The Electrical Activity of Embryonic Chick Heart Cells Isolated in Tissue Culture Singly or in Interconnected Cell Sheets

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ABSTRACT Embryonic chick heart cells were cultured on a plastic surface in sparse sheets of 2–50 cells mutually in contact, or isolated as single cells. Conditions are described which permitted conjoint cells to be impaled with recording microelectrodes with 75% success, and isolated single cells with 8% success. It is proposed that cells in electrical contact with neighbors are protected from irreversible damage by the penetrating electrode, by a flow of ions or other substances from connected cells across low-impedance intercellular junctions. Action potentials recorded from conjoint and isolated single cells were similar in form and amplitude. The height or shape of the action potential thus appears not to depend upon spatial relationships of one cell to another. As the external potassium concentration was increased from 1.3 mM to 6 mM, cells became hyperpolarized while the afterhyperpolarization was reduced. At higher potassium levels, the afterhyperpolarization disappeared, the slope of the slow diastolic depolarization decreased, and resting potential fell along a linear curve with a slope of 61 mv per 10-fold increase in potassium. In pacemaker cells the diastolic depolarization consists of two phases: (*a*) recovery from the afterpotential of the previous action potential and (*b*) the pacemaker potential. These phases are separated by a point of inflection, and represent manifestations of different mechanisms. Evidence is presented that it is the point of inflection (PBA) rather than the point of maximal diastolic potential, that should be taken as the resting potential.

With the advent of techniques for dissociating heart tissue into its component cells and growing these cells in culture (for review see DeHaan, 1967 *a*, *b*), a growing literature has developed on the electrophysiological properties of embryonic heart cells maintained in vitro (e.g. Fänge et al., 1956; Crill et al., 1959; Sperelakis and Lehmkuhl, 1966; Lehmkuhl and Sperelakis, 1967).

Numerous potential advantages have been cited in applying the techniques of electrophysiology to such cells in culture. However, these advantages have proven difficult to exploit, for two main reasons. First, the properties of dissociated heart cells in culture are sensitive to a wide variety of external variables, including the method of tissue dissociation, the density at which cells are plated, properties of the serum used in the medium, the ionic environment, and whether the cells are growing or nonmitotic (DeHaan, 1965, 1967 *a*, *b*).

Second, most of the benefits to be derived from applying electrophysiological recording techniques to heart cells depend upon being able to record at will either from an active cell, completely isolated from any neighbors, or from such a cell in electrical contact with a small group of neighbors, under circumstances in which the number of cells in the group may be determined and the points of contact examined. Although substantial valuable information about heart cells in culture has been obtained by impaling sheets or aggregates of cells, successful recordings have been obtained only from cells cultured at relatively high densities. Despite attempts in several laboratories, no successful recordings from actively beating isolated single cells have been published, although abortive impalements have yielded low-level resting potentials from such cells (Crill et al., 1959; Lehmkuhl and Sperelakis, 1965; Lieberman, 1967). Spontaneous action potentials from isolated single cells have not been reported.

Success in recording from cells in sparse sheets or those completely isolated from neighbors appears to depend upon at least three sets of variables: (*a*) Characteristics of the microelectrode, (*b*) components of the culture medium, and (*c*) level of oxygen in the ambient atmosphere. In the present work we describe techniques which have enabled us to impale isolated single cells successfully, and illustrate the fundamental similarity of the action potentials recorded from isolated and conjoint cells (those in contact with neighbors). In addition, we report on a study of the influence of the extracellular concentration of potassium ions $[K^+]_o$ on the electrical properties of conjoint cells.

METHODS AND MATERIALS

Culture Methods

Tissue culture techniques were similar to those described previously (DeHaan, 1967 *a*) with the exception that media with lower protein content and a low-oxygen atmosphere were employed. Hearts were dissected from chick embryos incubated for 7 or 12 days and dissociated into their component cells by three 8-min cycles of trypsinization (0.05% trypsin, pH 7.3, 37°C). Damaging effects of the enzyme were minimized by collecting freed cells at the end of each 8-min period in a trypsin-inhibitor medium (Table I). The final suspension was filtered through bolting silk, counted in a hemocytometer, and resuspended in growth medium (Table I). Cells

were plated in small plastic culture dishes (Falcon plastics, 35 mm diameter) at a density of 2 or 4 $\times$ 10^5 cells/plate. The cultures were incubated at 37.5°C in a water-saturated atmosphere of 5% CO_2, 10% oxygen, and 85% nitrogen.

All media employed (Table I) contained penicillin G (100 units per ml), streptomycin sulfate (50 μg per ml), and a small amount of phenol red (2 mg per liter). The modified Earle's balanced salt solution (Earle, 1943) and chick embryo extract were prepared as described previously (DeHaan, 1967 *a*). Embryo extract was dialyzed against K-free salt solution. Low-K media contained only that potassium (1.3 mM) contributed by the nutrient supplement (M-199) and nondialyzed horse serum. Solutions and media were sterilized before use by filtration through cellulose membrane (0.45 μ pores) which had been washed with hot water and saline to remove detergents. Embryo extract and sera were stored at −70°C. Sodium and potassium concentrations of all media were determined by flame photometry (Patwin model FC, Patwin Electronics, Waterbury, Conn.), and osmolarity on a Precision Systems "Osmette" (Precision Systems, Inc., Framingham, Mass.).

TABLE I

COMPONENTS OF STANDARD MEDIA

Component	Growth medium 729A	Trypsin inhibitor
	vol.%	*vol.%*
Horse serum (heat-inactivated)	4	10
M-199*	20	20
Chick embryo extract	6	—
Balanced salt solution (K-free)	69	69
Penicillin-streptomycin	1	1

* Grand Island Biological Co., Grand Island, N. Y.

Electrical Recording

Cultures of heart cells incubated for 1–4 days were used for recording. The culture dish was positioned in a warming chamber (Lieberman, 1967) mounted on the stage of an inverted phase optics microscope. Temperature in the dish was maintained at 35–37°C by circulating warm water through the walls of the chamber from a constant temperature bath. To maintain pH at 7.3 during the recording period, a mixture of 10% CO_2, 10% O_2, and 80% nitrogen was passed through holes in a toroidal brass gassing ring, to form a curtain of gas (mixed with room air) over the fluid layer. At the beginning of an experiment a layer of nontoxic mineral oil ("Klearol," Sonneborn Division, Witco Chemical Co., N. Y.) was pipetted over the culture medium to prevent evaporation. This oil is freely permeable to oxygen and CO_2 (DeHaan, 1967 *c*) and does not clog or otherwise damage recording electrodes passed through it. In this manner, cells could be maintained under optimal physiological conditions on the microscope stage during the course of an experiment lasting many hours.

Glass micropipette electrodes were drawn on a solenoid-activated pipette puller (Industrial Science Associates, Ridgewood, N. Y.) at maximal coil temperature.

This resulted in electrodes of 50–150 megohms tip resistance. Electrodes were filled with 3 M KCl (filtered through cellulose membrane) by boiling, tip down, at 80°C at a reduced atmospheric pressure for 5 min. The reference electrode was a glass tube filled with saline-agar bridging between the culture medium and a pool of 3 M KCl. Both electrodes were mounted in Leitz micromanipulators and connected to the recording system by means of chlorided silver wires dipped in 3 M KCl. Potentials were recorded with a high impedance preamplifier (Bioelectric Instruments DS2C), dual beam oscilloscope (Tektronix 502A), and an oscillographic camera (Grass C4). A voltage divider placed between the reference electrode and ground was used to inject accurate 50 mv pulses through the system for calibration.

When high-resistance electrodes were used, adequate shielding was essential to reduce background noise. To this end, the amplifier was placed within 5 inches of the recording electrode. The connecting lead and all others were shielded. All metal parts and shielding were connected to a common ground, and the entire recording setup was enclosed in a Faraday cage. Under these conditions, a noise level of 2 mv or less could be maintained.

To determine the effects of various levels of $[K^+]_o$ two methods were employed. In one, records were obtained from a number of cells growing in a medium containing a given $[K^+]_o$. The average magnitudes of the measured parameters could then be compared with those from different cells in a medium containing more or less K. This method has the advantage that records could be obtained from many cells rapidly, since each cell need only remain impaled long enough to record a few action potentials at two or three different sweep frequencies. However, large numbers of recordings are necessary to determine the significance of small differences recorded from different cell populations.

The second method, which is technically more difficult, was to impale a cell growing in low-K medium, and then increase the $[K^+]_o$ gradually, in stepwise fashion, while recording from that same cell. This required keeping an electrode sealed in a cell long enough to allow reequilibration after the addition of each aliquot of potassium. A cell was considered to have come to equilibrium when the recorded resting potential and action potentials had remained constant in form and amplitude for a minimum of 5 min. The $[K^+]_o$ was increased while recording from cells by adding saline containing 0.25 M KCl to the 2 ml of medium in the dish through the oil layer, with the aid of a micrometer-drive syringe. Addition of 10–20 μl droplets increased the $[K^+]_o$ by increments of 1.25–2.5 mM. Resultant changes in $[Cl^-]_o$ and osmolarity were not corrected.

RESULTS

Within about 4 hr after inoculation into standard medium, 25–50% of the cells adhere to the surface of the culture dish and take on their characteristic morphology as myocardial-like M cells, or F cells having properties more like those of fibroblasts (DeHaan, 1967 *a*). 24 hr after inoculation, nonadhering "floaters" are removed with the first change of medium, leaving behind healthy cells attached to the dish bottom at a density of 50–200 cells/mm^2. In most regions, the majority of the cells are completely isolated one from

another. However, some cells are found in groups or sparse sheets of 2–50 cells mutually in contact. Cells tend to settle at higher density in the center of the dish, and are more sparsely distributed at the periphery, especially in low-K media (DeHaan, 1967 *d*).

In growth medium the cells divide rapidly, increasing about 10-fold in 4 days of cultivation. Thus, by the end of that period cell densities as high as

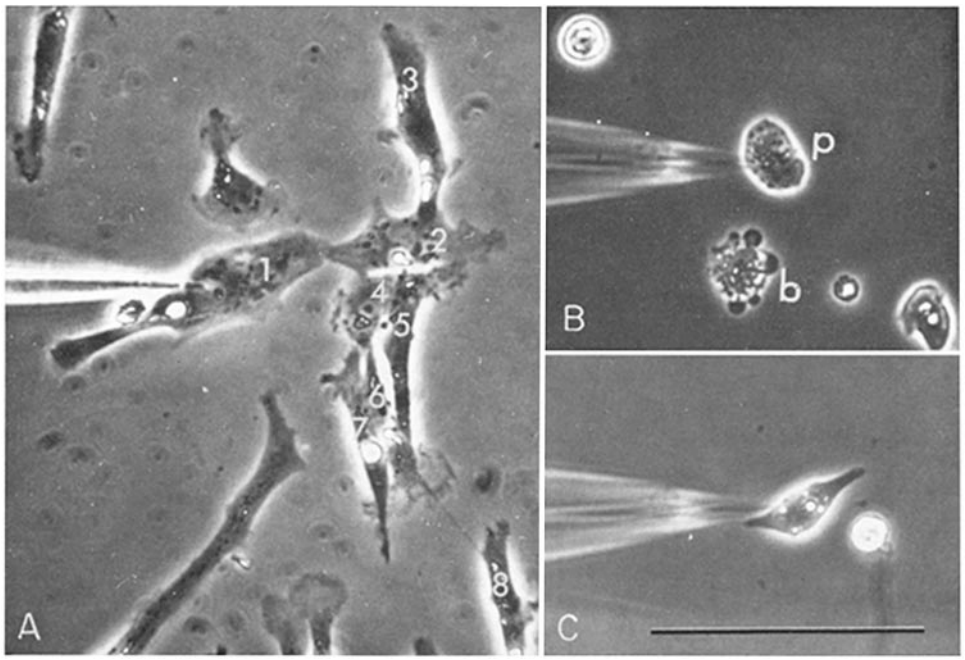

FIGURE 1. 7-day heart cells 48 hr in culture. Phase optics; scale = 100 μ. A, electrode penetrating an M cell in a group of seven conjoint cells (numbered); photographed while action potentials were being recorded. Cell 8, connected by a slender strand, was not beating. B, isolated single cells, unsuccessfully impaled. Penetrated cell (*p*) is showing early signs of deleterious effects of impalement. Cell *b*, impaled 4 min previously, is exhibiting extensive blebbing and vacuolization. C, isolated single M cell successfully impaled, photographed while recording.

1000 cells/mm^2 are not uncommon, and isolated cells are rare. Typical cells, photographed with phase optics while recording, are shown in Fig. 1. The cell impaled in Fig. 1 A is one of a group of seven conjoint cells. This cell and three others in the group (cells 1, 3, 4, and 7) are typical M cells with a single nucleolus. All were beating synchronously. Action potentials were being recorded at the time the photograph was taken. Several binucleolate F cells are also visible.

We refer here to "conjoint" cells as those adhering to, and electrically inter-

connected with one or more neighbors. Interconnection is generally manifested in synchronous rates of beating. Although F cells do not usually contract (DeHaan, 1967 *a*), they apparently can conduct a propagated impulse, or serve as a low-resistance bridge to permit the spread of excitation. All M cells in a conjoint group usually beat in synchrony even if their only connection is across an F cell. Neighboring cells beating at different rates indicate a lack of electrical interconnection. Cell 8 (Fig. 1 A), connected to the main group by a slender strand, was not beating. In other cases, however, cells have been seen to beat in synchrony when the only connection between them was a fiber of such size and appearance.

Impalability

Impalability refers to the ease with which cells may be penetrated by a microelectrode without irreversible damage. It was roughly quantitated by noting

TABLE II

IMPALABILITY OF ISOLATED SINGLE AND CONJOINT CELLS

	Conjoint cells			Isolated single cells		
	Attempted impalements	Successful	Unsuccessful	Attempted impalements	Successful	Unsuccessful
Experiment I	33	23	10	36	1	35
Experiment II	30	23	7	30	5	25
Experiment III	30	24	6	30	2	28
	—	—	—	—	—	—
Total	93	70	23	96	8	88
Mean %	—	75.3	24.7	—	8.3	91.7

the ratio of successful to unsuccessful attempts to impale cells under a given set of conditions. The criteria of success were that upon recovery from impalement (usually after an initial depolarization) the cell developed a resting potential (E_R) of at least −40 mv, and that the recording lasted a minimum of 10 sec. In the experiments designed specifically to test ease of impalability (Table II) action potentials were also recorded from every cell counted as a successful impalement.

Attempts to impale M cells in sheets using glass microelectrodes of the type commonly described (tip diameter 0.4–0.5μ; resistance 10–40 megohms) were moderately successful. Cells in nongrowth media and ambient atmospheres containing 40% oxygen could be impaled with 20–30% successs. These recordings lasted from a few seconds to several minutes, terminating when either the electrode became dislodged from the cell, or the resting potential diminished to near zero as the cell exhibited visible signs of damage, becoming

vacuolated and "blebby." Under these conditions, isolated cells were never successfully impaled.

In media containing 6% embryo extract, in which the cells grow rapidly, a greater degree of success was obtained from conjoint cells. The electrode healed in more readily, and recordings generally lasted several minutes. Comparison between cells grown in atmospheres of 90, 40 or 10% oxygen was also striking. In sparse cultures, cells in 90 or 40% oxygen were more vacuolated in appearance and difficult to impale. Groups of cells cultured in growth medium and 10% oxygen atmospheres for 48 hr could be impaled successfully in over 60% of the attempts. Once the electrode was sealed into a cell, recording could be continued for 15–20 min (frequently for 30 min or more) and was most often discontinued by the electrode becoming dislodged, rather than by death of the cell.

Under these circumstances, other cells in the same dish, differing only in that they were not in contact with neighbors, still could not successfully be impaled. At best, abortive impalements, yielding low-level membrane potentials of 15–20 mv could be obtained.

With the introduction of ultramicroelectrodes similar to those used by Tomita and Kaneko (1965), with resistances of 100–150 megohms, success in impalements of conjoint cells increased still further, and recording from isolated cells for the first time became feasible. The degree of success in impaling M cells in groups and isolated cells is indicated by the results of three experiments shown in Table II. In each case, the culture was prepared for recording, and cells were impaled one after another, systematically alternating between M cells in groups and isolated single cells. In most cases the electrode was allowed to remain in each cell no more than 3–4 min, by which time the impalement was declared successful or unsuccessful as defined above. All three experiments were done with cells grown 48 hr in 10% oxygen in growth medium (729A) containing 1.3 mM K^+. Under these conditions, cells in contact with two or more neighbors could be readily impaled about 75% of the time. This was about the same degree of success attained with dense cultures several cell layers thick. Moreover, a large portion of the remaining unsuccessful attempts were with cells in which good recordings were obtained but the resting potential did not meet the minimum criterion of −40 mv, or the electrode became dislodged a few seconds after recording began.

Under identical conditions, isolated single cells in these cultures permitted 8% successful recordings. Most commonly, upon impalement an isolated single cell exhibited a 15–20 mv resting potential. Within a few seconds it became dotted with small black spots at its periphery (Fig. 1 B, cell *p*). In most cases violent blebbing and vacuolization ensued (Fig. 1 B, cell *b*). In a successful impalement, the isolated single cell did not increase in number of intracellular vacuoles, nor did it withdraw pseudopodial extensions. It remained

smooth-surfaced and healthy in appearance throughout the recording period (Fig. 1 C). Furthermore, after withdrawing the electrode from a successfully impaled cell, that cell could often be impaled a second time with equal success.

Action Potential Characteristics

The properties measured on recorded action potentials are shown in Fig. 2: the maximal diastolic potential (MDP), the overshoot or maximal positive potential (MPP), and the duration (DUR) are self-explanatory. The potential at the point of sharp curvature between the end of the slow diastolic depolarization and the beginning of the rapidly rising phase of the action potential was termed the "potential at break phase 0" (PB0). It is considered to be roughly equivalent to the threshold potential in primary pacemaker cells.

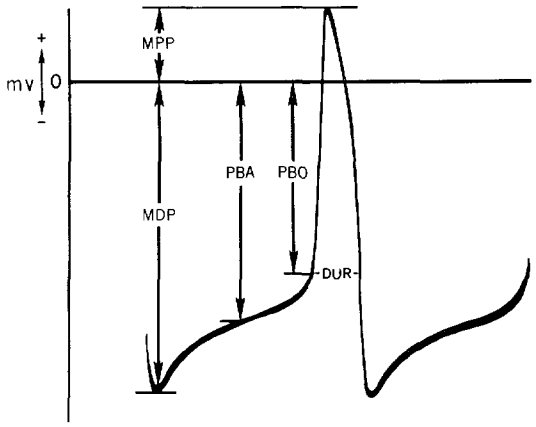

FIGURE 2. Diagrammatic action potential to show parameters measured. MDP = maximal diastolic potential; MPP = maximum positive potential; DUR. = duration; PBA = potential at break of afterpotential; PB0 = potential at break of phase 0.

In pacemaker cells, the period of gradual depolarization from maximal diastolic potential of one action potential to the rapidly rising portion of the next often consists of two phases separated by a more or less distinct point of inflection. The first phase is taken as a "positive afterpotential" or afterhyperpolarization[1] following the first action potential, while the second phase represents the slow diastolic depolarization or pacemaker potential leading to

[1] The term "positive afterpotential" originated in the older neurophysiological literature (Erlanger and Gasser, 1937) at a time when recordings were made with a pair of external electrodes, one placed on an intact region of a nerve fiber, the other on a damaged area. By convention, any inflection of the recording beam up was negative and down positive. If the membrane potential overshot the original resting level upon repolarization from a spike, the resultant downward sweep was called a positive afterpotential. With intracellular electrodes the old convention is reversed, negative membrane potentials being recorded downward from zero potential. A further downward deflection from the resting potential represents an increased negativity or hyperpolarization. Despite this fact, such a wave form following the repolarization of an action potential is still often referred to in literature as a "positive" afterpotential. To avoid the ambiguity, Woodbury (1962) recommends the term afterhyperpolarization. In the present work we use the term afterpotential or the abbreviation for afterhyperpolarization (AHP).

the next action potential. The potential at the point of inflection between these two phases was termed the "potential at break of afterpotential" (PBA). It approximated the resting potential, as indicated below.

The action potentials shown in Fig. 3 A–3 B were recorded from isolated single cells similar to the one shown in Fig. 1 C. They are typical in amplitude and wave form. In Fig. 3 A (R = 170 beats/min) the distinction between the afterhyperpolarization and diastolic depolarization is not obvious, although a

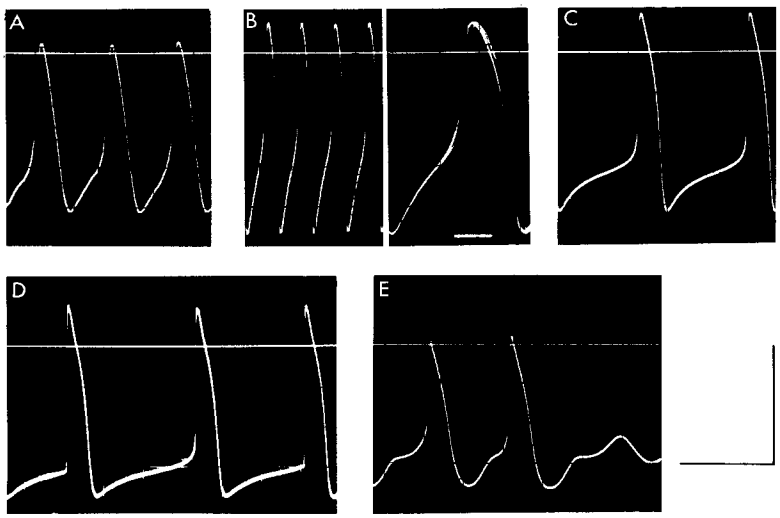

FIGURE 3. Representative action potentials recorded from 7-day heart cells, 48 hr in culture in medium 729A. A, from isolated single pacemaker M cell. MDP = −64 mv; PBA = −55 mv; DUR = 150 msec, rate = 170 B/M. B, from isolated single pacemaker M cell. MDP = −84 mv; PB = −49 mv; DUR = 70 msec; rate = 450 B/M. C, from conjoint pacemaker M cell, in sparse sheet. MDP = −64 mv; PBA = −50 mv; DUR = 140 msec; rate = 108 B/M. D, from conjoint M cell, alternating between primary and latent pacemaker. MDP = −63 mv; PBA = −53 mv; DUR = 130 msec. E, from conjoint M cell in a group of three; to show the distinction between AHP and pacemaker potential. MDP = −59 mv; PBA = −47 mv; DUR = 130 msec. Vertical scale = 50 mv; horizontal scale = 500 msec except for the single fast sweep-speed record shown in B. The horizontal scale shown in that panel = 50 msec.

slight inflection point is detectable on two of the three action potentials shown. This distinction is clearer in the records from the more rapidly beating (450/min) cell pictured in Fig. 3 B, especially at the higher sweep frequency shown (50 msec calibration). Because of the rapid pulsation rate of this cell, its action potential duration is shorter than usual (70 msec). However, it illustrates strikingly the wide range of durations and rates seen in isolated cells.

In Fig. 3 C are shown action potentials from a primary pacemaker in a sparse sheet of conjoint cells similar to those photographed in Fig. 1 A. Except

for pulsation rate (108/min) the form and amplitude of these action potentials are not substantially different from those recorded from isolated single cells (see below).

Active isolated single cells always exhibit a slow diastolic depolarization and the smooth transition into phase 0 characteristic of primary pacemakers. This is not necessarily true of cells in groups, as seen in Fig. 3 D, in which potentials characteristic of a primary pacemaker appear to alternate with those of a latent pacemaker. This record is from an impalement of one of a group of five cells in mutual contact.

TABLE III

MEASURED PARAMETERS (±1 SE) OF ACTION POTENTIALS OF HEART CELLS CULTURED IN DIFFERENT CONCENTRATIONS OF POTASSIUM

$[K^+]_o$, mM	1.3	2.2	4.2	6.1	8.1	12.0	22.0
Maximum diastolic potential, mv	−67.5 ±1.3	−69.5 ±0.7	−68.4 ±1.0	−65.1 ±0.7	−60.5 ±0.7	−51.3 ±1.0	−35.0 ±0.7
Overshoot, mv	15.4 ±0.6	15.3 ±0.5	22.0 ±0.9	20.5 ±1.0	20.9 ±0.9	18.4 ±0.8	—
PBA* (resting potential), mv	−56.0 ±0.8	−58.8 ±0.6	−63.0 ±0.9	−64.0 ±0.7	−60.0 ±0.6	—	—
Potential at beginning of phase 0‡, mv	−48.3 ±0.9	−52.8 ±0.6	−62.0 ±1.4	−62.8 ±1.1	−58.3 ±0.9	−47.3 ±0.9	—
Afterhyperpolarization§, mv	11.5	10.7	5.4	1.1	0.5	0	0
N	94	25	30	20	15	44	10
Primary pacemakers, %	73.4	44.0	36.7	10.0	13.3	13.7	0
With afterpotentials, %	94.7	100	57	55	30	0	—
E_K‖, mv	−110	−96	−78.8	−68.8	−61.3	−50.9	−34.8

* Potential at break of afterhyperpolarization refers only to action potentials exhibiting afterpotentials.
‡ Refers only to action potentials exhibiting pacemaker potentials.
§ Maximum diastolic potential minus PBA.
‖ $E_K = -61 \log \frac{82}{K_o}$.

Occasionally a recording was obtained in which the point of inflection between the afterpotential and slow diastolic depolarization was unusually sharp. This is illustrated in the first two action potentials in Fig. 3 E recorded from one of a group of conjoint cells. At the end of the second spike the impaled cell recovers from the afterpotential and develops a potential which represents either an electrotonic spread of current from neighboring cells or a subthreshold pacemaker potential. In the absence of a self-propagating action potential, the membrane potential subsides to the PBA, not to the maximal diastolic potential. In other recordings from conjoint cells, especially during the course of changes in $[K^+]_o$, transient periods of irregular beating were noted. In these cases, the pause between two action potentials came after recovery from the

after potential, with the membrane potential momentarily equilibrated at the level of the PBA. These results suggest that the PBA, rather than the maximal diastolic potential, is equivalent to the resting membrane potential.

Effects of $[K^+]_o$ on Electrical Parameters

All the records shown in Fig. 3 A–E were taken from cells maintained in medium containing 1.3 mM K^+. The magnitudes of measured parameters of action potentials recorded from such cells (including seven isolated single cells) are summarized in the first column of Table III. The action potentials of actively beating cells impaled in low-K medium are characterized by a pronounced pacemaker potential of relatively steep slope, a small overshoot, and a large afterhyperpolarization averaging 11.5 mv (Table III, column 1). The resting potential (PBA) has an average value of −56 mv. Mean values for the seven isolated single cells alone are: overshoot = 9.5 mv; maximum diastolic potential = −65.3 mv; PBA = −52.5 mv.

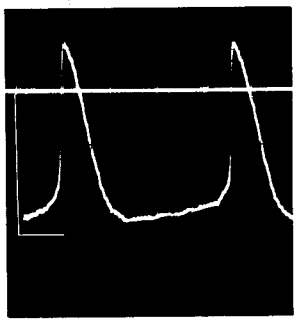

FIGURE 4. Action potentials recorded from an isolated single M cell. From a 12-day heart, 48 hr in culture, in medium 729A with 12 mM K^+. Calibration scales = 50 mv; 200 msec.

In media containing progressively more K^+, cells show consistent changes. Table III (columns 2–7) also summarizes the data from 137 impalements of conjoint M cells and 7 isolated single cells at higher K levels. As $[K^+]_o$ is increased to about 6 mM the cells become progressively hyperpolarized (PBA = 64 mv) while the afterhyperpolarization is gradually reduced. The effect on the slope of the pacemaker potentials is variable—in some cells it increases and the cells exhibit "tachycardia," in others it is depressed and the cells slow or stop firing completely.

As $[K^+]_o$ is increased beyond 6 mM, the afterpotential and the slow diastolic depolarization disappear altogether, and the resting potential, now equivalent to the maximum diastolic potential, progressively decreases. As the diastolic resting potential becomes stable, the cell continues to exhibit action potentials only if it is stimulated by a pacemaking conjoint neighbor.

However, some cells in high-K media, even though markedly depolarized, retain their pacemaker capacity. Fig. 4 shows a record obtained from an isolated single cell in a medium containing 12 mM K^+. The generator potential

arises smoothly from a very slow diastolic depolarization. The low resting potential (−46 mv) and relatively large overshoot (16 mv) are charcateristic for both isolated single and conjoint cells at this $[K^+]_o$.

The effects of increasing $[K^+]_o$ can be shown most dramatically by recording from the same cell while the extracellular potassium is changed. Fig. 5 shows action potentials recorded from a cell in a sparse group during an impalement which lasted for 40 min. During this time, seven aliquots of KCl solution were injected into the plate, after each of which the cell was allowed to reequilibrate. In this cell the maximal afterpotential occurred at a point between 1.3 and 4.4 mM K^+ (not shown). It became progressively smaller and virtually disappeared as $[K^+]_o$ was increased to 6.3 mM. This cell also illustrates an initial increase in slope of the diastolic depolarization immediately after the $[K^+]_o$ was brought to 6.3 mM (at 15 min). Upon equilibration at this K level,

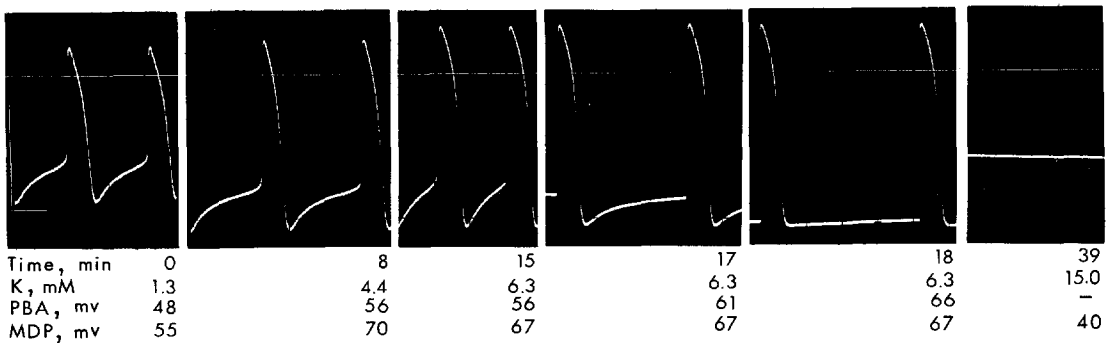

FIGURE 5. Action potentials recorded from a conjoint M cell, while potassium was increased from 1.3 mM to 15.0 mM over a period of 39 min. Time 0 = time of equilibrium 5 min after impalement. Vertical scale = 50 mv; horizontal scale = 200 msec.

the pacemaker potential declined (17 min) and finally disappeared (18 min) as the cell underwent the transition from a primary pacemaker to a driven cell. At the next increment of K^+ (to 8.2 mM) the sheet of cells became quiescent. From that point on, the membrane potential gradually declined to −40 mv as $[K^+]_o$ was elevated in three subsequent steps to 15 mM. The effects of alteration of $[K^+]_o$ on the measured diastolic potentials are summarized in Fig. 6. The data from cells grown at different K levels, taken from Table III, were used to plot the solid curves. The statistical significance of each of these points is shown by vertical bars representing two standard errors from the mean. The individual symbols represent measurements from records taken as the $[K^+]_o$ was elevated by one or more increments during the course of a single impalement.

The broken line, fitted by eye to the linear portion of the K curve (from 8.1–22 mM), has a slope of 61 mv per 10-fold increase in $[K^+]_o$. Solving the

Nernst equation

$$E_M = -61mV \log \frac{[K^+]_i}{[K^+]_o}$$

for $[K^+]_i$ yields a calculated value of 82 mM intracellular potassium.

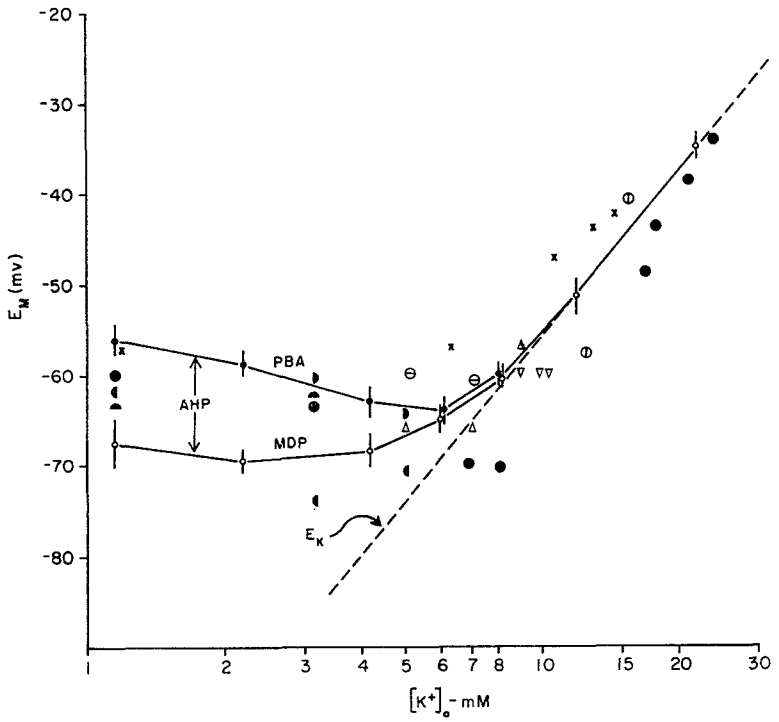

FIGURE 6. The diastolic membrane potentials plotted as a function of $[K^+]_o$. From 1–6 mM K^+, E_R is taken as equivalent to PBA. At higher concentrations E_R = MDP. Small closed circles are average values recorded from cells impaled at each $[K^+]_o$ (Table III). Vertical bars represent two standard errors from the mean. Individual symbols are E_R values obtained from cells impaled for extended periods, during which the $[K^+]_o$ was elevated in stepwise fashion. 30 points, taken from 10 different cells, are shown. The broken line (E_K) was calculated to have a slope of 61 mM per 10-fold increase of $[K^+]_o$.

The percentage of isolated 7-day embryonic heart cells which beat spontaneously in culture is a function of the $[K^+]_o$ (DeHaan, 1967 *a*). In the growth medium described in Table I, and in an atmosphere containing 10% oxygen instead of 40%, 50.5% of the cells which were attached to the dish after 24 hr were spontaneously active, whereas 63% were classified as M cells (20 experiments, 8300 cells counted). In Table III we have listed the percentage of M

cells successfully impaled which exhibited primary pacemaker potentials (% primary pacemakers). The figure, as before, is highest at 1.3 mM K^+ (73%) and drops off along a smooth curve as $[K^+]_o$ is elevated. Often an M cell which had been previously quiescent under visual observation began to contract spontaneously after penetration by the electrode.

We also reported earlier (DeHaan, 1967 *a*) that the depressive effects of K^+ were completely reversible. A culture with 50% of its cells beating in 1.3 mM K could be reduced to 5% by adding KCl to bring the $[K^+]_o$ up to 12 mM. If, after a few minutes, these cells were washed and the medium replaced with low-K culture fluid, the original 50% of the cells resumed spontaneous activity. In five experiments performed in the present series, cells growing in 4 mM or 12 mM K medium were washed with medium containing 1.3 mM K, and then impaled. Out of a total of 75 impalements, only 22 primary pacemaker potentials could be recorded. Thus, after 24–48 hr in high-K medium, the number of cells which manifest primary pacemaker activity drops from 80 to 29%. This apparently anomalous behavior remains unexplained.

DISCUSSION

Impalability

Investigators who have succeeded in recording electrical activity from heart cells in dense cultures have generally noted their inability to do so from isolated cells in sparse cultures. The abortive transient impalements that have been reported from such cells have not included spontaneous action potentials (Crill et al., 1959; Lehmkuhl and Sperelakis, 1965; Sperelakis and Lehmkuhl, 1964; Lieberman, 1967). Churney and Oshima (1963) experienced similar difficulties in attempting to record from strands of teased amphibian heart muscle. Cardiac muscle bundles containing many fibers were easily impaled and gave normal action potentials. Bundles containing 5–10 fibers were more difficult to record from and yielded only low-level, spikelike action potentials. They were unable to impale strands containing fewer than five fibers.

None of these investigators has attempted to offer an explanation for this difference in impalability, except the more or less tacit assumption that a cell surrounded on all sides by neighbors would be mechanically stronger and more stable than an isolated cell. Our experience would indicate, however, that mechanical support probably plays only a small role in the difference in ease of recording. This is shown by a comparison of Figs. 1 A and 1 C. The impaled conjoint cell (Fig. 1 A) is clearly not mechanically buttressed by any of the six neighbors with which it was electrically connected. Its only contact, in fact, is in a small region about 5 μ long where the end of the impaled cell adheres to an extension of the ruffled membrane of one of the other cells. Yet cells of this type could be impaled with approximately the same degree of

success as in very dense cultures, while recordings could be obtained only 8% of the time from isolated single cells (Table II).

We wish to propose an alternative explanation for this differential sensitivity to impalement, which is suggested by a consideration of the ultrastructure of intercellular connections. A variety of cell types, including cardiac muscle, exhibit "tight junctions" or "nexuses" at regions of intercellular contact (Dewey and Barr, 1964; Trelstad et al., 1966) where the outer leaflets of the apposed cell membranes fuse. These junctions have been associated with regions of low intercellular impedance in several cell types (Potter, Furshpan, and Lennox, 1966; Loewenstein and Penn, 1967). They are found in intercalated discs of mammalian cardiac muscle (Woodbury and Crill, 1961; Barr et al., 1965) and avian Purkinje fibers (Mizuhira et al., 1967), and appear to be present in electron micrographs of neonatal rat heart cells in tissue culture (Cedergren and Harary, 1964). Weidmann (1966) has presented compelling evidence that heart cells are connected across highly permeable junctions in the intercalated discs by showing that ^{42}K diffuses freely from cell to cell longitudinally along a strand of sheep ventricular fibers, with a longitudinal space constant of 1.55 mm (about 12 cell lengths). Intercalated discs and tight junctions are found only at the abutting ends of the cylindrical cardiac cells, not in the lateral membranes. He calculated the K resistance of a disc to be 3 ohm-cm^2, a value similar to that determined by Woodbury and Crill (1961).

Cells which have been dissociated, even by the gentlest treatments with proteolytic enzymes, undoubtedly suffer damage, both at the membrane surface and internally. Membranes of many cells become grossly permeable to dyes such as trypan blue (Levinson and Green, 1965), and myofibrillar structure may be disrupted (Wollenberger, 1964). Injection of enzymes inside excitable cells (Rojas, 1965) or external application of enzymes for extended periods (Holtzman and Agin, 1965) leads to depolarization and inexcitability. More moderate treatments can yield spontaneous action potentials in normally quiescent muscle cells (Holtzman and Agin, 1965).

When an electrode penetrates a cell membrane which is only 150–200 A thick, it seems reasonable to assume that some tearing of the membrane would occur, especially in a membrane weakened by trypsin. The "sealing-in" effect commonly observed within the first few minutes after cell impalement presumably represents the repair and adhesion of the membrane around the electrode. However, before this sealing in is completed, substantial intracellular material could be lost to the environment. If the impaled cell is in electrical contact with several neighboring cells, those neighboring cells might serve as a source of current and of intracellular ions which would tend to maintain the resting potential of the impaled cell. The adjoining cells might also serve as a source of other substances which could hasten membrane repair or synthesis. The isolated single cell, in contrast, would have no such external reservoir of

current or of cytoplasmic components. If its transmembrane pump mechanisms were inadequate to transfer materials from the extracellular milieu, the cell must succumb. In light of these considerations, the requirements for success in impaling isolated single cells, ultrafine electrodes and culture conditions favoring growth and membrane synthesis, are readily understood. It is interesting in this regard that 90% oxygen was reported to exert toxic effects on rat heart cells in vitro (Mark et al., 1967), while low-oxygen atmospheres have been found to foster growth of an established mammalian line (Brosemer and Rutter, 1961) and antigen-stimulated proliferation of plaque-forming spleen cells (Mishell and Dutton, 1967).

Percentage of Spontaneously Active Cells

Under the low-K conditions described, 50.5% of the isolated cells observed at 24 hr of culture were beating, and 63% had the M cell morphology. If we assume that M cells are true myocardial cells, and F cells are noncontractile fibroblasts, then 80% of the cells which could beat did so. This is slightly higher than the figure reported earlier (DeHaan, 1967 *a*), and corresponds well with the value cited by Mark and Strasser (1966) obtained from neonatal rat heart cells.

With contractile cells in sheets or groups, it is not possible to estimate visually how many of the cells are pacemakers. In a group beating in synchrony one cannot see which cells are driving and which are being driven. Of the 94 M cells tabulated in column 1 (Table III) all had a slow diastolic depolarization and 73% entered phase 0 with the smooth rounded "foot" typical of a primary pacemaker.

Sperelakis and Lehmkuhl (1964, 1966) have alluded to the dangers of using shape of the action potential as an assay for primary pacemakers. They have demonstrated the efficacy of using instead the response of cells to polarizing pulses (Lehmkuhl and Sperelakis, 1967). When a small depolarizing current is passed across the membrane of a true pacemaker, its frequency of discharge increases. A similar current passed across the membrane of a driven or dormant pacemaker may alter the shape of the recorded potentials but does not change the pulsation rate. These workers stated that 60–70% of all impaled cells were pacemakers. However, they have given no data on the relative numbers of primary pacemakers, latent pacemakers, and dormant pacemakers encountered in their cultures (Sperelakis and Lehmkuhl, 1964).

Form of Action Potentials

Perhaps the most noteworthy observation in the present study is the similarity in form and amplitude of action potentials recorded from isolated single and conjoint cells. Despite the difference in frequency of success, once an electrode was sealed in an isolated single cell and the cell had repolarized, it exhibited

action potentials which were similar to those of pacemaker cells in contact with a few neighbors, or in dense, multilayered cell sheets. The only quantitative difference in measured parameters was that the mean overshoot in isolated single cells fell below the average of conjoint cells. This resulted from two of the values which were very low (2 and 5 mv), whereas the overshoots of the remaining five isolated single cells fell well within the range of values for conjoint cells. The fact that earlier workers consistently found that action potential characteristics when recording from small groups of cells were different from intact tissue or thick cell sheets led some to conclude that these differences were real, not artifacts of the preparation. The common finding was that action potentials from cells in small groups or sparse sheets had low amplitude, little or no overshoot, and low resting potentials. However, these properties could also be explained in terms of current leakage around an improperly sealed electrode.

We assume here that a cell in an intact tissue or dense sheet would exhibit a large margin of safety, based upon such protective effects of the mass of cells surrounding it as their capacity to provide a reservoir of intracellular components, and the mechanical and nutritive support they could offer. A cell in contact with few neighbors is more sensitive to inadequacies in the milieu provided. Presumbably, this sensitivity may be manifested in many ways, but among them apparently is an impairment in the capacity of the cell to heal wounds in the surface membrane or seal around a penetrating electrode.

Thus, for example, Lieberman (1967) recorded action potentials from cells in dense cultures similar to those reported by others (Sperelakis and Lehmkuhl, 1966; Fänge et al., 1956; Crill et al., 1959) and illustrated here. He found, however, that cells in sparse areas gave action potentials which were smaller and more rounded, with slow rising velocities. He concluded that the action potential configuration may be contingent upon the "mass and geometrical arrangements of the cells" (Lieberman, 1967). However, for this study he employed our unimproved culture conditions (DeHaan, 1967 *a*) with high levels of oxygen (90%), deleterious concentrations of serum (30%), and microelectrodes with resistances of 10–40 megohms.

Similarly, from the fact that they could obtain only small, spikelike action potentials without plateau or overshoot, from bundles containing fewer than about five fibers of ventricular tissue, Churney and Oshima (1963) concluded that the typical configuration of the cardiac action potential might result from the "asynchronous firing of discrete portions of the myocardium"—that is, the action potential might be a summated response of many cells. Matsuda et al. (1967) have proposed a similar idea. The action potentials from isolated single cells shown in Fig. 3 A–3 B negate this idea. Alternative mechanisms for the production of spikelike action potentials by cardiac tissue have been suggested (Noble, 1962; Carmeliet, 1964; Pillat, 1967).

In this regard also, Weidmann has proposed (1956 *a*) that normal repolarization of the fiber could result from the accumulation of K^+ in intercellular spaces during the plateau phase. Woodbury (1962) has alluded to electron micrographic studies which have shown that the interstitial space is in fact much smaller in cardiac tissue than Weidmann thought—small enough to yield an increase in $[K^+]_o$ of up to 10 mM around each fiber. However, the fact that isolated cells, surrounded only by a liquid culture medium, exhibit relatively normal action potentials which are similar in form to those from pacemaker cells of the intact embryonic heart, indicates either that K^+ accumulation does not play an important role in repolarization, or that such accumulation is not a function of the size of the intercellular compartment.

Another aspect of action potential configuration which should be mentioned is our distinction between the afterhyperpolarization and diastolic depolarization, and our definition of resting potential. Large afterpotentials are apparently common in avian embryonic cells, both in the intact heart (Yeh and Hoffman, 1967) and in tissue culture (Sperelakis and Lehmkuhl, 1966). In a pacemaker cell, which does not exhibit a stable resting potential, the distinction between recovery from the afterpotential and the beginning of the slow diastolic depolarization may be obscured (Fig. 3 A). However, we have adduced evidence that such a distinction should nonetheless be made. The most crucial record is that pictured in Fig. 3 E, which shows that if an action potential, with its attendant drastic transients in sodium and potassium flux, does not occur, the membrane potential equilibrates at the PBA before beginning the next pacemaker depolarization. Thus, in cells exhibiting both an afterhyperpolarization and a pacemaker potential, the resting potential should be measured from the point of inflection between the two, not from the maximal diastolic potential. This point is important for an understanding of the effects of $[K^+]_o$ outlined in the next section.

Alterations of $[K^+]_o$

High $[K^+]_o$ depolarizes mature cardiac tissue and reduces its excitability (Greiner and Garb, 1950; Weidmann, 1956 *b*). A similar effect has been recorded from avian embryonic heart cells in culture (Sperelakis and Lehmkuhl, 1966) and is confirmed above (Fig. 6).

The response to decreases in $[K^+]_o$ below normal serum levels is less certain. Paes de Carvalho (1965) has reported that the cardiac transmembrane potential is maximal at about 5 mM K^+ and decreases (i.e. depolarizes) as $[K^+]_o$ is reduced. Lieberman (1967) recently obtained higher potentials from chick heart cells in culture in 4.4–5.6 mM K^+ than in media containing 1.4 mM. Weidmann (1956 *b*) found the peak resting potential at 2–5 mM K^+ in sheep and calf Purkinje fibers. On the other hand Vassalle (1965) has recently shown that the resting potential of mammalian Purkinje fibers is unaffected by a reduction of $[K^+]_o$ from 5.4 mM to 2.7 mM, although his records indicate

that the maximal diastolic potential increases substantially with this change. Furthermore, numerous workers, impaling a variety of cardiac preparations, have reported that membrane potential falls continuously along a more or less smooth curve, as $[K^+]_o$ is elevated from levels near zero (DeMello and Hoffman, 1960; Kanno and Matsuda, 1966; Antoni, Herkel, and Fleckenstein, 1963).

Our own results (Fig. 6) indicate that in embryonic heart cells, immediately accessible to changes in the environment, resting potential reproducibly reaches a peak of electronegativity at about 6 mM K^+. At $[K^+]_o$ below this level, as in high-K media, the membrane was depolarized. However, a second effect of $[K^+]_o$ must also be taken into consideration. As $[K^+]_o$ was decreased, more and more cells exhibited afterpotentials, and these were of progressively greater amplitude (Table III). That a decrease in $[K^+]_o$ from 5 mM to 1 mM should depolarize cells despite the marked increase in the ratio $[K]_i/[K]_o$, can be understood in terms of the influence of $[K^+]_o$ on membrane permeability to K^+. From the values provided by Carmeliet (1961), at -60 mv membrane potential, membrane resistance (R_M) drops by a factor of two as $[K^+]_o$ increases from near zero to 4.5 mM, and drops further, almost to zero, at 50 mM K^+. In the other direction, Hall, Hutter, and Noble (1963) and Vassalle (1965) both found high resting resistances in Purkinje fibers depolarized by low $[K^+]_o$. Weidmann (1966) has measured the flux of ^{42}K across the membranes of sheep ventricular fibers, and found a sharp decrease when $[K^+]_o$ was reduced from 5.4 mM to 0.54 mM. Thus at high $[K^+]_o$, membrane conductance in heart muscle is high, just as in the squid axon (Sjodin and Mullins, 1967). The major portion of this transmembrane ionic current is carried by K^+; i.e., the ratio $\frac{P_K}{P_{Na}}$ is large. Thus the cell acts essentially like a potassium electrode, where the membrane potential is equal to the potassium equilibrium potential (E_K). However, when $[K^+]_o$ is reduced below 6 mM and E_K becomes progressively more negative, the membrane potential does not follow. Instead the cell exhibits anomalous rectification, the situation in which an outward electrochemical potential gradient has the effect of reducing potassium permeability (Noble, 1965).

The fact that Antoni et al. (1963) depicted the membrane potential falling along a continuous curve of progressively steeper slope as $[K^+]_o$ was increased from 2.7 to 25 mM may be explained by the fact that they took the maximal diastolic potential as representative of the membrane potential, and also that they made no measurements at K levels between 2.7 and 10 mM. Similarly, Sperelakis and Lehmkuhl (1966) reported that most of the cells in their cultures became progressively depolarized as $[K^+]_o$ was increased from 1.7 to 25 mM. They also skipped observations between 2.7 and 10 mM. They did note, however, that some cells exhibited a slight hyperpolarization at 10 mM K^+.

As plotted in Fig. 6, a distinction is made between resting potential and

maximal diastolic potential, and measurements are made at close intervals. At $[K^+]_o$, of 8 mM and above, the cells behave as K electrodes. The measured values fall on the line of E_K having a slope of 61 mv per 10-fold change in $[K^+]_o$, calculated from the Nernst equation. No afterpotentials are present and the resting potential equals the maximal diastolic potential. These results lead to a calculated $[K^+]_i$ of 82 mM. This is slightly less than the value derived from similar data by Sperelakis and Lehmkuhl (1966) of 90 mM, and much lower than those obtained from the ^{42}K studies of Harsch and Green (1963) and of Burrows and Lamb (1962) whose data permit a calculation of about 180 mM $[K^+]_i$.

At 8.1 mM, one-third of the cells begin to show small afterhyperpolarizations, averaging only 0.5 mv, but the maximal diastolic potential remains at the level of E_K. As $[K^+]_o$ is decreased from 6.1 to 1.3 mM, more and more cells exhibit afterpotentials of progressively greater magnitude. Thus, both the maximal diastolic potential and the resting potential depart, respectively, further and further from E_K.

The mechanisms underlying the formation of afterhyperpolarizations are thought to be as follows (Woodbury, 1965; Noble, 1966). At the peak depolarization of the action potential, g_{Na} begins to fall as a result of "inactivation" of Na^+ channels in the membrane (Hodgkin and Huxley, 1952). After an immediate transient increase g_K begins a much slower time-dependent increase (McAllister and Noble, 1966, 1967) reaching a maximum at the end of the plateau. Since during this time g_{Na} has continued to fall, the major portion of the ion current is carried by K^+. Thus, the membrane potential moves toward E_K. This adds an immediate voltage-dependent decrease in g_{Na}, and a delayed decrease in g_K. Thus g_K is still above the resting level when repolarization is complete, and the membrane hyperpolarizes. Thereafter, g_K and the membrane potential fall slowly back to their resting values.

At low $[K^+]_o$, where values of E_K are high, the resting potential and the resting g_K are diminished as noted above. During phase 3 of the action potential, when g_K increases greatly over the resting level, large hyperpolarizations would be expected because of the great difference between the resting potential and E_K. On the other hand, at $[K^+]_o$ well above normal, the resting g_K should be so high that the resting potential is equivalent to E_K, and the cell should form no afterpotentials (Fig. 4). These are the results seen in Table III.

Finally, the effects of $[K^+]_o$ on pacemaker potentials deserve brief consideration. Our data confirm that at low $[K^+]_o$ most cells exhibit a diastolic depolarization, and that this is reduced in slope and disappears as $[K^+]_o$ is increased. Sperelakis and Lehmkuhl (1966) have described in detail the "conversion" of pacemakers into nonpacemakers by elevated $[K^+]_o$. The fact that increased K^+ abolishes both the afterpotential and diastolic depolarization might be taken as supporting the proposal of Dudel and Trautwein (1958) that these

two components of the diastolic membrane potential represent different manifestations of the same mechanism. However, high K can depress the pacemaker potential without affecting the afterhyperpolarization (Fig. 5, 17 min), or contrariwise, abolish the afterpotential but not the slow diastolic depolarization (Fig. 4), suggesting distinct mechanisms. Noble and Tsien (1968) have made a theoretical reconstruction of the decline in K current during the pacemaker potential in Purkinje fibers, basing their calculations on two separable underlying mechanisms. One is a declining kinetic variable (s) triggered by the previous action potential, which controls the time dependence of the K current. The other is a rectifier function (i_{K_2}) which is dependent upon E_K and produces a fall in g_K as a consequence of inward-going rectification. These authors also note a point in the course of the slowly falling diastolic potential, comparable to our PBA, at which membrane potential would stabilize if spontaneous excitation fails.

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