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Spatially non-uniform Ca^{2+} signals induced by the reduction of transverse tubules in citrate-loaded guinea-pig ventricular myocytes in culture

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1. Ratiometric confocal microscopy and the whole-cell patch clamp technique were used to simultaneously record intracellular Ca^{2+} transients and membrane currents from guinea-pig ventricular myocytes. Intracellular dialysis with the low-affinity Ca^{2+} buffer citrate enabled us to record and analyse Ca^{2+} transients caused by Ca^{2+} influx alone and by additional Ca^{2+} release from the sarcoplasmic reticulum (SR) in the same cell.
2. In freshly isolated adult myocytes (used within 1–4 h of isolation) both types of Ca^{2+} transients ('Ca^{2+} entry' and 'Ca^{2+} release' transients) were spatially uniform regardless of the Ca^{2+} current (I_{Ca}) duration. In contrast, Ca^{2+} transients in short-term cultured (1–2 days) myocytes exhibited marked spatial inhomogeneities. I_{Ca} frequently evoked Ca^{2+} waves that propagated from either or both ends of the cardiac myocyte. Reduction of the I_{Ca} duration caused Ca^{2+} release that was restricted to one of the two halves of the cell.
3. Analysis of the Ca^{2+} entry signals in freshly isolated and short-term cultured myocytes indicated that the spatial properties of the Ca^{2+} influx signals were responsible for the spatial properties of the triggered Ca^{2+} release from the SR. In freshly isolated ventricular myocytes Ca^{2+} influx was homogeneous while in short-term cultured cells pronounced Ca^{2+} gradients could be found during Ca^{2+} influx. Spatial non-uniformities in the amplitude of local Ca^{2+} entry transients were likely to cause subcellularly restricted Ca^{2+} release.
4. The changes in the spatial properties of depolarization-induced Ca_i^{2+} signals during short-term culture were paralleled by a decrease (to 65%) in the total cell capacitance. In addition, staining the sarcolemma with the membrane-selective dye Di-8-ANEPPS revealed that, in cultured myocytes, t-tubular membrane connected functionally to the surface membrane was reduced or absent.
5. These results demonstrate that the short-term culture of adult ventricular myocytes results in the concomitant loss of functionally connected t-tubular membrane. The lack of the t-tubular system subsequently caused spatially non-uniform SR Ca^{2+} release. Evidence is presented to show that in ventricular myocytes lacking t-tubules non-uniform SR Ca^{2+} release was, most probably, the result of inhomogeneous Ca^{2+} entry during I_{Ca}. These findings directly demonstrate the functional importance of the t-tubular network for uniform ventricular Ca^{2+} signalling.

During cardiac excitation–contraction coupling Ca^{2+} entry across the sarcolemmal membrane is amplified by the additional release of Ca^{2+} ions from the sarcoplasmic reticulum (SR) by a mechanism referred to as 'Ca^{2+}-induced Ca^{2+} release' (CICR; for reviews see Wier, 1992; Callewaert, 1992). In mammalian ventricular myocytes, Ca^{2+} release triggered by an action potential or a voltage clamp depolarization occurs simultaneously throughout the entire cell volume (Takamatsu & Wier, 1991; Lipp & Niggli, 1994; Cannell, Cheng & Lederer, 1994). Moreover, when the SR release function was irreversibly inhibited by ryanodine, Ca^{2+} transients associated with sarcolemmal Ca^{2+} influx alone were reported to show no spatial inhomogeneities (López-López, Shacklock, Balke & Wier, 1994; Cannell *et al.*

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1994). In contrast, in field-stimulated atrial cells, which lack t-tubules, marked Ca^{2+} gradients underlie the 'whole-cell' Ca^{2+} transient (Hüser, Lipsius & Blatter, 1996). Recent immunochemical and ultrastructural studies (Lewis-Carl *et al.* 1995) have provided evidence for the close association of sarcolemmal Ca^{2+} channels and ryanodine receptors in the SR membrane of ventricular cells. These proteins were found to be clustered along the surface and t-tubular membranes ensuring spatially uniform Ca^{2+} entry and release. In addition, elementary Ca^{2+} release events (Ca^{2+} sparks) originate at t-tubules (Shacklock, Wier & Balke, 1995).

In this study we provide evidence suggesting that the spatial uniformity of the Ca^{2+} entry and SR release signals in guinea-pig ventricular myocytes is lost after the reduction of t-tubular membrane during the short-term culture of the cells. This finding directly demonstrates the functional importance of the t-tubular network in ventricular Ca^{2+} signalling. Preliminary results have been published in abstract form (Lipp, Hüser, Pott & Niggli, 1994).

METHODS

Cell isolation and culture

Adult guinea-pigs (weight, 200–300 g) of either sex were killed by cervical dislocation after stunning. Ventricular myocytes were isolated using standard enzymatic procedures. Cells were either used for experiments within 1–4 h of isolation or transferred into culture medium (M199; Gibco) and stored in an incubator at 95% humidity, 3% CO_2 and 36 °C. Cultured cells were used 1–2 days after isolation. During this period no visible change in cell shape and cross-striation could be observed.

Current measurements

During the experiments a coverslip with cells attached was placed in a sandwich chamber mounted on an inverted microscope (TMD Diaphot; Nikon, Tokyo, Japan). Cell culture medium was replaced by Tyrode solution containing (mM): 140 NaCl, 4 KCl, 2 CsCl, 2 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 10 Hepes; pH 7·4 (adjusted with NaOH). Membrane currents were measured in the whole-cell configuration of the patch clamp technique using a patch clamp amplifier (AxoPatch 200; Axon Instruments). Cells were dialysed with an internal solution containing (mM): 65 caesium citrate, 10 CsCl, 10 NaCl, 1 $MgCl_2$, 0·04 $(NH_4)_5$fluo-3, 0·08 $(NH_4)_4$Fura Red, 10 Hepes; pH 7·2 (adjusted with CsOH). Both dyes were purchased from Molecular Probes Inc. (Eugene, OR, USA). All experiments were carried out at room temperature (20–22 °C).

Fluorescence measurements

Intracellular free Ca^{2+} concentration was measured using confocal microscopy in combination with a dye mixture of fluo-3 (F3) and Fura Red (FR). Details of the confocal set-up based on a BioRad MRC-600 confocal laser-scanning head stage (BioRad, Wetzikon, Switzerland) and the ratiometric $[Ca^{2+}]_i$ measurements using the F3/FR mixture have been described previously (Lipp & Niggli, 1993). Briefly, the 514 nm line of the argon ion laser was used to excite the F3/FR mixture. Emitted fluorescence was collected in the dual-emission mode of the microscope at 540 ± 15 nm (F3) and > 600 nm (FR), respectively. Fluorescence linescan images were converted to the F3/FR ratio and $[Ca^{2+}]_i$ images by applying an *in vivo* calibration curve obtained recently (Lipp, Lüscher & Niggli, 1996). The operational K_D for the F3/FR mixture was estimated at 1·6 μM. The linescan mode of the confocal microscope was used to allow sufficiently high temporal resolution.

For direct visualization of the sarcolemmal membrane the cells were incubated in extracellular solution containing 5 μM Di-8-ANEPPS (Molecular Probes Inc.), a dye known to selectively accumulate in the surface membrane. Di-8-ANEPPS was excited at 514 nm while fluorescence was collected at > 600 nm.

RESULTS

Cultured ventricular myocytes lose functional t-tubular membrane

It has been described recently that during short-term culture the electrical capacitance measured from isolated cardiac myocytes is reduced (Lipp *et al.* 1994; Mitcheson, Hancox & Levi, 1996). We used guinea-pig ventricular myocytes either freshly isolated or kept in short-term culture for 1–3 days to directly investigate the nature of this reduction. Figure 1*A* shows the time course of the membrane capacitance (C_m) in three groups of ventricular myocytes: freshly isolated (control) cells and cells kept for 24–30 h and > 48 h in culture. The mean control C_m of freshly isolated myocytes was 123·4 pF. In cells kept in culture for 24–30 h this value decreased to approximately 35% of the control level. After an additional 24 h in culture, the further decrease in C_m was small, indicating that the structural changes were almost complete after 24 h in culture.

The observation that the reduction of membrane area during culture was not accompanied by visible changes in cell shape suggested that the t-tubular membrane was selectively reduced. To demonstrate this more directly we stained the sarcolemmal membrane with the voltage-sensitive, membrane-selective fluorescent dye Di-8-ANEPPS. We preferred Di-8-ANNEPS to Di-4-ANEPPS since the former is believed to selectively stain the outer leaflet of the cell membrane and therefore only visualizes the cellular membrane fraction accessible from the outside (Haugland, 1992). A representative result is shown in Fig. 1*B*. These properties were typical for all cells investigated under the present experimental conditions. *n* was 50 for freshly isolated cells and 37 for ventricular cells in short-term culture. While t-tubules were readily stained in freshly dispersed myocytes, in cultured cells fluorescence was restricted to the peripheral sarcolemmal membrane. These data provide direct evidence that cultured ventricular myocytes reduce functional t-tubular membrane that is accessible from the extracellular side. In combination with the simultaneous reduction of the cell capacitance these results strongly suggest a functional loss of the t-tubular system during the first day of short-term culture. We believe that such a fast membrane reduction can most probably be explained by a simple disconnection of the t-tubular membrane from the surface membrane. Due to the rapid loss of this membrane fraction dramatic consequences are to be expected for subcellular Ca^{2+} signalling in these cells. Therefore we compared Ca^{2+} signals in freshly isolated

and short-term cultured ventricular myocytes to directly visualize these effects.

Ca^{2+} transients in freshly isolated cells are spatially uniform

Figure 2 shows the representative current (top traces) and Ca^{2+}_i signals (lower traces) recorded from freshly isolated ventricular myocytes. During acquisition of the confocal linescan images (bottom traces) the myocytes were stimulated by a train of step depolarizations from −50 mV to +5 mV with two different durations. In cardiac cells, Ca^{2+} influx triggers the additional release of Ca^{2+} from the SR by the CICR mechanism. However, due to the presence of the low-affinity Ca^{2+} chelator citrate in the dialysing solution, I_{Ca} initiated Ca^{2+} release from the SR in a highly regenerative ('all-or-none') manner (Callewaert *et al.* 1995). As a result, I_{Ca} with a long duration (above 125 ms) elicited Ca^{2+} release signals for every voltage step, while reduction of the duration (below 100 ms) evoked two types of intracellular Ca^{2+} transients: Ca^{2+} transients caused by Ca^{2+} entry alone (●) and those caused by additional SR Ca^{2+} release (○). Under conditions of citrate loading, release transients are characterized by the fact that $[Ca^{2+}]_i$ and the associated inward current continue to increase beyond the end of the depolarizing voltage step. If I_{Ca} fails to trigger SR Ca^{2+} release the rise in $[Ca^{2+}]_i$ is terminated upon repolarization. Inward currents following repolarization reflect Ca^{2+} extrusion by the sarcolemmal Na^+–Ca^{2+} exchanger current (I_{NaCa}; Mechmann & Pott, 1986; Lipp, Pott, Callewaert & Carmeliet, 1990). Therefore, Ca^{2+} entry and release transients were readily distinguished by their time course and the accompanying change in I_{NaCa}. This experimental

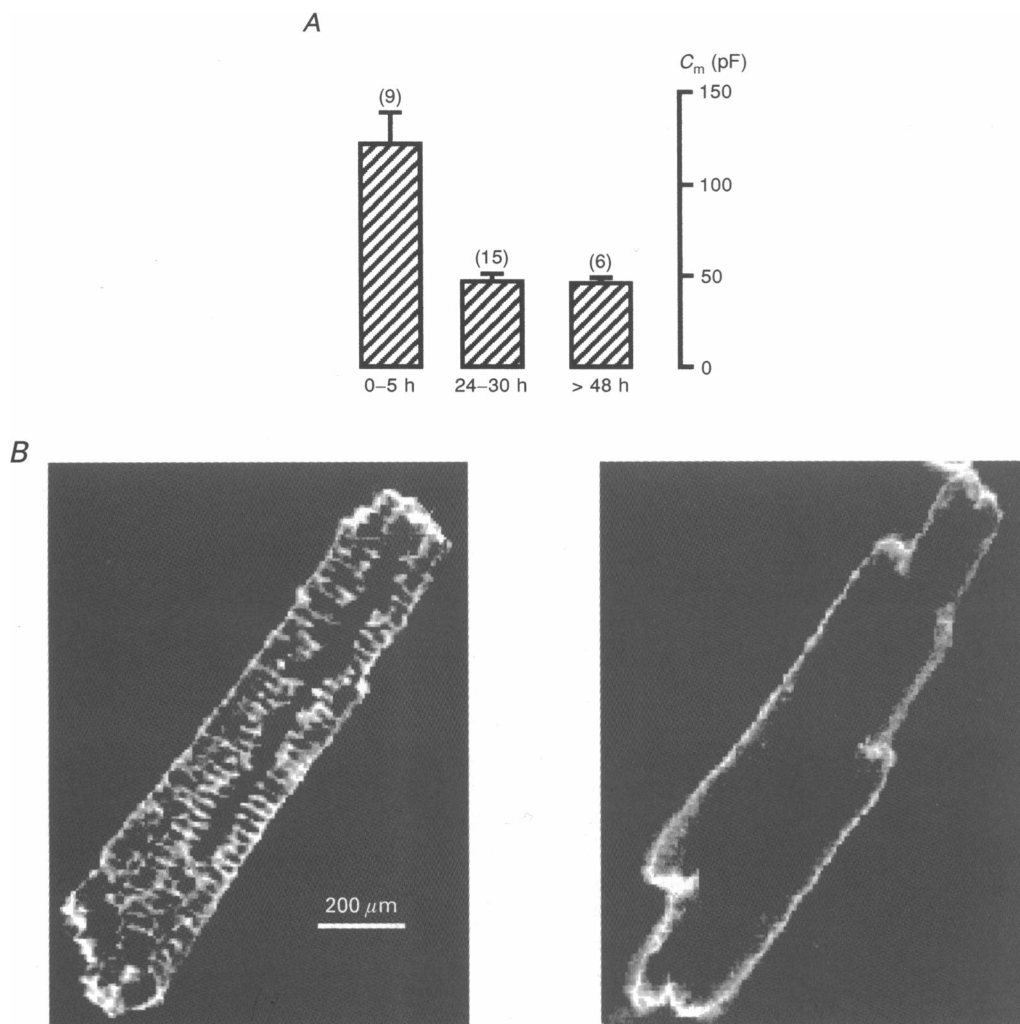

Figure 1. Ventricular myocytes lose functional t-tubules during short-term culture

A, membrane capacitance (C_m) in freshly isolated (0–5 h after isolation; $C_m = 123·4 \pm 17·7$) or cultured ventricular cells (either 24–30 h or > 48 h after isolation; at 24–30 h, $C_m = 46·9 \pm 3·5$; at > 48 h, $C_m = 44·31 \pm 5·0$). *B*, direct visualization of sarcolemmal membrane with Di-8-ANEPPS. While t-tubules were readily stained in acutely dissociated cells (left), in cultured cells (right, after 1 day) fluorescent staining was restricted to the peripheral sarcolemma. These cell images were representative for freshly isolated ($n = 50$) and short-term cultured ($n = 37$) myocytes.

separation of Ca^{2+} entry and additional SR Ca^{2+} release signals was used to study the spatial properties of both signals.

The linescan images shown in the bottom panel of Fig. 2*B* revealed that for both experimental conditions Ca^{2+} entry and release transients were spatially uniform, a property that was found in all cells investigated under the present experimental conditions ($n = 16$). Thus, the change of $[Ca^{2+}]_i$ occurred on a single vertical line, i.e. simultaneously across the scanned line, characteristic of homogeneous Ca^{2+} transients (see Lipp & Niggli, 1994). Even a further reduction of Ca^{2+} influx by (i) decreasing the I_{Ca} duration to 25 ms, or (ii) stepping to a different membrane potential at which I_{Ca} is expected to be smaller, did not induce any subcellular inhomogeneities, strongly indicating that I_{Ca}-triggered SR Ca^{2+} release and I_{Ca}-induced Ca^{2+} influx transients in ventricular myocytes with operating t-tubular systems were always homogeneous. An operating or functional t-tubular system was defined as a membrane system of t-tubules that were either electrically connected to the surface membrane or could be stained by a membrane indicator such as Di-8-ANEPPS.

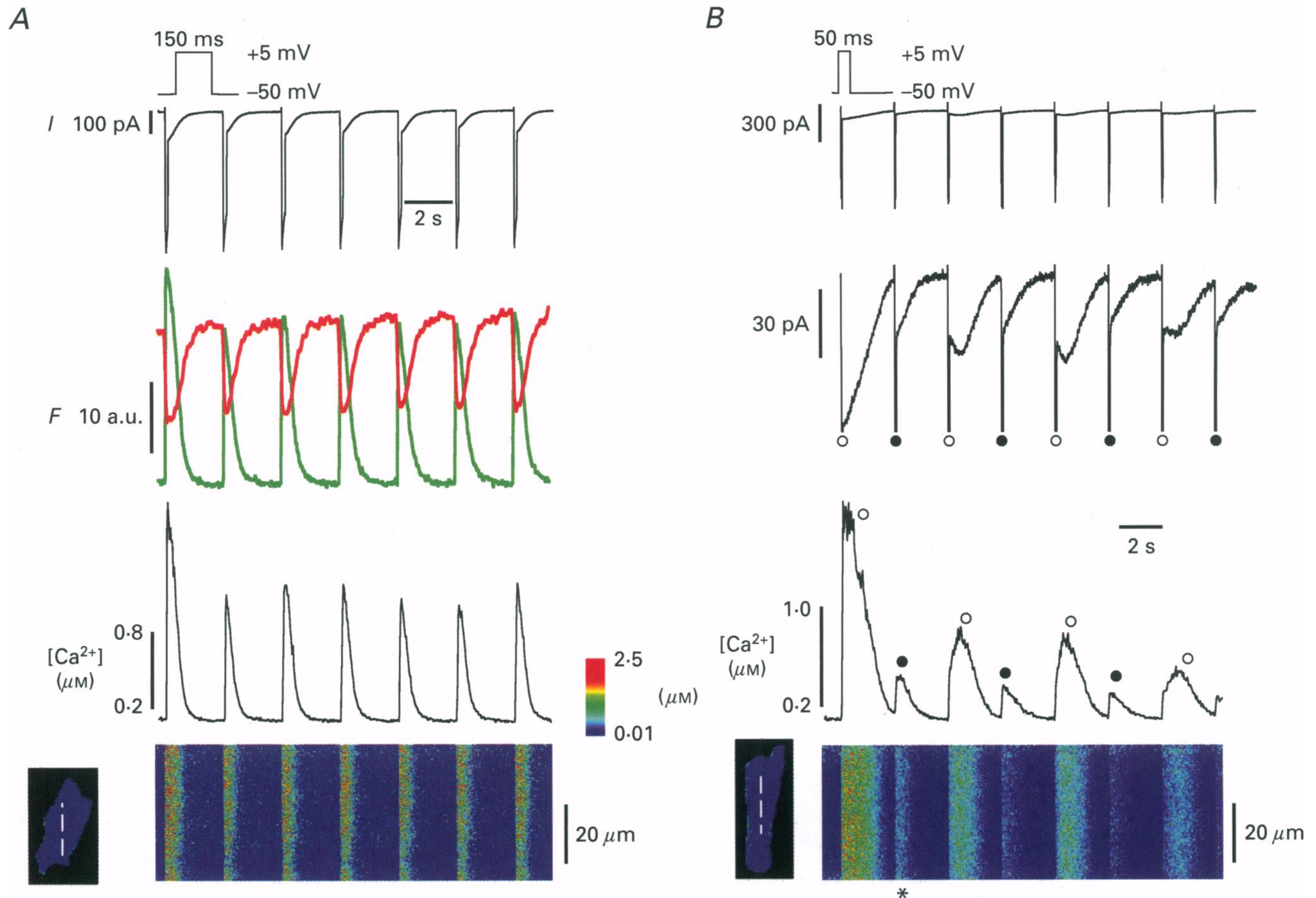

Figure 2. Spatially uniform $[Ca^{2+}]_i$ transients in freshly dissociated cells

Recordings from two representative freshly isolated guinea-pig ventricular myocytes dialysed with a citrate-based pipette solution. The cells were voltage clamped at a holding potential of −50 mV and step depolarizations were applied (duration, 150 ms (*A*) or 50 ms (*B*); frequency, ~0·5 Hz) that evoked I_{Ca} (amplitude, ~700 pA (*A*) and ~800 pA (*B*)). *A* shows (from top to bottom) the membrane current, the fluo-3 fluorescence (green trace) superimposed on the Fura Red fluorescence (red trace), the $[Ca^{2+}]_i$ recordings (averaged from the whole line) and the ratiometric linescan image ($[Ca^{2+}]_i$ was colour-coded according to the colour wedge). Analysis of the linescan image revealed homogeneous SR Ca^{2+} release signals. The additional image of the cell indicates the line chosen for this linescan (dashed white line). *B* presents (from top to bottom) the current recordings at two different magnifications (in the lower recording the amplitude of I_{Ca} was cut off), the $[Ca^{2+}]_i$ transients (averaged from the entire line) and the corresponding linescan image of $[Ca^{2+}]_i$. The ratiometric cell image indicates the line chosen for this linescan. In both traces, the membrane current and $[Ca^{2+}]$, two different groups of I_{Ca} could be identified: those triggering SR Ca^{2+} release (○) and those failing to induced SR Ca^{2+} release (●). The Ca^{2+} entry transient in the linescan images marked by * was chosen for further analysis in Fig. 4. The results shown in this figure were representative for all freshly isolated cells examined under the present experimental conditions ($n = 16$).

But what are the consequences of the loss of a functional t-tubular system for spatially reliable I_{Ca}-triggered SR Ca^{2+} release? The next series of experiments performed with short-term cultured ventricular myocytes was directed at this question.

Spatial inhomogeneities in Ca^{2+} release transients in cultured cells

Figure 3 shows representative recordings from a myocyte kept in culture for 24 h. During acquisition of the linescan image (bottom traces in Fig. 3) the cell was stimulated by a

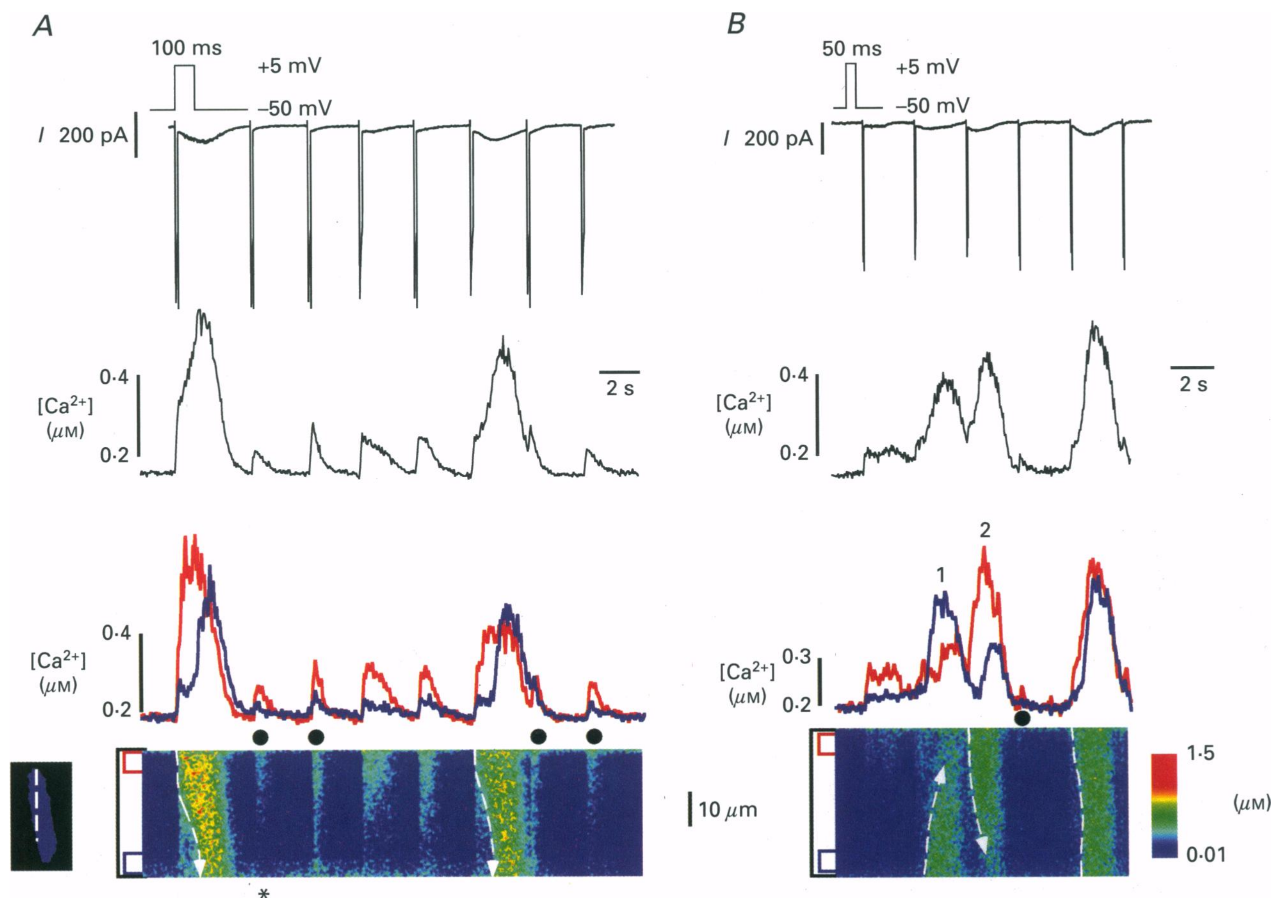

Figure 3. Spatial non-uniform $[Ca^{2+}]_i$ transients in cultured cells

A ventricular myocyte was kept in culture for 24 h and dialysed with a citrate-based pipette solution. The holding potential was set to −50 mV and trains of I_{Ca} were elicited (step potential, +5 mV; duration, 100 ms (*A*) or 50 ms (*B*); frequency, ~0·5 Hz). The traces represent (from top to bottom for both panels) membrane current, Ca^{2+} transients (averaged from the whole line), Ca^{2+} transients (averaged from regions of the linescan image as indicated in the linescan image) and the ratiometric linescan image ($[Ca^{2+}]_i$ was colour-coded according to the colour wedge). For both I_{Ca} durations not every depolarization was sufficient to trigger SR Ca^{2+} release. ●, Ca^{2+} entry signals. *A* shows recordings obtained when the duration of I_{Ca} was set at 100 ms. Two I_{Ca} were able to trigger SR Ca^{2+} release as evidenced by the inward change of I_{NaCa} after repolarization. The underlying Ca^{2+} transients showed properties of propagating Ca^{2+} waves the fronts of which were emphasized by the two white dashed arrows in the linescan image. The inset to the left of the ratiometric linescan image in panel *A* shows Ca^{2+} transients averaged from the upper (red) and lower (blue) part of the linescan image (corresponding to the upper and lower part of the cell, see ratiometric cell image) replotted to emphasize their subcellular properties. Note that each SR Ca^{2+} release transient averaged from the lower part of the cell was preceded by a Ca^{2+} entry transient indicating that Ca^{2+} was not triggered by I_{Ca} but instead corresponded to the propagating Ca^{2+} wave. The Ca^{2+} entry transient in the linescan images marked with the asterisk was chosen for further analysis in Fig. 4. *B* represents recordings from the same cell as in *A* during a train of I_{Ca} with a duration of 50 ms. While the Ca^{2+} transients marked 1 and 2 corresponded to SR Ca^{2+} release that was restricted to one of the two halves of the myocyte (as evidenced from the linescan image), the 5th I_{Ca} triggered a subcellular Ca^{2+} wave. Again the wavefronts of the subcellular Ca^{2+} waves were emphasized with dashed arrows (left) or a dashed line (right). The results shown in this figure were representative of all freshly isolated cells examined under the present experimental conditions ($n = 10$).

train of depolarizations to elicit I_{Ca} with a duration of 100 ms (*A*) and 50 ms (*B*). I_{Ca} failed to trigger SR Ca^{2+} release more frequently in cultured cells than in acutely isolated cells. SR Ca^{2+} release transients (and in parallel I_{NaCa}) in most cases displayed a rather complex time course. The linescan images revealed that the spatial uniformity of Ca^{2+} signals was lost in cultured cells. Note that Ca^{2+} release occurred earlier at the upper part of the scanned line, which corresponds to the upper cell end. Following repolarization the region of elevated $[Ca^{2+}]_i$ expanded into the lower part of the linescan image (i.e. lower part of the myocyte, as shown in the inset in panel *A*). The front of the Ca^{2+} transient during depolarization was not parallel to the scanned line (i.e. homogeneous Ca^{2+} transient, see Fig. 2)

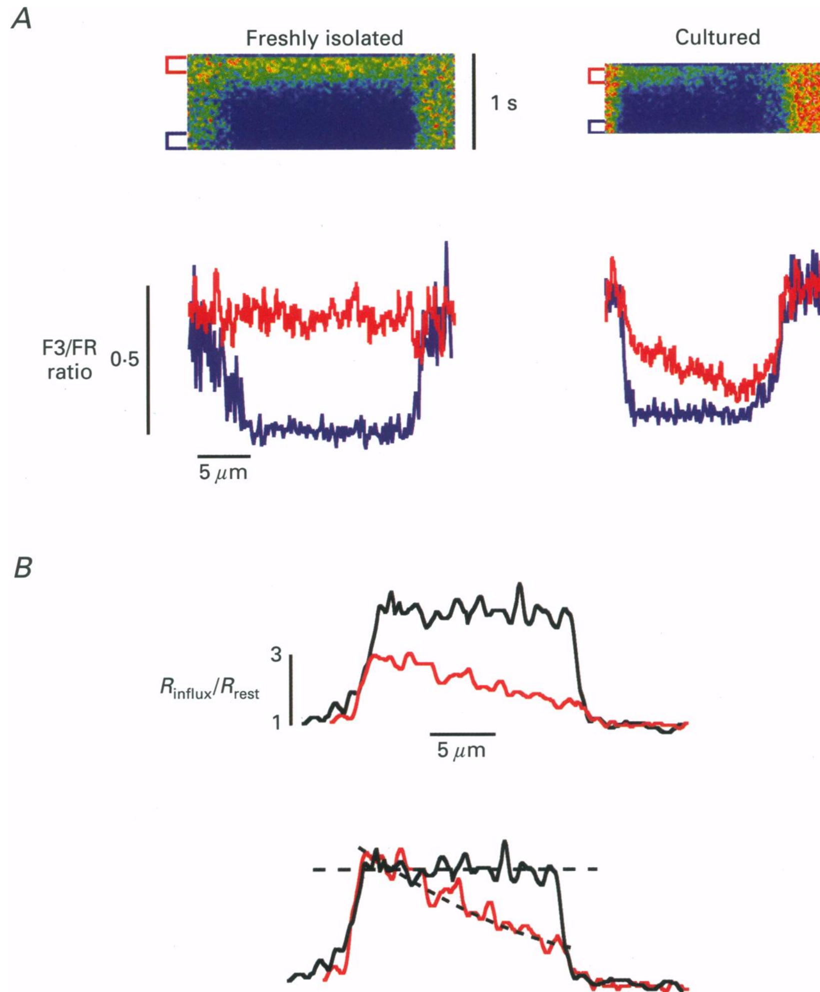

Figure 4. Comparison of Ca^{2+} entry transients in freshly isolated and short-term cultured guinea-pig ventricular myocytes

A shows original ratiometric linescan images from Ca^{2+} entry transients obtained in a freshly isolated ventricular myocytes (left image; taken from Fig. 2, ∗) and in a myocyte kept in short-term culture for 1 day (right image; taken from Fig. 3, ∗). The lower traces represent temporal averages of the linescan images during flow of I_{Ca} (red traces) and during the resting period (blue traces) The colours chosen for the line traces correspond to the brackets at the left side of the linescan images. Due to the ratiometric method used here to access $[Ca^{2+}]$, the cell fluorescence faded into the background for both indicator channels (i.e. fluo-3 and Fura Red) and therefore the F3/FR ratio approaches 1 with an increasing noise. Since the resting ratio is significantly less than 1 the ratio apparently increased at the cell boundaries ('edge effect'). *B* presents normalized Ca^{2+} entry transients derived from the original linescan images in *A*. The upper tracings show the spatial properties of Ca^{2+} entry transients after normalizing the F3/FR ratio during the flow of I_{Ca} with the resting conditions for freshly isolated (black traces) or short-term cultured (red traces) cells. In order to highlight the subcellular properties of the Ca^{2+} entry we normalized both traces to their amplitude and emphasized the shape of the subcellular Ca^{2+} distribution. While the Ca^{2+} distribution during I_{Ca} was homogeneous in freshly isolated myocytes (black trace) a Ca^{2+} gradient could be identified in cells lacking a functional t-tubular system (red trace). These spatial differences between Ca^{2+} entry from freshly isolated and short-term cultured myocytes were representative of all cells in the present study.

but showed deviations that are characteristic for subcellular Ca^{2+} waves (Lipp & Niggli, 1994). These characteristics were even more pronounced after repolarization.

The local Ca^{2+} signals (Fig. 3*A*, ● in bottom panel) showed that the Ca^{2+} entry transients in those regions where Ca^{2+} release was initiated (upper part of the linescan, red) were larger than those in other subcellular regions. To emphasize this difference the local Ca^{2+} transient averaged from the upper part of the cell (where Ca^{2+} waves were initiated in this cell) and that transient derived from the lower part of the cell (where propagation of Ca^{2+} waves occurred) were enlarged and replotted (inset in the lower part of Fig. 3*A*). These results were representative for all myocytes investigated under the present experimental conditions ($n = 10$) with respect to the subcellular properties of their I_{Ca}-induced Ca^{2+} signals.

Reduction of the I_{Ca} duration to values $\leqslant 50$ ms (Fig. 3*B*) often resulted in SR Ca^{2+} release that did not govern the entire cell. Instead it remained restricted to either of the two halves of the cultured ventricular myocyte (Fig. 3*B*; bottom panel). Detailed analysis of the linescan image revealed that the Ca^{2+} transients (marked 1 and 2 in Fig. 3*B*) reflected SR Ca^{2+} release that originated at either end of the cell but ceased propagation in the central region of the myocyte: Ca^{2+} waves with spatially restricted propagation (the direction of Ca^{2+} propagation was emphasized by the dashed, white arrows in the two linescan images). The last Ca^{2+} transient in this linescan image showed a Ca^{2+} wave again travelling through the entire cell demonstrating that spatially restricted Ca^{2+} release was not a property of the particular cell; instead differences in the local positive feedback of the CICR mechanism provided by the subcellular SR network have to be proposed (see also Lipp & Niggli, 1993).

In summary, short-term cultured ventricular myocytes without an operating t-tubular system always generated highly non-uniform SR Ca^{2+} release signals. What is the basis for the difference in the subcellular properties of the Ca^{2+} signals (Ca^{2+} entry and Ca^{2+} release) in freshly isolated and short-term cultured ventricular cells?

The loss of functional t-tubules results in spatially non-uniform Ca^{2+} entry

Figure 4 presents the spatial properties of Ca^{2+} entry signals recorded in freshly isolated (left column) and cultured cells (right column). The upper panel of Fig. 4*A* shows the original, ratiometric linescan images of the Ca^{2+} entry transients. Averages of parts of the linescan images in the temporal domain (i.e. along the time axis) during flow of I_{Ca} (shown in red) and during rest (shown in blue) were superimposed in the bottom panel of Fig. 4*A*. The Ca^{2+} entry transient obtained in freshly isolated cells was homogeneous while that derived from a cultured cell was characterized by a Ca^{2+} gradient from the left to the right side of the linescan image.

Figure 4*B* shows Ca^{2+} distribution during I_{Ca} (red profiles) normalized by the Ca^{2+} distribution during resting conditions (blue profiles). The upper traces represent the raw profiles while the bottom traces were scaled to identical peak amplitudes to emphasize the properties of the Ca^{2+} profiles. It became obvious that in freshly isolated myocytes (black traces in Fig. 4*B*) the Ca^{2+} entry transient was completely homogeneous but that the entry transient recorded from the cultured myocyte (i.e. lack of operating t-tubule membranes) was characterized by a steep Ca^{2+} gradient from left to right. To emphasize this important finding, we highlighted the subcellular Ca^{2+} distribution during flow of I_{Ca} by the two black dashed lines. Although the red trace indicated a steep subcellular Ca^{2+} gradient from the left to the right side of the chosen line for the linescan image it does not reach 1 (corresponding to no Ca^{2+} influx) at the right border of the cell.

Since the images of the subcellular Ca^{2+} distribution were calculated from linescan images the particular position of the chosen line with respect to the cell axis has to be taken into account. In freshly isolated ventricular myocytes the apparent Ca^{2+} entry profile did not depend on the position of the chosen line with respect to the cell axis. In contrast, in short-term cultured cells a larger Ca^{2+} entry signal could be observed in regions at the end of the cell than in regions that were chosen from central parts of the cell along the longitudinal axis. Differences between those amplitudes ranged from 500 to 1000%; the exact ratio depended on the precise position of the line. Therefore, we conclude that the Ca^{2+} entry was more likely to initiate SR Ca^{2+} release at the ends of the cell than in the central regions of the myocyte.

DISCUSSION

In the present study we used either freshly isolated or short-term cultured adult guinea-pig ventricular myocytes to directly investigate the role of the t-tubular system in subcellular Ca^{2+} signalling. The isolated cells were dialysed with a citrate-based pipette solution enabling us to separately record SR Ca^{2+} release and Ca^{2+} influx signals from the same cell without pharmacological suppression of SR Ca^{2+} release function. Application of ratiometric, confocal microscopy allowed us to measure the subcellular properties of $[Ca^{2+}]_i$ while avoiding problems arising from inhomogeneous distribution of a single-excitation single-emission Ca^{2+} indicator such as fluo-3 (see Lipp & Niggli, 1993; Lipp *et al.* 1996). During trains of step depolarizations eliciting I_{Ca} at various pulse durations homogeneous Ca^{2+} entry and SR Ca^{2+} release transients were recorded in freshly isolated myocytes while short-term cultured cells exhibited triggered Ca^{2+} waves either propagating through the entire cell or remaining spatially restricted. While freshly isolated myocytes were characterized by a complex microarchitecture of the t-tubular system, cells kept in short-term culture for 1–2 days showed a complete loss of functional t-tubular membrane as evidenced by a direct

visualization of the t-tubular system and determination of the electrical cell capacitance. These results directly demonstrate the importance of the t-tubular system for reliable and spatially uniform coupling between I_{Ca} and SR Ca^{2+} release in guinea-pig ventricular myocytes.

Subcellularly uniform Ca^{2+} release depends on the presence of functional t-tubular membrane

In previous studies using freshly isolated ventricular myocytes it has been shown that SR Ca^{2+} release is uniform on the microscopic scale as is the Ca^{2+} influx when SR Ca^{2+} release is irreversibly inhibited by ryanodine (Cheng *et al.* 1994). In this and other studies addressing this subject it was hypothesized that the complex t-tubular system of ventricular myocytes may be responsible for ensuring a spatially uniform Ca^{2+} release triggered by I_{Ca}. Moreover, terminal SR cisternae are in close contact with the t-tubular membrane, forming diads or triads (Sommer & Jennings, 1986). Thus, the ultrastructure of the ventricular myocyte is designed for subcellular uniform Ca^{2+} release from the SR.

In support, evidence has been presented that in field-stimulated cat atrial myocytes lacking a t-tubular system SR Ca^{2+} release is preferentially activated underneath the surface membrane and subsequently propagates into deeper cell layers later on (Hüser *et al.* 1996). The experimental results obtained using atrial myocytes indirectly supported the importance of the t-tubular system in ensuring homogeneous release of Ca^{2+} from the SR. Atrial myocytes lack a t-tubular system and except for peripheral couplings between the SR and the sarcolemmal membrane a large fraction of the SR does not exhibit close coupling to the surface membrane (e.g. corbular SR; Sommer & Jennings, 1986).

The present study demonstrates that the spatial uniformity of Ca^{2+} transients caused either by Ca^{2+} influx alone or additional SR Ca^{2+} release was lost during short-term culture of ventricular myocytes. To study Ca^{2+} entry and release transients without pharmacological inhibition of the SR function we dialysed the cells with a high concentration of the low-affinity Ca^{2+} buffer citrate. This intervention is known to partially uncouple SR Ca^{2+} release from Ca^{2+} influx, thereby causing Ca^{2+} release to be 'all-or-none' (Lipp, Pott & Callewaert, 1990; Callewaert *et al.* 1995). As reported previously (e.g. Takamatsu & Wier, 1991; Wier, Egan, López-López & Balke, 1994) Ca^{2+} transients in freshly isolated ventricular myocytes caused by SR Ca^{2+} release or trans-sarcolemmal Ca^{2+} influx are spatially homogeneous. However, in cells kept in culture for at least 24 h, I_{Ca} elicited rapid SR Ca^{2+} release in restricted subcellular regions. Following repolarization the region of elevated $[Ca^{2+}]_i$ subsequently expanded as a Ca^{2+} wave through the cytosol. Analysis of the spatial properties of Ca^{2+} entry transients revealed that Ca^{2+} release was preferentially initiated by I_{Ca} in those subcellular regions displaying larger 'local' entry signals. Interestingly, 'I_{Ca}-triggered Ca^{2+} waves' were also observed in freshly isolated and cultured atrial myocytes under identical experimental conditions (Lipp *et al.* 1994).

The loss of spatial uniformity in intracellular Ca^{2+} signalling was most probably caused by the reduction of functional t-tubules during culture. Reduction of functional (i.e. accessible either from the extracellular space or electrically) t-tubular membrane was visualized directly by staining the sarcolemma with Di-8-ANEPPS. Furthermore, we assessed the decrease in membrane area more quantitatively by measuring membrane capacitance, which dropped to about 35% within 48 h in culture. Qualitatively similar results on the reduction of t-tubules have been obtained by morphometric studies (Delcarpio, Claybomb & Moses, 1989; Decker, Behnke-Barcley, Cook, Lesch & Decker, 1991).

Triggered Ca^{2+} waves were caused by differences in the local surface-to-volume ratio

In this study we have been able to demonstrate for the first time the mechanism underlying subcellularly non-uniform SR Ca^{2+} release in ventricular myocytes that have lost their functional t-tubular system. While our results in freshly isolated citrate-loaded ventricular cells support the notion of a homogeneous Ca^{2+} influx on the microscopic scale, it was obvious that in myocytes lacking a functional t-tubular system Ca^{2+} influx was highly non-uniform resulting in steep gradients during the flow of I_{Ca}. Although inhomogeneous distribution of the Ca^{2+} channels cannot be excluded on the submicroscopic scale, it seems unlikely that such a phenomenon may be responsible for the non-uniform Ca^{2+} signals during I_{Ca} we observed under our experimental conditions.

In all myocytes short-term cultured for 1–2 days and dialysed with a citrate-containing pipette solution ($n = 10$) Ca^{2+} influx transients were always highest when recorded from the two cell ends as compared with more central regions of the cells along the longitudinal axis. This was most probably due to the elongated shape of the ventricular cell resulting in a relative increase of the Ca^{2+} influx per volume at the two ends when compared with more central regions of the cell. This non-uniform Ca^{2+} influx along the longitudinal cell axis resulted in a higher SR Ca^{2+} loading state and in a higher trigger signal for SR Ca^{2+} release at the two ends. Both processes cause the release probability to rise at locations with a larger Ca^{2+} influx. Therefore SR Ca^{2+} release is preferentially triggered at these locations and may subsequently propagate in non-excited regions of the cell in a wave-like fashion.

In summary, we have shown that in ventricular myocytes kept in short-term culture the t-tubular network was almost completely absent. This rapid morphological change (almost complete within 24 h) in turn greatly altered the spatial properties of the Ca^{2+} transients. After reduction of functional t-tubules the spatial uniformity of Ca^{2+} transients was lost, SR Ca^{2+} release showed up as propagating Ca^{2+}

waves triggered by I_{Ca}. Evidence was presented that this non-uniform Ca^{2+} release was caused by the loss of functional t-tubular membrane and subsequent non-uniform Ca^{2+} influx. Ca^{2+} influx during I_{Ca} was found to be highest at the ends of the cell where Ca^{2+} waves were always triggered. This finding supports the idea that *in vivo* (or in freshly dispersed cells) the t-tubular system ensures spatially reliable coupling between Ca^{2+} entry and SR Ca^{2+} release through tightly coupled sarcolemmal Ca^{2+} channels and SR Ca^{2+} release channels.

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