Differences between Pacemaker and Nonpacemaker Neurons of *Aplysia* on Voltage Clamping

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ABSTRACT The responses of pacemaker and nonpacemaker *Aplysia* neurons to voltage clamp commands of less than 200 msec duration are essentially identical. For moderate depolarizing commands there is an early inward transient current followed by a late outward current and an outward tail current when the membrane is clamped back to resting potential. On long (1–2 sec) commands in pacemakers there is a marked sag in the late current and an inward tail current. E_{tail}, the potential of the membrane at which there is no net current flow under the conditions prevailing at the end of the clamp, shifts from about −9.0 mv on short commands to +5.0 mv on long commands. In contrast there is no marked sag of the late current or inward tail current on long commands in nonpacemakers, and E_{tail} is near −9.0 mv for both short and long commands. The current sag and shift in E_{tail} can be ascribed to a decreased conductance (presumably to K^+) at the end of the long as compared to the short command in half of the pacemaker neurons. In the remaining cells the essential difference from nonpacemakers appears to be either a greater restricted extracellular space or a more active potential-dependent electrogenic Na^+ pump in pacemakers.

INTRODUCTION

Spontaneous action potentials occurring in the absence of obvious synaptic excitation are found in a number of invertebrate neurons (Bullock and Terzuolo, 1964; Hecht, 1965; Krygsman, 1952; Preston and Kennedy, 1962). These action potentials are believed to arise from a pacemaker mechanism inherent to the cell and to be independent of activity in other neurons (Bullock and Horridge, 1965).

The existence of endogenous pacemaker activity in molluscan neurons is well-documented (Alving, 1968; Arvanitaki and Chalazonitis, 1961; Strumwasser, 1965; Tauc, 1957). In *Aplysia* neurons there exists a difference, crucial for the present experiments, in the site of origin of pacemaker and synaptically initiated action potentials. All synapses in these neurons are located on

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distal cell processes while the essentially spherical soma is without synapses (Rosenbluth, 1963); moreover Tauc (1962) has shown for one identified cell that upon synaptic excitation a conducted action potential is initiated in these distal cell processes and that this spike causes the initiation of the action potential in the soma.

In contrast the action potentials generated by the pacemaker mechanism arise in the cell soma. This fact was demonstrated by Alving (1968), who placed a tight ligature between the cell body and its processes and found that all synaptic potentials and synaptically initiated action potentials disappeared, while pacemaker activity remained essentially unchanged.

In the visceral ganglion of *Aplysia* approximately 50–75% of the large neurons exhibit spontaneous pacemaker discharge (Carpenter, 1967; Frazier, Kandel, Kupfermann, Waziri, and Coggeshall, 1967). The pacemaker discharge may be regular (beating pacemaker) or may consist of periods of discharge alternating with periods of silence (bursting pacemaker). Over 30 neurons of the visceral ganglion have been identified by size, location, synaptic connections, and discharge pattern (Frazier et al., 1967). With minor variation the identified cells tend to exhibit the same form of discharge from preparation to preparation, both as to the presence or absence and the pattern of pacemaker discharge. At least one pacemaker neuron exhibits two or more circadian rhythms (Strumwasser, 1965).

Pacemaker discharge in *Aplysia* neurons has been examined by Strumwasser (1965, 1967, 1968) and Waziri, Frazier, and Kandel (1965). Waziri et al. have interpreted their results as being consistent with the observations of Dudel and Trautwein (1958) and Trautwein and Kassebaum (1961) on mammalian Purkinje fibers, who suggest that pacemaker activity is a result of a high resting Na^+ conductance with a voltage- and time-dependent decrease in K^+ conductance following the action potential. Strumwasser (1967, 1968) has emphasized that metabolically active processes may also be of importance in pacemaker patterning. In many *Aplysia* neurons pacemaker discharge is greatly reduced or abolished without loss of electrical excitability by a 50% replacement of external Na^+ with $Tris^+$ or sucrose but is not abolished by complete replacement of Na^+ by Li^+, NH_4^+, or guanidinium (Alving and Carpenter, unpublished data). Certain cells, however, may discharge spontaneously in the complete absence of Na^+ provided Ca^{++} is present (Carpenter and Gunn, 1969).

The experiments reported here examine differences between pacemaker and nonpacemaker neurons upon voltage clamping of the neuronal soma. A preliminary report has been given (Alving and Frank, 1966).

METHODS

Preparation The abdominal ganglion of *Aplysia californica* was taken from the animal, pinned to paraffin in a modified dark-field chamber (Alving, 1968), and im-

mersed in flowing artificial seawater (Marine Magic, Lampert Kay Co., Los Angeles, Calif.) at a temperature of 18–22°C. The chamber was situated under a Zeiss otoscope. The connective tissue surrounding the ganglion was cut, and in some cases removed, freeing the superficial cells. In this study, the cells used were from 100–400 μ in diameter.

Electrodes Glass capillary microelectrodes filled with 3 M KCl were used. The tips of the electrodes were 2-4 μ outside diameter, and the electrodes had a resistance of 1-2 MΩ. After filling with KCl, the electrode was washed in distilled water, and silver conducting paint (Silver Print 21-1, G. C. Electronics, Rockford, Ill.) applied to the electrode wall to within 500 μ of the tip. The tips of the electrodes were then placed in 3 M KCl and the paint allowed to dry overnight. The silver was insulated by coating it with a thin layer of polystyrene (Polystrene Q-Dope, G. C. Electronics, Rockford, Ill.) dissolved in benzene. The effectiveness of this insulating coat was tested by checking the resistance between the inside of the electrode and the wall while the electrode was submerged in saline. Electrodes were discarded if this resistance was less than 50 MΩ. Shields for the electrodes consisting of 008 gauge silver wire were wrapped around the electrodes over the insulating coat.

Equipment Two or occasionally three microelectrodes, each mounted on a Prior (Sobotka, New York, N.Y.) manipulator, were inserted into a neuron. In order to demonstrate satisfactory penetration all electrodes were initially used to record transmembrane potential. This was done through two unity gain (Bak M-4) amplifiers, with the outputs displayed on a Tektronix 565 oscilloscope.

After demonstration of satisfactory penetration, one electrode was used to supply transmembrane current from the clamping amplifier. The current electrode was required to be capable of passing 100 μamps for 2 sec in the extracellular solution. The remaining electrode was used to record membrane potential throughout the experiment. The shield of the recording electrode was driven by the cathode of the unity gain preamplifier. The shield of the current electrode was grounded.

The clamping system consisted of a Tektronix G unit plug-in amplifier (with Tektronix 133 power supply) and a Philbrick (USA-4-JX) high voltage–high current operational amplifier. The output from the Bak amplifier and the clamp command signal were fed to the G unit. The output of the G unit was fed through the 565 and the output of the 565 into the Philbrick, then to the current electrode. The output of this clamping system was proportional to the difference between the command pulse and the transmembrane potential and in a direction such as to decrease the difference between the two potentials by virtue of the IR drop across the cell membrane.

Current was measured through a heavily chlorided (low impedance) silver wire placed in the bath which was connected directly to the positive input of a high gain operational amplifier (Fairchild 702, current range used 10^{-6}–10^{-8} amp) with a source follower input (field effect transistor, 3821, Texas Instrument, Inc., Dallas, Tex.). The output of the current-measuring amplifier was displayed on the oscilloscope along with the outputs of the two Bak amplifiers.

Before clamping current was applied to the cell, the difference between ground and cell membrane potential was compensated by a voltage offset adjustment of the 702

through a potentiometer and battery. When this was accomplished there was no current flow through the cell when clamped at its resting potential.

Most of the capacitative coupling between the current and recording electrodes was eliminated by the shielding of the electrodes. This coupling was further reduced by a cross-neutralization amplifier (Fairchild 702 with source follower as above) (Nelson and Frank, 1964). When all neutralization and compensating circuits were optimally adjusted, the time required to clamp the membrane to a 100 mv step was less than 100 μsec.

The membrane was clamped intermittently, usually for a period slightly longer than the duration of the voltage step applied to the membrane. Just before the clamp was applied to the membrane the current electrode was switched from the grid of the unity gain amplifier and connected to the output of the clamping amplifier. With only intermittent voltage clamping of the membrane, the cell did not appear to be damaged by depolarization of 1 or 2 sec. Evidence for the lack of damage was derived from a comparison of the transient inward current-voltage relations and the spontaneous activity of the cell at the beginning and end of the experiment, neither of which was found to change in a consistent fashion.

Satisfactory insertion of two microelectrodes into a soma required that both electrodes record the same resting and action potentials and that insertion of the second electrode did not permanently alter either of these.

RESULTS

Spontaneous Activity of Neuron Recorded with Intracellular Electrodes

After successful placement of the two electrodes in a cell, the cell was classified as a "pacemaker" or "nonpacemaker" neuron according to its spontaneous activity (Alving, 1968). In Fig. 1, A 1 and 2 show the potentials recorded during spontaneous activity of a nonpacemaker; i.e., a cell which fires only when receiving synaptic input from another cell. In these cells the resting membrane potential remains constant except for alterations caused by synaptic potentials. Action potentials are generated only by excitatory synaptic potentials, and in this cell these appear as a convexity at the foot of the spike. Fig. 1, B 1 and 2 show the potentials recorded from a pacemaker neuron. The spikes arise from a slow depolarization of the membrane, and the rising foot of the spike is concave (Alving, 1968). Fig. 1 C shows the activity of a specialized form of pacemaker, which alternates between periods of discharge and periods of silence (Arvanitaki and Chalazonitis, 1961). Further criteria for distinguishing pacemaker and nonpacemaker spikes have been published (Alving, 1968), but for the purpose of these experiments discharge patterns were usually distinguished by examination of the rising foot of the spike.

In order to make meaningful measurements of the current-voltage relations obtained it was necessary to demonstrate that the soma was isopotential when the membrane was clamped (Cole, 1961). To do this three electrodes were inserted into the soma of a neuron. The clamping current was supplied with

one electrode while the membrane potential was fed back to the clamping amplifier with a second electrode. The third electrode recorded the transmembrane potential at different positions within the cell soma. The results of one experiment are shown in Fig. 2. In all such experiments, the potentials from the recording and monitoring electrodes were essentially identical.

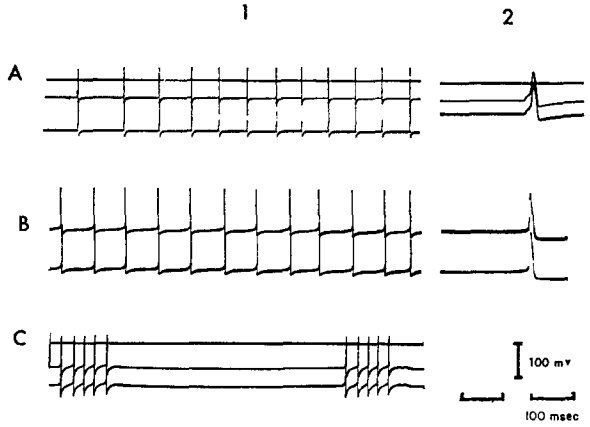

FIGURE 1. Types of spontaneous activity in *Aplysia* neurons. A 1 and 2 are records from an active nonpacemaker. The discharge is regular but spikes are always preceded by a synaptic potential. In such a cell the frequency of discharge is not affected by small changes of membrane potential. B and C show two types of pacemaker activity where no synaptic potentials are present (B 2).

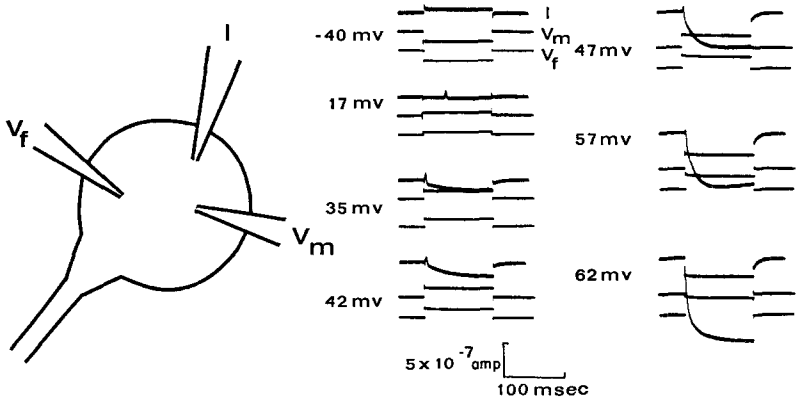

FIGURE 2. Evidence for soma isopotentiality under voltage clamp. Three electrodes were independently inserted into the neuron, one to measure current (I), one voltage electrode fed back to the clamping amplifier (V_f), and a second voltage electrode (V_m) to monitor voltage at other sites within the neuron. Records from each of these three electrodes are shown for seven different clamping commands. In all cases the potential recorded by V_m is identical to that recorded by V_f. In this and all following records outward, positive current is shown as increasing downward.

These experiments provided evidence that the soma membrane of the neurons studied could be assumed to be effectively clamped. The axon, however, was probably never clamped for any great distance, for frequently the clamping current trace showed small transients probably due to action potentials generated in the axon by the depolarizing clamp pulse.

Responses of Pacemaker and Nonpacemaker Somata to Short and Long Commands

Over 150 neurons were studied and complete current-voltage curves were obtained in 53. The current-voltage relations for pacemaker and nonpacemaker

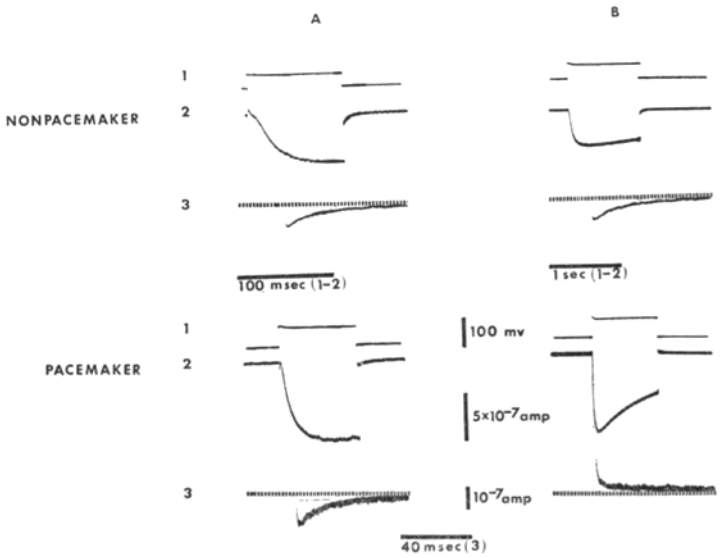

FIGURE 3. Representative records of responses from pacemaker and nonpacemaker neurons to short (A) and long (B) voltage commands. Trace 1 shows recording from the voltage electrode, trace 2 from the current-measuring system, and trace 3 is a higher gain faster sweep illustration of the tail current beginning at the moment at the end of the command pulse.

somata were similar for command potentials of 100 msec or less in duration, but striking differences became apparent upon longer commands. Representative illustrations of the membrane potential changes and associated clamping currents observed in pacemaker and nonpacemaker neurons are shown in Fig. 3. The upper trace in each record represents the potential measured by the recording electrode, while the middle trace shows the clamping current. The current illustrated is primarily the late, presumably K^+, current, since the large, brief current surge which charges the membrane capacitance is too rapid to be seen in these records, while the early current changes from inward to outward at commands of about this amplitude and is better seen in Fig. 4. The lowest trace is at higher amplification and expanded

sweep of that portion of the current trace beginning at the end of the clamping step. When the membrane was clamped back to the resting potential the clamping current did not immediately return to zero. The residual current, which will subsequently be called the tail current, implies that the membrane conductance does not instantaneously return to its resting value. Trace 3 illustrates the direction and time course of the tail current.

The 100 msec commands result in current flows which are essentially similar in the pacemaker and nonpacemaker cells. The K^+ current at these

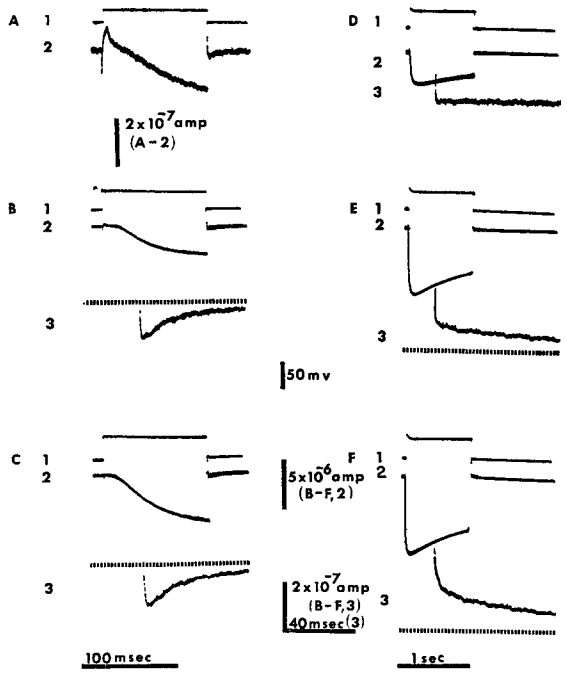

FIGURE 4. Short and long voltage clamp commands of different amplitudes in a pacemaker neuron. Trace 1 is voltage, trace 2 is current, and trace 3 is high gain, expanded sweep of the tail current.

large depolarizing commands is large and outward and reaches a plateau in about 50 msec. In both cells there is an outward tail current when the membrane is once again clamped to resting potential (trace 3). In B, currents resulting from 1 sec commands are shown. The response of the nonpacemaker is relatively little different from the response to the 100 msec command, but the current flow during the long command in the pacemaker differs from all the other records in two important ways. There is a dramatic sag in the current required to clamp the membrane with time (in this cell the current at the end of the command is about 50% that of the peak). In addition the tail current, which in the other records was outward, is now inward.

Fig. 4 illustrates the effect of variation of the amplitude of short and long commands in a pacemaker neuron. A-C show the responses to 100 msec commands, while D-F are 1 sec commands. As in Fig. 3, trace 1 represents voltage command, trace 2 is current, and trace 3 is an expanded, high gain trace of the tail current. The gain in trace 2 is greater in A than in the remaining sequences in order to show the early inward current.

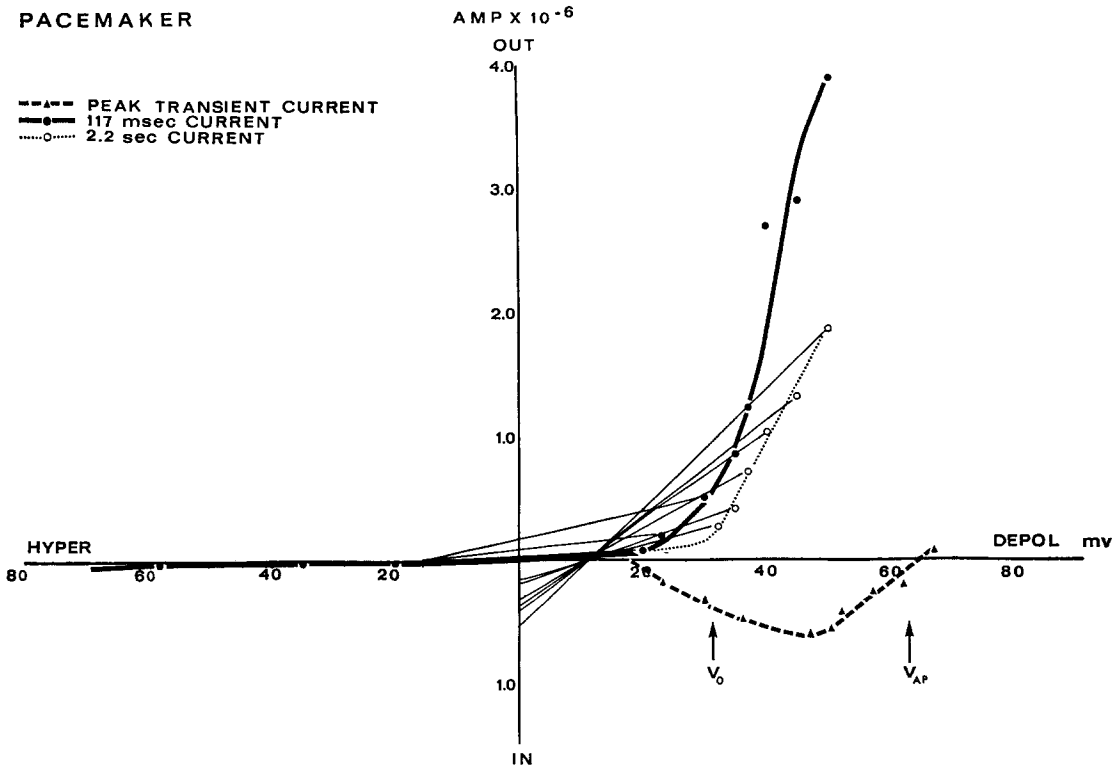

FIGURE 5. I–V relations for short and long commands in a pacemaker neuron (cell R_{15}). V_0 represents zero potential, V_{AP} represents the peak of the action potential, and the origin is set at resting potential. See text for further description. No leak corrections have been made.

At least for short commands (A-C) the current-voltage relation is a function of the membrane conductance after the capacitative transient. For hyperpolarizing command potentials the steady-state current is small and inward, and increases with increasing amplitude of command pulses. For small, depolarizing command potentials, the clamping current is steady and outward. With depolarization of over approximately 20 mv there appears an initial inward current surge followed by a delayed outward current which gradually increases to a steady value in approximately 100 msec (Fig. 4 A).

The time required for the delayed outward current to reach a steady value decreases slightly with increasing amplitudes of depolarizing command potentials. With still larger depolarizations, above 60–100 mv, both the early and late membrane currents were outward. For any cell, the membrane potential at which the initial current became zero was either the value of the membrane potential at the peak of the unclamped spike or slightly higher. These findings are similar to those reported by Frank and Tauc (1964) in *Aplysia* and by Hagiwara and Saito (1959) for *Onchidium*.

The amplitude of the tail current increased with increasing amplitude of the voltage command for short commands (A-C). However, at small but long commands the outward tail current became less as the command increased.

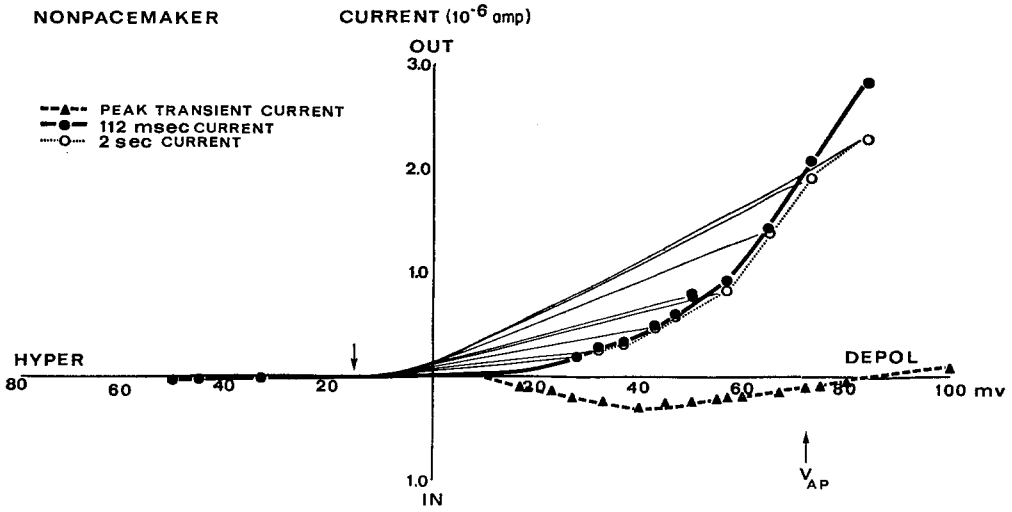

FIGURE 6. I–V relations for short and long commands in a nonpacemaker neuron.

In D, at a command of 35 mv, the tail current is essentially zero, and a sag in the level of the delayed current during the command is apparent. With larger commands the tail current becomes inward, and its value becomes progressively greater with further increases in command potential (E-F). Concomitant with the tail current changes there is an increase in the amount of sag of the delayed current, beginning after 50–100 msec. In nonpacemakers there was relatively little sag even when the clamp was maintained for up to 5 sec.

Figs. 5 and 6 are graphical representations of the current-voltage (I-V) relations obtained from typical pacemaker and nonpacemaker neurons, respectively. In both figures the triangles refer to the initial surge, the filled circles show the current-voltage relations at the end of the short command pulse, and the open circles the end of the long command pulse. In Fig. 5 the

sag in the current for long command pulses in pacemaker neurons is reflected in the shift to the right in the I-V plot for the long command pulses as compared to the short commands. In contrast the I-V plots for short and long commands in nonpacemaker neurons (Fig. 6) are essentially identical.

Since the membrane conductance does not immediately return to its resting level, it is possible to determine the potential at which there would be no net current flow under the conditions prevailing at the end of the command potential. The current at the end of the command potential and the initial tail current can be assumed to be transmembrane currents at two voltages with the same chord conductance. If it is assumed that there is no instantaneous rectification, the slope of a straight line connecting these points on the I-V plot gives an estimate of the membrane chord conductance at the end of the command potential,

$$Gm = \frac{I_c - I_t}{V_c - V_t}$$

where I_c is the current at the end of the command potential, I_t is the initial value of the tail current, V_c is the command potential, and $V_t = 0$ (i.e., resting potential). Extrapolation of this line to the abscissa gives the potential relative to resting potential at which no net current flows, E_{tail}, for the membrane under the conditions prevailing at the end of the command (Hagiwara and Saito, 1959).

The thin lines in Figs. 5 and 6 show the measured slope conductances at the end of short and long commands in pacemaker and nonpacemaker neurons. In the pacemaker neuron (Fig. 5) the tail currents are all outward for short commands and E_{tail} is -16 mv. For long commands all tail currents are inward and the conductance plots all cross the zero current axis at $+9$ mv. Thus, in this cell the equilibrium potential of the membrane at the end of the commands has shifted in the depolarizing direction by a total of 25 mv as the command duration was increased from 100 msec to 1 sec.

Current-voltage relations and values for E_{tail} were obtained for both short and long commands in 30 cells. Table I shows the average values for E_{tail} with range and standard errors for pacemaker, nonpacemaker, and pacemaker neurons with synaptic activity. In all three types of neurons the average E_{tail} for 100–200 msec commands was between -8 and -10 mv (hyperpolarized relative to resting potential). However, for long commands there was considerable difference in the responses of pacemaker and nonpacemaker neurons. There was a general tendency for E_{tail} for long commands in pacemakers to have values on the depolarizing side of the resting potential (average $+13.0$ mv). In contrast the E_{tail} for long commands in nonpacemakers was -5.9 mv. All but three cells classed as pacemakers showed a difference in E_{tail} between long and short commands of greater than 12 mv, and in but one

cell was the change less than 5 mv. In contrast the change in E_{tail} was greater than 9 mv in none. In no cell of either group was the E_{tail} for long commands more negative than for short commands.

In many *Aplysia* neurons action potentials are initiated by both synaptic and pacemaker mechanisms. Complete I-V relations were obtained for both long and short commands in 10 cells in which both pacemaker and synaptic spikes occurred spontaneously. Part C of Table I gives the average values and ranges of E_{tail} in these cells. As in the pacemaker cells, most of these cells showed some decrease in the clamping current with long commands, but the decrease was frequently not as marked as in pacemakers. The tail current, outward for short commands, was less and in some cases became inward on long commands. Also the E_{tail} for long commands was intermediate between that of the pacemaker neurons and the nonpacemakers (average +0.5 mv).

TABLE I

	Millivolts relative to resting potential
A. Pacemakers (8 cells)	
E_{tail} (short)	−8.0±1.4 (range −4 to −16)
E_{tail} (long)	+5.0±1.9 (range −3 to +12)
E_{tail} (short)−E_{tail} (long)	13.0±2.7 (range 4 to 25)
B. Nonpacemakers (12 cells)	
E_{tail} (short)	−9.8±0.2 (range −4 to −14)
E_{tail} (long)	−5.9±1.0 (range −1 to −13)
E_{tail} (short)—E_{tail} (long)	3.25±0.8 (range 0 to 9)
C. Pacemakers with synaptic activity (10 cells)	
E_{tail} (short)	−9.3±0.8 (range −5 to −12)
E_{tail} (long)	+0.5±1.2 (range −4 to +6)
E_{tail} (short—E_{tail} (long)	9.8±1.4 (range 3 to 16)

In the pacemaker neurons E_{tail} shifted gradually in a depolarizing direction either with increasing amplitude or with increasing duration of the command potential beyond about 200 msec. This suggests that the shift might be a function of the total charge crossing the membrane. Fig. 7 shows the values of E_{tail} in a pacemaker neuron plotted as a function of the total net charge lost by the cell at three different amplitudes of command potential. The total charge was taken from a planimeter measurement of the current trace during the command. The fact that the three curves do not superimpose indicates that changes in the equilibrium potential of the tail current are not simply dependent upon the total net charge lost by the cell.

The time taken for the membrane conductance to return to its resting value was determined in four cells. The return to resting conductance was measured by applying two command potentials separated by varying inter-

vals. The first command was of sufficient duration and amplitude to cause a decrease in the transmembrane current after the first 150 msec, and to cause the E_{tail} at the end of the command to become depolarizing relative to the resting potential (a command of 60–80 mv and 1–2 sec). The second command was of approximately equal amplitude but of 100 msec duration. The second command was given at varying times after the first, and the value of E_{tail} measured was compared to E_{tail} when the 100 msec command was given alone. In three neurons the interval between commands was not greater than 600 msec and in none of these cases had E_{tail} returned to control values. In one neuron, illustrated in Fig. 8, the interval between commands was in-

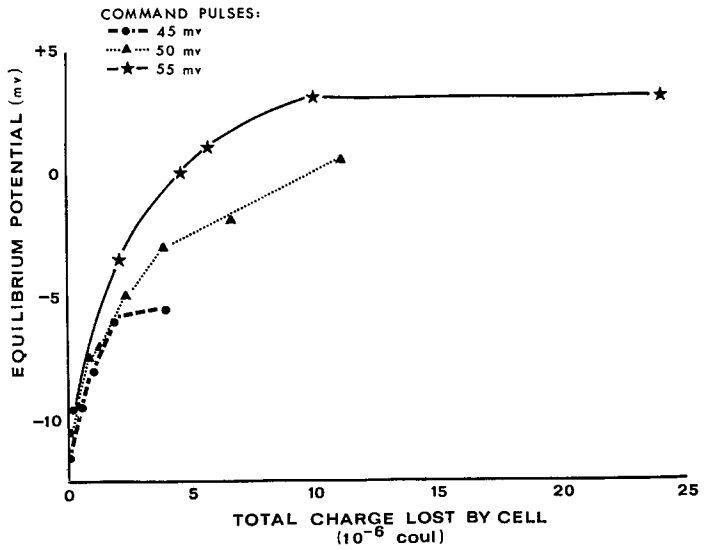

FIGURE 7. Shift of E_{tail} as a function of the total charge lost by the cell from clamps of varying durations and three different voltages from an experiment on a pacemaker neuron. Charge lost was measured by planimeter from the current trace.

creased in a stepwise fashion up to 1430 msec at which time E_{tail} had returned to its control value (filled circles). It is worthy of note that at 1200 msec after the long command E_{tail} was essentially the same as immediately after the long command.

As is also shown in Fig. 8, a marked difference in the behavior of the cell was observed depending upon whether the cell was voltage-clamped or current-clamped. A current clamp was applied by using a long constant current pulse in place of the long voltage clamp command, adjusted so that the total current which flowed was the same in both. The current pulse was followed as before by a 100 msec voltage clamp command. The results of this experiment are shown by the triangles in Fig. 8. Under conditions of the current clamp

the shift in E_{tail} was of much smaller amplitude and shorter duration, being essentially back to the control value within 400 msec.

Pacemaker and nonpacemaker cells did not show significant differences in the rate of development of delayed current. This was estimated roughly by determining the minimum depolarizing command at which the outward current was no longer increasing at the end of the 100 msec pulse. This is, of course, not a very precise measurement of this rate but any dramatic difference in the rate of turn-on of delayed rectification should have been made ap-

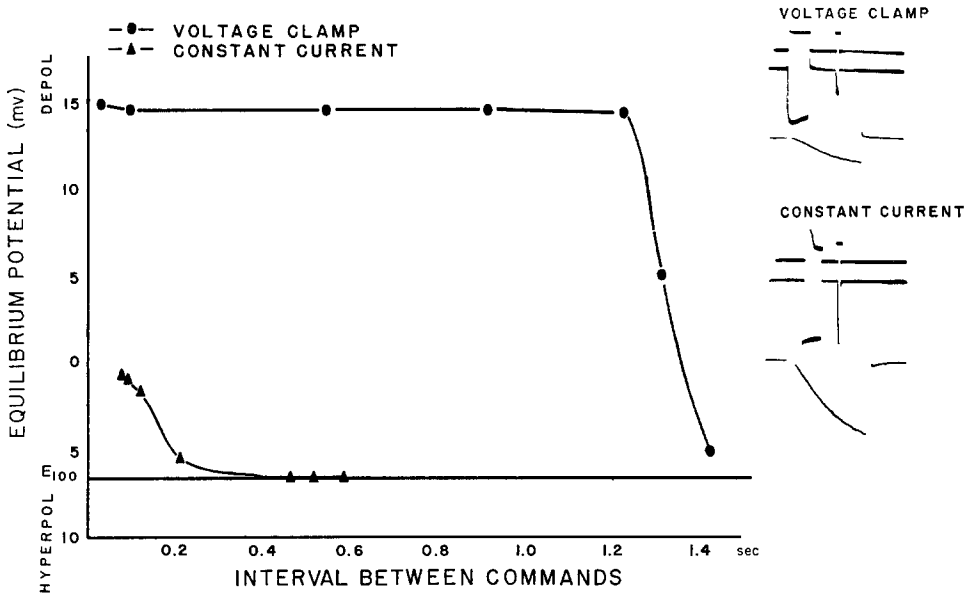

FIGURE 8. Time required for return of E_{tail} to initial values under voltage and current clamp conditions. In both cases a 1 sec clamp was followed at intervals by a 100 msec voltage clamp to test recovery. Initial E_{tail} is indicated as E_{100}. Insets show paired commands under voltage (upper) and current (lower) clamps. Upper trace of insets is voltage, middle is current, and lower is the expanded current trace for the 100 msec voltage command shown following the long command in the first two traces.

parent. Also there was no consistent difference between pacemaker and nonpacemaker in the ratio of peak inward current to the delayed (100 msec) current at a given command potential.

Membrane Resistance at the End of Short and Long Commands in Pacemaker Neurons

The essential differences between pacemaker and nonpacemaker neurons on long commands (i.e. the current sag and the shift in E_{tail}) could result from an increased conductance to a cation tending to move inward, such as Na^+,

or to a decreased conductance to a cation tending to move outward, such as K^+. They could also result from other mechanisms not associated with a conductance change. Therefore it is of interest to examine the chord conductance at the end of short and long commands in pacemaker neurons.

Table II shows measurements of E_{tail} for short and long commands and the ratios of membrane conductance at the end of 1–2 sec commands to the conductance at the end of 100 msec commands for 16 pacemaker neurons. The conductance ratio was obtained by comparison of commands of identical voltage at the two durations. The ratios shown in Table II represent the average of from one to three determinations taken at identical voltages. The average conductance at the end of long commands was 0.79 times that at the end of the short command. In seven cells the slope conductance for 2 sec commands was less than that for 100 msec commands by at least 25%, and in two cells it was 50% or less. However, in the remaining nine cells it is unlikely that the conductance change is large enough to explain the sometimes marked shift in E_{tail}. There was, however, no consistent difference in the amount of shift in E_{tail} between those cells with conductance ratios near 1 and those which showed a clearly decreased conductance with long commands.

TABLE II

	Pacemakers			Pacemakers with synaptic activity		
	E_{tail} short (Millivolts relative to resting potential)	E_{tail} long	$\frac{G_{2sec}}{G_{200msec}}$	E_{tail} short (Millivolts relative to resting potential)	E_{tail} long	$\frac{G_{2sec}}{G_{200msec}}$
	−6	+6	0.50	−5	−2	0.77
	−10	+10	0.89	−10	−3	0.59
	−8	−3	1.15	−8	+2	1.15
	−4	0	0.58	−10	+6	0.94
	−10	+6	0.62	−8	+5	0.29
	−6	0	1.03	−8	−5	0.67
	−4	+12	0.52	−14	−4	0.96
	−16	+9	1.10	−12	+4	0.90
Average	−8	+5	0.80	−9.4	+1	0.78

Fig. 9 shows plots of chord conductance at the end of 110 msec and 2.8 sec commands at three different voltages, from an experiment on a pacemaker neuron which exhibited some synaptic activity. The current sag on the long command is reflected in the difference between points at the same voltages. If this sag resulted from a conductance change, a conductance increase of about 16% would be necessary to explain the sag. The tail currents are plotted on the Y axis. In this cell E_{tail} moved in the positive direction on long commands as command voltage was increased. At each voltage the chord

conductances for commands of short and long duration are very similar in spite of the marked sag and shift in E_{tail}.

There were no obvious differences in the ratios of $G_{2\ sec}:G_{200\ msec}$ between pacemakers with and without synaptic activity, nor did the ratio appear to vary with the amplitude of shift of E_{tail}.

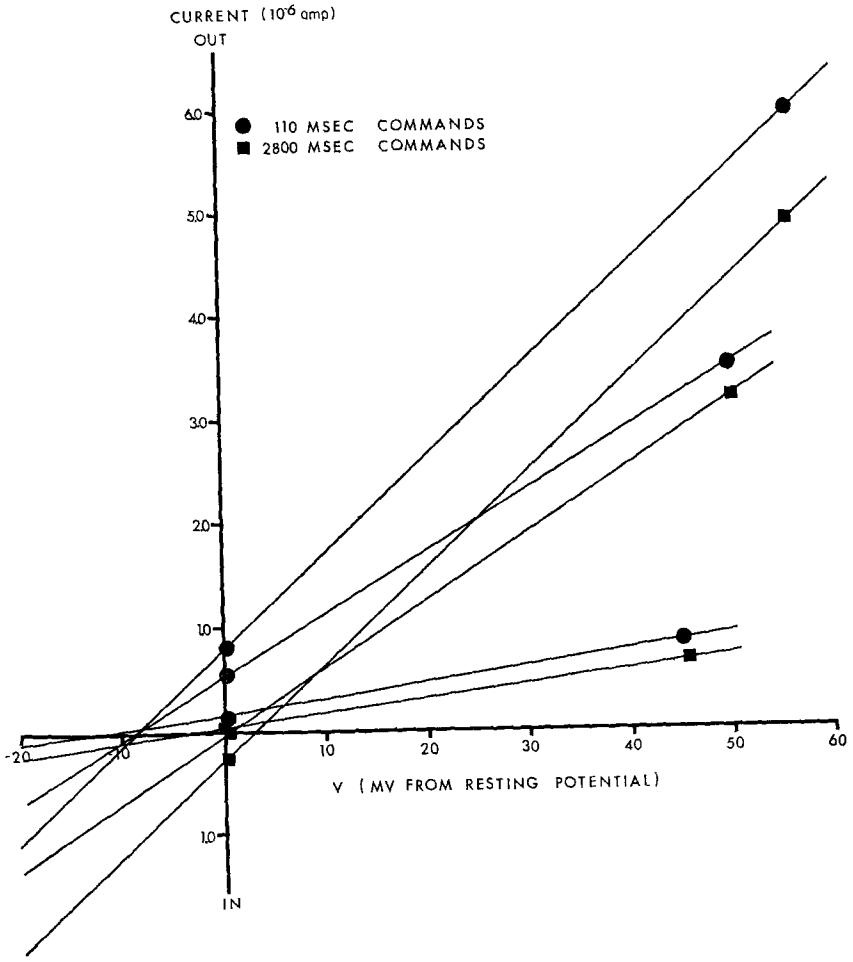

FIGURE 9. Chord conductance at the end of short and long commands in a pacemaker neuron. Points were measured from the end of the late current and from the initial tail current just after clamping back to resting potential ($V = 0$).

DISCUSSION

Two points are of particular interest with regard to the behavior of the pacemaker neurons under voltage clamp conditions. One is the decrease in clamping current and the shift in E_{tail} with long depolarizing clamping potentials.

The other is the chord conductance of these cells at the end of the short and long clamping depolarizations.

The Decrease in Clamping Current and the Shift in E_{tail} Could Be Explained on a Number of Bases

1. It could result from an increase in Na^+ (or Ca^{++}) conductance (late depolarizing Na^+ activation) (Grundfest, 1966 *a*). An increase in conductance to Na^+ would bring effective emf of the membrane at the end of the long command toward or even past zero potential, and the current required to clamp the membrane in the depolarizing direction might decrease despite an increase in membrane conductance. Just such a situation, of course, obtains at the peak of the inward early current.

2. A decreased K^+ conductance (depolarizing K^+ inactivation) (Grundfest, 1966 *a*) in the presence of another and more positive emf which contributes to the resting potential would also reduce membrane emf and would decrease clamping current for this reason. In addition the increased membrane resistance would result in a lower clamping current for a given clamping voltage. If the cause of the current sag and shift in E_{tail} is either an increased Na^+ or a decreased K^+ conductance, there should be a measurable change in slope conductance at the end of the command. If both events occurred simultaneously and equally, however, there would be no measurable conductance change.

3. Potassium accumulation outside the neuron resulting from restricted diffusion of the primarily K^+ outward clamping current would also decrease membrane emf with results similar to those observed in pacemakers on long commands. Frankenhaeuser and Hodgkin (1956) have suggested that the current sag and reversal of E_{tail} observed with commands of greater than 5 msec duration in clamped squid axon result from such a mechanism.

4. A potential-dependent source of current located in the cell membrane, such as an electrogenic Na^+ pump, might also explain the observations. If the electrogenic Na^+ pump, which is known to be present in these cells (Carpenter and Alving, 1968), were made less active or less electrogenic by depolarization, this would remove a hyperpolarizing current source located in the membrane of the cell, with the result that less depolarizing clamping current would be required to maintain a given depolarization of the membrane.

The observations on clamping current and shifts in E_{tail} do not directly or by themselves allow one to choose between these alternatives. Consideration of the chord conductances under different conditions is helpful. From Table II the observation that in none of the cells was the 2 sec chord conductance significantly more than the 200 msec chord conductance would rule out an

increase of Na^+ conductance as the primary basis for the late decrease in clamping current and shift in E_{tail}. Such a mechanism is unlikely in any case because of the large amount of current involved. In the cells with G_{2sec}: $G_{200\ msec}$ ratios significantly less than 1, it is most likely that the current sag and shift in membrane emf result from a potential- and time-dependent decrease in K^+ conductance. A combination of an increased G_{Na^+} and decreased G_{K^+} could explain the responses of the remaining pacemaker cells in which no differences were observed between conductances at the end of short and long commands. However, it seems rather unlikely that the two conductances would change by exactly equal but opposite amounts.

An accumulation of K^+ in a restricted space is a particularly attractive explanation for these observations, since that explanation has been used to explain current sag and change in tail curents with increasing duration of command potentials in squid axon (Frankenhaeuser and Hodgkin, 1956). Furthermore, in experiments performed at temperatures of 9°C or less, C. F. Stevens (personal communication) has found by varying the clamping voltage that the tail current reverses (i.e., is zero) at E_{K^+} in *Anisodoris* neurons. There are, however, several difficulties in invoking an extracellular accumulation of K^+ as an explanation for these results.

Frankenhaeuser and Hodgkin (1956) found that repetitive activity in nerve fibers caused a decrease in the negativity of the spike afterpotential and a slow depolarization of the membrane. They offered evidence that these observations resulted from an accumulation of K^+ in a restricted space, presumably just outside the cell membrane. The effect of repetitive activity was greater at cold than at warm temperatures, in agreement with Shanes (1954) observation that the amount of K^+ lost per impulse was threefold greater at 6° than at 24°C. The voltage clamp records illustrated by Frankenhaeuser and Hodgkin (their Fig. 14) show a current sag and a shift in the direction of the tail current on increasing duration of the command. They suggest that the cause is a K^+ accumulation similar to that which they propose to explain the effect of repetitive stimulation on the afterpotential. In agreement with this interpretation, the current sag and shift in tail current were found to be more prominent at 8° than at 20°C.

If the results of the present experiments are due to K^+ accumulation in a restricted space around pacemaker neurons, there should be other differences between pacemaker and nonpacemaker neurons due to this space. Repetitive activity of *Aplysia* neurons does result in a decrease in the negativity of the afterpotential which is more pronounced at 10° than at 20°C; however, consistent differences in the magnitude of this effect have not been found between pacemaker and nonpacemaker neurons (D. O. Carpenter, unpublished observations). These results provide no support for a functional difference in the restricted extracellular space between pacemaker and nonpacemaker neurons.

The present experiments were all performed at 18–20°C. If differences in the restricted extracellular space between pacemaker and nonpacemaker neurons are the cause of the disparity in the responses of the two types of neurons on voltage clamping there should be an even greater disparity at lower temperatures. Unfortunately this has not been tested.

Another difficulty in ascribing the different responses of pacemaker and nonpacemaker neurons to K^+ accumulation is the lack of anatomical evidence for differences in extracellular barriers to K^+. All *Aplysia* neurons have membrane indentations, which increase the surface area of the cell; however, except for a special group of neurosecretory cells not included in this study, there are no obvious differences in depth, width, or number of indentations between pacemaker and nonpacemaker neurons of the same size (Coggeshall, 1967 and unpublished observation). In addition no differences have been found in impedance loci between pacemaker and nonpacemaker neurons (Alving and Freygang, unpublished observations). This technique, using the methods of Falk and Fatt (1964), should detect significant differences in a pathway for current flow in series with the membrane (i.e., a restricted extracellular space).

There remains the possibility that the differences between pacemaker and nonpacemaker neurons on long voltage clamp commands result from differences in a membrane current source, such as an electrogenic Na^+ pump. Sizeable potentials may be generated by an electrogenic Na^+ pump in both pacemaker and nonpacemaker neurons of *Aplysia* (Carpenter and Alving, 1968). There is no evidence to date on the relative rates of Na^+ pumping in the two types of neurons. However, it is likely that the resting Na^+ permeability is higher in pacemaker than in nonpacemaker neurons, since a high resting Na^+ permeability is probably an important part of the mechanism of the pacemaker potential (Dudel and Trautwein, 1958). A higher rate of Na^+ entry in pacemaker neurons would necessitate a higher rate of Na^+ efflux.

If the electrogenic Na^+ pump is the current source which is responsible for the differences between these two classes of neurons the net pump current must decrease with depolarization. However, in muscle the rate of Na^+ efflux increases with depolarization (Horowicz and Gerber, 1965). The effect of potential on the electrogenic pump in *Aplysia* is not clear; however, the post-tetanic hyperpolarization of the crayfish stretch receptor, which is a result of an electrogenic Na^+ pump, is increased by hyperpolarization in spite of a decreased membrane resistance (Nakajima and Takahashi, 1966). In the photoreceptor of *Limulus* an electrogenic Na^+ pump contributes to resting potential (Smith, Stell, Brown, Freeman, and Murray, 1968). This pump appears to be activated by hyperpolarization and depressed by depolarization (T. G. Smith, Jr., personal communication). A potential dependence of the electrogenic pump of *Aplysia* neurons of this type could account for the ob-

served differences between pacemaker and nonpacemaker neurons simply on the basis of different rates of pump activity in the two types of neurons. The results of Smith et al. suggest that the electrogenicity of the pump, i.e., the ratio of the coupling of active Na^+ and K^+ movements, may also be potential-dependent. This factor may be of importance and may be the basis for the apparent discrepancy between the effect of membrane polarization on net Na^+ efflux and on the generation of potentials by the electrogenic pump. If the observed differences between pacemaker and nonpacemaker neurons are due to pump activity they probably reflect differences in the rate of pumping rather than a variable coupling ratio, however.

On the basis of the present information it is not possible to be certain of the reasons for the differences between pacemaker and nonpacemaker neurons on voltage clamping. The mechanism in the neurons which do not show a conductance change may be either a difference in the restricted extracellular space around the neuron, or a difference in the rate of an electrogenic Na^+ pump. In those cells which show a conductance change the current sag and shift in tail current probably reflect a time- and voltage-dependent decrease in G_{K+}. It has been suggested that a depolarizing K^+ inactivation of this type is an essential part of the mechanism of pacemaker generation in *Aplysia* neurons (Waziri et al., 1965) and other tissues (Dudel and Trautwein, 1958; Grundfest, 1966 *b*). The observation that a depolarizing K^+ inactivation is characteristic of pacemaker but not of nonpacemaker neurons supports this view.

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