Electrophysiology and Ultrastructure of Canine Subendocardial Purkinje Cells Isolated From Control and 24-Hour Infarcted Hearts

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Ventricular arrhythmias that accompany myocardial infarction in dogs may be secondary to the altered electrophysiological properties of the subendocardial Purkinje fibers that survive 24 hours after the coronary occlusion. To better understand the ionic mechanisms that underlie the altered electrical activity of these fibers, we have dispersed, using an enzymatic technique, Purkinje cells from the subendocardium of the infarcted ventricle (IZPCs) and compared their electrical and structural properties to Purkinje cells dispersed from fiber strands (SPCs) and from the subendocardium of the noninfarcted ventricle (NZPCs). Ultrastructural analysis of these cells shows that IZPCs contain an increased number of lipid droplets when compared with the SPCs and NZPCs. In addition, transmembrane action potentials of IZPCs have reduced resting potentials, action potential amplitudes, and upstroke velocity and are increased in duration when compared with either SPCs or NZPCs. Input resistance of IZPCs is increased over that measured in control cells (SPCs and NZPCs). Furthermore, the time course of the process of electrical restitution of action potential duration is altered in IZPCs with long action potentials. Finally, using K⁺-sensitive microelectrode techniques, we have determined that intracellular free K⁺ activity (aK⁺) in IZPCs (93.7±15 mM) is not significantly different from control aK⁺ measurements (SPC, 106±13 mM; NZPC, 103±12 mM). Thus a reduction in aK⁺ does not provide a basis for the reduced resting potentials observed in IZPCs. By studying the relation between the resting potential and log [K⁺], we determined that in IZPCs with reduced resting potentials, there is a significant increase in the PNa/PK ratio when compared with control. In summary, to better understand the cellular basis of ventricular arrhythmias postinfarction, we have developed a single cell model that will allow for more rigorous electrophysiological studies of the specific ionic currents that underlie the abnormal electrophysiology. (Circulation Research 1989;65:955-970)

Serious ventricular arrhythmias often occur in humans immediately after coronary artery occlusion and during the "in hospital" phase of patient therapy, as well as for weeks to years after the infarction.1,2 Several hypotheses maintain that the mechanisms of these cardiac arrhythmias can be understood in terms of specific alterations in cellular electrical activity that result from the myocardial infarction.3,4

Much of what is known about the cellular electrophysiological changes that occur during these phases of arrhythmias has been derived from experiments on isolated tissues, either excised at different times after the coronary artery occlusion or taken from normal hearts and superfused with solutions altered in such a way as to mimic conditions in ischemic hearts. Microelectrode studies on the isolated canine myocardium have shown 1) that Purkinje fibers survive on the endocardial surface of infarcts after the muscle has died; 2) that marked changes in the transmembrane action potentials of these surviving subendocardial Purkinje fibers occur at the time when the delayed phase of ventricular arrhythmias occurs; and 3) that the changes in the membrane potentials of these Purkinje fibers could cause arrhythmias.5-7 Specifically, the maximum diastolic potential, action potential amplitude, and maximum rate of depolarization are reduced in Purkinje fibers in the subendocardium of the ventricles 24 hours after the coronary artery occlusion when compared with control subendocardial fibers. The time for complete repolarization of the subendocardial Purkinje fibers in the 24-hour infarct is...
significantly prolonged. Even after prolonged in vitro superfusion these fibers remain significantly abnormal. This suggests that ischemic disease causes chronic changes in the membrane currents that underlie the action potential.

Specific changes in cell membrane properties of the Purkinje fibers surviving in these diseased and arrhythmic canine hearts are unknown. However, one recent study has described the passive membrane properties of these subendocardial Purkinje fibers. Some of the electrophysiological abnormalities of fibers in the multicellular infarct preparation may result from the reduction in viable subendocardial muscle cells immediately beneath the Purkinje fibers. For example, the abnormal prolongation of action potential of Purkinje fiber could result from changes in the cell-to-cell coupling between the surviving subendocardial Purkinje fibers. On the other hand, electrical abnormalities could result from alterations in specific ionic currents important in the depolarization and repolarization process. Voltage clamp studies have not been possible on the multicellular preparations of these subendocardial fibers because the multicellular infarct preparation is not structurally and functionally homogeneous.

Enzymatically dispersed cardiac myocytes are an essential preparation for the study of normal cardiac cell structure and function. As yet, no one has applied this technique to study the pathoelectrophysiology of single Purkinje cells isolated from infarcted hearts. Therefore, we developed a preparation of isolated subendocardial Purkinje cells from the 24-hour infarcted myocardium and then compared this group's general electrophysiological and ultrastructural characteristics with those of Purkinje cells isolated from normal subendocardium and from Purkinje fiber strands.

Materials and Methods

Healthy mongrel dogs (12–15 kg, 1–2 years old and of either sex) were used in these studies. Surgical production of myocardial infarction was done according to the Harris procedure under sodium pentobarbital anesthesia (30 mg/kg i.v.). With this method, the left anterior descending branch of the coronary artery was isolated and then occluded permanently by a two-stage ligation technique. At the time of the second ligature, lidocaine (2 mg/kg) was given intravenously if multiple ventricular beats occurred. The chest was closed, and no further antiarrhythmic agents were administered. Thirty-one dogs (approximately 75%) survived this procedure, recovered from surgery, and were used for cell study 24–26 hours after the occlusion. All veterinary procedures were approved by the Institutional Animal Care and Use Committee.

In addition, 41 dogs that had not undergone any previous surgery served as controls. This larger group was split into two smaller groups. Some hearts were used to prepare single cells from free-running Purkinje fibers (n=19 dogs), while the remaining were used to prepare subendocardial Purkinje cells from the noninfarcted myocardium (n=22 dogs).

Preparation of Cells Disaggregated From Cardiac Purkinje Fibers

At the time of cell disaggregation, each dog was anesthetized with sodium pentobarbital (35 mg/kg i.v.), and the chest was opened through a left thoracotomy. The heart was quickly excised from the chest. The method used for enzymatic disaggregation of canine Purkinje fibers has been modified from that of Sheets et al. For cells dispersed from the free-running Purkinje fiber bundles (single Purkinje cells [SPCs]), strands from both right and left ventricles were carefully dissected in a cool (12° C) zero-extracellular–calcium Krebs' solution. The technique used to disaggregate the subendocardial single Purkinje cells from control hearts (NZPCs) and from infarcted hearts (IZPCs) was the same except for the initial dissection. In both latter cases, small strips (4x2x2 mm) of endocardium containing longitudinally oriented Purkinje fiber bundles were carefully dissected from larger preparations that were removed from specific regions in the control heart and in the infarcted heart. Identification of the specific endocardial regions was done very carefully with a dissection microscope. All small strips prepared from the infarcted hearts were taken from the infarcted portion of the endocardial sections. The infarcted sections were identified grossly by the pale color (see Figure 1 of Friedman et al). After preparation of eight to 10 of these ministrips, the fiber bundles were subjected to the following general method for enzymatic disaggregation:

The Purkinje cells in fiber strands or ministrips were transferred to a 60-mm Petri dish containing 5 ml standard Hanks' balanced salt solution (minimum essential medium concentrated with amino acids and vitamins; GIBCO, Grand Island, New York), 5 mg/ml collagenase (Worthington Type II, 244 units/mg, Worthington Chemicals, Freehold, New Jersey), and 5 mM HEPES buffer (pH adjusted to 6.7 with 1N NaOH) (Ca++ content, 50–70 μM). The fibers were then cut with a razor blade into 2–3-mm lengths. The Petri dish was placed in a gyrator shaker bath with humidified 100% O₂ flowing over the dish. The bath was agitated (2–3 cps) for 40–60 minutes. Fibers were then washed twice in a high K⁺–saline solution (160 mM potassium glutamate, 5 mM HEPES buffer, 5.7 mM MgSO₄, pH adjusted to 6.7; Ca++ content, 50–70 μM) and then placed in the shaker bath (37° C) and allowed to recover for 10–15 minutes. Finally, individual cells were dispersed by gentle hand pipetting 20–60 times. The supernatant was carefully collected and centrifuged (Dynac II, RCF=71) for 40–60 seconds. The "loose" pellet was resuspended in 1–2 ml standard Hanks' solution without collagenase but containing 0.5 mM CaCl₂ (pH adjusted to 7.3).
Recording From Cells Disaggregated From Purkinje Fibers

Most single cell studies can be faulted because a process of selection is continuous and integral to experiments. Our experiments are no exception. Selection occurs immediately. After enzyme incubation and the dispersion protocol, viable SPCs and NZPCs were routinely obtained. The yield of SPCs was approximately 87,000 cells per heart, and 40% were calcium tolerant. For NZPCs and IZPCs, the yield depended on the number of minipreparations obtained in any one heart, and 37% of isolated cells were calcium tolerant. SPCs and NZPCs selected for electrophysiological study were those that under the light microscope exhibited morphology described for "healthy" Purkinje cells. That is, when viewed by light microscopy, the surface membranes of healthy cells appeared to be smooth and free of connective tissue. Blebs or disruptions of cell membrane were considered signs of cell injury, presumably secondary to the isolation procedure. Unlike suspensions of SPCs, cell suspensions of NZPCs included ventricular cells. The identification of healthy NZPCs was based on our studies of the SPCs; that is, Purkinje cells were rod-shaped and large in width (w) and length (l). (SPCs w=32±11 μm, l=125±26 μm, n=45; NZPCs w=34±11 μm, l=117±28 μm, n=64), possessed fine fingerlike processes at either end, and did not possess blebs. Electron microscopic studies of NZPCs confirmed that the healthy cells surviving the disaggregation procedures had structural characteristics of Purkinje cells and characteristics similar to SPCs (see "Results").

Cell suspensions from the subendocardium of infarcted myocardium included IZPCs and ventricular cells. However, often the ventricular cells appear as "ghosts," which is understandable since histological studies of ventricular muscle in the 24-hour infarct show that most ventricular muscle cells are dead. Furthermore, IZPCs were large (w=34±11 μm, l=106±32 μm, n=47), mostly rod-shaped cells, and under the light microscope, some cells had slightly corrugated membranes and appeared to be filled with "black" dots. Electron microscopic studies of IZPCs confirmed that these cells were Purkinje cells (see "Results").

For single microelectrode recordings of membrane potentials from single cells, a drop of the suspension of the disaggregated Purkinje cells was placed in a glass-bottomed superfusion chamber (volume, 0.5 ml) mounted on a Nikon inverted microscope stage. Cells were allowed to settle, and adherence to the bottom of the chamber was enhanced by placing the cells on a polylysine-coated (1 μg/ml) coverslip. Typically, cells were allowed to equilibrate in a normal Tyrode's solution (see Friedman et al for composition) (4 mM K+, 3 mM Ca2+) (bath flow rate = 5 ml/min). Temperature was continuously monitored (Bailey Instru-
mounted on a copper grid mesh and stained sequentially with saturated (7.7%) alcoholic uranyl acetate solution for 15 minutes followed by lead citrate stain for 10 minutes. Thin sections were examined using a Philips 300 electron microscope.

Up to four blocks per suspension were examined and photographed from the different preparations. The number of lipid droplets observed in each cell was determined for several cells in each group. The percentage of Purkinje cells examined that contained more than 15 lipid droplets per cell was determined for each experimental group. Fifteen lipid droplets per cell were chosen since the largest number of droplets counted in any one cell disaggregated from a free-running Purkinje fiber (a SPC) was 15 (total number of cells observed was 28).

"Steady-State" Membrane Properties

Input resistance of cells in each group was determined from the slope of the linear part of a "steady-state" current-voltage curve measured in normal Tyrode's solution (4 mM [K+]o). These measurements were made over a 5-10-mV range around the resting potential of the cell. Steady-state current-voltage curves were obtained in a single cell by applying a slowly depolarizing command ramp (1 mV/0.67 sec) from -120 mV to 20 mV using discontinuous single microelectrode voltage clamp (Pclamp, Axon Instruments). Microelectrodes used for these studies were filled with 3 M KC1 and had tip resistances of 30-50 MΩ. Switching frequency of the chop clamp was 3-4 kHz. In addition, the waveform of the voltage signal was monitored to ensure that the phase and sampling rate were set appropriately to allow for complete settling of the voltage before the current-injection phase.

[K+]o Studies

We determined the effects of changes in extracellular K+ ion concentration on the resting potential of SPCs, NZPCs, and IZPCs. Single cardiac cells are ideal preparations for accurately determining the effects of [K+]o on membrane potential since the resting potential (RP) is not an average of potentials of neighboring electrically coupled cells. Furthermore, single IZPCs are very appropriate for these studies since by removing the IZPC from the heterogeneous syncytium of the infarct we can accurately determine the resting potential of the cell. In addition, by carefully placing an extracellular K+ ion sensitive microelectrode (K-ISE) adjacent to the cell membrane from which the RP was being recorded, we precisely determined the [K+]o over the range of 1 to 100 mM during the solution changes. Measurement of RP was made only after equilibration of bath at each new [K+]o level. Then, for each cell we determined the relation between RP and log [K+]o, and fit data for [K+]o > 20 mM according to the Goldman12 and Hodgkin and Katz13 equation by varying [K+]i. In this way, we indirectly determined [K+]i for each specific cell. Using this

[K+]i value, a specific α = PNa/PK value was determined for that cell at the resting potential in 4 mM [K+]o using [K+]i + [Na+]i = 155 mM and assuming [Na+]i = 9 mM. Because the term (PNa/PK)[Na+]i would change the apparent [K+]i by less than 1 mM, our error in using this estimation of [Na+]i would not significantly alter the calculated α values.

 Intracellular K+ Determinations

In these studies, we measured intracellular free K+ activity (aK+) of cells from each group, SPC, NZPC, or IZPC. This required the insertion of two microelectrodes into the quiescent Purkinje cell (4 mM [K+]o). One electrode was the 3 M KC1 conventional microelectrode and measured the resting transmembrane potential of the cell. The other was a single barrel intracellular K-ISE. K-ISEs were made from the same micropipettes used to measure RP. These micropipettes were silanized with trimethylchlorosilane (Eastman Kodak, Rochester, New York) and then injected with a drop of Corning K+ liquid ion exchanger (WPI 477312, Corning, New Haven, Connecticut) and left overnight to allow the liquid ion exchanger to fill the micropipette tip. After backfilling the pipette with 160 mM KCl, K-ISEs were calibrated by the fixed-interference method (constant total Na+ and K+) before and after each impalement.14 The K-ISE electrode was connected via a Ag-AgCl wire to a 10^4-Ω impedance electrometer (Bloom Associates, Narberth, Pennsylvania). Intracellular K+ activity was calculated as follows:

\[ a_{K^+} = 10^{-k_{K^+}} \left( a_K^0 + k_{KNa} \left( a_{Na^+}^0 \right) \right) \]

where S is the electrode slope, V_K is the K-ISE potential minus the conventional microelectrode potential, a_K^0 is the extracellular Na+ activity, and kKNa is the K-ISE's selectivity coefficient for Na+.15 kKNa values ranged from 0.0103 to 0.0571 (x = 0.028 ± 0.015, n = 22). Mean K-ISE slope value (S) at 37°C was 60.3 ± 2.9 mV (range, 53.3-65.4 mV).

In general, the insertion of the second microelectrode into a cell caused membrane depolarization (to approximately -20 to -40 mV). Therefore, all values recorded and used to determine directly aK+ in a cell were made within several minutes of the second impalement so that error from diffusion of potassium chloride from the conventional microelectrode would be insignificant.16 Intracellular free K+ concentration was calculated from aK+ assuming a K+ activity coefficient of 0.74.17

Stimulation Protocols

Preliminary results indicated that cells that are dissociated from endocardium overlapping the infarct (IZPCs) have abnormally long action potentials. Therefore, to begin to understand the possible underlying causes for the abnormal repolarization of IZPCs, we determined the effects of different stimulation protocols on the action potential duration of
the NZPCs and IZPCs and compared the results with our previously published observations on SPCs.10

First, we assessed the effects of altering stimulation frequency on time course of repolarization at a steady state. For this protocol, repetitive stimuli were applied at constant cycle lengths ranging from 500 to 2,000 msec. Action potential duration at 50% and 100% repolarization at each cycle length at steady state (at 2–3 minutes) were determined and results within groups compared.

In addition, we determined the interval-duration relations for SPC, NZPC, and IZPC cells. In these experiments, an extrastimulus was interposed at variable S1-S2 intervals during the diastolic interval after every tenth basic drive stimulus (S1-S2=500 msec).10 The duration of the S2 action potential was measured at -60 mV level.18-19 The duration of the S2 action potential was then plotted as a function of the S1-S2 interval. The time course of change in action potential duration with this electrical restitution protocol was determined to be an exponential process. Fit of data to a single or double exponential curve was done using a simplex algorithm20 on an IBM-AT. Determination of best fit was done by analysis of mean residual squares data.

Data Analysis

All mean data are presented as mean±SD unless otherwise noted. Samples from each population were tested for normality of distribution using the Wilk-Shapiro and Kolmogorov-Smirnov tests. If a sample was shown statistically to be not normally distributed then a nonparametric test, the Mann-Whitney test, was used to compare samples. Parametric tests were used when comparing two normally distributed samples. In these cases, a statistical difference was evaluated first by analysis of variance test on the groups of data. Then, if appropriate, either an unpaired Student’s t test or a modified Student’s t test for samples with unequal variances was used.21 Differences with p<0.05 were considered significant. When making multiple comparisons, the significance level was corrected using the Bonferroni method.

Results

Structure of Cell Suspensions

Ventricular myocytes can occur in the suspension of NZPCs, but they usually do not survive the disaggregation procedure and appear as rounded cells. On the other hand, ventricular myocytes can exist in the IZPC suspension, but they appear as ghosts. That is, under modulation contrast optics, the ventricular cell membranes are not refractive and striations are not clearly identifiable, which is understandable since histological studies of ventricular muscle in the 24-hour infarct show that most endocardial ventricular fibers are dead.2 In contrast, at this level of magnification the membranes of the SPCs or NZPCs are smooth and refractive, and myofibrils are visible as striations. The membrane of an IZPC is also refractive and often seems to be covered by many small bumps that are not typical cellular blebs.22 This gives the membrane a ruffled appearance. Finally, all three types of Purkinje cells possess fingerlike projections at their ends, unlike the ventricular myocyte, which has a characteristic staircase configuration at its ends.

Using ultrastructural techniques, we determined that the subendocardial cells that survived the disaggregation procedure were intact Purkinje cells. Furthermore, IZPCs had the same structural characteristics as defined for subendocardial Purkinje fibers that survived in the infarcted regions of multicellular preparations.6 Electron micrographs illustrating these points about the fine structure are shown in Figures 1–3. Figures 1 and 2 illustrate a typical SPC and a NZPC. Cells from noninfarcted hearts displayed all the hallmarks of normal cell structure previously defined for the Purkinje cell in a multicellular preparation.6,23 Namely, SPCs and NZPCs contain extensive pools of glycogen, longitudinally oriented myofilaments, an absence of T tubules and a few lipid droplets. A small percentage of cells in the SPC (4%, total number of cells was 28) or the NZPC (12%, total n=67) suspensions contained more than 15 lipid droplets per cell (range, 15–30 droplets per cell). The sarcolemma of these cells is intact, and the basement membrane is not present at all sites around the cells. A portion of a cell-to-cell junction remains attached to a cell (Figure 1), and the fingerlike processes at the end of the cell are evident. On the other hand, although the IZPC has several distinguishing hallmarks of a normal Purkinje cell, it also contains an unusual number of lipid droplets (up to 10 times the number that occur in SPCs or NZPCs) (Figure 3). Thirty-eight percent of the IZPCs (n=106) contained more than 15 lipid droplets per cell (range, 15 to more than 70 droplets per cell). This is in agreement with an earlier study describing the ultrastructure of subendocardial Purkinje fibers in the 24-hour infarct.6

Electrophysiology of SPC, NZPC, and IZPCs

Representative transmembrane potentials from an SPC, an NZPC, and IZPCs are shown in Figure 4. The time course of the action potentials of IZPCs (panels c, d, and e) was heterogeneous. For instance, in some IZPCs, the action potentials lack a discrete plateau phase (panels c and d) while in others, small oscillations in potential occurred during the plateau phase. In all cases, the time course of repolarization was markedly prolonged (see below). RPs recorded from all cell types were compared. There was a slight decrease in mean RP of NZPCs (−85.0±4.0, n=85) when compared with the Purkinje cells dissociated from the free-running strands (SPCs) (−87.6±6.0 mV, n=44) (p=0.03). In addition, there was a significant decrease in mean resting potential of IZPCs (−75.5±11.0 mV, n=94) when compared
FIGURE 1. Electron micrograph illustrating a typical single Purkinje cell isolated from a canine Purkinje fiber strand (SPC in text). Note the extensive pools of glycogen granules (g), longitudinally oriented myofilaments (Mf), and the absence of T-tubules. Mitochondria (m) are intact, as is the sarcolemma (curved arrows). A portion of the junction remains attached to a cell fragment (large arrowhead). Few lipid droplets are present. Magnification, x7,820.

with NZPCs ($p=0.0001$, Mann-Whitney test). Resting potentials of IZPCs were also significantly reduced when compared with SPC data ($p=0.0001$, Mann-Whitney). A comparison of the frequency distribution graph of RP values for SPCs, NZPCs, and IZPCs is shown in Figure 5. Cells in the IZPC group have been divided into two groups depending on whether the RP is positive or negative to $-80$ mV. Forty-five percent of IZPCs had RPs greater than or equal to $-80$ mV (this is greater than one SD from the control mean value) while 55% of the cells had resting potentials less than $-80$ mV. IZPCs had proportionately more depolarized cells than NZPCs. Ninety-two percent of NZPCs had RPs more negative than or equal to $-80$ mV but only 8% had RPs less negative than $-80$ mV.

Resting potential data were collected for all cells. Then, subsets of these cells were used in at least one of the following protocols. Some cells in each group were stimulated at a basic cycle length (BCL) of 800 msec, and mean values of action potential parameters were compared. Mean resting potential and action potential amplitude (APA) of NZPCs were slightly reduced relative to the SPC values ($p<0.05$). In IZPCs, mean RP, APA, and upstroke velocity ($V_{\text{max}}$) were further reduced when compared with either control value (Table 1); however, comparison of the IZPC mean $V_{\text{max}}$ with the control value did not achieve statistical significance. Seventeen percent of the IZPCs had $V_{\text{max}}$ less than 40 V/sec.

To determine whether the refractoriness and/or restitution of excitability in the IZPCs was different
than control, action potential durations at 50% and 100% repolarization (APD<sub>50</sub> and APD<sub>100</sub>) of NZPCs and IZPCs were measured, and the mean values were compared (Table 2). NZPCs and IZPCs were selected in which the mean RPs were similar (−81.0±7.0 mV vs. −84.0±13.0 mV, NS) to exclude any voltage-dependent effect that might cause differences in the repolarization process between NZPCs and IZPCs.

Mean APD<sub>50</sub> of IZPCs was longer than APD<sub>50</sub> of NZPC at all BCLs except 500 msec; however, these differences were not significant (0.05<p<0.10). Mean APD<sub>100</sub> of IZPCs was longer than mean APD<sub>100</sub> of NZPCs at all BCLs studied (p<0.01.
except at BCL=1,500 msec). Interestingly, in NZPCs, the relative changes in APD_{90} and APD_{100} as a function of BCL were similar. In contrast, in IZPCs, the relative change in APD_{90} as a function of BCL was greater than the relative change in APD_{100} (Figure 6). APD_{100} of both cell types responded to a decrease in BCL with a decrease in APD_{100} (↓17% NZPC, ↓21% IZPC). However,
most changes in APD_{100} occurred between the 800- and 500-msec BCL for IZPCs, but the APD_{100} changes of NZPCs were more gradual as a function of cycle length.

**APD Changes During Electrical Restitution**

It was apparent that cells in both groups show a rate-dependent change in electrical activity at the steady state. At the rapid drive cycle length (500 msec), APD_{50} of IZPCs was not significantly different from APD_{50} of NZPCs. However, since APD_{100} of the IZPCs was prolonged compared with control at this rapid drive rate, we investigated whether there were differences in the time course of the change in the APD_{100} that accompanies the electrical restitution process in NZPCs and IZPCs. Results were then compared with our published data on SPCs.10

The changes in action potential duration accompanying the electrical restitution process in NZPCs (mean APD_{100} = 244 ± 41 msec, n = 5) were similar to those previously described for SPCs (APD_{100} = 240 ± 66 msec, n = 7) (see also Robinson et al10). In NZPCs, the time course of this process was best fit using a double-exponential equation where the fast component of the process was f_1 = 270 ± 134 msec (p<0.05 vs. SPC where f_1 = 195 ± 41 msec). On the

<table>
<thead>
<tr>
<th>Table 1. Transmembrane Action Potential Mean Values</th>
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<td>---------</td>
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<tr>
<td>n</td>
</tr>
<tr>
<td>SPC</td>
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<td>NZPC</td>
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<td>IZPC</td>
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RP, resting potential; APA, total action potential amplitude; V_{max}, maximum rate of depolarization of phase 0; n, number of cells; SPC, cells dissociated from Purkinje fiber strands; NZPC, Purkinje cells dissociated from subendocardium of noninfarcted ventricle; IZPC, Purkinje cells dissociated from subendocardium of infarcted ventricle.

*p<0.005 vs. SPC value.

†p<0.001 vs. SPC value.

‡p<0.02 vs. NZPC value.
TABLE 2. Action Potential Duration at Different Basic Drive Cycle Lengths

<table>
<thead>
<tr>
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<th>2,000</th>
<th>1,500</th>
<th>1,000</th>
<th>800</th>
<th>600</th>
<th>500</th>
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<tbody>
<tr>
<td>NZPC</td>
<td>174±56(11)</td>
<td>172±50(7)</td>
<td>150±25(21)</td>
<td>155±29(21)</td>
<td>146±21(14)</td>
<td>148±17(14)</td>
</tr>
<tr>
<td>IZPC</td>
<td>199±59(12)</td>
<td>223±57(8)</td>
<td>175±65(19)</td>
<td>177±54(25)</td>
<td>168±28(11)</td>
<td>140±43(10)</td>
</tr>
</tbody>
</table>

BCL, basic cycle length; APD_50 and APD_100, durations at 50% and 100% full repolarization in milliseconds; NZPC, Purkinje cells dissociated from subendocardium of noninfarcted ventricle; IZPC, Purkinje cells dissociated from subendocardium of infarcted ventricle. Mean±SD are shown. Numbers in parentheses indicate the number of cells.

*IZPC values are different from NZPC values as judged by Mann-Whitney test.

\[^{\text{1}}p<0.001.\]

other hand, in a group of IZPCs where mean APD_50_{mV}=261±57 msec (n=5, p>0.05 vs. NZPC value), the time course of the restitution process was altered (Table 3). In three of five IZPCs (APD_50_{mV}=295±35 msec), a single-exponential function best described the time course of the restitution process (T=473±61 msec, p<0.05 vs. NZPCs), and the extra parameters of a double-exponential curve did not improve the fit. The electrical restitution process in the other two IZPCs (APD_50_{mV}=210±42 msec) was similar to that seen in NZPCs (T=229 and 260 msec for these two IZPCs). A comparison of the restitution process in an NZPC and an IZPC is illustrated in Figure 7. The diastolic interval needed for the action potential (-60 mV) to attain 90% of its maximum steady-state APD value is shorter for this IZPC than for the NZPC. The time course of the process in this illustrated example from an NZPC.

![Figure 6](http://circres.ahajournals.org/)
TABLE 3. Action Potential Duration Restitution in NZPCs and IZPCs

<table>
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<tr>
<th>Cell</th>
<th>APDsi (msec)</th>
<th>r1 (msec)</th>
<th>r2 (msec)</th>
<th>A2/A1</th>
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<td>NZPCs</td>
<td>190</td>
<td>117</td>
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</tr>
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<td>2</td>
<td>280</td>
<td>279</td>
<td>4×10⁶</td>
<td>&lt;0.10</td>
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<td>3</td>
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<td>2,000</td>
<td>0.35</td>
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<tr>
<td>5</td>
<td>290</td>
<td>440</td>
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<td>0.53</td>
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<td>X = 244±41</td>
<td>270±134</td>
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<td>180</td>
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<td>5</td>
<td>310</td>
<td>469*</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>X = 261±57</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

APDsi, APD at -60 mV at steady state (basic cycle length, 500 msec); r1 and r2, time constants of recovery of APD_{-60mV} of S2 action potential; A2/A1, ratio of coefficients of the time constants; NZPCs, Purkinje cells dissociated from the subendocardium of noninfarcted ventricle; IZPCs, Purkinje cells dissociated from the subendocardium of infarcted ventricle.

*Single exponential provides best fit of data.

can be represented best by two time constants (τ1 = 157 msec, τ2 = 5,031 msec). On the other hand, for this IZPC, a single time constant (τ = 469 msec) best described the data. Note that even for short S1-S2 intervals, the APD_{-60mV} of the S2 in this IZPC is longer than the APD_{-60mV} of the NZPC (Figure 7).

In conclusion, Purkinje cells disaggregated from the subendocardium overlying the 24-hour infarct (IZPCs) have abnormal transmembrane potentials compared with the potentials of SPCs and NZPCs. IZPCs have reduced resting potentials, total action potential amplitudes, and V_{max}. The action potential duration of IZPCs is abnormally prolonged compared with NZPCs. In addition, the time course of electrical restitution of action potential duration in the IZPCs with abnormally long action potentials is altered.

"Steady-State" Current-Voltage Relations

The "steady-state" total membrane current-voltage relations of several SPCs (n=7), NZPCs (n=4), and IZPCs (n=9) were obtained using voltage clamp. A slowly changing voltage ramp was applied to a cell. This was repeated while the cell was bathed in different [K+]o solutions. In control cells and most IZPCs (66%), the current-voltage relations exhibited a region of inward rectification. The negative slope region was between -76 and -50 mV in control cells and between -74 and -30 mV in IZPCs. In the three IZPCs that showed no region of negative slope, the current-voltage relation was essentially flat over a range of 10 to 25 mV (Figure 8B). At the most negative resting potential (zero current level), the input resistance of the membrane/cell of SPCs (89±38 MΩ, n=10, Rp=-84±3 mV) was similar to that in NZPCs (92±49 MΩ, n=21, Rp=-84±3 mV) but it exists in 8 and 16 mM [K+]o. The inset shows the transmembrane potential of this Purkinje cell dissociated from infarcted ventricle in 4 mM [K+]o. The small black line is the zero potential. Vertical and horizontal calibration bars are 20 mV and 100 msec, respectively.
TABLE 4. Direct Determination of $P_{Na}/P_{K}$ in Cells of Each Group Using the Indirectly Determined [K$^+$]i Value

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>RP (mV)</th>
<th>$E_K$ (mV)</th>
<th>$P_{Na}/P_{K}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPC</td>
<td>10</td>
<td>-86±4</td>
<td>-97±3</td>
<td>0.014±0.006</td>
</tr>
<tr>
<td>NZPC</td>
<td>9</td>
<td>-82±6</td>
<td>-96±1.9</td>
<td>0.017±0.007</td>
</tr>
<tr>
<td>IZPC</td>
<td>9</td>
<td>-76.4±7*</td>
<td>-97±1.4</td>
<td>0.035±0.019†</td>
</tr>
</tbody>
</table>

$n$, number of cells; RP, resting potential of cell (4 mM [K$^+$]), $E_K$, K$^+$ equilibrium potential directly calculated based on indirect estimation of [K$^+$] (see "Materials and Methods"); $P_{Na}/P_{K}$ ratio, [K$^+$] - [K$^+$] (antilog $V_m$/-61.5)/[Na$^+$] (antilog $V_m$/-61.5)-[Na$^+$]; SPC, Purkinje fiber strand; NZPC, Purkinje cell dissociated from noninfarcted ventricle; IZPC, Purkinje cell dissociated from infarcted ventricle.

*p<0.001 vs. SPC or NZPC value.
†p<0.005 vs. SPC or NZPC value.

Resting Potential

To determine whether a reduction in [K$^+$] formed the ionic basis for the reduced resting potential in IZPCs, we did two types of experiments. In the first series, we determined the relation between RP and log [K$^+$], and then fit the data for [K$^+$] greater than 20 mM according to the Goldman equation by varying $E_K$ of NZPCs and SPCs (p>0.05). In this calculation, we indirectly calculated [K$^+$] at RP=0 mV for each specific cell (SPC, NZPC [K$^+$] range, 129-151 mM; IZPC range, 140-170 mM). For SPCs, NZPCs, and IZPCs the calculated $E_K$ values were not different (Table 4). Assuming [K$^+$] in a cell at RP=0 mV and at RP for [K$^+$]=4 mM are the same, we then calculated the $P_{Na}/P_{K}$ for that cell in 4 mM [K$^+$]. For NZPCs (mean RP= -82±6 mV, $n=9$), the calculated $P_{Na}/P_{K}$ value was 0.035±0.019 (range, 0.014-0.069). In IZPCs where mean RP= -76±7 mV ($n=9$), the calculated $P_{Na}/P_{K}$ value was significantly different ($p<0.005$) than the mean value in SPCs (0.014±0.006, $n=10$) or NZPCs. These data suggest that IZPCs with reduced resting potentials have a larger $P_{Na}/P_{K}$ ratio than NZPCs or SPCs (Figure 9). An increase in $P_{Na}/P_{K}$ could be due to an increase in $P_{Na}$ and/or a decrease in $P_{K}$ in IZPCs.

We also directly measured intracellular K$^+$ activity in a single Purkinje cell using an intracellular K-ISE as shown in Figure 10. Experimental results are presented in Table 5. Mean $a_K$ levels in SPCs ($n=8$) and NZPCs ($n=8$) are slightly greater than $a_K$ in IZPCs ($n=13$), but the difference is not significant. We therefore conclude that the $E_K$ of IZPCs was similar to $E_K$ of NZPCs and SPCs ($p>0.05$). In two cells, $E_K$ was determined by both the direct and indirect methods to see if both would yield similar $E_K$ values. First, a log [K$^+$] versus resting potential experiment was completed as described above. Then, a calibrated K-ISE was inserted into the same cell to permit a direct determination of $a_K$.

The estimated [K$^+$] values (167 and 166 mM in 4 mM [K$^+$]) were nearly the same as the directly measured values of [K$^+$] (163 and 162 mM in 4 mM [K$^+$]) for these two cells.

Discussion

The successful development of a preparation of isolated Purkinje cells from the subendocardium of the 24-hour infarcted myocardium (IZPCs) now provides an important model for the study of the pathoelectrophysiological changes in cells that survive myocardial infarction and cause arrhythmias. These cells have reduced RPs, APA, and $V_m$ when compared with control. In addition, the time course of repolarization and the process of restitution of action potential duration are abnormal.

Although it is difficult to compare precisely single-cell data with data from multicellular preparations, we must discuss our results in terms of published reports of similar electrophysiological studies on large multicellular preparations.5,8,24-25 In addition in this latter study,5 pooled data from over 250 cells were used to establish a mean value for each action potential variable. This is far in excess of our sample size. Nevertheless, when directly comparing these published results with ours, we can see the following:

1. Purkinje cells dissociated from the subendocardium of the normal heart (NZPCs), cells isolated from fiber strands (SPCs), and subendocardial Purkinje fibers of the multicellular preparation6 have similar ultrastructural characteristics.
2. There is a major ultrastructural change in Purkinje cells enzymatically dissociated from the subendocardium of the infarcted myocardium (IZPCs), namely, an accumulation of lipid droplets. Lipid droplet accumulation has been observed in...
the subendocardial Purkinje fibers in the multicellular infarcted preparation. The relation between cellular lipid droplet accumulation and abnormal cell electrophysiology has not been established. However, the lipid droplets are presumably secondary to the increased concentration of lipids in the plasma, which is known to occur during the altered metabolism associated with ischemia. Importantly, the dissociation procedure did not alter the characteristic presence of lipid droplets in the subendocardial Purkinje cells 24 hours after the coronary artery occlusion.

3. Mean RP and APA in NZPCs and SPCs were slightly different. Values of RP and APA in the multicellular Purkinje fiber strands and adult subendocardial Purkinje fibers show a similar difference.

Mean RP and APA in NZPC and IZPC were similar to values obtained in the multicellular preparations. Due to the time (2 hours) required to isolate single cells, it is appropriate to compare the mean IZPC data in the present study only with data collected from fibers in the multicellular preparation more than 2 hours after the start of superfusion. The RP, EK, and APA values of Purkinje fibers in the infarcted myocardium have a clear dependence on time after dissection and start of tissue bath superfusion.

4. We found that the Vmax values of Purkinje cells isolated from fiber strands (SPCs) were lower than published values for the multicellular Purkinje fiber (compare Robinson et al10 with Rosen et al28). Generally, Vmax values of NZPC were lower than Vmax of subendocardial fibers in multicellular preparations; however, note that the mean Vmax of IZPCs was similar to the mean Vmax of cells in the multicellular infarct preparation.

5. Transmembrane action potentials of NZPCs and IZPCs were prolonged (APD100) compared with the APD100 data from the multicellular data for subendocardial Purkinje fibers from infarcted and noninfarcted hearts. This finding was expected because Purkinje cells in tissue preparations are electrically coupled. This coupling can lead to interactions that change the measured properties of a cell within the syncytium. Furthermore, data from experimental and computer simulation studies also predict that action potential duration and the maximum rate of rise of the action potential will be greatly influenced by cell coupling.

6. At a BCL of 800 msec, APD90 of IZPCs was not significantly different from APD90 of NZPCs. At other drive cycle lengths (e.g., BCL=2,000 msec), APD90 of IZPCs is longer than NZPCs, yet at the shortest BCL (500 msec) APD90 of IZPC is shorter.
than ADP<sub>20</sub> in NZPCs. Comparable multicellular tissue data are not available for the various BCL.

There is a BCL-dependent action potential shortening in NZPCs, and it is similar to that observed for SPCs. The percent shortening in ADP<sub>20</sub> and ADP<sub>50</sub> in IZPCs is different from that of control. Significant BCL-dependent shortening of APD has also been observed for subendocardial Purkinje fibers in the multicellular control and infarcted preparations (K.P. Dresdner, unpublished observations). Thus, both the single and multicellular preparations of IZPCs show a BCL-dependent change in APD. The difference in the response of the APD to an increased heart rate in NZPCs versus IZPCs has important implications in the mechanisms of arrhythmias in the 24-hour infarcted heart. For instance these results might predict that there would be an enhanced disparity of action potential repolarization time course and hence refractoriness between the normal and infarcted myocardium during increased heart rates.

7. The electrical restitution process of action potential duration in NZPCs is similar to our published data for SPCs. For IZPCs with abnormally long action potentials, the time constant of recovery of action potential duration was altered. Two time constants best describe the process of electrical restitution in control cells, but the time course is described best by only a single time constant in IZPCs with long action potentials. This suggests that prolonged ischemia may have altered one process crucial to the restitution of the action potential duration. The background Na<sup>+</sup> current contributes to the electrical restitution process in normal Purkinje fiber strands since blockers of the inward rectifying current, tetrodotoxin and lidocaine, increase the fast time constant of this restitution process. On the other hand, studies show that lidocaine and tetrodotoxin only slightly shorten the APD of subendocardial fibers that survive infarction. This would suggest that action potential repolarization and APD restitution of IZPCs are not influenced by a background Na<sup>+</sup> current.

We found that the reduced resting potential of the IZPCs is not likely to be due to a direct change in a<sub>K</sub> (or E<sub>K</sub>) but is associated with an increase in the P<sub>N</sub><sub>Na</sub>/P<sub>K</sub> ratio. a<sub>K</sub> was determined by two experimental methods. Both methods found a<sub>K</sub> in SPCs and NZPCs to be similar to values reported for multicellular Purkinje fiber strands and control noninfarcted subendocardial fibers (112±19.8 mM=8<sub>K</sub>). This suggests then that although direct measurements of a<sub>K</sub> were made in cells after two microelectrode impalements (and depolarization to the second level of resting potential), large K<sup>+</sup> losses either due to the microelectrode impalements or due to the disaggregation procedure did not occur to alter the level of measured intracellular K<sup>+</sup>.

We found that a<sub>K</sub> was not significantly different in IZPCs when compared with NZPCs or SPCs. This observation is not necessarily contradictory to the results of Dresdner et al. In this latter study, both the maximum diastolic potential and the intracellular K<sup>+</sup> ion concentration of the subendocardial Purkinje fibers in the 24-hour infarcted preparation were significantly reduced during the first several hours of superfusion in the tissue bath. However, after the fourth hour of superfusion, intracellular K<sup>+</sup> activity in these fibers was no longer significantly different from control, yet maximum diastolic potential was still reduced. Nevertheless, even though intracellular K<sup>+</sup> returns to control levels, abnormalities in the transmembrane action potential exist in both the multicellular and the single cell preparations of the subendocardial Purkinje fibers that survive in infarcted hearts.

We conclude that IZPCs have an increased P<sub>N</sub><sub>Na</sub>/P<sub>K</sub> ratio (to a value of 0.069), which is almost one order of magnitude greater than that value measured in Purkinje fiber strands. This large α value in IZPCs could be due to an increase in P<sub>Na</sub> or a decrease in P<sub>K</sub> or both. Input resistance measurements of SPC, NZPC, and IZPCs indicate that there is an increase in input resistance in the IZPCs. An increase in input resistance (by 46%) has recently been described in a multicellular study of the passive membrane properties of canine subendocardial Purkinje fibers of the 24-hour infarcted myocardium. These authors suggested that the altered input resistance might be caused if cells became uncoupled in the multicellular Purkinje network after infarction or if there was an accumulation of a lysophosphatidylcholine type material in Purkinje membranes 24 hours after infarction. Voltage clamp studies have shown that low concentrations of exogenously applied lysophosphatidylcholine decrease membrane conductance in sheep Purkinje strands.

We suggest that a portion of the measured change in input resistance in the multicellular infarcted preparation may have been due to cell uncoupling, but since our results show that input resistance changes still exist in the isolated IZPC, we suggest that total membrane conductance has decreased in Purkinje cells surviving 24 hours after coronary occlusion. Based on our measured P<sub>N</sub><sub>Na</sub>/P<sub>K</sub> values, we suggest that the altered P<sub>N</sub><sub>Na</sub>/P<sub>K</sub> ratio in IZPCs is most consistent with a net decrease in P<sub>K</sub>. A reduction in P<sub>K</sub> may be due to an altered conductance of the inward rectifying current in the IZPCs. In control Purkinje cells, steady-state current-voltage relations suggest that, just as has been described for other cell preparations, an increase in [K<sup>+</sup>]<sub>i</sub> produces an increase in net outward current for a range of potentials positive to E<sub>K</sub>. Thus, the inward rectifying current is important in establishing the resting potential of these cells.

The multiform ventricular tachycardias that occur in the canine heart 24 hours after coronary artery occlusion are often used as reasonable models of the ventricular arrhythmias that occur in humans...
after myocardial infarction. In most cases in the canine heart, the origin of the impulses is thought to be in the subendocardial Purkinje fibers that survive in the infarcted heart. These ectopic impulses could originate from the nondriven rhythmic activity caused by an abnormally automatic or a triggerable Purkinje cell focus. However, in addition, the marked alteration in the repolarization time course of the action potentials of these Purkinje cells that survive and overlie the infarcted myocardium provide an ideal substrate for perpetuating reentrant arrhythmias (for recent review see Rosen et al41).

We have now provided the initial observations on the mechanisms of the altered electrophysiology of these Purkinje cells that survive in the infarcted heart. We found that when these cells are isolated from their environment they still show dramatic changes in cell electrophysiology suggesting that the changes are a result of altered function of membrane ion channels. We can conclude that the reduced RP of these isolated cells is not due to a decrease in aK but rather an increase in PNa/PK. Not considered in our studies of the PNa/PK ratio in these cells are the effects of an electrogenic Na⁺-K⁺ pump current on the Purkinje cell resting and action potential. It may be that the Na⁺-K⁺ pump function is depressed in IZPCs. Thus, less outward current is generated, which may then result in membrane depolarization and action potential prolongation. Future studies are needed to compare Na⁺-K⁺ pump function in IZPCs with pump function in control cells, SPCs, and NZPCs.

Furthermore, we have now established that electrophysiological and ultrastructural abnormalities still exist in subendocardial Purkinje cells of the infarcted myocardium after they have been dissociated into single cells. The development of a single cell preparation of these cells from the diseased heart now provides us with an appropriate model for more rigorous electrophysiological studies. For instance, we may now proceed using patch clamp techniques to understand the function of specific ionic currents in these cells from the infarcted heart.

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Key Words • myocardial infarction • Purkinje myocytes • heart cells
Electrophysiology and ultrastructure of canine subendocardial Purkinje cells isolated from control and 24-hour infarcted hearts.

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