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Cultured Heart Cell Reaggregate Model for Studying Cardiac Toxicology

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This review represents a summary of the technique of using cultured heart cells as a model system for studying the physiology, pharmacology, biochemistry and toxicology of myocardial cells. The general techniques and types of culture preparations commonly used are given and some of the advantages and disadvantages of working with cultured heart cells are summarized.

Introduction

Varieties of Tissues Cultured

Virtually all types of animal and plant cells have been cultured. These include connective tissue cells, such as fibroblasts, parenchymal cells from a variety of tissues such as kidney, and endocrine cells from glands such as the thyroid and pancreas (beta cells of the Islands of Langerhans). The cultured endocrine cells can synthesize and secrete their normal hormones, such as thyroxine and insulin. Neurones, both somatic and autonomic, and both sensory and motor, have been cultured, and neuroblastoma cell lines are also available. Muscle cells of all types have been cultured, including skeletal, cardiac and smooth muscles. Smooth muscle cultures that have been made include: intestinal, vas deferens, oviduct, iris, amnion, and vascular (aortic, mesenteric, umbilical). Co-cultures of nerve and muscle have been prepared, and functional neuromuscular contacts are made *in vitro*; this has most clearly been shown for skeletal muscle cells and vas deferens smooth muscle cells. With respect to vertebrate muscle cultures, for convenience, most preparations are made from chick or from small laboratory mammals, such as mouse, rat, and guinea pig. Usually embryonic or early post-natal tissues are used.

The first account of cultured heart cells was given by Burrows (*1*), who observed the independent pulsatory activity of single cells and saw its significance as direct confirmation of the myogenic theory of heart muscle. Although cardiac muscle was traditionally regarded as a branching syncytium, Lewis (*2*) rejected this concept in favor of cellular independence on the basis of his studies on cultured heart cells. Monophasic action potentials were recorded using relatively large-tipped electrodes (*3*), and larger potentials were obtained by impalement of cultured heart cells with finer microelectrodes (*4–7*). Details of the methodology for satisfactory intracellular recordings have been given by Sperelakis (*8, 9*).

Cultured cells in monolayer networks and sheets revert back to the young embryonic state. Such reverted cells are useful for studying certain properties, such as on the mechanism of the changes of type of cation channels and in K^+ permeability and on the electrogenesis of pacemaker potentials. In fact, reverted ventricular myocardial cells probably make a good model for study of the properties of cardiac nodal cells. In contrast, the cells in spherical reaggregate and strand preparations tend to retain their highly differentiated electrical properties [see book edited by Lieberman and Sano (*10*) for results from several laboratories]. The reaggregated cells also possess pharmacological receptors virtually identical to those of cells in the original myocardium from which they were derived; that is, exposure to trypsin, at the concentration and time necessary for cell dispersal, does not permanently damage the cell membrane or its receptors. Also,

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the reaggregates are easier to impale with one or two microelectrodes than are the monolayers. Hence, the discussion to be given below of the electrical properties of cultured heart cells will be divided into two major categories: (a) reverted monolayers, and (b) highly differentiated reaggregates. I will present here principally the results from my own laboratory, but the reader is referred to the book edited by Lieberman and Sano for articles by numerous experts.

Advantages of Using Cultured Heart Cells

The use of cultured heart cells in various multicellular organizations affords unique advantages over intact myocardium for answering certain types of questions. For example, the following types of studies can be performed on monolayer networks of interconnected cells. By using hearts of various embryonic ages for preparing the cultures, some fundamental questions of development can be answered. Cultured heart cells can be prepared which retain highly differentiated electrical properties and their normal pharmacological receptors. Cultured heart cells can also be prepared which have reverted ("partially dedifferentiated") electrical properties resembling young embryonic hearts, and these cells make a good model for cardiac nodal cells. Simultaneous recording of transmembrane potentials and contractions can be made on single cells by using microelectrode and photoelectric techniques. Since cardiac muscle is reduced to a one- or two-dimensional system, studies of electrotonic spread of current and cell-to-cell interactions are facilitated. The electrogenesis of various components of the action potential and electrogenesis of pacemaker potentials can be studied in isolated single cells where propagation from, or interaction with, contiguous cells is eliminated. Voltage clamp experiments can be done on isolated single cells in which there should be adequate voltage control over the entire cell membrane, and in which the membrane current densities can be measured. The problem of diffusion lag in the interstitial fluid space is reduced or eliminated, thus facilitating ion flux studies in a simple two-compartment system (*11, 12*). Since the cells are denervated, the effects of denervation can be studied, and the direct effect of various chemicals and pharmacological agents on the myocardial cells can be determined without complications due to neural and systemic influences. Since co-cultures of nerve and heart cells can be made, this makes a good preparation in which to study the process of neuromuscular transmission. Nearly pure muscle cultures can be produced, i.e., almost complete absence of fibroblasts; thus, for example, biochemical assays can be done solely on myocardial cells, which is not possible in the intact myocardium because of the various cell types present. Microelectrophoretic injection of various substances can be done while observing electrical and mechanical effects on the injected cell. The cultured cells can be maintained in various experimental media to attempt to change the composition of the cell membranes, and ascertain the concomitant changes in membrane transport and electrical properties. Cultured heart cells can be grown in chemically-defined media (*13*), and antibiotics may be omitted from the culture medium if careful sterile procedures are followed. Finally, the effects of prolonged exposure (e.g., for several weeks) to various toxicological agents and drugs (at various concentrations) on the myocardial cells can be determined.

As can be gleaned from the above, the use of cultured heart cells affords the unique opportunity to answer certain types of questions that are not readily answerable by using intact cardiac muscle.

Some Pitfalls of Using Cultured Heart Cells

Some pitfalls of working with cultured heart cells include the following. The state of electrical differentiation of the cells must be determined, i.e., it cannot be assumed that the cells will have highly differentiated electrical properties. The contractile apparatus, namely the myofibrils, may not be in a highly differentiated state, even if the cells are highly differentiated electrically; however, simple light microscopic examination often is sufficient to reveal whether a tight parallel packing of myofibrils is present. In reverted cells, the rate of spontaneous contractions is often highly variable from one hour to the next even within the same area of the same culture, and there is a similar variability from one culture to another; this problem makes studies on the effects of chemical agents on the rate of spontaneous contractions oftentimes difficult to interpret. In some cases, the cells become electromechanically uncoupled, i.e., they fire action potentials but do not visibly contract, particularly when the cultures are opened and placed in an experimental chamber for electrophysiological studies; this problem can often lead to erroneous conclusions unless the cells are impaled with microelectrodes for recording the electrical activity. One precaution cannot be overemphasized, namely, that conclusions on the effect of agents on contraction of the cells are equivocal unless the cells are electrically stimulated ("paced"), because the effect of the agent could be on the automaticity properties of the cell membrane (or on the action poten-

tial generation). Cultured heart cells, especially the monolayers, are much more difficult to satisfactorily impale with microelectrodes; this problem means that arbitrary criteria must be imposed for ascertaining adequate impalements from which the data are collected for the purpose of calculating mean values. With present technology, it is not possible to record the contractions of the cells quantitatively, e.g., force development. Under some conditions, such as harsh treatment during cell separation, some of the pharmacological receptors may be inactivated; hence negative effects of some chemical agents must be viewed with caution. In certain types of studies, such as biochemical assays of entire cultures, contaminant cells such as fibroblasts will be analyzed along with the myocardial cells; therefore, the experimenter must know the degree of contamination and endeavor to produce nearly pure cultures. For example, fibroblasts are known to respond to addition of beta-adrenergic agonists with an increase in cyclic AMP level, similar to the response of myocardial cells. The metabolism of the cultured cells may shift from more aerobic to more anaerobic, and from a preponderance of tricarboxylic acid cycle towards the hexose monophosphate shunt pathway (pentose cycle), with accompanying changes in the appropriate enzyme activities. The cells in culture may be in different states of cell division, depending on numerous factors such as the degree of confluency of the monolayers/multilayers and on various factors in the serum used in the culture medium. Different batches of serum can introduce variables in the functional status of the cultured cells.

As can be seen from the above comments, there are numerous problems and potential pitfalls concerning interpretation of the data collected on cultured heart cells, but if reasonable precautions are taken and the functional status of the cells is determined, the cultured heart cells can yield important new information not readily obtainable from intact cardiac muscle preparations.

Types of Cultures

The general term "tissue culture" can be divided into several subcategories: (a) organ culture, in which an entire isolated organ, such as a heart or endocrine gland, is placed intact into culture medium and kept alive and functional; (b) explant culture, in which a slice of or a minced fragment of a tissue is placed as such into culture medium and maintained; in many cases, cells "grow" out of the explant by a process of repeated cell division and cell migration; and (c) cell culture, in which the tissue is first minced, and then it is dissociated into its individual component cells by some means (usually in Ca^{2+}-free, Mg^{2+}-free solution, in combination with some proteolytic enzyme and continual stirring or agitation), and finally the cells are plated out in culture medium and allowed to grow (i.e., divide) and to re-associate in some fashion.

The cells that grow out from the explants or the enzyme-dispersed cells adhere to the substrate (bottom of the culture dish, usually glass or plastic) and form monolayers. Cell types that exhibit contact inhibition stop dividing when the monolayer cells form a confluent sheet one cell thick. Other cells types continue growing and form multilayers, at least in some regions of the culture dish, in which the cells pile on top of one another to form a thick mat of cells. It is common for monolayers and multilayers of cardiac muscle cells and smooth muscle cells, which contract spontaneously, to pull loose from the substrate and form three-dimensional assemblies of reaggregated cells, which usually assume a more-or-less spherical shape eventually. The formation of such spherical reaggregates can be expedited by growing the cells on a substrate to which they adhere poorly, such as cellophane (*14*). Spherical reaggregates can also be formed for other cell types besides muscle by spinning the enzyme-dissociated cells in a gyrotatory shaker (*15*). In this case, the cells make contact with one another in the vortex of the rotating flask and adhere, thus forming larger and larger reaggregates as a function of time. Rotation for 24–48 hr produces reaggregates varying between about 50 μm to 400 μm in diameter. Much larger reaggregates are not desirable because of a tendency for the cells in the core to become necrotic.

The enzyme-dispersed cells can be maintained in suspension by constant shaking which prevents them from adhering to the substrate or to one another, if desired for certain types of studies. One disadvantage of this preparation is that several cell types will undoubtedly be present and contributing to the data being collected, because the enzymatic dispersal is nonspecific, yielding a heterogeneous cell population.

For special types of experiments, usually electrophysiological in nature, the dissociated cells can be made to form long strands or thin bundles of aligned cells. For example, the cells can be plated into vessels containing glass fiber threads, to which they adhere and form a rather intact coat of cells along the entire thread (*16, 17*; and McLean and Sperelakis, unpublished data). The coated threads can then be removed and studied individually. An alternative method is to plate the cells in a vessel whose bottom has been coated with a material to which the cells will not adhere, but in which a thin

scratch has been made to expose surface favorable to cell adhesion, thus allowing the cells to align themselves in the scratch (etched substrate) (*16*).

So far, we have been discussing primary cultures of cells taken directly from a tissue. For some types of studies, whenever confluent monolayers are obtained, the cultured cells are shaken in the presence of low concentrations of proteolytic enzyme (e.g., trypsin) to free them from the substrate and to dissociate them again. These freed cells are then divided ("split") and plated into new culture vessels, to produce "secondary" cultures. When these cells become confluent (usually within 1-2 weeks), the process is repeated to produce "tertiary" cultures, and so on. If this process is repeated indefinitely over many months or years, the cultures become known as a cell line presumably of cells all having a common parentage, but the characteristics (both phenotype and genotype) of these cell lines may be quite different from those of the original cells. [A clone of cells is made by repeated divisions of a single cell so that all daughter cells derive from a single parent cell; for example, if skeletal myoblasts are plated in very dilute form, each myoblast that sticks forms a clone surrounding it. In those cases in which the cultured cells retain their original characteristics for a number of passages (e.g., six), these are known as a cell strain (but they have a finite life span *in vitro*). It should be noted that gradations in loss of some functional characteristics are seen in cells cultured for long periods.] Some cell types retain, more-or-less, their original characteristics for up to six such "passages" without significant "dedifferentiation" towards a stem-cell line. Other cell types may begin to dedifferentiate or to "revert" towards a more embryonic condition as a function of time while in primary culture. Fortunately, heart cells in primary culture can be made to retain a highly differentiated state for at least 6 weeks. All of the data on cultured heart cells to be discussed below is on such primary cultures.

Although cultured heart cells do undergo mitosis (*18–21*), there is no general agreement on whether the mitotic state affects the state of differentiation. Some investigators suggest that dividing cells are not in a highly differentiated state. Some investigators report that mitosis proceeds without loss, or with only transient loss, of myofibrils, whereas others (*20*) report that contraction ceases transiently during cell division. A good discussion of this problem of mitosis and differentiation is given in a recent review by Rumyantsev (*22*). Mitosis and organogenetic movements continue when the cells are depolarized in high K^+ (*23, 24*). It is not clear whether contact inhibition of cell division occurs among heart cells *in vitro*. Multilayer regions can be found in cultures produced at high plating density and cells do overgrow one another. The density of cells plated may influence their viability and function through secretion of factors into the medium ("conditioned medium") (*25*).

Culture Methods

General

The methods for culturing mature organs have been described by Trowell (*26*). The methods we used for making organ-cultured hearts, either completely immersed in culture medium or at an interface between culture medium and moist air, have been described in detail previously (*27–30*). Likewise, the methods we used for producing blood-perfused hearts grafted on to the choriollantoic membrane of a host embryonic chick have been described (*30*). In the present article, I wish to focus mainly on primary cell cultures produced from enzyme-dissociated cells. Embryonic or early postnatal tissue is generally used for preparing cell cultures because the myocardial cells are more easily freed from the tissue, although theoretically it is possible to culture adult tissues (although a clear demonstration of this has not yet been published).

Cell Dissociation

Fertilized chicken eggs were obtained from a local hatchery on a weekly basis, and incubated in an egg incubator (Sears Co.) at 37°C with daily rotation. The eggs were opened when the embryos were between 14 and 20 days old (hatching occurs at 21 days), and the hearts were removed. The hearts from about 12 embryos were pooled together (in chilled Ringer solution) for making one preparation. The hearts were washed free of blood in Ringer solution (5°C), and the atria were dissected off and discarded (except when it was desired to make cell cultures from atrial cells). The ventricles were then minced with a small scissors. Cell separation was carried out by stirring (magnetic stirring bar) in a Ca^{2+}-free, Mg^{2+}-free Ringer solution (37°C) containing glucose (100 mg%) and 0.05% trypsin (1:250, Nutritional Biochemicals Corp.). Other proteolytic enzymes may also be used, and in a few cases, we used collagenase (with or without hyaluronidase) to separate the cells; in general, there seems to be no important difference in the properties of the cultured cells regardless which enzyme is used to separate the cells from the intercellular matrix. [For vascular smooth muscle cell cultures, elastase or collagenase (with and without hyaluronidase) is sometimes used.]. What is important, however, is

that the concentration of trypsin not be too high or that the exposure period to trypsin not be too long.* The use of a Ca^{2+}-free, Mg^{2+}-free solution (contaminant Ca^{2+} and Mg^{2+} present only, e.g., about $10^{-6}M$) facilitates the separation of the cells; in fact, some types of tissues, e.g., some epithelial tissues, dissociate in Ca^{2+}-free, Mg^{2+}-free solution alone without the need of a proteolytic enzyme. Finally, mechanical shearing forces facilitate the cell separation, as for example, the vigorous stirring of the dissociation solution. Other methods of producing mechanical shearing forces have also been used in various laboratories.

During the dissociation process, at intervals of 5 to 15 min, the cloudy cell-containing supernatant is decanted into chilled normal culture medium (containing normal Ca^{2+} and Mg^{2+}). The combination of cold and serum rapidly inactivates the trypsin, thus the exposure of the already-freed cells to trypsin is minimized. Fresh dissociation solution is added to the undissociated tissue, and the process is repeated from five to seven times. The first one or two removals are discarded because they contain a great amount of nonmuscle cells, and the remaining ones are pooled together. The cells are washed by light centrifugation (50–200*g* for 5–10 min) into a pellet, aspirating or decanting the supernatant, replacing with fresh culture medium, and resuspending the cells. This washing process is often repeated for a second or third time to completely remove the dissociating enzymes.

The composition of the culture medium was usually 10% or 15% serum (fetal calf or horse), 40% nutrient solution (such as Puck's N-16 or Medium 199), and the remainder a balanced salt solution (Hank-Ringer). The chemical composition of the various synthetic media, such as Medium-199, Puck's N-16, NCTC-135, basal medium Eagle (BME), modified Eagle medium (MEM), Dulbecco's modified Eagle medium (DMEM), L-15, etc. are usually given in the catalogs of the companies that supply the media (e.g., Gibco, Microbiological Associates, etc.). In general, these media contain various amino acids, glucose, vitamins, inorganic salts, and a variety of other components. Some media are claimed to promote the growth and well-being of certain types of cells, therefore, the medium used is an important consideration. The type of serum used also may be important, and it is thought that fetal serum contains a higher concentration of a factor that promotes cell division. Some workers also add some chick embryo extract (CEE), bought commercially or prepared in their own laboratory, to the culture medium to promote cell growth and viability with respect to certain characteristics that the investigator is interested in.

Antibiotics, such as penicillin plus streptomycin (50 units/ml) or gentamicin, may be added to the medium also, but if careful sterile procedures are used throughout, antibiotics and fungicides (e.g., Fungizone) may be avoided if so desired. All solutions used are sterilized by filtration through filters with pore diameters of about 0.2 μm. All glassware and dissecting instruments are sterilized by autoclaving; plasticware used for culture vessels is purchased already sterilized and wrapped; and all dissections and procedures are carried out in a laminar-flow hood (presterilized with ultraviolet rays).

The washed pellet of cells is diluted with sufficient culture medium to give a concentration of about $0.5–1.0 \times 10^6$ cells/ml for plating into the culture vessels or for gyrotation. If the cells are plated at too low a density, they do not survive well, and the same seems to be true if the density is too high, i.e., there is an optimal plating density. The optimal plating density can be determined by serial dilution and assaying for some selected parameter, e.g., rate of cell proliferation. Before plating, some workers pass the cell suspension through a nylon mesh of small pore size to disrupt any large multicellular aggregations.

In some cases, the dissociation solution was modified so that it contained elevated K^+ (10–25mM) and ATP (5mM), because it seemed that this modification enhanced the production of highly-differentiated (electrically) cells, particularly when the K^+ concentration of the culture medium was also elevated (*35*). However, this procedure was not a requirement for producing highly differentiated cells.

Monolayers

For producing monolayer cultures, the cells are plated into the desired type of culture vessels (e.g., modified Carrell flasks with removable lids (Bellco), into plastic Petri dishes, or into plastic Falcon flasks). A glass coverslip can be placed in the Carrell flask so that the coverslip with adhering cells can be removed and placed into another chamber for experimentation. A volume of 1–3 ml of cell suspension is added per culture vessel, depending on its size and type. The cells settle down to the bottom of the culture dish over a period of 12 to 48 hr, if left undisturbed, and attach themselves to the sub-

*Numerous studies have shown the damaging action of trypsin on the cell membrane (*31*) and on intracellular proteins (*32*), and the metabolism of cultured cells may be affected by this damage, particularly during the first 24–48 hr in culture (*33*). However, trypsin-dissociated cells quickly recover adhesiveness to one another (*34*).

strate. They divide and make contact with one another to form various monolayer patterns, such as loose random networks, parallel strands, rosettes, and confluent sheets. Confluent sheets are usually produced when the cells are plated at the higher densities. At low plating densities, regions of isolated single cells are often found. The myocardial cells in suspension usually contract spontaneously, each with its own independent rhythm. Shortly after the monolayer cells make morphological contact with one another (e.g., within 10-50 min), they contract synchronously, meaning that they have formed junctions and that one or more cells acts as a pacemaker to drive the rest. Such monolayer cells can be impaled with one or two microelectrodes to examine their electrophysiological properties (*8, 9, 36, 37*).

Since fibroblasts and endothelioid cells stick to the substrate much faster than do the myocardial cells, if the plated cells are allowed to settle for only 30–90 min and then poured off carefully, many of the fibroblasts will remain stuck to the dish and can thus be discarded. If this procedure of differential adhesion is serially repeated for about 3–6 times (depending on the settling time), a myocardial-enriched, fibroblast-depleted culture can be thus produced (*38*). Such "pure" cultures are not necessary for most electrophysiological types of studies, but could facilitate interpretation of some biochemical types of experiments. But even without such procedures, the percentage of beating myocardial cells in a monolayer culture is often 50–90%, thus the proportion of nonmuscle cells may be quite low.

The cultures may be "fed" once or twice a week, i.e., fresh culture medium added, if desired, but primary cultures of heart cells survive quite well for several weeks without such feeding. [In fact, since some types of cells prefer "conditioned medium," i.e., medium in which other cells are growing or have grown, it is possible that feeding too often is actually deleterius. For example, it has been reported that the ratio of the number of cells to the volume of the medium has a critical value, below which the cells will not proliferate because they are unable to adequately "condition" the medium (*39*).]

*The oxygen tension may be important in determining the rate of cell proliferation (*41*) and relative activity of glycolytic versus oxidative metabolism. Cultured chick heart cells, while primarily dependent on glucose for energy metabolism, retain a capacity to utilize fatty acids, whereas cultured mammalian fetal heart cells lack this ability (*42*). Mammalian fetal heart cells in culture seem to adapt to low environmental oxygen tension by diminished synthesis of contractile proteins and a shift in lactic dehydrogenase isozyme pattern, without a decrease in cellular energy stores (*43*).

This is probably because the volume of culture medium (e.g., 3 ml) is relatively large compared to the volume of cells (wet weight of about 1–3 mg). If a pH indicator dye is added to the culture medium or if the pH of the medium is checked with a pH meter after 1–3 weeks in culture, one finds that there is almost no acidosis produced. Likewise, the cultures survive very well even if the culture vessels are sealed to the incubator atmosphere (compressed air and 5% CO_2, filtered and washed). Presumably the amount of oxygen available in the culture vessel (e.g., about 8 ml air in a Carrell flask) is sufficient to last the cells for several weeks.* Some laboratories place many open culture vessels (e.g., Petri dishes) in a large closed plastic box which can be "flushed" daily with any desired gas mixture.

Spherical Reaggregates

In addition to the monolayer/multilayer preparations described above, special reaggregations of cells can be prepared, as for example, the fiberglass strands mentioned above. However, the cells can also be reaggregated into small spheres of about 100–500 μm in diameter by either of two methods. The cells can be plated into glass culture dishes containing cellophane squares on the bottom. Since the cells do not adhere very well to cellophane, they pull free and form small spherical reaggregates (0.1–0.5 mm) spontaneously (*14, 40*). Alternatively, the cells can be placed into Ehrlenmeyer flasks and rotated on a gyrotatory shaker for about 48 hr (*15*). The cells make contact with one another in the vortex of the solution and stick together. The longer the gyrotation period, the larger the spherical reaggregates become. After the rotation period, the spherical reaggregates can be transferred to a regular culture vessel and cultured for up to 6 weeks. If the culture vessel has a glass substrate rather than cellophane, some reaggregates will stick lightly to the substrate (or tightly in some cases with outgrowth). The entire reaggregate contracts synchronously.

The spherical reaggregates are transferred to a heated (37°C) bath containing fresh culture medium or Ringer solution for microelectrode impalements. There are several advantages of spherical reaggregates over monolayers, the most notable of which is the much easier microelectrode impalements, presumably due to the three-dimensional shape and isolation from vibrations (shock-mounting). An advantage of spherical reaggregates ("mini-heart") over an intact heart is that the contractions are more feeble, such that it is often possible to remain in the same cell for a prolonged period. In addition, since there are no blood vessels in the reaggregate, the

effect of a drug under investigation cannot be on the rate of perfusion flow.

Reaggregates which are composed of highly differentiated (electrically) cells usually do not contract spontaneously, but do contract in response to electrical stimulation. Reaggregates which are composed of reverted cells generally contract spontaneously, at a rate of about 1 sec^{-1}. The properties of the cells do not seem to change very much, if at all, during incubation periods of 5 to 30 days.

Scanning electron microscopy has been done on heart reaggregates at various stages of formation by Shimada et al. (*44, 45*). One key finding was that a thin fibroblastic coat eventually completely covers the reaggregate. Fibroblasts from within the center of the reaggregate also worked their way out to the surface.

Changes in Properties of Intact Hearts During Development *in situ*

General Comments

The anterior half of the flat 16–20 hr old chick embryo blastoderm contains bilateral "precardiac" areas (mesoderm) whose cells are destined to form the heart (*46*). If the precardiac areas are dissected and placed into organ culture for several days, a tubular heart develops (within a vesicle) and it beats spontaneously, but further differentiation does not proceed *in vitro* (*47*). In normal development, twin tubular primordia are formed bilaterally from the precardiac mesoderm and fuse to form a single tubular heart (*48*). The tubular heart begins contracting spontaneously at 30–40 hr (9–19 somite stage), and begins to propel blood (*49*). The blood pressure is very low (1–2 mm Hg) at this stage, and the velocity of propagation of the peristaltic contraction wave is very slow (approx. 1 cm/sec in 3-day hearts) (*49*). Morphological changes occur so that chambers appear in the heart on about day 5. Circulation to the chorioallantoic membrane is established on day 5, so that metabolism of the embryo becomes aerobic at that time. The nerves also arrive at the heart on about day 5 (*49*), but they do not become functional with respect to neurotransmitter release until considerably later (*50, 51*). The heart rate of the chick embryo increases from about 50 beats/min on day 1.5 to the maximal value of about 220 beats/min on day 8, and the influence of temperature on heart rate is greatest in the young embryo (*49*).

Absence of Fast Na^+ Channels in Young Embryonic Chick Hearts

In order to determine the state of membrane differentiation of the cultured cells, comparison should be made with the properties of intact chick hearts at different stages of development *in situ*. The electrical properties of the heart undergo sequential changes during development (*52–55*). The myocardial cells in young (2–3 days *in ovo*) hearts possess slowly rising (10–30 V/sec) action potentials preceded by pacemaker potentials (Fig. 1*A*). Hyperpolarizing current pulses do not greatly increase the rate of rise of the action potential, and excitability is not lost until the membrane is depolarized to less than −25 mV. The upstroke is generated by Na^+ influx through slow Na^+ channels which are insensitive to tetrodotoxin (TTX) (Fig. 1*B*). Kinetically fast Na^+ channels which are sensitive to TTX make their initial appearance in the membrane on about day 5, and the maximal rate of rise of the action potential ($+\dot{V}_{max}$) suddenly jumps to about 50–70 V/sec. The fast Na^+ channels increase in density thereafter, until about day 18, when the adult

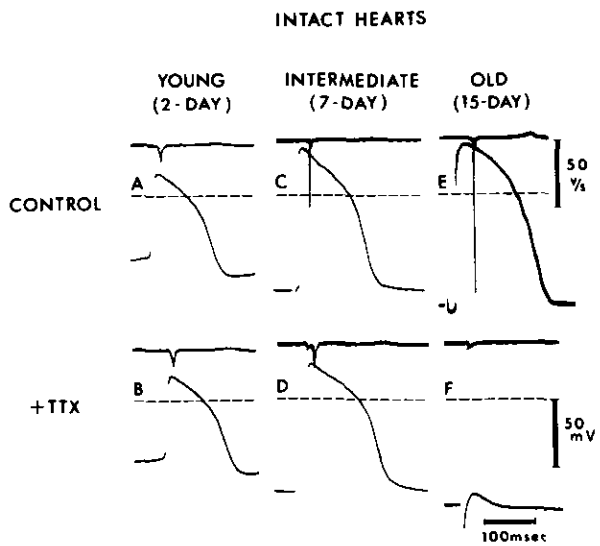

FIGURE 1. Development of sensitivity to TTX of intact embryonic chick hearts with increasing embryonic age: (*A-B*) intracellular recordings from a 2-day-old heart before (*A*) and 20 min after (*B*) the addition of TTX (20 μg/ml); (*C-D*) recordings from a 7-day-old heart before (*C*) and 2 min after (*D*) the addition of TTX (2 μg/ml) (note depression of the rate of rise in *D*); (*E-F*) from a 15-day-old heart prior to (*E*) and 2 min after (*F*) the addition of TTX (1 μg/ml). The action potentials were abolished and excitability was not restored by strong field stimulation in *F*. The upper traces give *dV/dt;* this trace has been shifted relative to the *V-t* trace to prevent obscuring *dV/dt*. The horizontal broken line in each panel represents zero potential. *dV/dt* calibration (in *E*) and voltage and time calibrations (in *F*) pertain to all panels. From Sperelakis and Shigenobu (*54*).

maximal rate of rise of about 150 V/sec is achieved (Fig. 1*E*). During an intermediate stage of development (from day 5 to day 8), fast Na^+ channels coexist with the large complement of slow channels. TTX reduces $+\dot{V}_{max}$ to about the value observed in 3-day hearts, i.e., 10–20 V/sec, but the action potentials and accompanying contractions persist (Fig. 1-C-D). After day 8, the action potentials are completely abolished by TTX (despite increased stimulus intensity) (Fig. 1*F*), and depolarization to less than −50 mV now abolishes excitability. This indicates that the action potential-generating channels consist predominantly of fast Na^+ channels, most of the slow Na^+ channels having been lost (functionally) so that insufficient numbers remain to support regenerative excitation. Addition of some positive inotropic agents increases the number of available slow channels (Ca^{2+}-Na^+) in the membrane, and leads to regain of excitability in cells whose fast Na^+ channels have been inactivated (*56, 57*).

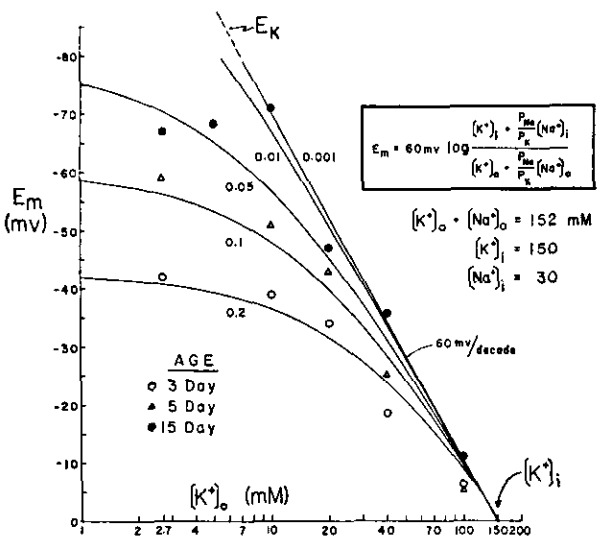

FIGURE 2. Resting potential E_m plotted as a function of $[K]_o$ on a logarithmic scale for data obtained from embryonic chick hearts at days 3 (○), 5 (△) and 15 (○). $[K]_o$ was elevated by substitution of K^+ for equimolar amounts of Na^+. Continuous lines give theoretical calculations from the constant-field equation (inset) for P_{Na}/P_K ratios of 0.001, 0.01, 0.05, 0.1, and 0.2. Calculations were made assuming $[K]_i$ and $[Na]_i$ values shown. For a P_{Na}/P_K ratio of 0.001, the curve is linear over the entire range with a slope of 60 mV/decade, i.e., it closely follows E_K. The data for the 3-day heart follow the curve for a P_{Na}/P_K ratio of 0.2, those for 5-day heart follow the theoretical curve for 0.1, and those for the 15-day heart follow the curves for 0.01 to 0.05. The estimated intracellular K^+ activities ($[K]_i$) obtained by extrapolation to zero potential are nearly the same for all ages. From Sperelakis and Shigenobu (*54*).

Low K^+ Permeability in Young Hearts

Young embryonic chick hearts have a low K^+ permeability (P_K) (*54*). This accounts for the low resting potentials of about −40 mV and for the high incidence of automaticity in the ventricular area of the tubular heart. As shown by resting potential versus log $[K]_o$ curves for hearts of different ages (Fig. 2), the low resting potentials in young hearts are caused by a high P_{Na}/P_K ratio of about 0.2, rather than to an internal $[K]_i$ greatly lower than that of old hearts. The $[K]_i$ level is about 130*mM* in 3-day-old hearts compared to 150*mM* in 15-day hearts; hence, the K^+ diffusion potential (E_K) is about −91 mV (at a $[K]_o$ of 4*mM*) in 3-day hearts. The P_{Na}/P_K ratio decreases to about 0.1 by day 5, and is between 0.05 and 0.01 by day 15. The increase in resting potential parallels the change in the P_{Na}/P_K ratio. The input resistance of the cells is high in young hearts (about 13 MΩ), and it declines to a final value of about 4.5 MΩ during development, and from this it was concluded that a low P_K was primarily responsible for the high P_{Na}/P_K ratio in young hearts. Carmeliet et al. (*58, 59*) have also shown from ^{42}K flux measurements that P_K is several fold lower in 6-day hearts than in 19-day hearts. As predicted from Figure 2, young hearts are less sensitive to elevation of $[K]_o$.

The presence of pacemaker potentials is determined by P_K in cells which have a low P_{Cl}, as myocardial cells do. The low P_K in young heart cells makes for automaticity as well as accounts for the low resting potentials. The incidence of pacemaker potentials in cells in young hearts is very high, and this incidence decreases during development, roughly in parallel with the increase in P_K (*54, 60*). Automaticity is absent in the ventricular cells of the older hearts.

Low (Na, K)-ATPase Activity in Young Hearts

The (Na, K)-ATPase specific activity is low in the young chick hearts and increases progressively during development, reaching the final adult value by about day 18 (*61*). Although the Na-K pump capability is thus low in young hearts, it is sufficient to maintain a relatively high $[K]_i$. The Na-K pump is aided in the task by the low P_K; that is, in young hearts, the pump capability is low but the ion leak is correspondingly low.

High Cyclic AMP Level in Young Hearts

The cyclic AMP level is very high in young hearts, and it decreases during development, first

rapidly and then more slowly, reaching the final adult level by about day 16 (*62, 63*). Since elevation of cyclic AMP leads to an increase in the number of available slow Ca^{2+}-Na^{+} channels (*56, 57*), the decrease in cyclic AMP level during development could be related to the concomitant decrease in density of available slow channels (in the absence of positive inotropic agents). We have proposed that a *protein constituent of the slow channel must be* phosphorylated in order for the channel to be available for voltage activation, and that this is the mechanism of action of those positive inotropic agents which elevate cyclic AMP (activation of cyclic AMP-dependent protein kinase).

Few and Nonaligned Myofibrils in Young Hearts

Electron microscopy of young chick hearts shows that there are only few and short myofibrils (Fig.

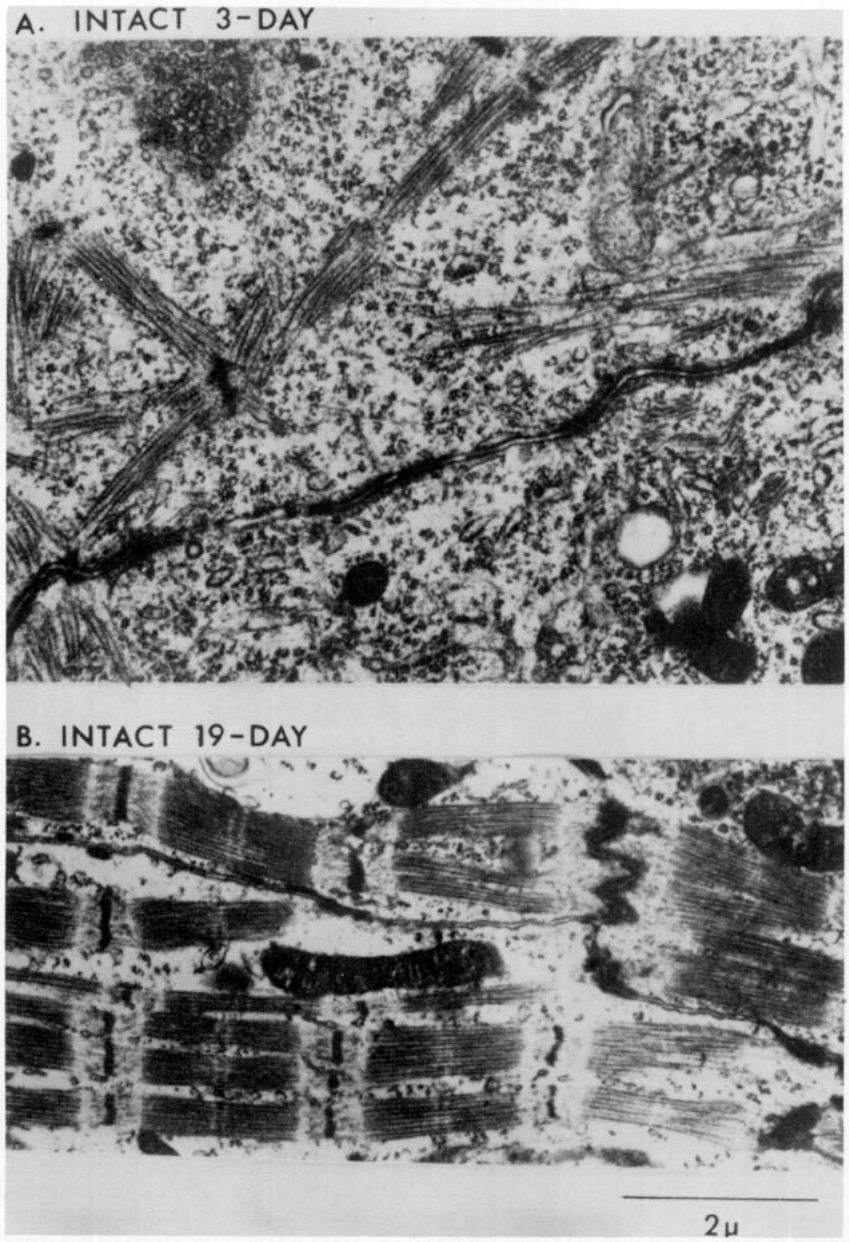

FIGURE 3. Cell ultrastructure of young (3 days *in ovo*) and old (19 days *in ovo*) intact embryonic chick hearts *in situ:* (*A*) 3-day ventricular cells demonstrating paucity and nonalignment of myofibrils; ribosomes are abundant in the cytoplasm; the contiguous cells are held in close apposition by desmosomes; (*B*) 19-day ventricular cells with abundant and aligned myofibrils. A convoluted intercalated disk appears between contiguous cells. Calibration given in *B* also applies to *A*.

3A). The sarcomeres are not complete, and the myofibrils run in all directions, i.e., they often run perpendicular to one another. There is an abundance of ribosomes and rough endoplasmic reticulum, and large pools of glycogen are found in the cells. As development progresses, the number of myofibrils increases and they become aligned, as illustrated in Figure 3B. By day 18, the ultrastructure of the myocardial cells is quite similar to that of cells in adult hearts.

Organ-Cultured Intact Young Chick Embryonic Hearts: Arrested Development

Organ culture of embryonic hearts *in vitro* provides a powerful means of analyzing the changes which occur during normal development *in situ*. For example, the embryonic age of the heart is one variable.

When 3-day-old chick embryonic hearts, which have not yet become innervated, are placed intact into organ culture for 10–14 days, these muscle cells do not continue to differentiate electrically or morphologically (*27, 28*). The impaled cells have low resting potentials and slow-rising TTX-insensitive action potentials (Fig. 4); that is, their properties are identical to those of fresh, noncultured 3-day-old hearts. They do not gain fast Na^+ channels. These hearts retain their slow Na^+ channels, as evidenced by the fact that Mn^{2+} (1*mM*) does not block the action potentials but D600 (verapamil analog) does (Fig. 4). In effect, the cells are arrested in the stage of differentiation attained at the time of explantation. The same was try of young hearts grafted on to the chorio-allantoic membrane of a host chick and thereby blood perfused (*30*) (Fig. 4). Therefore, something in the *in situ* environment of the heart in the developing embryo must control the appearance of the fast Na^+ channels. Since the innervation reaches the heart on about day 5, a trophic influence of the neurons could trigger the next step in differentiation.

When organ-cultured young hearts are incubated with RNA-enriched extracts obtained from adult chicken hearts, fast Na^+ channels appear *de novo*, the resting potentials increase, automaticity ceases, and the action potentials are rapidly rising and completely sensitive to TTX (*64*) (Fig. 5). There was a lag period of about 6 days before the effects became demonstrable. These findings indicate that further differentiation can be induced *in vitro*. Inducing potency is limited to extracts from hearts, and is destroyed by pretreatment of the RNA with ribonuclease. The induction was prevented by cy-

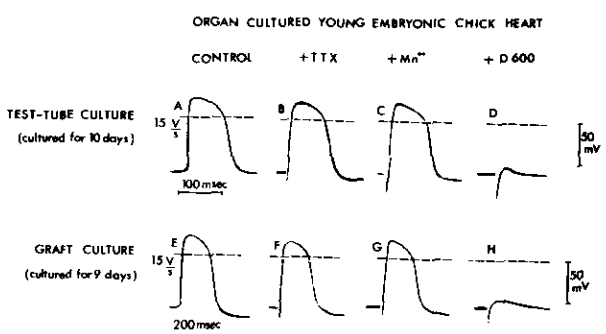

FIGURE 4. Lack of gain of fast Na^+ channels in organ-cultured young (3-day-old) embryonic chick hearts: (*A-D*) tissue in test-tube culture for 10 days. (*E-H*) tissue in graft culture for 9 days. As shown in (*A*) and (*E*) the control action potentials had slow rates of rise of about 15 V/sec. Addition of tetrodotoxin (TTX; 2 μg/ml) had little or no effect on the rate of rise of the action potential (*B, F*). Addition of Mn^{2+} (1*mM*), a known blocker of Ca^{2+} current, did not affect the action potentials (*C, G*). However, D600 ($10^{-6}M$), a known blocker of slow channels, did abolish the action potentials (*D, H*). Note the different time scale in *E-H* compared to *A-D*, and that the action potential durations were longer in the graft cultures compared to the test-tube cultures, for the same pacing frequency and temperature. Taken from Renaud and Sperelakis (*30*).

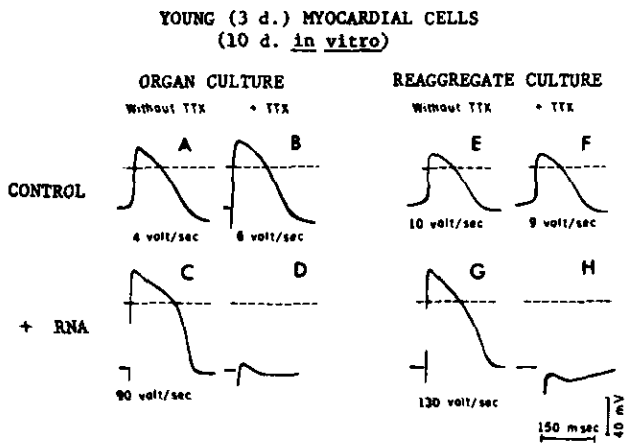

FIGURE 5. Arrest of development of young (3-day) embryonic myocardial cells in organ-cultured intact hearts (*A-B*) and in cultured reaggregates (*E-F*), and induction of further differentiation by culturing with RNA-enriched extracts from adult hearts (*C-D, G-H*): (*A-B*) records from one cell in a 3-day-old intact heart organ cultured for 10 days before (*A*) and after (*B*) addition of TTX (1 μg/ml), and showing the arrest of development; (*C-D*) records from one cell in an organ-cultured 3-day-old heart treated with RNA showing a fast rate of rise (90 V/sec) (*C*) and complete blockade by TTX (*D*); (*E-F*) records from one cell in a spherical reaggregate before (*E*) and after (*F*) TTX (1 μg/ml), illustrating the failure of membrane differentiation, including lack of fast Na^+ channels; (*G-H*) records from one cell in a spherical reaggregate culture treated with RNA showing a fast rate of rise (130 V/sec) and a high resting potential (−75 mV) (*G*); TTX (0.1 μg/ml) rapidly abolished the action potential (*H*). Electric field stimulation applied in *B, C, D, G, H*. Modified from McLean et al. (*64*).

cloheximide, an inhibitor of protein synthesis, thus suggesting that synthesis of new protein is required for the appearance of the fast Na^+ channels.

Survival of old embryonic hearts (nonperfused) is much more difficult than in the case of the young hearts, unless the hearts are minced into small fragments (of about 1 mm^3) (*27, 30*). In the latter case, the cells in the heart fragments tend to retain their highly differentiated electrical properties, including relatively large stable resting potentials with moderately fast-rising TTX-sensitive action potentials upon electrical stimulation (*30*). This was true whether the fragments were cultured in test tubes (immersed in culture medium) or grafted on to the choriollantoic membrane of a host chick. When whole hearts are cultured, either by immersion or at a fluid-air interface, survival was for a shorter period (e.g., up to 6 days), but again the cells tended to retain their highly differentiated state. Whole fetal mouse hearts in organ culture also survive for similar periods (*65*).

In summary, in organ culture, both young hearts and old hearts tend to retain the electrical properties that they originally possessed at the time of placement into culture.

Characterization of the Cultured Heart Cells

Spherical Reaggregate Cultures Prepared from Young Hearts

When spherical reaggregate cultures were prepared from 3-day-old embryonic hearts and cultured for 10 days, no evidence for differentiation was obtained (*64*). The impaled cells had low resting potentials, pacemaker potentials, and slowly-rising (about 10 V/sec) action potentials that were not sensitive to TTX (Fig. 6). Because of their importance, these experiments have been repeated, and similar results were again obtained (Pelleg et al., unpublished observations). In contrast, DeHaan and coworkers (*66*) reported that reaggregates of 4-day-old cells exhibited increasing rates of rise of the action potentials over several days in culture; since day 4 is on the edge of the intermediate period, it is possible that genes coding for the production of protein for fast Na^+ channels had been activated prior to culturing.

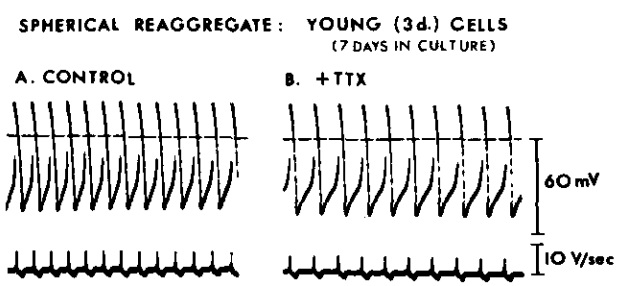

FIGURE 6. Spherical reaggregate culture prepared from young (3-day-old) embryonic chick hearts illustrating the lack of electrical differentiation of the cells *in vitro*: (*A*) Control before tetrodotoxin (TTX) addition showing the low resting potential with spontaneous slowly-rising action potentials, each preceeded by a pacemaker potential. (*B*) In presence of TTX (1 μg/ml, 10 min) showing no effect on the overshoot or rate of rise of the action potentials (TTX often depresses automaticity to varying degrees). Modified from McLean et al. (*64*).

As in the case of organ-cultured young hearts, when the spherical reaggregate cultures prepared from 3-day-old embryonic hearts were incubated with RNA-enriched extracts obtained from adult chicken hearts, the cells proceeded to differentiate after a lag period of several days. There were large stable resting potentials and rapidly-rising TTX-sensitive action potentials (*64*) (Fig. 5, *E-H*). These findings are consistent with the view that differentiation of immature cardiac myoblastic cells ordinarily does not proceed spontaneously *in vitro*, but that under appropriate conditions, the cells can be induced to do so.

Standard Monolayer Cultures Prepared from Old Hearts: Reversion to the Young Embryonic State.

Loss of Myofibrils. When ventricular myocardial cells are trypsin-dispersed from 14–20-day-old embryonic chick hearts and placed into standard monolayer cell culture, the cells lose many of their myofibrils within a short time. Cells in a monolayer culture are illustrated with light microscopy in Figure 7*A* and with electron microscopy in Figure 7*B*. As shown, after several days in culture, there are only few and incomplete myofibrils. Thus, the ultrastructure rapidly reverts to that characteristic of the young embryonic state (compare Fig. 7*B* with Figure 3*A*). However, the cells tend to regain more myofibrils after long periods in culture (*18*). It has also been found that the ultrastructure closely resembles the adult ultrastructure if the cultured cells are placed into media containing low serum concentrations (which presumably inhibits cell division and favors differentiation) (*67*). In addition, it has been found that even isolated single heart cells in culture may contain a dense packing of myofibrils (S. Chacko, personal communication). Collagenase-dissociated cells exhibit less myofibrillar disruption than trypsin-dissociated cells (*68*). Numerous enzyme and metabolic changes occur in cultured heart cells rapidly after culture (*9, 69, 70*).

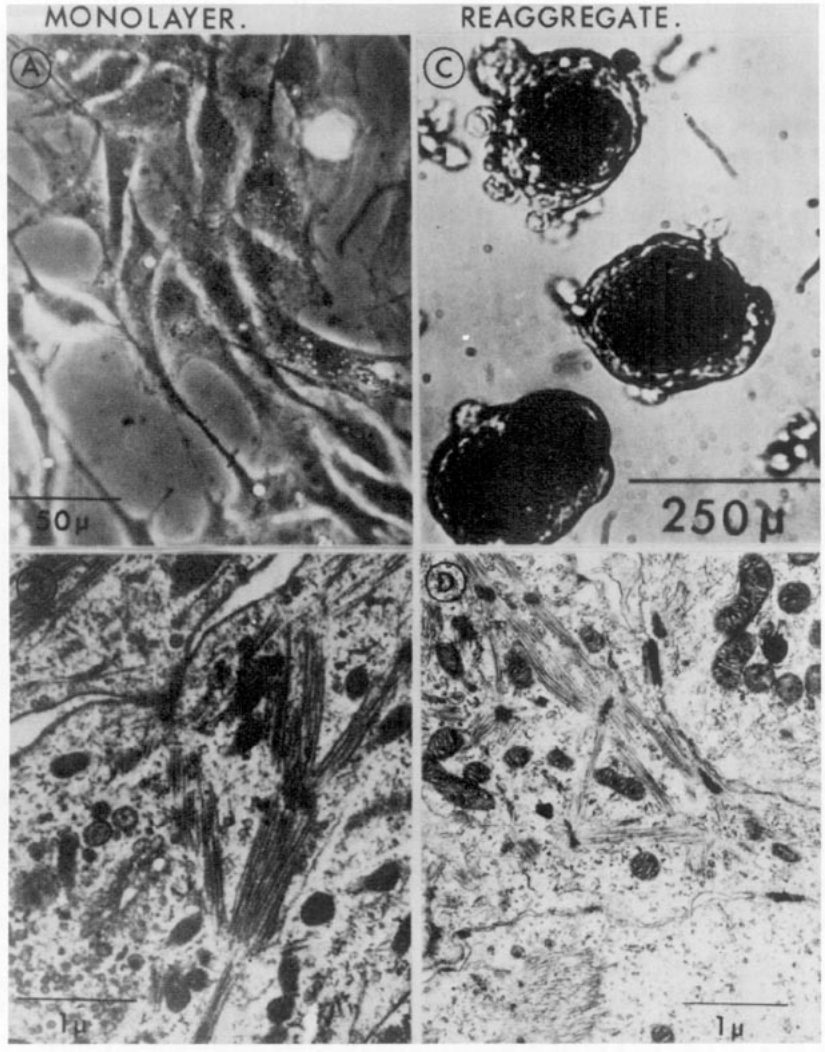

FIGURE 7. Micrographs of 19-day-old embryonic chick ventricular cells cultured as monolayers (*A-B*) and in spherical reaggregates (*C-D*); (*A*) low power phase contrast micrograph showing cell arrangement; (*B*) electron micrograph showing paucity and nonalignment of myofibrils in cells; (*C*) low power light micrograph showing several spherical reaggregates; (*D*) electron micrograph of several cells showing sparse, incomplete, and nonaligned myofibrils.

Loss of Fast Na^+ Channels and Gain of Slow Na^+ Channels. When the monolayer cells, cultured for 3–14 days, are impaled with microelectrodes, it is found that the resting potentials are low and the action potentials are slowly-rising ($+\dot{V}_{max}$ of 3-15 V/sec) and the overshoot is small (see Fig. 8*C*). Hyperpolarizing current pulses increase action potential amplitude (see Fig. 9*B*), but $+\dot{V}_{max}$ is increased only a relatively small amount. With depolarizing pulses, spike amplitude decreases (see Fig. 9*A*), and $+\dot{V}_{max}$ decreases and goes to zero at an E_m of about −20 mV. Spontaneous contractions cease at a $[K]_o$ of about 50*mM*, corresponding to a resting potential of about −20 mV (see Fig. 10). It was first shown by Sperelakis and Lehmkuhl (*7*) that TTX has no effect on the rate of rise or overshoot of the action potential (Fig. 8*D*). The loss of TTX sensitivity in monolayer culture has been confirmed in several laboratories (*71–73*). The action potentials closely resemble those found in young embryonic hearts (intact, noncultured) (compare Fig. 8*C* with Fig. 1*A*), and not those found in old embryonic hearts from which the cells were derived (compare Fig. 8*C-D* with Fig. 8*A-B* or with Fig. 1*E-F*). Thus, the ventricular cells taken from 14–20-day embryonic hearts revert back to the early embryonic state (or partially dedifferentiate) with respect to the action potential-generating mechanism, namely fast Na^+ channels are functionally lost and slow Na^+ channels are gained. This

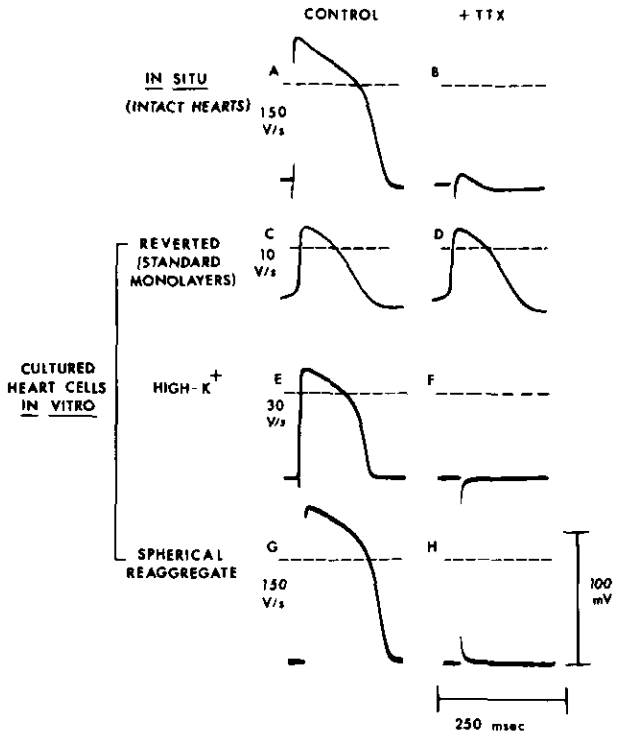

FIGURE 8. Comparison of electrophysiological properties of intact old (16-day) embryonic chick heart *in situ* (*A-B*) with those of trypsin-dispersed old ventricular myocardial cells in cultures prepared by three different methods (*C-H*) (*A-B*) intact heart; control action potential (*A*) was rapidly rising (150 V/sec), had a high stable resting potential (about −80 mV), and was completely abolished by tetrodotoxin (TTX; 0.1 μg/ml) (*B*); (*C-D*) standard reverted monolayers; control action potential was slowly rising (10 V/sec), was preceded by a pacemaker potential, the resting potential was low (about −50 mV) (*C*), and TTX did not alter the action potential (*D*). (*E-F*) partially reverted cells cultured as monolayers in media containing elevated K^+ concentration (25*mM*); control action potential had a rate of rise of 30 V/sec (*E*), lacked a pacemaker potential, had a moderately high resting potential (−60 mV), and was completely abolished by TTX (*F*); (*G-H*) highly differentiated cells in spherical reaggregate culture; control action potentials were rapidly rising (150 V/sec), the resting potentials were high (−80 mV) (*G*), and TTX abolished the action potentials (*H*). Modified from Sperelakis et al. (*35, 40, 54*).

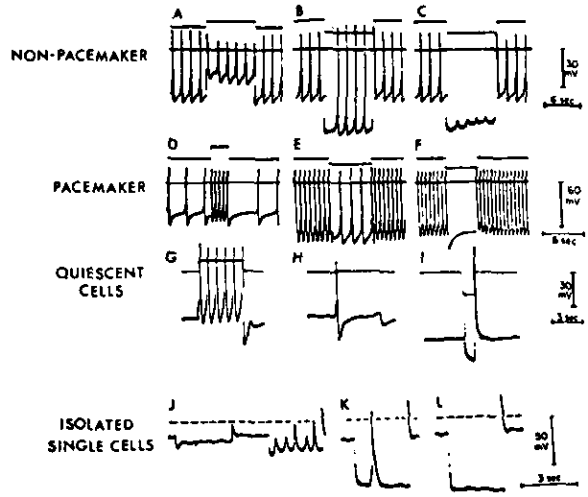

FIGURE 9. Effect of polarizing current on nonpacemaker and pacemaker reverted heart cells in monolayer culture; (*A-C*) nonpacemaker cell; frequency of firing unaltered by current pulses of 0.6 (*A*) and 1.4 nA (*B*); at 1.5 nA, action potentials prevented, but driving junctional potentials remain at unaltered frequency (*C*); (*D-F*) three pacemaker cells, depolarizing current pulse of 1.2 nA markedly increases rate of firing (*D*), whereas hyperpolarizing pulses slow the frequency (*E*, 0.6 nA) or cause cessation of firing (*F*, 1.2 nA); (*G-I*) quiescent cells can be induced to fire during depolarizing current pulses (1.4 nA in *G* and 0.2 nA in *H*), or at the termination of hyperpolarizing current pulses (*I*, 2 nA). (*J-L*) recordings from an isolated single cell in culture which was quiescent and had a very low resting potential. Hyperpolarizing current pulses of 0.2 and 0.4 nA (*J*), 0.8 nA (*K*), and 0.9 nA (*L*) elicited anodal-break responses as well as spontaneous firing during the pulse (*J-K*); the frequency of firing during the pulse was a function of the degre of hyperpolarization, indicating true pacemaker behavior. Modified from Sperelakis et al. (*77, 78*).

reversion can occur rapidly, often being complete within 24 hr (*35*).

The inward current during the action potential is carried mainly by Na^+ ion because the overshoot is a function of log $[Na]_o$ with a slope approaching the theoretical 60 mV/decade (*74*). $+\dot{V}_{max}$ is also dependent on $[Na]_o$, and excitability is abolished at 28*mM* Na^+ and below (*75*). Li^+ was first shown by Sperelakis and Lehmkuhl (*75*) not to substitute for Na^+ in carrying current through the slow Na^+ channels during the action potentials. A small inward Ca^{2+} current undoubtedly normally participates in the electrogenesis of the rising phase and overshoot of the action potential. In fact, in cells whose excitability is abolished in zero Na^+ (Li^+, choline$^+$, or sucrose replacement), elevation of $[Ca]_o$ to 10*mM* or more, or addition of Sr^{2+} (5–10*mM*) or Ba^{2+} (2–10*mM*), leads to rapid reappearance of action potentials (Fig. 11*A-E*). Thus, purely divalent cation spikes can be produced in cultured heart cells, as has been shown for Purkinje fibers by Vereecke and Carmeliet (*76*). In the presence of normal $[Na]_o$, addition of 2-12*mM* Sr^{2+} or Ba^{2+} produces much larger action potentials with greater overshoots (Fig. 11*F-H*). The divalent cations presumably pass through either slow Ca^{2+} channels or the slow Na^+ channels.

Decrease in P_K and Gain of Automaticity. Shortly after separation from the ventricle, many of the myocardial cells in suspension beat spontaneously at independent rhythms (*77*). This indicates that the normally nonpacemaker ventricular cells of old embryonic hearts rapidly gain automaticity upon cell separation. This suggests that a marked decrease in P_K occurs, because to

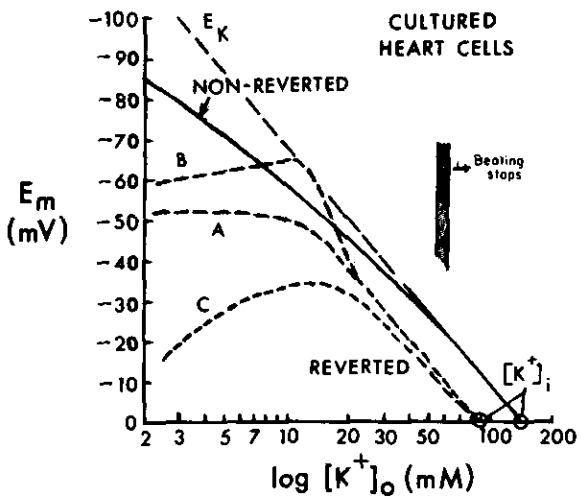

FIGURE 10. Resting potential E_m as a function of the external K^+ concentration for chick embryonic heart cells in monolayer culture (reverted) and in cultured spherical reaggregates (nonreverted). The line labelled E_K has a slope of 60 mV per tenfold change in $[K]_o$. For the reverted cells, with elevation of $[K]_o$ from 2.7 to 10–15*mM*, the resting potentials of some cells are almost unaffected (curve *A*), whereas those of other cells become slightly hyperpolarized (curve *B*). Isolated single cells which are not beating spontaneously usually have a much lower resting potential, but they become markedly hyperpolarized at 10–15*mM* $[K]_o$ (curve *C*). All cells depolarize as $[K]_o$ is elevated above 20*mM*, and extrapolation of linear region of curve to zero potential gives $[K]_i$ of 90–100*mM*. Spontaneous synchronous beating of cell groups ceases at a $[K]_o$ of about 50*mM*. For nonreverted cells the curve for cells in cultured spherical reaggregates fits a theoretical plot of values calculated from the constant-field equation for P_{Na}/P_K ratio of 0.02. The plot extrapolates to a $[K]_i$ of about 130*mM*. Modified from Sperelakis et al. (*40, 79*).

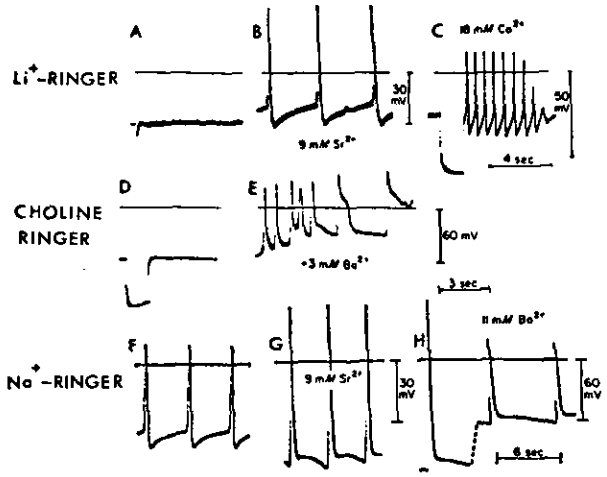

FIGURE 11. Demonstration of divalent cation action potentials in cultured heart cell monolayers: (*A*) absence of action potentials in Na^+-free Ringer solution (Li^+ substitution for Na^+) (because Li^+ cannot pass through slow Na^+ channels); (*B*) the appearance of spontaneous action potentials after addition of 9*mM* Sr^{2+} to the bath; (*C*) another culture in Li^+-Ringer developed excitability after elevation of Ca^{2+} in the solution to 18*mM* (a train of action potentials occurred at the end of a hyperpolarizing current pulse) (anodal-break excitation); (*D*) absence of excitability in Na^+-free Ringer solution (choline$^+$ substitution for Na^+); (*E*) the appearance of spontaneous action potentials after addition of 3*mM* Ba^{2+}; note the progression into prolonged action potentials; (*F*) the control action potentials in the presence of normal Ringer solution; (*G*) the much larger and faster-rising action potentials produced by addition of 9*mM* Sr^{2+} to the solution, suggesting that both Sr^{2+} and Na^+ ions contribute to the inward current; (*H*) illustration of the large action potentials produced when 11*mM* Ba^{2+} was added to the normal Ringer solution (voltage gain cut in half in the middle of the sweep depicted); the control action potentials are not shown, but were similar to those given in (*F*).

exhibit automaticity, cells must have a low g_k and a very low g_{ct}. When the cells are allowed to adhere to the glass for a few days and are subsequently impaled, the resting potentials are low and many cells exhibit pacemaker potentials (Figs. 8*C*; 9*D-F*; 12). The input resistance increases, the average value being close to double (10 MΩ) that of cells in intact hearts. These facts are consistent with a low P_K. A plot of resting potential versus log $[K]_o$ suggests that the extrapolated $[K]_i$ is 90–100*mM* in the reverted cells (Fig. 10), corresponding to an E_K (at a $[K]_o$ of 4*mM*) of −82 mV. This value is considerably greater than the measured average resting potential of about −55 mV, hence indicating that the P_{Na}/P_K ratio is high (presumably because P_K is low). Thus, P_K also tends to revert back towards the young embryonic state in cultured monolayers. In fact, in some isolated single cells in culture, P_K is so low that the cells are depolarized beyond the level that action potentials can be produced (beyond the inactivation potential for the slow channels); therefore, these cells do not contract spontaneously (*78*). However, if impaled with one or two microelectrodes, it is found that the membrane resistance is very high and that spontaneous action potentials and contractions appear upon application of hyperpolarizing current pulses (Fig. 9*J-L*). As shown in Figure 10*C*, these cells exhibit a prominent hyperpolarization when $[K]_0$ is raised to 10–15 *mM*.

Some of the monolayer cells behave as nonpacemaker cells (*77*). As illustrated in Figure 9*A-B*, application of depolarizing or hyperpolarizing current pulses does not alter the frequency of firing in these cells. Sufficient hyperpolarization causes failure of the action potentials, but unlike the true pacemaker cells, small driving junctional potentials continue at unaltered frequency during the pulse (Fig. 9*C*); the junctional potentials probably represent the interaction with contiguous firing cells. In contrast, true pacemaker cells respond to small depolarizing current pulses by an increase in firing rate, and to small hyperpolarizing pulses by a de-

crease in firing frequency (Fig. 9*D-F*); note the absence of junctional potentials in Figure 9*F* when firing is abolished. The slope of the pacemaker potential is a function of E_m. Quiescent cells can be induced to exhibit trains of action potentials during application of long-duration depolarizing pulses (Fig. 9*G-H*), and anodal-break excitation can be elicited by brief hyperpolarizing pulses (Fig. 9*I*). Some cells possessing automaticity function as latent pacemakers in that they are driven by true pacemaker cells which have greater intrinsic firing rates.

Large depolarizing pulses in some true pacemaker cells result in the occurrence of damped oscillations during the pulse (Fig. 12*A-C*). In some true pacemaker cells, application of a hyperpolarizing current pulse, large enough to shut off the train, but too small to elicit anodal-break excitation, turns off the train (Fig. 12*D*); subsequent application of a hyperpolarizing pulse sufficiently large to elicit anodal-break excitation (or a brief depolarizing pulse sufficient to trigger a single action potential) turns the train back on (Fig. 12*E*). This suggests that, in this form of automaticity, each action potential is responsible for triggering the next one. In some cases, the train turns off and on paroxysmally (Fig. 12*F*) and, in such cases, it can be seen that a depolarizing afterpotential follows the hyperpolarizing afterpotential.

Automaticity is suppressed by elevation of $[K]_o$ above 15*mM* (Fig. 12*G-I*), as expected by the increase in K^+ conductance (g_k) predicted from the constant-field equations. The g_k is a function of both P_K and $[K]_o$, and P_K is also increased in elevated $[K]_o$ (59). Distinction between suppression of automaticity and depolarization block of excitability is clearly demonstrated in Figure 12*I*, in which automaticity of the same cell is completely suppressed, yet the cell is capable of producing an action potential upon anodal-break stimulation. Note that spontaneous firing did not occur during the hyperpolarizing pulse itself, further indicating the suppression of automaticity (compare with Fig. 13*F*).

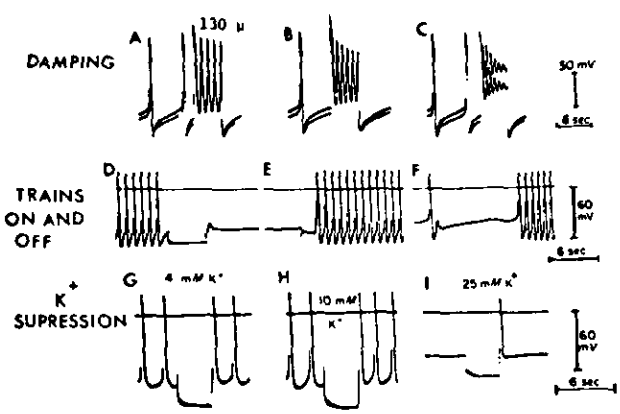

FIGURE 12. Impalement of one cell in a monolayer culture with two microelectrodes (130 μm apart) showing nearly identical potential changes in both electrodes when current is passed through one electrode; depolarizing pulses of 9 (*A*), 10.2 (*B*), and 11.6 (*C*) nA; note damping of responses. Bridge imbalance in C due to high current. (*D-E*) Trains of spontaneous action potentials turned off (*D*) and on (*E*) by small hyperpolarizing pulses. Automatic firing was stopped by a 1.2 nA pulse too small to cause anodal-break excitation; firing reinstated by a larger pulse (1.8 nA) which produced anodal-break excitation (*E*). (*F*) Spontaneous arrest of firing and resumption of firing in a cell; note depolarizing afterpotential following the hyperpolarizing afterpotential. (*G-I*) Recordings from one true pacemaker cell illustrating suppression of automaticity by elevation of extracellular K^+. In 4*mM* (*G*) and 10*mM* (*H*) K^+, automaticity was not suppressed, whereas elevation of K^+ to 25*mM* caused depolarization and cessation of spontaneous action potential generation, but anodal-break excitation elicited an action potential (*I*). Hyperpolarizing current pulses of 4.8 nA applied in each panel. Modified from Sperelakis et al. (79, 97).

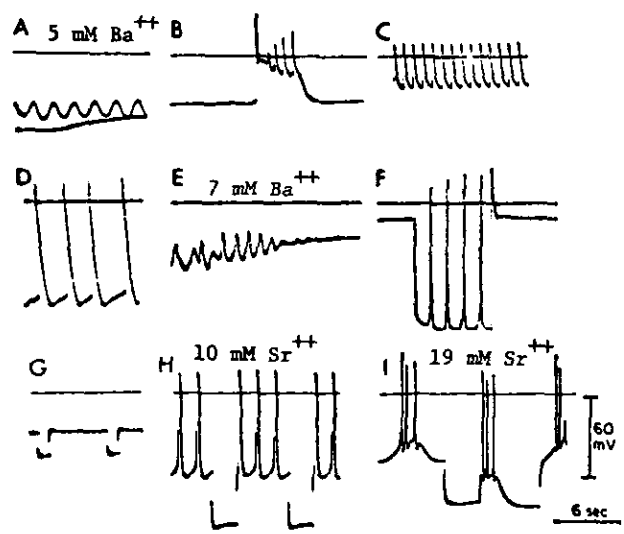

FIGURE 13. Effects of Ba^{2+} and Sr^{2+} on reverted cells in monolayer culture. In a quiescent cell, Ba^{2+} (5*mM*) depolarized and induced spontaneous firing: (*A*) two successive sweeps superimposed at 20 sec after addition of Ba^{2+} showing onset of depolarization and oscillations; (*B*) after 3 min, the action potentials acquired prolonged plateaus with repetitive discharges on the plateau; (*C*) at 5 min, sustained depolarization occurred with repetitive action potentials. In a firing cell (*D*), Ba^{2+} (7*mM*) rapidly depolarized and increased membrane resistance (*E*); repolarizing current pulse (1.2 nA) applied during the sustained depolarization initiated action potentials during the pulse (*F*). In a quiescent cell (*G*) 10*mM* Sr^{2+} produced hyperpolarization and converted the cell to a true pacemaker (*H*), as evidenced by cessation of firing during applied hyperpolarizing pulses of 4.8 nA. Elevation of Sr^{2+} to 19*mM* (*I*) induced sinusoidal-like oscillations with several action potentials superimposed and produced prominent depolarizing afterpotentials; current pulse of 6.0 nA applied. Modified from Sperelakis and Lehmkuhl (79).

Automaticity is rapidly induced by 2–5*mM* Ba^{2+} Fig. 13*A-C*), presumably by its effect of decreasing P_K (*79, 80*) and perhaps also by increasing the inward background depolarizing current through acting as a current carrier. Partial depolarization is produced, and membrane resistivity is greatly increased. If the depolarization produced is too great (beyond the inactivation potential for the slow channels), the cell becomes quiescent but will fire spontaneous action potentials (and contract concomitantly) during the application of hyperpolarizing current pulses (Fig. 13*D-F*). The frequency of the action potentials so produced is a function of the intensity of the hyperpolarizing current pulse, small hyperpolarizations giving a high frequency and larger hyperpolarizations giving a lower frequency (and even suppression of firing). Sr^{2+} (5–10*mM*) also has the ability to induce automaticity in quiescent cells concomitant with hyperpolarization (Fig. 13*G-I*) (*79*). At the higher Sr^{2+} concentrations, prominent depolarizing afterpotentials are produced, and the action potentials ride on top of large sinusoidal-like oscillations. Although the effect of Sr^{2+} could be due to stimulation of an electrogenic Na^+ pump potential contribution to E_m, Sr^{2+} also hyperpolarizes cells partially depolarized by ouabain or local-anesthetic inhibition of the (Na,K)-ATPase, thus suggesting that Sr^{2+} acts by decreasing P_{Na} (or increasing P_K). Ba^{2+} has a similar hyperpolarizing action, but this is often masked by its prominent depolarizing action due to decreasing P_k.

Decrease in (Na,K)-ATPase, and Cyclic AMP Level. The cultured monolayer cells were shown to have a lowered (Na,K)-ATPase specific activity by Sperelakis and Lee (*81*). In this respect also, there appears to be some reversion towards an earlier embryonic state.

In one study of cultured cells prepared from old embryonic hearts, the basal cyclic AMP level of the monolayers was about 50% higher than in the control intact hearts (of the same embryonic age) (*62*), whereas in a subsequent study, the basal cyclic AMP level in both monolayers and spherical reaggregates was significantly lower than in the control hearts (*63*). The decrease was even greater in reaggregates prepared from young hearts, because of the larger control basal level (see above). Thus, the change in the basal cyclic AMP level in the cultured heart cells does not appear to be consistent. On the other hand, what is consistent is the large increase in cyclic AMP level after addition of isoproterenol; this occurred in both studies quoted above, and in both monolayer and spherical reaggregate cultures, and in cultures prepared from old or young embryonic hearts. This confirms that functional beta-adrenergic receptors are present in the cultured cells, and also demonstrates that beta-receptors are already present in the heart by day 4 of embryonic life.

Partial Reversion in Elevated K^+. It was found that separating the cells and culturing them in media containing elevated K^+ (12–60*mM*) helped the cells to retain more highly differentiated electrical properties (when tested in normal K^+ solutions) (*35*). The resting potentials were higher (about −60 mV) and automaticity was absent in most cells, indicating that P_K was not as greatly reverted. Although the action potentials were only moderately faster rising ($+\dot{V}_{max}$ of about 30–40 V/sec), they were completely abolished by TTX (Fig. 8*E-F*). Thus, it appears that some fast Na^+ channels are retained and the number of slow Na^+ channels is not greatly increased. Hyperpolarization by current injection did not greatly increase $+\dot{V}_{max}$, indicating that it was not a case of a large fraction of the fast Na^+ channels being voltage inactivated. Addition of ATP (5*mM*) to the medium seemed to have a slight beneficial effect, especially in combination with elevated K^+, for retention or regain of differentiated membrane properties. Therefore, cell separation and culture in high K^+ media acted to prevent complete reversion to the young embryonic state. It has been reported that TTX sensitivity of cultured heart cells depends on cell associations (*82*).

Spherical Reaggregate Cultures Prepared from Old Hearts: Retention of Highly Differentiated Membrane Electrical Properties

Fast-Rising TTX-Sensitive Action Potentials and High Resting Potentials. Many cells in spherical reaggregates (see Fig. 7*C*) (and in cylindrical strands) retain fully differentiated electrophysiological properties identical to those of the intact old embryonic hearts *in situ* (Fig. 8) (*40*). Such cells had high resting potentials of about −80 mV, and pacemaker potentials were absent; this indicates that P_K was high. The plot of E_m versus log $[K]_o$ indicated a P_{Na}/P_K ratio of about 0.02, and a $[K]_i$ of about 130*mM* (Figs. 10 and 14*B*). The input resistance averaged about 5 MΩ (Fig. 14*D*). The action potentials had fast rates of rise (100–200 V/sec), and they were completely blocked by low concentrations of TTX ($3 \times 10^{-7}M$) (Fig. 8*H*). $+\dot{V}_{max}$ diminished as the cells were progressively depolarized by elevation of $[K]_o$, and all excitability was abolished at an E_m of about −50 mV (Fig. 14*A*). In addition, a short chronaxie of about 0.5 msec was found (Fig. 14*C*), indicative of high excitability comparable to that of adult cardiac muscle. These

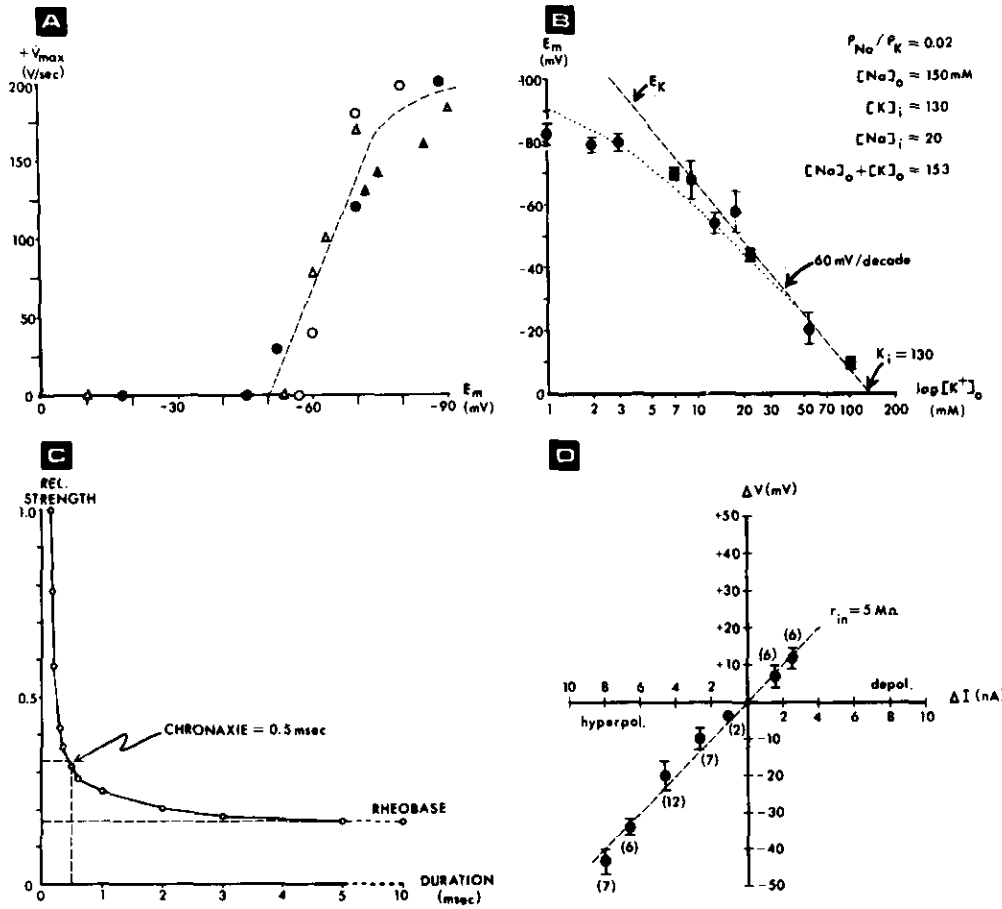

FIGURE 14. Summary of some electrophysiological properties of highly differentiated (electrically) cells in spherical reaggregate cultures. (*A*) Plot of $+\dot{V}_{max}$ versus membrane potential (E_m) from aggregates in four separate cultures (different symbols) with complete inactivation of the fast Na^+ channels at about −50 mV. Resting potential varied by elevation of $[K]_o$. Each point represents the mean of four to seven values obtained from different reaggregates within a culture. (*B*) Plot of E_m versus the log of external K^+ concentration $[K]_o$) (equimolar substitution of K^+ for Na^+) extrapolates to an internal $[K]_i$ of 130*mM*. The slope of the points in high $[K]_o$ is about 60 mV/decade; the straight line gives the K^+ diffusion potential (E_K) calculated from the Nernst equation. The dotted line was calculated from the Goldman constant-field equation for a P_{Na}/P_K ratio of 0.02 and for the ion concentrations given in the figure. (*C*) Strength-duration curve obtained from an impaled cell having a chronaxie of 0.5 msec. (*D*) Voltage/current relation gave an average input resistance of about 5 MΩ; data obtained from cells in several different aggregates (150 to 300 μm in diameter). Taken from McLean and Sperelakis (*40*).

results suggest that these reaggregated cells retained their full complement of fast Na^+ channels as well as a high P_K, and that, therefore, reversion did not occur to any significant extent in these cells. That is, by a number of criteria, many cells in cultured spherical reaggregates are adult-like in electrical properties.

On the other hand, not all cells were of this type. Some cells in the same reaggregate, or cells in another reaggregate in the same culture vessel, had partially or completely reverted. That is, there was a wide spectrum of degrees of reversion. Aging the cultures for several weeks sometimes improved the incidence of highly differentiated cells.

Pharmacological Receptors. To test for the retention of some pharmacological receptors in the reaggregates, experiments were done using the induction of slow channels (Ca^{2+}–Na^+) in the sarcolemma as an assay. To facilitate the detection of induction of slow channels, the fast Na^+ channels and excitability were blocked by using TTX (Fig. 15). Then, addition of agents, such as catecholamines and methylxanthines, which rapidly increase the number of slow channels available for activation upon stimulation, causes the appearance of slowly rising overshooting action potentials (the "slow responses") which resemble the plateau component of the normal action potential (Fig. 15*C*). The slow responses are accompanied by contractions, and it has been shown that both Ca^{2+} and

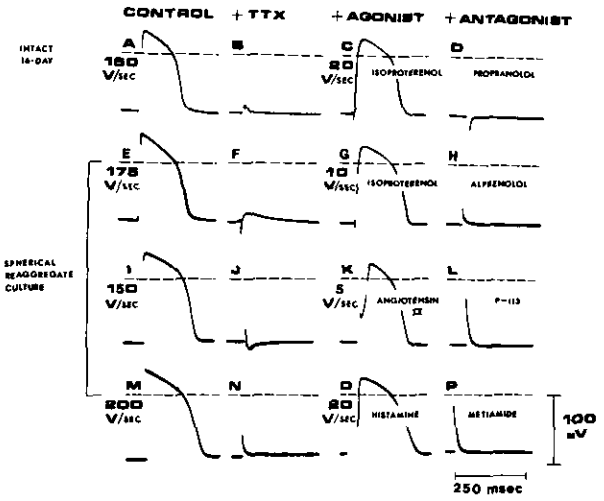

FIGURE 15. Demonstration of functional receptors for positive inotropic agents in intact old (16-day) embryonic chick hearts (*A-D*) and in cultured spherical reaggregates of trypsin-dispersed ventricular cells (*E-P*). Following blockade of the fast Na^+ channels by tetrodotoxin, positive inotropic agents induce slow responses which are blocked by specific antagonists. (*A-D*) Records from a ventricular cell in an intact 16-day-old heart. The control, rapidly-rising (150 V/sec) action potential (*A*) was abolished by tetrodotoxin (TTX; 0.1 μg/ml) (*B*). Addition of isoproterenol ($10^{-6}M$) induced a slowly-rising (20 V/sec) overshooting electrical response (*C*) which was blocked by propranolol ($10^{-5}M$) (*D*). (*E-H*) Records from one cell in a cultured spherical reaggregate showing control action potential (*E*) abolished by TTX (0.1 μg/ml) (*F*); isoproterenol ($3 \times 10^{-5}M$) induced a slow response (*G*) which was blocked by the specific beta-adrenergic receptor antagonist, alprenolol ($10^{-7}M$). (*I-L*) Records from one cell in another reaggregate showing control action potential (*I*) and its blockade by TTX (*J*); a slow response was induced by angiotensin II ($10^{-6}M$) (*K*) and was blocked by its competitive antagonist, P-113 ($10^{-5}M$) (*L*) (*M-P*) Records from one cell in another reaggregate showing control action potential (*M*) and its blockade by TTX (*N*); histamine ($10^{-5}M$) induced a slow response (*O*) which was abolished by the H_2 antagonist, metiamide ($10^{-5}M$) (*P*). (*56, 83, 84*).

Na^+ inward currents participate in the slow response (*56, 57*). The slow responses are blocked by agents which block inward slow Ca^{2+} current, including Mn^{2+}, La^{3+}, verapamil, and D-600. The effect of the catecholamines, such as isoproterenol (but not that of methylxanthines, such as caffeine), is blocked by beta-adrenergic blocking agents (Fig. 15), thus again indicating the presence of functional beta-adrenergic receptors. Angiotensin II also induces the slow response, and its action is blocked by specific angiotensin receptor blocking agents (P-113 and sar^1-ile^8-Ang II) (Fig. 15) (*83*). Finally, histamine also induces the slow response, and its action is blocked by histamine H_2-receptor blocking agents (but not by beta-adrenergic antagonists or by H_1-receptor antagonists (Fig. 15) (*84*). Thus, the cells possess functional receptors for a variety of positive inotropic agents.

Phosphorylated Slow Channels: An Hypothesis. Histamine and beta-adrenergic agonists, subsequent to binding to their specific receptors, are known to lead to stimulation of adenylate cyclase with resultant elevation of cyclic AMP levels. These positive inotropic agents also induce the slow responses, presumably by making more slow channels available in the membrane. Therefore, we postulated that the latter is brought about by phosphorylation of a protein constituent of the slow channels by means of a cyclic AMP-dependent protein kinase (*56, 57, 85*). That is, we believe that the phosphorylated slow channel is the form available for voltage activation. Consistent with this view, we found that cyclic AMP itself and its dibutyryl derivative also induced the slow responses, but only after a much longer lag period (peak effect in 15–30 min), as would be expected from slow penetration through the membrane. Another test of the hypothesis was done by using a GTP analog (5'-guanylimidodiphosphate [GPP (NH)P], an agent known to directly activate adenylate cyclase in a variety of broken cell preparations. The addition of GPP (NH)P (10^{-5}–$10^{-3}M$) induced the slow response in cultured reaggregates of chick heart cells within 5–30 min (Fig. 16) (*86*). These results support the hypothesis that the intracellular level of cyclic AMP controls the availability of the slow channels in the myocardial sarcolemma.

Angiotensin II, however, does not elevate cyclic AMP (Vogel et al., unpublished observations) but it does induce the slow channels; we suggested that angiotensin may directly activate protein kinase [see discussion and appropriate references in Freer et al. (*83*)]. Fluoride ion, in low concentrations

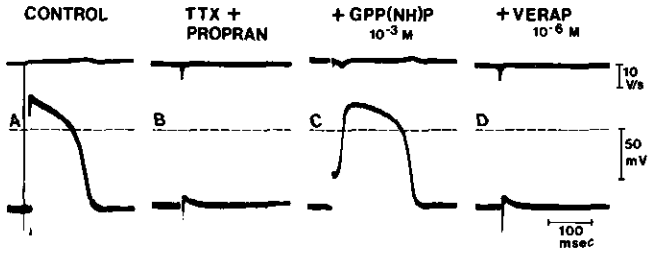

FIGURE 16. 5'-Guanylimidodiphosphate [GPP (NH)P] induction of a slow response in a cultured heart cell reaggregate: (*A*) control action potential recorded in normal Ringer solution; (*B*) the addition of TTX (10^{-6} g/ml) completely blocked excitability; propranolol ($10^{-6}M$) was then added to produce blockade of the beta-adrenergic receptors; (*C*) addition of GPP(NH)P ($10^{-3}M$) induced the slow response within 15 min; (*D*) addition of verapamil ($10^{-6}M$) abolished the slow response within 1 min. All recordings from one cell. The preparation was paced at a constant rate of 1/sec throughout. The upper traces give dV/dt. Calibrations for voltage, time, and dV/dt are given in *D*.

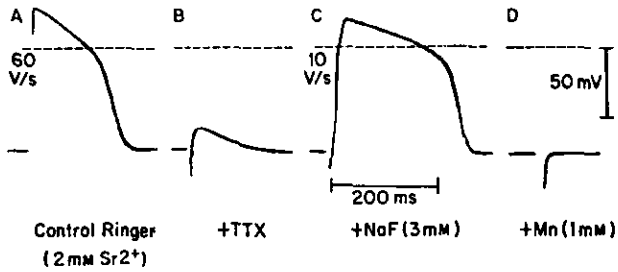

FIGURE 17. Fluoride induction of a slow response in cells in reaggregate culture of embryonic (16-day-old) chick ventricular myocardial cells. All recordings from one cell: (*A*) control action potential in control Ringer (containing 2.0*mM* Sr^{2+} because SrF is more soluble than CaF); (*B*) complete blockade of excitability by tetrodotoxin (TTX) (10^{-6} g/ml); (*C*) addition of NaF (3.0 *mM*) rapidly induced a slowly-rising overshooting electrical response; (*D*) The slow response was abolished within 2 min after the addition of Mn^{2+} (1.0*mM*). The preparation was paced at a rate of 0.5/sec throughout the experiment. Taken from Vogel et al. (*87*).

(<1*mM*) induces the slow response (Fig. 17) and acts as a positive inotropic agent, but does not elevate cyclic AMP; we suggested that fluoride ion acts by inhibiting the phosphatase which dephosphorylates the slow channel protein, thereby resulting in a larger fraction of phosphorylated channels [see discussion and appropriate references in Vogel et al. (*87*)]. Thus, the results with angiotensin and fluoride can be fitted within the framework of the phosphorylation hypothesis. However, this is not meant to imply that all positive inotropic agents exert (indirectly) all of their effects only on the slow channels of the membrane.

The induced slow responses are blocked by hypoxia, ischemia, and metabolic poisons (including valinomycin), accompanied by a lowering of the cellular ATP level (*88*). Under conditions in which the slow channels are blocked, the fast Na^+ channels are unaffected, thus indicating a differential dependence on metabolic energy. The slow responses blocked by valinomycin are restored by elevation of the glucose concentration (*89*). Thus, these findings are consistent with the phosphorylation hypothesis, since ATP is required for the phosphorylation. In summary, these peculiar properties of the slow channels allow the myocardial cells to exert control over the Ca^{2+} influx across the cell membrane and thereby the force of contraction; in addition, such control may serve as a protective mechanism to conserve ATP under adverse conditions of transient regional ischemia (*85*).

Slow Channel Blockade. As stated above, the slow channels are not blocked by tetrodotoxin (TTX), whereas the fast Na^+ channels are. The slow channels seem to be of three types: (a) slow Na^+, (b) slow Ca^{2+}, and (c) slow Ca^{2+}–Na^+. All three types of slow channels are blocked by verapamil and its analog, D600 (*90*). Mn^{2+} and La^{3+}, at low concentrations (e.g., 1*mM*), are more selective for the slow Ca^{2+} and slow Ca^{2+}–Na^+ channels (see Fig. 4). The slow channels induced by the positive inotropic agents seem to be predominantly of the slow Ca^{2+}–Na^+ type, as mentioned previously. These channels are blocked at acid pH, nearly complete blockade occurring at about pH 6.0, whereas the fast Na^+ channels are not significantly affected at this pH (*91*). The local anesthetics, in contrast, do not distinguish between the fast and slow channels, the dose-response curves for both types of channels being virtually identical (*92*) (Fig. 18). Ouabain (10^{-6}–10^{-4}M) suppressed the induced slow channels, $10^{-4}M$ being adequate to completely block the slow response induced in reaggregated cell cultures by isoproterenol (Fig. 19) (*57, 93*). This unexpected finding suggests that the positive inotropic effect of ouabain is not mediated by an increased electrogenic transmembrane Ca^{2+} inward current during

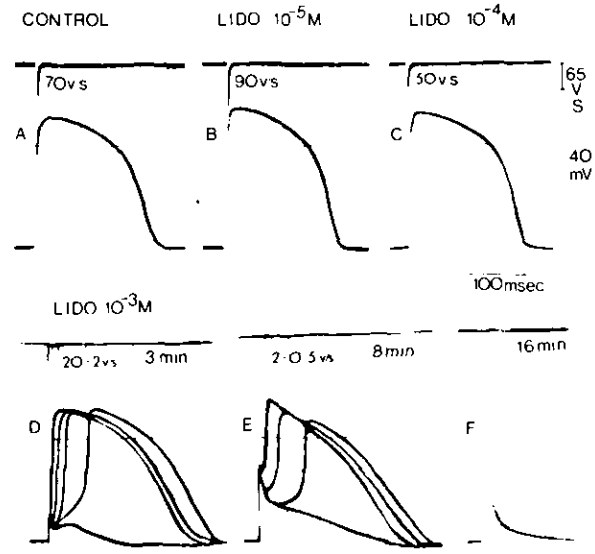

FIGURE 18. Effect of lidocaine on the normal action potential in reaggregate cell cultures of embryonic (16-day-old) chick ventricles. (*A*) normal action potential recorded from one cell bathed in normal Ringer solution, $+\dot{V}_{max}$ of 70 V/sec; (*B*) addition of lidocaine ($10^{-5}M$) increased $+\dot{V}_{max}$ to 90 V/sec; (*C*) elevation of lidocaine to $10^{-4}M$ reduced $+\dot{V}_{max}$ to 50 V/sec; (*D*) further elevation of lidocaine to $10^{-3}M$ caused failure of the action potential by 3 min, when stimulated at the same intensity (several consecutive sweeps superimposed in *D* and *E*); (*E*) increased stimulus intensity brought in responses again which again failed at 8 min after the addition of $10^{-3}M$ lidocaine; (*F*) excitability was completely abolished at stimulus intensities 10 times normal threshold at 16 min. Calibrations in *C* pertain to all panels. The maximum rate of rise of the action potential is given by the peak excursion of the *dV/dt* trace. Although not illustrated, lidocaine blocked the slow responses induced by isoproterenol at the same concentrations. From Josephson and Sperelakis (*92*).

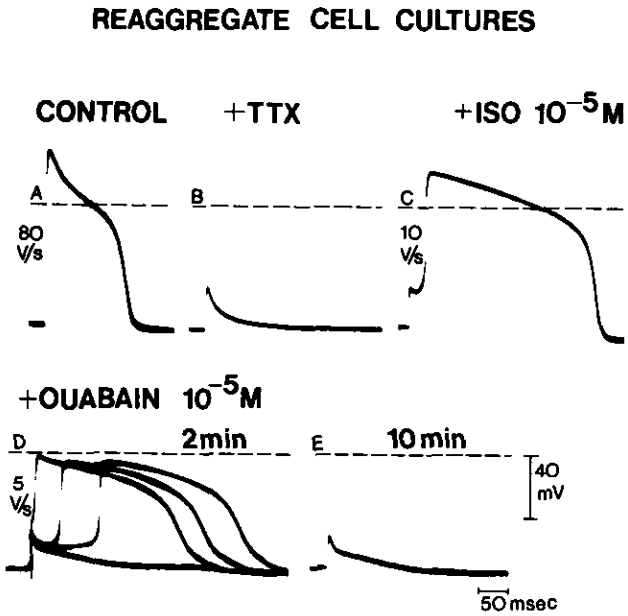

FIGURE 19. Ouabain blockade of slow responses induced by isoproterenol in a reaggregate cell culture of embryonic (16-day-old) chick ventricular cells: (*A*) normal action potential recorded from a cell bathed in normal medium; $+\dot{V}_{max}$ was 80 V/sec; (*B*) addition of 1 μg/ml of tetrodotoxin (TTX) abolished the action potentials despite electrical stimulation at intensities 10 times normal threshold. (*C*) addition of isoproterenol ($10^{-5}M$) rapidly induced slowly-rising (10 V/sec) overshooting electrical responses; (*D*) administration of ouabain ($10^{-5}M$) caused a reduction in the slow response (compare with *C*), with subsequent failure of the slow response within 2 min (several consecutive sweeps were superimposed); (*E*) excitability remained abolished at 10 min despite intense electrical stimulation. Taken from Josephson and Sperelakis (*93*).

the cardiac action potential, at least not at these high concentrations, but instead could partly account for the toxic effects of high concentrations of cardiac glycosides.

Reverted Morphology. Although it was found that, in a given reaggregate, the sarcolemmal electrical properties of most cells were highly differentiated, the ultrastructure of the cells in that same reaggregate exhibited the characteristic appearance of an immature cardiomyoblast (Fig. 7*D*). There were only few and incomplete myofibrils, and they were not aligned. There was a paucity of sarcoplasmic reticulum also. The morphology was similar to that of reverted monolayer cells (compare Figs. 7*D* and 7*B*) as well as to that of the 3-day-old intact hearts (compare Figs. 7*D* and 3*A*). The cell borders had numerous desmosomes, but no gaplike junctions could be found. Cultured strand reaggregates described by Lieberman's group exhibit a more highly differentiated morphology, including aligned myofibrils and well-developed intercalated disks. Furthermore, Jones et al. (personal communication) have achieved highly differentiated morphology (and electrophysiology) in multilayer cultures of chick heart cells incubated in media with reduced serum concentrations.

Electrical Coupling Studies. Evidence was obtained from double microelectrode impalements that cell-to-cell propagation occurs throughout the spherical reaggregates (50–400 μm in diameter). However, no evidence of low-resistance connections between cells could be obtained in aggregates containing the most highly differentiated cells. (McLean and Sperelakis, unpublished observations.) Two cells were impaled at various interelectrode distances (50–200 μm, using applied currents of up to 30 nA. The input resistance of these cells averaged about 5 MΩ. In aggregates containing reverted cells (with pacemaker potentials), moderate to strong electrotonic interaction between the two microelectrodes was obtained in about 50% of the cases; electrotonic interaction was weak or absent in the remaining 50% of the cell pairs. In contrast, DeHaan and Fozzard (*94*) have reported strong electrotonic interactions between all cells in their spherical reaggregates, and Lieberman (*95*) reported strong interaction in his strand preparations. Jongsma and van Rijn (*96*) also reported strong electrotonic interaction in monolayers of rat heart cells, whereas Lehmkuhl and Sperelakis (*97*) reported weak or absent electrotonic interaction in monolayers of chick heart cells, except in the case where exceptionally long and thick fibers or strands were impaled. Thus, it appears that cultured heart cells can be made to exhibit different degrees of electrotonic coupling depending on the condition. It has been reported that nonmyocardial cells, such as fibroblasts, may become electrically coupled to myocardial cells in culture (*38, 98*).

Spherical Reaggregate Cultures of Vascular Smooth Muscle Cells

By use of methods nearly identical to those we routinely use for heart cells, we were able to produce cultured reaggregates of vascular smooth muscle cells which contracted spontaneously (*99*). Single cells were trypsin-dispersed from arteries and veins (great vessels near the heart and mesenteric vessels) isolated from 10–20-day-old chick embryos, and induced to reaggregate into small spheres (100–500 μm in diameter) either by gyrotation or by plating on cellophane. Many of these spherical reaggregates contracted spontaneously or in response to electrical stimulation during culture periods of up to 6 weeks. When the reaggregates

were allowed to adhere to a glass substrate, cells emigrated from the spheres to form aprons of monolayered cells which continued to contract. Thick and thin myofilaments (mean diameters of 146 and 65 Å, respectively) and characteristic "dense bodies" were observed in a large fraction of the cells examined by electron microscopy. This ultrastructure is rather typical for smooth muscle cells, and the muscle cells can be readily distinguished from fibroblasts (which contain very extensive rough endoplasmic reticulum and do not contain thick myofilaments).

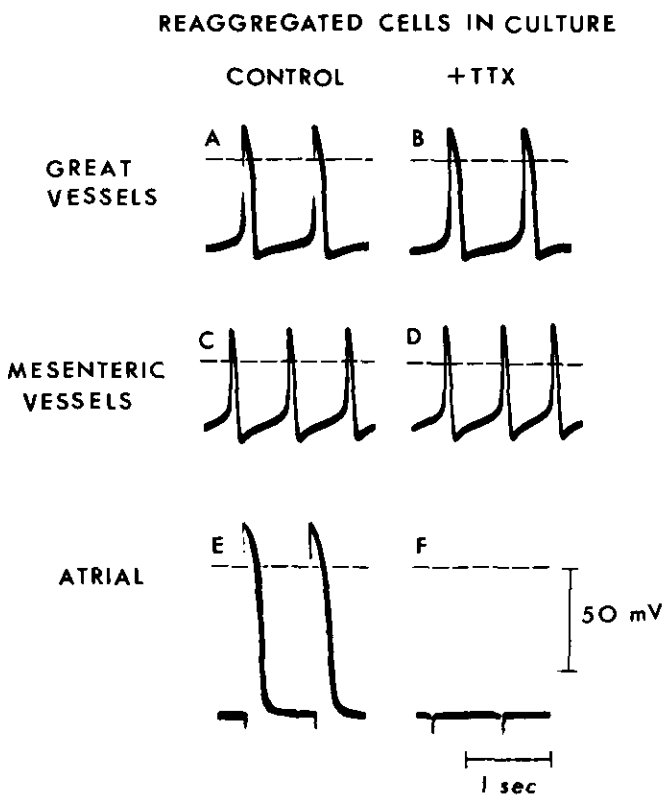

FIGURE 20. Comparison of action potentials recorded intracellularly from cells in cultured spherical reaggregates of trypsin-dispersed vascular smooth muscle cells and atrial cells. (*A, C, E*) Control before TTX; (*B, D, F*) In presence of TTX. (*A-B*) Recordings from a reaggregate of great vessel cells from 16-day chick embryos: (*A*) slowly rising (5 V/sec) action potentials fired spontaneously from a low membrane potential of about −50 mV; visible contractions of the aggregate accompanied the action potentials; (*B*) the action potentials and contractions persisted unchanged after prolonged exposure to tetrodotoxin (TTX; 1 μg/ml for 35 min). (*C-D*) recordings from an aggregate of cells from mesenteric vessels: (*C*) these cells exhibited action potentials with pacemaker potentials; (*D*) TTX did not affect the action potentials. (*E-F*) recordings from an atrial reaggregate; (*E*) action potentials with rapid rates of rise (100 V/sec) arose from a high stable resting potential (−75 mV) in response to extracellular field stimulation; contraction of the aggregate accompanied each stimulation; (*F*) TTX rapidly abolished both action potentials and contractions. Time and voltage calibration bars in *F* apply throughout. Taken from McLean and Sperelakis (*99*).

The vascular smooth muscle cells in these primary cultures had resting potentials of −40 to −60 mV and overshooting action potentials with maximal rates of rise usually about 4–10 V/sec (*99*) (Fig. 20*A, C*). Such electrophysiological properties are characteristic of smooth muscle cells. In addition, the action potentials were insensitive to tetrodotoxin, a blocker of fast Na^+ channels, as expected since smooth muscle cells do not possess fast Na^+ channels (Fig. 20*B, D*). The action potentials were preceded by pacemaker potentials, which are responsible for the spontaneous firing.

Reaggregates of cardiac atrial cells, prepared at the same time for comparison, had larger resting potentials (mean of −71 mV), and the action potentials had much faster rates of rise (mean of 84 V/sec). (Fig. 20*E*). The action potentials were completely blocked by tetrodotoxin, thus indicating their dependence on current through fast Na^+ channels (Fig. 20*F*). These properties are characteristic of myocardial cells, and are quite different from those of the cultured vascular smooth muscle cells.

Therefore, these results indicate that identifiable vascular smooth muscle cells can be successfully maintained in primary culture as reaggregates for several weeks, and that these cells retain electrical and contractile properties similar to those of smooth muscle cells in intact adult blood vessels. Thus, this preparation provides a convenient system for electrophysiological and pharmacological studies of vascular smooth muscle cells. Some obvious advantages of these cultured cells include the relatively easy impalement of the reaggregates by one or two microelectrodes (probably because of the absence of tough connective tissue) and the absence of innervation. One notable disadvantage is the great difficulty in producing cultured vascular smooth muscle cells capable of contracting compared to the relative ease with which cultured heart cells are produced.

We have recently been successful in producing and impaling cultured reaggregates of vascular smooth muscle cells from rat aorta (Harder et al., unpublished observations), and of smooth muscle cells from guinea pig vas deferens (McLean et al., unpublished). Monolayer cultures of vas deferens smooth muscle have also been prepared and studied in other laboratories (*100*). Spontaneously contracting monolayer cultures of vascular smooth muscle from rat aorta, which were passed several times and maintained for up to 3 months, also have been produced and characterized (*101*), and spontaneously contracting monolayer cultures of venous smooth muscle have been prepared (S. Chacko, personal communication). Spontaneously con-

tracting cultures of vascular smooth muscle cells from embryonic chick omphalomesenteric vessels have been prepared (*102*).

Summary

This article attempts to give a brief, and perhaps oversimplified, summary of cultured heart cells as a model system for studying the physiology, pharmacology, biochemistry, and toxicology of myocardial cells. The general techniques used are briefly described, and references are given so that the reader can go into greater detail on any topic. The types of culture preparations commonly used, such as monolayers and spherical reaggregates, are given, and some of the advantages and disadvantages of working with cultured heart cells are summarized. The terms in common usage relevant to tissue culture are loosely defined in an incidental manner in the introduction and methods section.

In order to provide the reader with a reference point with which to assess the functional state of the cells in culture, a brief description is given of some of the key properties of the cells that change during normal development of the heart *in situ*. It is demonstrated that the cells in standard monolayer cultures (primary) initially isolated from old embryonic hearts usually possess the characteristics of cells in intact young embryonic hearts, that is, they tend to revert back to the young embryonic state ("partial dedifferentiation"). Namely, they possess little or no functional fast Na^+ channels and have a low P_K, thus resulting in a low resting potential, automaticity, and slow-rising TTX-insensitive action potentials. In contrast, cells in cultures of spherical reaggregates often retain (or regain) their initial highly differentiated electrical properties; namely, they have large stable resting potentials and fast-rising TTX-sensitive active potentials. However, some spherical reaggregates can be made in which the cells possess reverted properties. That is, reaggregation, although an important factor, is not sufficient. Many factors, some unknown, appear to influence the degree of differentiation observed in the cultured cells. These factors probably include: embryonic age of the hearts, possible damage during cell separation, plating density, reaggregation, composition of the culture medium, period in culture, and perhaps presence of fibroblasts.

The cultured cells retain their pharmacological receptors; this is particularly clear for the highly differentiated cells. The ultrastructure of the cultured myocardial cells often reverts back to the early embryonic state, even in those cases in which the cell membrane remains highly differentiated; on the other hand, cultured heart cells (monolayers) can be made which possess a tight packing of parallel and completed myofibrils.

Immature myocardial cells placed *in vitro* do not continue to differentiate, either electrically or morphologically. This is true of reaggregate cell cultures prepared from young embryonic hearts and of organ-cultured intact young embryonic hearts. However, addition of an RNA-enriched extract from adult chicken hearts to the culture medium does allow electrical differentiation to proceed after a lag period of several days.

Spontaneously contracting spherical reaggregate cultures of smooth muscle cells can also be made from vascular smooth muscle as well as from other smooth muscles, such as intestinal or vas deferens. These cultured cells can be relatively easily impaled with one or two microelectrodes, and the cells retain the same properties they possessed in the intact muscle.

In conclusion, cultured heart cells can be used to study and to answer a wide variety of multidisciplinary questions which are difficult or impossible to answer in intact cardiac muscle.

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