SUBCELLULAR LOCALISATION OF INTRACELLULAR CALCIUM STORES IN EMBRYONIC MOUSE CARDIOMYOCYTES

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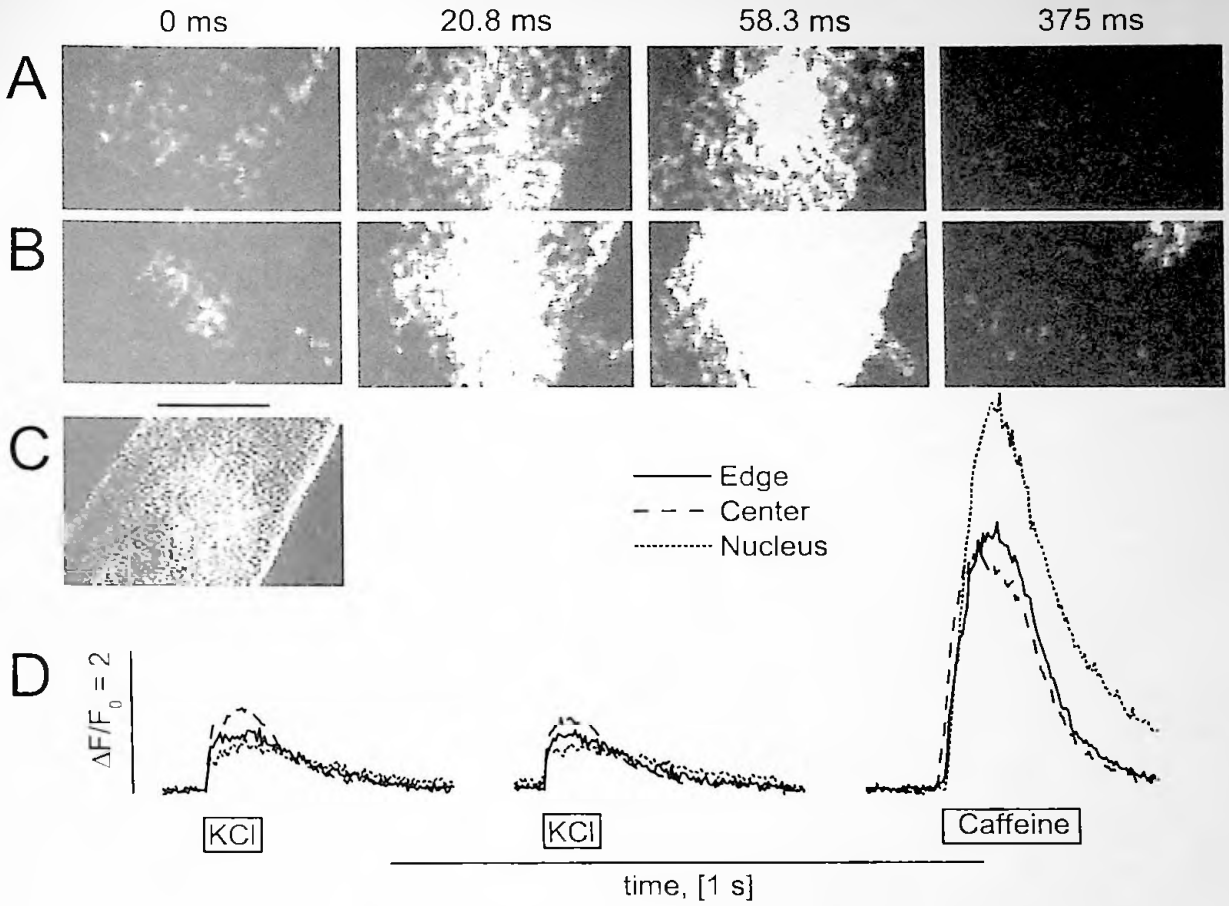
During murine gestation the heart is seen 8.5 dpc (days *postcoitum*) as a contracting tube that in the following days undergoes rapid morphological and cellular developments both prior to and following birth at 21 dpc. Postnatal development of adult excitation-contraction (E-C) coupling in ventricular cardiomyocytes includes formation a t-tubular system that forms dyadic junctions with sarcoplasmic reticulum (SR) thereby allowing function coupling between L-type Ca^{2+} channels and Ca^{2+} release channels (ryanodine receptors, RyRs). Before this transition, contraction may be activated directly by Ca^{2+} current (I_{Ca}), which is the major excitatory current during early (11-13 dpc) murine cardiogenesis (Davies *et al.*, 1996, *Circ. Res.* 78:15-25). It is also known, however, that mRNA of cardiac RyR (RyR-2) is present during early rat embryogenesis (Gorza et al., 1997, *J. Mol. Cell. Cardiol.* 29:1023-1036) and that mutant mouse embryos lacking this protein die ~10 dpc (Takeshima *et al.*, 1998, *EMBO Journal.* 17:3309-3316). It is possible therefore that RyRs and intracellular Ca^{2+} stores may play different roles during embryogenesis than following the post-natal maturation of the heart. To explore this possibility we examined Ca^{2+} signaling in mouse (*Mus musculus*) embryonic heart cells during early (10 dpc) and late (16-18 dpc) stages of embryonic development using rapid (240 frames per second) 2-dimensional confocal microscopy.

Pregnant mice at the desired stage of gestation were killed by cervical dislocation and the hearts of the embryos were dissected and enzymatically digested using 1 mg/ml collagenase B in solution also containing (in mM): NaCl 120, KCl 5.4, $MgSO_4$ 5, Na-pyruvate 5, glucose 20, taurine 20, HEPES 10 (pH 6.9 adjusted with NaOH). The dissociated cells were plated onto gelatin-coated glass slips and maintained in an incubator in standard DMEM (Dulbecco's modified medium) containing 20% fetal calf serum. The cells attached to the glass surface and started spontaneous contractions within 12 hours and were examined after 2-3 days at 37 °C using confocal fluorescence microscopy (Noran Inc.) following loading of the cells with the permeant Ca^{2+}-indicator dye Fluo-3AM (2 μM, 12 min, 37 °C). The standard Tyrode's solution contained (in mM): NaCl 137, KCl 5.4, $CaCl_2$ 2-5, $MgCl_2$ 1, HEPES 10, glucose 10, pH 7.4 (adjusted with NaOH). Cells were generally activated by rapid application (<50 ms) of a depolarizing solution containing 70-100 mM K^+ (iso-osmolar replacement of K^+ for Na^+ or KCl-addition) or a solution with 5-10 mM caffeine. The sarcolemma and internal membranes were visualized by staining with di-2-ANEPEQ. Some cells were voltage-clamped in the whole cell configuration using Cs^+ instead of K^+ in the external solution and a dialyzing solution containing (in mM): NaCl 15, Cs^+-aspartate 110, TEA-Cl 15, HEPES 20 Mg-ATP 5, cAMP 0.2, EGTA 2, K_5-Fluo-3 2, (pH 7.2 adjusted with CsOH). Spontaneously beating cells were selected for experiments.

To evoke Ca^{2+} signals in embryonic cells we used a depolarizing K^+-rich solution to trigger electrical activity and activate Ca^{2+} current while caffeine (5-10 mM) was targeted at



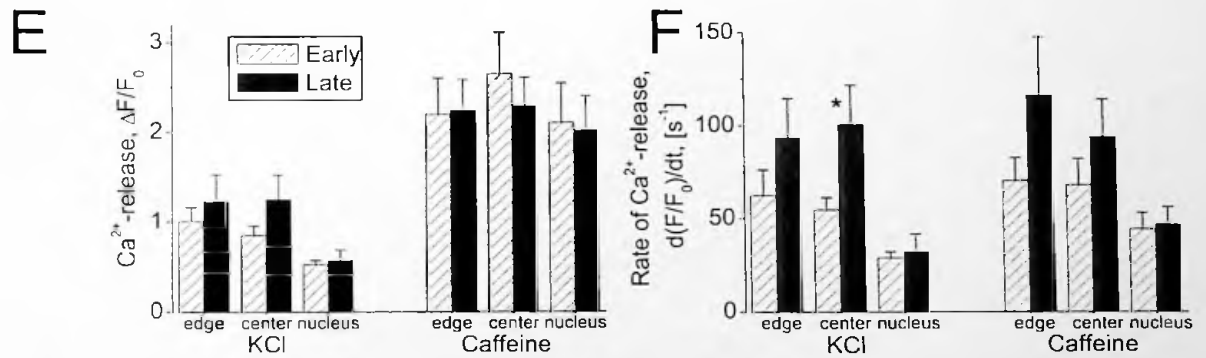


Figure 1. Ca^{2+}-signaling in mouse cardiomyocytes isolated early (10 dpc) or late (16-18 dpc) during embryonic development. Panels A to D show results from a single cell preloaded with Flou-3 AM. Panels A and B show samples of confocal images recorded at 240 Hz during rapid application of a K^+-rich solution (panel A) and a solution with 5 mM caffeine (panel B) at the times indicated following the onset of Ca^{2+} release (0 ms). Panel C: Confocal image of sarcolemma and internal membranes stained for 1 min with 5 μM di-2-ANEPEQ. N indicates nucleus. Scale bar is 10 μm. Panel D. Time course of local Ca^{2+}-transients measured at the edges and center, and in the nucleus during two brief KCl depolarizations followed by exposure to 5 mM caffeine. Panel E: Comparison of local Ca^{2+} transients (maximal $\Delta F/F_0$) produced by KCl depolarization (KCl) or 5 mM caffeine, at edge, center or nucleus of cardiac cells from early (10 dpc, n = 12-16) or late (16-18 dpc, n = 8) stages of development. F: Comparison of the rate of rise of local Ca^{2+} transients (maximal $d(F/F_0)/dt$) under the same conditions. The * indicates significance at the $p<0.05$ level.

RyRs to directly induce SR Ca^{2+}-release. Panels A to D in Figure 1 show typical responses recorded in a single embryonic cell in the late stage of development (16-18 dpc). Panel A shows selected frames of Ca^{2+}-dependent Fluo-3 fluorescence recorded during KCl depolarization. The first frame, labeled 0 ms, shows the onset of the Ca^{2+} transient occurring over wide areas. Based on subsequent staining of external and internal membranes with di-2-ANEPEQ (panel C) it appears that the initial Ca^{2+} signal occurs both at surface of the cell and in the interior, but not in the nucleus (upper right, panel A: 0 ms, 20.8 ms; N in panel C). In the following frames (20.8 ms, 58 ms), the Ca^{2+} signals got stronger, invaded the nucleus (58 ms), and then faded away (375 ms). The time-course of the Ca^{2+} signals detected at the edges (edge) of the cell, in central regions (center), and in the nucleus was plotted in panel D for two consecutive KCl depolarization and for the caffeine-induced Ca^{2+} release shown in detail in the confocal images in panel B. In this cell the caffeine-induced response was first seen (0 ms) in the center of the cell, from where it spread throughout the cell. The caffeine signal was much stronger than the KCl-induced rise in Ca^{2+}. The nuclear response was again delayed (frame 20.8 ms), but then displayed a strong afterglow (375 ms), typical of cardiac cells. The strong caffeine-induced Ca^{2+}- signal suggest the presence of prominent SR-Ca^{2+} stores gated by RyRs. Considering the lack of t-tubules in embryonic cells, it was surprising that Ca^{2+} signals appeared at many locations (panel A: 0 ms) throughout spontaneously beating and KCl-stimulated cells from one frame to the next (<4.167 ms), i.e. too fast and too uniform to be mediated by diffusion of Ca^{2+}.

To validate and expand these observations we performed similar experiments using myocytes harvested early (10 dpc) and late (16-18 dpc) during embryonic development. Figure 1E compares the maximal amplitude of the Ca^{2+} signals in different regions at the cell at the indicated stages of development. The caffeine induced-responses (maximal $\Delta F/F_0$) did not change in different regions and during development suggesting that the amount of releasable SR Ca^{2+} did not change, and that the response was of sufficient duration to allow equilibration between different regions. The smaller KCl-induced responses, on the other hand, showed a trend towards larger Ca^{2+} signal in late development both at the edges and centers of cells, but not in the nuclei. To probe the local Ca^{2+} signals occurring prior to regional equilibration, we measured the maximal rate of rise of Ca^{2+} ($d(F/F_0)/dt$, panel F) and found that the enhancement of the Ca^{2+} signal during developmental was more pronounced, especially in the central region of cells (*, $p < 0.05$), and was seen with both KCl and caffeine (panel E). Furthermore, it was a consistent finding that both the amplitude and rate of rise of Ca^{2+} signals, were of similar amplitude in the center and at the edges of cells.

A strikingly different picture was seen in voltage-clamped embryonic cardiomyocytes dialyzed with 1 mM Fluo-3 and 2 mM EGTA, to buffer intracellular Ca^{2+} and limit its diffusion (Fig. 2). In such experiments we found that the activation of a small Ca^{2+} current by depolarization to 10 mV was accompanied by Ca^{2+} signals that were strong near the surface membrane, but were equally weak at center and nucleus. The suppression of the central Ca^{2+} signal in dialyzed whole cell clamped myocytes suggests that the Ca^{2+} buffers may block the Ca^{2+}-induced Ca^{2+} release mediated by RyRs.

Experiments were typically terminated by staining the cellular membranes by rapid perfusion of a solution containing the voltage-sensitive and moderately lipophillic dye

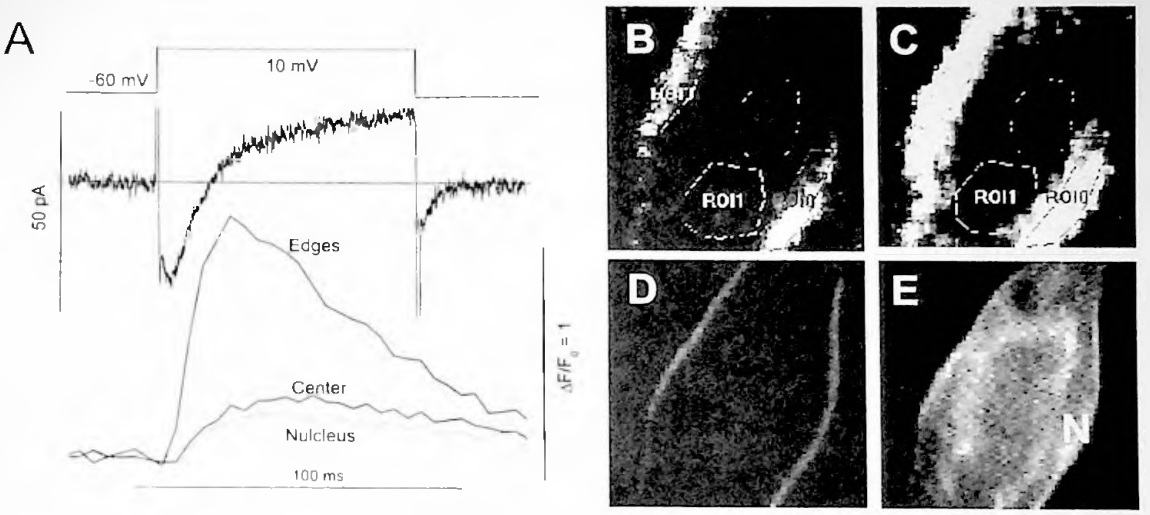


Figure 2. Ca^{2+} current and local Ca^{2+} transients in an embryonic cardiac cell dialyzed with 1 mM Fluo-3 and 2 mM EGTA. Panel A: Time course of Ca^{2+} current and local Ca^{2+} transients (Edges (ROI0+ROI3), center (ROI1), nucleus (ROI2)) activated by an 80 ms depolarizing voltage-clamp pulse from –60 to 10 mV. Panel B and C show confocal images of the Ca^{2+} signals in frames recorded 12.5 and 25 ms, respectively, following deopolarization. Panel D and E show confocal images of membranes after staining with di-2-ANEPEQ for 10 s (panel D) and 60 s (panel E). (Membrane capacitance 79 pF.)

di-2-ANEPEQ. This compound stained the surface membrane within seconds (Fig. 2D) and penetrated to internal membranes within a minute (Fig 1 C, Fig. 2E) revealing extensive internal membrane structures, at times clustered around the nucleus. Consistent with ultrastructural observation, we found perinuclear origin of Ca^{2+} releases and Ca^{2+} sparks in some cells.

The finding of equally large caffeine-induced Ca^{2+} transients at 10 and 16-18 dpc suggests that development of RyR sensitive Ca^{2+} stores is an early event in murine caridogenesis. These Ca^{2+} stores appear to cluster around the nucleus, but are also widely distributed throughout the cells (cultured 1-3 days), and appear to be activated rapidly (<4-10 ms) to release significant amounts Ca^{2+} both during KCl-depolarization and spontaneous beating. It is critical to note that the rate of Ca^{2+} release increased from the early to the late stage of embryonic development suggestive of growth-dependent organization of SR. The structural organization and activation of this Ca^{2+} pool can hardly be the same as in mature ventricular cardiomyocytes (dyadic SR Ca^{2+} release triggered by I_{Ca}) since the embryonic cells lack not only t-tubules but also the striation indicative of a regular myofilament array. The lack of t-tubules and sensitivity to Ca^{2+} buffers suggests some similarity to Ca^{2+} signaling events in atrial cells. We did not, however, observe the pronounced delays in the central Ca^{2+}-transient often associated with atrial E-C coupling.

We conclude that intracellular Ca^{2+} stores play a significant and increasing critical role in cardiac E-C coupling fairly early in embryonic development. The gating and modulation of these stores remains to be determined, but it is apparent that they differ from that observed in atrial and ventricular myocytes of the adult mammalian heart. Supported by NIH RO1 16152 and a grant to Philipp Sasse from the Maine Affiliate of the American Heart Association.