Voltage-sensitive dye mapping in Langendorff-perfused rat hearts

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Voltage-sensitive dye mapping in Langendorff-perfused rat hearts. Am J Physiol Heart Circ Physiol 284: H892–H902, 2003. First published November 7, 2002; 10.1152/ajpheart.00648.2002.—An imaging system suitable for recordings from Langendorff-perfused rat hearts using the voltage-sensitive dye 4-[2-(di-n-butylamino)-6-naphthyl]vinyl]pyridinium (di-4-ANEPPS) has been developed. Conduction velocity was measured under hyper- and hypokalemic conditions, as well as at physiological and reduced temperature. Elevation of extracellular [K⁺] to 9 mM from 5.9 mM caused a slowing of conduction velocity from 0.66 ± 0.08 to 0.43 ± 0.07 mm/ms (35%), and reduction of the temperature to 32°C from 37°C caused a slowing from 0.64 ± 0.07 to 0.46 ± 0.05 mm/ms (28%). Ventricular activation patterns in sinus rhythm showed areas of early activation (breakthrough) in both the right and left ventricle, with breakthrough at a site near the apex of the right ventricle usually occurring first. The effects of mechanically immobilizing the preparation to reduce motion artifact were also characterized. Activation patterns in epicardially paced rhythm were insensitive to this procedure over the range of applied force tested. In sinus rhythm, however, a relatively large immobilizing force caused prolonged PQ intervals as well as altered ventricular activation patterns. The time-dependent effects of the dye on the rat heart were characterized and include 1) a transient vasoconstriction at the onset of dye perfusion and 2) a long-lasting prolongation of the PQ interval of the electrocardiogram, frequently resulting in brief episodes of atrio-ventricular block.

MAPPING OF CARDIAC ELECTRICAL ACTIVITY using voltage-sensitive dyes has proven to be a valuable complement to extracellular electrode-based mapping. Recently, this approach has yielded important new information on normal and abnormal activation patterns in hearts from a variety of species (1, 10, 27, 39, 40). The voltage-sensitive dye approach offers the possibility of obtaining very high spatial resolution without the methodological complications involved in fabricating large high-resolution electrode arrays (12, 21). This is a particular advantage for recordings in small preparations, such as the hearts of rodents. In addition, this approach provides a direct measurement of the transmembrane voltage rather than extracellular potentials.

The increasing use of genetically engineered murine models in cardiovascular studies has resulted in a significant need for quantitative, reproducible techniques for evaluating the electrophysiological properties of the mouse heart. Voltage-sensitive dye mapping has proven to be a very useful tool in this context (1, 26, 27, 36). Although at present the mouse is the predominant experimental animal for genetically engineered models, there is an increasing interest in utilizing larger animals such as the rabbit (22) and the rat (8, 29). Voltage-sensitive dye mapping has been applied to cultured monolayers of rat cardiac cells (7, 30); however, it has not been applied to the rat heart in vitro. The rat heart is a widely used model in cardiac electrophysiological and hemodynamic studies. Rapid progress on the rat genome (13, 23) makes it likely that the use of genetically engineered rat models will increase substantially in the near future. Accordingly, our goal was to develop a voltage-sensitive dye-imaging system suitable for recordings from the Langendorff-perfused rat heart. This report describes the successful implementation of this technique with emphasis on issues that are specific to the rat heart. We present activation patterns in paced and sinus rhythm and also illustrate present limitations. We have also evaluated the effects of a commonly used method of reducing motion artifacts: mechanical immobilization of the heart by pressing it against the front glass of the imaging chamber.

METHODS

Experimental Preparation and Recording Methods

All experiments followed the guidelines of the Canadian Council for Animal Care. Rat hearts were isolated using a procedure very similar to that previously described for mouse hearts (18, 27). Male Sprague-Dawley rats (250–300 g) were injected with 300 U ip of heparin 15 min before the heart was isolated. The animals were anesthetized with isoflurane and euthanized by cervical dislocation, after which the heart was immediately excised and placed in ice-cold Krebs-Henseleit solution. After removal of extraneous tissues, the aorta was

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cannulated, and perfusion with Krebs-Henseleit solution (32°C) was started at a constant flow rate of 13 ml/min. Perfusion pressure in the cannula was monitored with the use of a pressure transducer (Harvard Apparatus; Saint Laurent, Quebec, Canada) and recorded throughout the experiment. The Krebs-Henseleit buffer consisted of (in mM) 118.0 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 1.0 CaCl₂, 25.0 NaHCO₃, and 11.1 glucose and bubbled with 95% O₂-5% CO₂ in a 37°C water bath for a pH of 7.4. All solutions were filtered using a 5-μm pore membrane filter (Millipore; Bedford, MA). The Krebs-Henseleit solution passed through a glass heating coil (Radnoti Glass Technology; Monrovia, CA) immediately before reaching the heart, and the heart itself was immersed in solution in a water-jacketed chamber throughout these recordings. The heating coil and water-jacketed chamber were warmed by using a thermocirculator (Harvard Apparatus). The temperature in the bath near the heart was monitored by using a thermocouple thermometer (Harvard Apparatus). The temperature in the bath near the heart was allowed time to recover from any AV block produced by the dye, and the perfusion (and bath) temperature was then slowly raised to 37°C before recordings were begun (unless otherwise noted).

**Experimental Protocol**

After perfusion was begun and the pacing and ECG electrodes were attached, the heart was immersed in the water-jacketed recording chamber and maintained at 32°C. A 30-min stabilization period followed during which the perfusion pressure and ECG was monitored and recorded. The solution was then switched to one containing 1 μM 4-[6-[2-[n-butylamino]-6-naphthyl]vinyl]pyridinium (di-4-ANEPPS, Molecular Probes; Portland, OR) for a period of 5 min. Di-4-ANEPPS was prepared as 10 mM stock solution in DMSO (stored frozen) and added to the standard Krebs-Henseleit solution (0.01% DMSO in the final solution). The concentration of di-4-ANEPPS was arrived at by trial and error: higher concentrations often evoked significant arrhythmias (ventricular tachycardia or fibrillation). We found it necessary to carry out the dye perfusion (loading) at 32°C because attempts to perfuse the dye at 37°C resulted in irrecoverable arrhythmias even at the 1 μM dye concentration. In most experiments, the PQ interval lengthened substantially during the dye perfusion, often resulting in a brief episode of some degree of atrioventricular (AV) block.

Once the dye-loading procedure was completed, the heart was perfused with the standard Krebs-Henseleit solution for the remainder of the experiment unless otherwise noted. The heart was allowed time to recover from any AV block produced by the dye, and the perfusion (and bath) temperature was then slowly raised to 37°C before recordings were begun (unless otherwise noted).

**Imaging System**

**Illumination.** Figure 2 is a block diagram illustrating the main features of this imaging system. Illumination is provided by a 250-W Quartz Tungsten Halogen light source (Oriel Instruments; Stratford, CT). The output from the light...
source is reflected off a cold mirror and filtered with a 500 ± 25 nm interference bandpass filter (Omega Optical; Brattleboro, VT). The resulting light is then directed onto the preparation using a dichroic mirror (Omega Optical). A shutter (Oriel Instruments), automatically controlled by the acquisition software, opens immediately before and closes immediately after image acquisition, thus minimizing the light exposure of the preparation. The light emitted from the preparation passes through the dichroic mirror and a long-pass (>590 nm) Schott glass filter (Melles Griot Canada; Ottawa, Ontario, Canada) before reaching the camera.

Image acquisition. This system is built around the Dalsa CA-D1–0128T camera (Dalsa; Waterloo, Ontario, Canada), used in a binning mode, resulting in 60 × 60 pixels acquired with 12-bit resolution at 950 frames/s. Equipped with a 25-mm focal length lens (CF25L, Fujinon; Wayne, NJ) and a 3-mm spacer, the camera produces a 15 × 15 mm field of view (250 × 250 μm/pixel). The camera is connected to a National Instruments PCI-1422 image acquisition board (National Instruments; Austin, TX) in a Dell Precision 420 Pentium III/800 MHz workstation equipped with 1 GB of random access memory (RAM) running Windows NT operating system (Microsoft; Redmond, WA). Image acquisition software written in LabVIEW (National Instruments; Austin, TX) acquires the ~7 MB/s data stream, which is held in RAM during the acquisition and subsequently written to hard disk.

Other data acquisition. ECG data, bath temperature, pressure, and the trigger signal for the stimulator are all recorded by using a National Instruments PCI-7030/6030E real-time data acquisition board. Because of its on-board processing capability, this board can handle data acquisition independently of the host computer for short periods of time. Temporarily storing the data in on-board memory allows the host computer to focus exclusively on the data-intensive image acquisition when necessary and thus prevents loss of data. Data acquisition software was written in LabVIEW RT and integrated into a single-user interface with the image acquisition. Synchronization of the image data to the ECG and stimulus data was achieved by 1) using the “frame valid” camera signal to clock data acquisition so that exactly one ECG sample was acquired for each image frame, and 2) recording the “acquisition in progress” signal from the image acquisition board so that the exact ECG/stimulus sample corresponding to the first image frame acquired could be identified. This approach resulted in reliable synchronization of the ECG and stimulus data and allowed these signals to be used as a reference for signal averaging (see Data Processing).

Data Processing

Data were processed offline using software developed in the interactive data language development environment (Research Systems; Boulder, CO), with external routines written in C++ (Visual C++, Microsoft) for the most computationally demanding tasks. The data recorded for each individual pixel were processed as follows: 1) the background fluorescence level was subtracted from the signal, 2) any linear trend in the data was removed, and 3) the sign of the data was changed so that a depolarization corresponded to a positive change in the signal. Areas of the field of view not covering the preparation were manually masked and excluded from further analysis. The activation time for each pixel was detected as the time of maximum rate of rise of the fluorescence signal (27). Activation maps were computed for individual cycles with activation times referenced to the stimulus pulse (paced rhythms) or the R (or S) wave of the ECG (sinus rhythm). Signal-averaged activation maps were then computed by averaging the activation times for individual pixels over all cycles. The analysis software allowed the operator to view the activation map for each individual cycle before including it in the average to ensure that the activation pattern remained constant from cycle to cycle. The activation maps shown in this paper are signal averaged over 5 s of recording (20–25 cycles). Isochrones were computed from the signal-averaged maps using a built-in contour plot routine in interactive data language. Local conduction velocity vectors were computed from the slope of a least-squares fit plane to 7 × 7 pixel areas of the activation maps as described previously (26, 27). To compare the conduction velocity in a preparation in paced rhythm under different conditions, a 15 × 15 pixel area was selected, and the average magnitude of the local conduction velocities within this area was computed. The following criteria were used to select this area: 1) the area was selected near the center of the imaging field where the quality of the signal was usually the best (less motion artifact due to firm contact with the recording chamber window), 2) the immediate vicinity of the stimulus location was avoided (to reduce influence of stimulus artifacts), and 3) the area was selected so that the conduction pattern within the area was approximately one dimension (isochrones straight and parallel). Although the process of manually selecting a subregion for analysis is somewhat arbitrary, in practice these criteria usually determined the placement of the region to within a few pixels.

Data are presented as means ± SE. Hypothesis testing was carried out by using unpaired or paired t-tests as appropriate in cases with only two groups and ANOVA or repeated measures ANOVA followed by the Holm t-test for three or more groups. A P value of <0.05 was considered statistically significant.

RESULTS

Di-4-ANEPPS Interferes With AV Conduction

As mentioned in METHODS, hearts frequently exhibited PQ interval prolongation and transient episodes of AV block in response to dye application via coronary perfusion. Figure 3A shows PQ intervals measured in eight hearts at three points during the study: 15 min into the stabilization period, immediately before dye perfusion (i.e., after ~30 min of stabilization), and immediately on return to sinus rhythm after completion of the dye perfusion. The PQ interval was prolonged by 38% after dye perfusion (53.66 ± 2.22 ms before dye perfusion and 74.06 ± 4.86 ms after dye perfusion). For comparison, we also measured the heart rate (RR interval) in the same eight hearts and at the same time points as in Fig. 3A. As shown in Fig. 3B, there was no statistically significant effect of di-4-ANEPPS on RR interval. The fact that the temperature in our experiments was increased from 32°C to 37°C after the dye perfusion made it difficult to quantify the time course of recovery from the PQ prolongation caused by the dye. When a temperature of 37°C was reached, the PQ interval was 51.94 ± 2.14 ms. For comparison, PQ intervals measured in three hearts subjected to solvent (0.01% DMSO) perfusion only were 51.18 ± 2.02 ms before DMSO perfusion (32°C), 49.90 ± 0.96 ms after DMSO perfusion (32°C), no significant difference), and 41.01 ± 0.57 ms on reaching a
temperature of 37°C. Thus the PQ interval was prolonged after dye perfusion both at 32°C and upon warming to 37°C, and this prolongation was due to di-4-ANEPPS and not to DMSO. The PQ interval recovered slowly from this prolongation, but the time course was so slow that it was impractical to wait for a complete recovery.

We also measured PQ intervals before and after dye perfusion in five hearts perfused with Krebs solution containing 2 mM Ca\(^{2+}\) rather than 1 mM. The higher Ca\(^{2+}\) concentration resulted in a smaller, but still statistically significant, PQ interval prolongation of 10% (50.74 ± 1.99 ms before dye perfusion and 56.05 ± 3.05 ms after dye perfusion). The hearts used for this series of measurements were part of a pilot study for a different project, which used pentobarbital rather than isoflurane anesthesia to isolate the hearts. To rule out the possibility that the choice of anesthesia influenced the result, we performed the following control experiments. First, PQ intervals were measured in one heart by using pentobarbital anesthesia and our normal (1 mM [Ca\(^{2+}\)]) Krebs solution. This yielded the normal, pronounced response to the dye (PQ intervals of 54.46 ms before and 87.23 ms after dye perfusion), demonstrating that the response was not suppressed by pentobarbital anesthesia. Second, PQ intervals were measured in one heart isolated by using isoflurane anesthesia and perfused using 2 mM [Ca\(^{2+}\)] Krebs solution. Consistent with the other five hearts perfused with 2 mM [Ca\(^{2+}\)], this yielded a much smaller PQ prolongation in response to dye perfusion (from 44.20 to 48.83 ms). To determine whether the attenuation of the dye effects observed with 2 mM [Ca\(^{2+}\)] could be reproduced using a different divalent cation, we measured PQ intervals in five hearts (isolated using isoflurane anesthesia) perfused with Krebs solution containing our normal 1 mM [Ca\(^{2+}\)] but with [Mg\(^{2+}\)] increased to 2.2 mM by addition of 1 mM MgCl\(_2\). PQ intervals in these hearts were 61.24 ± 2.17 ms before and 79.45 ± 5.37 ms after dye perfusion. We therefore conclude that the observed modulation of the dye effect on the PQ interval is specific to Ca\(^{2+}\).

Di-4-ANEPPS Causes Transient Vasodilation

A transient drop in perfusion pressure of 27.4 ± 3.9 mmHg was consistently observed at the onset of dye perfusion. However, the perfusion pressure returned to normal levels within a few minutes, i.e., with a much more rapid time course than the PQ interval prolongation. In three hearts subjected to solvent (0.01% DMSO) perfusion alone, this response was reduced but not completely absent (5.3 ± 1.5 mmHg), suggesting that DMSO contributes to the response but is not solely responsible. In five hearts perfused with solution containing 2 mM Ca\(^{2+}\) rather than 1 mM, the transient pressure drop in response to dye perfusion was reduced to 8.8 ± 2.0 mmHg. The response of the perfusion pressure to different levels of extracellular [K\(^+\)]\(_o\) in the perfusate was also measured. Elevated [K\(^+\)]\(_o\) is known to cause vasodilation in rat coronary arteries (17, 31). Figure 4A shows the perfusion pressure at different time points during the study for four hearts subjected to three different K\(^+\) concentrations. Note that elevated [K\(^+\)]\(_o\) produces a drop in perfusion pressure similar to that induced by the di-4-ANEPPS. The first data point labeled 5.9 mM [K\(^+\)]\(_o\) was recorded immediately on reaching a temperature of 37°C and was quite variable compared with the other measurements (note the large means ± SE). Part of the reason for this variability may have been that some of the hearts had additional brief episodes of AV block while the temperature was being increased, and AV block tended to result in elevated perfusion pressures. The measurement of perfusion pressure at 5.9 mM [K\(^+\)]\(_o\) was therefore repeated at the end of the study to avoid any influence of these effects. The discrepancy between the perfusion pressures measured in 5.9 mM [K\(^+\)]\(_o\) at the beginning and at the end of the study (second data point labeled 5.9 mM [K\(^+\)]\(_o\)) is therefore likely due to a transient pressure overshoot during the initial mea-
measurement due to the recent increase in temperature from 32°C to 37°C.

Detection of Reduced Conduction Velocity Because of Elevated Extracellular \([K^+]_o\)

Figure 4B shows conduction velocities measured in the four hearts subjected to selected perfusate \([K^+]_o\) concentrations. An elevation of \([K^+]_o\) to 9 mM from the baseline of 5.9 mM causes a statistically significant 35% reduction in conduction velocity from 0.66 ± 0.08 to 0.43 ± 0.07 mm/ms as would be expected from the depolarization caused by this maneuver. Figure 4C and D, shows the isochronal activation maps for one of the four hearts at 5.9 mM (Fig. 4C) and 9 mM (Fig. 4D) \([K^+]_o\), illustrating the region used to estimate conduction velocity.

Detection of Reduced Conduction Velocity Because of Reduced Temperature

In four hearts, the conduction velocity was measured at 32°C before the temperature was raised to 37°C. The average conduction velocity at 32°C was 0.46 ± 0.05 mm/ms, and at 37°C conduction velocity measured 0.64 ± 0.07 mm/ms. Although both this result and the observations in 9 mM \([K^+]_o\) are expected, they form essential control data for future, more detailed, studies.

Epicardial Breakthrough Patterns in Sinus Rhythm

Activation patterns during sinus rhythm in the isolated rat heart generally were similar to those we have previously reported in the mouse heart (27), i.e., distinct areas of early activation ("breakthrough") could usually be identified toward the apex in both the left and right ventricles (Fig. 5A). Of the four hearts studied with different levels of applied immobilization force, three exhibited this type of activation pattern. In two of these hearts, breakthrough on the right ventricle clearly occurred before that on the left ventricle. In the remaining heart, right and left ventricular breakthrough occurred simultaneously (within the 1-ms time resolution of our recording system).

Effects of Immobilization Force

Quantitative measurement of activation in selected regions of the ventricle requires reduction of the mechanical activity of the heart. We evaluated the influence of applied force on these recordings by using a balloon force transducer, as described in methods (Fig. 1B). Recordings were obtained in paced rhythm as well as in sinus rhythm in four hearts at three different levels of applied force. These three levels of force corresponded to pressures of 20, 30, and 40 mmHg as measured by the balloon transducer. Assuming that the area of contact between the balloon and the heart can be approximated by a circle 12 mm in diameter (113 mm² area), this corresponds to applied forces of 0.30, 0.45, and 0.60 N, respectively. Recognizing that we are unable to accurately measure the area of contact between the balloon and the heart, we have chosen
to report the applied “force” in terms of the balloon pressure.

**Paced rhythm.** Figure 6B shows the average conduction velocity in the four hearts measured in the same manner as in Fig. 4. There was no significant difference between the conduction velocities measured at any of these three levels of immobilization force.

**Sinus rhythm.** Figure 6, C and D, shows RR and PQ intervals measured at the three levels of immobilization force. The solid symbols represent measurements taken immediately (within 30 s) before application of the immobilization force, and open symbols are measurements taken with the immobilization force applied during the acquisition of imaging data. The heart rate (RR intervals) was insensitive to immobilization force within the range studied; there was no significant difference in RR intervals before and after application of force at any of these three levels of force. In contrast, the PQ interval was affected by the two higher (30 and 40 mmHg) levels of applied force, where there was a significant lengthening (3–4 ms) after application of these forces. These measurements were made at 37°C, which accounts for most of the difference between the PQ intervals reported here and those in Fig. 3A (re-
corded at 32°C). The slight downward trend in the PQ intervals before force application in Fig. 6D (solid symbols) probably reflects gradual recovery from the PQ interval prolongation caused by di-4-ANEPPS, because these data were acquired in order of increasing force.

In two of three hearts that exhibited a sinus rhythm activation pattern with clearly identifiable breakthrough sites on both the right and left ventricle, increased immobilization force affected the activation pattern in the way shown in Fig. 5. Note that increasing the applied force appears to delay the site of early activation on the left ventricle compared with that on the right ventricle. In the remaining heart, the activation pattern was very nearly identical at all three levels of force. This heart also showed an activation pattern with nearly synchronous breakthrough on the right and left ventricles. The increase in PQ interval with increasing force was also less pronounced in this heart than in the others.

**DISCUSSION**

**Detection of Changes in Conduction Velocity in Paced Rhythm**

The dependence of conduction velocity on [K+]o in cardiac tissue is well known. In most cases, increases in [K+]o cause a slowing of conduction velocity (34), although in some species there is a range of slightly elevated [K+]o, which gives rise to small increases in conduction velocity (14, 28, 33). We have used this response to verify the ability of our system to detect changes in conduction velocity in the isolated Langendorff-perfused rat heart, as well as to demonstrate that our preparations exhibit a normal response to this well-characterized intervention. Specifically, the conduction velocity in a selected area of ventricular myocardium was measured in three different [K+]o in a set of four hearts as described in METHODS. The result, shown in Fig. 4B, is a statistically significant reduction in average conduction velocity from 0.66 ± 0.08 to 0.43 ± 0.07 mm/ms (35%) when [K+]o is increased from normal (5.9 mM) to 9 mM. This compares favorably with the data obtained by Smeets et al. (34) with extracellular electrode mapping in the rabbit ventricle: 0.62 mm/ms at [K+]o = 5.6 mM and 0.33 mm/ms at [K+]o = 9 mM. For comparison, Suzuki et al. (35) measured a conduction velocity of 0.39 mm/ms in the left ventricle of the in situ rat heart. However, it has been previously reported that the conduction velocities measured in isolated hearts exceed those measured in situ (5). In addition, it is not clear whether the measurements of Suzuki et al. (35) represent conduction along or across the fiber direction.

The conduction velocities at two different temperatures, 32°C and 37°C, in a set of four different hearts were also measured. These results yield conduction velocities of 0.46 ± 0.05 mm/ms and 0.64 ± 0.07 at 32°C and 37°C, respectively, or a Q10 factor (change in response to a 10°C change in temperature) of 1.9. For comparison, Smeets et al. (34) reported conduction velocities of 0.34 mm/ms at 37°C and 0.21 m/ms at 27°C, or a Q10 of 1.6.

These measurements of the [K+]o and temperature dependence of ventricular conduction velocity agree closely with published values from measurements using extracellular electrodes. These measurements therefore provide strong evidence that our instrumentation and overall experimental approach render useful physiological results. The relatively simplistic approach of evaluating the conduction velocity within a selected area was chosen deliberately to avoid complications caused by different fiber orientations in various regions of the left and right ventricle. Conduction within the boundaries of the analysis area (selected according to the criteria described in METHODS) moved with an approximately straight activation wavefront, i.e., the conduction was effectively having one dimension. This is appropriate and necessary for this purpose so that the data obtained can be directly compared with existing data from essentially one-dimensional preparations (14, 34). However, more sophisticated methods that take into account both conduction velocities and the direction of conduction (26, 27) will be necessary for other purposes, such as assessing anisotropy of conduction velocities in models of hypertrophy and/or fibrosis (37).

**Epicardial Breakthrough Patterns in Sinus Rhythm**

Activation patterns in sinus rhythm in the rat heart consistently gave rise to separate sites of epicardial breakthrough on the right and left ventricle, similar to our previous results in the mouse heart (27). Breakthrough occurred slightly earlier on the right ventricle epicardium than on the left ventricle. Studies in the human heart (5, 15) as well as in rodents (35, 36) have previously shown that this is the normal sequence of ventricular epicardial breakthrough. It is important to note, however, that the delay between right and left ventricular breakthrough measured in the rat heart in this study is on the order of 1 ms, i.e., at the limit of temporal resolution offered by our imaging system. Mapping studies in the human heart (5, 15) have demonstrated that the endocardial activation sequence is the reverse, i.e., that the left ventricular endocardium activates earlier than the right ventricular endocardium. The fact that right ventricular epicardial breakthrough precedes left ventricular breakthrough thus appears to be due to a longer transmural conduction time in the left ventricle, presumably due to the greater thickness of the left ventricular wall. The ventricular walls of the rat heart are of course much thinner than those of the human heart, and the difference in absolute wall thickness between the right ventricle and left ventricle is therefore correspondingly much smaller as well. With the assumption of a transmural conduction velocity of 0.4–0.5 mm/ms (5) and a difference in wall thickness on the order of 1–2 mm, the difference in transmural conduction time should be on the order of 2–4 ms. Right ventricular breakthrough occurring 1 ms before left ventricular breakthrough...
would thus be consistent with left ventricular endocardial activation 1–3 ms before right ventricular endocardial activation. These values seem plausible, given that in the human heart (5) left ventricular endocardial activation precedes right ventricular endocardial activation by 10–15 ms, but right ventricular epicardial breakthrough precedes left ventricular breakthrough by 5–10 ms or more.

**Mechanical Immobilization to Suppress Motion Artifacts**

Mechanical immobilization of the preparation is a frequently employed technique for suppressing motion artifacts in cardiac voltage-sensitive dye recordings (6, 19, 27). Generally, the preparation is pressed against a glass (Plexiglas) surface with sufficient force to constrain its movement. Whereas this approach is effective (6, 9, 19, 27), the effects of subjecting the heart to this applied force have not been characterized in detail. Therefore, recordings were made during paced rhythm as well as in sinus rhythm in four hearts subjected to three different levels of immobilizing force. Force was measured by a balloon pressure transducer (Fig. 1B) located on the paddle used to push the heart against the front window of the chamber. The three levels of applied force corresponded to 20, 30, and 40 mmHg pressure in the balloon transducer and were chosen so that the lowest applied force (20 mmHg) corresponded to the minimum required for successful recordings. No arrhythmias were observed as a result of application of immobilization force, indicating that the range of force tested was sufficiently low to avoid wall stress-induced arrhythmias (4).

**Paced rhythm.** For the recordings during paced rhythm, conduction velocity was measured in the same fashion as for the results described in the previous section. There was no significant effect of the applied force on the conduction velocity over the range tested. We conclude, therefore, that conduction velocity in rat ventricular myocardium in paced rhythm is relatively insensitive to applied force in the range required for immobilization under our conditions. Mechanical immobilization can thus be considered an appropriate method for reducing motion artifacts in this type of experiment. It must be noted, however, that we have not evaluated the possible effects of the applied force on action potential duration. In fact, this range of applied force under our conditions does not immobilize the ventricle to the extent that action potential durations can be measured reliably. Figure 6A shows a typical action potential recorded from rat ventricle. This action potential was recorded by using an immobilization force corresponding to 20-mmHg balloon pressure in paced rhythm (pacing site at the base of the left ventricle) and is an average of the action potentials recorded for 24 consecutive pacing cycles. The example in Fig. 6A shows that mechanical immobilization reduces motion to such an extent that activation can reliably be quantified. However, it is also clear that there is a remaining motion artifact, as evidenced by the “hump” during final repolarization, which prevents measurement of the action potential duration. That is, although the action potential morphology in Fig. 6A seems normal, its duration is \( \sim 100 \) ms, i.e., more than twice the expected duration of a rat ventricular action potential (38).

**Sinus rhythm.** In sinus rhythm, the rat heart is considerably more sensitive to the applied immobilization force. In particular, AV conduction is affected even by moderate amounts of applied force, which may be related to the presence of mechanoreceptors in the AV junction of the rat heart (24, 25). We characterized the effects of the applied force on the RR and PQ intervals of the ECG by comparing these parameters measured immediately before and during the recordings at each level of force. There was no significant effect on the RR intervals (Fig. 6C) over the range of applied force tested. In contrast, the PQ interval was quite sensitive to applied force, and it was only at the lowest level tested (20 mmHg pressure in the balloon transducer) that there was no significant effect of force on the PQ interval. The corresponding isochronal activation maps (Fig. 5) also changed in response to increasing force. In the adult rat heart, the activation pattern in sinus rhythm was very similar to those we have previously reported for the mouse ventricle (27). In sinus rhythm, there are clearly distinguishable sites of early activation (breakthrough) located toward the apex in each ventricle. However, in two of three cases (see RESULTS), the breakthrough site on the left ventricle was gradually delayed as the applied force was increased, suggesting that the response may involve compression of the left bundle branch. The prolongation of the PQ interval with increased applied force was also more pronounced in the two cases that exhibited this response than in the one case that did not. We conclude, therefore, that AV conduction, and in particular conduction to the left ventricle, is quite sensitive to applied force under our conditions. Thus the applied force has to be kept to an absolute minimum during recordings in sinus rhythm to avoid influencing the results. The correlation between changes in the isochronal activation map and the PQ interval suggests that the PQ interval may be used as an independent indicator of effects on the atrioventricular conduction. Fortunately, the lowest applied force (20 mmHg balloon pressure) used in this study, which is sufficient for a successful recording, is sufficiently low to avoid effects on the PQ interval.

**Alternative approaches to motion artifact reduction.** As an alternative (or complement) to mechanical immobilization, it is worthwhile to consider additional approaches to motion artifact reduction. These alternative approaches fall into two categories: 1) use of motion-blocking pharmacological agents, and 2) improvements in instrumentation to correct for motion artifacts. The two most frequently used motion-blocking agents, 2,3-butanedione monoxime (BDM) and cytoskeletal inhibitors, have recently been shown to significantly alter the action potential duration in the mouse ventricle (1). Published data (3) show that BDM affects...
L-type Ca\(^{2+}\) current and transient outward K\(^{+}\) currents in the rat ventricle and produces a substantial lengthening of the rat ventricular action potential as well. Our preliminary data (unpublished observations) indicate that cytochalasin-D also lengthens the rat ventricular action potential. Although these agents are effective motion blockers, they may thus have a significant effect on the repolarization of the ventricular action potential. Whereas the use of BDM or cytochalasin-D is appropriate in many situations, the consequences of their effects on the action potential duration must be carefully considered. A significant recent advance is the application of ratiometric or dual-wavelength techniques (2) for the suppression of motion artifacts in voltage-sensitive dye recordings from cardiac preparations (16, 32). Although technically challenging, this approach offers the possibility of minimizing motion artifacts without the use of pharmacological agents or mechanical immobilization. Further development of dual-wavelength techniques may be therefore the most promising approach so far to the problem of motion artifacts in voltage-sensitive dye recordings from the heart.

Effects of Di-4-ANEPPS

Dilation of coronary arteries. Di-4-ANEPPS at 1 \(\mu M\) has a significant effect on perfusion pressure in the Langendorff-perfused isolated rat heart. Perfusion with 1 \(\mu M\) di-4-ANEPPS consistently caused a transient drop in perfusion pressure, which recovered in a few minutes. It is therefore unlikely that this response causes any persistent effects that would influence the results obtained in these imaging experiments. Perfusion with solvent (0.01% DMSO) alone also resulted in a transient pressure drop, albeit very much smaller. A small part of this response should therefore be attributed to DMSO rather than di-4-ANEPPS. Increasing the Ca\(^{2+}\) concentration in the perfusate from 1 to 2 \(mM\) also attenuated the response. Rat coronary arteries are known to dilate in response to elevated [K\(^{+}\)]\(_{o}\), the entrance of inward rectifier K\(^{+}\) channels to changes in [K\(^{+}\)]\(_{o}\). Elevated [K\(^{+}\)]\(_{o}\) increases the outward current carried by these K\(^{+}\) channels (20) and therefore hyperpolarizes smooth muscle cells, causing sustained dilation (17, 31). The ability of our instrumentation to detect changes in perfusion pressure due to dilation of coronary arteries was important in this study, because this provides information about the viability of the preparation. Thus we tested the response of perfusion pressure to changes in [K\(^{+}\)]\(_{o}\). Elevation of [K\(^{+}\)]\(_{o}\) to 9 \(mM\) from 5.9 \(mM\) caused a statistically significant drop in perfusion pressure similar to that detected on di-4-ANEPPS perfusion. Reduction of [K\(^{+}\)]\(_{o}\) to 4 \(mM\) (immediately following the period in 9 \(mM\) [K\(^{+}\)]\(_{o}\)) caused the perfusion pressures to return to baseline, demonstrating that our instrumentation is capable of detecting changes in perfusion pressure due to vasodilation or constriction. Perfusion pressure can therefore be used as an independent index of the state of the preparation to avoid recording from compromised hearts.

Effects on atrioventricular conduction. The effects of di-4-ANEPPS on the heart rate (RR interval) and AV conduction time (PQ interval) can be evaluated from the ECGs acquired during these imaging experiments. Whereas the heart rate was unaffected by di-4-ANEPPS (1 \(\mu M\)), there was a progressive effect on the PQ interval. Perfusion with 1 \(\mu M\) di-4-ANEPPS frequently resulted in brief episodes of AV block. On recovery from these episodes of block (or on completion of dye perfusion in the few cases where no block was observed), we observed a prolongation of the PQ interval, which averaged 38% (Fig. 3A) and recovered only very slowly. In contrast to the transient effect on perfusion pressure during dye perfusion, the PQ interval prolongation was due exclusively to di-4-ANEPPS because it was not observed in hearts perfused with solvent (0.01% DMSO) alone. Because of the slow and gradual recovery (shortening) of the PQ interval, it is important that measurements designed to compare AV conduction before and after an intervention (drug application, etc.) be done over a brief time span. The interpretation of the results will otherwise be confounded by the drift in baseline PQ interval (cf. our measurements of the influence of immobilization force in this article).

The mechanism by which di-4-ANEPPS affects the PQ interval in the rat heart is unclear. One possibility is that the dye affects the intracardiac ganglia that are known to be present in the rat AV junction (25), producing local release of acetylcholine at the AV node and thus a prolongation of the PQ interval. This explanation seems unlikely, however, given the long-lasting nature of the response. Another possibility is that the dye could compromise perfusion of the AV node and thus render it ischemic. However, the fact that no global signs of ischemia (arrhythmias, ECG changes) were observed and that there was no increase in perfusion pressure (in fact, di-4-ANEPPS causes a transient decrease in perfusion pressure, as discussed in Dilation of coronary arteries) makes this explanation unlikely as well. Perhaps the most plausible explanation is that di-4-ANEPPS affects either ion channels or intercellular coupling (connexins) in the AV node, resulting in slowed conduction. The observation that the PQ prolongation is attenuated when the heart is perfused with a higher concentration of Ca\(^{2+}\) (2 \(mM\) rather than 1 \(mM\)) does suggest that the response may be linked to ion channels. Perfusion with Ca\(^{2+}\) can affect inward currents important to conduction in at least two ways. First, lowering [Ca\(^{2+}\)] to 1 \(mM\) would be expected to reduce inward Ca\(^{2+}\) current by decreasing the electrochemical driving force for this ion. Second, surface potential “screening” effects of Ca\(^{2+}\) can shift the voltage dependence of ion channel gating (11). This would particularly affect the availability of Na\(^{+}\) channels at the maximum diastolic potential, which would be lower at 1 \(mM\) [Ca\(^{2+}\)] than at 2 \(mM\) due to a hyperpolarizing shift in the steady-state inactivation characteristics of this channel. Lowering [Ca\(^{2+}\)] to 1...
mM might therefore also reduce inward Na\(^+\) current. However, the observation that the prolongation of the PQ interval in response to di-4-ANEPPS perfusion is not attenuated if the concentration of a different divalent cation (Mg\(^{2+}\) instead of Ca\(^{2+}\)) is increased by 1 mM suggests that surface charge screening effects are not important in this context. One might speculate that if the dye reduces inward Ca\(^{2+}\) and/or Na\(^+\) current, this would have more serious consequences for conduction if inward current is already reduced due to a low [Ca\(^{2+}\)] in the perfusate. However, if inward Ca\(^{2+}\) or Na\(^+\) current is significantly reduced due to the low [Ca\(^{2+}\)], one would expect the PQ interval to be longer in 1 mM Ca\(^{2+}\) compared with 2 mM before as well as after dye perfusion. This is not the case in our observations, which show a significant difference in PQ intervals between 1 and 2 mM [Ca\(^{2+}\)] only after dye perfusion. It is also difficult to explain why an effect of the dye on ion channels would be local to the AV node. A reduction of Ca\(^{2+}\) current would be expected to affect the sinoatrial node as well and result in a slowing of the heart rate; however, the RR interval remained constant in atrial node as well and result in a slowing of the heart.

Further studies at the single-cell level are therefore required to elucidate the actual cellular mechanisms by which di-4-ANEPPS slows AV conduction in the rat heart. Nevertheless, this is an important finding, which must be taken into account when interpreting the results of measurements in sinus rhythm. Although increasing [Ca\(^{2+}\)] in the perfusate will result in more severe motion artifacts, the attenuation of the PQ prolongation may make this an acceptable tradeoff for some measurements in sinus rhythm.

To summarize, we have developed a voltage-sensitive dye-imaging system and integrated monitoring of the state of the preparation by recording ECG, perfusion pressure, and temperature in a synchronized manner. Our results demonstrate the feasibility of applying voltage-sensitive dye imaging with di-4-ANEPPS to the whole isolated rat heart and illustrate the advantages and limitations of this approach.

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