transmitter has been tentatively identified to be 5-HT or a 5-HT-like compound (Pax and Sanborn, 1967b); and (3) the intracellular electrical activity of unipolar cells of the cardiac ganglion has recently been characterized (Palese et al., 1969, 1970). Results of these experiments should provide information about the cardiac physiology of limulus and of arthropods in general.

Cardioregulatory Nerve Innervation of the Cardiac Ganglion

The cardiac ganglion is situated externally along the dorsal mid-line of the heart and extends almost the entire length of the heart. The ganglion is thickest in the fourth, fifth and sixth segments and gradually decreases in size as it extends both anteriorly and posteriorly (Patten and Redenbaugh, 1900). Several cell types are present in the

ganglion and include large unipolar, large and small bipolar cells and large and small multipolar cells (Bursey and Pax, 1970b).

Recent evidence suggests that the unipolar cells are motor in function. Palese et al. (1970) found that part of the electrical activity recorded intracellularly from the unipolar cells was derived from a presynaptic input. The activity of the cells is suggestive of the observations one would expect in a follower cell and not in a pacemaker cell.

The cardiac ganglion is connected with the central nervous system by means of the segmental cardiac nerves (6 through 13). Nerves 6, 7 and 8 arise from the hindbrain. Nerve 6 enters the cardiac ganglion near the second pair of ostia. Nerves 7 and 8 fuse together into one large nerve and enter the large intertergal muscles dorsal to the heart. The nerve then divides, the main branch forming the pericardial nerve which passes posteriorly in the epidermis lateral to the heart. Small branches of the fused nerve enter the cardiac ganglion just posterior to the third pair of ostia.

Abdominal cardiac nerves 9 through 13 are paired and arise from the abdominal ganglia of the ventral nerve cord. These nerves give off numerous branches to the epidermis, and also an important branch which goes anteriorly and unites with the pericardial nerve. The remaining branch continues dorsally and connects with the cardiac ganglion at the level of each of the last five pair of ostia.

Effects of the Cardioregulatory Nerves

Inhibition of the cells of the cardiac ganglion is accomplished by segmental nerves 7 and 8, which arise from the brain. While recording extracellularly from the cardiac ganglion, Heinbecker (1933) found that upon stimulation of the inhibitor nerves all activity in the cardiac ganglion could be abolished. This led him to the conclusion that these extrinsic nerves act on the ganglion cells directly.

Electrical stimulation of these nerves results in a decrease in heart rate and strength of contraction of the heart muscle (Carlson, 1905b; Pax and Sanborn, 1964). Pax and Sanborn (1964) and Bursey and Pax (1970a) noted that under certain conditions stimulation of these nerves caused a slight increase in rate. This suggested to them that some acceleratory fibers are carried in these nerves. Von Burg and Corning (1969) were unable to detect any evidence for the presence of acceleratory fibers, but recent evidence by Corning et al. (1971) suggests that acceleratory fibers may be present.

Acceleration of the heart is accomplished by the abdominal cardiac nerves. Stimulation of the ventral cord or any of the abdominal ganglia causes an increased rate of heartbeat (Carlson, 1905c; Heinbecker, 1933). The abdominal nerves appear to carry some inhibitory fibers along with the acceleratory fibers (Bursey and Pax, 1970a; Pax, 1969; von Burg and Corning, 1967, 1970).

Pharmacological Studies

A large number of pharmacologically active drugs have been applied to the isolated heart preparation of limulus. Table 1 summarizes some of these results. From the Table it can be seen that most of the drugs tested had an excitatory effect on the limulus heart.

Pax and Sanborn (1967a) perfused GABA through isolated limulus hearts and found that the drug mimicked stimulation of the cardioinhibitory nerves by decreasing the rate and strength of beating of the heart. Unlike the inhibitory nerves, GABA did not decrease the number of units discharging nor the total duration of each burst of electrical activity in the cardiac ganglion. Picrotoxin blocked the effects of stimulation of the cardioinhibitory nerves but not the effects of applied GABA.

Pax and Sanborn (1967b) reported that 5-HT slowed the heart rate and reduced the strength of beat in limulus. When applied to the isolated cardiac ganglion, 5-HT decreased the rate of rhythmic discharge, reduced the number of neurons discharging in each burst and decreased the duration of each burst. Bromlysergic acid diethylamide decreased the ability of the cardioinhibitory nerves to influence heart rate and it blocked the rate and strength changes produced by exogenously applied 5-HT. They suggested that 5-HT or a similar compound is the cardioinhibitory transmitter in the limulus heart.

Table 1. Summary of the effects of exogenously applied drugs on the heartbeat of limulus. An excitatory effect is indicated by (E), an inhibitory effect by (I), and no effect by (O).

Drug	Effect	Reference
Acetylcholine	E	Carlson, 1922; Garrey, 1942; Heinbecker, 1936
Epinephrine	E	Carlson, 1907
Gamma-aminobutyric acid (GABA)	I	Burgen and Kuffler, 1957; Pax and Sanborn, 1967a; Abbott et al., 1969; Parnas et al., 1969
L-glutamate	O	Pax and Sanborn, 1967a
	E	Abbott et al., 1969 Parnas et al., 1969
5-HT	I	Burgen and Kuffler, 1957; Pax and Sanborn, 1967b
	O	Abbott et al., 1969
Nicotine	E	Carlson, 1907, 1922; Armstrong et al., 1939
Strychnine	E	Carlson, 1907; Heinbecker, 1936

Von Burg and Corning (1968) injected 5-HT into ganglia of the ventral cord of limulus and observed a decrease in electrical activity of the dorsal roots of the abdominal nerves and an inhibition of the heart rate. They, too, suggested that 5-HT is a possible mediator of inhibition, but, in this case, the effects of 5-HT are central rather than peripheral.

Contrary results were reported by Abbott et al. (1969) and Parnas et al. (1969). These authors reported that GABA appeared to mimic stimulation of the cardioinhibitory nerves in every way. GABA decreased the frequency of the bursts of the ganglionic discharge, the length of the discharge and the number of units firing in a discharge. 5-HT was shown to have no effect on the electrical and mechanical properties of the peripheral neuromuscular system of the heart.

Abbott et al. (1969) and Parnas et al. (1969) suggested that glutamate may be the excitatory transmitter in limulus heart. In a deganglionated heart glutamate in low concentrations caused contractions of the heart muscle. In a spontaneously beating heart, higher concentrations increased the myocardial tone but decreased the strength of the beat pulsations. When glutamate was added to an isolated ganglion, the ganglionic discharges increased in both frequency and duration and firing appeared throughout the quiescent period between beats. On the other hand, Pax and Sanborn (1967a) reported that 10^{-5}M glutamate had no effect on the

spontaneously active heart. At higher concentrations glutamate caused a negative inotropic effect, leaving the rate unchanged.

Objectives of this Study

There is a large amount of information concerning the effects of stimulation of the cardioregulator nerves and application of exogenous drugs on the frequency and strength of contraction of the muscle of isolated spontaneously beating arthropod hearts. Only a few studies have been concerned with the effect of stimulation of the cardioregulator nerves and application of drugs on the intracellular electrical activity from cells located in the cardiac ganglion. Furthermore, with the exception of the crayfish stretch receptor neuron, few pharmacological studies have been performed on peripheral nerve cells. For these reasons electrophysiological and pharmacological experiments were performed on the large unipolar cells of the cardiac ganglion of limulus.

In view of the contradictory conclusions drawn by Pax and Sanborn (1967a, 1967b), Abbott *et al.* (1969) and Parnas *et al.* (1969) concerning the chemical nature of the cardioinhibitory transmitter, both GABA and 5-HT were applied to the ganglion in an effort to clarify this discrepancy. Besides GABA and 5-HT, other compounds which have been reported to be active in invertebrate nerve and muscle preparations were studied. It is hoped that the results of these

experiments will help to determine the chemical nature of synaptic transmitters involved in cardioregulation and to determine the possible sites of the excitatory and inhibitory effects produced by the exogenously applied drugs. Such information will provide a better understanding of the physiology of the limulus heart.

MATERIALS AND METHODS

Care and Maintenance of Animals

The experiments were performed on the horseshoe crab, Limulus polyphemus, obtained from the Gulf Specimen Co., Panacea, Florida and shipped periodically by Air Express. Mature females with carapace widths from 20 to 30 cm were used. The animals were stored until use in a Dayno Co. Model 703 artificial sea water aquarium at 13 to 16° C.

Isolation of the Heart and Cardiac Ganglion

Procedures used for isolating the hearts have been described previously (Pax and Sanborn, 1967a). A heart was exposed by making two longitudinal cuts through the dorsal carapace of the prosoma and opisthosoma, just lateral to the heart. Two transverse cuts, one just posterior to the median eyes and the other just posterior to the seventh entapophyses of the opisthosoma, were made connecting the two lateral cuts. The rectangular-shaped portion of the carapace made by the four cuts was lifted and dissected away from underlying muscles and connective tissue with a sharp probe. The internal extensor muscles of the opisthosoma just dorsal to the anterior half of the heart were carefully

cut away, completely exposing the heart and its cardiac ganglion.

A transverse cut was made in the cardiac muscle through the first pair of ostia. A dark glass rod (OD 7 mm) was inserted through this cut and was pushed posteriorly through the lumen of the heart. The dark rod makes it easier to distinguish the positions of the large unipolar cells of the ganglion. The rod was then lifted and the heart-cardiac ganglion preparation was dissected free of tissue in the pericardium. The isolated heart preparation was placed in a paraffin chamber, held in position with straight pins and washed with sea water ("Instant Ocean," Aquarium Systems, Inc.).

Connective tissue surrounding the cardiac ganglion was removed. After removal of this tissue, the isolated heart preparation was again washed in sea water. During the course of the experiments the sea water was changed every 60 minutes or after each drug application. Temperatures were maintained at room temperature of 22 to 26^{o} C.

Isolation of the Segmental Nerves

Segmental nerves 7 and 8 are usually found entering the cardiac ganglion as a single fused nerve just posterior to the third pair of ostia and just anterior to the connective tissue overlying the posterior two-thirds of the heart. This fused nerve does not require dissection. Stimulation of the

nerve was performed with a suction electrode located dorsally, that is, just as the nerve enters the ganglion.

Isolation of segmental nerves 10 through 12 was accomplished in a manner similar to isolation of the heart described above except for a slight modification. The pericardial nerve was first isolated near the hinge of the carapace. Since each of the segmental nerves 9 through 13 sends a branch to the pericardial nerve and since each nerve has a branch which crosses the pericardial nerve dorsally as it passes toward the cardiac ganglion, the segmental nerves can easily be located by following the pericardial nerve posteriorly. The segmental nerves can then be dissected free from the surrounding fat tissue.

Microelectrodes

Microelectrodes were made with Kimax glass capillary tubing (OD 0.8 to 1.0 mm) and were pulled on a Narishige horizontal micropipette puller. The electrodes were filled with a hot solution of 3M KCl under a controlled vacuum.

Recording

KCl-filled microelectrodes were placed on either a Ag-AgCl wire (No. 34) or a WPI right angle electrode holder which was connected to a WPI M-4 or M-4A Precision Electrometer. The preamplifiers led into a dual beam Tektronix

502A oscilloscope. The electrical activity viewed on the oscilloscope was recorded with a Grass Kymograph camera. The microelectrodes were positioned by Narishige micromanipulators. A large Ag-AgCl wire (No. 20) was placed in the paraffin chamber and used as the indifferent electrode.

Intracellular Stimulation

For intracellular current injection KCl-filled microelectrodes of 10 to 40 MΩ resistance were used. In these experiments current was applied by a Grass S4 stimulator through the M-4 preamplifier to the electrodes, which could be used simultaneously for both stimulating and recording. The strength of the applied current was measured from the oscilloscope.

Extracellular Stimulation

Extracellular stimulation was accomplished by means of a suction electrode applied to one of the segmental nerves. The suction electrode consisted of a piece of glass tubing (OD 4 mm) drawn to a narrow tip (ID 400μ). The suction electrode was positioned with a Narishige micromanipulator.

Stimulation of the segmental nerves 10 through 12 was accomplished by placing the suction electrode on one of the nerves just distal to the point where it crossed the pericardial nerve. In slightly less than half of these preparations stimulation of the segmental nerves failed to affect

the heart rate, possibly because of injury incurred to the nerves during the dissection (Carlson, 1905b). The fused segmental nerve 7 and 8 was stimulated dorsally.

Stimulus parameters were 5 to 9 volts, duration of 3 msec and frequencies ranging from 2 to 200 Hz for the fused nerve, and 5 to 9 volts, duration of 3 msec and frequencies of 8 or 20 Hz for nerves 10 to 12 (Pax, 1969; Pax and Sanborn, 1964). All stimulations were supramaximal.

Source and Preparation of Drugs

Stock solutions of the drugs were made by dissolving salts of a drug in artificial sea water ("Instant Ocean") as shortly before the experiment as possible. The following drugs were used:

Dopamine (as HCl)--Mann Research Labs.

Gamma-aminobutyric acid--Nutritional Biochemicals Corp.

L-glutamic acid--Sigma Chemical Co.

5-Hydroxytryptamine-creatinine sulfate--Sigma Chemical Co.

Picrotoxin--Nutritional Biochemicals Corp.

Strychnine sulfate--Sigma Chemical Co.

Experimental Procedures

Drugs were tested by either total perfusion of the ganglion or by local application to individual unipolar cells. Total perfusion was accomplished by one of two methods.

In the first method, after a unipolar cell had been penetrated with a microelectrode, all of the saline bathing the heart preparation was completely removed with a syringe. The saline was replaced by an equal amount of drug solution poured into the chamber and completely immersed the preparation. This method did not prove feasible because either the removal of the saline or the application of the drug tended to pull the cell away from the microelectrode in approximately 50% of the cases.

The second method involved simple addition of the drug solution to the saline bath. Since the amount and concentration of the added drug solution and the amount of saline in the bath (20 ml) were known, it was possible to calculate the new effective concentration of the drug solution. Initially, the concentration of the drug near the ganglion approximates the concentration of the added solution. After a time the processes of mixing and diffusion sufficiently lower the concentration to the new effective concentration.

When the preparation was washed while the cell was still being penetrated with a microelectrode, a measured amount of the bathing solution was removed and replaced with an equal amount of fresh saline. This ensured a constant level of saline in the bath. The washing procedure was repeated at least three more times. During removal of solution from the bath, care was taken to ensure that the level of the solution was sufficient to keep the entire preparation

completely immersed. Otherwise, the same difficulties in keeping the electrode in the cell were encountered as with the first method.

Local application of drugs to individual unipolar cell bodies was accomplished by means of a micropipette (OD 150μ). The pipette was made by drawing a piece of glass tubing to a fine point in a flame. The tip was polished and smoothed with an oilstone. The other end of the glass tubing was inserted into one end of a piece of polyethylene tubing. A 10 ml syringe was placed at the other end of the plastic tubing. The glass pipette was clamped to a plastic rod and the rod was secured to a Narishige micromanipulator. The tip of the pipette was positioned as close to the cell body as possible with the manipulator.

The pipette was filled with 1 μl of drug solution and, after positioning the pipette over a cell body, the drug solution was forced out of the pipette.

Data Reduction

The electrical activity from the unipolar cells has recently been described (Palese et al., 1969, 1970). For clarification a short description of the electrical activity will be given here, since the same terminology will be used. Figure 1a is an example of the spontaneous electrical activity recorded from the soma of a unipolar cell. Intracellular electrical activity begins with a large fast-rising

depolarization to some peak value. This level of depolarization is maintained for 23 to 450 msec. During this depolarization, spike-like potentials, which are irregular in both frequency and amplitude are seen. The depolarization (initial phase) is followed by a rapid partial repolarization of several mV, which, in turn, is followed by either a further slow repolarization to the base level or by a plateau and then a return to the resting level (recovery phase). The recovery phase is characterized by the appearance of spikes which are regular in amplitude and frequency.

The amplitude of the initial phase is measured from the resting membrane level to the peak level of the depolarization. The duration of this phase is measured from the onset of depolarization in the cell to the end of the partial repolarization (Figure 1a, first arrow). The amplitude of the recovery phase is measured from the resting membrane level to the peak level of the phase. In contrast to the initial phase, the amplitude of the recovery phase does not include the amplitude of the spike-like potentials which occur during this phase. The duration of the recovery phase is measured from the end of the initial phase to the time at which the cell is completely repolarized (Figure 1a, second arrow).

RESULTS

Inhibition

The segmental nerves (7 through 13) serve a cardioregulatory function in limulus. Stimulation of these nerves at various points along their processes, which extend from their origin in the central nervous system to their terminations in the cardiac ganglion, causes a change in both the frequency and the amplitude of the heartbeat. The rate and strength of beating are decreased by stimulation of nerves 7 and 8 (Carlson, 1905b; Heinbecker, 1933; Pax and Sanborn, 1964). However, these experiments dealt exclusively with the effects of stimulation of the cardioinhibitory nerves on the mechanical contraction of the heart muscle.

Only recently have investigations been made to examine the electrical activity recorded intracellularly from cells in the cardiac ganglion (Palese et al., 1969, 1970; Lang, 1971). The electrical activity from unipolar cells indirectly suggests that these cells are motor in function. Since these cells play an important role in the heartbeat, and since changes in both the rate and strength of contraction occur upon stimulation of the cardioregulatory nerves, the question arose as to how stimulation of the regulatory nerves

affects the rate, amplitude and duration of the electrical activity recorded from the unipolar cells. An answer to this question should be important in determining how the cardioregulatory fibers exert their influence on the heartbeat.

Fused nerves 7 and 8 were therefore stimulated dorsally as the nerve enters the ganglion. Pax and Sanborn (1964) reported that stimulation of the cardioinhibitory nerves at frequencies from 2.5 to 80 Hz caused slowing of the heart rate, and a frequency of 200 Hz caused an increase in the rate. In these experiments stimulation of the inhibitory fibers was performed at 5 V, 3 msec duration and frequencies of 2 to 60 Hz and 200 Hz. The nerves were stimulated for 15 to 30 sec. The electrical activity in the unipolar cells immediately prior to and immediately following the onset of stimulation were then compared. Table 2 summarizes the results of these experiments. Figures 1 and 2 give examples of the responses seen.

Stimulation of the inhibitors at frequencies between 2 and 60 Hz generally produced no marked changes in the amplitudes of the initial and recovery phases or in the number of spikes occurring on the initial phase, but changes were observed in the durations of both the initial and recovery phases, the number of spikes on the recovery phase and the bursting frequency.

The duration of the initial phase is increased by inhibitory nerve stimulation, the magnitude of the increase

Table 2. Summary of the effects of stimulation of the cardioinhibitory nerves on the electrical parameters of the activity recorded from the unipolar cells. (+) indicates an increase, (-) indicates a decrease, and (0) indicates no effect. Number in parentheses is the per cent of cells (excluding those showing blockade) exhibiting the change.

Frequency of Stimulation (Hz)	Number of Animals	Number of Cells	Number of Cells Showing Blockade	Initial Phase		
				Amplitude	Duration	Number of Spikes
2	4	12	0	0	0	0
5	7	16	2	0	+ (57)	0
10	6	14	4	0	+ (54)	0
20	6	16	5	0	+ (73)	0
40	7	18	5	0	+ (85)	0
60	4	7	0	0	+ (100)	0
200	2	5	0	0	0	0

continued

Table 2--continued

Frequency of Stimulation (Hz)	Recovery Phase			Bursting Rate
	Amplitude	Duration	Number of Spikes	
2	0	+ (75)	0	0
5	0	+ (50)	- (88)	- (100)
10	0	+ (100)	- (100)	- (100)
20	0	+ (92)	- (100)	- (100)
40	0	+ (69)	- (100)	- (100)
60	0	+ (71)	- (100)	- (100)
200	0	0	0	0

Figure 1. Effects of stimulation of the cardioinhibitory nerves upon the electrical activity of a unipolar cell.

A. Normal spontaneous electrical activity. The bursting frequency is 17.3 bursts/min.

B. The cardioinhibitory nerves were stimulated at 2 Hz, 5V, 3 msec duration. The amplitude of the largest ipsp is 5 mV. The bursting frequency is 15.0 bursts/min.

C. The cardioinhibitory nerves were stimulated at 5 Hz, 5V, 3 msec duration. The bursting frequency is 8.0 bursts/min.

D. The cardioinhibitory nerves were stimulated at 60 Hz, 5V, 3 msec duration. The activity is completely blocked.

E. The cardioinhibitory nerves were stimulated at 200 Hz, 5V, 3 msec duration. There is no apparent change in bursting activity.

Time scale: 500 msec. Voltage scale: 20 mV.

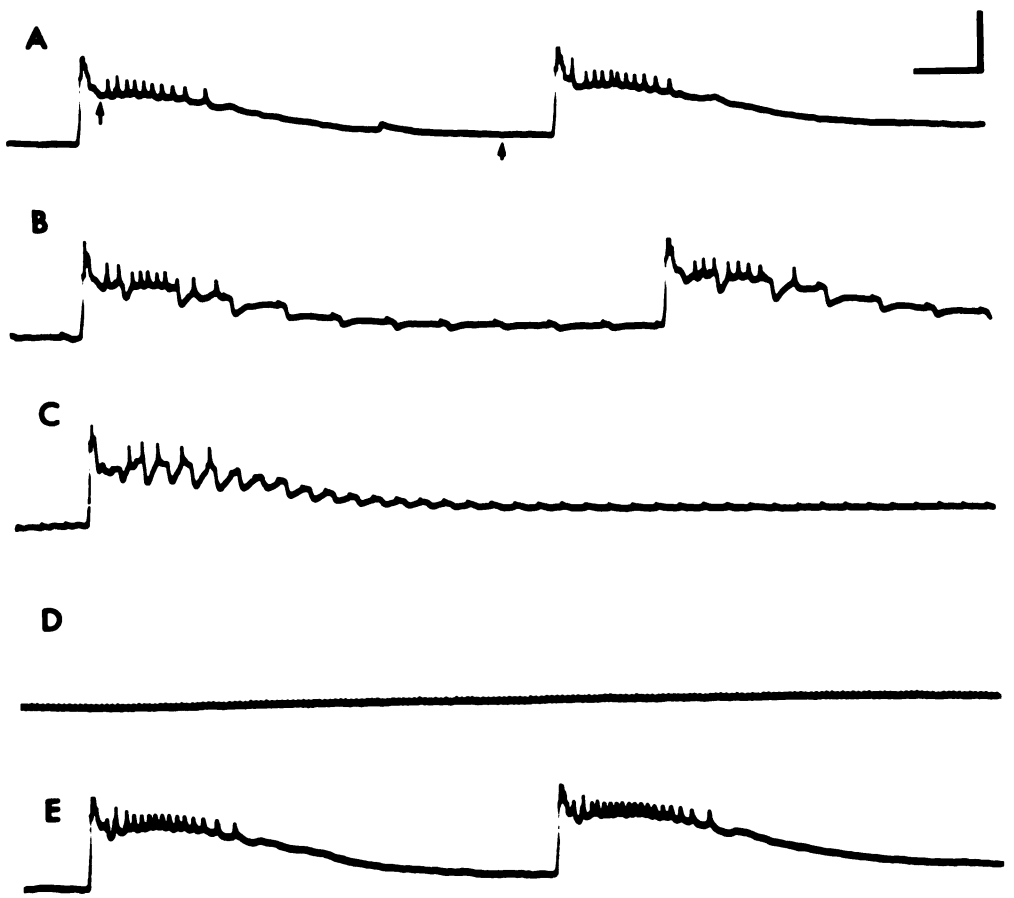

Figure 1

being dependent on the frequency of stimulation. At 2 Hz the changes were not measurable, at 5 Hz the increase averaged 14%. Larger increases in duration were obtained with higher frequencies of stimulation, such that at a frequency of 60 Hz there was a 70.3% increase in duration. In this case the duration increased from 138 msec to 235 msec. The number of cells exhibiting the increased duration appeared to be dependent upon the frequency of stimulation. At 5 Hz only eight of the fourteen cells examined showed an increased duration during stimulation; at 20 Hz eight of eleven cells showed an increased duration; and at 60 Hz all cells examined had an increased duration.

Increases in the duration of the recovery phase were also produced by stimulation of the cardioinhibitors at frequencies above 2 Hz. In contrast to the duration of the initial phase, the increases in the duration of the recovery phase became smaller with increasing frequency of stimulation. For example, at 5 Hz the increase in duration was from an average of 2.273 sec before stimulation to 3.566 sec during stimulation. This represents an increase of 57%. At 20 Hz the increase amounts to 26%, there being an increase from 2.699 sec to 3.406 sec. At 60 Hz the duration is increased by only 17%, from 2.618 sec to 3.043 sec. An increase was seen in the largest number of cells at a frequency of 10 Hz.

The number of spikes on the recovery phase was unchanged by stimulation at 2 Hz, but at frequencies above this the

number decreased. The average decrease in the number of spikes occurring on the recovery phase was least at a frequency of 5 Hz. There was an average decrease in the number of spikes from 7 to 5 (maximum decrease of 6 spikes). Fourteen cells showed a decrease. At the higher frequencies the spikes on the recovery phase were decreased during stimulation in all cells examined. In Figures 2c, 2d and 2e the decreases in spikes on the recovery phase are shown. The average decrease in spike number was 4 at a stimulating frequency of 40 Hz, 5 at both 10 and 20 Hz and 6 at 60 Hz. Decreases ranged from 1 to 17 spikes per burst.

Stimulation of the inhibitors also decreased the bursting frequency. All cells examined showed decreases in bursting frequency when the inhibitors were stimulated at frequencies ranging from 2 to 60 Hz. The decrease in bursting frequency was related to the frequency of stimulation, larger decreases in bursting frequency being generally obtained with higher frequencies of stimulation. Figure 2 shows the changes in bursting rate produced by stimulation of the inhibitors at various frequencies. The bursting frequency from a spontaneously bursting cell prior to stimulation was 28.7 bursts/min. At a frequency of 10 Hz, the bursting frequency was 23.8 bursts/min (Figure 2b); at 20 Hz the frequency was reduced to 20.1 bursts/min (Figure 2c); at 40 Hz the frequency was 20.3 bursts/min (Figure 2d); and at 60 Hz the frequency was 24.0 bursts/min (Figure 2e).

Figure 2. Effects of stimulation of the cardioinhibitory nerves upon the electrical activity of a unipolar cell, in which the activity was not blocked by the nerves.

A. Normal spontaneous electrical activity. The bursting frequency is 28.7 bursts/min.

B. Stimulus parameters 10 Hz, 5V, 3 msec duration. The largest ipsp seen in this cell was 1 mV. The bursting rate is 23.8 bursts/min.

C. Stimulus parameters 20 Hz, 5V, 3 msec duration. The bursting frequency is 20.1 bursts/min.

D. Stimulus parameters 40 Hz, 5V, 3 msec duration. The bursting frequency is 20.3 bursts/min.

E. Stimulus parameters 60 Hz, 5V, 3 msec duration. The bursting frequency is 24.0 bursts/min. In this case stimulation at this frequency is less effective than at 20 and 40 Hz.

Time scale: 500 msec. Voltage scale: 20 mV.

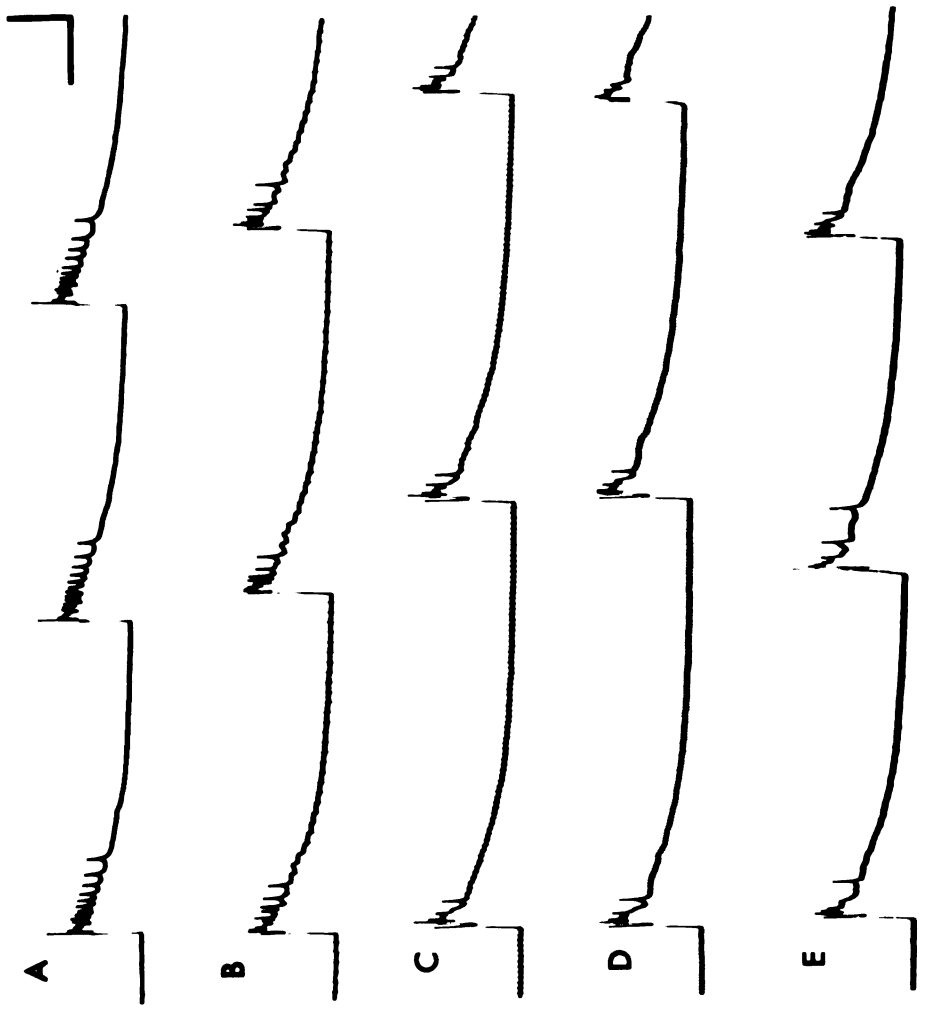

Figure 2

At 2 Hz the decrease in bursting frequency was small and variable. At 5 Hz the bursting frequency was decreased by 20%. There is a larger decrease at 10 Hz, the decrease being 27%. At 20 and 40 Hz, the decreases in bursting frequency are not markedly different, there being a 35% decrease in bursting frequency at 20 Hz and a 33% decrease at 40 Hz. The decrease was maximal at 60 Hz, there being an average decrease of 46% in the bursting frequency. Values of the decreases in rate ranged from 0.5 to 17.1 bursts/min.

In several cases stimulation of the inhibitors completely blocked the appearance of electrical activity in the unipolar cell body (Figure 1d). This phenomenon was seen in two cells while stimulating the inhibitors at 5 Hz, in four cells at 10 Hz, and in five cells at 20 Hz and 40 Hz. No blockade of activity was seen while stimulating at 60 Hz.

Pax and Sanborn (1964) found that stimulation of the inhibitors at a frequency of 200 Hz increased the rate of spontaneously beating limulus hearts. In these experiments five cells from two animals were examined. Stimulation of the inhibitors at this frequency failed to affect the electrical activity or bursting frequency from the unipolar cells (Figure 1e).

At stimulus frequencies of 2 to 5 Hz small inhibitory postsynaptic potentials (ipsp's) were apparent (Figure 1b and 1c). These potentials were usually in the hyperpolarizing direction but in rare instances were depolarizing. The maximum amplitude of the ipsp's from the 16 cells examined had a

mean value of 2 mV (range 1 to 6 mV; SD = 1.3 mV). The amplitudes of the ipsp's were largest near the peak of the recovery phase and the amplitudes gradually decreased in size as the cell repolarized, until only slight hyperpolarizing deflections were seen. This gradual reduction in size suggests the presence of an equilibrium potential for a chemical transmitter, which increases the membrane conductance to certain ions.

Reversal potentials for the ipsp's were obtained from a total of five cells and had a mean value of -72 mV (range -44 to -116 mV). In most cases, however, accurate values for the reversal potentials were not easy to obtain. The small amplitude of the ipsp's made accurate measurements impossible. Also, the ipsp's were quite insensitive to membrane polarization. As the membrane was hyperpolarized by 10 to 15 mV, the ipsp's which appeared during the quiet phase of the bursts became smaller and eventually disappeared. When the membrane was hyperpolarized by an additional 10 to 20 mV, no ipsp's were detectable. Upon further hyperpolarization of the membrane, the ipsp's reappeared, but they were in the depolarizing direction.

There appears to be a correlation between the latency of the ipsp and the distance of the unipolar cell from the point where the inhibitor nerves enter the ganglion ($r = 0.864$; $P < .01$). The latency increases about 9 msec for each segment increase in distance between the inhibitor nerves and

the cell penetrated. For example, if the latency for the ipsp's in a cell in cardiac segment 5 is 30 msec, then the latency for ipsp's in a cell in cardiac segment 6 would be 39 msec.

In five cells the effective membrane resistance was examined during stimulation of the inhibitors at high frequencies (60 Hz). There was found to be a 4% decrease in the effective membrane resistance (R_{eff}) during stimulation. This indicates that the membrane conductance is increased during stimulation.

Excitation

In this study abdominal cardiac nerves 10 through 12, the accelerator nerves, were stimulated pericardially near their junctions with the right pericardial nerve. Stimulation of these nerves individually for one minute periods resulted primarily in an increased bursting frequency (Table 3).

Increases in rate were seen in all cases examined. The greatest increase in rate was 9.9 bursts/min. Before stimulation of the abdominal nerves the mean bursting frequency was 16.6 bursts/min. One minute after onset of stimulation the average bursting rate was 20.8 bursts/min. This represents an increase of 25%. In Figure 3a the frequency of bursting just prior to stimulation was 18.1 bursts/min, and the frequency was 16.9 bursts/min following the onset of

Table 3. Summary of the changes in bursting frequency resulting from stimulation of the abdominal nerves.

Nerve	Number of Animals	Number of Cells	Number of Cells Showing Decreased Rate	Number of Cells Showing Increased Rate
10	2	6	0	6
11	4	13	6	13
12	1	1	0	1

stimulation. After 1 min of stimulation the bursting frequency had increased to 23.8 bursts/min (Figure 3b).

Initial rate decreases ranging from 0.5 to 1.2 bursts/min were seen in 46% of the cases in which nerve 11 was stimulated. The decrease in bursting rate is similar to the initial decrease in frequency of mechanical contraction following onset of stimulation of the abdominal nerves (Pax, 1969; Bursey and Pax, 1970).

Besides changes in frequency, other electrical parameters were examined. No significant changes in the electrical activity were apparent. Table 4 summarizes the results. The Friedman two-way analysis of variance test was used to determine statistically significant differences within each parameter. The two bursts immediately before onset of stimulation, the two bursts immediately after onset of stimulation, the two bursts immediately before cessation of stimulation and the two bursts immediately after cessation were

Figure 3. Effects of stimulation of abdominal cardiac nerve 11, a cardio-acceleratory nerve, upon the electrical activity of a unipolar cell.

A. Two bursts of activity just before and just following onset of stimulation (indicated by arrow). The bursting frequency prior to stimulation is 18.1 bursts/min, and following onset of stimulation, the bursting frequency has decreased to 16.9 bursts/min. Stimulus parameters 8 Hz, 5V, 3 msec duration.

B. Three bursts just prior to and just following cessation of stimulation (indicated by arrow). The bursting frequency has now increased to 23.8 bursts/min, following 1 min of stimulation of nerve 11.

Time scale: 500 msec. Voltage scale: 8 mV.

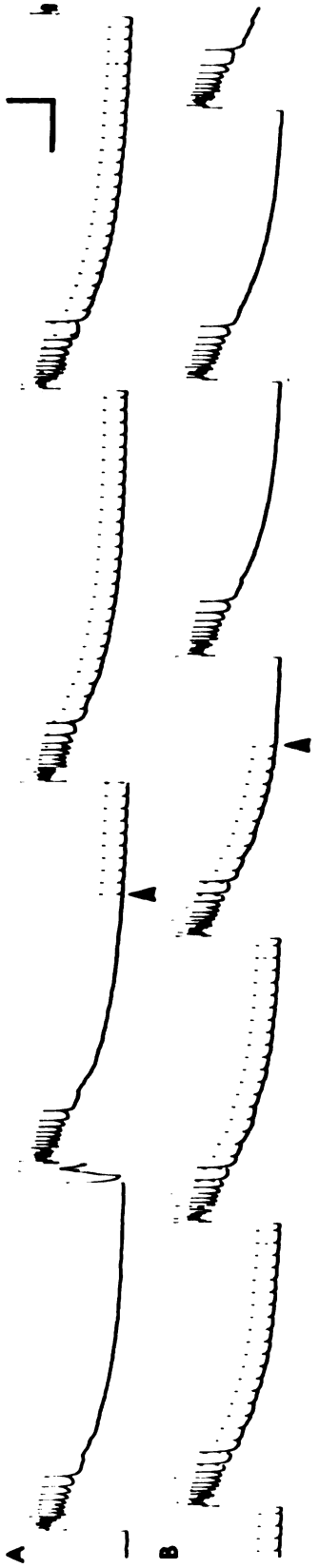

Figure 3

Table 4. Summary of statistical analyses used to examine differences in electrical parameters produced by stimulation of the abdominal nerves.

Electrical Parameter	Number of Cells	χ^2_t	Probability
Amplitude of Initial Phase	20	5.58	.20>P>.10
Amplitude of Recovery Phase	20	3.08	.50>P>.30
Duration of Initial Phase	20	6.13	.20>P>.10
Duration of Recovery Phase	20	29.34	P<.001
Number of Spikes on Initial Phase	20	3.80	.30>P>.20
Number of Spikes on Recovery Phase	20	6.40	.10>P>.05
Bursting Frequency	20	31.30	P<.001

compared. The amplitudes of the initial and recovery phases, the number of spikes on these two phases and the duration of the initial depolarizations do not appear to be affected by stimulation of the cardiac nerves. Any fluctuation in the parameters is random.

The only parameter other than frequency that was found to be markedly affected by the cardiac nerves was the duration of the recovery phase. In 17 of the 20 cells examined the duration of the recovery phase measured after stimulation of the nerves was less than the duration measured prior to stimulation. Before stimulation the average duration was 3.045 sec, and after stimulation the duration decreased to 2.746 sec. The largest decrease seen was 0.730 sec.

This decrease in duration can be explained solely on the basis of the increase in frequency brought about by stimulation of the cardiac nerves. Examination of the electrical activity from 20 spontaneously beating cells demonstrated an inverse correlation between the duration of the recovery phase and the frequency of the bursts ($r = -0.796$; $P<.002$). For every one burst/min decrement in the burst frequency, there is a 0.236 sec increment in the duration of the recovery phase.

Although not statistically significant, the number of spikes on the recovery phase seems to be somewhat affected by stimulation of the abdominal nerves. From the twenty cells examined nine cells showed an increase in the number of spikes. It is difficult to show a correlation between the number of spikes on the recovery phase and the duration of this phase, because movement of the electrode within a cell can cause a change in the spike number. It is possible that in the normal cell the number of spikes is dependent upon the duration of this phase.

Stimulation of the inhibitory nerves results in the appearance of postsynaptic potentials in the cell soma. However, whenever the abdominal nerves were stimulated, no postsynaptic potentials appeared, even in cells that showed inhibition shortly after the stimulation began (Figure 3a).

No changes in R_{eff} were seen upon stimulation of the abdominal nerves.

L-glutamate

In crustaceans and insects there is evidence suggesting that glutamate is a possible neurotransmitter (Takeuchi and Takeuchi, 1964). This drug can mimic the excitatory transmitter of some arthropod neuromuscular junctions, including limulus (Parnas et al., 1968; Robbins, 1959; Usherwood and Machili, 1966; Van Harreveld and Mendelson, 1959).

Pax and Sanborn (1967a) found that l-glutamic acid (10^{-3}M), perfused through the isolated heart, decreased the strength of contraction of limulus heart muscle, but had no apparent effect on the heart rate. L-glutamate (10^{-5}M) had no effect whatsoever. Abbott et al. (1969) reported that 10^{-6}M glutamate caused heart muscle contractions in a deganglionated heart. At 10^{-4}M, glutamate increased the myocardial tone of a spontaneously beating heart, but decreased the strength of contraction. Parnas et al. (1969) showed that, when 10^{-5}M glutamate was perfused through the isolated heart, the electrical discharge of the cardiac ganglion was altered and spike activity appeared throughout the normally quiescent period between beats. The heart rate was reported to increase by only 5%, although their records show a 17% increase in rate.

The increase in rate and the alteration in the discharge pattern of the cardiac ganglion observed by Parnas et al. (1969) suggested that glutamate acts directly on the cells of the cardiac ganglion. To determine the effects of glutamate on

the parameters of the electrical activity recorded from the unipolar cells l-glutamic acid (glutamate) was applied with a pipette to individual unipolar cells and by perfusion over the entire heart-ganglion preparation.

Application of 1 μl amounts of 10^{-3} M glutamate to 5 cells with a pipette had no apparent effects on the electrical activity, but perfusion of the ganglion with concentrations of 5×10^{-5} M and 5×10^{-4} M did affect several of the electrical characteristics of the ten unipolar cells examined from three animals. Figure 4 shows an example from one such experiment.

Glutamate affects both the durations of the initial phase and the recovery phase. For example, in Figure 4a the duration of the initial phase was 71 msec and the duration of the recovery phase was 3.126 sec in normal saline. In glutamate the duration of the initial phase was 58 msec and the duration of the recovery phase was 2.154 sec (Figure 4b). At a concentration of 5×10^{-5} M glutamate, there was a decrease in the duration of the initial phase from 133 msec to 95 msec. After three minutes there was a slight increase in the duration of the initial phase to an average of 109 msec. In the cells examined in 5×10^{-4} M glutamate there was a decrease in duration of about 35%. The decrease was from 152 msec in normal saline to 91 msec after glutamate had been applied. After three minutes there was essentially no change from the value immediately following application.

Figure 4. Effects of perfusion of l-glutamic acid upon the bursts of activity of a unipolar cell.

A. Normal spontaneous electrical activity. 10^{-4}M l-glutamate was added to the bathing solution at the arrow. Transient depolarizations are produced by intracellular current injections of 1.8×10^{-9} A applied through the microelectrode. R_{eff} is 11.7 MΩ, and the bursting frequency is 17.3 bursts/min.

B. Electrical activity following application of l-glutamate. The bursting frequency has increased to 25.9 bursts/min. R_{eff} is 11.7 MΩ. Figures 4A and 4B are continuous.

C. Spontaneous electrical activity of the same cell as in Figure 4A. The preparation has been washed four times. 10^{-4}M l-glutamate was added to the bath at the arrow. A current of 1.0×10^{-9} A was applied to the cell. R_{eff} is 15.8 MΩ.

D. L-glutamate produced an increase in frequency from 15.8 to 39.0 bursts/min. At this time some bursts are irregular in shape. R_{eff} is 16.2 MΩ. Figures 4C and 4D are continuous.

E. Electrical activity 5 sec following the end of Figure 4D. The bursts are regular in shape.

Time scale: 500 msec. Voltage scale: 20 mV.

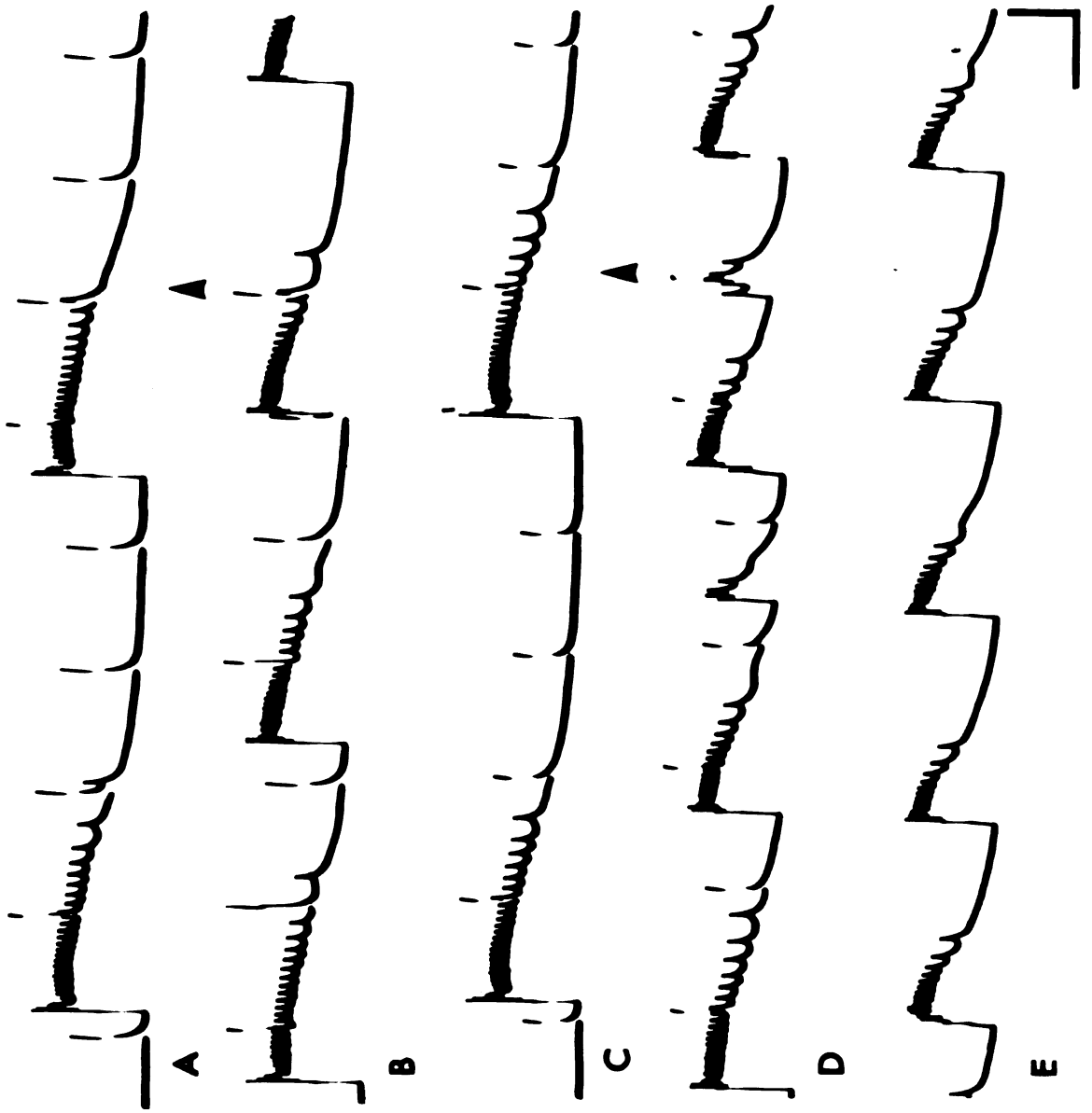

Figure 4

5×10^{-4}M glutamate decreased the duration of the recovery phase from 2.751 sec to 1.352 sec (maximum decrease 2.886 sec). All cells showed an increase in duration from 20 to 67% of their original durations during the three minutes following application. The decrease in duration of the recovery phase was not as pronounced in 5×10^{-5}M glutamate, there being a decrease from 2.793 sec to 1.789 sec (maximum decrease 1.982 sec).

The number of spikes occurring during the recovery phase decreased upon application of glutamate. In 5×10^{-5}M glutamate there was a reduction in the number of spikes from 13 to 10 (maximum decrease 5 spikes), but within three minutes the number of spikes returned to the pretreatment level. In 5×10^{-4}M glutamate the reduction in the number of spikes was more than twice that seen in the lesser concentration. In normal saline the number of spikes on the recovery phase had a mean value of 18. The number of spikes in glutamate averaged 7. After three minutes there was a partial recovery in the number of spikes, there being an increase of two spikes per burst.

Parnas et al. (1969) noted an increase in heart rate by perfusion of 10^{-5}M glutamate. In these studies glutamate (5×10^{-4}M) more than doubled the increase in frequency seen with the lesser concentration. At 5×10^{-4}M glutamate increased the bursting rate by 139% (range 42 to 319%). For example, in Figure 4c the bursting rate of a cell in

normal saline is 15.8 bursts/min. Application of 5 x 10^{-4}M glutamate increased the rate to 39.0 bursts/min (Figures 4D and 4E). After three minutes the frequency decreased to a rate only 40% greater than the pretreatment rate.

Changes in R_{eff} were examined by application of short depolarizing pulses (20 msec, 1 to 3 Hz) to determine whether glutamate changes the somal membrane conductance. In both concentrations of glutamate the R_{eff} of the cells did not differ significantly from the pretreatment level.

It appears that the effects of glutamate are readily reversible. Although there was no quantitative examination performed, it was apparent that the increases in frequency produced by glutamate are abolished after the heart preparation was washed several times with fresh saline.

3,4-Dihydroxyphenylethylamine (Dopamine)

Fourtner and Pax (manuscript in preparation) recently found that dopamine greatly enhances both the frequency and strength of muscle contraction in an isolated limulus heart. Experiments in which dopamine was applied to individual cells and was perfused on the entire ganglion were performed to determine the site of action of dopamine.

The effects of dopamine (10^{-3}M) locally applied to cell bodies by means of a pipette were examined in 10 cells from four different animals. Electrical activity was compared

from the bursts just preceding application of dopamine, from the second burst and from the sixth burst following application of the drug. Figure 5 shows the results of one such experiment. In this case Figure 5A represents spontaneous electrical activity from a cell immersed in normal saline and Figure 5B shows electrical activity after dopamine had been applied.

No marked differences were produced in the amplitude and duration of the initial phase, in the duration of the recovery phase, nor in the R_{eff}. In contrast, the amplitude of the recovery phase, the number of spikes occurring on both phases and the bursting frequency were affected. For example, in Figure 5A the amplitude of the recovery phase was 16 mV, the number of spikes on the initial phase was 10 and the number on the recovery phase was 12, and the bursting frequency was 23.5 bursts/min. After dopamine had been applied, the amplitude of the recovery phase decreased to 14 mV, the number of spikes on the initial phase was 5 and on the recovery phase was 8. The bursting rate was slightly decreased to 23.0 bursts/min (Figure 5B).

Five cells showed a decrease in amplitude of the recovery phase by 5 mV (range 2 to 9 mV). By the sixth burst after application the amplitudes had returned to their pretreatment level.

The number of spikes occurring on both the initial phase and recovery phase were decreased by dopamine. Dopamine

Figure 5. Effects of local application of dopamine upon the electrical activity of a unipolar cell.

A. Spontaneous activity from a cell in normal saline. The number of spikes on the initial phase is 10, and on the recovery phase is 12.

B. Dopamine (10^{-3} M) was applied to the soma with a pipette at the arrow. Dopamine hyperpolarized the membrane by 5 mV. The number of spikes on the initial phase is 5 and on the recovery phase is 8. Figures 5A and 5B are continuous.

Time scale: 500 msec. Voltage scale: 20 mV.

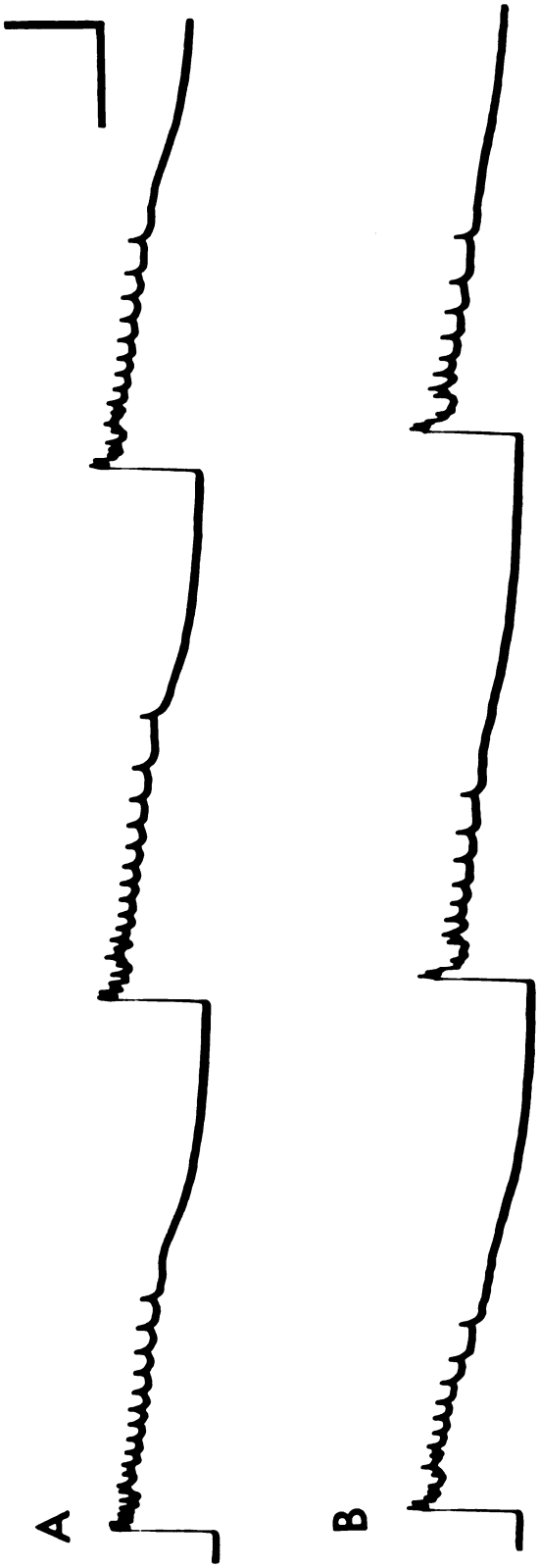

Figure 5

reduced the number of spikes on the initial phase by 5 per burst (range 2 to 9). This represents a decrease of 40%. The number of spikes on the recovery phase showed an even larger change, there being an average reduction of 7 spikes (range 1 to 23 spikes) per burst, representing a decrease of 50%.

The frequency of bursting generally was either unaffected or was slightly depressed by dopamine. By the sixth burst after dopamine was applied the rate had returned to the pretreatment level.

Dopamine produced a mean hyperpolarization of 3 mV (SD = 2.7 mV). The hyperpolarization could be the result of an increase in membrane conductance of the somal membrane, but none of the cells showed a significant change in R_{eff}.

Five cells were examined to determine the presence of an equilibrium potential. Steady hyperpolarizing or depolarizing currents were applied through the microelectrode. The drug was applied to the soma. Regardless of the potential at which the membrane was clamped, dopamine usually resulted in a hyperpolarization of 1 to 8 mV. The mean value of the potentials obtained in this manner was 61.5 mV, and the standard deviation within each of the cells was 28.8 mV. This standard deviation appears too large to indicate that an equilibrium potential is present.

Perfusion of the isolated heart preparation was examined in eight cells from five different animals. Concentrations

of 5 x 10^{-5}M and 5 x 10^{-4}M dopamine were used. The activity from bursts prior to application of the drug, the third and fourth bursts following application and bursts from 2 and 8 minutes following application of dopamine were compared.

Figure 6 shows the results of one such experiment. Figure 6A and 6C show the spontaneous electrical activity of a unipolar cell in normal saline. Figure 6B shows the activity from the first three beats following application of 5 x 10^{-5}M dopamine. Figures 6D and 6E show the activity from the five bursts following application of 5 x 10^{-4}M dopamine. Figure 6F shows five bursts four minutes after application of 5 x 10^{-4}M dopamine. In this instance dopamine hyperpolarized the cell by 5 mV.

Dopamine causes hyperpolarization of the cell membrane. There is a hyperpolarization of 2 mV (range 1 to 5 mV) with 5 x 10^{-5}M dopamine and of 4 mV (range 2 to 9 mV) with 5 x 10^{-4}M dopamine. Dopamine had no effect on R_{eff}.

Dopamine, at the higher concentration, affected more of the parameters of electrical activity than did the lower concentration. At 5 x 10^{-4}M the amplitudes of the initial and recovery phases and the number of spikes on the initial phase and the duration of the initial phase were not markedly affected. All other parameters were significantly changed.

Durations of the recovery phase were altered by dopamine (Figure 6). In normal saline the duration of the recovery phase was 2.446 sec (Figure 6C). Immediately following

Figure 6. Effects of perfusion of dopamine upon the electrical activity of a unipolar cell.

A. Electrical activity from a cell bathed in normal saline. At the arrow 10^{-4}M dopamine was added to the sea water bath, resulting in an effective concentration of 5 x 10^{-5}M. The bursting frequency is 13.8 bursts/min and the R_{eff} is 10.2 MΩ.

B. Dopamine hyperpolarized the cell membrane by 5 mV and increased the bursting frequency to 18.7 bursts/min. The R_{eff} is 9.4 MΩ. Figures 6A and 6B are continuous.

C. Electrical activity from the same cell as in Figure 6A. The increased frequency is due to residual action of dopamine. The bursting frequency is 20.9 bursts/min and the R_{eff} is 10.1 MΩ.

D. 10^{-3}M dopamine was added to the saline at the arrow. The cell membrane is again hyperpolarized by 5 mV. The bursting frequency has decreased to 10 bursts/min and the R_{eff} is 9.6 MΩ. Figures 6C and 6D are continuous.

E. Activity from the same cell 15 sec after the last burst in Figure 6E. The spikes on the recovery phase have been completely blocked in the first burst.

F. Activity from the same cell 4 min after dopamine was added to the bath. The bursting frequency has increased to 28.6 bursts/min, and the R_{eff} is 10.1 MΩ.

Time scale: 500 msec. Voltage scale: 20 mV.

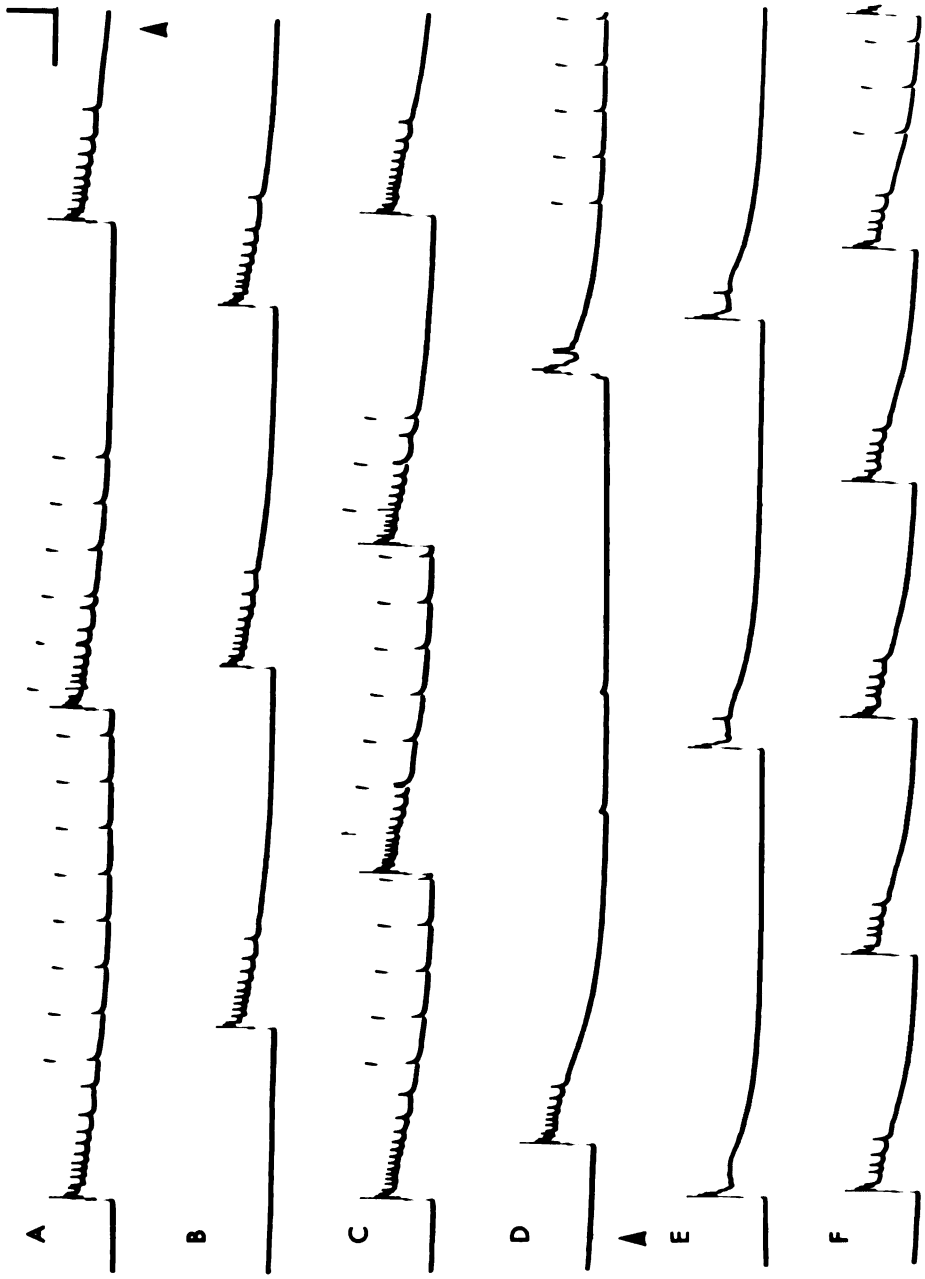

Figure 6

application of dopamine the duration increased to 3.391 sec (Figure 6D). At the end of the two and eight minute periods the duration of the recovery phase of this cell had decreased to 1.993 sec and 1.599 sec respectively. In the cells examined there was a tendency for the duration of the recovery phase to increase slightly from 1.900 sec to 2.120 sec. Within two minutes a 15% decrease in the duration from 2.120 sec to 1.723 sec occurred. At eight minutes the duration decreased by an additional 17% to 1.412 sec, so that the duration of the recovery phase at the end of the eight minutes was 26% less than the pretreatment duration.

Dopamine decreases the number of spikes on the recovery phase. In Figure 6C the number of spikes on the recovery phase is 10. Immediately following application of the drug, the number has decreased to 1 (Figure 6E). The mean number of spikes on the recovery phase of cells in normal saline was 11 (range 6 to 24 spikes). Immediately following drug application the number of spikes was reduced to a mean value of 3 (range 0 to 13 spikes). At the end of the two minute period the spikes had partially recovered to a value slightly higher than that of the bursts following application. At this time the spike number averaged 5 (range 2 to 11 spikes). After six additional minutes the number of spikes did not change markedly.

The frequency of the bursting activity was affected differently by 5×10^{-5} M dopamine than by 5×10^{-4} M dopamine.

In the lower concentration the rate increased by 16% (range 2 to 34%) immediately following application of dopamine. For example, the frequency of bursting was 13.9 bursts/min from a cell bathed in normal saline (Figure 6A). After application the frequency has increased to 18.8 bursts/min (Figure 6B). Within two minutes the rate has increased to a rate 25% greater than the pretreatment level (range 1 to 52%). The frequency returns to the original rate within six minutes.

In the higher concentration there was an initial decrease in rate followed by a return to the pretreatment frequency within three minutes of application of the drug. Cells perfused in the lower concentration did not show the initial decrease in rate.

Figure 6 shows the effect of 5×10^{-4} M dopamine on the bursting frequency. In Figure 6C the frequency of bursting in a cell in normal saline is 20.9 bursts/min. Subsequent to application of dopamine, the frequency has decreased to 10.0 bursts/min (Figures 6D and 6E). An increase to 28.6 bursts/min was seen within four minutes (Figure 6F). In the cells examined the rate decreased by 26% (range 5 to 55%) following application of dopamine. Within three minutes the bursting rate returned to normal. After an additional five minutes the frequency increased by 34% (range 10 to 97%).

5-Hydroxytryptamine (5-HT)

Pax and Sanborn (1967b) and Burgen and Kuffler (1957) reported that 5-HT reduced both the heart rate and the strength of beat in isolated limulus hearts. When 5-HT was applied to an isolated cardiac ganglion, Pax and Sanborn (1967b) found that 5-HT decreased the frequency of rhythmic discharge of the ganglion, reduced the number of neurons discharging in each burst, and decreased the duration of each burst. All of these effects were readily reversible. In contrast, Abbott et al. (1969) found that 5-HT had no effect on the electrical and mechanical properties of the peripheral neuromuscular system of the heart.

In an attempt to clarify this discrepancy 10^{-3}M 5-HT was applied to individual unipolar cell bodies and 5×10^{-5}M 5-HT was used to perfuse the entire limulus heart to determine the effects of 5-HT on the electrical activity of the unipolar cells.

Effects of local application were examined in twelve cells from five different animals. In all the cells the activity just prior to application and the second and third bursts following application were compared. Figure 7 shows an example from one such experiment. Unaffected by 5-HT were the amplitudes and duration of the initial phase, the number of spikes occurring on the initial phase and the duration of the recovery phase. The effective membrane resistance changed within the limits of the control levels.

Figure 7. Effects of local application of 5-HT upon the electrical activity of a unipolar cell.

A. The electrical activity from a cell bathed in normal saline. The bursting rate is 10.8 bursts/min and the R_{eff} is 23.8 MΩ.

B. 10^{-3}M 5-HT was applied to the cell soma at the arrow. 5-HT blocked the spikes on the recovery phase and decreased the bursting rate to 10.0 bursts/min. In this case the R_{eff} was reduced to 19.1 MΩ. However, this decrease in R_{eff} is uncommon. Figures 7A and 7B are continuous.

Time scale: 500 msec. Voltage scale: 20 mV.

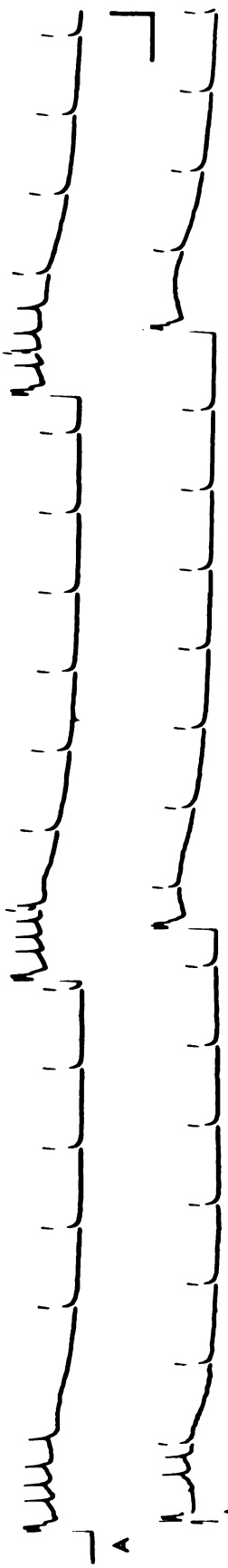

Figure 7

Each of the cells showed a decrease in amplitude of the recovery phase by an average of 3 mV. In Figure 7A the amplitude of the recovery phase is 16 mV. 5-HT reduced the amplitude by 2 mV (Figure 7B). The number of spikes occurring on this phase decreased by 5 (range 1 to 10 spikes).

5-HT tended to decrease the bursting rate slightly. The bursting rate decreased by 4% shortly after application.

The electrical activity of nine cells from five different animals was examined as the heart was immersed in 5×10^{-5} M 5-HT. This concentration was the minimal concentration which affected the electrical activity of the unipolar cells. Figure 8 demonstrates the results from one such experiment. In Figures 8A and 8C the electrical activity from a cell immersed in normal saline is shown. Figure 8B shows the activity three minutes following application of 5-HT.

Four of the nine cells examined were unaffected with respect to the amplitudes of the initial and recovery phases, the duration of the initial phases, and R_{eff}. The number of spikes appearing on the initial phase of the bursts from these cells showed an average decrease of 3 following application of the drug. Three minutes after drug application the number of spikes slightly exceeded the pretreatment levels.

5-HT completely blocked the appearance of spikes on the recovery phase. In Figure 8C, just prior to 5-HT application, the number of spikes on the recovery phase is 5.

Figure 8. Effects of perfusion of 5-HT upon the electrical activity of a unipolar cell.

A. The first three bursts of activity are from a cell in normal saline. At the arrow 10^{-4} M 5-HT was added to the bath, resulting in an effective concentration of 5 x 10^{-5} M. The membrane was hyperpolarized by 9 mV. The bursting activity is completely blocked. R_{eff} in normal saline is 16.4 MΩ, and after 5-HT R_{eff} measured 15.6 MΩ.

B. Same cell as in Figure 8A. Bursting activity taken 3 min after application of 5-HT. The bursting frequency has decreased by 39%. R_{eff} appears to have increased, but the change in amplitude produced by the current is the result of the bridge circuit in the preamplifier becoming unbalanced.

C. Electrical activity from another cell in normal saline.

D. Activity recorded after addition of 5-HT. There was a decrease in bursting frequency and a complete elimination of the spikes on the recovery phase.

Time scale: 1250 msec, Figure 8A; 500 msec Figures 8B, 8C, and 8D. Voltage scale: 20 mV.

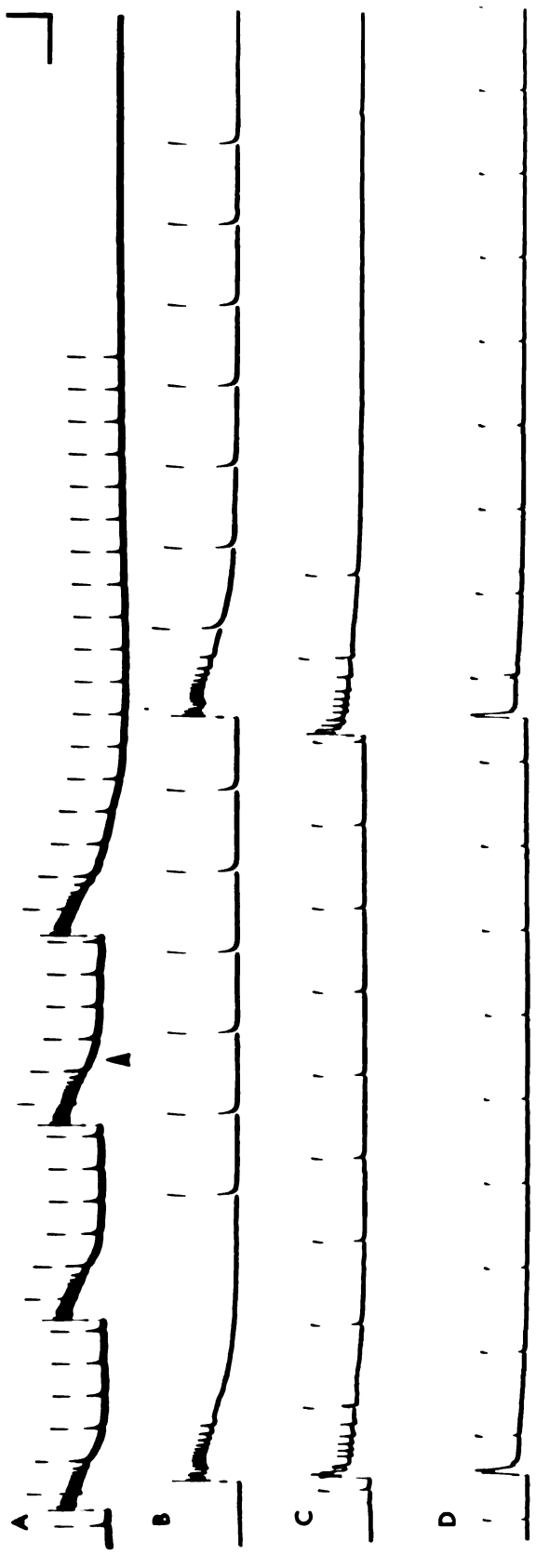

Figure 8

Immediately following application of 5-HT, the number of spikes is 0 (Figure 8D). In normal saline the average number of spikes on the recovery phase was 11. Following application the average was 0, and within three minutes 7 spikes reappeared on the recovery phase.

In the same four cells the durations of the recovery phase shows a decrease. From bursts preceding application of the drug, the durations had a mean value of 4.305 sec. After 5-HT was applied, the duration decreased to 3.156 sec, a decrease of 29%. Within three minutes the durations increased to 6.059 sec.

The bursting rates of the cells mentioned above showed an initial decrease of 9%. Within three minutes the bursting rate decreased to 54% of the control frequency.

The electrical parameters of the remaining five cells were markedly affected by 5-HT. In these instances 5-HT prevented the appearance of any changes in potential to be recorded from the soma (Figure 8A). No bursting activity of any sort is present. This blockade of bursting activity lasted from two to five minutes, and at the end of this time activity resembling the pre-treatment activity appeared (Figure 8B). The new activity differed somewhat from the controls.

The number of spikes on the initial phase either returned to the pretreatment value or was decreased by 6 to 8 spikes.

In these cells there was a tendency towards a decrease in the duration of the initial phase. Prior to treatment the durations had a mean value of 196 msec, and after the activity reappeared the value was 120 msec.

The rate for the recovered bursts was 48% below the control frequency. In Figure 8B the bursting rate was 50% of the control rate within 90 sec after activity had reappeared in the unipolar cell.

In five cells 5-HT produced an average hyperpolarization of 5 mV. There was no change in membrane potential produced in the remaining four cells. This hyperpolarizing response did not appear correlated with the type of change in bursting activity produced by 5-HT. As with dopamine, a reversal potential for 5-HT could not be determined.

There appears to be a desensitization of the cells in the ganglion to 5-HT. While monitoring the electrical activity from three cells, the 5-HT perfusion solution was replaced with fresh drug solution. The new solution did not affect the activity of the unipolar cell, despite the fact that the first solution blocked the activity completely.

Gamma-aminobutyric Acid (GABA)

Pax and Sanborn (1967a) perfused GABA through isolated limulus hearts and observed that GABA decreased both the rate and strength of contraction of the spontaneously beating

heart, thereby mimicking the cardioinhibitory nerves. But GABA failed to have any effect on the number of units discharging and the total duration of each burst of electrical activity in the cardiac ganglion. Abbott et al. (1969) and Parnas et al. (1969) reported that GABA did mimic stimulation of the cardioinhibitory nerves in that GABA decreased the frequency of the bursts of the ganglionic discharge, decreased the length of the discharge and decreased the number of units firing in a discharge, as well as depressing the amplitude of contraction and the heart rate.

In an effort to clarify this discrepancy GABA was applied to individual unipolar cells and the entire heart preparation to determine its effects on the electrical activity recorded from the unipolar cells.

GABA (10^{-3} M) was applied to 13 unipolar cells from five different animals with a pipette. GABA significantly altered all parameters of the electrical activity from the unipolar cells. Figure 9 shows the results of one such experiment.

An obvious change occurs in the effective membrane resistance. For example, for the cell in Figure 9C the R_{eff} measured in normal saline was 21.1 MΩ. Following local application of GABA the membrane resistance decreased to 13.0 MΩ (Figure 9D). Prior to application of this drug R_{eff} of the 13 cells averaged 23.0 MΩ. GABA caused a decrease in resistance to 18.5 MΩ. This represents an average decrease of 27.8%. Within one minute after application R_{eff} returned to its control value.

Figure 9. Effects of locally applied GABA upon the electrical activity of a unipolar cell.

A and B. The first burst is from a cell in normal saline. At the arrow 10^{-3}M GABA was applied to the soma with a pipette. GABA depolarized the cell membrane by 16 mV, and completely eliminated the slow depolarization upon which the spikes were superimposed. The membrane recovers quite rapidly.

C. Two bursts of activity from a second cell in normal saline. R_{eff} is 26.1 MΩ.

D. GABA (10^{-3}M) was applied to the soma at the arrow. In this case GABA depolarized the membrane by about 2 mV, and decreased R_{eff} to 21.3 MΩ. The sharp peaks indicative of spike activity have disappeared. Figures 9C and 9D are continuous.

E. Two bursts of activity from a third cell in normal saline. R_{eff} is 21.8 MΩ.

F. 10^{-3}M GABA was applied at the arrow. GABA hyperpolarized the cell membrane by 1 mV. R_{eff} is 20.9 MΩ.

G. The number of spikes on the initial and recovery phases are greatly reduced, but spikes are still evident on the initial phase. The amplitude of the recovery phase has decreased by 36%. Figures 9E, 9F and 9G are continuous.

Time scale: 500 msec. Voltage scale: 20 mV.

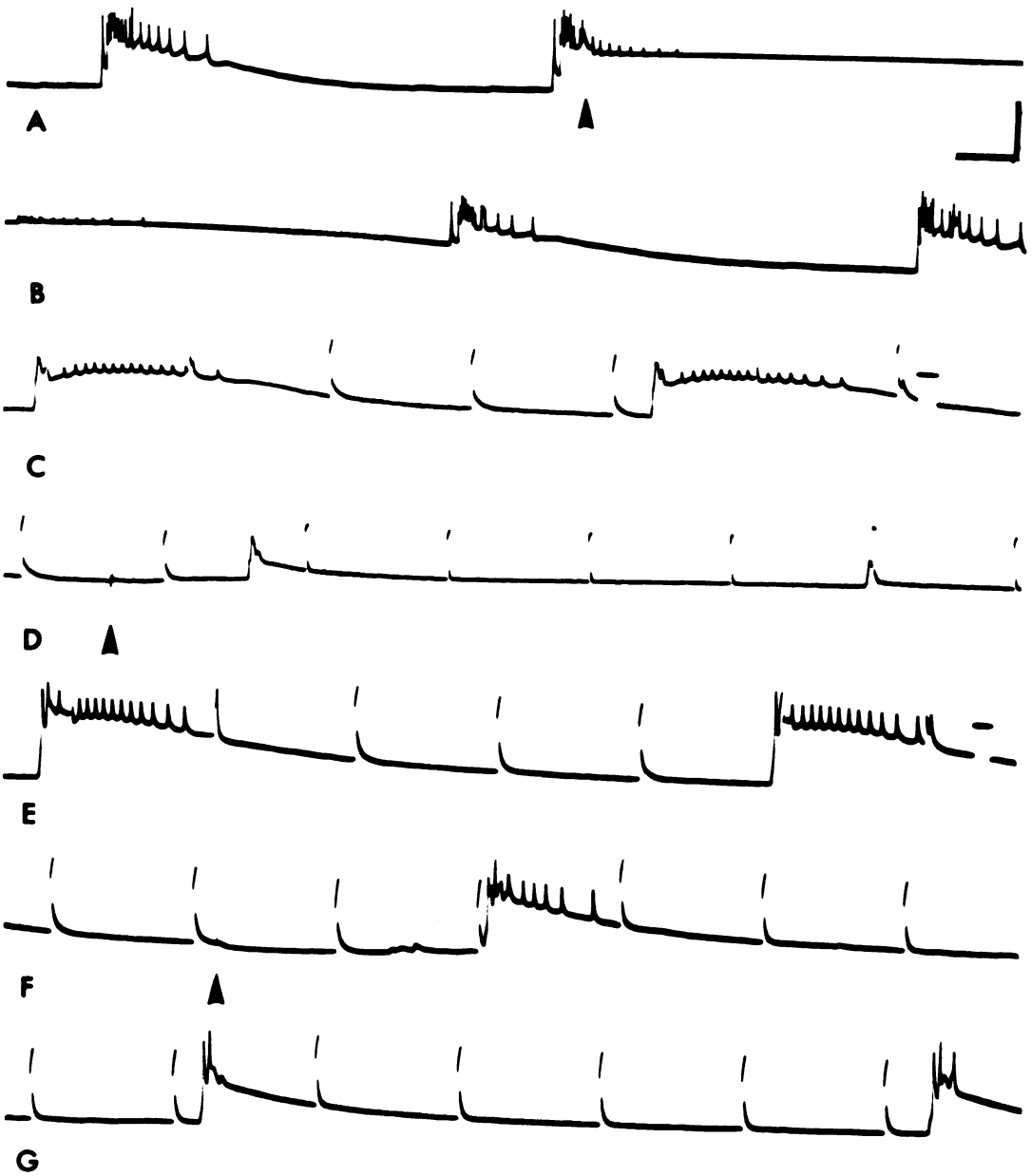

Figure 9

Upon application of GABA it was noted that the membrane potential shifted in either a depolarizing or hyperpolarizing direction, depending upon the cell being treated. Since there were large changes in R_{eff}, and because there was a shift in the membrane potential, the possibility of the presence of a reversal potential for GABA was investigated. Various current strengths were applied through the microelectrode to change the membrane potential to particular values. The drug was applied to the soma and the value to which GABA shifted the membrane potential was measured from the oscilloscope. This method was performed a minimum of six times for each of the ten cells examined. Table 5 summarizes the results from these experiments.

Table 5. A summary of the values obtained for the reversal potentials for GABA and the standard deviation for each cell.

Resting Membrane Potential (mV)	Reversal Potential (mV)	S.D.
55	65	2.3
43	41	1.8
43	47	2.1
62	55	4.8
69	62	6.0
37	53	3.9
45	49	3.9
45	39	1.5
52	41	6.6
33	52	1.3
$\bar{X}$ = 48	$\bar{X}$ = 50	$\bar{X}$ = 3.4

In contrast to the other drugs examined, a reversal potential for GABA can be determined. This suggests that GABA effectively increases the conductance of the somal membrane of the unipolar cells to a particular ion or ions. A chi-square test was used to determine that the values for the reversal potentials for GABA do not significantly differ from one another (.20>P>.10).

GABA causes significant decreases in the amplitudes and durations of both the initial and recovery phases, and the number of spikes. There was no apparent effect on the bursting rates.

The decrease in the amplitudes of the initial and recovery phases is brought about in one of three ways. There may be a total loss of the slow depolarization in the initial phase and the recovery phase. A number of spikes arising directly from the baseline are seen (Figures 9A and 9B). In these instances spikes appear to be in two groups similar to those occurring on the initial phase and recovery phase of a normal cell. The first group of spikes tends to be slightly larger in amplitude and higher in frequency than those appearing in the second group. The amplitudes of the spikes in either group was never more than 4 mV. An interval of 50 to 150 msec separates the two groups. Three cells exhibited this response.

A different response was seen in a second group of three cells. In this group there was a complete elimination of all

spike activity with a concomitant decrease in the amplitude of the slow depolarization upon which the spikes were superimposed. Figure 9 demonstrates an example of this type of response. Figure 9C represents the electrical activity from a unipolar cell in normal saline. Figure 9D represents the electrical activity following application of GABA. The amplitude of the initial phase has decreased from 17 mV in normal saline to 4 mV following application. The number of spikes is 17 in normal saline and 0 after GABA.

In the three cells examined the amplitudes of the initial phase decreased from 34 to 14 mV, and the amplitude of the recovery phase decreased from 19 to 3 mV. The number of spikes on the initial phase in normal saline averaged 4, but none occurred immediately following pipetting of the GABA onto the cell body. Again, there was a partial recovery, with two spikes reappearing on the initial phase within several bursts after drug application. In contrast the spikes on the recovery phase showed no recovery until at least five minutes after application.

The durations of both the initial and recovery phases were greatly decreased. The initial phase decreased by an average of 24% (from 122 msec to 93 msec). The recovery phase decreased by 69% (from 3.718 to 1.241 sec). In both phases there was approximately a 50% recovery to the control values within several beats after drug application.

The third response was seen in the remaining seven cells. This response was characterized by a simple reduction

of all parameters. An example of this response is seen in Figures 9E, 9F and 9G. Figure 9E shows the spontaneous activity from a cell in normal sea water. Figures 9F and 9G show the activity from the same cell after GABA was applied. Note the decrease in size and duration of the depolarizing bursts of the cell.

GABA decreased the amplitude of the initial phase by 63%. Prior to drug application the amplitudes of the initial phase had a mean value of 27 mV. The amplitude measured immediately following application was 10 mV. A slightly higher reduction was seen in the amplitude of the recovery phase. GABA reduced the amplitude of this phase by 75%, from an average of 16 mV before application to 4 mV following application.

The spikes occurring on the initial phase were reduced by 50%. There was a reduction from a mean of 6 spikes to 3 spikes. The number of spikes on the recovery phase was more drastically reduced. GABA completely blocked the spikes on this phase in five cells. In the remaining two cells the spike number was reduced by 65%.

Finally, GABA reduced the durations of the two phases. The duration of the initial phase was reduced by about 27%, from a mean value of 155 msec to 113 msec. An average decrease of 35% was seen in the duration of the recovery phase. There was a reduction in duration from 3.411 to 2.207 sec.

All effects of GABA were completely reversible. Within five to six beats following application of GABA the values for each of the parameters were approximately half way between the control values obtained in normal saline and the values obtained after GABA was applied. After flushing the ganglion with fresh saline, the values for the parameters appeared to approximate the control values.

Six cells from four different animals were perfused with 5×10^{-5} M GABA. The primary effect of the drug was seen in all cells. The electrical activity recorded from the cells was completely blocked by GABA for periods ranging from two to ten minutes after application. Figure 10 shows the results from one such experiment. In Figure 10A is shown the spontaneous activity from a cell immersed in normal saline. Figure 10B shows the record from a unipolar cell immediately following application of GABA. No depolarizing bursts or slow depolarizations are recorded from the unipolar cell. The activity recorded 10 min after application is shown in Figure 10C.

Approximately one minute after the first bursting activity reappeared, records were made of these bursts. This activity was compared with the activity preceding drug application. The amplitude of the initial phase was the same both before application and one minute after recovery. No significant changes were observed in the amplitudes of the recovery phases.

Figure 10. Effects of perfusion of GABA upon the electrical activity of a unipolar cell.

A. Three bursts from a cell in normal saline. At the arrow 10^{-4} M GABA was added to the saline with the resulting effective concentration of 5 x 10^{-5} M.

B. The bursting activity is completely blocked.

C. Electrical activity recorded 10 min after GABA was applied. The bursting rate is only 42% of the control value. No spikes appear on the recovery phase.

Time scale: 500 msec. Voltage scale: 20 mV.

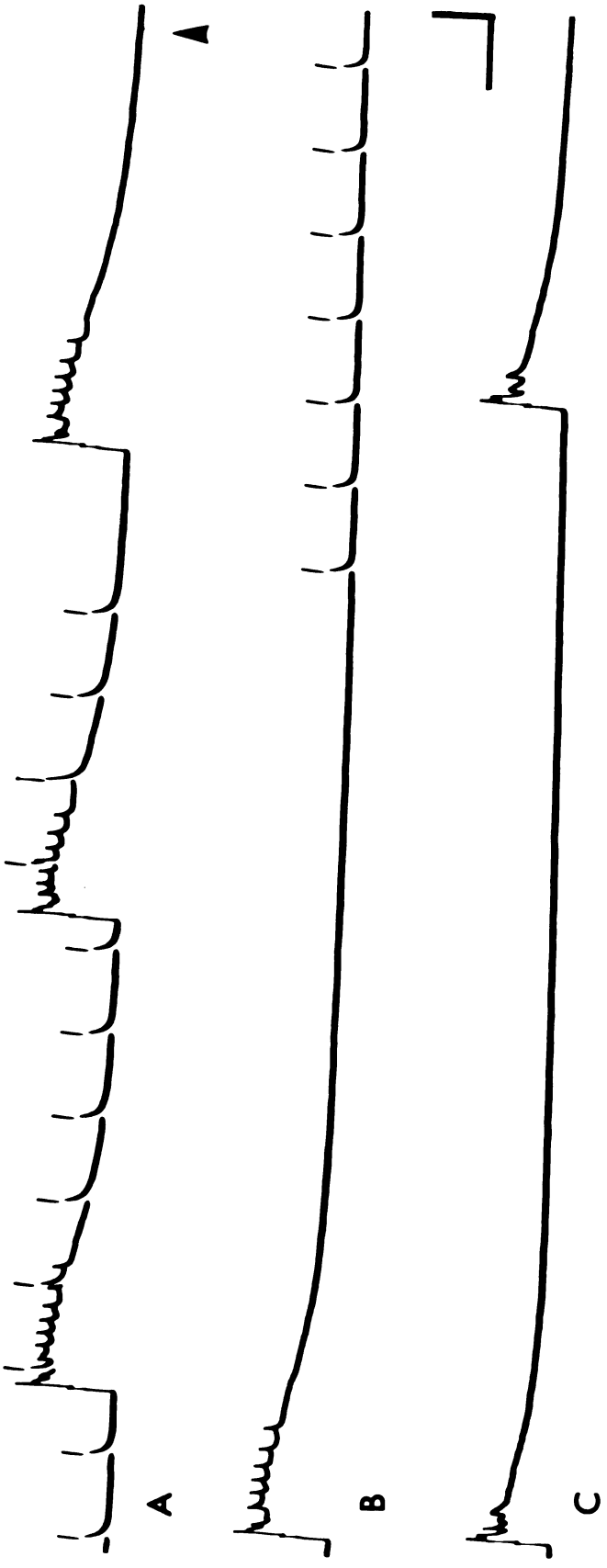

Figure 10

No significant changes were present in the number of spikes on the initial phase. The average number of spikes on the initial phase before treatment was 6, and after treatment 7.

The number of spikes on the recovery phase showed a marked reduction in the activity following the reappearance of the bursts. Prior to application the mean number of spikes was 8. After the activity returned to the cell, the mean number of spikes was only 3.

The durations of the two phases before and after GABA were compared. The duration of the initial phase showed a slight increase from 123 msec to 134 msec, but the increase did not appear significant. The durations of the recovery phase after GABA failed to show any marked changes from those measured before GABA.

Decreases in the bursting rates were caused by GABA. The bursting rate after bursting had reappeared was 30% less than the control rate.

As mentioned above, replacement of 5-HT solution with fresh drug-containing solution failed to affect the activity of the unipolar cells. After activity reappeared in the unipolar cells while the ganglion was still immersed in GABA, the bathing solution was agitated slightly. In all cases the electrical activity was again completely blocked.

Picrotoxin

In crustaceans picrotoxin has been shown to block several types of inhibitory synapses (Elliott and Florey, 1956; Robbins and Van der Kloot, 1958). In mammals Eccles et al. (1963) have shown that picrotoxin blocks presynaptic inhibition in the spinal cord.

Pax and Sanborn (1967a) found that picrotoxin effectively blocks the decrease in rate produced by the cardioinhibitory nerves in the limulus heart. Since picrotoxin blocks the inhibition produced by segmental nerves 7 and 8, picrotoxin was perfused over the heart to determine whether the drug blocks the ipsp's seen in the unipolar cells.

In these experiments a total of seven cells from seven different animals was examined. In each experiment the inhibitory nerves were stimulated for 15 sec at a frequency of 3 Hz and 30 Hz prior to application of picrotoxin. Picrotoxin (5×10^{-4} M) was applied to the heart preparation and a five minute period allowed to elapse before the inhibitory nerves were again stimulated.

Picrotoxin alone causes an increase in the frequency of bursting activity (Pax and Sanborn, 1967a). Prior to application the mean rate for the bursting activity was 19.8 bursts/min, and after perfusion the rate increased to 25.0 bursts/min, an increase of 26%. None of the other characteristics of the electrical activity of the unipolar cells was

significantly altered. There was no change seen in R_{eff} or in the membrane potential.

Figure 11 is an example of one such experiment. Figure 11A shows the activity from a spontaneously beating heart and Figures 11B and 11C show the effects of stimulating the cardioinhibitory fibers at 2 and 20 Hz, respectively. At 2 Hz ipsp's of 3 mV amplitude are seen. Stimulation at 20 Hz decreased the bursting rate to 0 bursts/min, the inhibitory nerves completely blocked the activity in this cell. Figure 11D represents the spontaneous activity five minutes after picrotoxin was applied. In this case the bursting frequency has increased from 19.0 to 26.5 bursts/min as a result of the picrotoxin. In Figure 11E no ipsp's can be seen during stimulation of the inhibitory fibers at 2 Hz. At a frequency of 20 Hz (Figure 11F) the inhibitory fibers are not as effective as they were prior to picrotoxin application. In this case the heart rate is reduced to 56% of the prestimulus rate.

In each of the seven hearts examined picrotoxin blocked the appearance of ipsp's in the somata of the unipolar cells. The mean amplitude of the largest ipsp from each cell before picrotoxin perfusion was 3.5 mV, while five minutes after picrotoxin was applied no measurable ipsp's were recorded from the unipolar cells.

The question arises whether the stimulus to the inhibitory fibers is effective throughout the period of stimulation

Figure 11. Effects of perfusion of picrotoxin upon the inhibition produced by segmental nerves 7 and 8.

A. Electrical activity from a cell bathed in normal saline.

B. The inhibitory nerves were stimulated at 2 Hz, 5V, 3 msec duration. The maximum amplitude of the ipsp was 3 mV.

C. Electrical activity is completely blocked when nerves 7 and 8 were stimulated at 20 Hz.

D. Electrical activity from the same cell 5 min after picrotoxin was applied. Note the increase in bursting rate. The effective concentration of the picrotoxin was 5×10^{-4} M.

E. Stimulation of the inhibitor nerve at 2 Hz failed to produce ipsp's in the unipolar cell.

F. Stimulation of nerves 7 and 8 at 20 Hz failed to block the electrical activity.

Time scale: 500 msec. Voltage scale: 20 mV.

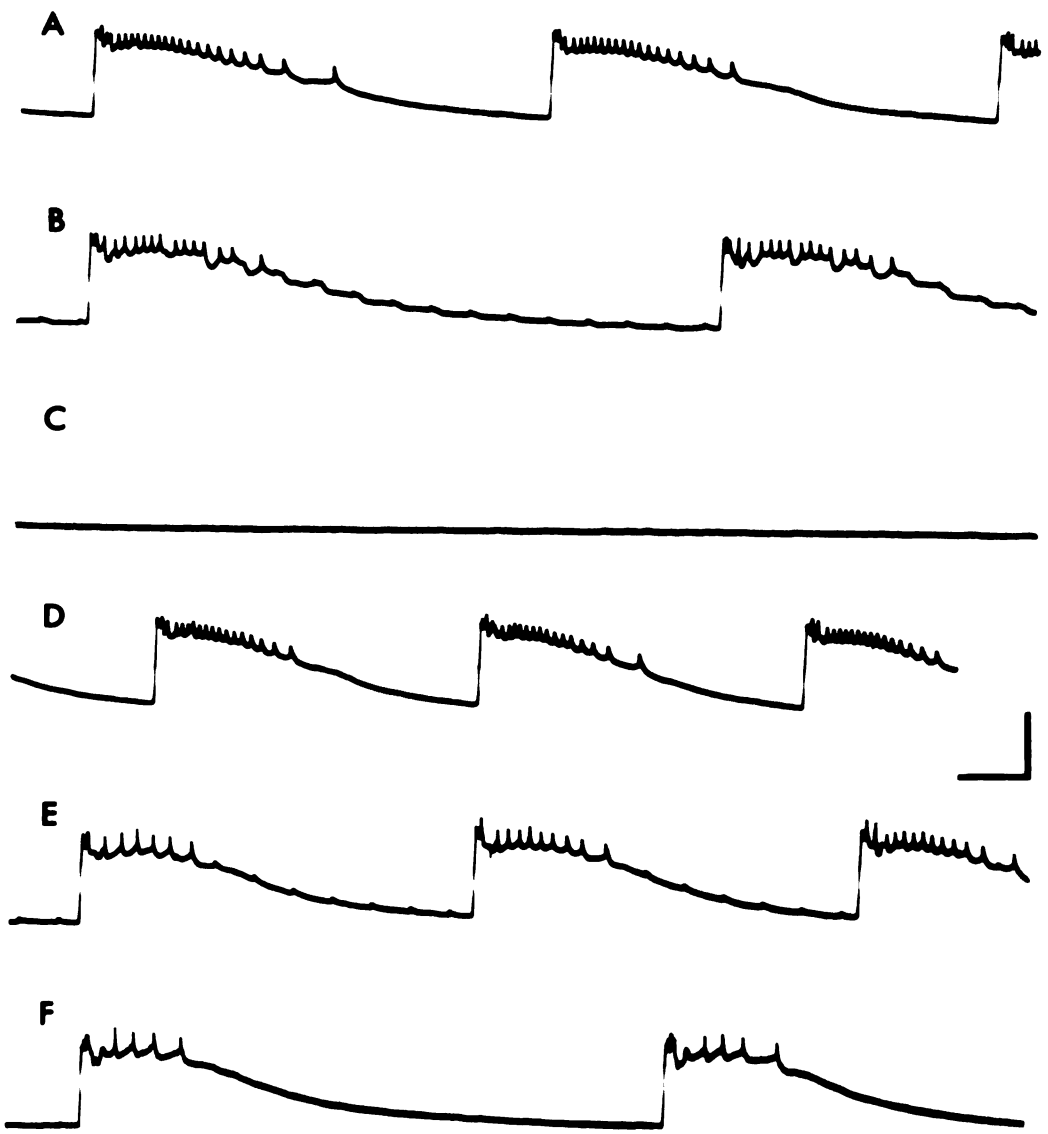

Figure 11

while the preparation is being perfused in picrotoxin. Should this be the case, then no ipsp's would appear in the cell soma. This problem is countered by the fact that stimulation of the inhibitory fibers could still produce a slowing of the bursting frequency, although the reduction in frequency was not as great in the presence of the drug as it was before treatment. This suggests that the inhibitory nerves are still responding to the electrical stimuli.

The relative bursting rates were computed by comparing the ratio of the bursting rate during stimulation to that obtained immediately before stimulation (Pax and Sanborn, 1967a). The mean relative rate for the stimulation frequency of 20 Hz was 0.15 (an 85% decrease in frequency) before application of the drug, while five minutes following application of picrotoxin, the relative rate increased to 0.62 (a decrease of only 38%).

Pax and Sanborn (1967b) reported that picrotoxin antagonized the decrease in heart rate produced by application of 5-HT. Three experiments were performed to determine whether picrotoxin blocked the action of 5-HT on the electrical activity of the unipolar cells. In these three instances picrotoxin (5×10^{-4} M) failed to antagonize 5-HT (5×10^{-5} M). These results with 5-HT are possibly due to the fact that fairly large concentrations of 5-HT were used in these experiments compared with the concentrations used by Pax and Sanborn. In my experiments these large

concentrations of 5-HT were required to produce changes in the parameters of the electrical activity from the unipolar cells.

Strychnine

Strychnine is known to cause blockade of spinal inhibition in mammals (Bradley et al., 1953; Curtis, 1959; Esplin and Zablocka, 1965). This drug interferes only with postsynaptic inhibition, which is produced by the action of a specific chemical transmitter substance on the postsynaptic neuronal membrane (Curtis, 1962; Kuno, 1957). Presynaptic inhibition is not reduced by strychnine.

Since strychnine blocks inhibition, this drug was applied to the isolated heart preparation of limulus to determine whether it also blocks the inhibition produced by segmental nerves 7 and 8. Strychnine (10^{-4}M) was perfused on four different hearts for a minimum of 15 minutes. The cardioinhibitory nerves were stimulated at frequencies of 4 and 40 Hz, and the effects of stimulation in the drug solution compared with the effects of stimulation prior to application of the drug.

Strychnine itself decreased the number of spikes on the initial phase, increased the duration of the initial phase and decreased the frequency of the bursting activity. None of the other electrical parameters were affected. There was a mean of 7 spikes on the initial phase in cells in normal

saline and 5 spikes following application of the drug. The duration of the initial phase increased from 187 msec in normal saline to 294 msec in strychnine. Finally, before strychnine was applied, the bursting frequency averaged 17.2 bursts/min, whereas the frequency decreased to 15.6 bursts/min 15 minutes after the drug was applied.

The effects of strychnine are best determined by examining the maximum amplitudes of the ipsp's appearing in the unipolar cells during stimulation of the inhibitors and the changes in relative bursting rate. Figure 12 shows the results from one experiment. In Figure 12A the electrical activity from a spontaneously beating heart in normal saline is shown. Stimulation of the inhibitors at 4 Hz results in the appearance of ipsp's of a maximum amplitude of 2 mV and a relative bursting rate of 0.78 (Figure 12B). At a stimulus frequency of 40 Hz the relative bursting rate has decreased to 0.47. In Figure 12C the burst of activity shown appeared 7.12 sec after onset of stimulation.

Figure 12D shows two bursts of activity 15 minutes after strychnine had been applied. In this case the bursting frequency was 15.4 bursts/min. In Figure 12E it is evident that stimulation of the inhibitor nerves still produces ipsp's of 2mV amplitude. At this frequency (4 Hz) the relative bursting rate is 0.74. When the frequency of stimulation was increased to 40 Hz, the relative bursting rate decreased to 0.52 (Figure 12F).

Figure 12. Effects of perfusion of strychnine upon the inhibition produced by segmental nerves 7 and 8.

A. Electrical activity from a cell bathed in normal saline.

B. The inhibitory nerves were stimulated at 4 Hz, 6V, 3 msec duration.

C. The burst frequency has been reduced to 6.2 bursts/min, a decrease of 53%. During the time between Figures 12C and 12D, the electrode came out of the cell, but it was re-inserted into the same cell.

D. Electrical activity from the same cell 15 min after 10^{-4}M strychnine was applied. This activity was atypical, since strychnine increased the bursting rate.

E. Stimulation of the inhibitory nerves at 4 Hz still produces ipsp's of 2 mV amplitude.

F. Stimulation of nerves 7 and 8 is just as effective as stimulation before addition of strychnine. The bursting rate was reduced by 48%.

Time scale: 500 msec. Voltage scale: 20 mV.

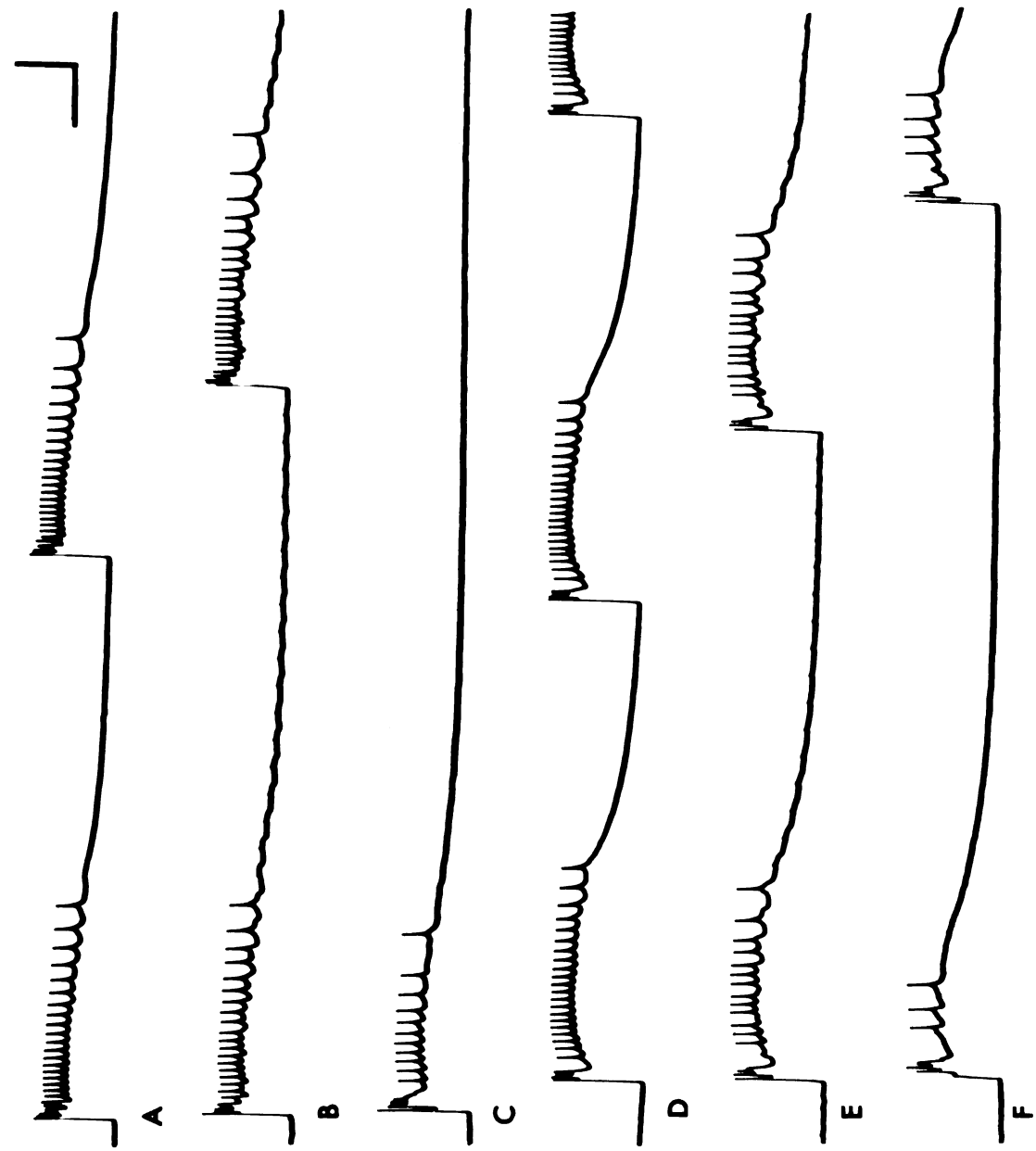

Figure 12

In the five cells examined in normal saline the mean maximum amplitude of the ipsp's recorded was 3.4 mV, while the amplitude was 3.9 mV for cells bathed in strychnine.

At a frequency of 4 Hz the relative bursting rates do differ slightly. In normal saline the mean relative bursting rate was 0.82, while in strychnine the mean relative rate was 0.88. This difference suggests that strychnine may possibly be blocking inhibition to a slight extent. The mean relative bursting rate was 0.58 in normal saline at a stimulus frequency of 40 Hz, and in strychnine the relative rate was 0.61, but the differences do not appear significant ($0.409 \geq P \geq 0.350$).

When the strychnine solution was replaced with fresh saline, the shape of the recovery phase changed from a simple repolarization to a shape which reached peak amplitude, followed by an extended plateau. During this plateau there was an increase in the number of spikes and in the spike frequency. The plateau was terminated by a sudden return of the membrane potential to its resting level.

This finding is similar to observations made by Washizu *et al.* (1961) who reported that strychnine produced a prolongation of the spike potential by a plateau in the soma of the crayfish stretch receptor neuron. The increase in the number of spikes in the unipolar cells of limulus could be the result of the plateau depolarization spreading to the spike-generating region and causing repetitive firing, as in the stretch receptor.

DISCUSSION

Mechanism of Cardioregulation

Acceleration

Stimulation of the accelerator nerves in both decapod crustaceans and limulus produces similar effects. Frequencies as low as 1 Hz produce noticeable increases in rate (Hagiwara, 1961; Pax, 1969). The change in heartbeat frequency apparently has an upper limit (9 beats/min in limulus). Pax (1969) noted that in limulus stimulation of the accelerators at frequencies in excess of 5 Hz produced no greater increase in rate than does stimulation at 5 Hz. Maynard (1953) obtained similar results in the lobster. The chronotropic and inotropic effects produced by the accelerator nerves always outlast the period of stimulation. These aftereffects are usually more pronounced in limulus than in the decapod crustaceans.

Wiersma and Novitski (1942) first hypothesized the mechanism of regulation of the crayfish heart. The frequency of the heartbeat was determined by the frequency of volleys (series of impulses) in the cardiac ganglion. During acceleration, the number of impulses in each volley, as well as the frequency of volleys, increased, thus accounting for the

increases in amplitude and heart rate seen during stimulation of the accelerator nerves.

When the electrical activity of cardiac ganglion cells of crustacean hearts was monitored with microelectrodes, there was an increase in the frequency of bursts and a decrease in the number of spikes of simple follower cells. There were no recognizable postsynaptic potentials (Otani and Bullock, 1959; Terzuolo and Bullock, 1958). In a second cell type (followers without sustained depolarization) there was observed an increase in the burst frequency and the appearance of excitatory postsynaptic potentials (epsp's), one epsp for each stimulus to the accelerator nerve. These epsp's could summate and lead to the production of spikes in the cell (Otani and Bullock, 1959).

The results obtained in this study are similar to those in the crustaceans. Supramaximal stimulation of the abdominal nerves resulted primarily in an increased frequency and a decrease in the duration of the recovery phase. No postsynaptic potentials were recorded.

Pax (1969) and Bursey and Pax (1970) found that the acceleratory nerves gave marked inhibitory effects with stimulus frequencies in excess of 10 Hz. Evidently, the acceleratory nerves also carry some inhibitory fibers. Similar findings have been observed in the segmental cardiac nerves of the cockroach (Miller and Usherwood, 1970). Florey (1968) reported that a single cardioregulatory nerve in the

crayfish may have both acceleratory and inhibitory fibers. In the study reported here six cells showed an initial decrease in burst frequency when nerve 11 was stimulated, but no ipsp's were ever recorded.

These results suggest that, as in the simple follower cells of crustaceans, the increases in burst frequency are probably due to an indirect effect through the pacemakers. The abdominal nerves have no direct input to the large unipolar cells of the cardiac ganglion.

The remote possibility that the abdominal cardiac nerves do have a direct input to the unipolar cells must not be excluded. It is feasible that the synapses are located at a distance from the soma. Because of the space constant of the unipolar cell membrane, postsynaptic potentials produced by the accelerators may not be seen in the cell soma.

Inhibition

The rate and strength of beating in both limulus and the crustaceans can be decreased by stimulation of the inhibitory nerves from the central nervous system at a frequency greater than 5 Hz. Using extracellular recordings from the cardiac ganglion Maynard (1953) found that the inhibitor of panulirus depresses the heart by direct action on the nerve cells, decreasing or stopping the normal burst of spikes associated with each burst. Impulses reaching the ganglion after a burst had begun caused a significant inhibition of the remaining portion of the burst. Impulses arriving in

the ganglion 0.1 sec before a burst began caused complete inhibition.

Florey (1957, 1960) suggested that the inhibitor nerves released a chemical mediator. He based his conclusion on the facts that (a) diastolic arrest, which could be induced by stimulation of the inhibitors, continued for several seconds after the end of inhibitor stimulation; and (b) picrotoxin reversibly blocked inhibition produced by the stimulation of inhibitory fibers. In limulus Pax and Sanborn (1964) showed that changes in heart rate were not tightly coupled to stimulation of the inhibitor nerves, and that a time lag in the response occurred both at the beginning and end of stimulation. This suggested that inhibition in limulus was also chemically mediated.

Three results from these experiments suggest that chemical synaptic transmission is present between the inhibitory nerves and the unipolar cells. First, the ipsp's which appear in the cells decrease in amplitude as the cell membrane repolarizes during the recovery phase and they are still in the hyperpolarizing direction at the resting membrane potential. This suggests that there is an equilibrium potential for the ipsp's, and the level of this potential is higher than the normal resting membrane potential.

Secondly, presetting the membrane potential at levels higher than the equilibrium potential for the ipsp's converted the ipsp's into depolarizing potentials. This reversal

of the postsynaptic potentials occurred in all the cells examined. However, as stated in the results, there was a 10 to 20 mV range of hyperpolarization in which the ipsp's were undetectable. The difficulty in reversing the ipsp's and their small size suggest that the synapses are located at a distance from the cell soma. Finally, the decrease in R_{eff} during high frequency stimulation of the inhibitor nerves is indicative of a change in membrane conductance produced by a transmitter substance.

Since there appears to be one ipsp per stimulus to the inhibitor nerve, there is reason to believe that the inhibitors have a direct input to the unipolar cells, without the intervention of an interneuron between the inhibitory fibers and unipolar cells. The long latency of the ipsp's can be explained by the time needed for conduction of the impulses along the axons of the inhibitory fibers rather than being due to synaptic delays over a number of synapses.

Terzuolo and Bullock (1958) found hyperpolarization and ipsp's in Type C cells (follower cells with spontaneity) when the inhibitor nerves were stimulated. In some Type C cells and in some simple follower cells they sometimes found depolarizing potentials. These depolarizing potentials may be due to the fact that they were using KCl-filled microelectrodes, which can change the intracellular concentration of chloride ions by simple diffusion of chloride from the electrode tip. If the synaptic potentials induced by the

inhibitory nerves are chloride-dependent, there could be a reversal in the ipsp's. In these studies depolarizing postsynaptic potentials were never seen in the unipolar cells of limulus. This observation suggests that the ipsp's in the unipolar cells are either not chloride-dependent or the synapses are located at such a distance from the soma that changes in the intracellular chloride concentration have not reached the synaptic regions.

Carlson (1905b) and Pax and Sanborn (1964) found that stimulation of the inhibitors decreased both the frequency and strength of the heartbeat. Intracellular recordings from the unipolar cells can explain these decreases. The reduction in burst frequency is due to the action of the inhibitors on the pacemaker cells. The appearance of pacemaker potentials in cells with an endogenous rhythm is a common phenomenon (for review see Bullock and Horridge, 1965). The membrane potentials of these cells undergo periodic decreases (pacemaker potentials) until the threshold potential is reached. It is possible that the stimulation of the inhibitory nerves slows the rate of rise of the pacemaker potentials so that there is a longer time interval between successive periods of activity. Modulation of the rhythm of spontaneously active cells by direct inhibition is seen in the central nervous system of Aplysia (Kandel et al., 1969).

In several of the unipolar cells there was a complete blockade of activity (Figure 1D). This blockade could be

the result of the inhibitory nerves hyperpolarizing the pacemaker cells to such an extent that the threshold potential is never reached. Thus, there would be no inputs to drive the unipolar cells. This blockade of firing seen in the unipolar cells could explain the cardiac standstill in limulus seen by Carlson (1905b).

The spontaneous activity recorded from the unipolar cells does not appear to have a simple origin. The spikes on the recovery phase appear to be endogenous to the cell because they can be eliminated by intracellularly applied hyperpolarizing current pulses or can be elicited by depolarizing pulses (Palese et al., 1970). The appearance of ipsp's in the unipolar cells are probably the cause of the decrease in the number of spikes on the recovery phase when the inhibitory nerves are stimulated. The ipsp's could transiently lower the level of the depolarization at the spike-generating region of the membrane below threshold. This decrease in spike number would reduce the input to the muscle cells, producing a decrease in the number of junction potentials which summate to give a sustained depolarization in the myocardial cells.

Pax (1969) concluded that the inhibitory fibers present in the abdominal cardiac nerves have characteristics similar to the inhibitory fibers, segmental nerves 7 and 8, which innervate the ganglion. However, in these studies no ipsp's were found to be present in the six cells which showed an

initial decrease in rate when nerve 11 was stimulated. Therefore, these two groups of inhibitory fibers have a marked difference in their innervation patterns. The inhibitory fibers in the abdominal nerves seem to have inputs only on the pacemaker cells, while the inhibitory fibers in segmental nerves 7 and 8 have direct inputs to both the unipolar cells and pacemaker cells.

Pharmacology of Inhibition

Among the invertebrates picrotoxin is known to block postsynaptic inhibition produced by stimulation of inhibitor nerves at the neuromuscular junction of crustaceans and some insects (Grundfest et al., 1959; Takeuchi and Takeuchi, 1969; Usherwood and Grundfest, 1964), the crayfish heart (Florey, 1957) and the crayfish stretch receptor (Elliott and Florey, 1956; Kuffler, 1960). Hichar (1960) found that picrotoxin caused a gradual increase followed by a depression in the externally recorded activity of the fifth abdominal ganglion of the crayfish, Orconectes virilis.

In these studies picrotoxin decreased the effectiveness of the inhibitory nerves in reducing the burst frequency and in all cases picrotoxin completely eliminated the ipsp's seen in the unipolar cells. Since there was no change produced by picrotoxin in the resistance of the unipolar cell membrane and the resting membrane potential, it appears that the conductance of the cell membrane is unaffected by picrotoxin.

There are at least three possibilities to explain the blocking action of picrotoxin. First, picrotoxin could cause a decrease in the transmitter released by the inhibitory nerve terminals. Secondly, picrotoxin could compete for the same receptor sites on the unipolar cell membrane as does the natural transmitter. Thirdly, picrotoxin could block the permeability changes induced in the postsynaptic membrane by the combination of the inhibitory transmitter and its receptors. The results of these experiments cannot support any of these possibilities.

The blocking effects of picrotoxin support the notion of chemically mediated synaptic transmission. To the best of my knowledge, this drug has no effect on transmission at electrical junctions.

Strychnine is known to be a potent blocker of postsynaptic inhibition in vertebrates. For this reason strychnine was used to perfuse the ganglion to determine whether it blocked the effects of the cardioinhibitory nerves. In these experiments strychnine had no effect on the decrease in rate and frequency produced by stimulation of nerves 7 and 8. This lack of effect of strychnine upon inhibitory transmission is quite common in invertebrate preparations, for example, the crayfish heart (Florey, 1957), lobster leg muscle (Grundfest et al., 1959), crayfish stretch receptor (Washizu et al., 1961) and molluscan DInhi cells (Gerschenfeld, 1964).

Other Pharmacological Studies

In these studies four drugs which have been implicated in synaptic transmission in invertebrates were tested for their effects on the electrical activity of the unipolar cells. Table 6 summarizes the effects of these drugs. The fact that these drugs produce physiological changes does not necessarily imply that they have a role in chemical transmission in the limulus heart.

5-HT

5-HT is omnipresent in the animal kingdom. Among the arthropods 5-HT is present in the nervous system of limulus and astacus, but in other arthropods it appears only in the pericardial organs (Welsh, 1968). For the most part 5-HT has an excitatory effect. In the hearts of crustacea, cockroaches and molluscs, 5-HT increases the frequency and strength of beating of the heart muscle (Carlisle, 1956; Cooke, 1966; Erspamer and Ghiretti, 1951; Loveland, 1963; Maynard, 1958; Maynard and Welsh, 1959; Twarog and Roeder, 1957; Welsh, 1954, 1956). Receptor sites sensitive to 5-HT have been found with the use of iontophoretic techniques in the central nervous system of molluscs (Gerschenfeld et al., 1967; Gerschenfeld and Stefani, 1965, 1966; Gerschenfeld and Tauc, 1961, 1964; Stefani and Gerschenfeld, 1969; Kerkut and Walker, 1962; Kerkut et al., 1970).

Inhibition of activity by 5-HT is uncommon. Sherman and Pax (1970) found that 5-HT evoked a decrease in heart

Table 6. Summary of changes produced by various drugs on the parameters of the electrical activity recorded from unipolar cells. (+) indicates an increase, (-) indicates a decrease and (0) indicates no change.

Drug	Number of Animals	Number of Cells	Initial Phase		
			Amplitude	Duration	Number of Spikes
L-glutamic acid					
Pipette	3	5	0	0	0
Perfuse	3	10	0	-	0
Dopamine					
Pipette	4	10	0	0	-
Perfuse	5	8	0	0	0
5-HT					
Pipette	5	12	0	0	0
Perfuse	5	9	0,-	0,-	-
GABA					
Pipette	5	13	-	-	-
Perfuse	4	6	-	-	-

continued

Table 6--continued

Drug	Recovery Phase				
	Amplitude	Duration	Number of Spikes	Bursting Rate	R_{eff}
L-glutamic acid					
Pipette	0	0	0	0	0
Perfuse	0	-	-	+	0
Dopamine					
Pipette	-	0	-	-	0
Perfuse	0	+	-	-,+	0
5-HT					
Pipette	-	0	-	-	0
Perfuse	0,-	-	-	-	0
GABA					
Pipette	-	-	-	0	-
Perfuse	-	-	-	-	-

rate and heart beat amplitude by its actions on the cardiac ganglion of the tarantula. The giant Retzius cells of the leech central nervous system are also inhibited by this drug (Kerkut et al., 1967). As previously mentioned, 5-HT reduces the heart rate and strength of beat in an isolated limulus heart.

The results from the pipetting experiments suggest that the somata of the unipolar cells are chemically insensitive to 5-HT. There was little or no change in the membrane properties of the cells examined. This is to be expected if the unipolar cell bodies do not play a significant role in nervous integration (Palese et al., 1970; Zollman and Gainer, 1970). In the ventral ganglia of limulus application of 5-HT also has no effect on the soma membrane of some large neurons (Nystrom et al., 1970).

Perfusion of 5-HT onto the whole ganglion blocked the appearance of spikes on the recovery phase of all the cells examined. In five cells the bursting activity was completely blocked and there was a decreased rate in four cells. This compares favorably with the external recordings of Pax and Sanborn (1967b). They found that 5-HT decreased the rate of rhythmic discharges and the number of units discharging in each burst. These effects mimic the effects of stimulation of the inhibitor nerves. But the main question to be answered is this: Are the effects produced by 5-HT due to the effects of the drug on sensitive sites on the unipolar cell membrane,

or does 5-HT stimulate the inhibitor nerves and indirectly inhibit the unipolar cell? This latter possibility is seen in the molluscan central nervous system (Gerschenfeld and Tauc, 1961). In this case 5-HT applied directly to an H cell resulted in excitation of the cell. Perfusion of the ganglion by 5-HT caused inhibition of the same cell because an inhibitory interneuron with an input to the H cell was excited by 5-HT.

It appears that 5-HT is acting on the receptor sites of the unipolar cells. This is based indirectly on the observation that, although activity could be blocked in the unipolar cell for 15 to 30 sec by high frequency stimulation of the inhibitors, some recovery of activity was seen no longer than a minute after onset of stimulation. This observation suggests that the inhibitory fibers are becoming less effective because there is either a decrease in transmitter release by the inhibitory nerve terminals or there is a desensitization of the receptor sites on the membranes of the pacemaker and motor cells.

In the five cells showing blockade of bursting activity by 5-HT, the blockade lasted a minimum of two minutes and in some cases the blockade lasted five minutes. If 5-HT were exciting the inhibitory nerves, recovery of activity in the unipolar cells would have appeared at least one minute earlier. Therefore, the recovery of activity during stimulation is probably due to desensitization of the postsynaptic

membrane and 5-HT mimics the cardioinhibitory transmitter by acting on the unipolar cell membrane. This lends further support to the idea that 5-HT or a similar compound is the chemical mediator of inhibition.

This idea is further supported by the fact that in some cells replacement of the 5-HT perfusion solution with new solution of the same concentration of the drug failed to depress the electrical activity in the unipolar cell. Such failure suggests a desensitization of the receptors of the postsynaptic membrane.

Further studies are needed before 5-HT is finally chosen to be the inhibitory transmitter. First, the action of compounds such as 6-HT and 5,6-dihydroxytryptamine should be examined. Kerkut and Price (1964) found that the heart of the crab was ten times more sensitive to 6-HT than 5-HT. Carlisle (1956) reported that the active substance of the pericardial organ was an ortho-dihydroxytryptamine. Second, a comparison should be made of the effects of various ion concentrations on the ipsp's produced by the inhibitor nerves and the hyperpolarization produced by 5-HT. Since the sites of the synaptic regions appear to be at a distance from the soma, it does not appear feasible at this point to make a comparison between equilibrium potentials for the natural transmitter and 5-HT. Thirdly, biochemical analyses of perfusates should be made to determine whether 5-HT is released by electrical stimulation of the cardioinhibitory nerves.

GABA

In arthropods GABA mimics the effects of stimulation of inhibitor nerves. This chemical compound is one of the constituents of Factor I, an extract of mammalian central and peripheral nervous tissue that inhibits synaptic transmission at various sites (Bazemore et al., 1957; Florey, 1957). GABA tends to have an inhibitory effect on nerve cells of central and peripheral nervous systems, including cells in the insect central nervous system (Kerkut et al., 1968, 1969), the D cells of molluscs (Gerschenfeld and Tauc, 1961), and the crayfish stretch receptor neuron (Edwards, 1960; Edwards and Kuffler, 1959; Kuffler and Edwards, 1958; McLennan, 1957). Inhibitory effects are produced in arthropod hearts, including the hearts of decapod crustaceans (Enger and Burgen, 1957; Florey, 1954, 1957) and the tarantula (Sherman and Pax, 1970).

Pax and Sanborn (1967a) suggested that GABA is probably not the chemical mediator of limulus cardioinhibitory nerves, since picrotoxin blocks the action of the inhibitory nerves, but not the effects of applied GABA. Abbott et al. (1969) concluded that GABA was the cardioinhibitory transmitter because of the pronounced effects of the drug on ganglionic discharge.

In this study local application of GABA with a pipette drastically altered the somal membrane resistance by about 28%. This decrease in R_{eff} indicates that the somal membrane

has receptor sites which are sensitive to GABA. When GABA combines with these sites, the membrane becomes permeable to certain ions. This change in conductance is substantiated by the presence of a reversal potential.

GABA affects not only the membrane of the soma, but also the spike-generating region of the cell. This statement is based on the observation that GABA could decrease the number of spikes in the soma. It is possible the hyperpolarization of the cell soma produced by GABA passively spread to the spike-generating region of the cell and decreased the excitability of the region. However, it is also possible that the GABA diffused to the region of the excitatory inputs to the unipolar cells and decreased the effectiveness of these inputs, by partially blocking synaptic transmission at these regions.

In the perfusion experiments the activity from all cells examined was completely blocked. These observations could result from one of two possibilities. First, the R_{eff} may have been decreased to such an extent that the electrotonic spread of spikes and the slow depolarization from distant regions of the cell does not reach the cell soma. However, in several of the cells examined the R_{eff} returned to the pretreatment level before any electrical activity was recorded.

Secondly, GABA may also be directly affecting pacemaker activity. This possibility cannot be substantiated, since no extracellular recordings were made at this time. But the

observations of Parnas et al. (1969), who found that GABA at 5 x 10^{-6}M blocked all electrical activity of the ganglion, would support this possibility. In addition, the decrease in relative bursting rate seen after bursting had re-appeared in the soma suggests that pacemaker elements are being affected.

The recovery of bursting activity and of the resting potential and R_{eff} while GABA is still present probably represents an inactivation of GABA in the solution which surrounds the cell and not a desensitization of the receptor sites. This statement is based on the observation that once the bursting activity re-appears, simple mixing of the solution once again blocks the bursts. Inactivating mechanisms occur in the lobster and crayfish slowly adapting stretch receptor (Kuffler and Edwards, 1958). The inactivation may be accomplished by the action of enzymes present on the external surface of the cell membrane or an active uptake of the GABA by nerve or glial cells.

Since there is no desensitization of the receptor sites and because Pax and Sanborn (1967a) found that picrotoxin did not block the effects of GABA, it is believed that GABA is not the inhibitory transmitter. GABA may simply act as a general inhibitor of neuroelectrical activity in limulus as proposed by von Burg and Corning (1968).

Dopamine

For a long period of time dopamine was overlooked as a possible neural transmitter, because it is a precursor of norepinephrine and epinephrine. Only recently has the presence of this compound been determined in the nervous system of molluscs (Cardot, 1963; Kerkut et al., 1966; Sedden et al., 1968; Sweeney, 1963). In arthropods dopamine appears in the thoracic nerve mass of the decapod crustacean Carcinus maenas and in crab pericardial organs (Kerkut et al., 1966; Cooke and Goldstone, 1970).

Studies of the action of this compound have been restricted to central and peripheral nerve cells. Dopamine inhibits the crayfish stretch receptor and leech Retzius cells (McGeer et al., 1961; Kerkut and Walker, 1967). In cells of the molluscan central nervous system dopamine inhibits some cells, especially DInhi cells (Gerschenfeld, 1964; Kerkut and Walker, 1962; Kerkut et al., 1968; Walker et al., 1968). Other cell types may be excited (Ascher et al., 1967; Gerschenfeld and Tauc, 1964). In limulus Fourtner and Pax (manuscript in preparation) found that, after an initial inhibition, dopamine markedly increased both the amplitude and frequency of contraction. To my knowledge, dopamine has not been tested on other invertebrate heart preparations.

In the pipetting experiments dopamine failed to appreciably affect the R_{eff} in a majority of the cells. In addition, a value for a reversal potential could not be determined.

This suggests that on the somal membrane there are no receptor sites that are sensitive to dopamine. Dopamine did produce a hyperpolarization of 3 mV. Since there was no change in membrane conductance of the soma, the hyperpolarization could be explained by diffusion of the dopamine to sensitive receptor sites found on another portion of the membrane, perhaps the axon hillock region. The hyperpolarization could explain both the decrease in amplitude of the recovery phase and the decrease in the number of spikes on the initial and recovery phases. The hyperpolarization could decrease the excitability of the spike-generating regions of the cell membrane by simply increasing the membrane potential in these areas.

Perfusion of dopamine caused a hyperpolarization of the unipolar cells. At a concentration of 5×10^{-5}M, the bursting frequency of all cells showed a 16% increase by the third burst following application of the drug.

These results lend further support to the notion that the unipolar cells are follower cells and not pacemaker cells as proposed by Heinbecker (1936) and Bullock et al. (1943). If the bursting activity of the unipolar cells is endogenous to the cells, then it is expected that a hyperpolarization of the cell membrane would decrease the bursting frequency. However, the bursting frequency in these cells increases. This suggests that dopamine excites the pacemaker cells and these cells respond with an increased frequency of activity.

The decrease in frequency seen with the higher concentration of dopamine can be explained by one of two possibilities. This concentration of dopamine could directly and transiently hyperpolarize the pacemaker cells. Secondly, it is possible that dopamine depolarizes the inhibitor nerves and indirectly reduces the frequency of bursting. Results from these experiments cannot support either of these possibilities.

Dopamine decreased the number of spikes in the unipolar cells. Fourtner and Pax observed an increase in amplitude of contraction of the myocardial cells. These results suggest that dopamine has a peripheral action on the motor nerve terminals or the membranes of the heart muscle cells.

L-glutamate

In arthropod neuromuscular systems glutamate has been proposed as the excitatory transmitter. In molluscs glutamate excites D cells and inhibits H cells (Gerschenfeld and Lasansky, 1964; Kerkut *et al.*, 1970). The effects of glutamate have been examined in isolated arthropod hearts. Enger and Burgen (1957) applied glutamate to the lobster heart and recorded a marked increase in the rate and strength of contraction. Brockman and Burson (1957), on the other hand, reported that glutamate decreased the frequency and amplitude of contraction of the crayfish heart. Sherman and Pax (1970) found that l-glutamate had little effect on the heart of the tarantula, but the strength of contraction was increased by

the action of glutamate on the muscle. Abbott et al. (1969) found that glutamate increased myocardial tone and decreased the strength of the beat pulsations of limulus hearts.

Pipetting of glutamate had no effect on the membrane conductance or membrane potential. Thus, the somata of these cells lack excitatory and inhibitory receptors for this compound.

Perfusion of glutamate resulted in a decrease in the number of spikes on the recovery phase. The decrease is possibly due to the decrease in the duration of the recovery phase.

The decreased duration of the recovery phase may be due to a reduction in the presynaptic input to the unipolar cell. This reduction can be accomplished in one of two ways. There could be a reduction of the total number of inputs to the unipolar cell. This would mean that glutamate has an inhibitory effect on some cells of the ganglion. A reduction could also be explained by a decrease in the number of action potentials in each of the presynaptic fibers. This would decrease the number of epsp's in the unipolar cell, especially those appearing late in the unipolar cell activity. An alternative explanation is that the postsynaptic membrane could become desensitized to the excitatory transmitter. It appears that the third explanation is the tenable one, since a decrease in duration occurs in spontaneously beating hearts in normal saline.

Parnas et al. (1969) noted an increase in heart rate produced by 10^{-5}M glutamate. In these studies 5 x 10^{-5}M glutamate increased the rate of bursting activity by 64%. Glutamate appears to be exciting the pacemaker cells, which seem to have some sensitive receptor sites.

These same authors also noted an increased myocardial tone. The increased tone could be the result of glutamate acting on muscle cell membranes or on the motor cells. Since there is a decrease in spike number in the unipolar cells, the increased tone must be due to the effect of glutamate acting on the muscle or motor nerve terminals, but not on the spike-generating region of the motor nerve.

Summary

The studies provided here serve as a basis for explaining the effects of stimulation of the cardioregulatory nerves and the application of a number of drugs on isolated limulus hearts. Few similar attempts have been made in other arthropod heart preparations.

The results from the stimulation of the cardioaccelerator nerves suggest that their action is primarily on pacemaker cells in the ganglion. The cardioinhibitory nerves appear to have direct inputs to both the pacemaker cells and the unipolar cells. Inhibition of the pacemakers causes a decrease in heart rate. Inhibition of the unipolar cells decreases the number of spikes in these cells and thus reduces

the input to the heart muscle. This causes a decrease in the strength of contraction.

The pharmacological studies indicate that different drugs exert their influence at different sites in the limulus heart--pacemaker cell, motor cell, muscle cell. Investigations into the type of electrical activity present in each of the other cell types in the ganglion and how drugs alter this activity will be necessary before a full understanding of the physiology of the limulus heart is obtained.

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