MICROELECTRODE STUDIES OF THE UNIPOLAR CELLS OF THE CARDIAC GANGLION OF THE HORSESHOE CRAB LIMULUS POLYPHEMUS

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ABSTRACT

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By

Vincent J. Palese, Jr.

The electrical activity from cells of the cardiac ganglion of L. polyphemus have not yet been examined. In order to determine the electrical properties and the functional relationship between these cells, the electrical activity of the unipolar cells was examined with microelectrodes.

The resting membrane potential in these cells averages 42 mv (SE = 1.8 mv). Intracellular depolarization begins with a fast-rising initial depolarization of 23 mv (SE = 1.0 mv), which is maintained for 25 to 120 msec. This is then followed by a slow repolarization of about 16 mv amplitude. The total duration of cellular depolarization, measured in the somata of the unipolar cells, has a mean value of 2.34 sec (SE = 0.16 sec).

A number of small (5 to 7 mv) spike-like potentials are superimposed upon the initial depolarization and the slow repolarization. The frequency of the "spikes" is high on the initial depolarization (50 "spikes"/sec; SE = 2.1 "spikes"/sec) and declines to a frequency of 15 "spikes"/sec (SE = 1.0 "spikes"/sec) on the slow repolarization.

Depolarizing currents of sufficient strength (mean = 1.1 X 10^{-9}A; range 0.6 to 2.5 X 10^{-9}A) can elicit "spikes" similar in size and shape to those recorded in a normal burst. Currents up to 5 X 10^{-9}A fail to excite the soma membrane. The data suggest that the "spikes" arise at an active membrane region within the cell process and invade the soma electrotonically.

The specific membrane resistance for the soma membrane averages 12,700 ohm-cm^2 (Range, 3750-26,000 ohm-cm^2). The time constant has a value of 19.6 msec (Range, 4-40 msec). The specific membrane capacitance measures 1.54 uF/cm^2 (Range, 0.15-7.47 uF/cm^2). These values are within the range normally found in other nerve cells.

The unipolar cells appear to have no functional connections between them. This was determined by means of recording simultaneously from pairs of unipolar cells and injecting current into one cell of the pair.

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INTRODUCTION

Carlson (1904; 1909) demonstrated that the heartbeat of Limulus polyphemus is neurogenic. Since this discovery, numerous studies have been undertaken concerning the external electrical recordings from the cardiac ganglion, the site of pacemaker activity, and the effects of ions and drugs on the ganglion and cardiac muscle. It is surprising that, despite the fact that the L. polyphemus heart preparation has been utilized for about sixty-five years, studies with intracellular electrodes on cardiac ganglion cells are lacking. Such studies are necessary for a complete understanding of the physiology of the heart of L. polyphemus.

A logical place to begin studies of intracellular activity in the cardiac ganglion of L. polyphemus is with the large unipolar cells (100 X 140 u) present in cardiac segments four through nine (Heinbecker, 1936; Bursey and Pax, in press). These cells protrude dorsally and laterally from the ganglion, are plainly visible under a dissecting microscope and are easily penetrable with microelectrodes. In addition, these cells may have a pacemaker function, generating the electrical discharge of the cardiac ganglion (see below). To further understand the role of these cells in the control of the heartbeat and their relation to the electrical activity of the cardiac ganglion, I have undertaken an examination of the intracellular electrical activity of the unipolar cells and the electrical properties of their soma membranes.

Anatomy of the Heart and Cardiac Ganglion

The heart of L. polyphemus is suspended in a pericardial sinus just below the dorsal carapace. It is a triangular-shaped tube which reaches a length of 10 to 17 cm in the adult. Eight pairs of slit-like ostia are located on the dorso-lateral surface of the heart and divide the heart into nine unequal segments.

The cardiac ganglion is situated externally along the dorsal midline 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. Several cell types are present in the ganglion and include large unipolar cells, large and small bipolar cells and multipolar cells (Bursey and Pax, in press).

Neurogenic Origin of the Heartbeat and Determination of the Site of Pacemaker Activity

The neurogenic origin of the heartbeat of L. polyphemus was demonstrated most convincingly by Carlson (1904). A lesion of both the cardiac ganglion and the two lateral nerves, which are connected with the cardiac ganglion by a plexus (Patten and Redenbaugh, 1900), in any segment of the heart was found to destroy the coordination of the two ends of the heart on either side of the lesion. If the cardiac muscle was sectioned in any segment, leaving the cardiac ganglion intact, coordination was maintained. Complete removal of the cardiac ganglion from the muscle resulted in cessation of the heartbeat. Samojloff (1930) substantiated Carlson's findings by demonstrating that the ganglion can be excited by extracellular electrical stimulation. This excitation induced an extra systolic contraction of the heart muscle

and this induction of an extra systolic contraction was likened to a similar activity seen in the pacemaker of the vertebrate heart.

A conflicting conclusion was drawn by Dubuisson (1931a; 1931b). He found that, if normal distension of the heart was maintained by air, a deganglionated heart would re-establish a beat after 15 to 30 minutes. Thus, the heart muscle of L. polyphemus seems in itself potentially rhythmic, that is, the heartbeat is myogenic, not neurogenic, in origin. Heinbecker (1933) confirmed Dubuisson's findings, but finally concluded that the heartbeat was definitely neurogenic, for he found that ganglion cell activity always preceded activity in the heart muscle.

Once it had been established that the cardiac ganglion was responsible for the initiation of the heartbeat in L. polyphemus the next step was to determine the specific location of pacemaker activity in the ganglion. Carlson (1905) observed that the greatest automatism was exhibited by the middle third of the heart. Edwards (1920) supported this when he found that cardiac segments four and five exhibited activity about 0.03 to 0.04 seconds before excitation of the anterior and posterior segments. Garrey (1930; 1932) applied local warming or cooling stimuli to the cardiac ganglion or stretched the ganglion with a thread to demonstrate that new pacemakers could be elicited in every part of the ganglion between the third and eighth segments. Bullock et al. (1943) reached the same conclusion by means of electrical stimulation.

Heinbecker (1933) observed that the large unipolar ganglion cells could initiate activity in small ganglion cells. He found that, when activity in the large cells was stopped in the presence of CO_2,

electrical stimulation of the small cells not affected by CO_2 did not change their pattern of activity. After the electrical activity of the large cells had returned in normal saline, externally applied electrical stimuli did cause a change in the rhythm of the small cells. On this basis and the fact that the unipolar cells always responded to electrical stimuli up to a frequency of five impulses/sec, Heinbecker suggested that the large ganglion cells acted as pacemakers.

With external electrodes Heinbecker (1936) and Prosser (1943) recorded slow negative waves from the midportion of the ganglionic trunk (pacemaker segments), but not from the first three cardiac segments which contain only small bipolar cells. Such waves were also recorded in the posterior portion of the ganglion, but were smaller in amplitude than those recorded from the pacemaker segments. Since Heinbecker observed that (1) the slow potentials may have been the result of single unipolar cells and not the smaller multipolar cells, which do not give rise to such long slow potentials even when other cell types are present; (2) the multipolar cells, which are incapable of spontaneous activity, were active in the presence of a unipolar cell, both Heinbecker and Prosser considered the large unipolar ganglion cells to be pacemaker cells. Bullock et al. (1943) suggested that the pacemaker of the heart was perhaps only one large unipolar ganglion cell. However, it was not clearly shown that the long slow potentials are present only when unipolar cells alone are present and that the slow potentials are independent of small ganglion cells. If this were the case, then the unipolar cells would appear to have an intrinsic spontaneous rhythm which is a requirement for a pacemaker mechanism.

Furthermore, it must be remembered that knowledge of the histology of the cardiac ganglion cells was incomplete at that time and Heinbecker may not have recognized all cell types present in the ganglion during his experiments.

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. The animals were stored in a Dayno Co. Model 703 artificial sea water aquarium and were maintained at a temperature of 13 to 16° C.

Isolation of the Heart and Cardiac Ganglion

The 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 entapophysis 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 cut away, completely exposing the heart and its cardiac ganglion. The hearts were measured from the bifurcation of the anterior arteries to a point at the level of the seventh entapophysis. Hearts of 11 to 17 cm in length and from both sexes were used.

A transverse cut was made in the cardiac muscle through the first pair of ostia. A glass tube (outside diam, 6.9 mm) with soft tips of Apiezon wax was inserted through this cut and was pushed posteriorly through the lumen of the heart. The tube 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 several times with artificial 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 several times in sea water. The entire isolation procedure took approximately 45 to 60 minutes. During the course of the experiments the sea water was changed every 45 to 90 minutes. Temperatures were maintained at room temperature of 22-26° C.

Microelectrodes

Microelectrodes were made with Kimax glass capillary tubing. Electrodes were filled with methanol followed by diffusion displacement with 3 M KCl or 0.5 M K_2SO_4 (modified from Tasaki et al., 1954). Only electrodes of 5 to 50 Mohms were used.

Recording

KCl-filled microelectrodes were placed on a Ag-AgCl wire which was connected to an Argonaut LRA 043 Negative Capacitance Electrometer or a WPI M-4 Precision Electrometer. The preamplifiers led into a dual beam Tektronix 502A oscilloscope. Intracellular electrical activity was recorded with a Polaroid or Grass Kymograph camera. The microelectrodes were positioned by means of Narishige micromanipulators. A large Ag-AgCl wire (#20) was placed in the paraffin chamber and used as the indifferent electrode.

Only unipolar ganglion cells were penetrated. These cells are distinguishable from other cell types in the cardiac ganglion by four morphological characteristics: (1) large size (100 X 140 u); (2) spherical shape; (3) asymmetrical pigment distribution; (4) dorsal or lateral protrusion from the fiber tract of the ganglion.

A total of twelve animals, in which a minimum of five cells per animal were penetrated, was used. Only the activity recorded in the first four penetrations of each animal was examined in order to exclude cellular activity of aged preparations (preparations more than five hours old).

Intracellular Stimulation

Both KCl- and K_2SO_4-filled microelectrodes of 5 to 40 Mohms resistance were used in intracellular current injection. Electrodes of low resistance are preferable in intracellular stimulation, but penetration of the unipolar cells with such microelectrodes proved difficult. Current was applied through the electrode by means of the M-4 Electrometer and a Grass S4 (square wave) Stimulator. The strength of the applied current was measured from the oscilloscope.

DATA AND OBSERVATIONS

Description of Intracellular Electrical Activity

Large unipolar cells of the cardiac ganglion of L. polyphemus were penetrated with microelectrodes in order to characterize the electrical activity which occurs in these cells during a spontaneous burst of the ganglion. Figure 1 is an example of the electrical activity recorded from the soma of one of the large unipolar cells. The cell whose activity is represented in the figure was situated in the posterior third of the fifth cardiac segment.

Table I summarizes the data obtained from such experiments. The resting membrane potential for the unipolar cells ranged from 20 to 65 mv (inside negative). Intracellular activity begins with a sudden, large, fast-rising depolarization (initial depolarization) of 9 to 43 mv. This level of depolarization is maintained for 25 to 120 msec and a number of spike-like depolarizations ("spikes") are seen throughout the duration of the initial depolarization. Following this sustained level of depolarization there is a fairly rapid partial repolarization of five to ten mv. No "spike" activity occurs during this repolarization.

Immediately following this rapid repolarization there is an additional repolarization, which follows a slower time course. In many instances, one sees a further slow depolarization in place of the slow repolarization (Figure 2). Superimposed upon the slow repolarization there is a series of 5 to 38 small, transient "spikes" which have an amplitude of five to seven mv. These "spikes" are quite evident throughout much of the slow repolarization. The frequency of these

Table I. A summary of the electrical properties of unipolar cardiac ganglion cells.

	Total Number of Animals Examined	Total Number of Cells Examined	Range	Mean	S.E.
Resting Membrane Potential (mv)	12	48	20 - 65	42	1.8
Amplitude of Initial Depolarization (mv)	12	48	9 - 43	23	1.0
Rise Time of Initial Depolarization (msec)					
Type II	4	11	13.6 - 17.3	14.8	
Type III	5	9			
Before Notch			3.0 - 11.6	6.4	
After Notch			10.9 - 39.1	21.6	
Rate of Rise of the Initial Depolarization (mv/msec)					
Type II	4	11	1.1 - 2.9	2.0	
Type III	5	9			
Before Notch			1.3 - 5.7	2.4	
After Notch			0.6 - 2.2	1.2	
Amplitude of Slow Repolarization (mv)	10	40	4 - 32	16	1.2

Table I. Continued

	Total Number of Animals Examined	Total Number of Cells Examined	Range	Mean	S.E.
Number of Small Spikes Occurring During Cellular Activity	10	40	5 - 38	18	1.5
Frequency of Spikes Before Slow Repolarization (spikes/sec)	10	40	30 - 70	50	2.1
Frequency of Spikes Upon Slow Repolarization (spikes/sec)	10	40	8 - 26	15	1.0
Duration of Cellular Depolarization (sec)	10	40	1.16 - 3.80	2.34	0.16
Duration of Spike Activity (sec)	10	40	0.16 - 1.92	1.04	0.10
Duration of Quiet Period (sec)	10	40	0.41 - 1.96	1.20	0.07
Specific Membrane Resistance (ohm-cm^2)	6	21	3750 - 26,000	12,700	
Time Constant (msec)	4	9	4 - 40	19.6	
Specific Membrane Capacitance (uF/cm^2)	4	9	0.15 - 7.47	1.54	

"spikes" during the initial depolarization is relatively high (70 "spikes"/sec) in the record of Figure 1, and declines to a frequency of 15 "spikes"/sec during the later stages of the slow repolarization. The duration of "spike" activity (measured from the first "spike" of the initial depolarization to the last "spike" on the slow repolarization) ranged from 0.16 to 1.92 sec for the cells examined.

Following this period of "spike" activity there is a quiet period which is characterized by the absence of "spikes" and by a further repolarization of the soma membrane to the resting membrane potential. This quiet period lasts for 0.408 to 1.960 sec. In 28 of 40 cells examined the duration of the quiet period exceeded the duration of the "spike" activity just mentioned.

There seems to be a direct correlation between the duration of the quiet period and the duration of the entire depolarization of the cell soma ($r = 0.374$). The duration of the entire depolarization ranged from 1.16 to 3.80 sec. For every 0.55 sec increment in the duration of the quiet period, there is a 1.00 sec increment in the duration of the entire depolarization. However, no evident correlation was found between the duration of the quiet period and the duration of "spike" activity ($r = -0.050$).

Figure 1 shows an initial depolarization that has more than one step on the rising phase of the initial depolarization before the peak level of depolarization is reached (Type I). In this case three steps appear on the rising phase. These steps appear to represent the summation of three spike-like potentials. Usually no more than three steps were seen. Two other forms of initial depolarization also occur. Figure 2 shows an initial depolarization that is a simple straight

depolarization from base line to its peak value (Type II). One step does appear on the rising phase, but in most cases no such steps appear.

In other cases there is a slightly more complicated depolarization beginning with a simple depolarization which ends in a notch and is followed by a depolarization which has a slower rise time (Type III). Examination of this last type of activity suggests that the notch is possibly formed by the summation of two separate depolarizations with different rise times. Types I and II were each encountered 43% of the time, whereas Type III was found only 14% of the time.

The rise times of the initial depolarizations of Types II and III were examined. In the eleven cells examined from Type II, the peak depolarization was reached within 13.6 to 17.3 msec, mean 14.8 msec. The rate of rise ranged from 1.1 to 2.9 mv/msec, with a mean value of 2.0 mv/msec. Nine cells were examined from Type III. Two measurements were made. The first measurement included the rise time and the rate of rise to the notch in the initial depolarization. Here, the rise times ranged from 3.0 to 11.2 msec, mean 6.4 msec, and the rate of rise ranged from 1.3 to 3.0 mv/msec, with a mean value of 2.4 mv/msec. The second measurement was made to the peak of the initial depolarization from the beginning of the initial depolarization. In this case the rise times ranged from 10.9 to 39.1 msec, mean 21.6 msec, and the rate of rise from 0.6 to 2.2 mv/msec, mean 1.2 mv/msec.

No overshoot of the initial depolarization above the resting membrane potential was ever observed, there being a minimum of five mv difference between the resting potential and the peak of the initial depolarization. There is a direct linear relationship between the initial depolarization and the resting membrane potential ($r = 0.652$).

Figure 1. Spontaneous intracellular electrical activity recorded from the soma of an unipolar cell. Two bursts of activity are shown. In this record the initial depolarization has three steps on its rising phase (Type I). Resting membrane potential 40 mv. Time scale: 500 msec. Voltage scale: 10 mv.

Figure 2. Spontaneous intracellular electrical activity recorded from the soma of a unipolar cell, in which the initial depolarization is simple and straight (Type II). Resting membrane potential 55 mv. Time scale: 200 msec. Voltage scale: 5 mv.

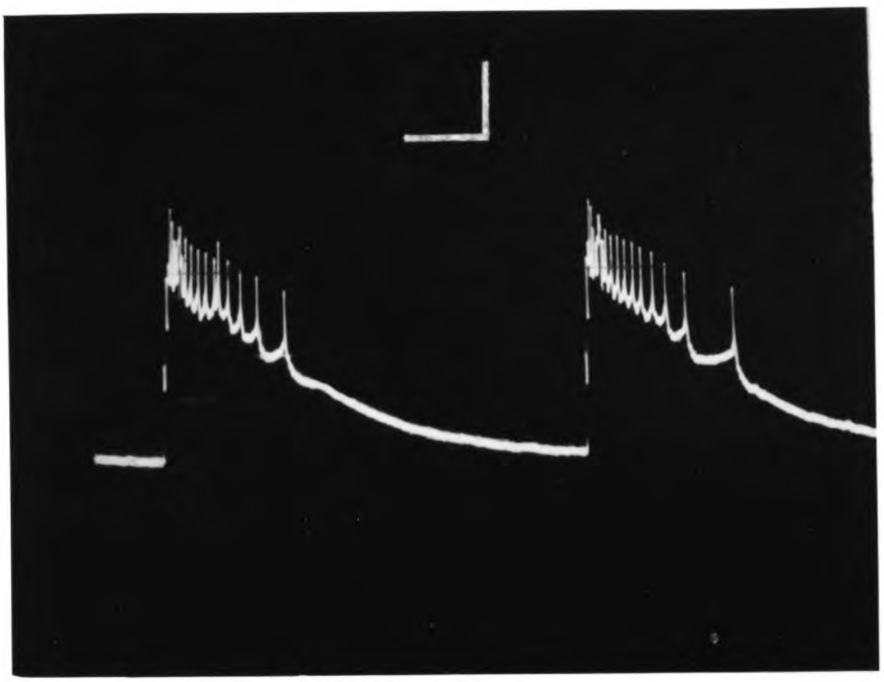

Figure 1

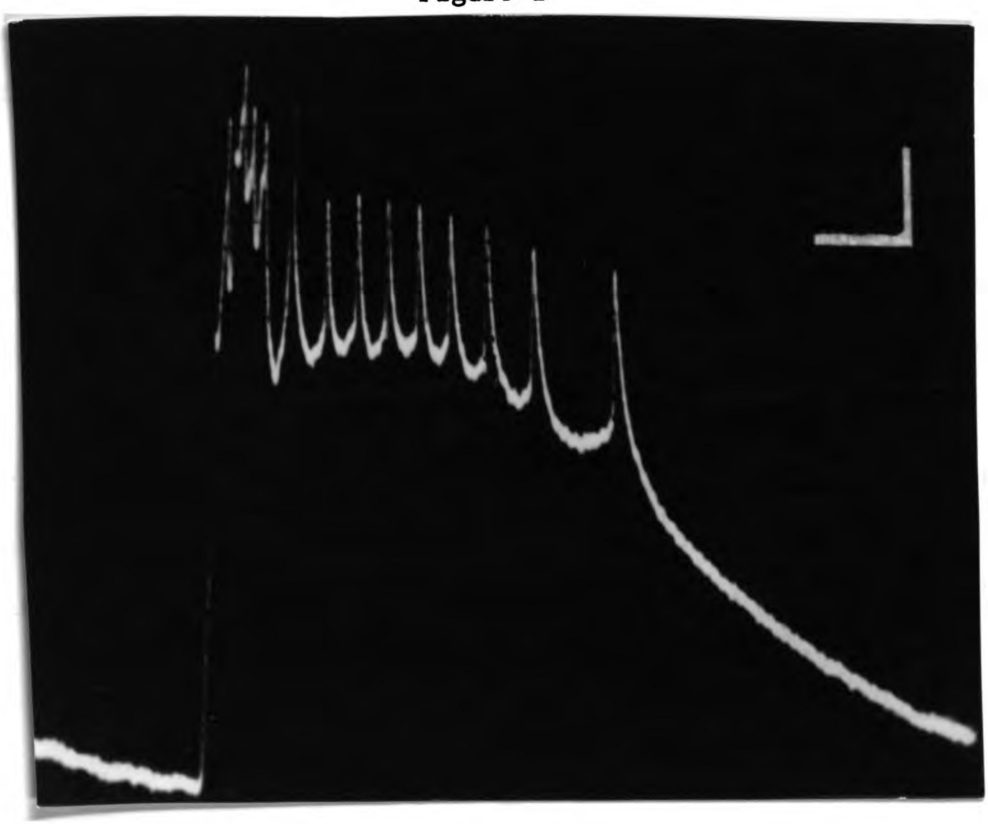

Figure 2

Figure 3 shows that for every 8.8 mv increment in the size of the initial depolarization there is a 10.0 mv increment in the resting potential.

With respect to the "spikes", an average of 18 "spikes" (SE = 1.5) occurred on the slow repolarization. The duration of "spike" activity for all cells had a mean value of 1.04 sec (SE = 0.10 sec). Initially the "spike" frequency is quite high, 50 "spikes"/sec (SE = 2.1 "spikes"/sec) on the initial depolarization. This is followed by a repolarization of 25 to 120 msec, and a decline of the "spike" frequency to about 15 "spikes"/sec (SE = 1.0 "spikes"/sec) on the slow wave of repolarization. No linear relationship was evident between the number of "spikes" on the slow repolarization and the maximum amplitude of the slow repolarization ($r = -0.061$).

During penetration of the unipolar cells it was sometimes observed that the cell responded with a continuous firing of "spikes" similar to those seen during a normal burst. These depolarizations occurred continually from one intracellular burst of activity to the next, even though there was no sustained depolarization. In such cases, when the microelectrode was slightly withdrawn, the continuous activity would cease and there would be a return to the activity normally found. It appears likely that the microelectrode was pressing against the soma membrane from within the cell and that withdrawal of the electrode brought it out of contact with the soma membrane into the soma interior.

Figure 3. Graph of the relationship between the amplitude of the initial depolarization and the resting membrane potential. The line was calculated by the method of linear regression.

Figure 4. Effects of intracellularly applied current upon the membrane potential. The membrane polarization of a cell was changed by passing current through the recording microelectrode. The upper beam represents membrane potential changes; the lower beam, current pulses. Depolarization is in the upward direction. The currents applied were 0.20×10^{-9} A, 0.30×10^{-9} A and 0.40×10^{-9} A. Resting membrane potential 55 mv. The input resistance measured 37 Mohm. Time scale: 50 msec. Voltage scale: 5 mv.

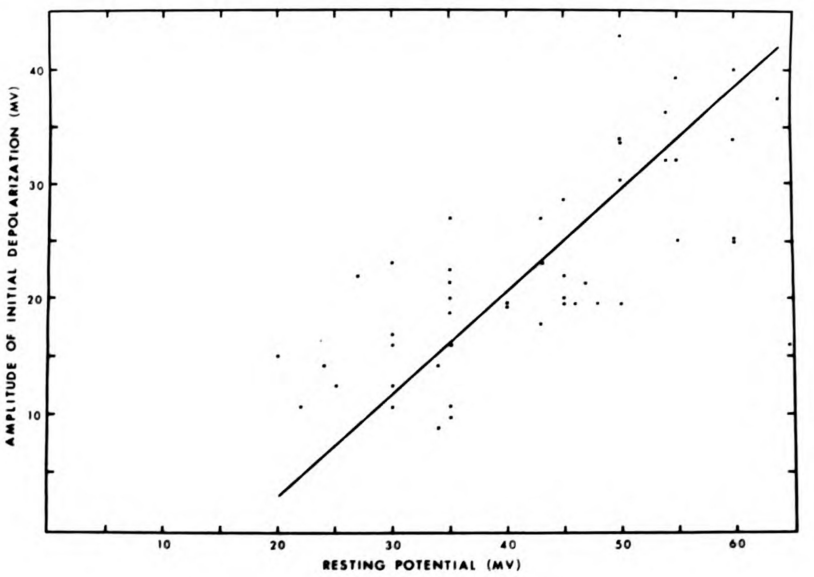

Figure 3

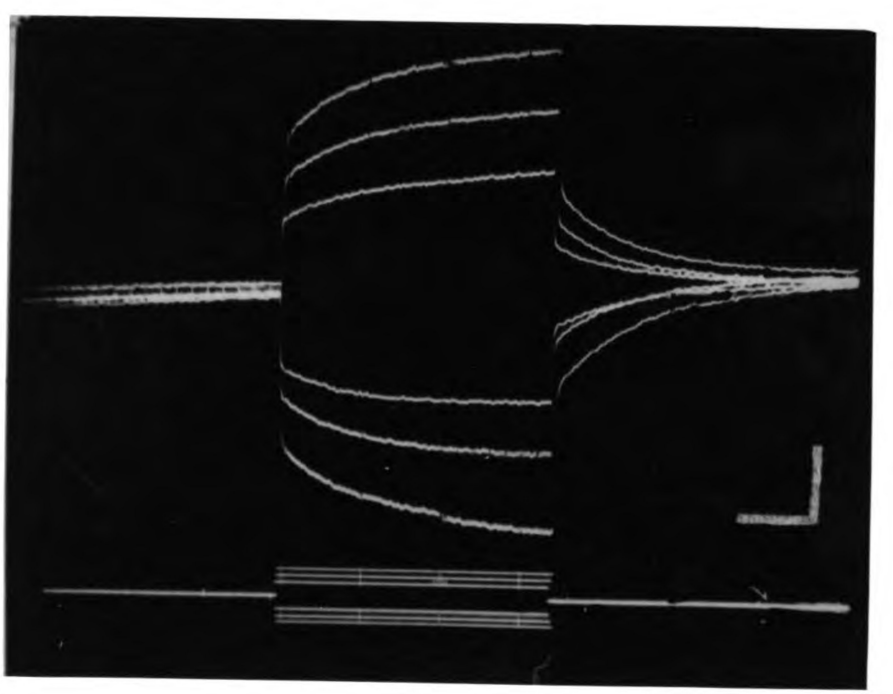

Figure 4

Intracellular Current Injection

Depolarizing current

Figure 4 (upper tracings) shows one example of the results obtained from the application of an outwardly directed current to a cell membrane. Here, a series of current pulses ranging from 0.20 X 10^{-9} A to 0.40 X 10^{-9} A was applied to the cell. The change in potential produced by the current is linear, and increases from 7 to 15 mv. The input resistance in this case measured 37 Mohms.

In most instances an outward current of sufficient intensity resulted in small "spikes", similar in size and shape to those described above, to be recorded some finite time after onset of the stimulus. As is evident from Figure 8B, the induced "spike" in cell VI occurred while the depolarizing current was applied. However, if the duration of the depolarizing current was decreased, the current intensity had to be increased. The small "spike" then occurred on the repolarization phase after the cessation of the current pulse. In order to initiate these "spikes" with an outward current, a current intensity of 1.1 X 10^{-9} A (range 0.6 to 2.5 X 10^{-9} A; duration of 800 msec) was required. An increase in current strength above threshold resulted in a decreased latent period (measured from the beginning of the current pulse to the initiation of the small depolarization) and an increase in the number of induced "spikes". Although small depolarizations could be induced by outward currents, an overshooting potential (one whose amplitude was greater than the magnitude of the resting potential) could never be elicited in the soma, regardless of the strength of the current. Current intensities up to 5 X 10^{-9} A failed to excite the soma membrane.

In no case was it necessary to completely depolarize the soma membrane to zero membrane potential to induce the transient "spikes". However, it was often necessary to depolarize the soma to a value greater than the amplitude of the intracellular depolarization at which "spike" activity occurred in a normal burst. From 20 cells examined, 17 had to be depolarized to a potential greater than that at which spontaneous "spikes" occurred. In two cells induced "spikes" occurred at a level less than that at which "spikes" normally occurred and in one cell no "spikes" were induced by a current pulse.

Depolarizing currents applied during the period of "spike" activity on the slow repolarization often increased the number of "spikes" (Figure 8C, second burst of cell VI). Subthreshold currents -- currents lower than those required to elicit "spikes" during the quiet period -- were often capable of doing this as long as the current pulses were applied shortly after the last "spike". For example, in one case a current of 2.2×10^{-9} A failed to induce a "spike" when a current pulse was applied 550 msec after the last "spike" on the slow repolarization. However, when an input of the same strength was applied during normal "spike" activity on the slow repolarization, the number of "spikes" increased from five to nine. Similarly, in another case a current input of 1.5×10^{-9} A induced one "spike" when stimulation was begun 520 to 600 msec after the spontaneous "spikes" had ceased. When the same current intensity was applied to the same cell during spontaneous "spike" activity, an increase in the number of "spikes" from eight to twelve resulted.

Hyperpolarizing current

Inwardly directed currents result in a hyperpolarization of the soma membrane (Figure 4, bottom tracings). As with the depolarizing current, hyperpolarizing currents resulted in a rapid initial change in membrane potential, a leveling off to a plateau, and a repolarization to the resting membrane potential upon cessation of the current impulse. An increase in intensity of the applied current resulted in an increase in the change of the membrane potential.

In contrast to depolarizing currents, inward currents failed to induce any transient changes in potential similar to the "spikes". The activity during a spontaneous burst of intracellular activity, however, could be changed by applying inward currents. A hyperpolarizing current of sufficient strength applied as the small "spikes" occurred on the slow repolarization reduced the number of "spikes" by one or two. By increasing the current intensity the number of "spikes" would be gradually reduced until none occurred on the slow repolarization as long as the current was applied. In such experiments a rebound "spike" occurred after the termination of hyperpolarizing current in 14 of 15 cases. In such instances the magnitude of the current had a minimum value of 1.0×10^{-9} A.

When applied at the beginning of an intracellular burst of activity, hyperpolarizing currents increased the amplitude of the initial depolarization. This is in contrast to the effects observed upon the small "spikes", which could be eliminated. At no time could the initial depolarization of a spontaneous burst be eliminated, even during a hyperpolarization of the soma membrane by 115 mv more than the resting potential.

Specific membrane resistance

The changes in membrane potential induced by an intracellular current are dependent upon two factors: (1) the strength of the applied current; (2) the resistance of the cell membrane. The following experiment was performed to determine the specific membrane resistance of the cell somata of the unipolar ganglion cells.

In order to determine the total membrane resistance, a number of depolarizing and hyperpolarizing currents were injected into the somata of the unipolar ganglion cells and the changes in membrane potential were recorded. To ensure that the microelectrode did not become polarized, the polarity of the currents was reversed for every stimulation. The current intensities were randomly distributed.

Changes in membrane potential were plotted on a graph against the current required to produce these potential changes (Figure 5). Since $R = E/I$ from Ohm's law, the effective (total, input) resistance (R_{eff}) is equal to $\Delta E/\Delta I$. In other words, R_{eff} can be measured as the slope of the regression line through the experimental points. For the data presented in Figure 5, the R_{eff} had a value of 25.1 Mohms. R_{eff}, measured from 21 cells from six different animals, had a mean value of 28.1 Mohms (Range, 8.3 to 56.1 Mohms).

Specific membrane resistance (R_M) is the product of the surface area of the object whose resistance is measured and the effective resistance ($R_M = R_{eff} \times \text{Area}$) (Hodgkin and Rushton, 1946). If it is assumed that the unipolar cells of the cardiac ganglion of L. polyphemus are spherical in shape and have a diameter of 120 u, then the surface area of the soma membrane is approximately 4.52×10^{-4} cm^2. The specific membrane resistance of these cells is then

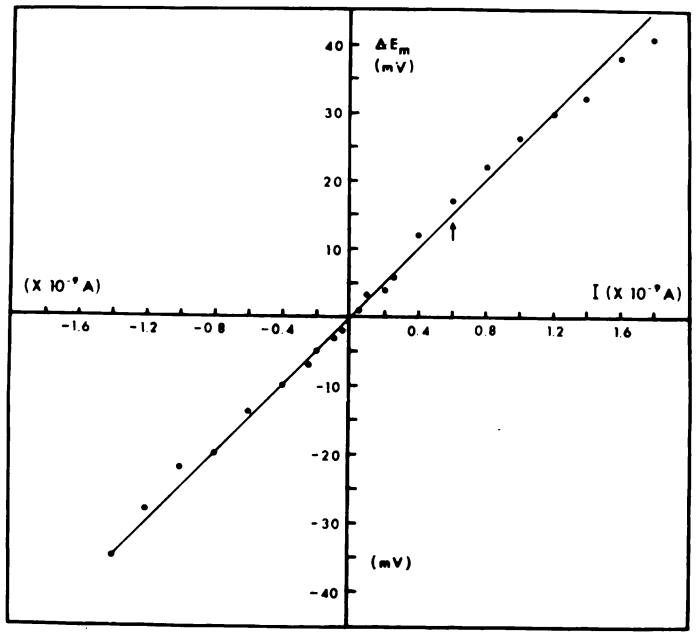

Figure 5. Current-voltage relationship of the soma of a unipolar cell. Note that the relationship is linear in both the hyperpolarizing and depolarizing regions. Arrow indicates the current intensity at which "spikes" were induced in the cell. The line was calculated by the method of linear regression.

$$R_M = R_{eff} \times \text{Area}$$
$$= (28.1 \times 10^6 \text{ ohms}) \times (4.52 \times 10^{-4} \text{ cm}^2)$$
$$= 12{,}700 \text{ ohm-cm}^2.$$

Values for the specific membrane resistance ranged from 3750 to 26,000 ohm-cm^2, with a mean value of 12,700 ohm-cm^2.

Time constant and specific membrane capacitance

Further measurements were made to determine the time constant of the membrane. It was possible to obtain an approximate measurement from only nine cells from four different animals because the response of the membrane recorded by the simultaneously stimulating and recording electrode and the speed of the film made the time constant difficult to measure. The value of the time constant averaged 19.6 msec (Range, 4-40 msec).

Once the specific membrane resistance and the membrane time constant are known, it is possible to calculate the specific membrane capacitance from the equation $C_M = \tau/R_M$. Therefore, $C_M = 19.6 \times 10^{-3}$ sec/$12{,}700 \times 10^3$ ohm-cm^2 = 1.54 uF/cm^2, with a range of 0.15 to 7.47 uF/cm^2. Although the variability between the minimum and maximum values for C_M is large, the values fall within the range of values for the specific membrane capacitance recorded from other nerve cells (Coombs et al., 1959; Prosser and Brown, 1961).

Records from Simultaneously Impaled Cells

Simultaneous recording

In order to determine the presence or absence of junctions between unipolar cells, pairs of unipolar cells were penetrated simultaneously.

In such experiments the electrical activity in 43 pairs of cells from 11 animals was examined. Figure 6 is an example of the activity recorded from a pair of cells.

Spontaneous bursts of activity occur at the same frequency in the cells of a pair, but activity does not begin at exactly the same time in each. The difference in time between the beginning of activity in each cell is linearly related to the distance between cells ($r = 0.862$). For each 7.3 msec increment in the phase difference, there is an increment of approximately one third of a cardiac segment between cells.

There are also several other differences between the electrical activities recorded from pairs of cells. The number of transient "spikes" on the slow repolarization phase is almost never the same, and there is no evident synchronization in the firing of the "spikes". The durations of "spike" activity of a spontaneous burst and the duration of the total depolarizations also differ between cells (Figure 7).

A few instances occurred in which an infrequently observed type of electrical activity was recorded from simultaneously penetrated cells. In such cases small depolarizations preceded the initial depolarization of a spontaneous burst. When this abnormal activity occurred, it was generally recorded in both cells, but there were a few instances in which such activity occurred in one cell but not the other.

As stated in the description of intracellular activity, it was observed that it was possible to induce a continuous firing of "spikes" in some cells by manipulation of the microelectrode. During penetration of pairs of cells it was possible to obtain one cell with normal electrical activity and the second cell with a continuous firing of "spikes". However, no evident effect of the continuously firing cell upon the normal cell was seen.

Figure 6. Spontaneous intracellular electrical activity recorded simultaneously from a pair of unipolar cells. Three bursts of activity are shown. Cell I (top trace) has 12 "spikes" on its slow wave of repolarization, while Cell II (bottom trace) has 11 "spikes". The durations of the total depolarizations measure 3.6 sec for Cell I and 3.2 sec for Cell II. Note the slow depolarization in Cell I, while only a slow repolarization is present in Cell II. Time scale: 1 sec. Voltage: 10 mv.

Figure 7. Simultaneous activity recorded from a pair of cells in which the durations of "spike" activity differ. The duration of "spike" activity for Cell III in the top trace is about 1500 msec, while that in Cell IV (bottom trace) is about 1400 msec. Time scale: 500 msec. Voltage scale: 10 mv.

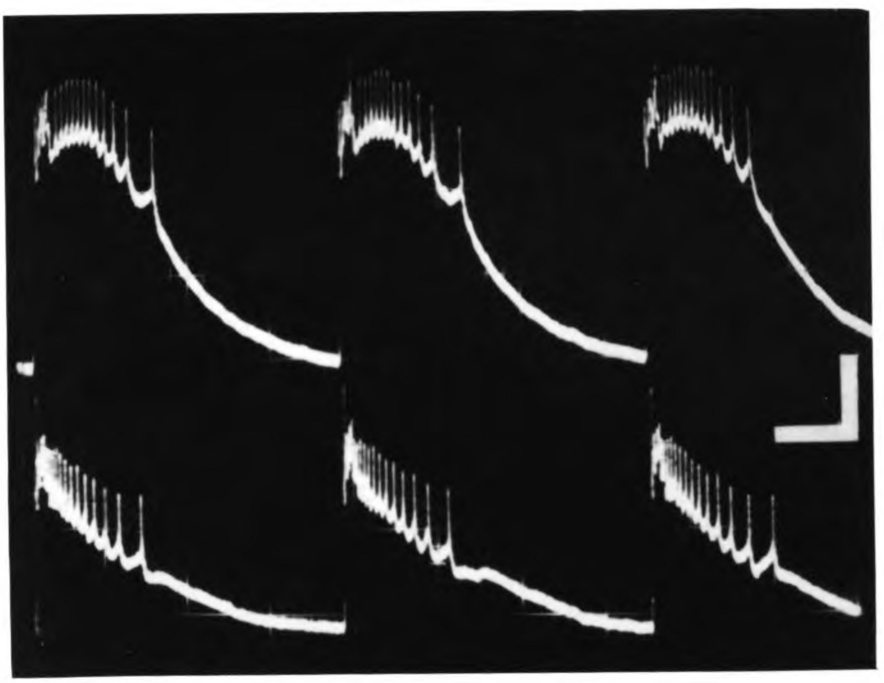

Figure 6

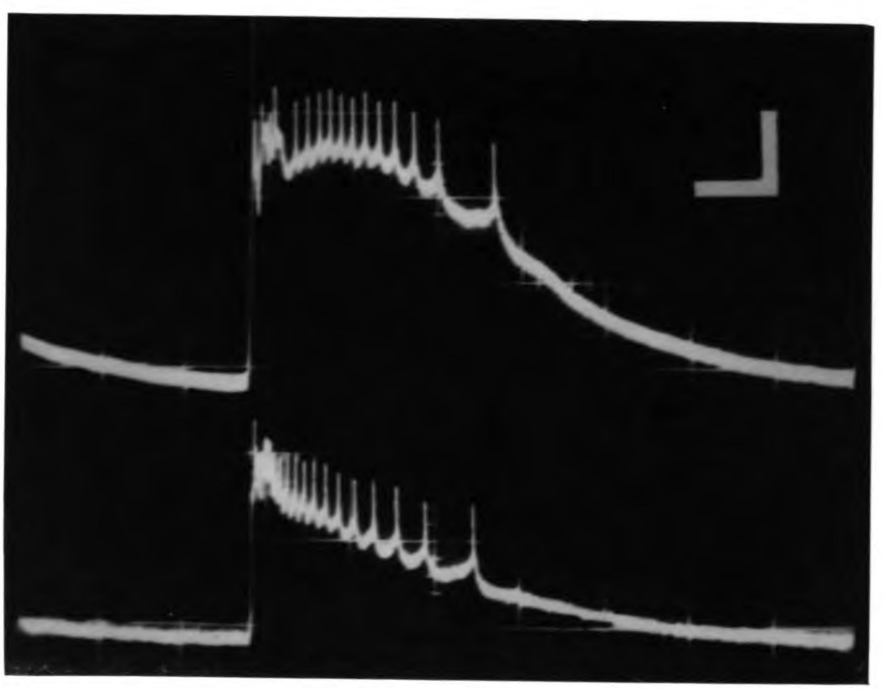

Figure 7

Injection of current into one of two impaled cells

In order to obtain more information concerning junctions between unipolar ganglion cells, depolarizing and hyperpolarizing currents were passed through a microelectrode into one of two simultaneously impaled cells. The intracellular activity of the second unipolar cell was examined during the passage of current. A total of six animals were used in these experiments. The effects of depolarizing currents were examined in 20 pairs of cells; the effects of hyperpolarizing currents in 17 pairs of cells.

If tight (electrical) junctions exist between cells, then it is expected a priori that there would be an electrotonic spread of applied current from one cell to the other through the tight junctions. However, if synapses exist between the cells, and, if it is assumed that the depolarizations induced by depolarizing currents are action potentials in the axons (see Discussion), then the induced depolarizations would result in some sort of potential change in the non-stimulated cell.

Figure 8 shows a typical result of experiments of this kind. Depolarizing currents of subthreshold strength and hyperpolarizing currents of varying strengths applied to Cell VI during the quiet period produced no visible change in the non-stimulated cell (Cell V). As mentioned above, depolarizing currents applied during "spike" activity on the slow repolarization phase increased the number of "spikes" in the cell being stimulated, but no corresponding increase in the number of "spikes" was observed in the non-stimulated cell. Conversely, hyperpolarizing currents applied to Cell VI during the period of "spike" activity did not lead to a decrease in the number of "spikes" in Cell V (Figure 8C, 8D).

Finally, depolarizing currents of threshold strength were applied to one of the cells during a quiet period. These currents were able to induce transient "spikes" in the cell being stimulated, but no transient changes in potential were seen in the second cell.

Figure 8. Simultaneous recordings from a pair of unipolar cells in which current is applied intracellularly to one cell of the pair.

Figure 8A. Normal spontaneous intracellular electrical activity. In the following traces the activity is from this same pair of cells. Cell V (non-stimulated cell) is represented in the top trace, and Cell VI (stimulated cell) in the bottom trace.

Figure 8B. A current of 1.0×10^{-9} A was applied to Cell VI. The current was sufficient to induce "spikes" in Cell VI, but no evident change in potential is seen in Cell V.

Figure 8C. The applied current was increased to 2.0×10^{-9} A. The number of "spikes" resulting from the current input into Cell VI is larger than for a normal burst, and the latent period decreased when compared to Figure 8B.

Figure 8D. A hyperpolarizing current of 1.3×10^{-9} A was applied to Cell VI. Again, no evident change is seen in Cell V.

Time scale: 1 sec. Voltage scale: 60 mv, Figure 8A, 8B and top trace of 8C; 120 mv, bottom trace of Figure 8C, and Figure 8D.

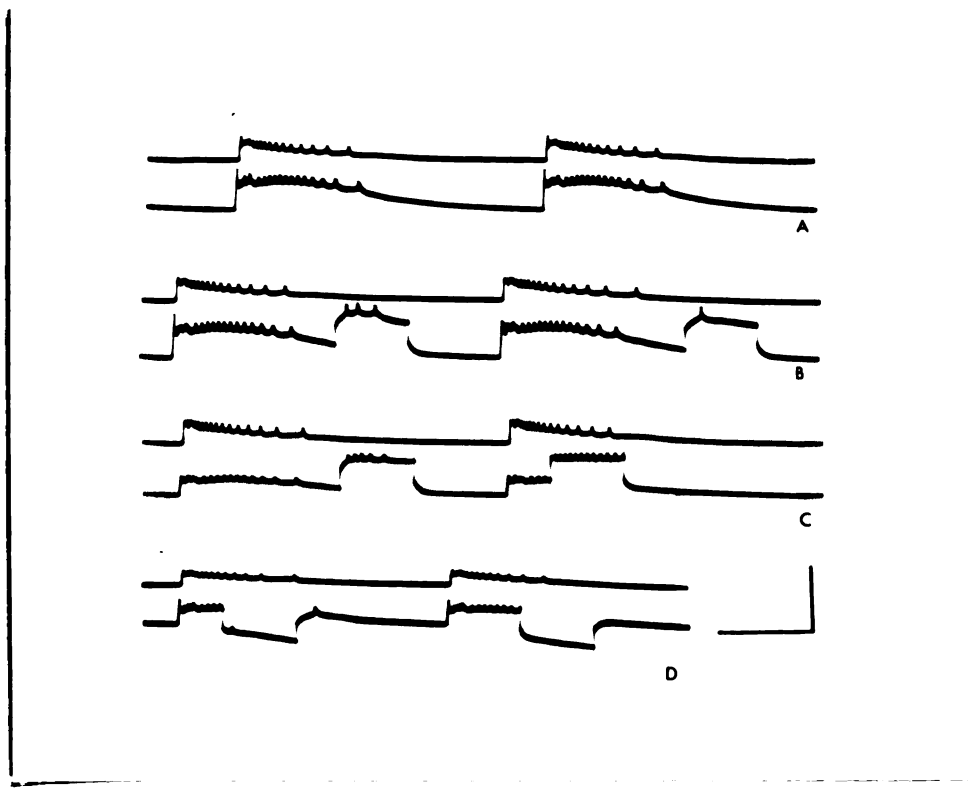

Figure 8

DISCUSSION

Origin of "Spikes"

The electrical activity of single units recorded extracellularly by Prosser (1943) is quite like that which I have recorded intracellularly. Prosser observed that the pattern of discharge of an axon usually had an initial high frequency of impulses, sometimes a pause and finally a discharge at decreasing frequency. Table I shows that the spike-like depolarizations have an initial frequency of 50 "spikes"/sec. A fast repolarization phase with no attendant "spikes" was sometimes recorded intracellularly and varied in duration from a few to about 120 msec. This may possibly explain why Prosser sometimes found a pause during spike activity. After the repolarization, the frequency of "spikes" declined to 15/sec.

The similarities between my intracellular recordings and the extracellular recordings of Prosser suggest that the "spikes" recorded in the soma are action potentials which are being conducted in the axonal processes of the unipolar cells. If this is the case, then the small size of these depolarizations in the soma is probably due to a lack of active propagation of the action potentials into the soma -- the potentials seen being a simple electrotonic spread of the "spikes" into the soma.

As previously mentioned, it was often seen that "spikes" could be made to fire continuously from one intracellular burst to another. This is possibly due to the fact that some stretch was exerted on the cell membrane. Bullock and Terzuolo (1957) observed that some cells in the lobster which initially had simple follower activity subsequently

acquired interburst spikes of a presumably spontaneous sort. In at least one case this was associated with manipulation of the microelectrode. Thus, it is possible that such a situation could have resulted in our observations. This lends support to the suggestion that the "spikes" are initiated within the unipolar cell which is being penetrated.

From the data obtained by intracellular recordings, a model of the unipolar cell can be derived. It seems that the unipolar cell of the cardiac ganglion of L. polyphemus is a case in which the "spikes" are neither initiated in nor invade the cell soma. An active membrane region of high excitability is present at some distance from the soma. When this region is excited by a decrease in membrane potential, the region responds by producing a number of "spikes" which then spread into the soma electrotonically.

This model is a fairly common observation in nerve cells. Coombs et al. (1959) found that in the cat motoneuron depolarization elicits a spike in the axonal membrane and this spike eventually results in the spike in the soma by means of electrotonic spread. Ito (1957) obtained similar results with spinal ganglion cells of the toad.

According to Edwards and Ottoson (1958) initiation of the spike potential of the stretch receptor of the crab also occurs in the axonal membrane some distance away from the soma. The large cardiac ganglion cells of the lobster and the giant neuron of Aplysia are further examples (Hagiwara, 1961; Hagiwara and Bullock, 1957; Hagiwara et al., 1959; Otani and Bullock, 1959; Tauc, 1962a, 1962b; Watanabe, 1958). Since the somata of the unipolar cells in L. polyphemus are distant from sites of initiation of the "spikes", and because they probably do

not actively conduct action potentials under normal physiological conditions, it is possible that the cell bodies have little role to play in the processes of impulse initiation and transmission.

On the basis of this model the data from intracellular current injections can be readily explained. Depolarizing currents of sufficient intensity resulted in transient depolarizations similar to the "spikes" appearing in the soma. Because these induced "spikes" are similar in size and shape to the intracellularly recorded "spikes", it is presumed that both of these depolarizations are initiated in the same region of the neuron. Since the "spikes" are not overshooting in the soma, they probably arise in the axon at some distance from the cell soma and spread into the soma electrotonically.

The depolarization of the soma membrane caused by the application of depolarizing currents spread electrotonically into the active membrane region of the cell. The active region became excited and responded by producing a number of "spikes". Increases in depolarizing current would cause the active region to become more excitable and to respond with a decreased latent period and an increased number of "spikes".

Hyperpolarizing currents resulted in a decrease in the number of "spikes", but increasing the intensity of the hyperpolarizing current failed to increase the amplitude of the "spikes". No reversal in the polarity of the "spikes" was ever observed with depolarizing currents. These results can be explained on the basis that when the membrane potential of the nerve cell is increased there is sufficient spread of the current, such that the "spikes" are blocked at the active region of the cell, because the active region becomes less excitable.

From these observations it is concluded that the "spikes" are probably not postsynaptic potentials. The only other alternative is that the "spikes" represent action potentials occurring at some location in the cell other than the soma. In addition, since the excitability of the active region is changed fairly easily by intracellular current inputs, it seems possible that the active region is either located fairly closely to the cell soma or is extremely sensitive to small changes in membrane potential.

Origins of Sustained Depolarization

The sustained depolarization recorded in the cell soma appears to be biphasic and is suggestive of two separate events occurring in the unipolar cell. The first event is the initial depolarization and its subsequent repolarization. This is superimposed upon the second phase, the slow repolarization.

In such a situation the biphasic activity may be the result of two or more different types of input to the cell or one type of input to the cell with a number of inputs located at various distances from the cell soma. A third possibility is that one or both of the sustained depolarizations is endogenous to the cell itself. In the first case one input would cause the fast-rising initial depolarization and another input the slow repolarization. These postsynaptic responses then summate to give the activity seen in Figure 2. In the second case the initial depolarization could be the result of one or more inputs located at varying distances from the cell soma. The postsynaptic responses of the unipolar cells to the distant inputs would summate to

form the slow repolarization seen in the soma. The third possibility suggests that one or both of the depolarizations is initiated within the cell itself. If only one of the depolarizations is endogenous, the other may be the result of a synaptic input.

Although no quantitative measurements could be made, the suggestion that the initial depolarization and slow repolarization phases are postsynaptic responses is based on the observations that depolarizing currents caused the amplitudes of the two activities to decrease. On the other hand, hyperpolarizing currents caused the amplitudes of the two responses to increase. These results appear contradictory to the results expected if these activities were endogenous to the nerve cells themselves, but agree with the results expected if the initial depolarization and slow repolarization are responses of the unipolar cells to a synaptic input.

Interconnection Between Cells

In the cardiac ganglion of the lobster the interactions among neurons occur through synapses and electrical connections (Hagiwara, 1961; Hagiwara et al., 1959; Watanabe, 1958; Watanabe and Bullock, 1960). In Squilla, on the other hand, no synaptic activity is present between cells in the ganglion. Transmission occurs solely by electrical connections (Watanabe and Takeda, 1963). Between the unipolar ganglion cells of L. polyphemus no connections appear to be present. When electrodes were implanted in two unipolar cells and one of the cells was stimulated through the microelectrode, no potential changes were observed in the second cell. Watanabe (1958) did find an attenuation

factor of 0.16 to 0.48 between two large cardiac ganglion cells of the lobster. If electrical connections are present between unipolar cells in L. polyphemus, the attenuation factor would have a value much lower than this. Thus, the connections would probably be of little or no physiological significance. In addition, potential changes of a fast time course, such as "spikes", do not appear in a one to one synchrony between cells, indicating that these also are not conducted across electrical connections.

Another alternative is synaptic connections between unipolar cells. If it is true that the depolarizations induced by a depolarizing current are action potentials, then it is expected that the spikes would be conducted along the axon to the nerve endings. The synaptic excitation or inhibition process initiated in the stimulated cell would cause post-synaptic responses in the non-stimulated cell which would lead to an increased or decreased number of "spikes" in an intracellular burst. However, no such events were seen. If there were a monosynaptic connection between the two cells, a one to one ratio of presynaptic impulses to postsynaptic responses is expected, which would result in changes in the non-stimulated cell. This does not appear to be the case. These observations seem to eliminate the possibility of synapses between cells.

The last alternative is that synchronization of the intracellular electrical activity of the unipolar cells is a result of a common input. The abnormal activity mentioned in the results supports this suggestion, for abnormal activity preceding an intracellular burst is generally present in both cells. However, a situation such as this does not shed further light on the role of the unipolar cells as either pacemaker cells or motor neurons.

As previously stated, the cardiac ganglion of L. polyphemus has a number of potential pacemakers. It is possible that the unipolar cells recorded from are potential pacemakers, but are subservient to a primary pacemaker. Thus, these cells would receive a common input. The same situation occurs if the cells are motor neurons with common inputs from the pacemaker.

Specific Membrane Resistance

The specific membrane resistance of the unipolar cells of L. polyphemus is much larger than that reported for many vertebrate cells (Araki and Otani, 1955; Coombs et al., 1959; Frank and Fuortes, 1956; Hagiwara and Saito, 1959; Ito, 1957), but is of the same order of magnitude as those observed in some invertebrate nerve cells (Fessard and Tauc, 1956; Otani and Bullock, 1957, 1959). The wide range of values for unipolar cells of L. polyphemus is probably due to differences in the sizes of the cells. Furthermore, the specific membrane resistance of cells of L. polyphemus are the same as those from the Crustacean cardiac ganglion cells (Otani and Bullock, 1957). There is possibly some relationship between the membrane resistance of a cell and whether an actively propagated response occurs in the soma.

Figure 5 shows that the change in potential is directly related to the amount of current applied. In nerve cells of other animals a good linear relationship is observed until a certain level of depolarization is reached. Beyond this level the depolarization is lower than that expected from linear proportionality. This phenomenon is sometimes referred to as delayed rectification (Hagiwara et al., 1959; Kandel and

Tauc, 1966; Otani and Bullock, 1959; Tauc and Kandel, 1964). The fact that the unipolar cells of L. polyphemus do not show delayed rectification may be due to the fact that the cells are surrounded by a capsule of connective tissue which could contribute to the effective resistance of the membrane (Bursey and Pax, in press).

Kusano (1966) determined that the resistance of a myelin sheath layer of 10 u thickness, which encompasses the giant nerve fibers of a kuruma shrimp, to be on the order of 2.3×10^5 ohm. A value of one order of magnitude larger than this as a result of the connective tissue capsule could be significant in measurements from the unipolar ganglion cells. Measurements of the resistance of the capsule surrounding the unipolar cells in L. polyphemus proved difficult. Such a study is necessary to conclude whether delayed rectification does occur in these cells.

Function of the Unipolar Cells

The intracellular electrical activity recorded from unipolar cells of the cardiac ganglion of L. polyphemus is extremely similar in size and shape to the electrical activity of a simple follower (Type A) cell obtained from crustacean cardiac ganglion cells (Bullock and Terzuolo, 1957; Hagiwara and Bullock, 1955, 1957; Watanabe, 1958). However, the electrical activity from L. polyphemus appears to lack the slow depolarizing postsynaptic potentials found in the Crustacea. This is possibly due to the fact that the site of synaptic input to the unipolar cells of L. polyphemus is further removed from the cell body. Thus, due to the electrical properties of the cell membrane, the postsynaptic

potentials may be so attenuated that no deflections are visible in the record from the cell soma. Another possible explanation is that the unipolar cells have no synaptic input, thus no synaptic potentials are expected. The results from these experiments cannot give support to either possibility.

Although the evidence is by no means conclusive, the results suggest that the unipolar cells in L. polyphemus are of the follower type. The initial depolarization and slow repolarization may be the result of a synaptic input. These postsynaptic responses would cause an excitation of an active membrane region within the cell. A series of action potentials is then set up within an axon and may eventually result in contraction of cardiac muscle. The action potentials spread electrotonically into the soma and are seen as small deflections of five to seven mv.

Since connections between unipolar cells have not yet been shown to exist, the idea that these cells are pacemakers remains a remote possibility. If new sites of pacemaker activity can be set up throughout most of the ganglion, as shown by Garrey (1932), then it is expected that the new pacemaker would excite the remaining unipolar cells by some physiological connection. However, no evidence to this effect has yet been found.

If, indeed, these cells are motor in function, there is still the possibility that these cells do have an endogenous rhythm which is prevalent only if the normal pacemaker has been destroyed. This may explain Heinbecker's (1933) observation that activity in large nerve fibers and in large ganglion cells can initiate activity in the small ganglion cells and his conclusion that the large ganglion cells act as

pacemakers. Before the function of the unipolar cells in the heartbeat of L. polyphemus is determined, the origin of the initial depolarization and slow repolarization has to be found by means of pharmacological experiments. In addition, neuronal processes of unipolar cells should be studied morphologically by intracellular dyes and physiologically by intracellular and extracellular recording techniques to determine the pathways of the neuronal processes.

SUMMARY

1. The electrical activity of unipolar cells of the cardiac ganglion of L. polyphemus was examined with microelectrodes to determine the electrical properties and the functional relationship between the unipolar cells.

2. Resting membrane potential in these cells averages 42 mv (SE = 1.8 mv).

3. Intracellular depolarization begins with a fast-rising initial depolarization of 23 mv (SE = 1.0 mv), which is maintained for 25 to 120 msec. This is then followed by a slow repolarization of about 16 mv amplitude. The total duration of both depolarizations has a mean value of 2.34 sec (SE = 0.16 sec).

4. A number of small (5 to 7 mv) spike-like potentials are superimposed on the depolarization. The frequency of the "spikes" is high on the initial depolarization (50 "spikes"/sec; SE = 2.1 "spikes"/sec) and declines to 15 "spikes"/sec (SE = 1.0 "spikes"/sec) on the slow repolarization.

5. Depolarizing currents of sufficient strength (mean = 1.1 X 10^{-9} A; range 0.6 to 2.5 X 10^{-9} A) can elicit "spikes" similar to those recorded in a normal burst. Currents up to 5 X 10^{-9} A fail to excite the soma membrane. The data suggest that the "spikes" arise at an active membrane region within the cell process and invade the soma electrotonically.

6. The specific membrane resistance for the soma membrane averages 12,700 ohm-cm^2. The time constant has a value of 19.6 msec. The specific membrane capacitance measures 1.54 uF/cm^2.

7. The unipolar cells appear to have no functional connections between them. This was determined by means of recording simultaneously from pairs of unipolar cells and injecting current into one cell of the pair.

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