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Imaging Cellular Calcium Dysfunction in the Heart Using Multi-Modal Optical Mapping

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I. INTRODUCTION

It is well recognized that intracellular calcium is an important mediator of cardiac myocyte contraction and molecular signaling. Recently, the importance of intracellular calcium in cardiac electrical instability and arrhythmogenesis has gained significant interest. Calcium mediated arrhythmogenesis has been linked to T-wave alternans [1], heart failure [2], and mutations of calcium regulatory proteins [3]. The strong association between calcium regulation and arrhythmogenesis can be appreciated by recognizing the direct effect intracellular calcium has on membrane ionic currents such as those associated with the sodium-calcium exchanger, L-type calcium channels, and calcium mediated potassium channels [4]. Complicating this relationship is the fact that membrane ionic currents, by their effect on the action potential, can also influence intracellular calcium. Despite the growing importance of calcium mediated arrhythmogenesis, the underlying mechanisms are not well understood due to the complex interactions between membrane currents and intracellular calcium and, importantly, the difficulty of measuring both transmembrane potential and intracellular calcium from intact cardiac preparations.

Optical mapping provides the ability to map cellular function at the level of the whole heart, and has proven to be an essential tool for studying the cellular mechanisms of cardiac arrhythmias [5]. Given that optical mapping is based on the principles of fluorescence, it is possible to use multiple fluorescent probes (i.e. dyes) of cellular function, as long as there is sufficient spectral separation between fluorescence emission of each dye such that one does not overlap with the other (i.e. crosstalk). Our laboratory and others have used these principles to simultaneously measure intracellular calcium and transmembrane potential from intact cardiac preparations [6,7]. Dual calcium-voltage optical mapping is, however, not sufficient to completely understand calcium mediated arrhythmogenesis. Intracellular calcium concentration is a very important parameter, but due to heterogeneities of dye distribution and excitation light, absolute fluorescence cannot be used to assess its value. Borrowing from calibration methods developed for single cells [8], we have developed a ratiometric optical mapping system that can measure relative intracellular calcium levels that are independent of dye and excitation light heterogeneity [9]. The combination of dual calcium-voltage and ratiometric calcium optical mapping can provide a very powerful tool for understanding the mechanisms of calcium mediated arrhythmogenesis.

II. METHODS

To study calcium mediated arrhythmogenesis, we have developed a multi-modal optical mapping system that is able to perform dual calcium–voltage [6] and ratiometric calcium optical mapping [9] in the same cardiac preparation. While these techniques have been used separately, creating a multi-modal system that allows quick and easy mode changing confers a considerable advantage over single mode systems.

Experimental Preparations

Our multi-modal optical mapping system has been designed to accommodate a wide variety of cardiac preparations, ranging from whole murine hearts to canine atrial and ventricular preparations. Briefly, intact cardiac preparations are cannulated and perfused at a constant flow with oxygenated Tyrode's solution, placed in a Lexan chamber and positioned in front of an imaging window. To maintain physiologic temperature, the preparations are immersed in the coronary effluent, which is maintained at a constant temperature equal to the perfusion temperature by a heat exchanger located in the chamber. The volume-conducted electrocardiogram (ECG) is monitored using three silver disk electrodes fixed to the chamber in positions roughly corresponding to ECG limb leads I, II, and III. To ensure physiological stability of the preparation, the ECG, coronary pressure, coronary flow, and perfusion temperature are continuously monitored throughout each experiment. Preparations

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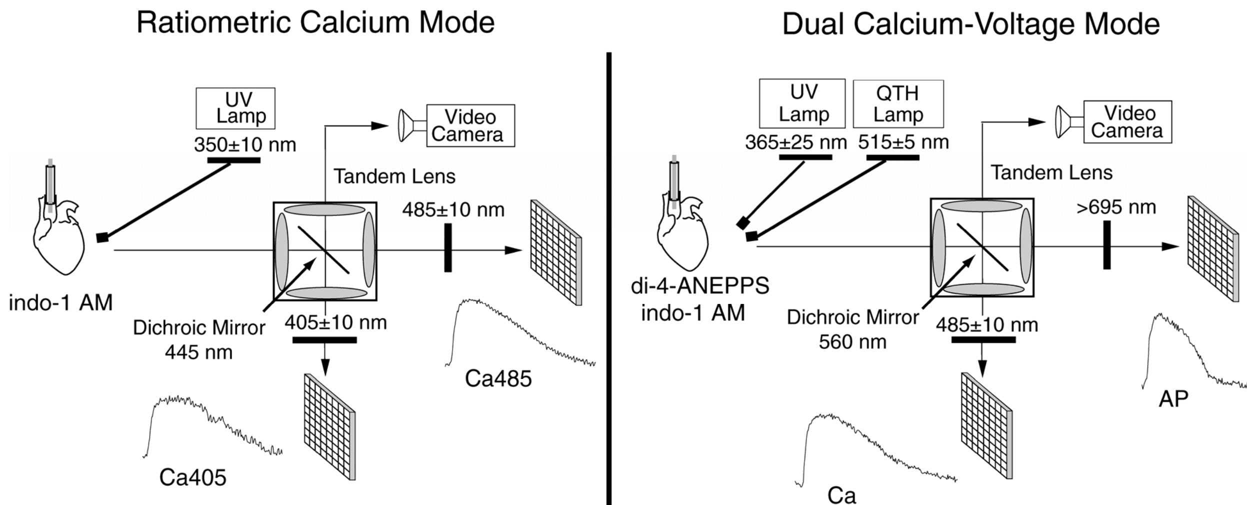

Figure 1: Schematic diagram for the multi-modal optical mapping system. Shown are the setups for ratiometric calcium mode (left) and dual calcium-voltage mode (right). See the text for a detailed description of each mode. For ratiometric calcium mode (left), one of 256 actual calcium transients at each wavelength (Ca405 and Ca485) are shown adjacent to the photodiode array from which they were obtained. Offline, ratiometric calcium signals (Ca Ratio) are calculated from the background corrected Ca405 and Ca485 signals. For dual calcium-voltage mode, one of 256 actual action potentials (AP) and calcium transients (Ca) are shown adjacent to each detector array (see text for details).

last no longer than 2 – 3 hours. remain viable for 3 – 4 hours, but experiments typically

Ratiometric Calcium Mode

Shown in Fig. 1 (left) is the system setup for ratiometric calcium optical mapping. Preparations are perfused with the dual emission fluorescence, calcium-sensitive dye Indo-1 AM (Molecular Probes) dissolved in 1 mL solution of DMSO and Pluronic (20%w/v) at a final concentration of 10 μmol/L for 45 minutes at 37°C. The two emission wavelengths for Indo-1 AM are at 405 nm and 485 nm, corresponding to calcium bound and unbound states, respectively. Both peaks are excited to a similar extent using a liquid light guide (Thermo-Oriel) to direct excitation light from a 500 W mercury arc lamp (Thermo-Oriel) filtered at 350±10 nm (Chroma Technology). Light fluoresced from the preparation is collected by a tandem lens assembly consisting of four high-numerical aperture photographic lenses (Nikon, Tokyo, Japan) which can be interchanged to control optical image magnification.

To separate fluorescence corresponding to the unbound and bound state of Indo-1 AM, a 445 nm dichroic long pass mirror (Chroma Technology) is placed at a 45 degree angle within the tandem lens assembly such that all wavelengths above 445 nm are transmitted and filtered at 485±10 nm (Chroma Technology) to one 256 element photodiode array and wavelengths below 445 nm are reflected and filtered at 405±10 nm (Chroma Technology) to a second photodiode array. The two detector arrays are in perfect register such that corresponding channels measure signals from the same location on the preparation with an error < 35μM [6]. Finally, the dichroic mirror within the tandem lens assembly can be momentarily rotated 90 degrees to direct an image of the preparation to a CCD video camera (Pulnix) to view the preparation.

Photocurrent from the 256 photodiodes of each detector array is passed through low-noise current to voltage converters (Hamamatsu, Hamamatsu City, Japan) and then undergo postamplification (1, 50, 200, 1,000) with AC coupling (10 s time constant), followed by low-pass antialias filtering (500 Hz). Signals recorded from each photodiode array and ECG signals are multiplexed and digitized with 12-bit precision at a sampling rate of 1,000 Hz/channel (Microstar Laboratories, Bellevue, WA). Offline, calcium transients recorded at 405 nm (Ca405) and 485 nm (Ca485) are used to calculate a ratiometric calcium signal (Ca Ratio) that is insensitive to heterogeneities of excitation light and dye staining as we have described previously [9].

Dual Calcium-Voltage Mode

The multi-modal optical mapping system has been designed so that it can be easily switched from ratiometric calcium mode (as described above) to dual calcium-voltage mode (Fig. 1, right). Upon completion of ratiometric calcium measurements, the preparation is perfused with a second fluorescent dye, Di-4-ANEPPS (Molecular Probes), which is sensitive to transmembrane potential. The order in which dyes are used is important because the voltage sensitive dye may interfere with ratiometric imaging. Di-4-ANEPPS is

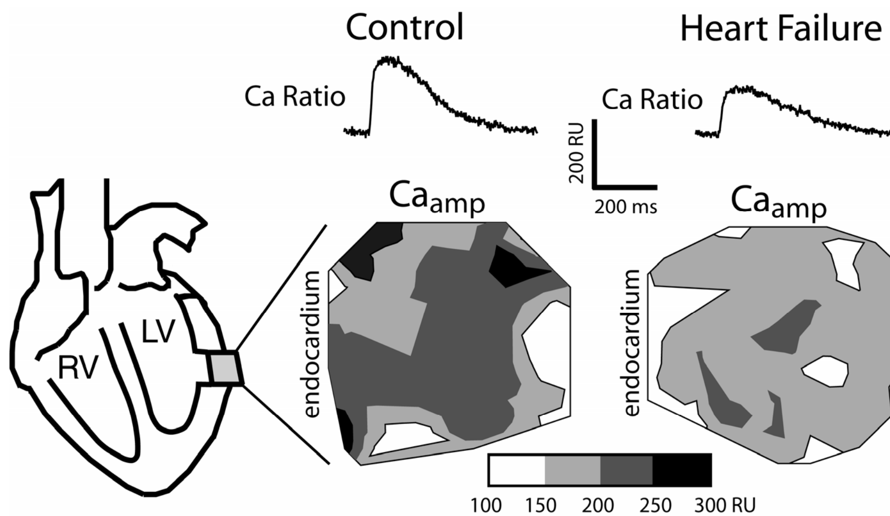

Figure 2: Shown are ratiometric calcium transients (Ca Ratio) from the transmural surface of a canine left ventricular wedge preparation isolated from a control canine (left) and a canine with rapid pacing-induced heart failure (right). Both signals are calculated from Ca405 and Ca485 recorded near the endocardial surface. The amplitude (max-min) of the Ca Ratio signal is much larger for the control preparation compared to the heart failure preparation. Scale bar shows time in ms and amplitude of transient in ratiometric units (RU). At the bottom are contour plots depicting the spatial distribution of Ca ratio amplitude (Ca_{amp}) across the transmural surface, where darker shades of gray depict larger Ca_{amp}. Each contour map represents an area of approximately 1.4 cm x 1.4 cm, where the endocardial surface is located on the left side and the epicardium is on the right. Overall, Ca_{amp} was smaller in the heart failure preparation compared to the control.

dissolved in 0.19 mL of ethanol at a final concentration of 15 μmol/L and perfused for approximately 10 minutes at 37°C. Excitation light for the voltage dye requires a 180 W quartz tungsten halogen lamp light source (Thermo-Oriel), filtered at 515±5 nm (Chroma Technology), and directed to the preparation by a second liquid light guide (Thermo-Oriel). Excitation light for Indo-1 AM is the same as that for the ratiometric calcium mode with the exception that we use a lower power light source (250 W vs. 500 W) filtered at 365±25 nm.

To minimize crosstalk between dyes, fluorescence is measured at 485 nm for Indo-1 AM (Fig. 1, Ca) and > 695nm for Di-4-ANEPPS (Fig. 1, AP). First, to separate these wavelengths a 560 nm dichroic long pass mirror (Chroma Technology) replaces the 445 nm mirror used for the ratiometric calcium mode. Then, transmitted light corresponding to optical action potentials passes through a long pass filter with a cutoff at 695 nm to the first detector. Reflected light is filtered at 485±10 nm, thereby directing optical calcium transients to the second detector. The total time required to change from ratiometric calcium mode to dual calcium voltage mode, including dye perfusion, is no more than 15 minutes.

III. RESULTS

Using multi-modal optical mapping techniques, we have characterized calcium dysfunction and arrhythmogenesis associated with heart failure (HF) induced by chronic, rapid ventricular pacing [10]. Shown in Fig. 2 are ratiometric calcium transients (Ca Ratio) for a single beat recorded near the endocardium of the transmural wall from the isolated canine left ventricular wedge preparation [11]. Calcium transients demonstrate a rapid increase in intracellular calcium corresponding to normal calcium induced calcium release from the sarcoplasmic reticulum (SR) via the ryanodine receptor (RyR). The calcium transient amplitude (Ca_{amp}), which corresponds mainly to the amount of calcium released by the SR, was approximately 30% larger in the control preparation compared to the HF preparation. Shown below each Ca Ratio trace is the spatial distribution of Ca_{amp} across the transmural wall as calculated from the calcium transients recorded at all 256 sites. Overall, Ca_{amp} was smaller (lighter gray) in the HF preparation (170 ± 27 RU) compared to control (194 ± 41 RU). Similar results were observed in 11 HF and 10 control preparations.

Shown in Fig. 3 are representative examples of action potentials (AP) and calcium transients (Ca) recorded near the endocardium in the same control (left) and HF preparations (right) shown in Fig. 2 using dual calcium-voltage optical mapping. When pacing the preparation at a cycle length of 2000 ms, the duration of the action potential (APD) from the HF preparation (300 ms) is much longer than that recorded from the control preparation (220 ms). The decay portion of the calcium transient corresponds to calcium uptake by the SR and extrusion by the sodium-calcium exchanger. When fit to a single exponential, a much slower decline to resting diastolic calcium levels is observed in HF (290 ms) compared to control (230 ms). Shown below each trace is the spatial distribution of APD across the transmural wall as calculated from the action potentials recorded at all 256 sites. In the control preparation, APD is longest (darker gray) near the endocardial surface. Similarly, APD is longest near the endocardial surface in the HF preparation; however, on average APD is longer compared to control (note the different gray scale).

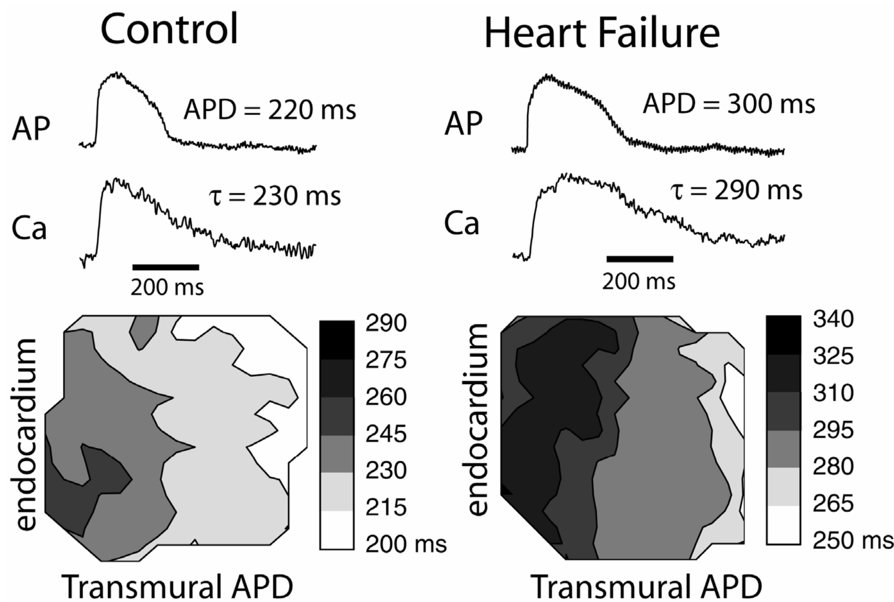

Figure 3: Shown at the top are representative action potentials (AP) and calcium transients (Ca) measured from the same control and failing heart preparations as in Fig. 2, using dual calcium-voltage mode. All signals are recorded from the center of the mapping field. Values are given for action potential duration (APD) and the exponential fit of the decay of the calcium transient (τ). At the bottom are contour plots depicting the spatial distribution of APD across the transmural surface, where darker shades of gray depict longer APD. Each contour map represents an area across the transmural surface as indicated in Fig. 2.

To determine the arrhythmogenic consequence of calcium dysfunction in HF preparations, the same wedge preparations were stimulated rapidly (cycle length = 140 - 100 ms) during Isoproterenol (Iso) administration (0.2 µm) to simulate exercise conditions and induce ectopic activity. In control preparations with Iso administration, immediately upon termination of rapid pacing an ectopic (i.e. unstimulated) beat was observed 20% of the time. In contrast, every HF preparation exhibited ectopic activity during Iso administration. Shown in Fig. 4 is a representative example of an action potential (AP) and calcium transient (Ca) recorded simultaneously during the induction of an ectopic beat. Immediately after halting rapid pacing (S1), an ectopic beat (asterisk) occurred, followed by a slow depolarization in membrane potential, known as a delayed afterdepolarization (DAD). The ectopic beat immediately following rapid pacing was also reflected in the calcium transient recording. In addition, following the ectopic beat is a slow increase in intracellular calcium that mirrors the DAD. This likely represents a spontaneous calcium release (SCR) from the SR. Furthermore, just preceding the ectopic beat, transmembrane potential and calcium levels slowly increase (arrows) and have an initial time course and amplitude comparable to the DAD and SCR. This is consistent with an ectopic beat triggered by an SCR-mediated DAD. Shown at the bottom of Fig. 4 is an ECG recording of ventricular tachycardia induced by rapid pacing and Iso administration in a different HF preparation.

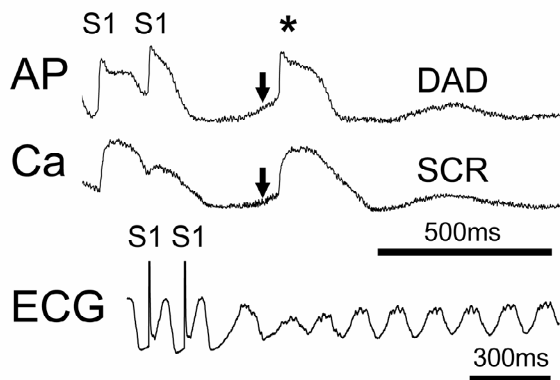

Figure 4: Representative action potential (AP) and calcium transient (Ca) recorded using dual calcium-voltage mode from a heart failure wedge preparation during the induction of ectopic activity. After cessation of rapid pacing (S1), an ectopic beat is evident (asterisk) followed by a delayed-afterdepolarization (DAD) in the AP recording and a spontaneous calcium release (SCR) in the Ca recording that did not elicit an ectopic beat. The arrows indicate slow depolarization and slow intracellular calcium release that precedes the ectopic beat, a hallmark of calcium mediated ectopic activity. Shown at the bottom is an ECG recording of the induction of ventricular tachycardia in a heart failure wedge preparation under similar conditions.

Using ratiometric calcium optical mapping, we were able to determine the spatial distribution of SCR amplitude in the absence of a triggered beat. Shown in Fig. 5 is a representative example of SCR amplitude across the transmural wall in a HF wedge preparation (left). Ca Ratio transients are also shown (right) from which SCR amplitude was calculated. Regions of largest calcium release are indicated by darker shades of gray. An interesting finding was that an SCR event did not occur from a single cell but from a region of myocardium comprised of many cells, as indicated by SCRs occurring over a significant portion of the transmural wall. These data indicate that SCR events can occur simultaneously from a large group of cells. This may explain how an SCR can depolarize a sufficient amount of tissue and create a DAD strong enough to trigger an ectopic beat.

IV. CONCLUSION

In this manuscript, we describe a novel multi-modal optical mapping system that can be used to investigate

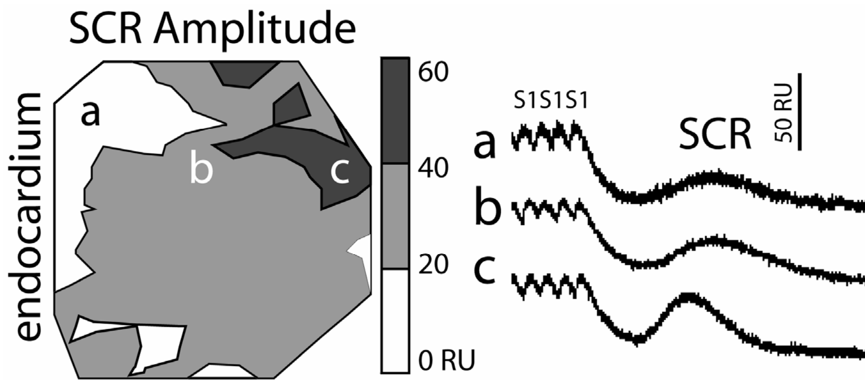

Figure 5: Using ratiometric calcium mode, the transmural pattern of SCR amplitude in a failing heart is shown (left). In this example, an ectopic beat did not occur. The contour map represents an area across the transmural surface as depicted in Fig. 2. On the right are three Ca Ratio signals measured from sites (a, b, c) across the transmural surface. Scale bar is provided to show relative amplitude of SCRs. Note that the SCR occurs over a relatively large area of the transmural surface.

calcium-mediated arrhythmogenesis. To this end, the ability to measure intracellular calcium and transmembrane potential simultaneously with high spatial and temporal resolution from intact cardiac preparations is essential. Moreover, to assess cellular calcium dysfunction, it is essential to measure changes in calcium concentration using ratiometric techniques.

In HF preparations, we found that the decay of intracellular calcium to resting diastolic levels and the magnitude of calcium release from the SR were slower and smaller, respectively, compared to controls. Similar results have been reported by others [12]. Previously, we have found that a slow decay of intracellular calcium can cause calcium overload during rapid pacing and lead to SCR and DAD activity under conditions of enhanced calcium entry [13]. Alternatively, RyR function may be impaired (e.g. high open probability) in HF compared to control, as evidenced by reduced calcium transient amplitude. As a result, RyR may be “leaky” and, thus, promote SCR and DAD activity [14]. However, HF is associated with multiple structural and ion channel lesions, so arrhythmogenesis is likely to be multi-factorial and require further investigation.

While our multi-modal system is very robust, several precautions must be noted. The ratiometric calcium signals must be interpreted with caution because they are not calibrated to account for the non-linear relationship between calcium and fluorescence at high intracellular calcium levels. Therefore at high calcium levels, the change in calcium may be underestimated. Additionally, dual calcium-voltage optical mapping is susceptible to overlap in fluorescence emission. For example, changes to the system that preferentially enhance fluorescence from one dye versus the other could result in significant error due to fluorescence crosstalk. Therefore, if any such changes are made, verification of minimal or no crosstalk should be performed. As long as such precautions are made, multi-modal optical mapping is a very powerful technique for investigating calcium mediated arrhythmogenesis.

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