Validation of formamide as a detubulation agent in isolated rat cardiac cells

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Brette, Fabien, Kimiai Komukai, and Clive H. Orchard. Validation of formamide as a detubulation agent in isolated rat cardiac cells. Am J Physiol Heart Circ Physiol 283: H1720–H1728, 2002. First published June 20, 2002; 10.1152/ajpheart.00347.2002.—Kawai M, Hussain M, and Orchard CH. Am J Heart Circ Physiol 277: H603–H609, 1999 developed a technique to detubulate rat ventricular myocytes using formamide and showed that detubulation results in a decrease in cell capacitance, Ca\(^{2+}\) current density, and Ca\(^{2+}\) transient amplitude. We have investigated the mechanism of this detubulation and possible direct effects of formamide. Staining ventricular cells with di-8-ANEPPS showed that the t tubule membranes remain inside the cell after detubulation; trapping of FITC-labeled dextran within the t tubules showed that detubulation occurs during formamide washout and that the t tubules appear to reseal within the cell. Detubulation had no effect on the microtubule network but resulted in loss of synchronous Ca\(^{2+}\) release on electrical stimulation. In contrast, formamide treatment of atrial cells did not significantly change cell capacitance, Ca\(^{2+}\) current amplitude, action potential configuration, the Ca\(^{2+}\) transient or the response of the Ca\(^{2+}\) transient to isoprenaline. We conclude that formamide washout induces detubulation of single rat ventricular myocytes, leaving the t tubules within the cell, but without direct effects on cell proteins that might alter cell function.

transverse tubules; rat ventricular cell; di-8-ANEPPS; rat atrial cell; Ca\(^{2+}\) current; Ca\(^{2+}\) transient

THE SARCOLEMMAL OF MAMMALIAN cardiac ventricular myocytes has invaginations, called t tubules, which occur perpendicular to the longitudinal axis of the cell at intervals of \(\approx 2 \mu m\) (19). Interest in the physiological role of the t tubules has grown in the last few years, although evidence for their function is still sparse. One approach to the investigation of their function has been to use confocal microscopy which, used in conjunction with Ca\(^{2+}\) indicators, has shown that Ca\(^{2+}\) sparks arise more frequently around the t tubules than else-where in the cell (21), and immunocytochemistry has shown that several key proteins involved in excitation-contraction coupling are concentrated at the t tubules (4), suggesting an important role for this structure in excitation-contraction coupling (20). Another approach has been to compare data from ventricular myocytes with data from preparations that lack t tubules, e.g., atrial cells (15), Purkinje cells (6), or cultured ventricular cells, which lose their t tubules after 1–3 days (18). However, differences in protein expression in these systems may also contribute to observed differences.

In 1999, Kawai et al. (14) developed a new technique in their laboratory to investigate the role of the t tubules by inducing acute detubulation of freshly isolated rat ventricular myocytes. They showed that incubation of these cells in 1.5 mol/l formamide (a membrane permeant agent) for 15 min, followed by washout, induces loss of the t tubules, confirmed by staining the cell membrane with the lipophilic dye di-8-ANEPPS. They also showed that this procedure decreased Ca\(^{2+}\) current density by \(\approx \)75%, decreased the amplitude of the Ca\(^{2+}\) transient, and decreased cell capacitance by \(\approx \)26%, a value close to the proportion of the cell membrane in the t tubules determined with electron microscopy (19).

However, several questions remain. First, how does detubulation occur? Second, what are the functional consequences of detubulation on the spatial and temporal distribution of the Ca\(^{2+}\) transient? Third, does formamide have direct effects that might account for the observed changes? The present study was designed to address these questions.

MATERIALS AND METHODS

Isolation of single rat myocytes. Myocytes were isolated from rat hearts according to the method of Boyett et al. (3), which enabled atrial and ventricular myocytes to be isolated...
from the same heart. Male adult Wistar rats (250–300 g) were euthanized by stunning, followed by cervical dislocation, in accordance with the Home Office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986. The heart was quickly removed and mounted on a Langendorff apparatus and retrogradely perfused with isolation solution containing (in mmol/l) 130 NaCl, 5.4 KCl, 0.4 NaH2PO4, 1.4 MgCl2·6 H2O, 0.5 CaCl2, 10 HEPES, 10 glucose, 20 taurine, and 10 creatine, pH set to 7.3 using NaOH. When the coronary circulation had cleared of blood, perfusion was continued with Ca2+-free isolation solution (in which CaCl2 had been replaced with 0.1 mM EGTA) for 4 min, followed by perfusion for a further 10 min with Ca2+-free isolation solution containing 0.8 mg/ml collagenase (type I; Worthington Biochemical; Lakewood, NJ) and 0.1 mg/ml protease (type XIV; Sigma; St. Louis, MO). The atria and ventricles were then excised from the heart. Male adult Wistar rats (250–300 g) were perfused repetitively (1,000 lines of 512 pixels) at 5-ms intervals across the width of the cell. Image analysis was performed off-line using Zeiss LSM 5 Image Examiner Software V2.81 (Zeiss, Germany). Linescan images (8-bit) are presented as the original signal. Traces showing the time course of fluorescence are presented as a ratio of fluorescence/background fluorescence (F/F0). Analysis of Ca2+-transients recorded from the subsarcolemmal space (SS) and cell center (CC) was performed using Origin software. The initial rate of rise of the fluo 3 transient was calculated by fitting a linear function to the upstroke and is expressed as change in fluorescence intensity per ms (dF/dt).

Electrophysiological recording. Currents and voltages were recorded from rat atrial cells using the whole cell configuration of the patch-clamp technique. Pipettes were made from borosilicate glass tubing (GC150TF-15, Clark Electromedical Instrument) with a vertical puller (PP-83, Narashige), re-polished (MF-83, Narashige). When filled with the pipette solution (see below), pipette resistance was 1–2.5 MΩ. Junction potentials between the pipette solution and the reference electrode were cancelled before obtaining a tight gigaseal (>1 GΩ). For measurement of membrane current, cell capacitance and series resistance were compensated (60–80%). Cell membrane capacitance was measured by integrating the capacitance current recorded during a 10-mV hyperpolarizing pulse from a holding potential of −80 mV.

The voltage-clamp amplifier was an Axopatch 1D (Axon Instruments), which was controlled by a Pentium personal computer connected through a CED 1401plus A/D interface (Cambridge Electronic Design), which was also used for data acquisition and analysis with Signal software (Cambridge Electronic Design). Signals were filtered at 2 kHz with an eight-pole Bessel low-pass filter before digitization at 10 kHz and storage.

L-type Ca2+ current (I_{Ca,L})-voltage (V) curves were obtained using 300-ms depolarizing pulses from a holding potential of −80 mV, to voltages between −40 and +50 mV, in 5-mV increments, at a frequency of 0.125 Hz. A 50- or 100-ms prepulse to −40 mV was used to inactivate sodium current and T-type Ca2+ current. I_{Ca,L} was measured as the difference between the peak inward current and the current at the end of the depolarizing pulse currents are expressed as current density (pA/pF). The pipette solution used for these experiments was the same as that used by Kawai et al. (14) and contained (in mmol/l) 120.0 CsCl, 20.0 KCl, 10.0 NaCl, 5.0 Mg-ATP, 1.0 BaPATA, and 10.0 HEPES, adjusted to pH 7.2 with CsOH. Cs-based pipette solutions and 5 mmol/l CsCl experiments was the same as that used by Kawai et al. (14) and contained (in mmol/l) 130.0 KCl, 3 Mg-ATP, 0.4 Na-GTP, 10 EGTA, and 25 HEPES, adjusted to pH 7.2 with KOH. Stimulation frequency was 0.33 Hz. Analysis was performed using Origin software (Microcal).

Recording Ca2+ transients in atrial cells. Rat atrial cells were loaded with the Ca2+-sensitive fluorescent indicator fura 2-AM (3 μM; Molecular Probes) for 10 min at room temperature. Cells were electrically field stimulated at 0.4 Hz. The ratio of fluorescence emitted at 510 nm in response to alternate excitation with 340- and 380-nm light was used
as an index of intracellular Ca$^{2+}$. Analysis was performed using Signal software (Cambridge Electronic Design) by averaging 20 signals at steady state.

**Solutions.** The control bathing solution used in these experiments contained (in mmol/l) 113.0 NaCl, 5.0 KCl, 1.0 MgSO$_4$, 1.0 CaCl$_2$, 1.0 Na$_2$HPO$_4$, 20.0 sodium acetate, 10.0 glucose, and 10.0 HEPES and 5.0 U/l insulin, pH adjusted to 7.4 with NaOH. Acetate was included in the bathing solution because it is an important metabolic substrate for cardiac muscle (1). To induce detubulation by osmotic shock, cells were exposed to this solution plus formamide (1.5 mol/l) for 15–20 min, before returning the cells to control solution, as described by Kawai et al. (14). All solutions were made using ultrapure water supplied by a Milli-Q system (Millipore). All solution constituents were reagent grade and were purchased from Sigma unless stated otherwise. Di-8-ANEPPS was kept as a stock solution of 1.0 mmol/l in DMSO plus 20% pluronic acid, which was added to the bathing solution. Isoprenaline was added directly to the bathing solution from an ampoule (Guy’s Hospital; London, UK) to give a final concentration of 0.5 μmol/l.

**Statistics.** Data are presented as means ± SE. Unpaired t-tests were used to compare data from control and formamide-treated cells; paired t-tests were used to test the effect of isoprenaline. For the microtubule confocal data, which are not normally distributed (10), the Mann-Whitney rank sum test was used. P < 0.05 was taken as significant.

**Results.** Formamide treatment induces detubulation of rat ventricular myocytes. Figure 1A, top, shows a confocal image of a representative control cell stained with di-8-ANEPPS. The x-y image clearly shows the t-tubule network and is representative of 31/31 cells. The x-z image, reconstructed from a z-stack of x-y images (see MATERIALS AND METHODS), shows that the t-tubule network is present throughout the depth of the cell. A plot of the fluorescence intensity along the longitudinal axis of the cell (Fig. 1A, middle) shows peaks of fluorescence at ~2-μm intervals; a fast Fourier transformation of this plot (Fig. 1A, bottom) in 17 cells gave an interpeak interval of 0.5386 ± 0.0091 μm$^{-1}$, i.e., 1.86 ± 0.03 μm.

Figure 1B, top, shows that cells stained with di-8-ANEPPS after treatment with formamide (14) show staining of the surface membrane but little staining within the cell. The fluorescence versus distance plot (Fig. 1B, middle) clearly shows that only surface membrane staining is present; little staining is present in the interior of the cell, and the fast Fourier transformation (Fig. 1B, bottom) shows no periodicity in the fluorescence profile. This result is representative of

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**Fig. 1.** The effect of detubulation on t-tubule structure of ventricular myocytes. Top, representative x-y and x-z confocal images of rat ventricular cells stained with the lipophilic dye di-8-ANEPPS. Middle, fluorescence intensity (FI) profile along dotted line indicated on the longitudinal axis of each cell at top in arbitrary units (AU). Bottom, the results of a fast Fourier transformation of the fluorescence profile. A: control ventricular cell is shown; the t-tubule network is clearly visible. Similar staining was obtained in 31 cells. The fluorescence profile shows peaks at ~2-μm intervals; for this cell, the fast Fourier transform gives a peak at 0.5183 μm$^{-1}$ i.e., 1.93 μm. B: rat ventricular cell stained after formamide treatment. Staining is visible predominantly on the surface membrane (top and middle; n = 28/34), and the fast Fourier transformation does not show a peak. C: rat ventricular cell that was first stained and then detubulated. The confocal image shows staining in the interior of the cell (n = 36/47) but with no clear periodicity (middle and bottom).

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28/34 cells. Similar results were obtained in cells stained for 5 h (n = 6/6) or 6 h (n = 4/4) after detubulation, indicating that the detubulation is not reversed during this time.

T tubules remain inside cell after detubulation. To investigate what happens to the t tubules during detubulation, we exposed the cells to formamide after the membrane had been stained with di-8-ANEPPS. Figure 1C, top, shows a representative image from a cell after this procedure, showing staining on the cell surface and within the cell. However, the intracellular staining is not as well organized as in control cells (compare Fig. 1C, which is representative of 36/47 cells, with Fig. 1A); the profile of fluorescence versus distance shows that fluorescence is present within the cell (Fig. 1C, middle) but shows no periodicity (Fig. 1C, bottom; n = 11).

Thus it appears that the t tubule membrane remains inside the cell after detubulation. To investigate whether the t tubules form vacuoles inside the cell, we described after detubulation in skeletal muscle (16), 50–100 µg/ml FITC-dextran was added to the bathing solution for different parts of the detubulation procedure. Figure 2A shows a confocal image of a control cell bathed in control solution containing FITC-dextran for 2 h, showing the this dextran does not cross the cell membrane: fluorescence can only be seen in the extracellular solution, not within the cell (n = 5/5). Figure 2B shows a confocal image of a representative cell that had undergone the detubulation procedure with FITC-dextran (see MATERIALS AND METHODS) present in all the solutions used, followed by resuspension in dextran-free solution 30 min after the removal of formamide. Fluorescence is clearly visible within the cell (n = 12/15) indicating FITC-dextran trapped within the cell after detubulation; the fluorescence appears to be localized within the cell, but showing a longitudinal pattern, rather than the transverse pattern shown by the t tubules in control cells (Fig. 1A). When this experiment was repeated, except that formamide was washed out with a FITC-dextran-free solution, no fluorescence was visible within the cell (Fig. 2C, n = 10/10). Thus it appears that the FITC dextran becomes trapped within the cell on washout of formamide and thus that detubulation occurs at this point in the protocol.

Microtubule network remains unchanged after detubulation. The cytoskeleton plays an important role in cell structure and in the localization of proteins (23). Thus it seemed possible that the observed changes could be secondary to changes in the cytoskeleton. We therefore stained the microtubule network in control and formamide-treated cells using a specific antibody to β-tubulin and a FITC-conjugated secondary antibody (see MATERIALS AND METHODS). Figure 3A, left, shows a confocal image of the microtubule network in a representative control cell (n = 116). The pattern of staining appeared the same after detubulation (n = 107; Fig. 3A, right), and the mean fluorescence signal was not significantly different between control and detubulated cells (17.27 ± 1.37 arbitrary units (AU) in control, n = 116, vs. 17.56 ± 1.35 AU in detubulated cells, n = 107; not significant (NS); Fig. 3B). In addition, cell area, which is measured on confocal images through the center of the cells, was not significantly different after detubulation (2,681.9 ± 106.8 µm² in control cells, n = 53, vs. 2,632.6 ± 71.1 µm² in detubulated cells, n = 63; NS).

Effect of detubulation on intracellular Ca²⁺ distribution in ventricular myocytes. Because I_Ca is concentrated in the t tubules (see Introduction), detubulation would be expected to alter Ca²⁺ distribution after electrical stimulation. Figure 4A shows transverse line scan images from a representative control cell (left) and detubulated cell (right) during electrical stimulation. In the control cell, electrical stimulation resulted in a rapid and synchronous rise in intracellular Ca²⁺ concentration ([Ca²⁺]i) across the width of the myocyte; this is clearly shown by the Ca²⁺ transients recorded from the SS and CC (Fig. 4B, left), which have almost identical amplitudes and time courses (F/F₀: SS, 2.7 ± 0.2; CC, 2.7 ± 0.6; NS and dF/dt: SS, 3.4 ± 1.0; CC, 3.3 ± 1.0 F U/ms; NS, n = 7). In contrast, in the detubulated cell, Ca²⁺ initially increases at the edge of

Fig. 2. The accessibility of FITC-dextran to the interior of ventricular myocytes. A: representative image of a ventricular myocyte that was bathed for 2 h in FITC-dextran. Fluorescence can be seen in the solution but not within the cell; similar results were obtained in 5 cells. B: representative image of a ventricular myocyte in which the formamide-containing and washout solutions contained FITC-dextran, showing that fluorescence is visible within the cell (n = 12/15). C: representative image of a ventricular myocyte in which the formamide-containing solution contained FITC-dextran, but the washout solution was dextran free, showing that no fluorescence is present within the cell (or the solution); similar results were obtained in 10 cells. Scale bar is 10 µm for A–C.
Fig. 3. The effect of detubulation on the organization and density of microtubules in ventricular myocytes. A: immunofluorescence confocal images of sections taken at the level of the nuclei in representative control (left) and detubulated (right) ventricular myocytes labeled with an antibody to β-tubulin, showing no apparent difference in the organization of the microtubule network. Scale bar is 20 μm. B: fluorescence intensity (normalized to cell section area) of β-tubulin staining in control (open bar) and detubulated (hatched bar) ventricular myocytes. Means ± SE, n = 116 and 107 observations, respectively (not significant, NS). AU, arbitrary units.

the cell and then propagates to the center of the cell at 46 ± 6 μm/s (n = 10). Figure 4B, right, shows that Ca^{2+} recorded from the SS rises earlier and more rapidly than that recorded from the CC (dF/dt: SS, 3.5 ± 0.3; CC, 0.6 ± 0.1 F U/ms; P < 0.05; n = 10), although peak systolic [Ca^{2+}] was the same in SS and CC (F/F0: SS, 2.2 ± 0.2; CC, 1.9 ± 0.1; NS; n = 10).

The amplitude of the Ca^{2+} transient was significantly (P < 0.05) smaller in detubulated cells than in control cells (Fig. 4, B and C), as reported previously (14). However, the rate of rise of Ca^{2+} in SS was not statistically different in control and detubulated cells (3.4 ± 1.0 and 3.5 ± 0.3 F U/ms, respectively; NS) but in CC was significantly faster in control cells than in detubulated cells (3.3 ± 1.0 and 0.6 ± 0.1 F U/ms, respectively; P < 0.05).

Figure 4C shows global Ca^{2+} transients obtained from the whole scan. In contrast to the Ca^{2+} transient recorded from the control cell, in 7 of 10 detubulated cells, the Ca^{2+} transient showed a biphasic rising phase, as reported by Kawai et al. (14), as a consequence of the asynchronous rise of Ca^{2+} across the width of the cell.

Atrial cells lack t tubules. To investigate the possible direct effects of formamide, the detubulation procedure was performed on atrial cells, which lack t tubules (24); this is confirmed in Fig. 5A, which shows a confocal image of a representative rat atrial myocyte stained with di-8-ANNEPS, showing the absence of a t tubule network (n = 22). A similar result was obtained after incubation of atrial cells with formamide (15–20 min), followed by washout (Fig. 5B, n = 20), although measurements of cell length and width during addition and removal of formamide showed that atrial cells undergo the same changes in size as described for ventricular cells by Kawai et al. (14) (n = 3, data not shown).

Effects of formamide treatment on electrophysiologi- cal parameters of atrial myocytes. We repeated in atrial myocytes the experiments that Kawai et al. (14) carried out in ventricular myocytes. The results are shown in Fig. 6. Cell capacitance measured with the whole cell configuration of the patch-clamp technique did not change significantly after formamide treatment (Fig. 6A; 48.6 ± 2.2 pF in 38 control cells vs. 46.1 ± 2.4 pF in 26 formamide-treated cells, NS). The I_{Ca-V} curve, measured as described by Kawai et al. (14), did not change
significantly after formamide treatment of atrial cells (Fig. 6B): \( I_{Ca} \) density during a test pulse to \(-5 \text{ mV} \) was \(-2.34 \pm 0.39 \text{ pA/pF} \) in control cells (\( n = 10 \)) versus \(-2.50 \pm 0.47 \text{ pA/pF} \) in formamide-treated cells (\( n = 8 \); NS). These data are different from those obtained in rat ventricular cells in which formamide treatment significantly decreased cell capacitance and \( I_{Ca} \) density (see Introduction).

To investigate whether formamide treatment affects other physiologically important currents, we recorded action potentials from single atrial rat cells before and after exposure to formamide. Figure 6C shows action potentials recorded from a representative control atrial cell (left) and a representative formamide-treated atrial cell (right). Mean data from 10 cells in each group show that the resting membrane potential, action potential amplitude, and times for repolarization of the action potential (APD25, APD50, and APD90) were not significantly different in control and formamide-treated cells (Table 1).

Effects of formamide treatment on \( Ca^{2+} \) cycling in atrial myocytes. To investigate further the possible direct effects of formamide on proteins and cell function, we recorded \( Ca^{2+} \) transients from control and formamide-treated atrial cells, in the absence and presence of the \( \beta \)-adrenergic agonist isoprenaline (0.5 \( \mu \text{mol/l} \)). Figure 7A shows representative \( Ca^{2+} \) transients recorded with the fluorescent dye fura 2 from control (left) and formamide-treated (right) atrial cells in the absence and presence of isoprenaline. The amplitude of the \( Ca^{2+} \) transient was not significantly different in control and formamide-treated cells (0.0607 \( \pm 0.0093 \) ratio units (RU) for control cells, \( n = 11 \), vs. 0.0622 \( \pm 0.0151 \) in formamide-treated cells, \( n = 8 \); NS; Fig. 7B). Similarly, treatment with formamide had no significant effect on the half-time of decay of the \( Ca^{2+} \) transient (\( T_{1/2} \)), which was 371 \( \pm 34 \) ms in control cells, \( n = 11 \), versus 379 \( \pm 34 \) ms in formamide-treated cells, \( n = 8 \); NS (Fig. 7C). This is different from the result obtained in rat ventricular cells by Kawai et al. (14) and reported above, in which formamide treatment significantly decreased \( Ca^{2+} \) transient amplitude (see Introduction).

Isoprenaline-induced a significant inotropic and lusitropic effect in both control and formamide-treated cells (\( P < 0.01 \), Fig. 7, B and C, respectively), but there was no significant difference in the amplitude or \( T_{1/2} \) of the \( Ca^{2+} \) transient between the two populations of cells in the presence of isoprenaline (amplitude was 0.0986 \( \pm 0.0199 \) RU in control cells, \( n = 11 \), vs. 0.0962 \( \pm 0.0205 \) RU in formamide-treated cells, \( n = 8 \); NS).
The present study shows that treatment of NS. T/H11002 potential parameters in atrial myocytes H1726 DETUBULATION OF SINGLE RAT MYOCYTES

ruption of the t tubules, in agreement with the report rat ventricular myocytes with formamide produces dis-
et of the plasma membrane and fl maintained in the outer lea-
42 ms in control cells, n = 11, vs. 312 ± 42 ms in formamide-treated cells, n = 8; NS).

DISCUSSION

Formamide-induced detubulation of rat ventricular myocytes. The present study shows that treatment of rat ventricular myocytes with formamide produces dis-
estranged in the outer leaflet of the plasma membrane and does not cross the cell membrane (8); the observation that t-tubular staining was not observed in formamide-
treated cells, despite the presence of t-tubular membranes within these cells (see below) supports this idea. Thus the membranes stained by the dye are those accessible from the extracellular space. The loss of t-tubular staining after treatment with formamide suggests, therefore, that the t tubules are no longer open to the extracellular space. This disruption was maintained for at least 6 h.

The present experiments help elucidate the mechanism and nature of this disruption. The use of FITC-labeled dextran in the extracellular solution showed that this dextran became trapped within the cell only when it was present in the solution used to wash out formamide (Fig. 2). This agrees with previous suggestions (14) that it is the rapid expansion of the cell that occurs on formamide-washout that causes the t tubules to detach from the cell surface. It also agrees with the observation that incubation of cells in formamide for up to an hour, without washout, does not disrupt the t tubule system (not shown), and is compatible with previous data (14) showing that the major functional changes occur on washout of formamide.

The pattern of fluorescence from the trapped FITC-dextran suggests that this indicator is trapped in discrete compartments within the cell, which have a different spatial organisation from the original t tubules. This is consistent with the t tubules resealing within the cell, thus forming vacuoles, as suggested after detubulation of skeletal muscle (16). The idea that the t tubules reseal within the cell is also supported by the observation that the cells maintain their shape after formamide treatment is also supported by the observation that the cells maintain their shape after formamide treatment does not cross the cell membrane (8); the observation that the cells maintain their shape after formamide treatment is also supported by the observation that the cells maintain their shape after formamide treatment (19). It is unclear, however, whether the t tubules that have resealed within the cell remain capable of ion transport.

Detubulation does not affect cytoskeleton. The cytoskeleton plays an important role in determining cell structure and shape and in anchoring proteins (2, 23). It seemed possible, therefore, that the changes produced by formamide could be secondary to effects on the cytoskeleton. However, the observation that there is no apparent disruption of the cytoskeleton (Fig. 3) and no change in cell size after formamide treatment makes this unlikely.

Detubulation changes spatial distribution of Ca2+ after electrical stimulation. In control cells, Ca2+ increased homogeneously across the cell on electrical stimulation. However, in formamide-treated cells, Ca2+ initially increased close to the cell membrane and then propagated into the cell. This is similar to the pattern observed in atrial and Purkinje cells, which

Table 1. Effect of formamide treatment on action potential parameters in atrial myocytes

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 10)</th>
<th>Formamide Treated (n = 10)</th>
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<tbody>
<tr>
<td>Resting potential, mV</td>
<td>−80 ± 1</td>
<td>−81 ± 1</td>
</tr>
<tr>
<td>Amplitude, mV</td>
<td>124 ± 3</td>
<td>119 ± 4</td>
</tr>
<tr>
<td>APD25, ms</td>
<td>3.0 ± 0.2</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>APD50, ms</td>
<td>6.3 ± 0.9</td>
<td>8.5 ± 1.7</td>
</tr>
<tr>
<td>APD90, ms</td>
<td>28.5 ± 6.5</td>
<td>29.7 ± 4.1</td>
</tr>
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Values are means ± SE; n, no. of cells in each group. APD, action potential duration.
lack t tubules (6, 11), and is consistent with the form-
amide-treated cells being detubulated and with an
important role for the t tubules in ensuring rapid and
synchronous Ca\(^{2+}\) release throughout the cell. Inter-
estingly, the whole cell Ca\(^{2+}\) transients calculated from
these line-scan images showed a biphasic rising phase
as a consequence of this asynchronous release (Fig. 4);
similar Ca\(^{2+}\) transients have been reported previously
in detubulated cells by Kawai et al. (14), who, in agree-
ment with the present data, suggested that it might be
caused by initial Ca\(^{2+}\) release at the cell surface, fol-
lowed by propagation into the cell.

It seems likely that the inward propagation of Ca\(^{2+}\)
is due to propagated Ca-induced Ca release (CICR)
because the rate of propagation is within the range
reported previously for CICR (12), the amplitude of the
Ca\(^{2+}\) transient is the same at SS and CC (Fig. 4), and
propagation is inhibited by the sarcoplasmic reticulum
inhibitors ryanodine and thapsigargin (not shown). This
propagation is, therefore, similar to that in atrial cells
(15) but different from that in Purkinje cells, in which
buffered diffusion of Ca\(^{2+}\) appears to be responsible for
the inward movement of Ca\(^{2+}\) from the cell surface
so that the amplitude of the Ca\(^{2+}\) transient is smaller
in the center of the cell than at the periphery (6).

**Formamide has no direct effect on proteins.** As previ-
ously described for the atrial cells of other species, e.g., cat (15), rabbit (4), and guinea pig (7), the rat
atrial cells used in the present study lacked t tubules
(Fig. 5). This preparation therefore enabled us to in-
vestigate the possible direct effects of formamide on
protein and cell function in the absence of t tubules.
Formamide treatment of atrial cells did not induce a
significant decrease in cell capacitance, or in the am-
plitude or density of the Ca\(^{2+}\) current, or in the config-
uration of the action potential or Ca\(^{2+}\) transient. Thus
formamide appears to have no direct effect on \(I_{\text{Ca,L}}\), or
on the other currents, and hence presumably the chan-
nels that underlie the atrial action potential or Ca\(^{2+}\)
transient. Similarly, the response of the \(\beta\)-adrenergic
pathway to isoprenaline was unaltered after form-
amide treatment. These data are consistent with the
idea that the effects seen in rat ventricular myocytes
are due to detubulation rather than direct effects of
formamide on proteins.

**Significance of this technique.** Several different tech-
niques have been used previously to investigate the
role of the t tubules in cardiac muscle. Immunocyto-
chemistry has been widely used to identify protein
distribution and has shown, for example, that the \(I_{\text{Ca,L}}\)
channel is concentrated in the t tubules (4). However,
the observed distribution may depend on the antibody
used and the accessibility of the epitope (17). Although
this technique can yield useful information about pro-
tein distribution, it cannot give information about lo-
calized protein function, which may depend on other
local factors, such as accessory proteins and modulators.
The patch-clamp technique has been used to study
localization of functional channels. Jurevicus and Fischmeister (13) used a double patch-clamp technique
and a double-barreled microperfusion system to show
that Ca\(^{2+}\) and Na\(^{+}\) channels are uniformly distributed
on the sarcolemmal membrane of frog ventricular myo-
cytes. However, this technique does not have access to
the t tubules. An alternative approach has been to use
the diffusion delay between the bulk extracellular so-
lution and the t tubules. This approach has been used
to investigate the distribution of Na\(^{+}\) and Ca\(^{2+}\) chan-
nels in guinea pig ventricular myocytes (22) and has
shown that 64% of \(I_{\text{Ca}}\) and \(I_{\text{Na}}\) change slowly after a
rapid change of extracellular [Ca\(^{2+}\)] and [Na\(^{+}\)], indicat-
ing that \(\sim 64\%\) of functioning Na\(^{+}\) and Ca\(^{2+}\) channels
are in the t tubules. This approach has also been used
by Christé (5) to investigate the distribution of K\(^{+}\)
inward rectifier current in rabbit ventricular myocytes.
Comparison of preparations lacking t tubules with
those having t tubules has also been used, although it
is difficult to exclude differences in protein expression
(see Introduction). Acute detubulation provides a com-
plimentary technique to investigate the localization of
membrane functions.

In summary, it appears that washout of formamide
causes the t tubules to become uncoupled from the
surface membrane and reseal within the cell. It seems
most likely that the effects of formamide on the func-
tion of rat ventricular myocytes are due to this detu-
bulation, rather than secondary to direct effects of
formamide on the cytoskeleton or proteins. This tech-
nique therefore provides an additional tool to study the
functional role of the t tubules in ventricular myocytes.

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