Calcium and mechanically induced potentials in fibroblasts of rat atrium

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Abstract

Objectives: Electrically non-excitable cardiac fibroblasts in the sino-atrial node region are mechano-sensitive. Rhythmic contraction of adjacent myocardium, or artificial stretch of the tissue, produce a reversible change in the membrane potential: mechanically induced potentials (MIP). Stretch of normal cardiomyocytes can be associated with intracellular calcium changes. The purpose of this study is to use pharmacological interventions to investigate the possibility that stretch-induced Ca2+ entry through ion channels in the sarcolemma and Ca2+ release from internal stores play a role in MIP generation. Methods: Isolated spontaneously contracting or artificially stretched preparations of right atrium of rat heart were superfused with physiological solutions. An intracellular floating microelectrode recorded fibroblast MIPs and was also used for injection of current. A dye, Lucifer yellow, applied through the micropipette, identified recording sites. We assessed the role of extracellular Ca2+ using EGTA in the bathing solution. For the role of intracellular Ca2+ in the generation of MIP, several substances that influence [Ca2+] handling were applied intracellularly by diffusion from the recording microelectrode. These include: BAPTA (to chelate intracellular Ca2+); BHQ, thapsigargin and CPA (to deplete Ca2+ from intracellular stores by inhibition of the endoplasmic reticulum (ER) ATP Ca2+ pump), and caffeine and ryanodine (to induce ER Ca2+ release). Results: All the pharmacological compounds which were introduced intracellularly, and EGTA applied extracellularly, decreased the amplitude of the MIP to variable degrees. Only thapsigargin induced a bi-phasic response with an initial increase in MIP amplitude, followed by a decrease. MIP duration was reduced by most interventions, exceptions being low extracellular Ca2+, BHQ and ryanodine. Short duration extracellular application of caffeine, which was added to the perfusate as a secondary contractile stimulus, partly restored the MIPs by activation of cardiac contraction. Intracellular current injection, before any intervention, linearly altered both membrane potential (E,) and MIP amplitude (Vm). Application of compounds listed above introduced non-linearity to the E m/V m relationship. Conclusion: We suggest that mechanically induced Ca2+ influx, induced through stretch-activated channels in the plasma membrane, and release of Ca2+ from the endoplasmic reticulum, play key roles in the mechanism of MIP generation. Further, our results demonstrate the existence of functional ryanodine/caffeine-sensitive Ca2+ stores in cardiac fibroblasts.

Keywords: Excitation-contraction coupling; Stretch; Fibroblasts; Ryanodine; Thapsigargin; Calcium, intracellular concentration; Rat, atrium

1. Introduction

Electrophysiological characteristics of non-muscle cells of the atrium have been described in isolated hearts of frog [1,2] and rat [3]. Electrophysiological and histological studies [4,5] demonstrated that these cells are fibroblasts. Their resting membrane potential typically is about -20 to -25 mV, and the membrane resistance may exceed 1GΩ. These cells are electrically non-excitable and mechano-sensitive. Spontaneous contraction of the surrounding myocardium, or mechanical changes evoked by artificial stretch of the tissue, elicit changes in the membrane potential—mechanically induced potentials (MIP). Intracellular polarisation changes the amplitude but has no effect on the frequency of MIP. These potentials could be explained by the operation of stretch-activated channels, described for cultured fibroblasts [6]. This idea is supported by the finding that gadolinium, a stretch-activated cation channel blocker, is capable of reducing and removing MIP [7]. Gadolinium is a non-specific blocker of stretch-activated channels and blocks also some Ca2+ and potassium chan-
nels [8-10]. If stretch-activated channels exist in fibroblasts, however, they may not directly determine the cell’s response to stretch at the membrane potential levels observed in fibroblasts.

Membrane stretch increases [Ca\(^{2+}\)]\(_i\) in a variety of cells [11,12]. Mechanically induced elevation of [Ca\(^{2+}\)]\(_i\), can arise from two sources: Ca\(^{2+}\) influx [13,14] from the outside and Ca\(^{2+}\) release from internal stores [15-17]. The endoplasmic reticulum may serve as a Ca\(^{2+}\) store. The Ca\(^{2+}\)-ATPase and the Ca\(^{2+}\) release channels are the two major structures of the endoplasmic reticulum enabling it to serve as an intracellular source of Ca\(^{2+}\). Net movement of Ca\(^{2+}\) between store and cytosol is the difference between active Ca\(^{2+}\) transport by the ATPase and passive Ca\(^{2+}\) release through the release channels [18]. We propose that [Ca\(^{2+}\)]\(_i\), plays an important role in MIP generation in electrically non-excitable cells. Calcium release from internal stores, and ATP-driven Ca\(^{2+}\) re-uptake have been observed in a large variety of cells [19-22] and have been reported to cause oscillations in membrane potential [22]. In particular, [Ca\(^{2+}\)]\(_i\), oscillations have been shown in different lines of fibroblasts [23-25].

We examined the importance of the endoplasmic reticulum in MIP production by intracellular application of relatively specific blockers of the endoplasmic Ca\(^{2+}\)-ATPase. To test our hypothesis that Ca\(^{2+}\) release plays a role in the production of MIP in cardiac fibroblasts, a number of substances that influence [Ca\(^{2+}\)]\(_i\), handling were applied intracellularly: the Ca\(^{2+}\)-specific chelator—BAPTA [26,27]; substances which deplete Ca\(^{2+}\) from intracellular stores by inhibition of the Ca\(^{2+}\)-ATPase of the sarcoplasmic reticulum—BHQ [18,28], thapsigargin [18,29,30] and CPA [30,31]; and ryanodine, which binds to the SR Ca\(^{2+}\) release channels and alters Ca\(^{2+}\) release properties [32]. Recently, ryanodine receptors have been demonstrated in electrically non-excitable cells [32,33]. Caffeine has been used to release Ca\(^{2+}\) from intracellular organelles [34]. All these compounds were introduced by diffusion from the microelectrode into the cells. This was done to exclude any influence on the whole tissue preparation and to investigate only the direct intracellular influence. After application of the compounds, caffeine was added to the perfusate to increase the contractile strength of the preparation and provide a secondary mechanical stimulus. Finally, as an oscillating influx of extracellular Ca\(^{2+}\) may be an important cause of MIP generation, we also studied the effects of extracellular EGTA in Ca\(^{2+}\)-free perfusion.

2. Methods

2.1. Preparation and electrical recording

Experiments were performed on spontaneously contracting and externally stretched preparations of right atria from hearts of rats (Rattus norvegicus) of either sex. The animals were killed by decapitation, the hearts were removed and the opened atria fixed to the bottom of a thermostatically controlled (37 ± 0.2°C) constant-flow perfusion chamber. The standard perfusate was composed of (mM) 118 NaCl, 2.7 KCl, 1.2 CaCl\(_2\), 1.2 MgSO\(_4\), 2.2 NaH\(_2\)PO\(_4\), 25 NaHCO\(_3\), 5 Glucose and had an osmolality of 293 ± 3 mosM and a pH of 7.4. During the experiments the solution was bubbled with carbogen (5% CO\(_2\), O\(_2\)).

Cellular electrical activity was recorded using conventional electro-physiological techniques as previously described [35]. Briefly, an Ag/AgCl wire in a glass micropipette (with KCl as electrolyte) served as a reference electrode in the perfusion chamber. A short Ag/AgCl pin, connected to the headstage of the amplifier via a 30 μm platinum–iridium wire, was partly inserted into a floating glass microelectrode containing KCl. In experiments with extracellular application of compounds we used standard microelectrodes (1.5 M KCl), made from standard glass capillaries. For intracellular diffusion of the compounds, glass capillaries were made from “Pyrex” glass tubules. Glass tubules were flushed for 30 min with HCl (centrifuged), washed for 60 min and rinsed during the last 10 min with de-ionised water. From these tubules, capillaries (outside diameter 1.1 mm, inside diameter 0.9 mm) were made. The capillaries contain glass microfilaments. Micro-electrodes were pulled and filled with 140 mM KCl, containing one of the compounds. These microelectrodes had tip resistances of about 6–9 MΩ. The recording microelectrode and the reference micropipette were connected through a high-input impedance amplifier. This was designed for simultaneous voltage measurements and current injections (current-clamp scheme) with an output (gain = 1) to an oscilloscope and a digital tape recorder. Any voltage drop across the microelectrode produced by current injection (negative rectangular impulse, usually 10⁻⁹ A) was not compensated. This was because the microelectrode tip resistance is considerably lower (two orders of magnitude) than the input membrane resistance of the investigated cells. A micromanipulator, with the aid of a light microscope, slowly moved the microelectrode into the chamber. In the solution-filled chamber the electrode potential was compensated to zero. On lowering the tip of the microelectrode to the endocardial surface of the preparation, a sudden shift of the potential to a value negative to −5 mV was interpreted as possible cell penetration. Sometimes, spontaneous oscillations in membrane potential occurred synchronously with atrial contraction. These oscillations were considered as possible mechanically induced potentials (MIP). The standard test for intracellular location of the electrode tip was the cellular reaction to current injection as described earlier [1]. Briefly, intracellular hyperpolarization increases the MIP amplitude towards its reversal potential. Final proof of the recording site was obtained by intracellular dye injection as described below. Data were digitised by an A/D converter.
(BE490, Bakker Electronics) plugged into a 486 personal computer and analysed off-line using commercial software (Signalys 3.0).

2.2. Mechanical recording and stimulation

The measurement of force (F) was achieved using the force transducer and the bridge amplifier of the Plugsys system 603 (Hugo Sachs Electronic, Germany). The isolated sinus node was fixed between the force transducer and a solenoid for extraneously applied short-duration stretch. The solenoid was fixed to a micrometer which was used for the adjustment of pre-load (pre-stretch). The standard low preload of 1 mN produced a stable force development during the entire experimental protocol. Under these conditions the amplitude of the mechanogram of each of the investigated fragments of atria was usually at about 0.5 mN. The contractile activity of the preparation provided a physiological mechanical stimulus to the non-muscle cells [1-3]. This was possible since the mechanical activity of the isolated atrial fragments did not change significantly during 2 h of perfusion with standard solution. Pharmacological manipulations which change the mechanical properties of the cardiac preparation naturally modify the parameters of the mechanically induced potential. Investigations on the influence of EGTA, applied extracellularly (which abolishes contractile activity), were therefore performed under controlled extraneously applied stretch. The stretch applied extraneously by the solenoid was constant and similar (about 0.5 mN) in all experiments with EGTA. Length changes were carefully calibrated. Clearly, the degree of stretch to which individual cells of the tissue were subjected will still have differed—a situation inherent in the study of cells in situ. Careful calibration of the artificial mechanical stimulus, localised intracellular rather than extracellular application of drugs, and spontaneous contraction will have minimised the variability of the stretch to which individual cells were exposed during the experiments.

2.3. Pharmacological interventions (see also Table 1)

Pharmacological substances applied intracellularly were: BAPTA (1,2-bis(2-aminophenoxy)ethane-N,N',N'',N''-tetraacetic acid, Sigma); BHQ (2,5-di-tert-butylhydroquinone, Sigma); thapsigargin (Sigma); CPA (cyclopiazonic acid, Sigma); caffeine (Sigma); ryanodine (BIOMOL Research Laboratories). Stock solutions of BHQ, CPA, thapsigargin, and BAPTA were made up in DMSO (anhydrous dimethylsulfoxide, Sigma). Each substance was tested in several experiments (n = 7-12). All reagents were introduced into the cell by diffusion from the inserted microelectrode. The concentration of reagents in the microelectrode was usually 100 μM. We used this concentration because it is the minimal concentration which is effective at a microelectrode resistance between 6 and 9 MΩ. Concentrations less than this or microelectrode resistances higher than 10 MΩ were not effective. The time between the insertion of the microelectrode and the onset of an effect of the compounds tested varied. This was mainly because of the resistance of the microelectrode tip to the efflux of the compound. In control experiments-DMSO (up to 0.4%) had no effect on MIP of fibroblasts. In the experiments, the concentration of DMSO in the microelectrode was kept below 0.1%. Thapsigargin is light-sensitive [36]; experiments with application of thapsigargin were therefore carried out in the dark. Control MIPS were recorded during the first few seconds after insertion of the microelectrode. Although the drugs had started diffusing from the microelectrode, we rarely saw changes in the membrane potential (V_m), MIP amplitude (V_m) or MIP duration during the first minute. This is likely to be due to relatively slow diffusion of components from the microelectrode. Moreover, the first MIPS in experiments with drug application were found to be similar to the first MIPS in control recording. EGTA (Sigma) was added into the Ca^{2+}-free bathing solution. In several experiments caffeine (10 mM) was added to the perfusate subsequent to other pharmacological interventions as a secondary stimu-

| Table 1 |
| Summary of drugs applied and electrophysiological effects. (mean ± s.d.) |

<table>
<thead>
<tr>
<th>Drug</th>
<th>Control</th>
<th>Drug effect</th>
<th>After caffeine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Em (mV)</td>
<td>V_m (mV)</td>
</tr>
<tr>
<td>Saline solution</td>
<td>20</td>
<td>-50</td>
<td>36.6 ± 3.5</td>
</tr>
<tr>
<td>BHQ</td>
<td>8</td>
<td>-50</td>
<td>36.5 ± 2.2</td>
</tr>
<tr>
<td>CPA</td>
<td>12</td>
<td>-50</td>
<td>37.1 ± 3.8</td>
</tr>
<tr>
<td>Thapsigargin</td>
<td>8</td>
<td>-50</td>
<td>35.4 ± 3.3</td>
</tr>
<tr>
<td>Ryanodine</td>
<td>8</td>
<td>-50</td>
<td>34.1 ± 2.9</td>
</tr>
<tr>
<td>Caffeine</td>
<td>6</td>
<td>-50</td>
<td>35.4 ± 2.8</td>
</tr>
<tr>
<td>EGTA</td>
<td>5</td>
<td>-40</td>
<td>11.2 ± 1.9</td>
</tr>
</tbody>
</table>

Em = fibroblast membrane potential (adjusted by current command to determined take off level; see Methods); V_m = amplitude of stretch induced depolarization; t = duration of stretch-induced depolarization. Statistical analysis was performed using Student’s unpaired t-test to compare control with the effect of subsequent experimental interventions. Confidence intervals are marked as Δ for 0.95 and ΔΔ 0.99.

* * * Experiments with EGTA were performed with a background of externally applied stretch, where the duration of the stimulus defined MIP duration.
Fig. 1. Representative recording of a mechanically induced potential (MIP, current-clamp mode) from an atrial mechanosensitive fibroblast during hyperpolarization to about ~50 mV. (A) Background activity directly after insertion of the microelectrode. The MIP excursion is from about ~50 to about ~17 mV. (B) MIP after 60 min of perfusion of the tissue with standard solution. (C) Effect of caffeine (10 mM) subsequently added to the perfusate (arrow) on a train of MIPs (lower curve) and mechanogram of spontaneously contracting atria (upper curve). (D) Graph showing the dependence of the MIP (V_m) on the membrane potential (E_m) at the beginning of the experiment (top graph) and after 60 min of recording (bottom graph). The ‘x’ on the vertical dashed line shows the value of V_m (E_m about ~50 mV) after caffeine was added to the bathing solution as well. (E) The light photomicrograph demonstrates the dye-marked non-muscle cell. Scale bar = 100 μm.
Fig. 2. Effect of BAPTA (100 μM in the microelectrode) on MIP in cardiac fibroblasts. (A) MIP during hyperpolarization to about −50 mV, recorded immediately after insertion of the microelectrode into the cell. (B) MIP reduction after 10 min of intracellular diffusion of BAPTA. (C) Effect of caffeine (10 mM) subsequently added to the perfusate (arrow) in a train of MIPS. (D) Graph showing dependence of $V_m$ on $E_{m}$ at the beginning of the experiment (top graph) and after 10 min of diffusion of BAPTA when steady state is reached (bottom graph). The ‘X’ on the vertical dashed line shows the value of $V_m$ ($E_m$ about −50 mV) after caffeine was added to the bathing solution.

lus applied extracellularly in order to increase the mechanical activity of the preparation (mechano-stimulation). The lag-time of action of extracellular caffeine was very short. After application of this compound the wash-out was therefore introduced almost immediately.

2.4. *Location of microelectrode tip and cell identification*

To verify the location of the microelectrode tip during measurements, in control experiments the recording site was marked by microiontophoretic application of the fluorescent dye, Lucifer Yellow (4%). The micro-iontophoresis protocol [37,38] was a 2-min train of hyperpolarizing rectangular pulses of 1-s duration, with an amplitude of $1.5 \times 10^{-9}$ A. Before, during and after injections the biocell activity of the cell was recorded to check the unchanged location of the microelectrode tip. On completion of 5 to 8 injections of Lucifer Yellow, a small fragment of myocardium (about 1 mm$^3$) containing the sites of injection was immediately fixed with 4% paraformaldehyde in 0.05 M phosphate-buffered saline solution (PBS) pH 7.3 for 6–12 h at 4°C. A series of cryostatic slices (10 μm) was mounted on microscopic slides in glycerol. Preparations were examined with a fluorescence microscope.

3. Results (see also Table 1) 1

Fig. 1 shows a typical MIP recorded from a mechano-sensitive fibroblast of rat right atrium immediately after

1A summary of the different drugs applied and their effect on the electrophysiological characteristics of cardiac fibroblasts is provided in Table 1.
insertion of the microelectrode into the cell (Fig. 1A), and after 60 min of tissue perfusion (Fig. 1B). The mechanically induced oscillation of the membrane potential, which is caused by spontaneous contraction of the atrium, was stable as long as the atrial contraction remained stable. This was so over several hours, and allowed us to use the contraction of the tissue as a physiological mechanostimulator [1-3]. Fig. 1C shows the simultaneous recording of the mechanogram (F) and bio-potentials (MIPs) of a fibroblast during spontaneous contraction of the tissue. Subsequent addition of caffeine (10 mM) to the perfusate significantly increased contraction and MIP amplitude. In all cases the increase of MIP amplitude after extracellular caffeine application was directed towards zero voltage. This increase of MIP amplitude is the effect of increase of contractile strength of the atrial muscle after extracellular application of caffeine. Because caffeine was washed out almost immediately after application, the registered increase of MIP amplitude is likely to be caused by the increase of contractile strength of the tissue rather than diffusion of the drug into the fibroblast. Under control conditions, intracellular injection of a constant current to hyperpolarize the cell to different values between 0 and −100 mV produced a linear relationship between the MIP amplitude (V_m) and the resting membrane potential (E_m) (Fig. 1D). This relationship was stable throughout the duration of control experiments. Membrane hyperpolarization to about −100 mV usually increased V_m to a variable degree from 50 to 90 mV. This variation is determined by the degree of mechanical stimulation of the contracting atrium on each investigated cell. Similar data were obtained in 20 equivalent experiments. Intracellular dye application demonstrated that these recordings were made from non-muscle sino-atrial cells—fibroblasts (Fig. 1E).

Fig. 2 demonstrates the results of diffusion of BAPTA from the microelectrode into a mechanosensitive fibroblastic. Effect of BHQ (100 μM in the microelectrode) on MIP in a cardiac mechanosensitive fibroblast. (A) MIP during hyperpolarization to about −50 mV, recorded shortly after insertion of the microelectrode. (B) MIP taking off from about −50 mV after 15 min of diffusion of BHQ. (C) Effect on V_m of 10 mM caffeine subsequently added to the perfusate arrow). (D) Graph showing the dependence of V_m on E_m at the beginning of the experiment (top graph) and after 15 min of diffusion of BHQ (bottom graph). The 'X' on the vertical dashed line shows the value V_m (E_m about −50 mV) on subsequent addition of caffeine to the bathing solution.
last. The control recording of MIP (Fig. 2A) was performed immediately after insertion of the microelectrode into the cell. In all pharmacological experiments depicted in Figs. 2–7, the first MIPs (recorded on the background of a uniform, slightly hyperpolarized take-off potential of −50 mV) served as control. Within 10 min, BAPTA reduced $V_m$ by up to 80% of the initial value, and decreased the duration of the MIP by up to 70%. Thereafter, a steady state was reached (Fig. 2B). After a steady state had been reached, subsequent mechanical activation by addition of caffeine (10 mM) to the perfusate significantly increased the amplitude of the MIP which was initially depressed by BAPTA (Fig. 2C). Internal BAPTA, at $E_m$ between 0 and −20 mV, abolished the MIP. At $E_m$ negative to −20 mV, the dependence of $V_m$ on $E_m$ was linear (Fig. 2D). Membrane hyperpolarization to about −100 mV produced MIPs of about 28 mV amplitude (compared with control levels of 88 mV recorded immediately after insertion of the microelectrode into the cell). Similar data were obtained in 8 further experiments.

Fig. 3 illustrates the effect of intracellular BHQ. A control MIP was recorded from the fibroblast (Fig. 3A). Within 15 min BHQ reduced $V_m$ by up to 55%, and decreased its duration as well (Fig. 3B). After reaching a steady state, caffeine (10 mM) added subsequently to the perfusate increased $V_m$ (Fig. 3C). The effect was less pronounced than that in the previous series (Fig. 2C). BHQ reached its maximal effect in about 15 min and virtually abolished the MIP at $E_m$ between 0 and −15 mV. The dependence of $V_m$ on $E_m$ negative to −15 mV was non-linear, and plateaued at $E_m$ of about 60 mV (Fig. 3D). Membrane hyperpolarization to −100 mV produced a MIP of about 22 mV (compared with 81 mV immediately after insertion of the microelectrode into the cell). Similar data were obtained in 12 further experiments.

A similar protocol applied to CPA. Fig. 4A shows the

![Graph A](image)

![Graph B](image)

![Graph C](image)

![Graph D](image)

**Fig. 4.** Effect of CPA (100 μM in the microelectrode) on atrial fibroblast MIP. (A) MIP taking off from about −50 mV. (B) MIP after 10 min of diffusion of CPA. (C) Effect of 10 mM caffeine subsequently added to the bathing solution (arrow). (C) Graph showing dependence of $V_m$ on $E_m$ at the beginning of the experiment (top graph) and after 10 min of diffusion of CPA at steady state (bottom graph). The 'X' on the vertical dashed line shows the value of $V_m$ ($E_m$ about −50 mV) on subsequent addition of caffeine to the bathing solution.
control MIP. CPA, after 10 min, reduced $V_m$ by up to 55% and decreased its duration by 20% (Fig. 4B). During the steady state, addition of caffeine (10 mM) slightly increased $V_m$ (Fig. 4C). After CPA had reached its maximal effect, there was no MIP at $E_m$ potentials positive to $-22$ mV. Again, the dependence of $V_m$ on $E_m$ negative to $-22$ mV was non-linear and reached a maximum when $E_m$ was approximately $-50$ mV (Fig. 4D). Membrane hyperpolar-

![Graphs showing MIP and $V_m$ vs. $E_m$](image)

Fig. 5. Effect of thapsigargin (100 μM in the microelectrode) on MIP in mechanosensitive atrial fibroblasts. (A) MIP during hyperpolarization to about $-50$ mV, recorded after insertion of the microelectrode. (B) MIP 5 min after the onset of diffusion of thapsigargin. (C) MIP after 30 min thapsigargin. (D) Effect of caffeine (10 mM) subsequently added to the perfusate (arrow) on the amplitude of the MIP. (E) Graph showing dependence of $V_m$ on $E_m$ (top graph) at the beginning of experiment (bottom graph) after 5 min and (c) after 30 min of diffusion of thapsigargin. The 'X' on the vertical dashed line shows the value of $V_m$ ($E_m$ about $-50$ mV) on subsequent addition of caffeine to the bathing solution.
ization to $-100$ mV produced MIPs of about 15 mV (compared with 69 mV immediately after insertion of the microelectrode into the cell). Similar data were obtained in 8 further experiments.

Fig. 5 shows similar protocols to the previous experiments, this time using thapsigargin. Control MIP was about $-50$ mV (Fig. 5A). In this case, after 5 min, thapsigargin increased $V_m$ to 130% (Fig. 5B). The $V_m/E_m$ relationship was linear (Fig. 5E, line b). Membrane hyperpolarization to $-100$ mV produced a MIP of about 95 mV (compared with 75 mV immediately after insertion of the microelectrode into the cell). However, during the following 25 min the mechanically induced electrical activity in the cardiac fibroblasts decreased to a new steady state. MIP was reduced by up to 45% compared to the initial value. The duration of the MIP decreased by 15% (Fig. 5C). Finally, MIPs disappeared between $E_m = 0$ mV and $-10$ mV. The relationship between $V_m$ and $E_m$ negative to $-10$ mV was non-linear, with a plateau near hyperpolarization values of $-85$ mV (Fig. 5E, line c). Membrane hyperpolarization to approximately $-100$ mV produced MIPs of about 35 mV. After steady state, caffeine (10 mM) significantly increased MIP (Fig. 5D). Similar data were obtained in 8 further experiments.

Fig. 6A shows a control MIP. Intracellular application of caffeine, within 5 min, reduced the MIP and its duration by 70 and 45%, respectively (Fig. 6B). The MIP disappeared at $E_m$ between 0 and $-12$ mV. The relationship between $V_m$ and $E_m$ more negative than $-12$ mV was non-linear, reaching a plateau at $E_m$ negative to $-70$ mV (Fig. 6C). Here, membrane hyperpolarization to about $-100$ mV produced a MIP of about 15 mV (compared with 65 mV during control recordings). Similar data were obtained in 6 further experiments.

Fig. 7A shows control recordings for ryanodine which, after 20 min, reduced $V_m$ by up to 70% with no change in MIP duration (Fig. 7B). Thereafter there was no MIP at $E_m$ between 0 mV and $-25$ mV (Fig. 7C). The relationship between $V_m$ and $E_m$ negative to $-25$ mV was non-linear, reaching a plateau at $-60$ mV hyperpolarization (Fig. 7C). Membrane hyperpolarization to about $-100$ mV produced a 14 mV MIP (compared with 78 mV in

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Fig. 6. Effect of caffeine (100 μM in the microelectrode) on MIP in atrial mechanosensitive fibroblasts. (A) MIP taking off from about $-50$ mV. (B) MIP after 5 min of diffusion of caffeine. (C) Graph showing dependence of $V_m$ on $E_m$ at the beginning (top graph) and at the end (bottom graph) of the experiment.
Fig. 7. Effect of ryanodine (100 μM in the microelectrode) on a MIP in a fibroblast of the right atrium. (A) MIP taking off from a hyperpolarizing potential of about −50 mV, immediately after insertion of the microelectrode. (B) MIP after 20 min of diffusion of ryanodine. (C) Graph showing the dependence of $V_m$ on $E_m$ at the beginning (top graph) and at the end (bottom graph) of the experiment.

control). Similar data were obtained in 8 further experiments.

Fig. 8 shows the influence of lowered extracellular Ca$^{2+}$ on a series of typical fibroblast MIPs. This time mechanical stimulation was performed via artificial stretch of the tissue to sustain a stable mechanical environment. Fig. 8A shows MIPs caused by spontaneous contractions of the tissue and the membrane potential shift caused by artificial stretch (AS) of the tissue. These artificially caused MIPs have an amplitude of about 10 mV mostly, from a steady potential of −40 mV. EGTA (2 mM) in Ca$^{2+}$-free solution gradually decreased the MIP amplitude caused by artificial stretch to 40% of control (Fig. 8B). After EGTA washout and re-perfusion with extracellular Ca$^{2+}$, the MIP reappeared (Fig. 8C). Similar data were obtained in 5 further experiments.

Because one of the mechanisms of MIP generation could be related to Na$^+$/Ca$^{2+}$ exchange across the plasmalemma [22], extracellular Na$^+$ was reduced or fully substituted by choline chloride. Reduction of extracellular Na$^+$ amplifies calcium oscillation, which may cause membrane potential oscillations [22]. In our experiments, reduction or substitution of sodium by choline did not change MIPs caused by artificial stretch of the tissue.

4. Discussion

Our data demonstrate complex mechanisms involved in the generation of MIPs in mechanosensitive cells of the heart. Intracellular dye injection confirmed that recordings were made from non-muscle sino-atrial cells. Lucifer Yellow was injected exclusively into cells which displayed MIPs, not action potentials. Moreover, the cells which were marked were different from cardiomyocytes in size and morphology. These findings, together with earlier histological studies using electron microscope techniques after intracellular injection of colloidal gold indicate that the cells which have this type of electrical activity are fibroblasts [5].

Control experiments show a linear dependence of $V_m$ on $E_m$ (artificially shifted by intracellular injection of a
constant current). Thus, under normal conditions, the hyperpolarization-induced increase in $V_m$ is apparently not caused by a hyperpolarization-induced increase in the number of activated ion channels, as expected in cardiomyocytes. It is mainly by an increase in the number of ions passing through each channel that is open during mechanical stimulation. This effect could be caused solely by the increase in the gradient of the electric field.

Our data also demonstrate that the MIP depends on extracellular and intracellular Ca$^{2+}$ concentrations. Intracellular BAPTA, which reduces the concentration of cytosolic Ca$^{2+}$, dramatically reduced both amplitude and duration of MIP. These data show that [Ca$^{2+}$], plays a key role in the generation of MIPs. CPA, BHQ and thapsigargin—substances which deplete internal Ca$^{2+}$ stores by inhibiting the re-uptake of Ca$^{2+}$—led to a similar final effect. These data demonstrate the role of the endoplasmic reticulum as an intracellular source of Ca$^{2+}$ in the generation of MIP. Thapsigargin produced a different behaviour during the first minutes of the experiment. There was an increase in the MIP amplitude that might be explained by a temporary increase in the cytosolic Ca$^{2+}$ concentration. Such a response has been reported for endothelial cells [39]. Perhaps the dynamics of the thapsigargin-induced changes in Ca$^{2+}$ handling are faster than those of CPA and BHQ. This would produce an increase in cytosolic Ca$^{2+}$ concentration by an immediate inhibition of the re-uptake of Ca$^{2+}$ into the endoplasmic reticulum. Thapsigargin binds stoichiometrically to endoplasmic reticulum Ca$^{2+}$-ATPase [40] and causes an essentially irreversible inhibition of activity by blocking the enzyme [41]. Thapsigargin acts both with high affinity and high specificity. Of the other inhibitors, BHQ [42,43] shares with thapsigargin its mechanism of action, albeit with a significantly lower affinity [41]. CPA is also selective for endoplasmic reticulum Ca$^{2+}$-ATPase inhibition with a significantly lower affinity [44]. Thus, CPA and BHQ have largely comparable inhibitory actions, although they are less potent than thapsigargin.

In all the experiments discussed above, caffeine was

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**Fig. 8. Example of the influence of zero extracellular Ca$^{2+}$ on a MIP of an atrial fibroblast, caused by artificial stretch (horizontal bar) of the tissue of 0.5 mN.**

(A) Control MIP recording. The figure shows the typical MIPs of an atrial fibroblast, caused by spontaneously contraction of atria, and membrane potential shift (MIP), caused by artificial stretch of the tissue. (B) Reduction in MIP amplitude after 2 min of perfusion with 2 mM EGTA in Ca$^{2+}$-free solution. (C) MIP after 2 min of wash-out.
added to the bathing solution after an initial steady state was reached. The aim of this intervention was to increase the contractile strength of the tissue. This response served as a secondary, mechanical stimulus for fibroblasts after intracellular injection of drugs. In our control experiments (microelectrodes filled with KCl only) caffeine (10 mM) added to the bathing solution significantly increased the contractile strength of the isolated right atria and, in parallel, the amplitude of MIPs. The increase in MIP amplitude was directed towards zero voltage. This was independent of MIP amplitude before application of caffeine. In experiments with intracellular diffusion of drugs, the degree of increase in MIP amplitude induced by the caffeine-induced increase in contraction varied, and was smaller than in non-drug control experiments. This is presumably due to the overall decrease in the fibroblast's responsiveness to mechanical stimulation during intracellular exposure to the drugs tested.

The effect of intracellular diffusion of caffeine and ryanodine demonstrates the caffeine/ryanodine sensitivity of the Ca\(^{2+}\) stores involved in the generation of MIPs. Ion channels characterised by their sensitivity to ryanodine and caffeine are present in the membrane of Ca\(^{2+}\) stores of a number of cells [45,46] including non-cardiac fibroblasts [23]. Ryanodine keeps the channel open and thus calcium is released from the endoplasmic reticulum [47]. Since both Ca\(^{2+}\) release and re-uptake by the endoplasmic reticulum influence MIPs, it seems possible that intracellular Ca\(^{2+}\) oscillations are involved in MIP generation.

In general, BAPTA, thapsigargin, CPA and caffeine, which change [Ca\(^{2+}\)], decreased both the amplitude and duration of MIP. BHQ and ryanodine decreased only its amplitude. These drugs also changed the cell's response to hyperpolarization. MIPs did not reach the initial maximal value and the relationship between artificial membrane polarisation (\(E_m\)) and MIP amplitude (\(V_m\)) became non-linear, reaching plateau levels at high negative potentials. In several cases, at low \(E_m\), there was no MIP visible at all. During the initial phase of thapsigargin injection into the mechanosensitive fibroblasts when, as presumed earlier, the cytosolic Ca\(^{2+}\) concentration might temporarily have risen, the functional relation between \(V_m\) and \(E_m\) remained linear. These observations suggest that, while intracellular Ca\(^{2+}\) concentration is reduced, the mechanically induced ionic current ceases to be directly proportional to the electric field. This might be due to a Ca\(^{2+}\)-dependence of the ion channels involved or to the disclosure and activation of previously masked or inactive ion channels, the gating of which is voltage-dependent.

Application of EGTA in Ca\(^{2+}\)-free perfusate decreased the amplitude of MIP, caused by artificial stretch of the tissue, by up to 40%. That is, the extracellular calcium concentration also plays a role in MIP generation. One possible mechanism would involve increased loss of calcium from the cell into the calcium-depleted extracellular medium. The fact, however, that extracellular EGTA in Ca\(^{2+}\)-free perfusate did not totally abolish MIPs suggests either that Ca\(^{2+}\) may oscillate within the cell or that other ions contribute to MIP generation.

Stretch-induced oscillations in membrane potential of electrically non-excitable cells are linked to oscillations in intracellular Ca\(^{2+}\) activity [22]. Since a number of stretch-activated channels are known to be permeable to sodium ions, stretch-induced changes in intracellular calcium could be caused indirectly by Na\(^{+}\)/Ca\(^{2+}\) exchange mechanisms. So far, Na\(^{+}\)/Ca\(^{2+}\) exchange across the plasmalemma has not been firmly established in electrically non-excitable cells. Although in some studies variations in extracellular or intracellular Na\(^{+}\) concentration have not been found to affect Ca\(^{2+}\) flux and Ca\(^{2+}\) concentration [48], in others with fibroblasts expressing the ras-oncogene extracellular Na\(^{+}\) modulates Ca\(^{2+}\) oscillations [49]. In our experiments we did not observe changes in MIP when extracellular Na\(^{+}\) was reduced or fully replaced by Choline. Our data so far do not support Na\(^{+}\)/Ca\(^{2+}\) exchange (even if present) in playing a key role in MIP generation in cardiac fibroblasts.

Thus, generation of MIP, elicited by the spontaneous mechanical activity in the heart or by externally applied stretch, is related to changes in extracellular and intracellular Ca\(^{2+}\) concentrations. Still, it is not clear yet which mechanisms initiate MIP generation. To test whether this response could be triggered by the operation of mechanosensitive ion channels [50,51], gadolinium, a blocker of stretch-activated ion channels, was applied in an earlier study [7]. It blocked MIP generation. We suggest that stretch-activated channels may contribute to the increase in free cytosolic Ca\(^{2+}\) inherent to MIPs. Such a contribution, even if small, could trigger Ca\(^{2+}\)-induced Ca\(^{2+}\) release from the endoplasmic reticulum. This interpretation is in line with the results of this investigation which show Ca\(^{2+}\) release from the endoplasmic reticulum to be a key mechanism in MIP generation.

There is, however, an alternative explanation. Calcium release from the endoplasmic reticulum could involve direct mechanical modulation of properties of the intracellular calcium stores. This hypothesis is in line with the observation that the total storage capacity for calcium ions of the endoplasmic reticulum decreases with stretch [52]. Thus, a reticulum fully filled at a short cell length would possibly not be able to keep all its content when subject to the consequences of stretch. This response could possibly be promoted by the cytoskeleton. Evidence for this form of mechano-electric feedback has been gained in experiments where the cytoskeleton was destroyed during recordings of Ca\(^{2+}\) in cardiac fibroblasts, resulting in decreased MIP activity [7].

The evidence we present here shows that changes in intracellular calcium handling are the key mechanism in generation of mechanically induced potentials in cardiac fibroblasts. Our findings are in keeping with the idea of intracellular calcium oscillations, caused by the interplay
of uptake and release of calcium by the endoplasmic reticulum. An important, but not necessarily exclusive, trigger for MIP generation is stretch activation of ion channels. We demonstrate, further, the existence of functional ryanodine/caffeine-sensitive Ca^2+ stores in cardiac fibroblasts.

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