Genesis of life-threatening ventricular arrhythmias during the delayed phase of acute myocardial ischemia. Role of cellular electrical coupling and myocardial heterogeneities
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DELAYED VENTRICULAR ARRHYTHMIAS DURING ACUTE MYOCARDIAL ISCHEMIA RELATE TO MACROREENTRANT ACTIVATION.


Summary.

Introduction. A temporal association between ischemia-induced cellular uncoupling and the occurrence of ventricular arrhythmias during the delayed phase of ischemia was demonstrated, and micro reentry has been proposed as the underlying mechanism. We tested the hypothesis that micro reentrant activation causes ventricular fibrillation (VF) during this phase of ischemia.

Methods. In isolated regionally ischemic blood perfused porcine hearts refractory periods were measured. High resolution activation mapping (142 extracellular electrograms, electrode distance 1 mm) permitted measurement of longitudinal and transversal conduction velocity. Wavelengths were calculated. Isolated ischemic left ventricular preparations were produced by superfusing tissue parts (5.7±2.4 gram) with warmed Tyrode's solution (37°C). Ten minutes after start of superfusion, attempts to induce VF were undertaken with programmed stimulation. Activation was mapped at high resolution with a 105 terminal multi electrode (electrode distance 1 mm).

Results. In isolated hearts, longitudinal and transversal conduction velocity decreased to minimal values of 45.0±19 (after 40 min) and 16.2±3.3 cm/s (after 20 minutes) respectively. Calculated wavelength was in the centimeter range. In isolated ventricular preparations no sustained arrhythmias occurred. Non sustained reentry occurred around lines of activation block. Conduction velocity was 27±13 cm/s in the third premature beat (between 10 and 60 minutes of ischemia) and 23±4 cm/s during spontaneous reentry, wavelength was 4.8±2.4 cm during the third premature beat and 2.3±0.3 cm during reentry.

Conclusions. No VF was induced in isolated left ventricular preparations. The wavelength is too long to be compatible with microreentry, both in isolated ventricular preparations and in regionally ischemic intact hearts. Therefore, microreentry cannot be the underlying mechanism of sustained VF during the delayed phase of acute ischemia, but macroreentrant activation occurs around lines of block.
Introduction.

Lethal ventricular arrhythmias during acute ischemia arise in two distinct phases, of which the mechanism of the second phase is unknown. Recent studies have described a temporal relation between the onset of cellular uncoupling and the occurrence of delayed arrhythmias, and it has been suggested that heterogeneous cellular uncoupling causes conduction slowing and block at a cellular level.

Cellular uncoupling has indeed been shown to cause very slow conduction in both cell cultures and in computer models. In combination with the formation of cellular heterogeneities, such very slow conduction could give rise to microreentrant activation, defined as activation propagating around a few myocytes. However, as long as cellular coupling is (partially) intact, the occurrence of local heterogeneities is prevented and the substrate for arrhythmias can only arise over larger distances, and can form the substrate for macro reentry.

We have recently demonstrated that subepicardium partially survives ischemia, whereas ischemic midmyocardium becomes inexcitable. Indeed, in that study, planar waves were frequently encountered in the ischemic zone during ventricular fibrillation (VF) during the IB phase of acute ischemia which is compatible with large (reentrant) circuits, and no evidence for microreentry was observed. Therefore, we hypothesized that partially intact cellular coupling prevents local heterogeneities during the IB phase of acute ischemia. Thus, the mechanism for VF during this phase of ischemia should relate to macro reentry.

To quantify the minimal size of the arrhythmogenic substrate, we calculated the wavelength during the IB phase of acute ischemia. The wavelength, the mathematical product of conduction velocity and refractory period, describes the distance that the activation front propagates during the refractory period of a certain site, and thus defines the minimal size of the reentrant circuit. Herewith, we can predict whether conditions for microreentry are present and whether microreentry can be responsible for VF during the delayed phase of acute ischemia. Thus, we measured conduction velocity and refractory periods in the Langendorff blood perfused porcine hearts during 60 minutes of ischemia.

To directly test the alternative hypothesis, that VF during the IB phase of acute ischemia is caused by microreentry, we attempted to induce VF in ischemic isolated porcine left ventricular preparations, with a programmed electrical stimulation protocol that assures the occurrence of VF in intact, regionally ischemic hearts. This approach can falsify the alternative hypothesis: if VF cannot be induced in this model in which there is no interaction between the ischemic and the non ischemic tissue (as is the case in the regionally ischemic intact heart), microreentry cannot be the underlying mechanism of VF during the IB phase. However, if microreentry is the underlying mechanism of delayed ventricular arrhythmias, the critical mass of the subepicardium in these preparations should be large enough to allow sustained VF.

Methods.

Langendorff perfused porcine hearts.

Pig hearts (n=5) were extirpated, connected to the Langendorff perfusion apparatus and made regionally ischemic as described previously. Details are described in chapter 2. Basic stimulation (BCL 450 ms, rectangular current pulses of 2 ms duration and twice diastolic threshold) was applied from an electrode in the center of the multi electrode terminal during 60 minutes of ischemia. Every 5 minutes, excitation threshold of this site was determined by stepwise increasing excitation current. The refractory period (1 ms accuracy) of this site was determined with the extra
stimulus technique at twice diastolic threshold. Electrograms were stored and activation maps constructed off line as described in chapter 2. Conduction velocity was measured in the longitudinal and transversal direction as described previously. Conduction velocity was measured in subsequent activation maps during ischemia in the same direction as before coronary occlusion. Conduction velocity could not be measured when many sites became inexcitable during ischemia or when activation evidently propagated in a different direction as before coronary occlusion.

Isolated left ventricular preparations.

From another 6 hearts, isolated left ventricular preparations were prepared as described in chapter 2. With a dermatome, a thin (approximately 0.4 mm thick) cut of approximately 3 mm length was made parallel to the epicardium. This created a normoxic epicardial slap connected to the rest of the wall-thick preparation at its boundary, but not coupled to the underlying midmyocardium. Hence, this procedure allowed pacing of the preparation from a normoxic, non depressed site.

The preparation was superfused with warmed (37°C) Tyrode’s solution, such that the non perfused midmural part became ischemic, while the superfused subepicardium and the thin cut remained well oxygenated. A programmed stimulation protocol consisted of three premature stimuli that were as shortly coupled as possible. The stimulation protocol started 10-15 minutes after start of the superfusion, which was used as the onset of ischemia, and was performed every 5 minutes during 90 minutes of ischemia.

In 4 preparations, tissue impedance, a measure for cellular uncoupling, was measured every 30 seconds with the four electrode technique (electrode distance: 2 mm, alternating current: 30 μA, frequency 1 kHz).

Activation maps were constructed from recordings from a 105 terminal multi electrode (9x12 grid, inter electrode distance 1 mm) that recorded from the subepicardium overlying the ischemic core of the preparation, as described in chapter 2.

Definitions

Ventricular fibrillation in the isolated left ventricular preparation and in the Langendorff heart was defined as an irregular, polymorphic tachycardia that did not terminate spontaneously. Regular monomorphic tachycardias were assumed to be sustained when lasting longer than 30 seconds; shorter duration subsequently defined non sustained ventricular tachycardia. In the Langendorff heart, atrioventricular dissociation (determined either visually during the experiment or from the on line extracellular electrograms) was required before the diagnosis ventricular arrhythmia was established.

Electrograms were assumed to represent inexcitability if they were either monophasic or when the steepest negative deflection of the intrinsic deflection (dV/dt max) was less negative than -1 V/s. Lines of block were defined as a region between adjacent electrodes where activation delay was more than 20 ms with a minimal length of 2 mm. In addition, both sides of the line of block should be normally excited either around the line of block, or parallel from outside the mapped region. Regions of conduction block were not included in this definition. Very slow conduction was defined as a conduction velocity <5 cm/s in the dominant direction of propagation. Hence, the tissue distant to the region of very slow conduction should be excited via this region, and not from a parallel direction.

Statistics

Unless stated otherwise, data are expressed as mean±SD. An unpaired t-test was used to test differences between groups; when data were not normally distributed, a Mann-Whitney Rank Sum
Excitation threshold (µA)

Duration of ischemia (min)

Refractory Period (ms)

Duration of ischemia (min)

Figure 5.1A. Course of excitation threshold of the stimulated site within the central ischemic zone in 5 Langendorff perfused porcine hearts during 60 minutes of ischemia. B. Course of refractory periods (symbols as in A) during 60 minutes of ischemia.

test was used as a non-parametric alternative. Multiple comparisons were tested with ANOVA. Significance was defined as p<0.05.

Results.

Refractory periods in Langendorff perfused hearts

We measured refractory periods and excitation thresholds. Figure 5.1A shows the course of excitation thresholds in 5 individual experiments. Inexcitability of the central ischemic tissue or an excitation threshold of more than ten times the control value occurred in 3 out of 5 experiments, after 37±8 minutes of ischemia. In the other two, excitation threshold remained relatively unchanged. In one heart, the central ischemic zone could not be excited after 60 minutes of ischemia. Thus, a large variation is present in the course of excitation thresholds during ischemia, where a dramatic increase most likely represents inexcitability under the stimulating electrode with subsequent remote activation. Panel B shows the course of refractory periods of the site under the stimulating electrode in these experiments. On average, refractory periods were reduced to 173±15 ms after 45 minutes of ischemia, compared to 268±8 before ischemia (p<0.05), and reached a minimal value of 166±21 ms after 50 minutes (p<0.05 vs before ischemia). Hence, there is a remarkable decrease in refractory periods during the delayed phase of acute ischemia.
A Pre occlusion  

B 45 minutes of ischemia

During determination of refractory periods, spontaneous activity occurred in 4 out of 5 hearts following the extra stimulus. VF occurred in 3 hearts between 40±3 and 57±3 minutes of ischemia, and was not confined to experiments with increased excitation threshold (VF occurred 2x in experiments with unchanged excitation threshold and once in an experiment where excitation threshold had dramatically increased). VF or sustained VT occurred following 12 out of 49 extra stimuli (3x VF, 9x sustained VT).

**Longitudinal and transversal conduction velocity in Langendorff perfused hearts**

The central ischemic zone of the regionally ischemic Langendorff heart was stimulated from the central terminal of the composite electrode. By means of stimulating centrally within the electrode, longitudinal and transversal conduction velocity were measured from the resulting elliptic activation patterns. Figure 5.2A shows an example of an activation map recorded prior to LAD occlusion. The anisotropy is clear, and longitudinal conduction velocity and transversal conduction velocity were measured from this activation map in the direction of the arrows. Mean longitudinal and transversal conduction velocity before coronary occlusion were 68±9 cm/s and 29±2 cm/s respectively. Panel B shows an activation map from the same experiment after 45 minutes of ischemia. Note that many sites within the ischemic zone are now inexcitable and that heterogeneous crowding of isochrones occurred. Conduction velocity was almost unchanged in longitudinal...
direction but could not be measured in the transversal direction, because activation clearly propagates at the left side around the line of block. If transversal conduction velocity were determined from the slowed conduction in the upper right side of the map, a value of approximately 7 cm/s is found, which is still larger than very slow conduction. Moreover, these low values of conduction velocity are present only in a small region within the ischemic zone, whereas in the remainder conduction velocity is normal or only slightly decreased. In 4 out of 5 experiments, conduction velocity could not be reliably measured between 45 and 60 minutes of ischemia because of the reduction of excitable sites and the increase in excitation threshold. Longitudinal conduction velocity decreased to a minimal value of 45±19 cm/s after 40 minutes (p<0.05 vs before occlusion), whereas the transversal conduction velocity dropped to 20±8 cm/s (p<0.05 vs before occlusion) during the 1B phase of acute ischemia. Minimal transversal conduction velocity was observed after 20 minutes of ischemia (16±3 cm/s).

Figure 5.3A shows the course of mean change in longitudinal and transversal conduction velocity during 60 minutes of ischemia. Note that the values reported after more than 45 minutes of ischemia are derived from one experiment only, and that these values might thus be less representative than those obtained at earlier stages of ischemia.

From conduction velocity and refractory periods, wavelengths were calculated. Panel B shows the course of wavelength calculated from longitudinal conduction velocity (“longitudinal wavelength”) and from transversal conduction velocity (“transversal wavelength”). The smallest wavelength measured was 2.8 cm. This observation is not compatible with microreentry.
Isolated left ventricular free wall preparations

To test the hypothesis that microreentry underlies VF during the delayed phase of acute ischemia, we attempted to induce VF with an aggressive programmed stimulation protocol in isolated left ventricular preparations ($n=6, 99$ attempts). Mean mass of the preparations was $5.7\pm 2.4$ gram (range 2.3-9.2 gram). Time course and amplitude of Rt rise in this model did not differ from published data in the intact heart\(^4\) (time to maximal Rt rise: $39\pm 10$ min and $30\pm 6.0\%$ of final value increase in this model vs $37\pm 1.4$ and $44\pm 28\%$ increase in the ischemic border zone of the intact heart\(^4\)). The subsequent arrhythmias that arose following the programmed stimulation protocol are shown in figure 5.4. The majority of attempts ($85\%$, D) resulted in capture of the paced beats, but no resulting spontaneous beats. In $9\%$, one spontaneous beat succeeded the paced train (C), in $1\%$ a couplet was observed (A). More than three spontaneous beats followed the pacing protocol in $5\%$ of cases (B), but it should be noted that these arrhythmias were monomorphic and never lasted longer than 10 seconds and that the coupling interval of the first beat exceeded the refractory period in all cases, indicating that, if reentry is the underlying mechanism, an excitable gap is present. No sustained arrhythmias were observed.

Hence, whereas a single premature beat caused ventricular fibrillation in the intact hearts in 12 out of 49 episodes; three short coupled premature stimuli could not induce any sustained arrhythmia in the superfused left ventricular preparation (0 out of 99 attempts, $p<0.001$). Three

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Figure 5.4A. Representative electrograms from one of the isolated left ventricular preparations. A. Three premature beats induce two spontaneous beats (1% of episodes). B. Premature stimulation is followed by a non sustained monomorphic tachycardia (5% of episodes). C. Three premature beats induce one spontaneous depolarization (9% of episodes). D. No spontaneous arrhythmias follow on premature stimulation (85% of episodes). Pie shows the distribution of induced spontaneous arrhythmias in 99 attempts to induce VF.
short coupled premature stimuli cause VF in 90% of cases in the intact heart during this phase of ischemia.

Wavelength in isolated free wall preparations

Because these preparations were stimulated from the normoxic epicardial cut, we measured lowest conduction velocity in the dominant conduction direction (and not parallel or perpendicular to the fiber direction) during the S4 premature paced beat and in spontaneously occurring reentrant beats. Conduction velocity was 27±13 cm/s between 10 and 60 minutes of ischemia during S4. No significant change in conduction velocity occurred during this time. Combined with a refractory period of 173±20 ms, this resulted in a wavelength of 4.8±2.4 cm. We also measured conduction velocity in the four out of nine single spontaneous beats following the programmed stimulation protocol that were reentrant (not shown). These reentrant beats had a conduction velocity of 23±4 cm/s (p=NS vs S4) but a significantly shorter revolution time: 105±26 ms (p<0.05). However, also in these cases the wavelength of the arrhythmia remained in the centimeter range (minimal 2.3±0.3 cm). Thus, both during premature stimulation and during spontaneously occurring beats, wavelength is too long to be compatible with microreentry.
Lines of conduction block were found in 14 out of 96 basic paced beats between 5 and 90 minutes of ischemia. Figure 5.5A shows an example of a line of conduction slowing around which the activation meanders and the wave fronts merge behind the barrier. Note that in the three consecutive activation maps (panel A-C, recorded after 50, 60 and 70 minutes of ischemia respectively) the line of block arises parallel to the fiber direction and that this line of block remains at the same location, indicating an anatomic (in this case enhanced anisotropy) rather than a purely functional origin.

The number of lines of block increased during S4 compared to basic stimulation: in 48 out of 82 beats lines of block were found (p<0.0001 vs basic stimulation). Figure 5.6 shows an example of increased number of lines of conduction block during premature stimulation. Panel A displays the same basic beat as displayed in figure 5.5A. Panel B shows that upon premature stimulation (coupling interval 182 ms), conduction velocity decreased (increased crowding of isochrones) and another line of block developed. The curvature of the activation front around the upper line of block increased compared to basic stimulation, as is evident from the increased angle of isochrones with respect to the line of block. Were these lines of block caused by decreased gap junctional
conductance, one would expect no influence of premature stimulation on their size and location. Lines of block were also observed during premature stimulation in another experiment (panel D) when no conduction slowing was present during basic stimulation (panel C). In this case, the line of block developed perpendicular to the fiber direction upon premature stimulation (coupling interval 160 ms) after 30 minutes of ischemia. Because here a line of block arose that was not previously present, in this example a purely functional mechanism most likely underlies the formation of this line of block. Complete revolutions around a line or region of block were found in 6 out of 82 third premature beats, whereas incomplete revolutions was observed in 29 out of 82 third paced beats ($p<0.001$).

Discussion.

We measured conduction velocity and refractory periods in the regionally ischemic, blood perfused porcine heart. The calculated wavelength remained within the centimeter range, which makes microreentry an unlikely mechanism for VF during the delayed phase of acute ischemia. With an aggressive programmed stimulus protocol, assuring VF in 90% of intact hearts, we were not able to induce sustained ventricular fibrillation in superfused left ventricular preparations of pig hearts. Herewith we have falsified the hypothesis that microreentry is the principle mechanism for the maintenance of ventricular fibrillation during the IB phase of ischemia. In this model, conduction velocity and refractory period were also insufficiently reduced in this model to be compatible with microreentrant activation: wavelength remained within the centimeter range. Rise in tissue impedance, however, was not different from that in the intact heart, underlining the notion that severity and time course of ischemia is not different in this model.

Wavelength

Quantification of conduction velocity in the Langendorff perfused regionally ischemic porcine heart revealed that conduction velocity was too high to be compatible with microreentrant activation: we found that (transversal) conduction velocity did not fall below 16 cm/s. We measured conduction velocity in the same direction throughout the experiment. Often, conduction velocity could not be measured because activation propagated in a different direction than before occlusion,
or too many sites were inexcitable. This could have led to an underestimation of conduction velocity. However, only in small regions within the ischemic zone conduction velocity importantly decreased, whereas it decreased only modestly in the remainder. The calculated wavelength was in the order of centimeters. These data strongly suggest that microreentry is not the underlying mechanism of VF during the delayed phase of acute ischemia. However, in the majority of experiments, VF occurred after only one premature beat that was applied to the ischemic subepicardium to measure refractory periods.

In the isolated left ventricular ventricle preparations, mean conduction velocity did not decrease to an extent compatible with microreentry, and consequently, wavelength remained in the centimeter range. In the presence of a refractory period of 170 ms, a conduction velocity of less than 0.6 cm/s would be required to result in microreentry. Such slow conduction was never encountered. However, in these experiments the area of interest was stimulated from the "normal" zone, i.e. the non-ischemic epicardial slice. Breakthrough activation of the field under the electrode could theoretically have contributed to the conduction velocity measured. Breakthrough activation, however, was never observed. We and others have shown that the midmyocardium during this stage of ischemia is for the largest part inexcitable, but theoretically this method of measuring conduction velocity could have led to an overestimation.

The data on conduction velocity and wavelength indicate that, given the fact that sustained VF needs several wavelets or one rapidly moving single source rotor, induction of sustained VF is physically impossible in the amount of tissue available in the isolated left ventricular preparation.

Cellular uncoupling and microreentry during the 1B phase of acute ischemia

Although the precise mechanism of delayed ventricular arrhythmias during acute ischemia remains largely not understood, various studies have suggested or demonstrated a relation between the occurrence of these arrhythmias and the onset of cellular uncoupling. Results obtained in models with diminished cellular coupling and an increased occurrence of arrhythmias during ischemia further strengthen this association. Dekker et al. have shown that in pressure and volume load induced heart failure start of cellular uncoupling is earlier and duration prolonged. In the same model, arrhythmias occurred earlier during ischemia and number and severity of arrhythmias was increased. Moreover, in connexin43 deficient mice the incidence of ischemia-induced arrhythmias was increased.

Hence, since electrical cellular coupling in multicellular preparations has a certain duration, it was hypothesized that microscopic heterogeneities that arise from ischemia-induced cellular uncoupling lead to microreentry. Rohr et al. have indeed demonstrated that in cultured cellular preparations palmitoleic acid, a specific gap junctional uncoupler, conduction velocity decreased to values that allow reentrant activation around one or a few cells, and model studies by Shaw and Rudy predicted that reduction of gap junctional conductance can reduce conduction velocity to approximately 1% of control values. However, in the setting of acute ischemia, microreentry has never been demonstrated. We have previously demonstrated that the ischemic subepicardium plays an important role in the substrate of these arrhythmias, that terminates at a critical amount of cellular coupling. Although in that study microreentry was not observed, its absence could not be determined with certainty. The ultimate proof for the presence of microreentry can be obtained if ventricular fibrillation can be induced in a relatively small piece of left ventricle. Conversely, if no ventricular fibrillation occurs with a programmed pacing protocol that assures ventricular fibrillation in 90% of cases in the intact heart during ischemia, microreentry cannot be the underlying mechanism of delayed ventricular arrhythmias during ischemia. We demonstrated that no sustained arrhythmias occurred in isolated left ventricular preparations weighing up to 9 gram. Despite that reentry and fibrillation have been reported in hearts as small as mouse hearts of approximately 0.2 gram, the critical mass for sustained arrhythmias was insufficient in our isolated left ventricular
preparations. Indeed, the example displayed in figure 5.7 shows that although reentry can occur, no sustained tachycardias arise. One single premature stimulus delivered centrally within the ischemic zone in the Langendorff perfused intact heart, however, caused ventricular fibrillation in 3 out of 5 hearts. In our previous study we applied short coupled premature stimuli from the normal side of the ischemic border, because during ischemia ectopic beats originate from that side of the ischemic border\textsuperscript{15}, which resulted in VF with only one premature stimulus in no more than 20% of the experiments\textsuperscript{4}. Stimulation from within the ischemic zone thus produces more fatal triggers than stimulation from the normal zone. This effect was independent from excitation threshold, making activation of a remote region with a short refractory period with large current pulses unlikely as the only cause for VF\textsuperscript{1}. Hence, during the 1A phase, when refractory periods are prolonged compared to control, premature stimuli from outside the ischemic zone are most arrhythmogenic\textsuperscript{15,15}. However, we show that during the 1B phase refractory periods are shortened. Thus, premature stimuli from inside the ischemic zone are most arrhythmogenic in this phase\textsuperscript{4}.

We demonstrated that no sustained arrhythmias occur in the isolated left ventricular preparation, whereas VF is a common finding in the intact regionally ischemic heart. Hence, these data strongly suggest that for the maintenance of sustained VF the non-ischemic zone is of particular importance.

**Lines of conduction block**

Lines of conduction block were found frequently, and in many instances complete or incomplete reentrant revolutions took place around such lines. In figures 5.6 and 5.7 we demonstrated that both functional as structural properties add to the occurrence of those lines. It has been reported that similar lines of conduction block are necessary for the induction of ventricular tachycardia in the epicardial border zone of the 5 day old infarct\textsuperscript{26}. Also, structural complexities anchor reentry in normoxic canine intraventricular septum\textsuperscript{27}. We found that lines of block occurred parallel to the fiber direction and that in subsequent recordings the location of the lines of block remained the same. This suggests a structural origin for the conduction slowing. We did not investigate the nature of such structural changes, but speculate that several mechanisms might underlie this phenomenon. First, epicardially running small blood vessels can become a line of block when the midmyocardium becomes inexcitable. Second, enhanced anisotropy can lead to load mismatch and preferential activation around a line of block\textsuperscript{28}. Enhanced anisotropy and redistribution of gap junctions have indeed been demonstrated in the 5 day infarct model\textsuperscript{29}. Third, residual coupling at the location of the line of block might be better, whereby the well coupled tissue is depressed by the severely depolarized midmyocardium\textsuperscript{30}. In these cases, no influence of premature stimulation on size and location of block is expected.

In addition, we also found purely functional lines of conduction block that were absent during basic stimulation, but could be provoked by premature stimulation. In this situation, local differences in refractory periods are the most likely mechanism.

The functional role of these lines of conduction block in combination with the absence of sustained VF in the isolated left ventricular preparations, strongly suggest that although reentrant beats that could initiate sustained arrhythmias can arise in the ischemic zone, the presence of the non ischemic zone in the intact regionally ischemic heart is required for maintenance of sustained VF.

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