Activation of Cardiac Tissue by Extracellular Electrical Shocks

Formation of ‘Secondary Sources’ at Intercellular Clefts in Monolayers of Cultured Myocytes

Vladimir G. Fast, Stephan Rohr, Anne M. Gillis, André G. Kléber

Abstract—This study investigated the activation of cardiac tissue by “secondary sources,” which are localized changes of the transmembrane potential ($V_m$) during the application of strong extracellular electrical shocks far from the shock electrodes, in cultures of neonatal rat myocytes. Cell monolayers with small intercellular clefts (length, 45 to 270 µm; width, 20 to 70 µm [mean±SD, 54±13 µm]; $n=46$) were produced using a technique of directed cell growth. Changes in $V_m$ relative to the action potential amplitude ($AV_m/APA$) were measured using a fluorescent voltage-sensitive dye and a $10\times10$ photodiode array. Shocks with voltage gradients of 4 to 18 V/cm were applied across the clefts during either the action potential (AP) plateau or diastole. During the AP plateau, shocks induced secondary sources in the form of localized hyperpolarizations and depolarizations in the regions immediately adjacent to opposite sides of the clefts. The strength of the secondary sources, defined as the difference of $AV_m/APA$ across a cleft, increased with increasing cleft length or increasing electrical field gradient. For shocks with a gradient of 8.5 V/cm, the estimated critical cleft length necessary to reach a $V_m$ level corresponding to the diastolic threshold of excitation was 171±7 µm. Accordingly, shocks with average strength of 8.2 V/cm applied during diastole produced secondary sources that directly excited cells adjacent to the clefts when the cleft length was 196±53 µm ($n=14$) and that failed when the cleft length was 84±23 µm ($n=9$, $P<0.001$). The area of earliest excitation in such cases coincided with the area of maximal depolarization induced during the plateau phase. These data suggest that small inexcitable obstacles may contribute to the $V_m$ changes during the application of strong extracellular electrical shocks in vivo. (Circ Res. 1998;82:375–385.)

Key Words: stimulation | defibrillation | optical mapping | voltage-sensitive dye | cell cultures

Although strong electrical shocks are commonly used to terminate atrial and ventricular fibrillation, the mechanisms by which the extracellular electrical field affects $V_m$ in a large mass of cardiac tissue and terminates fibrillation are not fully understood. Linear cable theory predicts that shock-induced changes in $V_m$ will decay exponentially with distance from the stimulation sites. Between these regions of exponential decay, no transmembrane current is generated, and no changes in $V_m$ occur.

To explain the defibrillatory effects of electrical shocks, the hypothesis of “secondary sources” was proposed. This hypothesis suggests that microscopic resistive nonuniformities in the tissue structure force the electrical current to be redistributed between extracellular and intracellular spaces and to produce changes in $V_m$, so-called secondary sources, far from the shock electrodes. If these secondary sources are sufficiently large in amplitude, they may excite or defibrillate a large mass of tissue.

Cardiac tissue contains resistive discontinuities at different spatial scales. On the smallest scale, barriers to electrical current flow are created by boundaries of individual cells and by high-resistance gap junctions. Resistive obstacles of larger dimensions are imposed by connective tissue sheets and the vasculature separating cell bundles and cell layers. The suggestion that boundaries of cardiac cells may induce secondary sources came from theoretical studies and from experiments on isolated ventricular myocytes in which the application of an extracellular electrical field produced large hyperpolarizations and depolarizations at opposite cell ends. However, this hypothesis was not confirmed by experiments carried out in two-dimensional networks of electrically coupled cells. In densely packed strands of cultured myocytes, no secondary sources were found at cell borders. At the same time, large changes in $V_m$ were observed at boundaries of cell strands and at inexcitable obstacles produced by intercellular clefts.

The purpose of the present study was to systematically investigate the formation of secondary sources at inexcitable obstacles in cultures of heart cells. First, we analyzed the relationship between the obstacle dimensions and the magnitude of the shock-induced changes of $V_m$ during the plateau...
Selected Abbreviations and Acronyms

- AP = action potential
- APA = amplitude of the AP
- \( V_m \) = transmembrane potential
- \( \Delta V_m/\Delta A \) = changes in \( V_m \) relative to APA
- \( \Delta V_m/\Delta A \) = difference of \( \Delta V_m/\Delta A \) measured across middle of obstacle

phase of the AP. Second, we tested the prediction of the “secondary sources” hypothesis that during application of extracellular shocks in diastole, small obstacles can lead to the direct excitation of cardiac tissue at sites distant from the shock electrodes.

**Materials and Methods**

**Directed Cell Growth**

Cell monolayers with desired growth patterns were produced on glass coverslips according to a previously published procedure, with some modifications (S. Rohr and R. Frickiger, unpublished data, 1997) that allowed localized coating of glass coverslips with collagen (type IV, Sigma). The growth pattern that is schematically shown in Fig 1A consisted of a rectangular cell monolayer (black) incorporating small intercellular clefts (white) of variable dimensions. The length of the clefts varied between 45 and 270 \( \mu m \); the width was 54±13 \( \mu m \) (mean±SD, \( n = 46 \)), varying from 20 to 70 \( \mu m \). The separation between clefts was 0.93 and 0.5 \( mm \) in the horizontal and vertical directions, respectively. In the direction parallel to the extracellular field gradient (see below), the distance between the clefts was significantly larger than the electrotonic space constant (350 \( \mu m \)), ensuring that changes in \( V_m \) caused by individual clefts were not interfering with each other.

Fig 1B shows a phase-contrast image of a small portion of a cell monolayer, including an individual cleft. In the immediate vicinity of the cleft, cells were oriented parallel to the cleft border, similar to the cell alignment at borders of cultured cell strands described previously. The region of partial cell alignment extended for <60 \( \mu m \), encompassing fewer than four cells. Beyond this region, cells were poly- and oriented randomly, similar to the isotropic cell cultures described previously. Since the dimension of the region with partial cell alignment was much smaller than the electrotonic space constant (see above), the effect of such cell alignment on changes in \( V_m \) was likely to be minor.

Measurements were performed between the third and the seventh day in culture. During measurements, cells were superfused with a Hanks’ balanced salt solution (GIBCO) with a composition of (mmol/L) NaCl 137, KCl 5.4, KH2PO4 0.4, NaH2PO4 0.4, MgSO4 0.8, CaCl2 1.3, NaHCO3 4.2, HEPES 5.0, and glucose 5.1. The pH of the solution was 7.4, and the temperature was kept constant at 35°C. The depth of the solution in the perfusion bath was \( \approx 3 \) mm.

**Optical Recordings of \( V_m \)**

\( V_m \) were measured from the change in fluorescence of the voltage-sensitive dye RH-237 (Molecular Probes). The dye was stored in a 2 mmol/L stock solution of dimethyl sulfoxide and diluted to yield a final dye concentration of 2 to 3 \( \mu m \) in the superfusion solution.

Cell cultures were superfused with the dye solution for 3 to 4 minutes. The experimental setup for multisite optical recording has been described elsewhere in detail. Briefly, fluorescence measurements were made using an inverted microscope (Axiovert 35M, Zeiss) with objectives of \( \times 40 \) (numerical aperture, 1.3; Plan-Neofluar) and \( \times 60 \) (numerical aperture, 0.75; Fluar, Zeiss) and a 10\( \times 10 \) photodiode array (Centronic). Cells were exposed to excitation light for 80 ms. An area of \( 28\times28 \) \( \mu m^2 \) (\( \times 40 \) magnification) or \( 14\times14 \) \( \mu m^2 \) (\( \times 60 \) magnification) of cell culture corresponded to each photodiode, with a center-to-center distance of 30 and 15 \( \mu m \), respectively. The photocurrents from the 96 diodes were converted to voltages, amplified, multiplexed, and digitized at a 12-bit resolution and a sampling rate of 25 kHz for each of the 96 channels.

**Stimulation and Application of Electrical Shocks**

Electrical stimulation of cells was performed at a cycle length of 500 ms via a bipolar electrode composed of a glass pipette (tip diameter, 80 to 120 \( \mu m \)) filled with Hanks’ solution and a silver wire coiled around the pipette tip. The pacing electrode was positioned at a distance of >1 mm from the recording site.

Extracellular electrical shocks were applied via two platinum plate electrodes positioned at opposite ends of the perfusing bath (Fig 1A). The bath measured 2.2\( \times \)2.2 \( cm^2 \), and the electrode dimensions were 2\( \times \)0.2 \( cm \). Monophasic truncated exponential shocks with a duration of 10 to 12 ms were delivered using one of two custom-built shock generators. The first shock generator was built with a discharge capacitor of 122 \( \mu F \). Pulses delivered from this generator had time constants of voltage decay of 35 to 38 ms independent of the shock.
strengths. The second generator was built with a capacitor of 220 μF and had a stabilization circuit to ensure slower decay of shock voltages. In this case, time constants of the voltage decay varied with the shock strength. At shock strengths of 5 and 20 V/cm, the average time constants were 318 and 76 ms, respectively. With both shock generators, the distribution of shock-induced changes in membrane voltage were similar at equal shock strengths. The generators were triggered by the stimulus and could produce shocks at preselected times during the cardiac cycle. In most of the measurements, the extracellular voltage gradient produced by the shock in the bath was measured simultaneously with the optical recordings of V_m by two silver electrodes with diameters of 0.2 mm and interelectrode distances of 3.5 mm. The electrodes were positioned near the mapping area and aligned with the direction of electrical field.

Shocks of variable strength were delivered 20 ms after a stimulation pulse that initiated an AP (plateau phase, Fig 1C) or 450 ms after the previous excitation (diastole). Shocks of opposite polarities were examined at the same locations. In total, the effects of electrical shocks on V_m and patterns of activation spread were measured at 46 intercellular clefts in seven cell dishes obtained from four cultures.

Data Analysis
Fig 1C shows an example of an AP recorded during application of an electrical shock. The APA was taken as the difference in fluorescence intensity measured before the onset of the AP and immediately after the AP reached the plateau. The change in fluorescence induced by the shocks, ΔV_m, was determined as the difference between light intensities measured 1 ms before and 3 ms after the onset of the shock. Shock-induced changes in the membrane potential were expressed as a change (percentage) in fluorescence intensity relative to the APA, i.e., ΔV_m/APA. In such a way, the spatial variability in fluorescence intensity due to inhomogeneous dye staining and nonuniformity of illumination was corrected for. When an average APA of 100 mV is assumed,12 the relative ΔV_m/APA values directly translate into changes of V_m in millivolts. To calculate local activation times, signals were digitally filtered using a gaussian low-pass filter (cutoff frequency, 1.5 to 2 kHz).15 The activation times were determined at 50% of the APA using linear interpolation between the nearest sampling points.16 Activation maps and isopotential maps of ΔV_m/APA distribution were constructed using linear interpolation and triangulation algorithms.

Data were expressed as mean±SD. Differences were compared using the two-tailed nonpaired t test. They were considered statistically significant at P<.05.

Results
Spatial Distribution of Shock-Induced Changes of V_m at Inexcitable Obstacles
The effect of an electrical shock on the distribution of ΔV_m/APA near an intercellular cleft is demonstrated in Fig 2. Panel A shows an image of the cell monolayer and the grid indicating the position of the photodiodes. The intercellular cleft is delineated with a dashed line. The length and the width of the cleft were ~240 and 60 μm, respectively. The stimulation electrode was located above the mapping area, and the shock electrodes were located on the left and the right sides. Panel B shows the isochronal map of activation spread initiated by the stimulus. The activation wave was split by the cleft into two parts. Around the cleft, the activation spread was homogeneous, suggesting no significant discontinuities in intercellular electrical coupling11 in the immediate vicinity of the cleft.
The whole mapping area was activated within $\approx 900$ ms, which corresponded to a conduction velocity of 30 cm/s.

In Fig 2, panels C and D depict isopotential maps of the relative changes in $V_m$, $\Delta V_m/\text{APA}$, caused by electrical shocks of opposite polarities. Shocks with a duration of 11 ms were applied $\approx 5$ ms after the onset of APs. The shock strength was 7.5 V/cm (panel C) and 8.5 V/cm (panel D). In panel C, cells were depolarized on the right side and hyperpolarized on the left side of the cleft. The isopotential map contained two localized regions of maximal depolarization (blue) and maximal hyperpolarization (red) adjacent to the obstacle, corresponding to current sources and current sinks, respectively. The polarity of the shock-induced changes in $V_m$ reversed across the obstacle. The changes in $V_m$ on opposite sides of the obstacle were only slightly asymmetrical, with maximal values of hyperpolarization and depolarization being $-42\%$ and $39\%$, respectively. With the reversed shock polarity (panel D), the regions of depolarization and hyperpolarization were interchanged. In this case, the distribution of $\Delta V_m$ became more asymmetrical: maximal hyperpolarization was considerably larger than maximal depolarization ($-60\%$ versus $34\%$). Such levels of asymmetry were smaller than in our previous study carried out in cultured strands, which was likely due to relatively small values of $V_m$ changes in Fig 2. The reasons for the difference between effects of shocks of opposite polarities is less clear. It could be due to presence of discontinuities outside the mapping area (see “Discussion”).

Individual recordings illustrating changes in $V_m$ across the obstacle from the same experiment are shown in Fig 3A and 3B for opposite shock polarities. The shape of $\Delta V_m$ traces in most cases corresponded to the truncated exponential field pulse in both depolarized and hyperpolarized areas. In a few cases, signals deviated from this shape, which is exemplified by trace 5 in panel B, which was likely to correspond to a motion artifact. Panels C and D illustrate profiles of $\Delta V_m/\text{APA}$ along the horizontal axis (corresponding to the row of diodes from 1 to 8 in Fig 2) for the two shock polarities. In the first case (Fig 3C), the profile was symmetrical, with depolarization and hyperpolarization decaying within a short distance from the obstacle. On both sides, the $V_m$ changes decreased from $-40\%$ to almost zero over a distance of 90 $\mu$m. Such a voltage change is much larger than the voltage drop predicted from the exponential decay, assuming a value for the one-dimensional electrotonic space constant of 350 $\mu$m. It can be attributed to two factors. First, the secondary current source produced by the obstacle was localized within a small cell area, and current was dispersed in many directions, whereas in a one-dimensional structure, current flows in only one direction. Second, the voltage drop near the current source was further augmented by the proximity to the current sink on the opposite side of the obstacle. A very steep voltage decay was also observed when shock polarity was reversed (Figs 3B and 3D).

The effects of shocks on $\Delta V_m/\text{APA}$ distribution during the plateau phase of AP was assessed at 20 clefts in four cell dishes obtained from two cell cultures. In 14 of 20 cases, the
ΔV_m/APA distribution pattern exhibited an abrupt transition from hyperpolarization to depolarization across an obstacle at both shock polarities, corresponding to the pattern shown in Figs 2 and 3. The location of the maximal voltages was usually symmetrical, near the middle portion of obstacles. Sometimes, however, one or both of the ΔV_m/APA extrema were moderately shifted from the central position. This suggests that the voltage distributions in these cases were affected by discontinuities in the monolayer structure other than a given intercellular cleft. This effect was prominent in 6 of 20 cases where the sign of the ΔV_m/APA did not change across an obstacle with one or both shock polarities. An example of this type is shown in Fig 4 (shock strength, 18 V/cm). With one shock polarity (panels A and B), changes of V_m near the obstacle (length, ≈100 μm) were predominantly negative. Large negative changes of V_m were recorded on the right side of the obstacle facing the cathode. On the left side of the obstacle, small positive voltage deflections were transiently recorded after the shock onset (traces 5, 6, and 7); these deflections were then followed by negative changes. Across the obstacle, there was an abrupt change of the ΔV_m/APA value from −88% to −16% (voltage gradient of 72%). With the opposite shock polarity (panels C and D), ΔV_m/APA values on the right side became positive, and there were practically no shock-induced changes of V_m on the left side. Nevertheless, across the obstacle, the ΔV_m/APA changed by 48% (from 41% to −7%).

**Strength of Secondary Sources Produced by Obstacles**

Fig 5 demonstrates the dependence of the magnitude of secondary sources produced by inexcitable obstacles as a function of the obstacle length and shock strength. As shown schematically in Fig 5A, the magnitude of a secondary source was defined as (ΔV_m/APA)_diff. This procedure permitted exclusion of the contributions of remote secondary sources (eg, see Fig 4) to the local V_m changes. For a given obstacle, the average of two (ΔV_m/APA)_diff values, which were measured in response to shocks of opposite polarities, was taken. Two shock strengths were evaluated. In the first group, the average
Secondary Sources

To test the prediction that resistive discontinuities cause direct excitation of cardiac tissue during application of extracellular shocks, shocks were delivered during diastole, and the isochronal maps of activation spread initiated by shocks were analyzed. Three shock strengths were tested in these experiments with average values of 4.7 V/cm (n=8), 8.2 V/cm (n=28), and 18 V/cm (n=4). In all three groups, shocks produced one of the following effects: (1) shocks of both polarities caused direct excitation of cells adjacent to intercellular clefts; (2) shock of one polarity resulted in direct excitation, whereas the shock of the opposite polarity failed to excite cells; and (3) shocks of both polarities failed to excite cells near clefts.

Fig 6 illustrates direct activation of myocytes by a secondary source created by an inexcitable obstacle (same experiment as shown in Fig 2). Panels A and C show isochronal maps of activation spread initiated by shocks of opposite polarities, and panels B and D show the corresponding recordings of $V_m$ from the sites surrounding the obstacle. Shock strengths were 8.3 V/cm (panels A and B) and 8.2 V/cm (panels C and D). With one shock polarity (panel A), a small cell region adjacent to the obstacle on the left side was directly activated by the shock. This was evident from the fact that the cells in this region had the earliest activation times and that AP upstrokes (panel A, traces 1 and 2) exhibited biphasic shapes: the shock caused initial rapid membrane depolarization (shown by arrows), which was followed by excitation. This area of earliest activation could be superimposed on the region of maximal depolarization produced by shock during the plateau phase of AP (Fig 2D). Away from this area, the amplitude of initial depolarization decreased (Fig 6D, traces 3 through 6). Cells on the right side of the obstacle were transiently hyperpolarized by the shock, with hyperpolarization gradually increasing toward the center (traces 7 through 10). The initial membrane hyperpolarization was followed by depolarization, which resulted from the propagating wave initiated on the left side of the obstacle. An almost symmetrically reversed activation pattern was observed when the shock polarity was reversed (Fig 6C and 6D). The site of the earliest activation in this case was located on the right side, superimposed on the area of maximal depolarization during application of shock in the plateau phase (Fig 2C). Since multiple clefts of similar size were present in the cell monolayer, such clefts should simultaneously activate cells at multiple locations, resulting in a multicentric overall pattern of activation spread.

Fig 7 demonstrates an example of asymmetric excitation when a shock of only one polarity was able to directly excite cells near an obstacle (the same experiment as in Fig 4). With assumed that a shock produces symmetric changes in $V_m$ and that cells are depolarized by 25% APA above the resting level (corresponding to a depolarization of 25 mV), cells will be directly activated when the $(\Delta V_m/\text{APA})_{\text{diff}}$ value is >50%. From the linear functions in Fig 5B, this estimated critical obstacle length is $85 \pm 8 \text{ \mu m}$ for a shock strength of 18.0 V/cm and $171 \pm 7 \text{ \mu m}$ for a shock strength of 8.5 V/cm.
one polarity (Fig 7A and 7B; shock strength, 18 V/cm), shock induced an initial depolarization of ≈20% of APA on the left side of the obstacle (Fig 7B, arrows at traces 4 and 5). Despite this depolarization, cells were not activated for ≈2 ms. After that, activation appeared at two sites almost simultaneously. The earliest activation was registered at diode 1, which was likely caused by an excitation wave originating outside of the mapping area and propagating from left to right. The delay between the beginning of the shock and the appearance of the excitation wave in the mapping area was ≈2.5 ms (Fig 7B, traces 1 and 2). If a velocity of 30 cm/s was assumed, this indicated that the origin of this wave was located at a distance of 0.75 mm from the obstacle. The mechanism of initiation of this wave excitation is not known (see “Discussion”). At the time of arrival of the excitation wave at the obstacle, a spatially isolated area of depolarization was registered at a second site (diodes 3 and 4). Because of this temporal coincidence with the arrival of the propagating wave, the activation at this site was likely a result of two factors: (1) arrival of the excitation wave initiated at the distant source and (2) facilitation by local shock-induced membrane depolarization.

With shock of reversed polarity (Fig 7C and 7D), a large initial depolarization (shown by arrows in Fig 7D) was induced on the bottom right side of the cleft, and cells were directly activated at the shock onset. In addition, a second smaller source of excitation was registered at the top right part of the mapping area. The larger area of earliest activation corresponded to the area of maximal depolarization produced by shock during the AP plateau (Fig 4C and 4D).

The stimulating efficacy of secondary sources depended on both shock strength and obstacle length. With an average strength of 8.2 V/cm (number of obstacles, n=28), shocks of both polarities directly excited cells when the obstacle length was 196±53 μm (n=14). Shocks of only one polarity resulted in direct activation when the obstacle length was 134±49 μm (n=5, P<.05). No activation by shocks of both polarities was observed at obstacles with lengths of 84±23 μm (n=9, P<.001). Thus, the critical obstacle length necessary for the
direct cell activation with shocks of 8.2 V/cm was between 84 and 196 μm. The estimate of critical length of 171±7 μm obtained from shock-induced changes of V_m during the plateau phase of AP (Fig 5) falls within this range.

With an average shock strength of 4.7 V/cm (n=8), shocks of both polarities excited cells at obstacles with a length of 210±42 μm (n=2); shocks of only one polarity directly excited cells at obstacles with a length of 202±53 μm (n=2); and shocks of both polarities failed to produce excitation at obstacles with a length of 114±65 μm (n=4). At an average shock strength of 18 V/cm (n=4), shocks of both polarities excited cells in two cases (obstacle length, 98±53 μm); shocks of only one polarity excited cells in one case (obstacle length, 100 μm); and shocks of both polarities failed in another case (obstacle length, 45 μm).

**Discussion**

In the present study, we investigated the formation of secondary sources of excitation at small inexcitable obstacles during the application of extracellular electrical shocks with a strength of 4 to 18 V/cm, which is similar to shocks used for defibrillation in patients. Two main results were obtained: (1) Extracellular shocks induced secondary sources at inexcitable obstacles, which were characterized by abrupt transition from hyperpolarization to depolarization on the opposite sides of the obstacles. (2) These secondary sources were able to directly excite localized cell areas adjacent to the obstacles far from the shock electrodes. The critical obstacle length necessary for direct activation was between 84 and 196 μm for a shock strength of 8.2 V/cm.

**Cell Culture as a Model for Studies of Defibrillation Mechanism**

The mechanism of defibrillation has two important aspects that are not fully understood. First, it has not been clarified how an
extracellular field shock changes the $V_m$ of the cells located between the shock electrodes. Second, it is not clear how shock-induced changes of $V_m$ interact with irregular electrical activity and interrupt fibrillation. Several hypotheses have been proposed to explain this process.\textsuperscript{20–27}

Regarding the first aspect, it has been proposed that microscopic discontinuities in tissue structure play a crucial role in defibrillation by inducing secondary sources of excitation far from the shock electrodes.\textsuperscript{24} Until recently, the effects of the microscopic discontinuities on shock-induced changes of $V_m$ could be investigated only by using computer models.\textsuperscript{3–6,28,29} Experimental studies in whole cardiac tissue are extremely difficult because of the three-dimensional structure of cardiac muscle, which prevents precise correlation of voltage changes with tissue structure at the microscopic level. To overcome this difficulty, we used two-dimensional cultures of neonatal rat myocytes, where shock-induced changes in $V_m$ can be monitored with a very high spatiotemporal resolution and correlated to the microscopic structure. Another advantage of cell cultures is that their geometry can be modified using techniques for directed cell growth. By use of these techniques, many types of structures encountered in whole tissue can be created in cell cultures, including cell strands of variable widths,\textsuperscript{12,15} tissue expansions,\textsuperscript{16,30} anisotropic arrangement of myocytes,\textsuperscript{17} and monolayers with intercellular clefts of pre-defined dimensions.

There are several differences between cell cultures and intact cardiac tissue. Some of them, including the anisotropic bidomain properties of cardiac tissue,\textsuperscript{31} were discussed in a previous study.\textsuperscript{11} Another difference relates to the distribution of gap junctions. Gap junctions are distributed regularly within the cell perimeter in cultured neonatal cells,\textsuperscript{17,32} whereas gap junctions are more concentrated at cell ends in adult myocardium.\textsuperscript{33,34} In this respect, cultured cells more closely resemble neonatal heart tissue\textsuperscript{34,35} and remodeled cardiac tissue found in the infarction border zone.\textsuperscript{34,36} The nonuniformity of gap junction distribution found in the adult myocardium might affect the distribution of shock-induced changes of $V_m$ at the subcellular scale. However, changes of $V_m$ on a larger scale, which were investigated in the present work, are not likely to be significantly modified by this type of resistive nonuniformity.

**Secondary Sources Produced by Inexcitable Obstacles**

Electrical shocks applied to cell cultures produced localized changes of $V_m$ at the inexcitable obstacles far from the primary stimulating electrodes. Typically, regions of hyperpolarization and depolarization were observed at the obstacles. As expected from secondary sources, there was an abrupt transition from maximal hyperpolarization to maximal depolarization across obstacles and a more gradual voltage change with distance away from the obstacles (Fig 2). In several cases, no change of $\Delta V_m$ polarity across obstacles was observed; ie, voltage changes were either all positive or all negative (Fig 4). Such a voltage profile may be the result of the summation of voltage changes produced by two secondary sources: one that was created by the cleft located within the mapping area and one or more additional sources located outside the mapping region. The mechanism for these additional sources, which were also observed previously,\textsuperscript{11} is not known. One possibility is that they might be due to discontinuities in intracellular resistance resulting from nonuniform expression of gap junctions.\textsuperscript{17}

The strength of the secondary sources, which was defined as the difference of $\Delta V_m/\text{APA}$ across an obstacle (Fig 5), was dependent on obstacle dimensions and on shock strength. Within the range of cleft length investigated in the present study (45 to 270 $\mu$m), the source strength was linearly related to the cleft length (Fig 5). With increasing cleft length, this dependence is expected to become nonlinear, and then the $\langle \Delta V_m/\text{APA} \rangle_{\text{diff}}$ values are expected to reach a steady level. In addition to the cleft length, the source strength is expected to be dependent also on the cleft width, because changing cleft width changes the total circumference of the resistive boundary and, therefore, modulates the electrotonic interaction between regions of current source and current sink on the opposite sides of a cleft. However, our data indicated no significant correlation with the cleft width. This finding does not necessarily mean that such a dependence is absent. More likely, it was masked in the present experiments by stronger dependence of the source strength on the cleft length and by the fact that cleft width showed relatively minor variations.

The dependence of $\langle \Delta V_m/\text{APA} \rangle_{\text{diff}}$ on cleft length shown in Fig 5 was used to estimate the critical cleft length that is necessary for direct cell excitation during diastole. The parameter $\langle \Delta V_m/\text{APA} \rangle_{\text{diff}}$ was used rather than the maximal level of depolarization for the following reason: As it was shown previously in cultured cell strands, shock-induced changes of $V_m$ could be asymmetric during the plateau phase and symmetric during diastole.\textsuperscript{34} However, the sum of hyperpolarization and depolarization in strands, which corresponds to $\langle \Delta V_m/\text{APA} \rangle_{\text{diff}}$ measured in the present experiments, was the same during both phases of the AP. Thus, the depolarization level to be expected during diastole is better represented by half of $\langle \Delta V_m/\text{APA} \rangle_{\text{diff}}$ measured during the plateau phase than by the maximal level of depolarization in this phase. The diastolic threshold of activation in adult guinea pig myocytes is $\approx 25$ mV more positive than the resting potential.\textsuperscript{37} By using the same excitation threshold for cultured neonatal rat myocytes, sufficiently large changes of $V_m$ were obtained during the application of shocks with a strength of 8.5 V/cm at clefts that were $171 \pm 7$ $\mu$m in length. We would like to point out that this estimate is approximate. Based on knowledge of the exact value of the threshold potential for activation of sodium channels in neonatal rat myocytes and on inclusion into consideration of the concept of “liminal length,” which postulates that a certain amount of tissue has to be activated in order to get a propagated response, a more precise analysis could be performed.\textsuperscript{37–41} However, the fact that the estimate provided above is quite consistent with the results obtained from the direct evaluation of the obstacle length critical for excitation (see below) suggests that this simplified analysis takes into account the major factors determining the activation process.

**Stimulation by Secondary Sources**

Depending on the shock strength and cleft dimensions, shocks applied during the diastolic phase directly excited cells near the clefts. The direct cell excitation was initiated on the leading edge of shock pulses (“make” response) in regions that coincided with areas of maximal depolarization.
recorded during the application of shocks in the plateau phase of the AP. It was not possible to test the effects of the falling edge of the shock pulses ("break" response), because even with weaker shocks, the cells were always excited before the end of the pulses, apparently by the "make" excitation at distant locations.

The critical cleft length necessary for direct cell excitation during the application of shocks with a mean gradient of 8.2 V/cm was in the range of 84 to 196 μm, which includes the estimate obtained from the ΔV_m/ΔPA measurements in the plateau phase. It is interesting how these estimates extrapolate to the intact adult tissue. To make such an extrapolation, differences in passive properties and fiber orientation between cell cultures and intact cardiac tissue have to be taken into account. By assuming continuous representation of cardiac tissue and considering a case with a cleft oriented longitudinally and an extracellular field gradient applied in the transverse direction, the critical cleft length can be predicted by scaling the longitudinal electrotonic space constant. In arterially perfused papillary muscle, the longitudinal space constant was 357 and an extracellular field gradient applied in the transverse direction, the critical cleft length can be predicted by scaling the longitudinal electrotonic space constant. In arterially perfused papillary muscle, the longitudinal space constant was 357 or 528 μm, depending on the presence of superfusion solution. Scaling from a space constant of 350 μm in cell cultures, the critical length value for longitudinally oriented clefts in ventricular myocardium is predicted to be <200 or 300 μm, depending on the experimental conditions. Discontinuities with dimensions of several hundred micrometers and larger are common in ventricular myocardium. In human pectinate muscle, connective tissue septa with such dimensions were found in ventricular tissue from young individuals, and much larger (up to 1 mm) septa were found in the aging myocardium. Our data suggest that such inexcitable obstacles may contribute to tissue excitation and defibrillation during the application of extracellular electrical shocks in the whole heart. The definitive answer to the question about the role of such discontinuities in the whole heart will require taking into account the three-dimensional tissue structure as well as the specific geometrical arrangement of clefts. In particular, clefts in the whole tissue might be closely spaced, allowing for strong interaction between areas of hyperpolarization and depolarization at neighboring clefts. Also, extracellular fields can be oriented at different angles relative to the cleft axis during shock application in the whole tissue. Future studies are necessary to elucidate how these factors affect the formation of secondary sources in the whole heart.

Acknowledgments

This study was supported by the Swiss National Science Foundation and the Swiss Heart Foundation. Dr A.M. Gillis is a Scholar of the Alberta Heritage Foundation for Medical Research and was the recipient of the NASPE Bigelow Travelling Fellowship. We thank Regula Flückiger for help with preparation of cell cultures.

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Circ Res. 1998;82:375-385
doi: 10.1161/01.RES.82.3.375

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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