Synthetic Strands of Cardiac Muscle: Growth and Physiological Implication

Abstract. *Cardiac muscle cells obtained from disaggregated embryonic chick hearts were cultured on differentially treated oriented substrata. Subsequent cell reaggregation, growth, and attachment produced linearly organized strands of cardiac muscle with dimensions suitable for electrophysiological analysis. Along the strand, areas that contained few muscle cells demonstrated reduced conduction velocity and were subject to propagation failure.*

The complexity and morphologic inhomogeneity of isolated preparations of cardiac muscle make it extremely difficult to analyze and compare the extent to which the basic physiologic mechanisms involved in cardiac muscle cell function, namely, excitation and contraction, truly reflect the membrane and contractile properties of the isolated cardiac muscle cell (*1*). Ultrastructural studies of cardiac muscle from a variety of animals failed to uncover an ideal preparation of cardiac muscle for voltage clamp studies, namely, a single, long cylindrical cell (*2*) and a preparation free of collagen for mechanical studies (*3*). Although several methods for preparing "individual cardiac cells" from adult mammalian hearts have been described (*4*), the physiologic value of these preparations is somewhat questionable (*1*). Furthermore, although preparations of embryonic and neonatal heart muscle in tissue culture are suitable for studying development and function in vitro (*5*), these preparations either have been large cell populations grown in mono- and multilayers, or have lacked the organizational arrangement of cardiac muscle, or have had both drawbacks. In this regard, however, the tendency of confluent sheets and networks that contain beating rat heart cells to form fiber-like (*6*) and spherical (*7*) masses has been described.

We describe a systematic attempt to cultivate the differentiation and orient the growth of cultured cardiac cells to produce a synthetic preparation that is more suitable, morphologically, for a given physiologic study [for example, the double-insulated gap method of voltage clamp (*1*)] than is naturally occurring cardiac muscle. Prototype versions of the definitive preparation (a single column of cardiac muscle cells) have provided us with insight into the mechanism of slow propagation like that which normally occurs in the atrioventricular node of the heart (*8*).

The culture techniques are modifications of those used earlier (*9*). The culture media contained Eagle's minimum essential medium or medium 199 supplemented with 10 percent fetal calf serum, 2 percent chick embryo extract, and 1 percent of a solution containing penicillin G (99 unit/ml) and streptomycin sulfate (36 μg/ml) (*10*). The components of the culture media, as well as the final solution, were passed through washed 0.45 μm Millipore filters to ensure sterility. Some of the cultures were grown in conditioned media containing medium 199 (*11*).

The orientation techniques reflect the ability of the surface of cultured cells to differentially or preferentially adhere to an oriented substratum (*12*). Culture dishes were coated with rat tail collagen (*13*), dried, and coated with 2 percent washed agar, which was then dried to a film. Differential surfaces were obtained by cutting through the agar film and thereby creating grooves or channels of exposed collagen approximately 25 μm wide. Alternatively, slits were cut in a Millipore filter to produce a template, which was then placed over the agar base. Deposition of a thin film of palladium by vacuum evaporation formed lines about 25 μm wide on the agar surface. After both procedures, the culture dishes were sterilized by exposure to ultraviolet light (2537 Å) for 15 to 20 minutes.

Freshly dissociated, beating heart cells do not readily adhere to the agar-coated surfaces. Within 12 to 24 hours, cells (either singly or as aggregates) preferentially attach and spread within the agar channels or on the lines of deposited palladium. After 3 to 6 days in culture, the cell bundles (strands) vary from 1.0 to 30.0 mm in length and 25 to 400 μm in width. The width of a strand is either variable (Fig. 1, part 1) or uniform Fig. 1, part 2) along its entire length.

With the aid of time-lapse cinematography, we established that bulbous regions of the strand develop from large aggregates of cells, which settle within the channels, coalesce either with other aggregates or with a basal layer of cells, and spread along the groove. A more uniform orientation is obtained when small aggregates and single cells are the majority of cells that settle initially into the channels and coalesce. Strands formed on palladium lines are uniform in width, and the cellular interrelations are clearly visible during early stages of development. However, with development, the forcefully beating strands tend to detach the deposited palladium lines from the agar surface; the preparation then floats in the medium. On the other hand, strands grown along channels adhere securely and although the cellular interrelations are somewhat obscured when

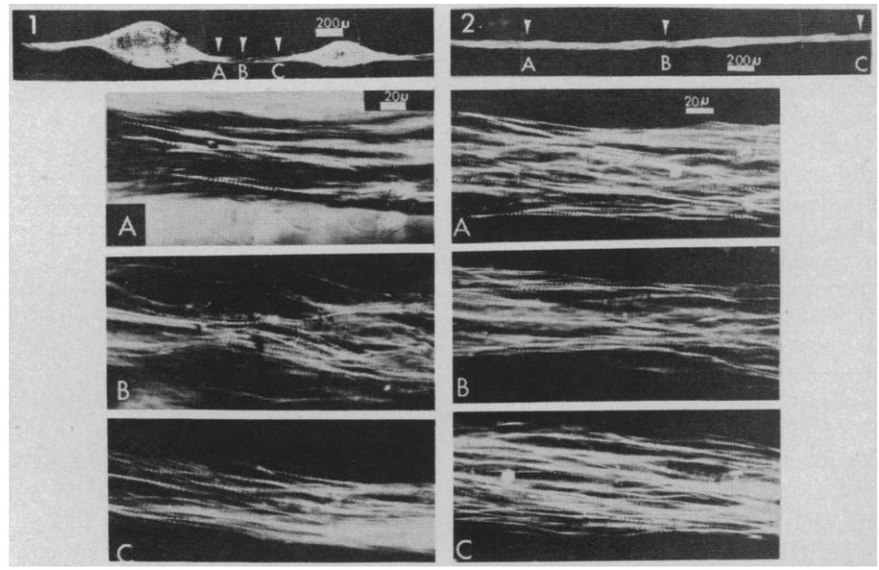

Fig. 1. Polarization photomicrographs of segments of cardiac muscle strands formed along channels cut in agar. The strands were fixed in Bouin's fluid after 5 (part 1) and 6 (part 2) days in culture. Both preparations were spontaneously beating, and impulse propagation was continuous. Inserts A, B, and C, which are higher magnifications of areas indicated by arrows in parts 1 and 2, demonstrate the linear organization of the myofibrils. The extensive continuity of muscle cells between the tapering ends of two bulbous areas is clearly shown in part 1B.

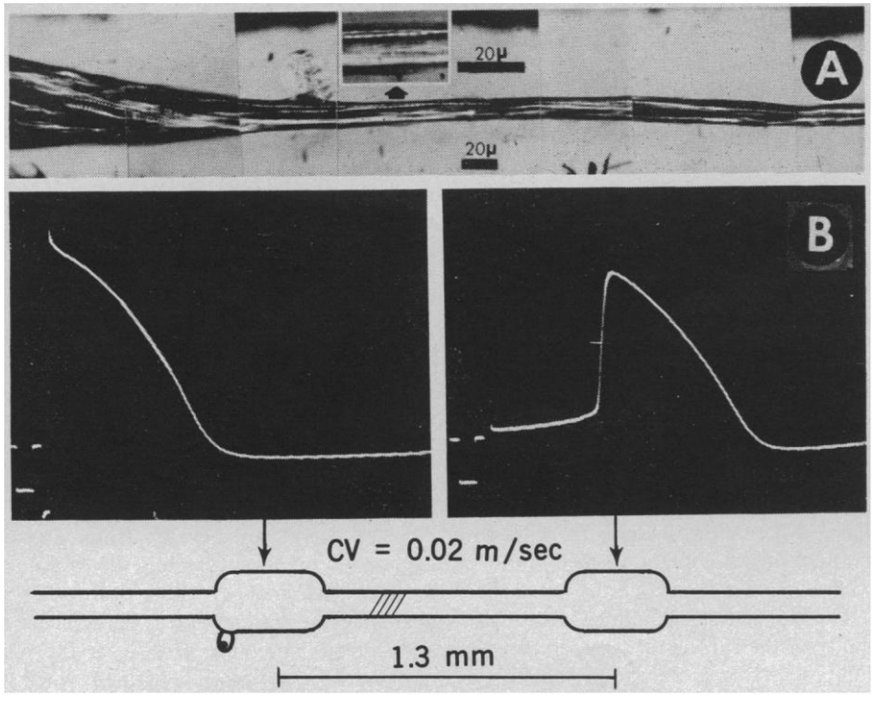

Fig. 2. (A) Composite polarization photomicrograph of a segment of cardiac muscle strand in which conduction block occurred when the area below the arrow received high rates of stimulation. The insert above the arrow is a high-power magnification of this region. Note the diminution in the number of myofibrils in the tapered region. (B) Transmembrane action potentials recorded from bulbous portions of a strand (stimulation, 120 min^{-1}). These bulbous portions bordered a region in which stimulation greater than 200 min^{-1} produced conduction block, which could be localized by direct vision. This region is represented by slanted lines in the schematic drawing below. Note the marked delay in conduction velocity (CV) across this region at the lower rate of stimulation (120 min^{-1}) (calibration: 20 msec, 20 mv).

using phase microscopy (optical interference is introduced by the cut surface), this visual problem prevents neither the observation of contractile motion nor the accurate placement and visualization of microelectrodes.

A majority of the strands are spontaneously active, but fewer beat synchronously along their entire length. The extent of spontaneous activity is markedly enhanced when the strands are grown in conditioned medium. The strands appear to contract uniformly, and beating tends to be more vigorous when the strands are maintained in conditioned media for as long as 3 weeks. On the contrary, the contractile activity of strands grown in fresh media usually decreases after 7 to 10 days in culture. These differences cannot be attributed to alterations in the ionic content of the media, since Na^+, K^+, and Ca^{2+} concentrations do not vary significantly in the two types of media (*14*).

In order to correlate structural features of the strand with impulse propagation, culture dishes were placed on a temperature-controlled (37°C) stage of an inverted phase microscope, and a layer of nontoxic, light mineral oil was floated over the surface of the medium to prevent evaporation (*9*). The preparation was stimulated extracellularly by a bevel-edged, glass microelectrode (50-μm tip diameter), which was filled with 0.5 percent agar in saline G. The unipolar electrode was connected via a salt bridge and silver wire to the output of a stimulus isolation unit, which was driven by a pulse generator. The culture dish was grounded through a silver wire positioned at the periphery of the dish. Frequency and duration of stimulation was selected so as to control the spontaneously beating strand. The preparation was then scanned with high-power television microscopy to verify continuous propagation along its entire length.

By gradually increasing the rate of stimulation, conduction failure was induced at a given locus along the strand. (Conduction failure was identified by a desynchronization of contraction within 100 μm.) The electrode was then moved to a position distal to the block to verify that conduction beyond the block was normal at higher frequencies (that is, that conduction block was not due to impingement on the refractory period of cell excitability) and to see whether retrograde block could be induced by rapid stimulation at the new locus. The entire preparation was scanned in this manner and photographed through the microscope to document areas of conduction block along its entire length. Preparations were fixed immediately for examination by polarization microscope (Fig. 2A). The most significant finding was that muscle cell content in blocked regions was less than that in areas capable of responding to increased rates of stimulation. These findings were corroborated by examination by electron microscope of preparations that showed similar patterns of impulse propagation (*15*).

Intracellular recording techniques were used to characterize impulse propagation in the synthetic strand and to localize conduction delay and block within selected areas of the preparation. In areas along the strand which have a high content of muscle cells, propagation is continuous and uniform (0.2 to 0.5 m/sec); whereas in those areas with fewer muscle cells, conduction velocity is much lower (0.002 to 0.02 m/sec). Figure 2B shows the propagation delay between two regions along a fiber that exhibited conduction block at high rates of stimulation (greater than 200 min^{-1}). The marked slowing of conduction velocity is comparable to that recorded from cells in the atrioventricular junctions of intact embryonic and adult hearts (*16*). Thus, normally occurring delays in conduction can be observed in a relatively simple, linearly aligned preparation of cardiac muscle—in contrast to the complex anatomy of the intact atrioventricular region (*17*). The preparation serves as a useful model system for questioning the factors contributing to slow conduction as it is known to occur in specialized regions of the intact heart (*8*).

We conclude that, regardless of the mechanisms involved by which isolated cardiac cells (i) interact with the substrate to give rise to a linearly aligned preparation and (ii) reaggregate and sort out with respect to cell type to form an inner core of muscle cells surrounded by an outer sheath of fibroblast-like cells (*15*), it is feasible to grow a synthetic strand of cardiac muscle with dimensions suitable for electrophysiological analysis.

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9. M. Lieberman, *Circ. Res.* **21**, 879 (1967). Hearts from chick embryos, ages 11 to 13 days, were excised aseptically in saline G, placed in a petri dish, and minced into small fragments (smaller than 0.5 mm.) The fragments were transferred to a 25-ml Erlenmeyer flask containing 10 ml of warmed 0.1 percent trypsin in saline G and gently agitated in a shaker bath at 37°C. After 5 minutes the suspension was triturated slowly (five passes through a 5-ml pipette) and returned to the shaker bath for a 5-minute period of agitation. The cell suspension with trypsin was then transferred to 20 ml of cold culture medium, and the dissociation procedure was completed by slowly pipetting the suspension ten times. After being filtered through multiple layers of sterile gauze, the suspension was centrifuged for 10 minutes at 1200 rev/min. After the supernatant was discarded, the pellet was resuspended in 2 ml of culture medium. A cell count was made with a hemocytometer, and the suspension was rediluted as necessary to obtain concentrations of 10^5 and 10^6 cell/dish. The cultures were incubated from 6 to 21 days in a humidified chamber at 37°C with a continuously freshened atmosphere of 5 percent CO_2 and 95 percent air. Fresh media was added after 3 to 5 days, and the total volume in the dish was brought to 5 ml or less. Subsequently, the media was replaced every 3 to 5 days.
10. Materials were obtained as follows: trypsin 1-300, Nutritional Biochemical Corp.; tissue culture dishes No. 3002, Falcon Plastics; Eagle's minimum essential medium, North American Biological Inc.; medium 199 and selected lots of fetal calf serum, Grand Island Biological Co.; penicillin G and streptomycin sulfate, E. R. Squibb Inc.
11. Dissociated chick heart cells (2×10^6 cells per 100-mm dish) were seeded in 10 ml of fresh culture medium. After 2 days, the medium was changed and 20 ml of fresh medium was added. The medium was then collected after 4 days and centrifuged for 20 minutes at 1200 rev/min, and the supernatant was filtered through 0.45 μm Millipore filters and stored at 4°C.
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14. Ionic concentrations of all media were determined with a Coleman model 21 flame photometer and were as follows: Na^+, 140 meq/liter; K^+, 5.6 meq/liter; and Ca^{2+}, 3.2 meq/liter.
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Δ^9-Tetrahydrocannabinol: Aversive Effects in Rat at High Doses

Abstract. *Water-deprived rats were administered a single dose of Δ^9-tetrahydrocannabinol either orally or intraperitoneally immediately after their first taste of a saccharine solution. In tests beginning 47 hours after drug administration, a dose-related reversal of rats' normal preference for saccharine was found. The data suggest that the drug produces aversive effects at doses of 1 to 32 milligrams per kilogram.*

Several investigators (*1*) have found a decrease in food intake by animals treated with Δ^9-tetrahydrocannabinol (Δ^9-THC), the principal active constituent of marihuana (*2*). Perhaps the clearest demonstration of this phenomenon was made by Manning *et al.*, who showed that Δ^9-THC produced a decrease in food consumption over a 30-day chronic administration period (*3*). This finding contradicts both a body of folklore concerning the effects of marihuana on human appetite for food and a recent study by Hollister (*4*), who showed that about half the human subjects given oral doses of Δ^9-THC increased food intake. A possible explanation of the discrepancy lies in differences in doses given to human and animal subjects, for animal subjects are typically given doses 10 to 100 times larger than human subjects are given. Studies in which high doses of THC are given to humans report that these doses may be slightly toxic in the sense that they produce unpleasant somatic effects (*5*). The toxicity of Δ^9-THC is also suggested by animal studies in which cats given doses from 0.5 to 4.0 mg/kg vomited and monkeys given doses from 16 to 64 mg/kg died (*6*).

We have used the "bait shyness" phenomenon to assess possible aversive effects of Δ^9-THC. "Bait shyness" or conditioned food aversion occurs when an animal ingests some food, usually a novel-tasting one, and then gets sick within the next 8 hours or so. In the laboratory, this phenomenon has been demonstrated for a variety of different tastes and various types of illness-inducing agents, including x-irradiation, injections of drugs such as apomorphine, methamphetamine, and various anesthetics. These studies have shown that rats will avoid novel-tasting substances that are followed by illness, even when the illness occurs as much as 8 hours after ingestion of the substance; the amount of aversion is related to the degree of illness (*7*).

Water-deprived rats were given various doses of Δ^9-THC from 0 to 32 mg/kg immediately after a period of exposure to a saccharine solution. They were then given preference tests between saccharine solution and tap water each day for 3 days, beginning 47 hours after drug administration. Saccharine preference was found to be inversely related to dose of Δ^9-THC, strengthening the view that THC may be aversive in high doses.

Forty experimentally naive male albino rats from the Walter Reed colony served as subjects (*8*). All weighed between 200 and 250 g at the start of the experiment and were singly housed in metal metabolism cages. The animals had continuous access to 45-mg rat food pellets (Noyes). After 2 days of free access to food and water, the animals were changed to a 23-hour water deprivation schedule in which tap water was available for 1 hour a day from a standard drinking tube. On the fourth day of this restricted drinking schedule, a 0.1 percent solution of saccharine in tap water, rather than pure tap water, was made available during the watering period. Immediately after this exposure, various doses of Δ^9-THC were administered either directly into the stomach with an oral administration needle or by intraperitoneal injection (*9*). The drug was diluted to concentrations of 2.25, 9.0, and 36.0 mg/ml with propylene glycol for administration to three groups of 10 animals each, with half the animals of each group getting oral administration and the other half, intraperitoneal administration. These concentrations were selected so that the dose volume would be approximately 0.2 ml when doses of 2, 8, and 32 mg/kg were given to the three groups. A fourth group of 10 animals received a placebo dose consisting of propylene glycol containing ethanol in the same proportion as the 36.0 mg/ml propyl-