Genesis of life-threatening ventricular arrhythmias during the delayed phase of acute myocardial ischemia. Role of cellular electrical coupling and myocardial heterogeneities

de Groot, J.R.

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EXPERIMENTAL MODELS, ANIMALS, MATERIALS, AND METHODS

Joris R. de Groot
Anesthesia and Surgical Procedures.

All of the described procedures were approved by the institutional ethical committee on animal research and conformed to the Guide for Care and Use of Laboratory Animals (NIH publication No 85-23, revised 1996).

**PIGS**

Pigs of either sex, weighing 18-25 kg, were premedicated with azaperone (200 mg i.m. Janssen-Cilag) and metomidate (100 mg i.m.) or with ketamine (350 mg i.m. Eurovet), azaperone (80 mg i.m.) and atropine (0.5 mg i.m. Eurovet) and anaesthetized with pentobarbital (30 mg/kg i.v. Sanofi). After endotracheal intubation artificial ventilation with room air was maintained.

**Isolated Langendorff perfused hearts**

Warmed (37°C) modified Tyrode's solution (composition in mmol/l: Na⁺ 157, K⁺ 4.7, Ca²⁺ 1.5, Mg²⁺ 0.7, H₂PO₄⁻ 0.5, Cl⁻ 137.6, HCO₃⁻ 28.0, glucose 11.0, dextran 4% and insulin 10E) was infused intravenously in a superficial vein. After a midsternal thoracotomy, heparin 5000 IU (Leo Pharmaceuticals) was injected intravenously. Approximately 1.5 liter of blood and Tyrode's mixture was collected from the anterior vena cava. After induction of ventricular fibrillation (VF) with direct current, the heart was rapidly excised and immersed in ice cold (4°C) Tyrode's solution. The aorta was cannulated and the heart was connected to the perfusion setup and retrogradely perfused with recirculating blood Tyrode's mixture. The perfusion setup consisted of a funnel that collected the effluent blood from the heart. A bubble oxygenator oxygenated the blood with O₂ 95%/CO₂ 5% and kept pH within physiological limits (7.35-7.45). A feedback controlled roller pump adjusted flow to a constant, preset pressure, usually chosen to maintain a flow at about 150 ml/min and pumped the blood through a feedback controlled heat exchanger to maintain temperature of the perfusate at 37°C. Myocardial temperature was kept within physiological limits during ischemia with an infrared lamp. A filter (pore size 40 micrometer) was interposed between the heat exchanger and the aortic cannula.

After connection of the heart to the perfusion setup, both ventricles were vented and the heart was allowed to regain a physiological temperature. Hearts were defibrillated with a DC shock. The left anterior descending artery was prepared free distal to the first diagonal branch and a suture was placed underneath it. The artery was occluded by gently lifting up the suture and placing an occluder across the vessel. Cyanotic discoloration after a control occlusion of 30 s defined the location of the ischemic border zone.

The heart was stimulated with rectangular current pulses of 1 ms duration at twice diastolic threshold through a pair of bipolar hook electrodes, inserted in the myocardium approximately 5 mm from the cyanotic border of the ischemic zone (visual inspection) with a Biotronik UHS 2000 stimulator. Typically, basic cycle length was 450 ms and premature stimuli were applied at a coupling interval just longer than the refractory period (1 ms accuracy). To prevent induction of IA ventricular arrhythmias¹², pacing was interrupted during the first 10 minutes of coronary occlusion. In case of induction of ventricular fibrillation, the heart was DC defibrillated, and allowed to recover for at least three minutes before premature stimulation resumed.

**Open chest in situ experiments**

After induction of anesthesia with pentobarbital as described above, anesthesia was maintained with isoflurane (1% Abbott) and sufentanyl (0.02 mg/hour continuous i.v. infusion,
Janssen-Cilag) was used as analgetic. A saline filled catheter was advanced through the right carotid artery to measure aortic blood pressure continuously. Lead II of the ECG was monitored continuously. The chest was opened after a bolus injection of sufentanyl (0.025 mg) via a midsternal thoracotomy. Body temperature was kept within physiological limits with a heated pad and an infrared lamp. The heart was suspended in a pericardial cradle, and the left anterior descending artery was isolated and ligated as described above. The right atrium was stimulated through a pair of bipolar hook electrodes with a Biotronik UHS 2000 stimulator with rectangular current pulses of 1 ms duration at a basic cycle length of 450 or 500 ms.

Superfused left ventricular preparations

Following the procedures described above in the "Anesthesia and surgical procedures" and "Isolated Langendorff perfused hearts" sections, the heart was immersed in cold Tyrode's solution. The left ventricle was cut open parallel to the left anterior descending artery and a square shaped transmural biopsy (typically 4 cm²) was cut from the left ventricular free wall. The papillary muscle was removed and a thin cut was made with a dermatome parallel to the epicardial surface. 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 left ventricular preparation was pinned on the silicon base of a heated (37°C) tissue chamber and superfused with warmed Tyrode's solution (37°C), such that the midmural part of the preparation became ischemic and the epicardium remained well oxygenated. A bipolar hook electrode was introduced in the thin epicardial sheet and the preparations were stimulated with a Biotronik UHS 2000 stimulator with rectangular current pulses of 1 ms duration at a basic cycle length of 450 ms. Ten to 15 minutes after start of the superfusion, programmed stimulation with up to 3 premature beats as shortly coupled as possible (1 ms accuracy) was begun.

RABBITS

Induction of hypertrophy

New Zealand white rabbits of either sex were premedicated with intramuscular ketamine 200 mg (Eurovet) and xylazine 20 mg (Bayer). An i.v. cannula was introduced in the ear vein. Atracuriumdibesilate 1.5 mg (Glaxo Wellcome) was given i.v. as a muscular relaxant. After endotracheal intubation, the chest was opened via a left lateral thoracotomy and one or two ribs were removed. The heart was exposed by gently pushing away the lungs overlying the pericardium. A prolene 6-0 suture with an atraumatic needle was jabbed through the pericardium and the myocardium around the large marginal branch of the circumflex artery close to its origin from the left main coronary artery. The suture was tied around the artery ten minutes after administration of 6 mg of lidocaine (Fresenius) i.v., and the presence of ischemia was confirmed by the cyanotic discoloration of part of the left ventricular free wall. The chest was left open for 60 minutes to allow immediate resuscitation in case of ischemia induced ventricular arrhythmias. After 60 minutes, the chest was closed in layers. The animals received antibiotics (Baytril 17.5 mg i.m. Bayer) and analgetics (Temgesic 0.09 mg, i.m. Schering-Plough) during seven days following the operation. After 8-16 months the animals were sacrificed and myocytes were isolated as described below.
Isolation of left ventricular myocytes

Control rabbits and rabbits with left ventricular hypertrophy (induced as described above) were anesthetized with intravenous administration of pentobarbital 100 mg/kg (Sanofi) and heparinized (1000 IU, i.v., Leo Pharmaceuticals). The chest was opened and the heart was extirpated and immersed in cold Tyrode's solution (composition see below).

Left ventricular myocytes were isolated as described previously. In short, the aorta was cannulated and the heart was perfused at 37°C with modified Tyrode's solution with the following composition in mmol/l: Na\(^+\) 155.5, K\(^+\) 4.7, Ca\(^2+\) 1.45, Mg\(^2+\) 0.6, PO\(_4\)\(^3-\) 0.4, Cl\(^-\) 136.5, HCO\(_3\)\(^-\) 27.0, glucose 11.0. After 15 minutes, perfusion was changed to a nominally calcium free medium containing HEPES 16.8, Na\(^+\) 155, K\(^+\) 4.7, Ca\(^2+\) 0.01, Mg\(^2+\) 2.0, PO\(_4\)\(^3-\) 1.4, Cl\(^-\) 149, HCO\(_3\)\(^-\) 4.3, glucose 11.0, and pressure was adjusted to 50 mm Hg. After 15 minutes, collagenase, hyaluronidase and trypsin inhibitor were added to the perfusion fluid, and perfusion was maintained until pressure had decreased to 0 mm Hg. After removal of the infarcted tissue and the right ventricle, the left ventricle was chopped up in pieces and shaken in a Gyrotory water bath shaker. Cells were allowed to sediment for 10 minutes, after which they were resuspended in HEPES buffer with albumin (1% w/v) and [Ca\(^2+\)] of 1.3 mmol/L. Cells were stored at room temperature in vials with 3 ml HEPES solution with albumin.

Experimental procedures and protocols.

Extracellular electrograms and activation maps

Unipolar epicardial extracellular electrograms were recorded from different composite electrodes. Sample frequency was 1 or 2 kHz, and signals were 40x amplified, filtered (low pass 0.16 Hz (3 dB point), high pass 1 kHz (3 dB point)), and stored on the hard disk of a personal computer for off line analysis. The steepest slope of the intrinsic deflection of the unipolar extracellular electrogram reflects the moment of local activation. Time between stimulus artifact and intrinsic deflection, the activation time, was measured for each electrode with the use of an interactive computer program. Activation times were plotted in the same matrix and isochrones were drawn between sites with equal activation times. Such maps were constructed with a custom-made computer program, using the MATLAB\textsuperscript{®} library.

Several multi electrodes were used. An 11x11 matrix, interelectrode distance 2 mm and a 9x12 matrix (interelectrode distance 1 mm) of stainless steel electrodes were used in the experiments described in chapter 3. The latter multi electrode was also used in the studies described in chapter 5. In that chapter, also a silver multi electrode, 11x14 matrix, interelectrode distance 1 mm was used for determination of conduction velocities. For the experiments described in chapter 7, an 11x11 matrix of stainless steel electrodes with interelectrode distance 5 mm was used.

Bipolar electrograms were constructed offline by subtracting two adjacent unipolar electrograms from each other. Conduction velocity was measured from activation maps that were constructed from pacing from a central terminal of the composite electrode. Hence, the propagation in longitudinal and transversal direction can be determined.

Induction of ventricular fibrillation

Starting 10 minutes after coronary occlusion in pigs or after submerging the porcine left ventricular preparation in the tissue bath, programmed stimulation was begun. From a bipolar hook electrode, inserted in the non-ischemic tissue close to the ischemic border zone rectangular current
pulses of twice diastolic stimulation threshold and 2 ms duration were applied from a Biotronik
UHS 2000 stimulator unit. Eight stimulated beats (at basic cycle length of 380-450 ms) were
followed by up to three premature stimuli with the shortest possible coupling interval (1 ms
accuracy). In subsequent cycles, the number of premature stimuli was stepwise increased until
ventricular fibrillation occurred or three premature beats failed to induce ventricular fibrillation.

Ventricular fibrillation was defined as non self terminating, irregular, polymorphic ventricular
tachycardia diagnosed from the extracellular electrograms, and was terminated with DC
defibrillation. Sustained ventricular tachycardia was defined as a sustained (>30 s duration) regular,
monomorphic ventricular rhythm. Refractory period denotes the shortest possible coupling interval
at which capture occurred.

Theoretical background of tissue impedance measurements

Tissue impedance (Rt) is defined as the voltage difference across the measured area divided
by the current applied,

\[ Z = \frac{V}{I} (\Omega) \]  \hspace{1cm} (1)

Where Z denotes impedance (\( \Omega \)), V is voltage (V) and I is current (A). Myocardium is composed of
both resistive and capacitive components, thus when an alternating current is applied, the voltage
difference is measured with a time delay causing a shift in phase angle, \( \phi (°) \), in which case the
impedance Z is a complex number,

\[ Z = R + jX (\Omega \cdot \text{cm}) \]  \hspace{1cm} (2)

where R is the resistance (\( \Omega \)), j is the imaginary unit (-1 exp 0.5) and X is the reactance, the
quadrature of voltage (V) and current (I). The resistive part of the myocardial impedance is
composed of intracellular and extracellular resistance. Changes in resistance in either of these
compartments that occur during ischemia, such as the collaps of the extracellular space and the rise
in intracellular resistance through the closure of the gap junctions, cause an increase in total
impedance. The reactive part of myocardium consists of the capacitors formed by the lipid bilayer.
The increase in \( \phi \) during ischemia reported by some authors indeed indicates changes in the
capacitive properties of myocardial membranes6.

Measurement of tissue impedance with the four electrode technique has been studied by
Rush et al7. The theoretical basis for using the four electrode technique in cardiac tissue which
constitutes an anisotropic bidomain, has been provided by Plonsey and Barr8. Fleischhauer et al.
demonstrated in the rabbit papillary muscle, in which cable analysis applies, that reality is more
complex: the vascular compartment is to a large extent electrically insulated from the interstitial
compartment. Thus, the heart is not a bidomain but consists from at least three different conductive
compartments9.

Tissue impedance measurements

In this study impedance was measured in pigs with a linear array of four equally spaced
platinum pins of 0.7 mm diameter, 5 mm length and an inter electrode distance of 2 mm. The
proximal ends of the pins were electrically insulated to prevent short-circuiting. The arrays were
introduced in the heart perpendicular to the epicardial surface and secured with two 3.0 mersilene
sutures. The outer electrodes in this electrode configuration used can be regarded as point sources
for current delivery10,11, epicardial insulation confines the measurement to the midmyocardium.
Alternating current of 30 \( \mu \text{A} \) amplitude and a frequency of 1 kHz was applied between the outer
two electrodes of the array. The voltage difference was measured between the inner two electrodes,
and impedance was calculated via Ohm's law. Measurement were taken automatically every 30
seconds, and stored on the hard disk of a personal computer.
The system was calibrated in 0.9% sodium chloride solution with known resistivity (65 \( \Omega \cdot \text{cm} \)). Since the individual electrodes had different resistive properties due to small differences in electrode surface, values reported are normalized to control values for each individual electrode. After insertion of the electrodes, the heart was allowed to recover for at least 30 minutes. Impedance was constant during this equilibration period. Since the absolute increase in tissue resistance depends on the location within the ischemic zone\(^{12}\), relative rise in tissue resistance was calculated by:

\[
\Delta R_t = (R_{t_{\text{meas}}} - R_{t_{\text{con}}}) / (R_{t_{\text{con}}} - R_{t_{\text{inf}}})
\]

in which \( R_{t_{\text{meas}}} \) is the measured \( R_t \) value, \( R_{t_{\text{con}}} \) is the control \( R_t \) measurement and \( R_{t_{\text{inf}}} \) is \( R_t \) at 90 minutes coronary occlusion. This equation returns a value between 0 (control) and 1 (90 minutes occlusion). Consequently, when impedance does not change as in the non-ischemic tissue, \( \Delta R_t \) remains zero. During the course of ischemia, \( R_t \) rises in a biphasic pattern within the ischemic zone. An instantaneous small increase occurs immediately after coronary occlusion as a consequence of the collapse of the vascular space\(^{13}\), whereas a second rise approximately 15 minutes after occlusion indicates the onset of cellular uncoupling. Onset of second rise was measured by calculating the maximal value of the first derivative of the \( R_t \) recording. Electrodes with drifting values or scattering of the measurements were excluded from analysis.

**Biochemical and histological analysis**

After completion of the electrophysiological experiment (after 60 or 90 minutes of ischemia), pig hearts were cut in approximately 1 cm thick slices from apex to basis. Slices were submerged for 8 minutes in 150 ml phosphate buffer with 2,3,5-triphenyltetrazoliumchloride (composition in mmol/L: Na\(_2\)H\(_2\)PO\(_4\) 2H\(_2\)O 67.1, Na\(_2\)HPO\(_4\) 2H\(_2\)O 142, 2,3,5-triphenyltetrazolium chloride 6.3, room temperature) to inspect the presence of LDH, a measure of cell integrity, in the subepicardial and subendocardial layers\(^{14,15}\). The reaction was terminated by rinsing the slices in cold saline after which they were stored in formaldehyde. Then, tissues were photographed and a transmural biopsy of approximately 1 cm\(^3\) was taken from the centrally ischemic part of two slices from each of these hearts. From these parts, 7 \( \mu \)m thick micro sections were stained for the presence of glycogen with periodic acid Schiff (PAS)\(^{16}\). In all sections, epicardium and endocardium were clearly discernable. To quantify the amount of viable tissue, we investigated four randomly chosen sections from the ischemic zone of each heart studied (100x magnification). Total subepicardial surface and subendocardium with glycogen within the section were measured with a vernier. The size of glycogen containing subepicardium was divided by the total subepicardial surface; thus, a percentage is expressed of total subepicardial surface within the section. Hence, a semi quantitative estimate of the percentage of glycogen containing subepicardium is derived.

**Potentiometric measurement of action potentials**

Langendorff, blood perfused pig hearts were electro-mechanically uncoupled with diacetylmonoamine (DAM) (15 mmol/l) and loaded with the voltage sensitive dye di-4-ANEPPS (15 \( \mu \)g/ml). The optical setup consisted of a video imaging system and was described previously\(^{17}\). Images (64x64 pixels) were acquired at a frame rate of 300 or 400 frames per second, and background fluorescence was subtracted from each frame. A 12-bit CCD camera (DALSA, CA-D1-0128T-STDL) was connected via a frame grabber (RoadRunner Model 24) to a Pentium II personal computer.

Tissue was illuminated with bandpass-filtered excitation light (520±30 nm) from two 250W tungsten-halogen light sources. Emitted light was transmitted to an emission filter (640±50 nm) and projected onto the video camera. Typically, movies of 10 second duration were recorded. Upon offline analysis, spatial and temporal filtering was applied, after which the spatial resolution was between 0.3-0.9 mm. For further improvement of the signal to noise ratio in the analysis of APD
and conduction velocity, up to 10 action potentials during basic pacing were averaged after confirmation that repolarization alternans was absent.

Analysis was performed with a custom-made analysis program based on the PV-Wave® library. Optical action potential duration was calculated at 70% repolarization. Activation moments were automatically determined at 50% of the amplitude of the upstroke of the action potential. From the subsequently constructed activation maps, local conduction velocity was measured as described above, in the direction where the spacing of isochrones was largest, thus conduction velocity was maximal, before occlusion.

Phase maps were produced as described previously. In short, the fluorescence of each pixel was plotted against the fluorescence of that same pixel offset by a time interval earlier. The angle of the coordinate of the fluorescence at both time points around the mean fluorescence describes the phase, \( \theta(t) \), with values between -\( \pi \) and \( \pi \). A new field \( \theta(x,y,t) \) was constructed with all pixels. Phase singularities were detected as described previously.

Ventricular fibrillation was induced by bringing the poles of a 9 V battery in contact with the ventricular myocardium, distant from the mapped area. With Fast Fourier transform, the dominant frequency of ventricular fibrillation was calculated, as described previously. Also, the number of epicardial breakthrough activations and the life span of phase singularity points was measured from phase maps.

Microelectrode measurement of action potentials

Action potentials from hypertrophic rabbit myocytes, normal rabbit myocytes and normal rabbit cell pairs were measured with the perforated patch clamp technique at 37°C. Pipettes were pulled from borosilicate glass and heat polished. The pipette solution consisted from (mmol/l): HEPES 16.8, K⁺ 140, Na⁺ 10, Ca²⁺ 0.01, Mg²⁺ 2, Cl⁻ 149.7, HCO₃⁻ 4.3, PO₄³⁻ 1.4, EGTA 0.1, glucose 11. pH was adjusted to 7.1 with KOH. The bath solution consisted from (mmol/l): HEPES 16.8, Na⁺ 155, K⁺ 4.7, Ca²⁺ 2.6, Mg²⁺ 2.0, PO₄³⁻ 1.4, Cl⁻ 149, HCO₃⁻ 4.3, glucose 11.0. Metabolic inhibition was produced as described below. Recordings were filtered on line (1 kHz), digitized at 2 kHz and stored on the hard disk of a personal computer for off line analysis. Action potentials were elicited at 2 Hz by 2 ms current pulses (1.5x diastolic threshold). Cell capacitance was calculated as described previously. No correction for the liquid junction potential was made.

Measurement of cytosolic calcium and rigor

Isolation of rabbit ventricular myocytes and intracellular calcium measurements have been described previously. In short, myocytes were loaded with the acetoxymethyl ester of Indo-1 (5 \( \mu \)mol/l, Molecular Probes) during 30 minutes, after which they were washed with fresh HEPES buffer without albumine. All procedures were performed at 37°C. Only rod shaped myocytes with clear striations (yield typically around 75%) were selected for measurements. Cells were attached to a poly-D-lysine coated (0.1 g/l) glass cover slip, placed upon a temperature controlled stage (37°C) of an inverted fluorescence microscope (Nikon Diaphot). A temperature controlled perfusion chamber (volume 30 \( \mu \)l) was tightly positioned over the glass cover slip. Chamber content could be changed completely within 0.1 second. Myocytes were superfused with HEPES solution containing \([Ca^{2+}]\) of 2.6 mmol/l. Field stimulation was applied with bipolar rectangular current pulses (2 Hz, 40 V/cm, 200\( \mu \)s duration) through two platinum electrodes positioned parallel to the chamber at 8 mm distance. Single myocytes or myocyte pairs were selected with top illumination, and a rectangular diaphragm was set to the measurement area. In cell pairs, de combined fluorescence of both myocytes was recorded. Cells were excited at 340 nm and Indo-1 fluorescence was recorded in dual emission ratio mode at 410 and 516 nm at 1 kHz sampling rate and stored on the hard disk of a personal computer for offline analysis. Upon offline analysis, recordings were corrected for
background fluorescence and for auto fluorescence. The moment of terminal rise in cytosolic calcium was arbitrarily defined as a 10% increase in diastolic calcium over the mean diastolic calcium during the first 10 minutes of metabolic inhibition.

**Induction of metabolic inhibition**

After control recordings were obtained, rabbit myocytes were superfused with warmed (37°C), normoxic HEPES solution, containing 2.6 mmol/l Ca²⁺, 2 mmol/l NaCN and no glucose. In some normal myocytes glycolysis was blocked by adding 1 mmol/l iodoacetate to the NaCN containing HEPES solution. By blocking glycolysis the contribution of the anaerobic reserve, defined as the glycogen content, on differences in time to rigor among cells could be investigated. To compare differences in time to rigor between two electrically coupled paired cells with single myocytes, we determined differences in time to rigor between the arbitrarily chosen groups of even and odd numbered single myocytes.

During the application of metabolic inhibition, 5 second recordings of Indo-1 fluorescence were taken every 2 minutes. Myocyte shape was monitored visually between recordings. Occurrence of rigor was defined as the rapid change in shape from rod shaped into square or round shaped, which occurred within seconds. The measurement area was adjusted to the new cell dimensions after rigor development.

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