TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY SHOWS Ca^{2+}-SPARKS IN CARDIOMYOCYTES FROM *RATTUS NORVEGICUS*, BUT NOT FROM *SQUALUS ACANTHIAS*

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Mammalian ventricular cardiomyocytes contract when Ca^{2+} is released from the sarcoplasmic reticulum (SR) in response to Ca^{2+} influx through L-type Ca^{2+} channels (I_{Ca}). This calcium-induced calcium release (CIRC) is thought also to generate the brief local Ca^{2+} releases which are seen as "Ca^{2+} sparks" with confocal fluorescence microscopy in resting cells. In contrast, ventricular myocytes from amphibians and elasmobranchs have a poorly developed SR and are thought to be activated directly by Ca^{2+} influx through the Ca^{2+} channel. To examine if they also differ from mammalian cells by lacking Ca^{2+} sparks we examined both types of cells using a novel technique of total internal reflection fluorescence (TIRF) microscopy and compared the results to similar measurements using confocal microscopy.

TIRF has been used to study focal and near adhesions of cells to glass surfaces which reflect totally a beam of light coming the other side (Truskey, Burmeister, Grapa and Reichert, J. Cell Science, 103: 491-499, 1992). When the critical angle of incidence is exceeded there is, ideally, no net energy flux out of the glass, yet an electromagnetic field is created near the surface and is strongly attenuated as the distance to the interface is increased to a fraction of a wavelength. This evanescent illumination can be seen as defining a single focal plane with a depth of resolution ($\cong$ 200 nm) which compares favorably to that of confocal microscopes ($\cong$ 1000 nm). We hypothesized that this type of illumination might be used for fluorescence excitation of intracellular Ca^{2+}-indicator dyes in live cells adhering to a glass surface. In that case, it might be possible to observe Ca^{2+} sparks with improved resolution using an inexpensive CCD camera.

Cells from ventricles of shark (*Squalus acanthias*) and rat (*Rattus norvegicus*) were prepared by enzymatic dissociation. Batches of cells were transferred to a perfusion chamber which had a glass prism as bottom and was mounted on the stage of an upright microscope (Olympus BX50WI) equipped with a long distance (2 mm) water immersion objective (LUMPlanFl, 60x, N.A. 0.9). The cells were allowed to settle in the chamber and were incubated 30 to 60 min at room temperature with Tyrode solutions containing 20 μM Fluo-3AM. A focused, attenuated laser beam (λ = 488 nm, argon ion, Omnichrome) was aimed through the side of the prism hitting the glass/fluid interface at the center of the microscope's field of vision obliquely from below. The angle of incidence was adjusted with a mirror mounted on a translation stage and was typically 5 to 10 degrees larger than the critical angle (θ_c = 66°, $\sin(\theta_c) = n_{water}/n_{glass}$, n_{water} = 1.38, n_{glass} = 1.58). The microscope was equipped also with lamps (incandescent and Hg-arc) and filters (488 nm interference excitation filter, barrier filter: > 510 nm) for bright field and epi-fluorescence microscopy. Images were detected with an intensified CCD camera (Attoflour) and acquired as individual frames by an IBM compatible computer or

stored on video tape (sVHS/NTSC, 30 frames/sec) for later analysis on a Silicon Graphics work station (Indy, Unix). The pixel size was 0.3 μm.

TIRF images teemed with fluorescence signals of a type which indicated a high sensitivity to minute translations in the vertical direction. The fluorescence of occasional bacteria flashed on and off as they moved rapidly in and out of the focal plane defined by particles resting on the on the bottom of the chamber. Spontaneously contracting cardiomyocytes often produced flashes where their freely moving ends appeared to hit the glass surface. Some streaks of fluorescent and excitation light, in the general direction of the laser beam, were found to penetrate into the fluid phase at air bubbles, imperfection in the glass surface, and, to a lesser degree, at all points of cellular contact. The background illumination resulting from this violation of the total internal reflection condition was minimized by focusing the attenuated laser beam to a small elliptical spot (100 μm x 200 μm) within the field of vision.

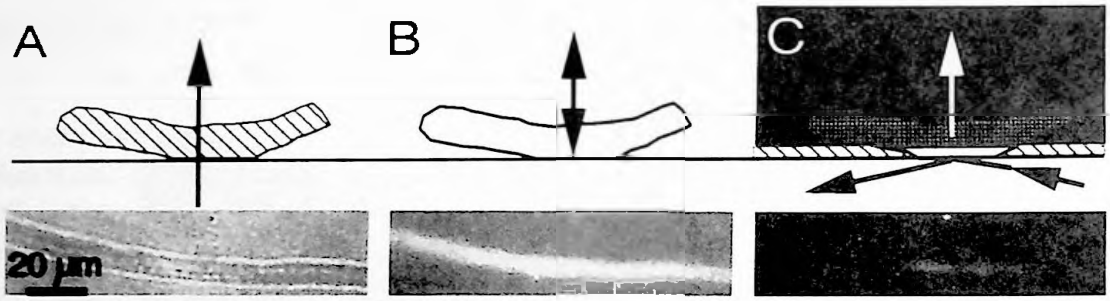

Figure 1. Shark ventricular myocyte images with bright field illumination (panel A), epi-fluorescence illumination, (panel B), and evanescent illumination (panel C). The diagrams show the directions of the light beams. The cell was incubated with 20 μM Fluo-3Am for 30 min.

Figure 1 compares images obtained from a shark ventricular myocyte using bright field illumination (panel A), epi-fluorescence illumination (panel B), and evanescent illumination (panel C). The bright field image shows the outline and striation pattern of the cell and was used to assess viability and provide a framework for the interpretation the fluorescent images. Epi-illumination produced bright uniform fluorescence within the entire outline of the cell. In contrast the TIRF images showed only small patches of fluorescence which initially had a diffuse outline and changed position during contractions. These fluorescent patches were generally oriented in the longitudinal direction of the cell and often included corners and ends. This pattern may simply reflect areas where the cell is resting on the glass surface. Within 30 to 90 min the cells often developed well defined areas of intense fluorescence which had sharp outlines, roughly reproduced the sarcomere pattern, and remained stable during contractions. These patches, were seen in ventricular myocytes from both rat and shark, and, we believe, represent stable cellular adhesions that could provide domains where Ca^{2+} signals may be observed without complications resulting from motion artifacts.

Within areas of adhesion, rat ventricular myocytes showed Ca^{2+} waves and Ca^{2+} sparks similar to those seen with confocal microscopy (Fig. 2). At a recording frequency of at 30 Hz, individual Ca^{2+}-sparks in rat myocytes typically lasted for only one or two frames. Ca^{2+} sparks were never seen in shark ventricular myocytes. The Ca^{2+} sparks in rat cells were particularly prominent when Ca^{2+}-overloaded states were developing. Shark cardiomyoctes, when subjected to Ca^{2+}-overloaded states, showed a slow continuous rise in the fluorescence signals without any occurrence of sparks.

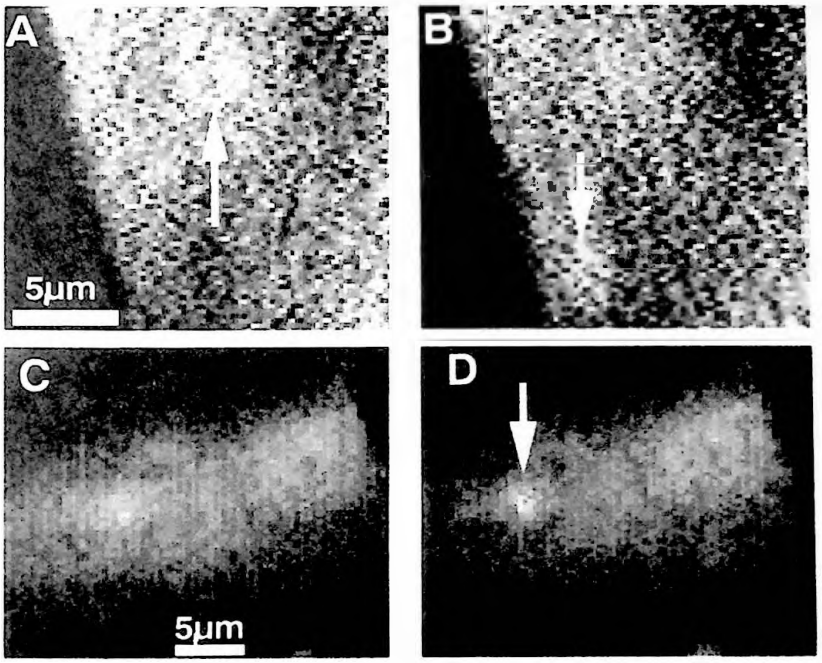

Figure 2. Comparison of Ca^{2+} sparks (arrows) measured in rat ventricular myocytes with confocal (panels A and B) and TIRF (panels C and D) microscopy. In both cases, the panels show two consecutive frames which were recorded at 30 Hz in cells incubated with fluo-3 AM.

These results show that TIRF can be successfully applied to live cardiomyocytes and can measure brief, local Ca^{2+} transients of the type labeled as "sparks" in confocal microscopy. The presence of such signals in ventricular myocytes from rat, but not in those from sharks, support the notion that these transients depend on the presence of functional internal Ca^{2+} stores. We estimate the vertical resolution of the technique to approximately 200 nm, which is significantly better than that achieved with confocal microscopy.

TIRF is a) less expensive than confocal microscopy, b) is likely to give less bleaching and toxicity since only the "focal plane" is illuminated, c) has a frame rate determined by the readout of the camera, not the scan-rate of the illumination/detection beam, and d) has superior depth resolution, albeit, only in a single superficial layer.

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