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Datum: 03/08/2013

ESC First Contact Initiative Grant - Report

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Host: **Prof. Dr. W. Jonathan Lederer, Department of Physiology, Laboratory of Molecular Cardiology, University of Maryland, Baltimore, USA**

Period: **February 1st – February 15th, 2013**

Background

Atrial fibrillation (AF) is the most common sustained arrhythmia and is associated with increased cardiovascular morbidity and mortality, particularly due to embolic stroke.[1] Re-entry is the most accepted pathomechanism of AF. Ectopic activity can trigger reentrant excitation in a vulnerable substrate, thereby contributing to the initiation and maintenance of arrhythmias.[1] It is assumed that Ca^{2+}-handling abnormalities contribute to ectopic activity, but the molecular basis is poorly understood. Based on Ca^{2+}-spark measurements, increased spontaneous Ca^{2+} releases from the sarcoplasmic reticulum (SR) have been observed in AF (SR Ca^{2+} leak).[2, 3] During diastole the released Ca^{2+} is pumped out of the cell via the Na^{+}-Ca^{2+}-exchanger (NCX), which brings 3 Na^{+}-ions (three positive charges) into the cell per extruded Ca^{2+} ion (two positive charges), thereby creating a depolarizing inward current. This may cause membrane depolarizations (delayed afterdepolarizations, DADs), which may provide a trigger for AF. In a recent publication[4] we used a tetracaine protocol (previously established by Shannon et al.[5]) to quantify SR Ca^{2+} leak in atrial myocytes from patients with sinus rhythm or chronic AF (cAF). We found again higher SR Ca^{2+} leak in cAF despite comparable SR Ca^{2+} load. In addition, we showed that cAF is associated with an increase in open probability of the ryanodine receptor (RyR, RyR2=cardiac subtype), the main Ca^{2+} release channel in the SR. These data point to an intrinsic RyR2 dysfunction in AF. Furthermore, we provided evidence indicating that CaMKII-mediated hyperphosphorylation of RyR2 may underlie the RyR2 dysfunction in cAF.[4]

Until now, we used simultaneous recordings of epifluorescent Ca^{2+} signals together with membrane currents or membrane potentials (Patch-clamp). In the future, we would like to establish confocal microscopy in our lab in addition to these Ca^{2+} measurements with the epifluorescence technique. This will enable us to combine confocal microscopy and patch-clamp methodology to record local Ca^{2+}-release events (sparks) and waves simultaneously with membrane currents or membrane potentials. Using this technique we would like to further characterize the molecular basis of the increased Ca^{2+}-voltage coupling gain, which we have identified previously in cAF.[4]

The aim of my visit to Dr. Lederer's laboratory was to gain knowledge and skills about confocal microscopy, which will enable me to establish this technique in our laboratory.

Part 1: Setting up the microscope

I worked on a Zeiss LSM510 confocal microscope (**Figure 1**) equipped with three single photon lasers (Ar, HeNe 543, HeNe 633). During the first days in the lab I obtained insight into the basics of confocal microscopy.

We used 0,22 µm fluorescent TetraSpeck™ beads, a size that is just above the resolution limit of visible light, to obtain a point spread function and to adjust the pinhole size (**Figure 2**).[6] Furthermore I dealt with special configurations of confocal microscopy, including fluorescence recovery after photobleaching (FRAP) and z-stack imaging with 3D reconstruction.

Part 2: Ca^{2+}-spark measurements

During the second half of my visit I recorded and analysed Ca^{2+}-sparks in murine ventricular myocytes. Murine ventricular myocytes were isolated as previously described.[7] Cells were loaded with Fluo-4 by 10 min incubation with 3 µM of fluo-4-acetoxymethyl (AM) ester (Invitrogen) and 0.01% Pluronic® F127 (a poloxamer made by BASF, Florham Park, NJ, USA) and allowed an additional 30 min for de-esterification. During experiments cells were perfused at room temperature with bath solution containing (in mM): NaCl 140, KCl 5, $CaCl_2$ 1.8, $MgCl_2$ 0.5, HEPES 5, Glucose 5, NaH_2PO_4 0.33. To load the SR with Ca^{2+}, cells were field stimulated at 0.5 Hz. During follow-up, Ca^{2+}-sparks were recorded along a longitudinal subsarcolemmal line (**Figure 3A**) using the 488 nm argon ion laser in line-scan mode at 0.96 ms/line. For analysis of Ca^{2+} spark a custom routine written by Dr. Christopher Ward in Interactive Data Language (IDL, version 7.1, ITT Visual Information Solutions, Boulder, CO, USA) was used.

9 out of 15 murine ventricular myocytes showed detectable Ca^{2+} sparks. **Figure 3B** shows a representative line scan image from a murine ventricular myocytes together with the corresponding three-dimensional reconstruction. **Figure 3C** shows a single Ca^{2+}-spark on an enlarged scale and illustrates the analysis of key Ca^{2+} spark parameters: amplitude ($\Delta F/F0$), full-width at half-maximal amplitude (FWHM) and full-duration at half-maximal amplitude (FDHM). These parameters reflect the gating kinetics of RyR2 and were comparable to previous data (Table 1).[8]

Table 1: Spark properties in murine ventricular myocytes.

Spark frequency (Hz)	Spark amplitude ($\Delta F/F0$)	FWHM (µm)	FDHM (ms)
0.45±0.12	**2.70±1.25**	**2.77±0.37**	**19.27±2.25**

Mean±SEM were calculated from individual data obtained in n = 9 myocytes from 4 animals. FWHM, full-width at half-maximal amplitude; FDHM, full-duration at half-maximal amplitude.

For analysis the Ca^{2+}-levels were estimated by the fractional change in fluorescence intensity relative to baseline ($R = \Delta F/F0$). This Ca^{2+} fluorescence ratio (R) can be converted to a Ca^{2+} concentration by using the following equation:[9]

$$[Ca^{2+}]_i = K \cdot R \cdot (K/[Ca^{2+}]_{rest} + 1 - R)$$

Where K is the dissociation constant of Fluo-4 and $[Ca^{2+}]_{rest}$ is the resting Ca^{2+} concentration. Using the mean fluorescence ratio (R=2.70±1.25; see Table 1) and assuming that the dissociation constant for Fluo-4 AM at room temperature is 345 nM and the resting $[Ca^{2+}]_i$ of a cardiac myocyte is 100 nM, the mean amplitude of Ca^{2+} sparks can be estimated to be around 289 nM, which is consistent with previous publications.[9]

Spontaneous Ca^{2+} waves were recorded in 2 cells with transversal line scan (**Figure 4**). These spontaneous Ca^{2+}-waves usually occurred at the end of experimental days pointing to impaired cell quality.

Future directions

We will establish the technique of confocal Ca^{2+} measurements together with simultaneous measurements of membrane currents or membrane potential in our lab in Essen. Dr. W. Jonathan Lederer agreed to advice us during setup and planning of the system.

During my visit in Baltimore I got to know the powerful programming language IDL, which is especially designed for imaging purposes. This software will certainly be a powerful tool for my future work. To facilitate future visits and to get the approval to use software from Dr. Lederer's lab independently, I became an external Member of the Center for Biomedical Engineering and Technology (BioMET) at the University of Maryland School of Medicine, Baltimore, USA.

Acknowledgement

I would like to thank the members of the ESC council for providing me the ESC first initiative contact grant in September 2012.

I would like to express my very great appreciation to Dr. W. Jonathan Lederer for his continuous help and support. I enjoyed the open-minded atmosphere in his lab and the extensive discussions with him. Dr. Dobromir Dobrev exempted me from my daily work in the laboratory and from teaching obligations, making my visit to Baltimore possible.

I also would like to offer my special thanks to Dr. Christopher Ward for the time-consuming introduction into the IDL software and for providing me the routines he developed for spark detection and analysis. My special thanks are extended to Dr. Brian Hagen and Dr. Maura Greiser for their valuable help regarding practical skills in using confocal microscopy and their daily support in the lab. Finally, I wish to acknowledge the organizational help provided by Pamela Wright.

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Figures

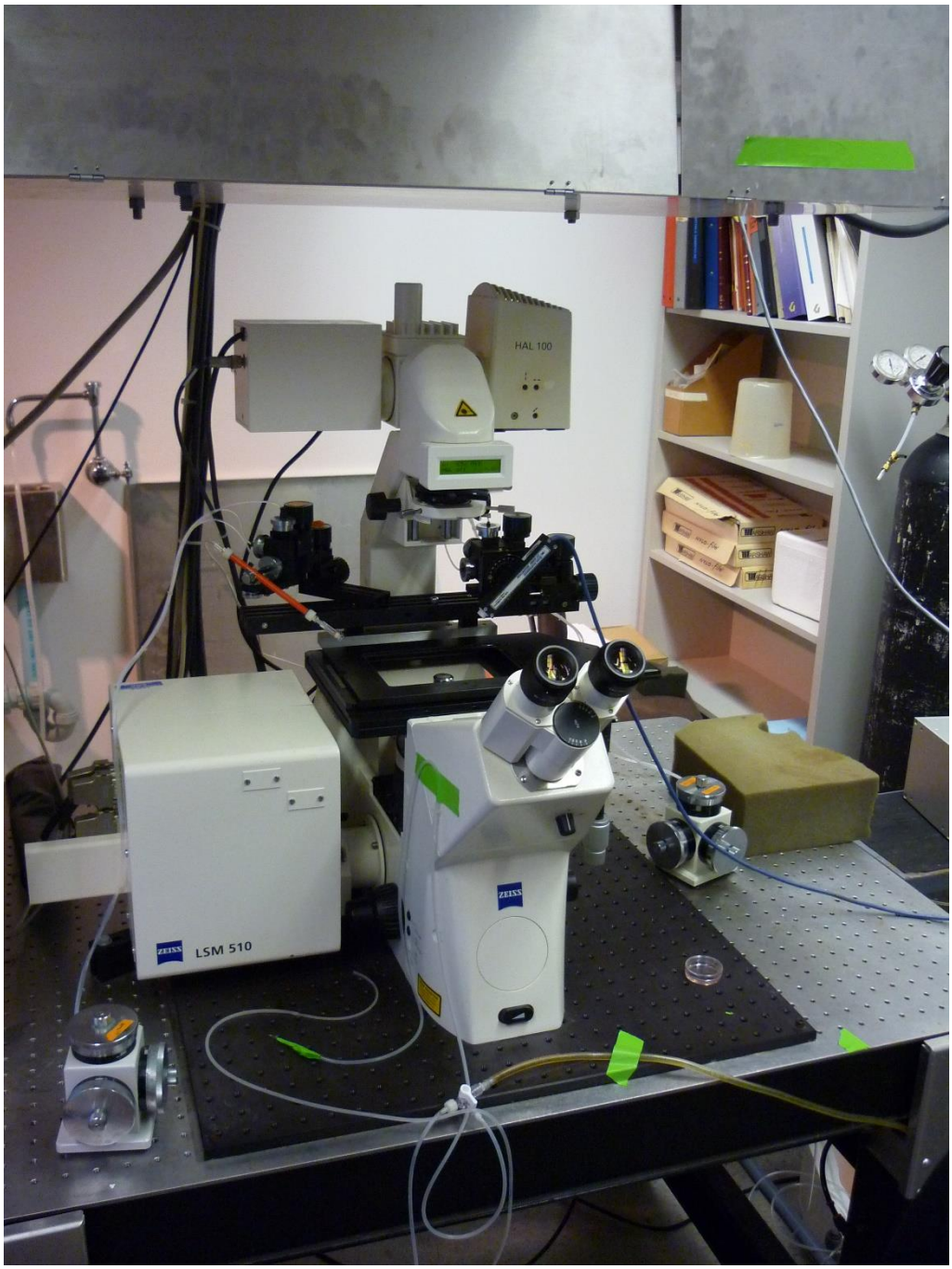

Figure 1, Setup with confocal microscope and patch-clamp equipment

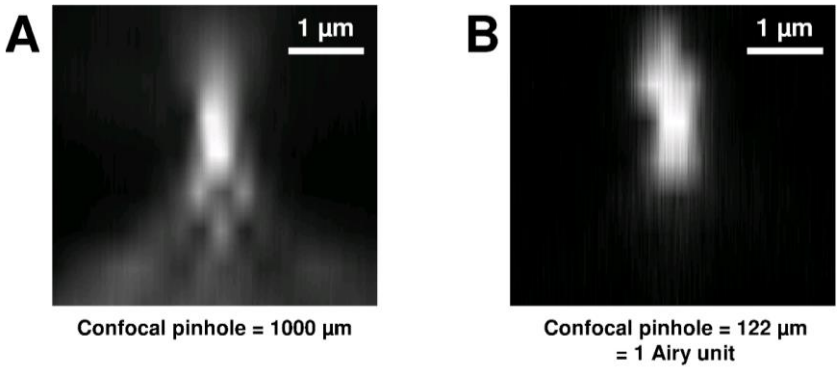

Figure 2, Microsphere images. Confocal images of a 220-nm-diameter red microsphere were collected using a x63/1.4 NA oil-immersion objective lens (Excitation: 633 nm; Emission: >650 nm). **A,** Using a large pinhole (1000 µm) produces an hourglass shape of the axial point-spread-function (PSF) due to the diffraction pattern outside the focal volume. **B,** Using a small pinhole (122 µm) results in a compact PSF and the absence of diffracton rings outside the focal volume. The confocal microscope has a much higher resolution along the z axis when the pinhole is set to 1 Airy unit.

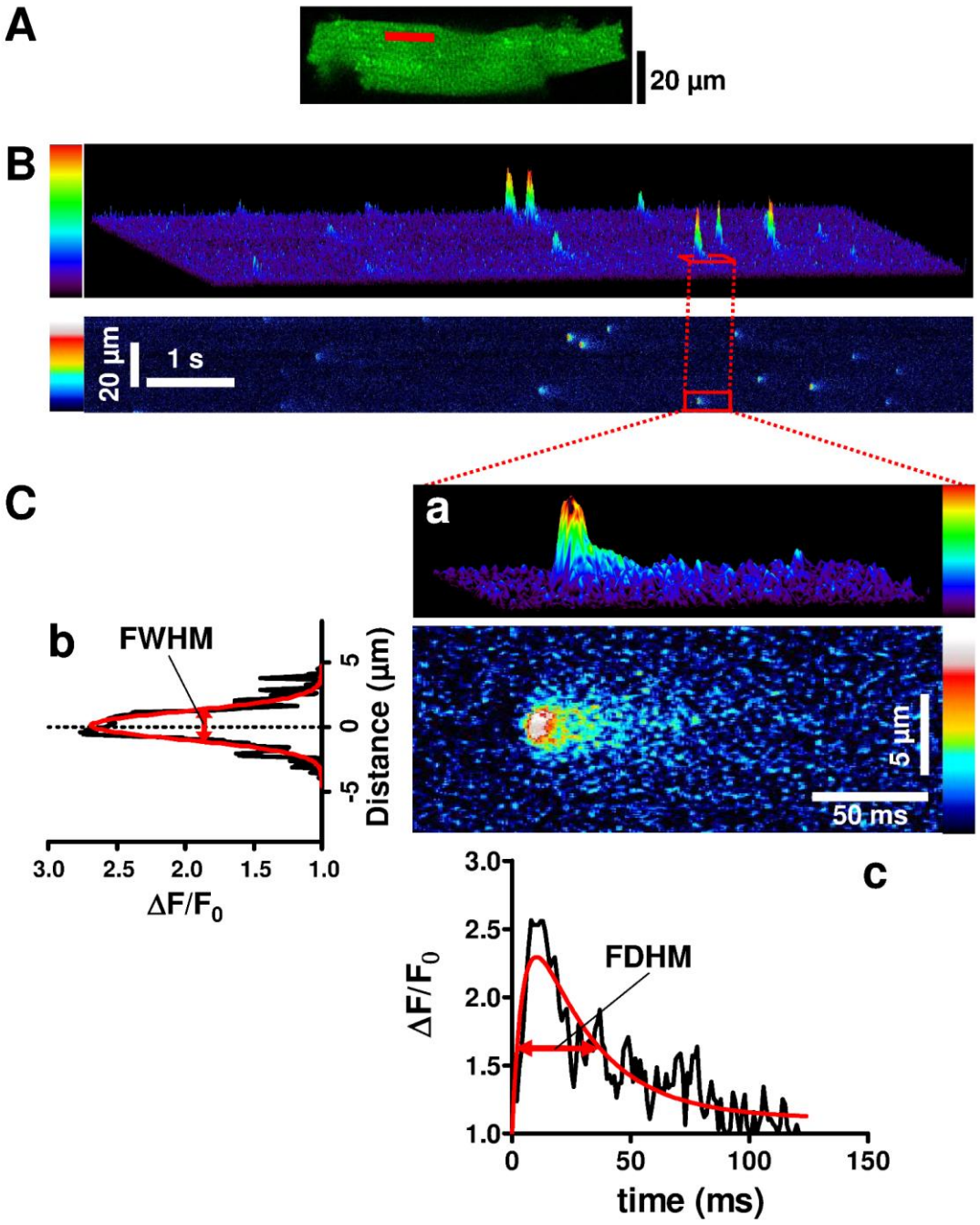

Figure 3, Recoding and analysis of Ca^{2+} sparks in murine ventricular myocytes. A, Confocal xy-image of a murine ventricular myocyte loaded with the fluorescent Ca^{2+} indicator Fluo-4 AM. Red bar indicates the scanned line used to obtain the representative longitudinal line-scan image shown in panel B. **B,** Three-dimensiol reconstruction together with the corresponding line-scan showing representative Ca^{2+}-sparks. **C,** Typical Ca^{2+} spark (**panel a**) and analysis of basic Ca^{2+}-spark characteristics (FDHM, full-duration at half-maximal amplitude, **panel b**; FWHM, full-width at half-maximal amplitude, **panel c**).

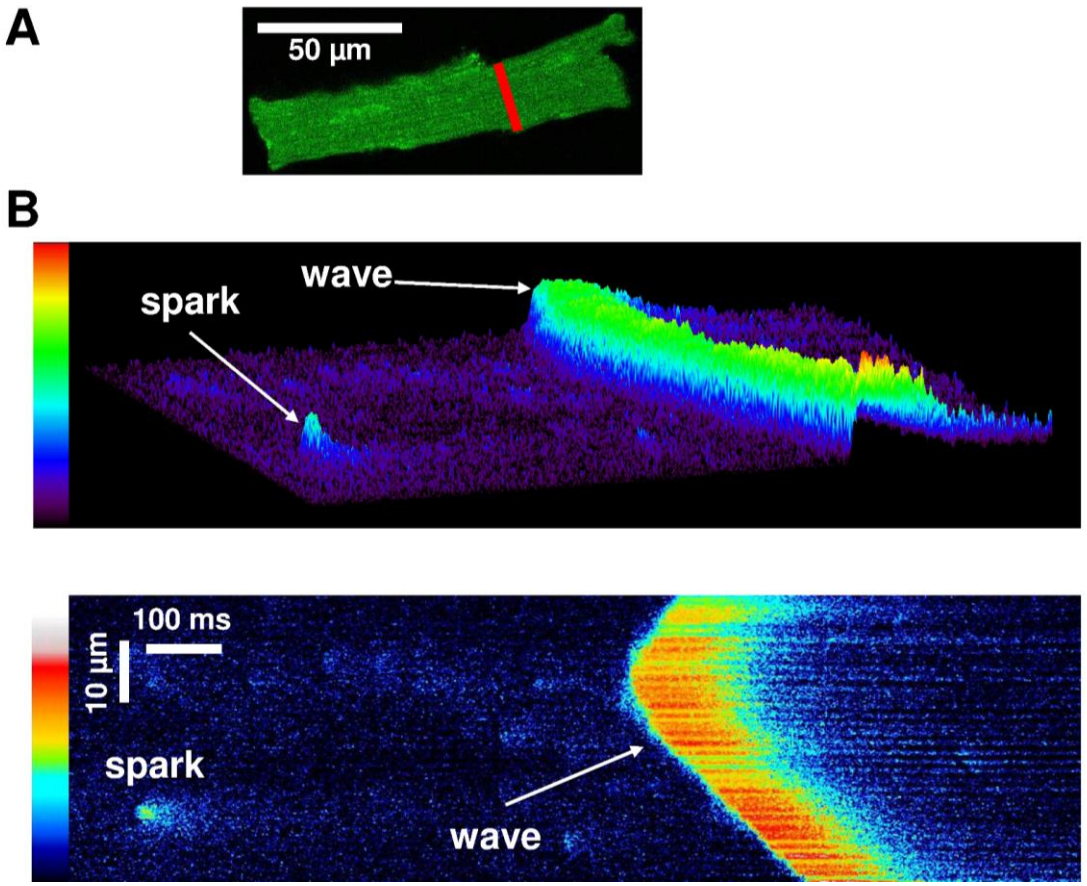

Figure 4, Recoding of a representative Ca^{2+} -wave in transversal line-scan mode. A, Confocal xy-image of a murine ventricular myocyte loaded with the fluorescent Ca^{2+} indicator Fluo-4 AM. Red bar indicates the scanned line used to obtain the transversal line-scan image shown in panel B. **B,** Three-dimensional reconstruction together with the corresponding line-scan recording of a Ca^{2+}-wave.