Electrophysiological Properties of Cultured Dog Myocytes Obtained by Endomyocardial Biopsy

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Right ventricular cardiac tissue (10–20 mg wet weight) was obtained from anesthetized adult dogs by endomyocardial biopsy. The biopsy could be repeated in one dog every 2 weeks for up to 3 months. Fifty to 200 cardiomyocytes, dispersed with collagenase and trypsin, were collected by centrifugation of the cells with 50% polysucrose–sodium diatrizoate solution (Ficoll-Paque). Single cardiomyocytes were suspended in a minimum essential medium containing 20% fetal bovine serum and 8-bromoadenosine 3′:5′-cyclic monophosphate (0.1 mM) for up to 3 weeks. Approximately 70–80% of the cultured cardiomyocytes were rod shaped after 24 hours (10–20% after 7 days). Cytoplasmic organelles of the cultured cells, examined with a transmission electron microscope, were within the normal range of canine heart morphology in vivo. Resting membrane potential of the cells was about −80 mV when superfused with a Krebs’ solution containing 4.7 mM potassium ions. The action potential lasted for 300 msec and had a peak amplitude of about 120 mV. Voltage-clamp experiments demonstrated the presence of an inward calcium current (=0.9 nA at +9 mV), which was facilitated by isoproterenol (0.1–1 μM). The background potassium current showed typical inward rectification at potentials more negative than −80 mV. The results indicate that morphological, electrophysiological, and pharmacological properties of the cultured cardiomyocytes were intact. We propose that the culture techniques we have developed can be useful for repeated investigation on functional aspects of cardiac muscles in myocardial disease. (Circulation Research 1989;64:203-212)
Tokyo, Japan) was advanced to the heart by way of the right femoral vein. Cardiac tissue (1–2 mg wet weight per specimen) was excised from the right ventricular endomyocardium in each biopsy. Numbers of specimens ranged from eight to 12 in one dog. The biopsy was repeated several times in one dog every 2 weeks for up to 3 months. About 60 biopsies were done on 20 dogs.

Histological changes in the endomyocardium of the right ventricle were examined after isolation of the whole heart from the anesthetized dog by open heart surgery. Only mild thickening of endomyocardium was observed in all three dogs in which the biopsy was repeated at least 100 times.

Cell Dispersion

Ten to 20 mg cardiac tissue was placed in a 5-ml glass vial filled with a nominally calcium-free solution (2 ml) having the following millimolar composition: NaCl, 137; KCl, 5.4; NaHCO₃, 2.4; dextrose, 1.1; and N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid (HEPES), 2.1 (adjusted with NaOH to pH 7.4). This solution also contained 0.05% collagenase (Sigma Type I, Sigma Chemical, St. Louis, Missouri) and 0.25% trypsin (Sigma Type XI). The enzymatic digestion was facilitated by magnetic stirring (30–60 rpm) in a water bath at 37°C. After each initial and second digestion (30 minutes each), the enzyme solution was discarded since it contained mainly erythrocytes and severely damaged cardiomyocytes. A fresh enzyme solution was added to the remaining tissue for further digestion; this procedure was repeated six times, every 25 minutes. After each digestion, the enzyme solution containing the dispersed cardiomyocytes and 2 ml Eagle's minimum essential medium (MEM) (Nissui, Tokyo, Japan) were layered over 4 ml polysucrose–sodium diatrizoate solution (Ficoll-Paque) (Pharmacia, Uppsala, Sweden) in a centrifuge tube.⁷⁸ Eagle's MEM contained 20% fetal bovine serum (FBS) (GIBCO Labs, Grand Island, New York). The suspension was centrifuged at 80g for 8 minutes.

Cell Culture

After the centrifugation, the supernatant was discarded and sedimented cells were suspended in 2 ml Eagle's MEM containing 20% FBS. Most cells settled to the bottom of the plastic multiwells (Falcon, 3047, Becton Dickinson, Lincoln Park, New Jersey) in about 10–15 minutes, and about half of the medium was exchanged with fresh MEM containing 20% FBS. The exchange of the medium was repeated five times. Finally, the cardiomyocytes were suspended in 1.5 ml Eagle's MEM containing 20% FBS and 0.1 mM 8-bromoadenosine 3':5'-cyclic monophosphate (8-Br-cAMP). The concentration of the electrolytes sodium, potassium, chlorine, and calcium in the culture medium was 143.8 mM, 7.3 mM, 132.5 mM, and 2.2 mM, respec-

![Figure 1](http://circres.ahajournals.org/)

**Figure 1.** Light micrographs of cultured right ventricular cells of adult dog. Rod-shaped cells have clear cytoplasm and cross-striations. Rounded cell and cellular debris are also shown. Magnification, ×490.
tively. Isolated cardiomyocytes were cultured in a CO₂-incubator (5% CO₂) at 37°C. The culture medium was gently exchanged every 24 hours. The cultured cardiomyocytes were observed on a stage of an inverted phase contrast microscope (Nikon, Garden City, New York) placed in a closed box gassed with 5% CO₂/95% air at 37°C.

**Ultrastructure**

For transmission electron microscopy, the suspended cells in the multiwells were transferred to a centrifuge tube and centrifuged for 4 minutes at 170g. The cells were fixed at 4°C in a cacodylate buffer (0.2 M) (pH 7.4) containing 2.5% glutaraldehyde for 30 minutes and postfixed in a cacodylate buffer (0.1 M) containing osmium tetroxide for another 30 minutes at 4°C. The cells were dehydrated serially in 70–100% ethyl alcohol and finally embedded in Epon (Nisshin E.M., Tokyo, Japan). The thin sections were obtained with a microtome and stained with uranyl acetate and lead citrate. Micrographs were taken with an electron microscope (JEM 100S, Nihon Denshi, Tokyo, Japan).

**Electrophysiology**

Cultured cardiomyocytes were pipetted into a glass-bottomed recording chamber (total volume ~1 ml) placed on a stage of an inverted differential interference contrast optic (magnification ×100). The chamber was continuously superfused (2 ml/min) with preheated (36–37°C) Krebs' solution composed of (mM) NaCl 117, KCl 4.7, CaCl₂ 2.5, MgCl₂ 1.2, NaH₂PO₄ 1.2, NaHCO₃ 25, and glucose, 11, and gassed with 95% O₂/5% CO₂.

Intracellular recordings were made from single cardiomyocytes cultured for 2 to 5 days. Glass microelectrodes filled with potassium chloride (2 M) having DC resistance of 8–15 MΩ were selected. Membrane currents were measured by means of sample-and-hold/voltage clamp (model 8100, Dagan, Minneapolis, Minnesota, or Axoclamp-2A, Axon Instruments, Burlingame, California). Switching frequency ranged from 3 to 7 kHz with a 50-50 duty cycle. A head stage of the clamp amplifier was monitored continuously on an oscilloscope. Microelectrodes filled with cesium chloride (2 M) (DC resistance 10–20 MΩ) were used to clamp the slow inward calcium current. The cells were held at -40 mV in the presence of 1–3 μM tetrodotoxin (TTX). Cesium (2 mM) was also present in the superfusate in order to block the background (inward rectifying) potassium current (Cs/Cl⁻-condition). The peak amplitude of the slow inward current was defined as a difference between the peak amplitude of the inward current and the amplitude of the current at the end of the commands (visual estimation method). The steady-state current-voltage (I/V) curve was constructed in two ways: 1) The amplitude of membrane currents at the end of long-lasting (100–200 msec) command pulses was plotted, and 2) slow (1–2 mV/sec) ramp commands were used instead of pulse commands. In the latter case, the I/V curve was displayed on an X/Y recorder. Mem-
brane potential was differentiated (dV/dt) by use of the 4094 Math Package (Nicolet, Madison, Wisconsin).

Drugs used were TTX (Sankyo, Tokyo, Japan), (±)-isoproterenol bitartrate (Sigma), noradrenaline bitartrate (Sigma), DL-propranolol hydrochloride (Sigma), forskolin (Sigma), 8-Br-cAMP as a sodium salt (Sigma), 4-aminopyridine (Sigma), verapamil hydrochloride (Eizai, Tokyo, Japan), and anhydrous caffeine (Wako Pure Chemicals Osaka, Japan).

Results

Morphology

Fifty to 200 cells were obtained from 10–20 mg (wet weight) cardiac tissue. The fractions from the third or fourth harvest (see "Materials and Methods") yielded the largest number of cardiomyocytes. About 70–80% of the cells obtained were rod shaped when suspended in a culture medium for 24 hours; none of them were stained by trypan blue. The myocytes gradually lost their sharp and irregular cell contours at the ends. Finally, the myocytes began to become round and lost the cross-striations. Some of the spheroidal cells that did not have smooth cytoplasmic membranes were stained by trypan blue and, thus, were identified as severely damaged cells. The proportion of rod-shaped cells decreased to about 10–20% after 7 days of culture.

The rod-shaped cardiomyocytes were $143 \pm 2 \mu m$ (range 69–242 \mu m) long and $28 \pm 1 \mu m$ (range 12–48 \mu m) wide ($n=220$). The thickness of the cells (typically 3–5 \mu m) was measured when they were transferred from the multiwells to the recording chamber. Figure 1 is an example of the light microscopy showing that the rod-shaped cardiomyocytes have clear cytoplasm and cross-striations. The fine structure of the cardiomyocytes was examined with transmission electron microscopy (Figures 2 and 3). Myofibrils and myofilaments were arranged perpendicularly. Mitochondria were of normal shape and size, and their cristae were sharp. Z, M, and A bands were evident; I bands were not clearly visible, presumably because of the contraction of myofibrils. Part of the sarcoplasmic reticulum was dilated. The results indicated that the structure of the myocytes in rod shape was intact or at least within the normal limits of canine heart morphology.10–13

Electrophysiology and Pharmacology

Results described in the following paragraphs were obtained from rod-shaped cardiomyocytes suspended in a culture medium for 2–5 days. It was not possible to transfer the "older" cells from the multiwells to the recording chamber since they were surrounded by nonmuscle cells. The cells

FIGURE 3. High-power electron micrograph of cultured cardiomyocyte. Micrograph was taken on second day of culture. Sharp cristae of mitochondria and glycogen granules are shown. I bands are not clearly visible because of contraction of myofibrils. Scale represents 1 \mu m. Z, Z bands; A, A bands; M, M bands; I, I bands; G, glycogen granules; MT, mitochondria. Magnification, \times 21,000.
FIGURE 4. Resting potential and action potential in cultured ventricular cardiomyocytes. Panel A: Relation between membrane potential (mV in ordinate) and logarithm of potassium concentration (mM in abscissa). Circles and vertical bars denote mean±SEM for numbers of cells tested. A straight line is best fit. Panel B: Sample recordings of values plotted in Panel A, which were obtained from a single cultured cell of fourth day. Superfusate was changed from normal Krebs' solution (resting potential was −75 mV) to a solution containing different concentrations of potassium ions (0–47 mM) for periods indicated by bars. Panel C: Shortening of action potential in potassium-rich (9.4 mM) solution. Upper and lower traces are membrane potential (v) and current injected (i), respectively. Cell membrane was depolarized from −79 mV to −64 mV in potassium-rich solution. Data are from a single cell of second culture day.

were continuously superfused with a normal Krebs' solution after they settled to the bottom of the recording chamber.

Resting and action potential. Resting membrane potential of the cardiomyocytes was −78.3±1.9 mV (range −70 to −92 mV) when the potassium concentration in the superfusate was 4.7 mM (n=60). The time constant of the resting membrane, determined from the final exponential decay phase of the electrotonic potential recorded in response to small (50–100 pA) hyperpolarizing current pulses, was 3.9±0.2 msec (n=10). The action potential evoked by passing brief (3–5 msec) depolarizing current had a peak amplitude of 117.8±2.3 mV (n=32) (Figure 4). The maximum rate of rise of the action potential was 204.7±13.1 V/sec (n=10). Phase 1 repolarization was very small (3–6 mV), and a notch between phase 1 and the plateau could not be clearly detected. 4-Aminopyridine (4-AP) (0.3–1.0 mM) did not significantly affect the rising phase of the action potential and the phase 1 repolarization (n=3). Duration of the action potential at 0 mV and at 90% repolarization level was 129.6±9.8 msec and 250.0±19.8 msec (173.8±11.1 msec for 50% repolarization), respectively (n=32).

In eight cells, resting membrane potential was shifted by 59.4 mV for one-decade changes in potassium concentration (Figure 4A). This implied that the concentration of potassium ions in the cytoplasm was about 100 mM, a finding compatible with concentrations observed in freshly isolated canine ventricular cells.14 In three cells, resting potential was −134.8±3.9 mV when the cells were superfused with Krebs' solution containing no potassium ions (sample tracing in Figure 4B). The action potential was always of shorter duration in a solution containing 9.4 mM potassium than in control (Figure 4C). The maximum rate of rise of the action potential in a solution containing 23.5 mM potassium ions was about one fifth of its respective control (n=3).

Background potassium current. The steady-state I/V curve in all 15 cardiomyocytes tested showed inward rectification at potentials more negative than −80 mV (Figure 5). Membrane conductance between −80 and −120 mV was 20.2±2.3 nS but was 2.1±0.3 nS between −80 and −40 mV (n=13). Slope con-
FIGURE 5. Inward rectification. Cultured cardiomyocyte of second day was clamped at —70 mV and subjected to hyperpolarizing or depolarizing commands (100 msec in duration) in presence of 1 µM TTX. Panel A: Sample recordings in response to four successively increasing hyperpolarizing commands and a depolarizing command. Upper and lower traces are membrane current (i) and membrane potential (v), respectively. Panel B: Steady-state I/V curve obtained from data in Panel A. Amplitude of membrane current at termination of command pulses (ordinate in nA; inward current downward) was plotted as a function of membrane potential (abscissa in mV). Panel C: Slow ramp command (1.5-2 mV/sec) was used to construct steady-state I/V curve in the same cell as in Panels A and B. Curve is indistinguishable from that in Panel B.

ductance at —80 mV was 15.5±0.7 nS (n=3), implying that input resistance was about 65 MΩ at resting potential. The rectification reached a steady state within 5-10 msec of the onset of hyperpolarizing commands and was subsequently independent of time over several hundred milliseconds (Figure 5A). However, small (<10%) outward relaxation on a current trace was observed when the hyperpolarizing commands were larger than 60 mV. Step commands (100-200 msec in duration) and slow ramp commands (1-2 mV/sec) gave essentially the same I/V curve (compare Figure 5B with Figure 5C). The steady-state I/V curve showed crossover in solutions containing different concentrations of potassium ions (n=3) (Figure 6A).

Cesium (0.1-2 mM) blocked the background potassium current (Figure 6B); the steady-state I/V curve at potentials less negative than —90 mV was not affected by 2 mM cesium. The action potential remained unchanged in the presence of 2 mM cesium when it was observed at potentials less negative than —90 mV (n=4). Cesium-induced blockade of the potassium current was concentration dependent and voltage dependent. At low concentrations (<1 mM), cesium-induced blockade of the current was greater at —130 mV than at —100 mV (n=4) (Figure 6B).15,16 The background potassium current was also blocked by 1-3 µM barium (Figure 6B).

It was concluded that the properties of the background potassium current or the I_{Kp}17 in cultured canine ventricular cells are indistinguishable from those that have recently been reported in freshly isolated canine ventricular myocytes14 or freshly isolated ventricular18-21 and atrial22-23 myocytes of other species (see Irisawa5 for review). Delayed rectifier potassium current. Long-lasting (≥200 msec) depolarizing commands from the holding potential between —30 and —40 mV evoked an inward current followed by an outward current. This inward/outward current during the commands was followed by an outward tail current after the termination of the commands, which decayed over the next several hundred milliseconds (Figure 7A). As will be described in the next section, the initial inward current was carried by calcium ions since it was blocked by 2 mM cobalt but not by 1-3 µM TTX. Properties of the outward tail current can be summarized as follows. First, optimal holding potential was between —30 and —40 mV. The minimum duration of the commands was 200 msec. Second, the threshold for the tail current was —20 mV. In other words, command pulses from —45 mV to —40 mV or to —22 mV (Figure 7C) failed to activate the tail current. Third, the peak amplitude of the tail current became larger as the amplitude of the command pulses became larger (Figure 7B). Fourth, the reversal potential of the tail current became more negative than —45 mV (Figure 7C). Finally, the outward tail current was never observed when cesium ions were injected into the cells. Bath application of 2 mM cesium did not affect the tail current. Therefore, it is most likely that the outward
Inward and outward tail currents were observed in the cultured canine cardiomyocytes. The inward current was induced by depolarizing commands from -40 mV to potentials greater than +2 mV (n=4) (Figure 8). The peak amplitude of the inward current increased as the command steps were made more positive. The maximum inward current (940±90 pA) occurred at +9±2 mV (n=3), the clamping current during the commands was net outward (Figure 8). The slow inward current was reversibly blocked by manganese (0.3-1 mM), cobalt (0.3-1 mM), or verapamil (30 µM) (n=2 for each calcium blocker) and, therefore, was identified as a calcium current.

**Inward calcium current.** Slow inward current was evoked by subjecting the cells to depolarizing commands (100-200 msec) greater than 19±2 mV from -40 mV (n=4) (Figure 8). The peak amplitude of the slow inward current increased as the command steps were made more positive. The maximum inward current (940±90 pA) occurred at +9±2 mV (n=3), the clamping current during the commands was net outward (Figure 8). The slow inward current was reversibly blocked by manganese (0.3-1 mM), cobalt (0.3-1 mM), or verapamil (30 µM) (n=2 for each calcium blocker) and, therefore, was identified as a calcium current.

**β-Adrenoceptors.** Isoproterenol (0.1-1 µM) and noradrenaline (1-3 µM) facilitated the inward calcium current (n=5 and 2, respectively) (Figure 9A). Neither the holding current at -40 mV nor the membrane conductance between -55 and -45 mV was affected by isoproterenol (Figure 9A). The effects of isoproterenol on the action potential were examined under the physiological condition in which the cells were impaled with microelectrodes filled with potassium chloride and superfused with normal Krebs’ solution. Propranolol (1 µM) reversed the facilitation of the plateau of the action potential by isoproterenol (1 µM).

The results indicated that intact β-adrenoceptors occur in the cultured cardiomyocytes. It is most likely that adenylate cyclase was also intact since forskolin (10 µM) or caffeine (1-3 mM) took the plateau to higher membrane potential (n=2 for each drug) (not shown).

A small proportion of dispersed myocytes showed spontaneous beating immediately after the dispersion. However, almost all the cells were quiescent after 24 hours. Cultured myocytes began to beat spontaneously when isoproterenol (100 nM-10 µM) was added to the culture medium but not when noradrenaline (up to 300 µM) was added. The beating rate (10-100/min) was dependent on the concentration of the drug applied; a rate of about 50 beats/minute was observed with addition of 1 µM.
isoproterenol. Spontaneous beating indicates that pacemaker currents such as \(i_{K,T-1}\) may have occurred in the presence of isoproterenol. However, such a phenomenon was never encountered when the cells were used for intracellular recordings in the Krebs' solution instead of the culture medium (Figure 9). The reason for this discrepancy under different experimental conditions is unknown at present. We have not tested the effects of isoproterenol on the action potential in the culture medium, which was slightly potassium-rich (7.3 mM). We have applied isoproterenol to the voltage-clamped cells under Cs/Cs-stat-condition at which \(i_{K,T-1}\) has already been eliminated.\(^5,27\)

Cyclic AMP. Another purpose of our experiments was to study the effects of 8-Br-cAMP, with which the cells were cultured. All the experiments were carried out in the cultured cardiomyocytes of the second and third days.

The action potential in all 14 cardiomyocytes suspended in a culture medium without 8-Br-cAMP was characterized by a short-lasting plateau (compare Figure 10A with Figure 10B). The duration of the action potential at 0 mV and at 90% repolarization was 15.1±3.2 msec and 89.3±23.3 msec, respectively. The peak amplitude of the action potential was 117.9±2.0 mV at the resting potential of -80.7±1.7 mV. The maximum rate of rise of the action potential was 211.5±10.5 V/sec (\(n=6\)). In all three cells tested, the action potential could be evoked in the presence of TTX (1 \(\mu M\)) (Figure 10C); threshold for the TTX-sensitive component to the action potential was -48.4±2.3 mV (\(n=14\)). Threshold of the TTX-resistant action potential was about -20 mV, and peak amplitude of the spike graded depending on the magnitude of the current injection. The TTX-resistant action potential was reversibly blocked by manganese (1 mM) (Figure 10C) or cobalt (0.3 mM), implying that an inward calcium current occurs even in those cells having the short-lasting plateau. The short-lasting plateau was greatly augmented by isoproterenol (0.1–1 \(\mu M\)) (Figure 10D).

**Discussion**

The results in the present study demonstrate for the first time that a small amount of ventricular tissue (10–20 mg) obtained by endomyocardial biopsy of the adult canine can be suspended into intact single cells and maintained in culture for 3 weeks.

**Ficoll-Paque**

In our preliminary study, the dispersed cells were centrifuged for 10 minutes at 170–240g without a polysucrose gradient centrifugation technique. This resulted in significant deterioration of the microscopic structure of the cardiomyocytes into mostly round cells without any clear cross-striations.
**Life Span of the Cultured Myocytes**

About 5–10% of myocytes survived for up to 3 weeks until they began to become rounded and finally lose their cross-striation. The consistent observations in these “old” cells were dilation of the sarcoplasmic reticulum, mitochondrial swelling, and lipid droplets. Although intracellular recording from these “old” cells was not practicable, the cells responded to isoproterenol (1 μM) with spontaneous beating. This implies that the essential steps for the excitation-contraction coupling were maintained for 2 to 3 weeks.

**Limitations in the Space Clamp**

A single-electrode voltage clamp did not allow perfect control of the membrane potential during the inward calcium current, especially when the current was evoked near +10 mV (Figure 8). Because of this imperfect space clamp, the maximum amplitude of the current (=1 nA) may have been underestimated significantly even though it was measured only in those cells in which the loss of voltage control during the initial 20–30 msec for 200-msec depolarizing command steps from −40 to +10 mV was less than 10% with respect to the steady level of the command pulses.

**Cyclic AMP and the Calcium Current**

Recent study shows that intracellular application of cAMP or subunits of cAMP-dependent protein kinase facilitates the inward calcium current in both atrial and ventricular cells. Bath application of 8-Br-cAMP also facilitates the calcium current. The results in the present study (Figure 10) led us to speculate that culture of the cells for 2 to 3 days would lead to a depletion of intracellular cAMP and that presence of 8-Br-cAMP (0.1 mM) in the culture medium simply overcame the depletion. In this context, observations by Fischmeister and Hartzell are of some interest. They observed that 2 μM isoproterenol increases the calcium current when the patch-pipette contains 0.1 μM cAMP but decreases the current when the pipette contains 5 μM cAMP. This implies that the intracellular level of cAMP after culture of the cells with 0.1 mM 8-Br-cAMP for 3 days may not exceed that upper limit, because 0.1–1.0 μM isoproterenol still enhances the calcium current (Figure 9).

In conclusion, the present study reports for the first time that the endomyocardial biopsy can be applied for the tissue culture of the adult canine ventricular cells for up to 3 weeks. Cultured ventricular cells appear to be intact in terms of their ultrastructure and their ability to generate normal action potential. The combination of the biopsy and the cell culture we have developed could be useful in studying the functional aspects of cardiac muscles in human heart disease.
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