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SOME ELECTROPHYSIOLOGICAL PROPERTIES OF
CULTURED CARDIOMYOCYTES ISOLATED FROM THE ADULT RAT

by

CAMERON BRUCE KENNEDY, B.Sc. (Hon.)

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfillment
of the requirements for the degree of
Master of Science

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ABSTRACT

Intracellular electrophysiological measurements were made on cultured cardiomyocytes from the adult rat heart. Cells were isolated and cultured by the method of Jacobson (1977). The electrophysiological properties of cultured cells changed in a characteristic way with time in culture. Cells upon isolation and up to one day in culture were difficult to impale and they exhibited low transmembrane potentials. Cells in culture from four to eight days were less difficult to impale but still exhibited low resting membrane potentials and were electrically inactive. Cells in culture from twelve to twenty-eight days were attached to the culture substratum and were selected for study on the basis of their being spontaneously contractile. The maximum diastolic potential for attached cells was less than that reported for cells in vivo but was the largest of those in vitro. In these attached cells spontaneous action potentials usually accompanied contractile activity. The upstroke of action potentials of the attached cells also had a slower rate of rise than that of cells in vivo and exhibited a plateau phase during repolarization.

The electrophysiological measurements indicate that in general, the cells may be grouped into three developmental phases. (1) Newly isolated: These cells are spontaneously contractile but failed to show any spontaneous electrical activity. An applied electrical current will elicit an action potential and a contraction. The newly isolated phase lasts from isolation through at least one day in culture.

(2) Quiescent: The majority of cells go into a state of quiescence. This phase is characterized by the lack of spontaneous contractions or action potentials. As well, these cells could not be electrically

excited to elicit an action potential. The quiescent phase lasted from at least the fourth through eighth days in culture. (3) Established: The final phase that the cells were observed to enter was characterized by the cells giving rise to spontaneous action potentials in synchrony with spontaneous contractions. This phase was determined to last from about the twelfth day to at least the twenty-eighth day in culture. These phases, which were determined electrophysiologically, are correlated with the in vitro morphological changes which have been previously reported for the cells (Schwarzfeld, 1979). In addition, the electrophysiological response exhibited during the established phase is compared to the in vivo state and discussed in terms of the possible ionic currents which underly it.

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INTRODUCTION

I Action Potential Configuration

Myocardial cells and Purkinje fibres are the two main electrically active tissues of the ventricle. Purkinje fibres are part of the specialized conducting system and are formed by the peripheral ramifications of the bundle of His and spread over the ventricular myocardium from the interventricular septum.

Each tissue has a characteristic electrical activity. In Purkinje fibres the action potential (Figure 1) has an initial rapid depolarization, termed the upstroke. This is followed by a rapid initial phase of repolarization during which the membrane potential returns to about zero. Subsequent phases of repolarization bring the membrane potential to its most negative value. When repolarization is complete, a slow depolarization brings the membrane potential back to a threshold level, at which time another action potential occurs. The slow depolarization is termed the pacemaker potential or diastolic depolarization and is responsible for the spontaneous electrical activity observed in Purkinje fibres. The pacemaker potential in Purkinje fibres is dependent on membrane potential and tends to decrease in rate at more hyperpolarized values (Noble, 1975).

In the bulk of ventricular myocardial cells (ventricular myocardium) action potentials are slightly different in form (Figure 2). The upstroke is followed at its peak by a phase of slow repolarization called the plateau. The initial rapid repolarization of Purkinje tissue is absent (Noble, 1975). As well, there is normally no pacemaker potential and the transmembrane potential difference is termed the resting potential. This resting potential difference is roughly

FIGURE 1

Action potential from sheep Purkinje fibre
(from Weidman, 1956, Figure 4.E)

- 0 = Phase 0. Upstroke of action potential
- 1 = Phase 1. Initial phase of repolarization
- 2 = Phase 2. Plateau of repolarization phase
- 3 = Phase 3. Tail of repolarization phase
- 4 = Phase 4. Diastolic depolarization.

Scale: 60 mV; 500 msec.

FIGURE 2

Action potential record from dog ventricle
(from Weidman, 1956, Figure 4.B)

Scale: 60 mV; 100 msec.

FIGURE 3

Action potential record from rat ventricle
(from Weidman, 1956, Figure 4.C)

Scale: 60 mV; 100 msec.

Fig. 1

Fig. 2

Fig. 3

equal to the maximum diastolic potential (MDP) (i.e. the most negative potential, see Figure 8) in Purkinje fibres (Noble, 1975).

The upstroke of the action potential is very rapid lasting less than a msec. (Dudel, 1972). In Purkinje fibres the maximum rate of depolarization, $dV/dt_{\max}$, (termed $+\dot{V}_{\max}$, the maximum rate of rise) during the upstroke can be as high as 600 V/s from a MDP of about -90 mV. $+\dot{V}_{\max}$ decreases if the membrane potential immediately preceding the action potential upstroke is briefly (1 msec.) depolarized and the action potential is initiated from this depolarized potential; $+\dot{V}_{\max}$ becomes zero (i.e. no action potential) if the membrane is briefly depolarized to about -50 mV (Weidman, 1955). The upstroke of the action potential in all types of myocardial tissue usually brings the membrane potential to about +20 to +30 mV. The amount by which the membrane becomes positive during the action potential is termed the overshoot. The magnitude of overshoot also depends on the value of membrane potential immediately before initiation of the action potential. The overshoot is less for more depolarized membrane potentials (Weidman, 1955). Membrane repolarization usually lasts about 500 msec. in both adult Purkinje fibres and ventricular myocardium from most mammalian species (Noble, 1975).

The action potential of adult rat myocardium differs from the foregoing generalized description of the mammalian cardiac action potential. In prenatal and early postnatal life the rat myocardial action potential is similar in configuration to that of adult members of other mammalian species (i.e. similar to Figure 2). As maturation takes place, the duration of the action potential is decreased by a

progressive loss of the plateau phase of repolarization (Bernard and Gargouil, 1963; Couch, West and Hoff, 1969; Langer, 1975, 1978). The action potential duration in the adult rat is only about 100 to 150 msec. and the configuration is distinctly different from most other adult mammalian species (Figure 3) (Langer, 1978).

II Ionic Currents Responsible for the Action Potential

A. Technical Considerations.

To understand the nature of the cardiac action potential it is necessary to study the ionic currents responsible for the different phases of the action potential. Although the voltage clamp technique (Hodgkin and Huxley, 1952a,c) has allowed ionic currents of squid giant axon to be studied accurately, the technique has serious limitations when used with cardiac tissue. The double microelectrode voltage clamp (Deck, Kern and Trautwein, 1964) has been applied to short segments of Purkinje fibres, and the sucrose gap (or double sucrose gap) voltage clamp (Stampfli, 1955; Rougier, Vassort and Stampfli, 1968) has been used on bundles of atrial or ventricular (papillary muscle) tissue. However, with each technique problems of voltage control arise when large surges of current are passing through the membrane (Fozzard and Beeler, 1975). As well, there is a non-homogeneous spread of current (and hence voltage) associated with the double microelectrode technique when inhomogeneous tissue preparations are used (Johnson and Lieberman, 1971). With the sucrose gap technique problems arise with leaching of ions from cells bathed by sucrose, or when sucrose penetrates into the intercellular clefts of ventricular tissue bundles. Both problems result in an increase in longitudinal resistance and errors in ionic current measurement (Beeler and Reuter,

1970a; Johnson and Lieberman, 1971; Goldman and Morad, 1977), especially when large ionic currents are flowing.

These limitations of the voltage clamp technique have raised doubts about measurements of the initial inward ionic current since it is a large current with a rapid onset. Johnson and Lieberman (1971) have also noted that few studies have successfully separated the capacitive current from the early ionic currents. In cardiac Purkinje fibres for example the capacitive current may last for 2 to 4 msec. Because that is longer than the period before the initial inward current (Fozzard, 1966), capacitive current must be mathematically separated from ionic current measurements.

B. Fast Inward Current.

In spite of limitations of and problems arising from present voltage clamping experiments, various attempts have been made to use voltage clamping to identify and characterize the ionic currents responsible for the cardiac action potential. The existence of two inward currents in mammalian myocardium has been proposed (Masher and Peper, 1969; Beeler and Reuter, 1970; Trautwein, 1972; Kohlhardt et al., 1972). Chapman, Kootsey and Johnson (1979), however, have produced a mathematical model based on data from various sources to mimic an action potential for a non-specific cardiac tissue. This model utilizes only a single inward current in addition to an electrogenic active transport of sodium and potassium ions in place of two inward currents. Although this introduction shall consider the model which utilizes two inward currents during the cardiac action potential, it should be remembered that an alternative model has been proposed. In the "two-inward-current" system the first ionic current during the

cardiac action potential has been shown to be quite large in magnitude and responsible for the rapid upstroke of action potential. Colatsky and Tsien (1979), using the double microelectrode technique, have shown inactivation parameters to be dependent on membrane potential in a manner similar to nerve axon sodium current (Hodgkin and Huxley, 1952b,c). In the rabbit Purkinje fibre used by Colatsky and Tsien (1979), the fast inward current shows no inactivation at membrane potentials more negative than about -100 mV and complete inactivation at membrane potentials less negative than about -50 mV. (Colatsky and Tsien (1979) mathematically separated the capacitive current from the fast inward current.) The fast inward current was found to be sensitive to both temperature (Dudel and Rudel, 1970) and external calcium concentration (Beeler and Reuter, 1970a) in sheep Purkinje fibres and dog ventricular myocardium respectively. Reversal potentials for the fast inward current have been shown to be near the assumed equilibrium potential for sodium in both dog (Beeler and Reuter, 1970a) and cat (Kohlhardt, et al., 1972) ventricular myocardium. In addition to these features, the fast inward current has also been shown to be sensitive to tetrodotoxin (TTX) (Rougier et al., 1969), a known sodium channel blocker (Kao, 1966). Furthermore, sodium-free perfusion studies have also shown that the fast inward current can be abolished in the presence of a sodium-free bathing medium (sheep and dog ventricular myocardium) (Masher and Peper, 1969; Beeler and Reuter, 1970b). Recovery of the fast inward current from inactivation is slower than for squid axon (Hodgkin and Huxley, 1952b) in a variety of mammalian cardiac preparations (Gettes and Reuter, 1974), and the fast inward current is less sensitive to TTX than in squid axon

(Narahashi, 1974). Nonetheless, the majority of data would suggest that the fast inward current is carried by sodium ions in a manner similar to that for squid axons, and is responsible for the rapid rate of rise of the upstroke of the action potential. As well, there does not seem to be any difference between Purkinje fibre fast inward current and ventricular myocardium fast inward current mechanisms from a variety of species.

C. Slow Inward Current.

To study the slow inward current alone, the fast inward current must be eliminated by the use of TTX, sodium-free perfusion, by depolarizing the membrane to values where the fast inward current is inactivated (see above) or by some other method. Voltage clamp data show that the slow inward current in a variety of species (dog, sheep, adult cat) is similar. The threshold potential for activation is at a membrane potential between -35 mV and -20 mV, with maximum current occurring at a membrane potential between about -17 mV and 0 mV (Masher and Peper, 1969; Beeler and Reuter, 1970b; New and Trautwein, 1972a,b). The activation of this current is complete at a membrane potential of -10 mV while inactivation begins at a membrane potential of about -55 mV to -60 mV and is complete at a membrane potential of about +10 mV in adult cat (Trautwein, McDonald and Tripathi, 1975) or cow (Reuter and Scholz, 1977) ventricular myocardium. Thus, potentials for complete activation and inactivation for the slow inward current are shifted to less negative membrane potentials than those for the fast sodium current. The slow inward current has been shown to have reversal potentials at slightly more positive potentials than the fast inward current in both dog ventricular myocardium (Beeler and Reuter, 1970a,b) and cat ventricular myocardium (Kohlhardt et al., 1972). Furthermore,

the strong dependence of this current on external calcium ion concentration in cat ventricular myocardium suggests that the slow inward current is being carried by calcium ions (New and Trautwein, 1972b; Kohlhardt et al., 1975). The slow inward current is insensitive to TTX (Beeler and Reuter, 1970b), but can be abolished in the presence of verapamil (α -isopropyl- α -[(N-methyl-N-homoveratryl)- γ -aminopropyl]-3,4-dimethoxyphenylacetonitrile·HCl) and D-600 (a methoxy derivative of verapamil), neither of which have any effect on the fast inward current. The divalent cations strontium and barium neutralize the effect of verapamil and D-600 on the slow inward current in cat ventricular myocardium (Kohlhardt et al., 1973). These observations are consistent with the hypothesis that the slow inward current in the ventricular myocardium of most mammalian species is carried by calcium ions.

Conversely, the data do not exclude the possibility that the slow inward current is carried by sodium ions as well as calcium ions. Vereecke et al. (1975) have shown that lowering the external sodium ion concentration tends to increase the amplitude of the slow inward current in cat ventricular myocardium under voltage clamp conditions. If sodium and calcium ions were competing charge carriers for existing ion channels, lowering the ratio of sodium ions to calcium ions would allow calcium ions to compete more effectively.

As more calcium ion was substituted for sodium ion current carriers, the magnitude of the current would also increase even though the same number of ions would be traversing the membrane (per unit time and area). That at least a part of the slow inward current is carried by calcium ions is suggested from the dependence of contraction

on the presence of the slow inward current and on the presence of external calcium ions. Gibbons and Fozzard (1975) found a direct correlation between the peak amount of slow inward current and the peak tension developed for a given action potential in sheep cardiac Purkinje fibres. As well, New and Trautwein (1972b) found in adult cat ventricular myocardium that when the slow inward current was eliminated by voltage clamping to the reversal potential for this current there was no development of tension (see Appendix 1). These experiments were carried out in the absence of fast inward current. When a slow inward current was allowed to flow by voltage clamping to membrane potentials more negative than the equilibrium potential for the slow inward current tension was produced. It would therefore seem that the presence of a slow inward current is required for the production of tension. The possibility that the presence of a calcium current may initiate the release of bound calcium from internal stores has also been suggested (Fabiato and Fabiato, 1975; Gibbons and Fozzard, 1975). The measurements of Fabiato and Fabiato (1975) indicate that calcium ions were released from internal stores in proportion to the concentration of free "intracellular" calcium ion in cellular preparations which had the sarcolemma removed. The initial free "intracellular" calcium ion concentration was not great enough to initiate tension production, although considerable tension could be measured in preparations where the sarcoplasmic reticulum had not been destroyed. An analogous situation in intact tissue would be where a calcium current would increase the intracellular calcium ion concentration to levels below those required for the production of tension. The increase in intracellular calcium ion

concentration would initiate the release of bound calcium from internal stores (i.e. sarcoplasmic reticulum) which in turn would initiate the production of tension.

These data, from the analysis of slow inward current, do not completely identify any single ion or ions as the principal charge carriers. They do, however, suggest that both sodium ions and calcium ions are responsible for this current, and that the slow inward current is an integral part of excitation-contraction coupling.

D. Outward Currents.

The repolarization phase of the cardiac action potential of the Purkinje fibre depends primarily on outward currents. As the time-dependent slow inward current inactivates and the time-dependent outward current activates, the net current will become outward and repolarization will occur. There appear to be at least three outward currents responsible for repolarization in the Purkinje fibre action potential (Fozzard, 1975). Two of these are time-dependent and one is time-independent. The main outward current, i_{K1} , is activated very quickly, at levels of depolarization comparable to the peak of the action potential and is time-independent (McAllister and Noble, 1966). This current is of low magnitude and contributes significantly to the repolarization phase. Two other outward currents, i_{x1} and i_{x2} , have activation kinetics which are much slower than i_{K1} , but which are still thought to contribute to the repolarization phase (Noble and Tsien, 1969a,b). Although these outward currents have been assumed to be primarily potassium currents based on reversal potential data, Goldman and Morad (1977) have shown that some inward chloride current is flowing during the plateau phase of the action potential in frog

heart and is responsible for a significant portion of the repolarization.

E. Currents Producing the Pacemaker Potential.

A fourth outward current activated by the Purkinje fibre action potential, termed i_{K_2} , has kinetics of inactivation much slower than any of the other outward currents (Noble and Tsien, 1968). This current has been found to inactivate over the duration of the diastolic depolarization, leaving an increasing net inward current responsible for the depolarization (Vassalle, 1966, 1977). Since the overall conductance falls during the slow diastolic depolarization (pacemaker potential), there is apparently no inward current increase until threshold is reached (Vassalle, 1977) and another action potential is initiated. Noble and Tsien (1968) used voltage clamp analysis to show that the current i_{K_2} displayed a large inward-going rectification. When the membrane was clamped to largely depolarized potentials there was very little i_{K_2} current. However, this current did activate when the membrane was re-clamped at potentials comparable to those where the diastolic depolarization occurs (Noble, 1975).

The experiments which have produced the data regarding outward currents have been performed on sheep Purkinje fibres. Ventricular myocardium does not normally have a pacemaker potential and therefore probably does not have the current i_{K_2} . Neither does ventricular myocardium have a phase 1 during-repolarization. For this reason the outward currents described for sheep Purkinje fibres may not necessarily occur in ventricular tissue.

III Tissue Culture Preparations

Voltage clamp analysis of excised tissue preparations has been

plagued with the problems of non-homogeneous spread of current due to variable resistance pathways between cells. Problems such as this are more prevalent in ventricular myocardium because of the presence of T-tubules (Johnson and Sommer, 1967) which may greatly increase the "surface" membrane area (i.e. the sarcolemma and all other membrane which separates the interior and exterior of the cell). T-tubules may also represent regions of inaccessible extracellular compartments. The presence of such compartments would hinder the rapid exchange of perfusion media in pharmacological or ionic testing experiments. Furthermore, such compartments would be likely sites for accumulation or depletion of ionic species involved in action potential fluxes. Lee et al. (1979) have overcome this problem by using the enzymes collagenase and hyaluronidase to isolate single myocytes from the ventricles of adult rats. These isolated single cells studied under voltage clamp conditions are free from non-homogeneous current spread associated with variable resistance intercellular pathways. The sodium current data of Lee et al. (1979) compare favourably with that of Colatsky and Tsien (1979) in spite of the tissue differences (rat ventricular myocardium versus rabbit Purkinje fibre respectively). It should be noted that Lee et al. (1979) suppressed ionic current flow (except sodium) by substituting impermeant ions for potassium, calcium and chloride ions. These authors also artificially hyperpolarized the cells by the removal of external chloride. Although the cells of Lee et al. (1979) were in an unnatural ionic environment and had at least one feature not inherent to in vivo or excised preparations (i.e. low resting membrane potential) they still produced fast inward current data comparable to the excised tissue preparations of Colatsky and Tsien (1979).

Thus, using single isolated cells is one approach to alleviating the problems associated with voltage clamping preparations of natural myocardium (Purkinje fibres or ventricular myocardium). Single isolated cells would be more appropriate for study if they could be isolated with normal properties in physiological concentrations of salts and at physiological temperatures. Another approach to solving the problem is utilization of a synthetic preparation of cardiac tissue as a model of myocardium. With adequate control of in vitro development it may be possible to synthesize a preparation which does not have the limitations inherent in the natural preparations. Desired properties of a synthetic preparation are a simple geometry of desired size, and of a limited number of homogeneous cells. Synthesis of such a preparation would require maintenance in culture of isolated cells for several days to weeks. In addition to the possibility of developing a better preparation for voltage clamp and pharmacological studies, the synthesized preparations might also be especially suited for studies of ~~intercellular~~ conduction.

Cavanaugh (1955) and Harary and Farley (1963) pioneered the culture of single isolated cells of avian and mammalian myocardium. Since that time numerous investigators have attempted to develop in vitro models of myocardium for electrophysiological studies. Three types of preparations have been used as models of the myocardium. These are monolayer or thin multilayer cultures of mixed cell type (Sperelakis, 1972), spherical reaggregates of mixed cell type (Sperelakis, 1978), and linear arrays or synthetic strands of mixed cell type (Lieberman et al., 1975; Sachs, 1976). Culturing methods such as these allow passive electrical properties (Lieberman et al.,

1975) as well as the action potential to be studied. In addition, voltage clamp analysis has been performed on spherical reaggregates of embryonic chick heart (Nathan and DeHaan, 1979; Ebihara et al., 1980). Nathan and DeHaan (1979) were unable to separate the activation of the fast inward current from capacitive transients. Ebihara et al. (1980), however, were able to adequately voltage clamp the fast inward current. The findings of Ebihara et al. (1980) are consistent with those of Lee et al. (1979) for single isolated cells and Colatsky and Tsien (1979) for Purkinje fibres.

The range of species and age of individuals used in the preparation of tissue cultures has varied from study to study. In addition to extensive reports regarding chick embryo cultures, there are reports on embryonic guinea pig (Auclair, 1976) and neonatal (up to seven days post partum) rat heart cell cultures (Mark and Strasser, 1966; Hyde et al., 1969; Schanne, 1972; Auclair, 1976; Kitzes and Berns, 1979; Schanne et al., 1979). Attempts have also been made to isolate heart cells from adult animals but most of these have been utilized in biochemical studies which did not require that the cells be established in culture (Vahouny et al., 1970; Bloom, 1971; Glick, Burns and Reddy, 1974). Isolation methods presently used for adult tissue have resulted in severely damaged myocytes (Berry, Friend and Scheuer, 1970; Moses and Kasten, 1979) which have survival times of only about up to 36 hours when perfused at physiological temperatures with normal physiological levels of calcium (Nag et al., 1977; Clark et al., 1978; Fry, Scales and Inesi, 1979). A viable myocardial model derived from adult tissue would be a valuable system for studies of biochemistry, pharmacology and electrophysiology, particularly since

it is often only in the adult state that idiopathic disease processes are fully manifest. Such a model might more fully approximate naturally occurring myocardial tissue of mature animals.

This study reports some electrophysiological measurements on cultures of myocytes derived from the heart of the adult rat. Isolated cells were grown in flat mono- or ~~thin~~ multilayer cultures and were incubated at normal physiological temperatures and in a medium containing normal physiological levels of all salts, including calcium, for periods up to sixty days.

METHODS AND MATERIALS

A Nikon inverted phase contrast microscope with long working distance optics was used to locate cells to be examined and to maintain visual control over placement of the microelectrode (probe) into the cell. The microscope was equipped with a heater stage which maintained the cell culture medium at 35°C . The microscope stage (Figure 4) and micromanipulator system (Appendix 2) were designed to allow the microelectrode to be advanced towards a cell at an angle of 10° to 12° elevation from the culture substratum. The low angle was required as a consequence of the relatively slight thickness (5 to 10 μm) of the myocardial cells which had established in culture. A probe advanced at too steep an angle simply pushed the upper cell membrane against the culture substratum before impalement was achieved.

An oblong teflon culture chamber fitted into a well in the heater stage. The chamber contained a 25 mm. diameter teflon ring whose centre was located over a glass viewing port in the bottom of the chamber. The ring was magnetically coupled to a perspex control ring located beneath the microscope stage. Rotation of the control ring caused a like rotation of the teflon ring. The teflon ring was used to support the glass coverslip on which the cultured cells grew. Rotation of the substratum permitted selected areas of the culture to be scanned in order to locate cells for impalement. The remainder of the chamber to one side of the culture ring was hollowed out to allow the microelectrode to be advanced at the required low angle.

Myocardial cells were isolated from the apical third of ventricles of rats $3\frac{1}{2}$ to 4 months of age. Isolated cells were grown in sterile cultures, prepared according to the method of Jacobson

(1977). Cultures were grown on 22 mm. square glass coverslips maintained in 34 mm. plastic petri dishes. In preparing a culture for examination, the heater stage was prewarmed, and about 80% of the growth medium from the culture's petri dish was transferred to the culture chamber so that the top of the culture ring was just submerged. The coverslip was then transferred to the culture ring and enough fresh growth medium was added to bring the total volume of medium in the chamber to approximately 4 ml. A sheet of light mineral oil (Klearol, Witco Chemical Co.) was layered on the culture medium to prevent evaporation. A jet of pure CO_2 from a manifold surrounding the culture chamber was adjusted so that the pH of the culture medium was maintained at 7.2 to 7.3. The mineral oil layer did not prevent the CO_2 from reaching the culture medium.

Glass capillary microelectrodes filled with 2 M KCl were used to impale cells whose transmembrane potentials were to be measured. Initially, the probes were manufactured using borosilicate glass of 0.85 - 0.90 mm. outside diameter (O.D.) pulled on a two stage micro-pipette puller (Industrial Science Associates) and were filled by vacuum boiling in the filling solution. Probe impedance was in the range of 35 - 60 M Ω . In subsequent studies borosilicate capillaries of 1.0 mm. O.D. containing a glass fibre were used to manufacture probes (Omega Dot, Cat. # 30-30-1; Frederic Haer & Co.). The glass fibre probes tended to allow more successful impalements of cells. Probes were mounted in a perspex collet-chuck holder. The probe contacted a silver-silver chloride (Ag-AgCl) electrode in the holder via a 2 M KCl salt bridge. The probe holder was clamped to the receiver unit of a hydraulically actuated micromanipulator. A glass

FIGURE 4. Heater Stage Assembly

- a. aluminum heater stage
- b. teflon culture chamber
- c. teflon culture ring
- d. glass viewport
- e. CO₂ manifold
- f. microelectrode (probe)
- g. probe holder
- h. reference electrode
- i. microscope condenser lens assembly.

Fig. 4

macropipette which dipped into the medium in the culture chamber served as a reference electrode. The front two-thirds of the reference electrode contained 5.4 mM KCl in 2% Agar (Difco), which made contact with the culture medium. The back third of the reference electrode was filled with 2 M KCl and into this was inserted a Ag-AgCl electrode. The Ag-AgCl electrodes of the reference electrode and probe holder were connected to an electrometer input amplifier (Mentor N-950 intracellular probe system). The Mentor probe system was also used to polarize the cells by means of transmembrane current injection. The current source was either one internal to the Mentor system or an external source (Grass SD5 stimulator; Medistor, microvolt calibrator, CIA).

Signals from the amplifier and current monitor of the Mentor system were displayed on an oscilloscope (Tektronix, 564B, or Gould digital storage, OS 4000) and fed into an audio monitor (Grass, AM5). The signals were also encoded by frequency modulation (Carleton University Workshop) and stored on magnetic tape (Sony, 854 recorder). For later analysis, the tape recorded signals were played back on the oscilloscope. Permanent visual records were made by photographing the oscilloscopic traces or by use of an ink-jet chart recorder (Siemens, Oscillomink B). In order to determine the rate of change of transmembrane potential during the action potential, action potential signals were electronically differentiated (Carleton University Science Workshop) for analysis (see Appendix 3 for details).

In experiments involving newly isolated cells, 0.5 mg/ml of deoxyribonuclease (DNase, Sigma type 1; Cat. # D4763) was added to the test medium to reduce the stickiness of cells and cell debris. In

cultures not treated with DNase, debris and red blood cells adhered to the tip of the probe hindering attempts to impale cells.

RESULTS

I Morphology

The isolated myocardial cells followed a characteristic sequence of morphological development in culture. Newly isolated cells were normally elongated (about $20 \times 100 \mu\text{m}$) with obvious cross-striations. Cells which survived in culture underwent a transition from elongated to round morphology (about $40 \mu\text{m}$ in diameter). During the transition, cross-striations disappeared gradually and the fully rounded cells became optically dense. The transition was complete within about 72 hours. About 20% of the rounded cells attached to the glass substratum and began to flatten and spread out. The process took several days to complete and cells were found to be more firmly attached towards the end of the eighth day than earlier on. Further development was characterized by the establishment of single myocardial cells as contracting loci in a contiguous sheet of fibroblast-like cells. The in vitro morphological development of cells can be divided into three stages: newly isolated, elongated and striated cells (up to 3 days); rounded phase of optically dense cells (3 - 8 days); and established phase of flattened, spread and spontaneously contractile cells (greater than about the ninth day). Photomicrographs of a typical sequence of in vitro development is shown in Figure 5. The spontaneous contractile activity and electrophysiological responses of the cells in culture could be correlated with the stages of morphological development and time in culture.

II. Spontaneous Contractions

Cultures which were transferred to the culture chamber from their petri dish contained cells which continued to contract spontaneously

FIGURE 5 Phase contrast photomicrographs of myocytes isolated from adult rat heart at three different stages of tissue culture development.

- A. Newly isolated phase shortly after plating
- B. Rounded phase; 3 days in culture
- C. Established phase; 13 days in culture.

(from Jacobson, 1977, Fig. 2)

Fig. 5

after the transfer. Newly isolated cells and those in culture for up to one day showed contractions of two types. One type involved the propagation of a wave-like contraction in either one or both longitudinal directions. The waves could originate at various points along the cell with the possibility that more than one could be propagating at the same time. A similar type of contraction, termed "escalation", has been observed in cultures of skeletal muscle (Griffin et al., 1972). A second type of contraction involved a quick, forceful, longitudinal shortening that involved the entire cell at once. Cells in the rounded phase (four to eight days in culture here) were seldom seen to contract spontaneously. When contractions were observed they usually resembled the escalation type in that they usually were restricted to a part of the cell. As the cells spread out in culture, waves of contractions were seen to propagate down the arms of the cell. Often in cells in the late transition stage of development, propagating waves would diminish before reaching the extreme locations of the cell. As cells more than eight to ten days in culture (into the established phase) continued to spread out, waves of contraction appeared more forceful and involved more of the cell area. Although in some cells waves of contraction still arose at various locations and propagated to other regions of the cell, it was common to see cells whose only type of contraction involved a shortening of the processes of the cell in a whole-cell fashion.

III Impalement Records

Initial attempts at impalement using probes manufactured from 0.85 to 0.90 mm. O.D. borosilicate capillaries proved unsuccessful in spite of probe impedances of 35 to 60 M Ω and tip diameters of less than

0.25 μm as determined by examination in the scanning electron microscope. Usually probe-tip pressure against a cell surface was observed to coincide with measurement of a slight (up to 5 mV) positive potential. As impalement occurred, a slowly developing negative potential was recorded. This usually reached 10 to 30 mV in several seconds. In response to this type of impalement (coinciding with the beginning of a negative potential) the cell would be observed to change its form of contraction to one in which the contractions were quick, forceful and at a frequency of 5 to 10 per second. These contractions, here termed "fibrillation", arose from various sites on the cell but usually from a site at about the area where the micropipette entered the cell. A fibrillating cell often changed shape and appearance, becoming smaller and more optically dense as the amplitude of contractions diminished. If no shape change occurred the fibrillations usually diminished in one to two minutes with the cell remaining quiescent afterwards. Action potentials were rarely recorded using these probes.

In contrast to the foregoing results, impalements using the Omega-dot capillaries were obtained regularly. With newly isolated cells the impalement records were usually of a consistent form. As the probe tip contacted the cell a negative potential up to 5 mV developed. This was followed by a rapid increase in negative potential to a value between -22 and -35 mV. This potential often diminished by up to 14 mV in from 2 to 18 seconds (Figure 6), becoming stable at the new level. A reversion to fibrillatory contractions from either a quiescent state or twitch type contractions occurred in response to penetration of the probe into the cell. The fibrillations would some-

FIGURE 6. Impalement record of a newly isolated cell.
Scale: 20 mV, 1 sec.

FIGURE 7. Impalement record of a 4 day cell culture.
Scale: 20 mV, 1 sec.

times diminish with time, but could be stopped by applying a hyperpolarizing current.

Cells which were in the rounded phase (4 to 8 days in culture) showed impalement of the fast developing type (see Figure 7). As the probe neared the cell, a 1 to 3 mV negative potential was recorded, followed by a rapid increase in negative potential to values of -32 to -52 mV. Occasionally, a small (approximately 1 mV) positive potential was seen just prior to impalement. After the initial rapid increase in negative potential a slower hyperpolarization of up to 4 mV to a steady level. The average steady level was -42 ± 2.3 mV ($n=12$)*. Cells which were impaled were not spontaneously active.

In established cells (older than 12 days), the impalement records were of three types. The first type (Figure 8a) was a slowly developing negative potential which reached a maximum in several seconds. Spontaneous action potentials were sometimes, but not always, observed as the potential neared its maximum. In many cases the slowly developing potential was accompanied by the cell reverting from either quiescence or whole-cell contractions to fibrillatory contractions. Action potentials were never associated with this process. The end result was a shape and appearance change of the type noted under similar circumstances for younger cells.

The second type of impalement was observed only in beating cells. In this case a slight (1 mV) negative potential was seen immediately before the onset of a fast developing negative potential which was accompanied by an immediate appearance of action potentials (Figure 8b). The maximum diastolic potential (MDP) of each action potential

* means given as $\pm$ standard error of mean; n = no. of observations.

FIGURE 8A Impalement record from 28 day culture. This record shows the slow development of potential after impalement and the subsequent spontaneously generated action potentials.
Upper trace $\dot{V}$. Scale: 10 V/sec.
Lower trace cell. Scale: 20 mV, 3 sec.

FIGURE 8B Impalement record of 14 day culture. This record shows the rapid increase in potential and immediate registration of action potentials which increase in amplitude after impalement.
Upper trace $\dot{V}$. Scale: 10 V/sec.
Lower trace cell. Scale: 20 mV, 1 sec.
Maximum diastolic potential (MDP)

FIGURE 8C Impalement record of 19 day culture. Immediately upon impalement action potentials of consistent amplitude were recorded on a steady most negative potential.
Upper trace $\dot{V}$. Scale: 10 V/sec.
Lower trace cell. Scale: 20 mV, 1 sec.
Maximum diastolic potential (MDP)

and overshoot value increased with each successive action potential. As the MDP increased, the frequency of action potentials decreased. The MDP and overshoot values reached a constant after several seconds. One whole-cell contraction was observed to occur in synchrony with each action potential. The frequency of action potential generation and contraction became fairly constant once the MDP was constant.

The third type of impalement was one where action potentials of consistent amplitude and frequency were immediately observed on a steady MDP (Figure 8c).

For cells in the established phase (greater than 12 days in culture), the MDP varied from -30 mV to -60 mV with a mean of -50 ± 5.6 (n=5).

IV Input Resistance

Cell input resistance was measured as the ratio of change in membrane potential for a change of applied transmembrane current (i.e. $R_{in} = \Delta E_m / \Delta i$). The input resistance in cells at all stages of development was variable from cell to cell as well as over the duration of recording from individual cells. Although there are few data, the input resistance of newly isolated cells was fairly consistent, having a mean value of $19 \pm 1.7 \text{ M}\Omega$ (n=4). Cells 4 to 8 days in culture generally showed lower input resistances, but had a larger range. For these cells the range was from 5 to 21 $\text{M}\Omega$ with a mean of $11 \pm 2.3 \text{ M}\Omega$ (n=8). For cells greater than 12 days in culture the input resistance was most variable. Values ranged from 5 to 44 $\text{M}\Omega$. For these cells the input resistance varied considerably during the recording period (which may have been the case as well for other groups that were not tested). No pattern could be determined for the

TABLE #1

Input resistance (R_{in}) measurements from one
spontaneously contractile myocyte in a 28 day
cell culture. Results in chronological order.

TABLE #1

POLARITY	APPLIED CURRENT i (nA)	MEMBRANE POTENTIAL CHANGE ΔE_m (mV)	INPUT RESISTANCE R_{in} (M Ω)
HYPERPOLARIZE	0.5	12	24
DEPOLARIZE	0.5	15	30
HYPERPOLARIZE	0.3	4	13
DEPOLARIZE	0.3	5	17
HYPERPOLARIZE	0.4	2	5
DEPOLARIZE	0.4	7	18
HYPERPOLARIZE	0.5	8	16
DEPOLARIZE	0.5	12	24
HYPERPOLARIZE	0.6	16	27
DEPOLARIZE	0.6	18	30
HYPERPOLARIZE	0.7	18	31
DEPOLARIZE	0.7	10	14
HYPERPOLARIZE	0.8	26	33
DEPOLARIZE	0.8	23	29
HYPERPOLARIZE	0.9	31	34
DEPOLARIZE	0.9	31	34

variability in terms of polarization history when cells were polarized more than once during a single impalement. This is exemplified in Table 1 for one such cell. No systematic control was attempted for polarization duration or nonpolarized recovery period. As a result of the large variability of input resistance no plot of change in membrane potential (ΔE_m) versus applied current (i) was made to calculate the slope resistance of these cells.

V Action Potentials

A. Cells in Newly Isolated Phase.

Although no spontaneous action potentials were observed from newly isolated cells, it was possible to hyperpolarize them with constant current injection, and then to briefly depolarize from the hyperpolarized level to evoke an action potential (Figure 9). Action potentials elicited in this manner were of short duration, ending well before the end of the 200 msec. depolarizing pulse. In one cell (6 hours in culture) the membrane was hyperpolarized from -26 to -90 mV by a steady current and depolarizing shocks brought the membrane potential to about -80 mV. The action potentials that resulted had overshoot values of $+8 \pm 0.4$ mV ($n=40$) and maximum rates of rise of 130 ± 0.6 V/s ($n=40$). When this cell had been hyperpolarized to -65 mV no action potentials could be evoked. A second cell (one day culture) behaved much the same way when hyperpolarized from -21 to -110 mV. Action potentials could be evoked when the membrane was depolarized to between -80 and -90 mV. The action potentials undershot the zero potential by about 20 mV and had maximum rates of rise of 124 ± 0.8 V/s ($n=30$). However, in several other cells which were newly isolated and displayed typically low resting membrane potentials (-22 to -34 mV)

FIGURE 9

Evoked action potential from a newly isolated cell. This cell was hyperpolarized by constant current injection and briefly depolarized to evoke an action potential.

Upper trace $\dot{V}$. Scale: 50 V/s. Arrow denotes peak.

Middle trace cell. Scale: 30 mV, 100 msec.

Lower trace stimulus monitor. On at single arrow, off at double arrow.

Fig. 9

hyperpolarizing to levels up to -76 mV would not allow action potentials to be evoked by depolarizing pulses.

B. Cells in Rounded Phase.

Cells which were in the rounded phase (4 to 8 days) were never observed to generate action potentials spontaneously. Furthermore, when these cells were hyperpolarized to levels as great as -104 mV and briefly depolarized (to as much as 0 mV) no action potentials could be evoked.

C. Cells in Established Phase.

i) Spontaneous Action Potential.

Spontaneous action potentials were recorded only from cells in culture for greater than 12 days. These cells showed slowly developing, depolarizing prepotentials resembling pacemaker potentials. When the prepotentials reached a threshold value an action potential occurred which always overshoot zero potential. A slight plateau could be seen in the membrane repolarization. That is, the rate of repolarization was not maximum immediately after the peak of the overshoot, but only after repolarization had proceeded for several tens of msec. (Figure 10). The prepotentials had variable rates of rise, from about 50 mV/sec. to about 260 mV/sec. There was a direct relationship between the slope of the pacemaker potential and the frequency of spontaneous action potentials.

The observed range of frequencies for the cells varied from an occasional isolated action potential to a maximum of about 250 per minute. The range for any one cell was less than this. For a given spontaneously firing cell the slope of the prepotential and frequency of action potential generation were both related to the MDP. As a

FIGURE 10

Spontaneous action potentials from a 19 day cell culture. Note the slight plateau to the repolarization phase (between arrows).

Upper trace $\dot{V}$. Scale: 10 V/s.

Lower trace cell. Scale: 20 mV, 100 msec.

Fig. 10

membrane was successively hyperpolarized the slope of the prepotential and the frequency both decreased. For depolarization the converse was true (Figure 11).

Action potential amplitude was a monotonic function of the MDP as shown in Figure 12. Most of the change in amplitude was due to a change in MDP and a slight portion was due to an overshoot change. Figure 13 shows that the overshoot varied in a non-linear fashion with the MDP. There was little change over a wide range of MDP. Overshoot values from +7 to +30 mV were recorded.

Action potential duration, measured to 90% repolarization, was also a monotonic function of MDP. Duration of action potential varied from 76 ± 0.8 msec. (n=30) to 210 ± 2.1 msec. (n=6). Figure 14 shows that as the MDP was increased the duration of the action potential increased.

The maximum rate of rise of the upstroke of action potentials was also a monotonic function of the MDP. Figure 15 shows that for the three cells represented the relationship was linear for each cell, although the slopes were different for different cells. Maximum rates of rise observed varied between 2.4 ± 0.1 V/s and 17 ± 0.2 V/s (n=30).

After-potentials of an oscillatory nature were sometimes associated with action potentials. In a few instances, the oscillatory afterpotential brought the membrane potential to threshold and an additional action potential was generated (Figure 16).

ii) Evoked Action Potentials.

Action potentials could also be evoked from cells which were capable of spontaneously generating action potentials. No cell was observed to spontaneously fire an action potential when hyperpolarized

FIGURE 11 The effect of current injection on spontaneous action potentials from a 19 day cell culture. A brief 0.5 nA depolarizing current (D) was applied followed by a recovery period and then 0.5 nA hyperpolarizing current (H).

Upper trace $\dot{V}$. Scale: 10 V/s.

Middle trace cell. Scale: 20 mV, 1 sec.

Lower trace stimulus monitor. Current injection onset at single arrow, offset at double arrow. Depolarizing current is represented as an upward deflection, hyperpolarizing as downward.

Fig. 11

FIGURE 12

Plot of the absolute amplitude of spontaneous action potentials versus the maximum diastolic potential (MDP).
Filled circles 19 day cell.
Open circles 28 day cell.
Curves fitted by least squares method. Arrows indicate non-polarized potentials.

Plot of spontaneous action potential overshoot
versus the maximum diastolic potential (MDP).
Filled circles 19 day cell.
Open circles 28 day cell.
Curve fitted by eye. Arrows indicate non-
polarized potential.

FIGURE 13

FIGURE 14

Plot of the duration of spontaneous action potentials versus the maximum diastolic potential (MDP).
Filled circles 14 day cell.
Filled squares 19 day cell.
Open circles 28 day cell.
Curves fitted by least squares method. Arrows indicate non-polarized potentials. The 14 day cell had spontaneous MDP changes.

FIGURE 15

Plot of the maximum upstroke velocity ($+V_{\max}$) of spontaneous action potentials versus the maximum diastolic potential.

Open circles 19 day cell.

Filled circles 14 day cell.

Open squares 28 day cell.

Curves fitted by least squares method. Arrows indicate non-polarized MDPs. The 14 day cell had spontaneous MDP changes.

FIGURE 16A Spontaneous action potentials from a 14 day cell culture showing an oscillatory afterpotential which generated an arrhythmic action potential (arrow).

Upper trace $\dot{V}$. Scale: 10 V/s.

Lower trace cell. Scale: 20 mV, 1 sec.

FIGURE 16B Spontaneous action potentials from a 16 day cell culture showing an oscillatory afterpotential which generated a short burst of subnormal action potentials and created an arrhythmia (arrow).

Upper trace $\dot{V}$. Scale: 10 V/s.

Lower trace cell. Scale: 20 mV, 1 sec.

Fig. 16A

Fig. 16B

to greater than -68 mV. These evoked action potentials could overshoot by as much as $+18 \pm 0.3$ mV ($n=30$), although the overshoot value decreased as the membrane was increasingly hyperpolarized and in many cases there was no overshoot. Duration for evoked action potentials was about 20 to 40 msec. The maximum rate of rise of the upstroke was greater for more hyperpolarized membranes (Figure 17) reaching a maximum of 84 ± 0.6 V/s ($n=30$) at a maximally hyperpolarized membrane potential of 130 mV.

VI. Periods of Quiescence

In cultures older than twelve days, spontaneously active cells sometimes became quiescent and subsequently restarted with no apparent external stimulus. The transmembrane electrical recording of one such event appears in Figure 18. This cell, from a twelve day old culture, had been spontaneously active, both electrically and mechanically, in a stable fashion for about four and one-half minutes before the brief period of quiescence occurred. The quiescent period lasted for approximately five seconds before another spontaneously generated action potential occurred. The first several action potentials after the quiescence were larger in amplitude and had greater upstroke velocities than those immediately before the quiescence or after the initial rhythm was resumed. As well, the delay between these action potentials was increased. It is noteworthy that the MDP was not greatly different before or after the quiescence and thus the increased amplitude of the first several action potentials after the quiescence was due to an increase in overshoot potential.

FIGURE 17

Plot of upstroke velocity ($+V_{\max}$) of spontaneous and evoked action potentials versus the maximum diastolic potential (MDP). All points of the curve plotted with open circles (except for uppermost point) represent spontaneous action potentials. All other points represent evoked action potentials.

Triangles 13 day.
Filled circles 16 day.
Open circles 28 day.

FIGURE 18

Spontaneous action potentials from a 12 day culture showing a quiescent period during repetitive firing.

Upper trace $\bar{V}$. Scale: 10 V/s.

Lower trace cell. Scale: 20 mV, 0.5 sec.

Fig. 18

TABLE 2. Summary of electrophysiological findings.

DAYS IN CULTURE			
	NEWLY ISOLATED	4 - 8 (ROUNDED PHASE)	12 - 28 (ESTABLISHED PHASE)
SPONTANEOUS CONTRACTIONS	ESCALATION WHOLE CELL	ESCALATION	ESCALATION WHOLE CELL
MEMBRANE POTENTIAL (mV)			
MEAN		-42 ± 7.9	-50 ± 12.4
RANGE	-22 to -35	-32 to -52	-30 to -60
SPONTANEOUS A.P.s	NOT OBSERVED	NOT OBSERVED	OFTEN
PREPOTENTIAL	-	-	PACEMAKER TYPE
+V _{max} (V/s)	-	-	2 to 15
OVERSHOOT (mV)	-	-	5 to 30
DURATION (msec.)	-	-	75 to 210
AFTERPOTENTIALS	-	-	HYPERPOLARIZING OSCILLATORY
EVOKED A.P.s		NONE	
MAXIMUM MEMBRANE POTENTIAL (mV)	-115		-130
+V _{max} (V/s)	131		84

DISCUSSION

To assess the utility of the present system of cell culture it is useful to compare the electrophysiological characteristics of the cells in vitro to the electrophysiological characteristics of cells in vivo. To do this well, however, it is desirable to consider from which type of myocardial cell measurements were made because the electrophysiological response from the cultured cells should be compared to data appearing in the literature for similar cells in vivo. Unfortunately, the literature has little quantitative analysis of the cardiac action potential of the adult rat. Data are only available in the literature for membrane potential, and action potential overshoot and duration. Qualitative data are available on the configuration of the ventricular action potential of adult rat. Beyond that, however, comparisons must be made with neonatal and embryonic rat ventricular myocardium. While extrapolation of some features of the neonatal rat cardiac action potential to the adult state seem plausible, they are not necessarily accurate or valid.

Further difficulty arises when the spontaneous action potentials recorded in vitro are analyzed in terms of their possible ionic currents. There are few data available for voltage clamp analysis of rat cardiac action potentials and few pharmacological studies of rat heart tissue. Because the configuration of the ventricular action potential of the rat is so different from most other mammalian species (Langer, 1978), interspecies extrapolation of ionic current data is tenuous. However, where rat heart ionic current data are scarce, such extrapolations have been made.

The following discussion attempts to assess the present cell,

culture in terms of its electrophysiological response by comparing it to values in the literature for in vivo measurements as well as by comparing it to other heart cell cultures. In addition, the electrophysiological response is correlated to a previously reported sequence of morphological changes which are characteristic for cell cultures of different ages (Jacobson, 1977; Schwarzfeld, 1979).

I Identification of Cell Type

Although the large majority of cells of the ventricle are myocardial, one might expect the isolation procedure to have liberated cells of various types (i.e. fibroblast, endothelial, smooth muscle, nerve, etc.). That most of the newly isolated cells were spontaneously contractile and striated is a clear indication that they were myocytes. Purkinje fibres and ventricular myocardium are the only types of cardiac muscle cells expected to be isolated, since only the apical portion of the ventricle was used in the preparation of cultures. ~~My~~ definite differential identification of these cells is made difficult because of the gradual transition of cellular morphology and action potential configuration between Purkinje fibres and ventricular myocardium in situ (Hoffman, 1961). However, it is worthwhile to consider whether individual cells from which action potentials have been recorded in this study are most likely to have been Purkinje fibres or ventricular myocardium.

That the preparation consisted largely of Purkinje fibres is supported by Sachs (1978) who stated that single cells enzymatically isolated from pure canine Purkinje fibre preparations were not different in their overt physical appearance from the cells isolated by Jacobson (1977). Further support for the cultured cells being

Purkinje fibres is the presence of spontaneous contractions and action potentials. Under normal circumstances Purkinje fibres are spontaneously active while ventricular myocardium is not. However, ventricular myocytes have been reported to be spontaneously active in vivo when depolarized, either by elevated external potassium ion concentration (Katzung and Morgenstern, 1977) or by depolarizing current injection (Katzung, 1974). The magnitude of membrane potentials from cells which exhibited spontaneous action potentials (-30 to -60 mV) were lower than those reported for measurements in vivo (-73 to -85 mV) (Bernard and Gargouil, 1963; Couch, West and Hoff, 1969; Langer et al., 1975; Marvin et al., 1979). The cultured cells are therefore in a depolarized state compared to in vivo. If these cells were ventricular myocytes the spontaneous activity would be expected as a result of the depolarization.

A definite identification of the cell type is not possible from the foregoing discussion. However, providing that there is no selectivity of cell type in the isolation procedure and assuming a very much greater number of ventricular myocytes compared to Purkinje fibres in the apical section of the ventricle, it is likely that most of the isolated cells were ventricular myocytes.

II Electrophysiological Characteristics

A. Membrane Potentials.

Membrane potentials recorded in vitro were lower than those recorded in vivo. Membrane potentials ranged from -21 to -60 mV in vitro while in vivo membrane potentials were from -73 to -85 mV (Bernard and Gargouil, 1963, Couch, West and Hoff, 1969; Langer et al., 1975; Marvin et al., 1979). It is important to consider whether the

low membrane potentials which have been recorded throughout the duration of the culture are inherent to isolated cells in culture or whether they are an artifact of the impalement of cells by micro-electrodes.

i) Depolarization due to impalement.

Impalement records of newly isolated cells and the contractile response associated with impalement may indicate that these cells were damaged by impalement. Newly isolated cells which were impaled were never observed to produce spontaneous action potentials and never exhibited membrane potentials more negative than -35 mV. Furthermore, the presence of fibrillations leading to partial or complete contracture immediately after impalement is also evidence for membrane damage. Fibrillations may have been similar to a type of postulated calcium-induced calcium-release contraction observed by Fabiato and Fabiato (1975) when myoplasmic calcium ion concentration is elevated. An elevation of myoplasmic calcium ion concentration could be brought about by a membrane which is excessively permeable (i.e. "leaky") to ions. A leaky cell would also likely have a low membrane potential due to the loss of intracellular potassium ions.

The records for cells in the rounded phase (4 - 8 days in culture) show relatively clean impalements and the quiescence was usually not disturbed by impalement. The relatively stable membrane potential and the lack of fibrillations are consistent with non-damaging impalements.

Records for cells in the established phase (in culture longer than 12 days) suggest some impalement damage (Figure 8a,b) but the subsequent increase in membrane potential and appearance of

spontaneous action potentials with whole-cell contractions indicate that the damage was repaired. Lehmkuhl and Sperelakis (1963) reported similar impalement records in cultures of embryonic chick ventricular myocytes. In some established cells the impalement appeared to cause no membrane damage (Figure 8c). These cells also had membrane potentials lower than cells in vivo. It is apparent that some impalements did result in membrane damage. However, the majority of impalements, particularly from older cell cultures (older than 4 days), appear to be with minimal membrane damage. Those cells from which impalement damage clearly is not indicated have membrane potentials which are lower than in vivo membrane potentials.

ii) Depolarization due to isolation.

Depolarized membrane potentials in newly isolated ventricular myocytes from adult rat have been reported previously (Powell, Terrar and Twist, 1978a,b; Lee et al., 1979). Some workers have circumvented the problem of low membrane potentials by artificially hyperpolarizing the cells. Powell, Terrar and Twist (1978b) added valinomycin to increase the mean membrane potential from -23.5 mV to -69.6 mV. Valinomycin is an ionophore which increases the resting membrane potassium conductance (Mueller and Rudin, 1967; Shigenobu and Sperelakis, 1975). Lee et al. (1979) hyperpolarized their cells by removing chloride ion from the perfusate. Although no values for membrane potential were given before chloride removal, in low or zero external chloride solutions the membrane potentials ranged from -70 to -90 mV.

Isolation of single heart cells from the adult rat by enzymatic

digestion has been observed to cause membrane damage (Clark et al., 1978; Fry, Scales and Inesi, 1979; Moses and Kasten, 1979) as well as disorganization of myofilaments (Moses and Kasten, 1979). Membrane damage typically involves the loss of the glycocalyx (Moses and Kasten, 1979; Isenberg and Klockner, 1980). The features which indicate membrane damage after isolation are:

- a) changes of the membrane ultrastructure
- b) low membrane potentials
- c) spontaneous action potentials and contractions which are consistent with a depolarized membrane.

The low membrane potentials might be the result of one or more of several features. For example:

- a) the exposure of cells to enzymes might cause removal of the glycocalyx and leave the membrane leaky
- b) isolation of cells from contact with other myocytes which, under normal conditions, might have a "conditioning" effect on one or another
- c) mechanical disaggregation, which might tear apart intercellular junctions of the intercalated discs and rupture the membrane at these sites.

One report which is inconsistent with the above observations is that of Isenberg and Klockner (1980). These workers, using isolated ventricular myocytes from adult rat, have shown that the glycocalyx was removed from their cells. The membrane potentials, however, ranged from -75 to -85 mV. It is not clear whether Isenberg and Klockner (1980) artificially hyperpolarized their cells in a manner similar to Powell, Terrar and Twist (1978b) or Lee et al. (1979), although Isenberg and Klockner did compare their membrane potentials to the artificially hyperpolarized membrane potentials recorded by these two groups. The cells isolated by Isenberg and Klockner (1980)

were not spontaneously contractile. Another similar report is that of Bustamante (1979). Enzymatically isolating single ventricular myocytes from adult guinea pigs Bustamante (1979) also found large membrane potentials (-80 to -100 mV) and no spontaneous contractions. Interestingly, Claycomb and Palazzo (1980) used a method similar to Bustamante (1979) to isolate ventricular myocytes from adult rats. The cells isolated by Claycomb and Palazzo (1980) did exhibit spontaneous contractions.

These findings indicate that some techniques can produce isolated adult ventricular myocytes which have "normal" electrophysiological properties. As well, some techniques which may be suitable for some species (i.e. guinea pig) may cause damage and depolarization in other species (i.e. rat). From these observations it is likely that the low membrane potentials recorded from the present cell culture are inherent to the cultured cells and may result from the isolation procedure or isolation itself. This is particularly true for cells in culture longer than four days where there was no obvious damage induced by impalement. In newly isolated cells there may have been impalement induced damage as well as inherent low membrane potentials.

B. Input Resistance.

Input resistance measurements from cells in all ages of culture are greater than those reported for rat ventricular cells in vivo. Coraboeuf (1969) reported ~~input~~ resistance of 1.5 M Ω in rat ventricular tissue when measured with single microelectrodes. No values could be found for input resistance of isolated adult rat cells. It is important to realize that parallel resistances for bundles of cardiac tissue could allow a low input resistance to be measured when the

input resistance for a single cell might be larger. A more meaningful measurement is the specific membrane resistance. Estimates of specific membrane resistance (see Appendix 4 for calculation) for newly isolated cells studied here are intermediate between values reported for embryonic chick heart cell monolayer cultures and embryonic chick heart cells cultured as linear arrays. The estimates for newly isolated cells were $1.2 \times 10^3 \Omega\text{cm}^2$. Values for embryonic chick heart cell monolayer cultures ranged from $480 \Omega\text{cm}^2$ to $630 \Omega\text{cm}^2$ (Sperelakis and Lehmkuhl, 1964; Sperelakis, 1972) and for embryonic chick heart cell cultured strands ranged from $16 \times 10^3 \Omega\text{cm}^2$ to $136 \times 10^3 \Omega\text{cm}^2$ (Lieberman *et al.*, 1975; Sachs, 1976). No report of specific membrane resistance for rat ventricular tissue *in vivo* was found.

The high specific membrane resistance of cultured chick embryo heart cells has been attributed to a low potassium conductance (Pappano and Sperelakis, 1969). A low potassium conductance should also cause low membrane potentials since the membrane potential is largely determined by the conductance for potassium (Noble, 1975). Powell, Terrar and Twist (1978b) also thought their isolated ventricular myocytes from the adult rat had low potassium conductance. Addition of valinomycin which should increase potassium conductance, restored the membrane potentials of their cells to near *in vivo* values (mean of -69.6 mV). It is therefore possible that the high input resistance and specific membrane resistance of the present cells is due to a low potassium conductance (which also results in low membrane potentials).

C. Spontaneous Action Potentials.

Spontaneous action potentials could be recorded only from cells

in culture for more than twelve days. The characteristics of the action potentials in vitro differ from those recorded from adult rat ventricle in vivo. Although no spontaneously generated action potentials have been reported for rat ventricle in vivo (nor have reports of recording from rat Purkinje fibres been found) it is possible that the spontaneity observed here was induced by the depolarized membrane potential (Katzung, 1974; Katzung and Morgenstern, 1977). The presence of a slight plateau in the repolarization phase (compare Figure 10 to Figure 3) is not consistent with adult rat ventricular tissue in vivo (Kell~~l~~ and Hoffman, 1960; Bernard and Gargouil, 1963; Couch, West and Hoff, 1969; Langer, 1975, 1978). The presence of this plateau gives the action potential the general appearance of an in vivo action potential from a neonatal rat (Langer, 1978). Overshoot values of about +7 to +30 mV appear similar to in vivo recordings (Coraboeuf, 1960). The maximum upstroke velocity of action potentials was slower than in neonatal rat ventricle in vivo, with those from cultured cells being from 2 to 18 V/s. Those recorded from neonatal rats in vivo were from 50 to 80 V/s (Bernard and Gargouil, 1968). There are no specific reports in the literature for maximum upstroke velocities from adult rat. It is noted, however, that the membrane potential measurements show no significant difference from the third to the twentieth day of post-natal life (Langer et al., 1975). Because the upstroke velocity is closely coupled to membrane potential (Weidman, 1955) it is possible that during the time when no membrane potential changes occur, that there also is no significant change in upstroke velocity. On the other hand development of new ion channels might not affect membrane potential but still alter upstroke

velocities. Excluding the possibility of ion channel changes from early post-natal to adult life, the upstroke velocity in early post-natal life may be assumed to be similar to that in adult life. If this assumption is true then the spontaneous action potentials differ from in vivo by virtue of low upstroke velocities. The largest maximum upstroke velocity recorded was 18 V/s at a membrane potential of -59 mV (Figure 15). The upstroke velocity increased at more hyperpolarized membrane potentials. However, action potentials had to be evoked at membrane potentials more negative than -69 mV.

D. Evoked action potentials.

The evoked action potentials recorded from newly isolated cells or from cells in culture longer than twelve days were similar. In both, the action potentials could overshoot and had maximum upstroke velocities greater than for spontaneous action potentials. In newly isolated cells, however, the action potentials tended to have shorter durations than in older cultures. The ability to evoke an action potential in newly isolated cells is a clear indication that they were still excitable and therefore maintained membrane integrity. The evoked action potentials from cells in culture for more than twelve days had higher velocities of upstroke than for spontaneous action potentials in the same cell (Figure 17). The evoked action potentials were evoked from a more negative membrane potential than that from which spontaneous action potentials occurred. The evoked action potentials had maximum upstroke velocities of up to 50 V/s at membrane potentials comparable to in vivo membrane potentials (-85 mV). This upstroke velocity is close to the values observed in neonatal rat hearts and may also be assumed to be close to in vivo values for

adult rat hearts, (see above).

Electric current pulses which could evoke action potentials in both newly isolated cells and cells in culture for more than twelve days did not elicit action potentials in cells in culture from four to eight days. This inexcitable period coincides with the time during which the cells are round in morphology and just beginning to become attached to the substratum. During this phase of attachment the cells may have undergone membrane reorganization which facilitates attachment but which electrically inactivates the membrane. Interestingly, this period is also the time when the lowest percentage of cells are observed to spontaneously contract (Schwarzfeld, 1979).

E. Arrhythmia.

The spontaneous action potential records from some cells in culture exhibit small irregularities in what is otherwise a stable, rhythmic train of action potentials. These brief arrhythmias occurred when a subthreshold oscillation of membrane potential appeared regularly between action potentials. These oscillations appear similar to oscillatory afterpotentials noted in whole heart tissue (Cranefield, 1977; Hiraoka, Okamoto and Sano, 1979) which are thought to depend on the slow inward current (Zipes, Besch and Watanabe, 1975). The arrhythmia was induced when one or more of the normally subthreshold oscillations reached threshold and an extra action potential or burst of action potentials was induced (Figure 16).

F. Quiescence.

A phenomenon noted in cultures of adult rat ventricular myocytes is that the cells may go through periods of quiescence which interrupt periods of rhythmic contraction (Schwarzfeld, 1979). The transmembrane

voltage record of a cell in culture for twelve days, which illustrates one short period of quiescence appears in Figure 18. This cell ceased generating spontaneous action potentials for a brief period before it resumed its former rate. No external stimulus was known to be responsible and there was no significant change as might have been expected in the MDP immediately preceding the period of quiescence. It may be assumed that some intracellular stimulus triggered the quiescence and that it was subsequently removed or inhibited. Contractions were not evident during the period of electrical quiescence.

III Nature of Spontaneous Action Potentials

The type of electrophysiological response noted here from spontaneously active ventricular myocytes in culture for more than twelve days is not normally observed in vivo. However, under certain conditions a response such as that noted here may be observed. These conditions include depolarization- or drug-induced fast inward current blockage in the presence of physiological levels of extracellular calcium ion. In addition to being spontaneously generated at depolarized membrane potentials in ventricular tissue (Katzung, 1974; Katzung and Morgenstern, 1977), the action potentials recorded from the cultured myocytes reported here have characteristics which are consistent with the slow inward current (from cardiac tissue of an unspecified species, measured by Zipes, Besch and Watanabe (1975)) being the charge carrier. The characteristics of the response of the cultured adult cells are compared to the action potential characteristics reported by Schanne et al. (1979) for cultured neonatal rat heart cells and the slow inward current in Table 3. The great similarity of results of this study with the results of Schanne et al.

TABLE 3 Comparison of electrophysiological characteristics
from adult rat ventricular myocyte cultures with
neonatal rat ventricular myocyte cultures and the
characteristics of the slow inward current in
intact heart tissue.

ELECTROPHYSIOLOGICAL CHARACTERISTICS	ACTION POTENTIALS (THIS STUDY)	SLOW RESPONSE* (SCHANNE <i>et al.</i> , 1979; BKAILY <i>et al.</i> , 1979)	SLOW CURRENT (ZIPES, BESCH, WATANABE, 1975)
TYPE OF TISSUE	ADULT RAT VENTRICLE	NEONATAL RAT VENTRICLE	NOT SPECIFIED
SPONTANEOUS ACTION POTENTIALS	PRESENT	PRESENT	PRESENT
MAXIMUM DIASTOLIC POTENTIAL (mV)	-30 to -60	-44.8	-40 to -70
ACTION POTENTIAL AMPLITUDE (mV)	45 to 80	67.4	35 to 75
OVERSHOOT POTENTIAL (mV)	+7 to +30	+22.4	0 to +15
MAXIMUM RATE OF RISE (V/s)	2.4 to 17.6	8.6	1 to 10
THRESHOLD FOR ACTION POTENTIALS (mV)	APPROX. -40**	-30.8	-30 to -40
SENSITIVITY TO DRUGS	NOT TESTED	INSENSITIVE TO TTX OR LIDOCAINE	INSENSITIVE TO TTX, SENSITIVE TO VERAPAMIL OR D-600
DEPENDENCE ON EXTRA-CELLULAR ION CONCENTRATION	NOT TESTED	Na ⁺ , Ca ²⁺	Ca ²⁺
SLOPE OF DIASTOLIC DEPOLARIZATION (mV/sec)	50 to 260	109	NOT STATED

* values are means; ** taken from Figure 10.

(1979) with neonatal rat in vitro and the characteristics of the slow inward current suggest that the primary inward current in the cultured adult myocytes is slow and perhaps carried by calcium ions. The dependence of some features of the slow response reported by Schanne et al. on sodium ion concentration would indicate that there may also be some sodium current associated with those action potentials.

If the present cells do have primarily calcium current during their action potentials, it would represent a deviation from in vivo. The lack of slow calcium current in adult rat ventricular myocytes in vivo is inferred from the loss of the plateau during the repolarization phase of the action potential throughout development (Langer, 1978). In most other mammalian species the plateau has been shown to be dependent on external calcium ion concentration. Also, the insensitivity of adult rat action potentials to manganese ions (Coraboeuf and Vassort, 1967; Bernard and Gargouil, 1968) which have been shown to block the slow inward current is evidence for the absence of this current in adult rat ventricular tissue in vivo. Isenberg and Klockner (1980) have analyzed a slow inward current in isolated ventricular myocytes of adult rat by voltage clamp methods. This current was independent of TTX application, and had kinetics of activation and inactivation and reversal potentials similar to slow inward current in bovine ventricular tissue (Reuter and Scholz, 1977). Although Isenberg and Klockner (1980) stated that the action potentials they recorded from isolated myocytes were similar to in vivo, their tracings (cf. Figure 2c) do show a significant plateau to repolarization. This plateau may be induced by isolation or isolation methods and could be due to a slow inward current.

The isolated ventricular myocytes from adult rat of Isenberg and Klockner (1980) show that complete de-inactivation of the slow inward current was achieved at membrane potentials more negative than about -35 mV, while complete inactivation was complete at membrane potentials more positive than zero. Activation began at membrane potentials more positive than about -50 mV and was complete at membrane potentials more positive than about zero. Unfortunately, these data cannot be compared with those of Lee et al. (1979) because they do not give specific values for activation-inactivation parameters for the fast sodium current in isolated ventricular myocytes of adult rat.

The similarity of the electrophysiological parameters of the present cells and those of Schanne et al. (1979) as well as the slow inward current described by Zipes, Besch and Watanabe (1975) would indicate that the spontaneous action potentials reported here are mediated by slow inward current with calcium as a likely charge carrier. The fact that these spontaneous action potentials occurred at membrane potentials where slow inward current is operant in isolated ventricular myocytes from adult rat is further support for this hypothesis. As well, the MDP for which spontaneous action potentials were recorded in culture (maximum of -68 mV) is at a membrane potential where the fast inward current is largely inactivated in other mammalian species (Beeler and Reuter, 1977), although an extrapolation of this inactivation data to rat may not be valid. However, where the present cells were hyperpolarized to membrane potentials more negative than -90 mV action potentials could be evoked when the membrane was quickly depolarized to -30 mV. From the data of

Isenberg and Klockner (1980), at -80 mV there should be no activation of slow inward current. It is therefore possible that hyperpolarization of the present cells to membrane potentials comparable to or more negative than in vivo values allows fast current channel to be deactivated. Upon evoking an action potential from this hyperpolarized membrane potential a fast inward current may be responsible for the upstroke of the action potential. Some support for this hypothesis appears in Figure 17. From this graph it may be seen that the maximum upstroke velocity, $+V_{\max}$, increased sharply when cells were hyperpolarized to membrane potentials more negative than -69 mV. An increase in upstroke velocity may indicate an increase in fast inward current (Hondgehem, 1978).

In summary, it is thought that the spontaneous action potentials recorded from the present cells in culture are mediated by slow inward current which may be carried by calcium ions. A fast current might be initiated by hyperpolarization of the membrane potential and subsequently evoking an action potential. Further data, such as voltage clamp analysis or pharmacological testing, is required to help identify the currents responsible for action potentials in cultured cells.

IV Comparison with Other Tissue Cultures

It is not possible to compare the present cell culture preparation with other adult cultures since data available in the literature are inadequate. However, it may be compared with embryonic and neonatal rat heart cell cultures. One limitation of such a comparison is that some investigators use the entire heart to derive single cells. Individual cells in culture are, therefore, not necessarily of

ventricular origin. The preparation of Athias et al. (1977) was one which used the entire heart to derive single cells. Membrane potentials recorded by Athias et al. (1977) were larger (-50 to -75 mV) than for the present culture and the action potentials had greater maximum upstroke velocities (up to 300 V/s at a membrane potential of -75 mV). Because these upstroke velocities are several times larger than for neonatal rat ventricle in vivo and because Athias et al. (1979) later report low membrane potentials and low upstroke velocities in a similar system, the large upstroke velocities of Athias et al. (1977) are suspect. Auclair (1976) also reported large membrane potentials (-70 mV) with upstroke velocities of 100 V/s. The neonatal rat preparation of Auclair (1976) also utilized the entire heart in the isolation procedure. It is possible that these reports of high upstroke velocities are related to the fact that the entire heart was used and that recordings were made from non-ventricular cells.

Kitzes and Berns (1979) used only ventricular tissue from neonatal rats. Their preparation had large membrane potentials (mean -68 mV; maximum -98 mV) but had low upstroke velocities (as judged from action potential tracings; no values given). Schanne et al. (1979) reported low membrane potentials (mean -45 mV) and low upstroke velocities (mean 8.6 V/s) for cultured cells isolated from neonatal rat ventricles. The cells of Schanne et al. (1979) also exhibited a plateau to the repolarization of action potentials. Unfortunately, there are no data for the effect of hyperpolarization on V_{max} and excitability in general.

In spite of the large age differences and associated developmental

differences, the cell cultures derived from adult ventricular sources and those from neonatal ventricular sources yielded cells with similar electrophysiological parameters. It is worthwhile to consider the possibility that the isolation and culturing of cardiac cells results in a particular type of membrane electrophysiology. Cultures prepared from chick embryo ventricles show changes from in vivo (Sperelakis and Shigenobu, 1972) which conform to those observed here and by Schanne et al. (1979) for pure ventricular cell cultures and by Athias et al. (1979) for mixed cultures. These features are low membrane potential and spontaneous action potentials with low upstroke velocities (Fänge, Persson and Thesleff, 1956; Crill, Rumery and Woodbury, 1959; Lehmkuhl and Sperelakis, 1963). The phenomena of low membrane potential and low maximum upstroke velocity have been shown to be influenced by the density of myocytes in the growth area of a culture. While in monolayer culture with relatively low density the cells exhibit low membrane potentials and low upstroke velocities. When grown in high density such as in myocyte enriched monolayer cultures (Lompre, Poggioli and Vassort, 1979) or in close apposition such as spheroidal reagggregates (McLean and Sperelakis, 1976; Sperelakis, 1978) the cultured cells can sometimes have electrophysiological parameters that are similar to those in vivo. It would therefore appear that cells may be conditioned after isolation through contact with other cardiac myocytes. This conditioning may result in the ability of isolated cells to maintain or regain electrophysiological features which resemble the in vivo state. Conversely, the reports by Isenberg and Klockner (1980) and Bustamante (1979) mentioned previously show that cells may be isolated from adult animals

and, in isolation, maintain electrophysiological parameters similar to in vivo.

V Classification of Cells

The morphological development and electrophysiological response of cells at different stages of culture growth allow the cells to be grouped into three categories. Initially upon isolation the cells were spontaneously contractile, had low membrane potentials, did not exhibit spontaneous action potentials but were excitable when briefly depolarized from hyperpolarized levels. These cells were typically elongated and striated in appearance. This phase lasted up to approximately 72 hours.

The second phase, called the "rounded phase", was characterized by the transition of elongated cells into round cells. This was accompanied by the loss of striations and the cells becoming optically more dense. The membrane potentials were generally greater than in the newly isolated phase but were still low compared to in vivo. The cells were less likely to be spontaneously contractile than earlier on and tended to be inexcitable. Spontaneous action potentials were never observed. This phase lasted up to about eight days in culture. During this time the cells began to attach to the substratum.

The third phase was estimated to begin after about eight days in culture. During this phase the cells became firmly attached to the substratum and began to send out pseudopod-like processes. The contractions became more forceful and involved the whole cell. Membrane potentials were the largest of those recorded in culture but were lower than in vivo. Spontaneous action potentials were frequently observed in synchrony with contractions. This phase was termed

the "established phase".

CONCLUSIONS

The present adult rat heart cell culture was found to exhibit some electrophysiological parameters different from intact tissue. The primary difference was a reduced membrane potential at all stages of tissue culture tested. Other differences such as reduced upstroke velocity of the action potential, and the presence of spontaneous action potentials may have been due to the reduced membrane potential. The similarity between these spontaneous action potentials and the action potentials reported by Schanne et al. (1979) or the slow inward current reported by Isenberg and Klockner (1980) and Zipes, Besch and Watanabe (1975) suggests that some membrane inward calcium current is involved in production of the action potential. The cells in culture exhibited three distinct stages of electrophysiological development which were related to stages of morphological development. The newly isolated phase lasted less than about 72 hours and contained spontaneously contractile, elongated striated cells. They did not exhibit spontaneous action potentials but an action potential could be evoked. The second phase, called the rounded phase, lasted from about the third to eighth day in culture and contained rounded, attaching and generally quiescent cells. These cells never spontaneously generated action potentials nor were they excitable. The third phase, called the established phase, began once attachment to the substratum was nearly complete and the cells had spread out. This phase was characterized by spontaneous action potentials in synchrony with forceful, whole-cell contractions.

The suggestion that the tissue culture development represents a "dedifferentiation" (Claycomb and Palazzo, 1979) in that the highly

organized and differentiated adult state regresses in an orderly fashion to an earlier embryonic state is not supported by the electrophysiological measurements reported here. That a loss of organization does occur has been shown by the loss of cross striations during the transition from elongated to rounded morphology. The presence of organized arrays of myofilaments in the established phase, as shown by transmission electron microscopy (Harley, 1979), indicates that a type of redifferentiation may occur between the rounded phase and the established phase. "Redifferentiation" does not imply that the cells have reorganized into an adult in vivo type of ultrastructure. In fact the organized arrays of myofilaments into contractile units were not packed into parallel bundles but were more scattered with respect to each other. It would, therefore, appear that the tissue culture development is structured, resulting in a particular morphology and electrophysiology. The extent to which electrophysiological features in vitro deviate from those in vivo requires further investigation.

APPENDIX 1

The elimination of an ionic current by voltage clamping to the reversal potential for that current can be inferred from the expression for ionic current:

$$i_{\text{slow}} = \bar{g}_{\text{slow}} (E_m - E_{\text{slow}}),$$

where i_{slow} = slow inward current

$\bar{g}_{\text{slow}}$ = the maximum instantaneous conductance

E_m = the voltage clamped membrane potential (command potential)

E_{slow} = the reversal potential for the slow inward current.

Thus, when the membrane is clamped to the equilibrium potential for the slow current (i.e. when $E_m = E_{\text{slow}}$) the coefficient $(E_m - E_{\text{slow}})$ = 0. At this time there will be no current flowing.

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APPENDIX 2

The micromanipulator was manufactured by coupling a 1.0 ml., 0.25 ml., and 100 μ l syringe (Becton-Dickson, serialized) together in a "Y" fashion using nylon tubing. The syringes and tubing were filled with light mineral oil (Klearol) and tested to ensure that a sealed system existed. The rack and pinion drives of a Narashigi micromanipulator were connected independently to the pistons of both the 0.25 ml. and 100 μ l. syringes. These syringes and the rack and pinion drives were mounted on a brass plate for stability, and acted as the control portion of the micromanipulator. The 1.0 ml. syringe was mounted on a small brass plate attached to a vertically aligned rack and pinion drive, and acted as the receiver of the micromanipulator. This rack and pinion drive was fastened to a stiff support column and was positioned so that the piston of the receiver syringe was aimed at the glass coverslip in the test vessel. The angle at which the receiver advanced towards the coverslip could be adjusted. The minimum drive of this system was approximately 0.23 μ m.

APPENDIX 3

Schematic diagram of apparatus set-up. The cell and test chamber are represented at left, with a microelectrode and reference electrode connected to the inputs of the amplification system.

APPENDIX 4

Specific membrane resistance, r_m , was calculated from the equation:

$$r_m = R_{in} \times A$$

where R_{in} = the cellular input resistance (Ω)

A = surface membrane area (cm^2).

- A. Estimation of surface membrane area for an average newly isolated cell assuming the cell to be cylindrically shaped.

$$A = 2 \pi r l$$

where r = radius = $10 \mu\text{m} = 10 \times 10^{-4} \text{ cm}$

l = length = $100 \mu\text{m} = 100 \times 10^{-4} \text{ cm}$

$$\begin{aligned} \therefore A &= 2 \pi \times 10 \times 10^{-4} \times 100 \times 10^{-4} \text{ cm}^2 \\ &= 0.6 \times 10^{-4} \text{ cm}^2. \end{aligned}$$

- B. Using the mean value of input resistance for newly isolated cells of $18.5 \text{ M}\Omega$, the specific membrane resistance was calculated as:

$$\begin{aligned} r_m &= 18.5 \times 10^6 \Omega \times 0.6 \times 10^{-4} \text{ cm}^2 \\ &= 1.2 \times 10^2 \Omega \text{ cm}^2 \end{aligned}$$

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