Abolishing Myofibroblast Arrhythmogeneicity by Pharmacological Ablation of α-Smooth Muscle Actin Containing Stress Fibers

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Rationale: Myofibroblasts typically appear in the myocardium after insults to the heart like mechanical overload and infarction. Apart from contributing to fibrotic remodeling, myofibroblasts induce arrhythmogenic slow conduction and ectopic activity in cardiomyocytes after establishment of heterocellular electrotonic coupling in vitro. So far, it is not known whether α-smooth muscle actin (α-SMA) containing stress fibers, the cytoskeletal components that set myofibroblasts apart from resident fibroblasts, are essential for myofibroblasts to develop arrhythmogenic interaction with cardiomyocytes.

Objective: We investigated whether pharmacological ablation of α-SMA containing stress fibers by actin-targeting drugs affects arrhythmogenic myofibroblast-cardiomyocyte cross-talk.

Methods and Results: Experiments were performed with patterned growth cell cultures of neonatal rat ventricular cardiomyocytes coated with cardiac myofibroblasts. The preparations exhibited slow conduction and ectopic activity under control conditions. Exposure to actin-targeting drugs (Cytochalasin D, Latrunculin B, Jasplakinolide) for 24 hours led to disruption of α-SMA containing stress fibers. In parallel, conduction velocities increased dose-dependently to values indistinguishable from cardiomyocyte-only preparations and ectopic activity measured continuously over 24 hours were completely suppressed. Mechanistically, antiarrhythmic effects were attributable to myofibroblast hyperpolarization (Cytochalasin D, Latrunculin B) and disruption of heterocellular gap junctional coupling (Jasplakinolide), which caused normalization of membrane polarization of adjacent cardiomyocytes.

Conclusions: The results suggest that α-SMA containing stress fibers importantly contribute to myofibroblast arrhythmogeneicity. After ablation of this cytoskeletal component, cells lose their arrhythmic effects on cardiomyocytes, even if heterocellular electrotonic coupling is sustained. The findings identify α-SMA containing stress fibers as a potential future target of antiarrhythmic therapy in hearts undergoing structural remodeling. (Circ Res. 2011;109:00-00.)

Key Words: arrhythmia ■ conduction ■ fibroblast ■ gap junction ■ myocardial structure

Fibrotic remodeling of the working myocardium is a common consequence of various insults to the heart like pressure/volume overload, infarction, genetic predisposition, and old age. Apart from compromising mechanical function, fibrotic remodeling is a major cause of cardiac arrhythmias. Arrhythmogenesis in fibrotically remodeled hearts is thought to be attributable to the disruption of the normally dense and orderly three-dimensional cytoarchitecture of parenchymal cells by excessive amounts of extracellular matrix produced by stromal cells. The resulting disorganization of the electrically excitable substrate causes slow conduction (discontinuous or “zig-zag” conduction or both) and conduction blocks that contribute to the precipitation of arrhythmias. Stromal cells responsible for fibrotic remodeling include so-called myofibroblasts (MFBs), which contribute to fibrosis by hypersecretion of extracellular matrix proteins. MFBs, which are not normally present in healthy myocardia, typically appear in the context of the pathologies mentioned and tend to locally persist for extended periods of time.

Apart from contributing to fibrotic remodeling, MFBs recently came into focus as a cell type that might directly contribute to arrhythmogenesis based on adverse electrotonic interaction with cardiomyocytes (CMCs). MFBs were shown in vitro to establish heterocellular gap junctional coupling with CMCs based on connexin 45 and connexin 43 expression. Because MFBs have a reduced membrane potential as...
compared to CMCs, heterocellular gap junctional coupling leads to depolarizing current flow from MFBs to CMCs that causes a reduction of the resting membrane potential of CMCs similar to the concept of "injury current flow" in the setting of an infarction. This results in slow conduction because of sodium channel inactivation and induces ectopic activity based on “depolarization-induced automaticity.” By inducing both slow conduction and ectopic activity, MFBs might contribute importantly to arrhythmogenesis in hearts undergoing fibrotic remodeling.

MFBs differ from normal resident cardiac fibroblasts by de novo expression of α-smooth muscle actin containing stress fibers (α-SMA-SFs). Given that fibroblasts in intact healthy hearts obviously exert no arrhythmogenic effects on neighboring CMCs, we hypothesized that disruption of α-SMA-SFs by appropriate pharmacological tools not only might revert the MFB phenotype toward fibroblast-like cells but also might particularly abolish their arrhythmogenic interaction with CMCs. Stress fibers are subject to constant turnover with dynamics determined by the rate of addition of monomeric actin (G-actin) to the fast-growing (barbed) end and the dissociation of actin at the opposite (pointed) end of filamentous actin (F-actin). This process, referred to as “treadmilling,” can be modulated by a number of actin-targeting compounds. Among these, Latrunculin B (LatB) blocks formation of actin fibers by sequestering G-actin, whereas Cytochalasin D (CytoD) interacts with the barbed ends of actin filaments (“capping”), thereby inhibiting G-actin binding. By contrast, Jasplakinolide (Jasp) stabilizes F-actin and facilitates actin polymerization. Using these three agents in models of fibrotic cardiac tissue containing α-SMA and vimentin. Nuclei were counterstained with 4',6-diamidino-2-phenylindole. Statistic values are given as mean±SD. Data were compared using the Student t test (two-tailed; homoscedastic or heteroscedastic when appropriate), and differences were considered significant at P<0.05.

Results

Effects of Actin-Targeting Drugs on MFB Morphology

Cardiac fibroblasts cultured on rigid glass substrates underwent a rapid phenotype switch to MFBs, which accounted for >90% of the initially seeded cells after 24 hours in

Materials and Methods

A detailed description of the methodologies used in this study is provided in the Online Supplement (available at http://circres.ahajournals.org).
Actin-Targeting Drugs Rescue MFB-Induced Slow Conduction

As shown before, MFBs cultured on top of monolayer strands of CMCs induce slow conduction based on their depolarizing effect on CMCs after establishment of heterocellular electrotonic coupling. We hypothesized that MFBs might lose this arrhythmogenic effect after disruption of their structural hallmark, ie, of α-SMA-SFs. For this purpose, strands of CMCs coated with MFBs were subjected for 24 hours to LatB, CytoD, and Jasp, and resulting effects on \( \theta \) and \( \dV/dt_{\text{max}} \) of propagating action potentials were assessed optically. Figure 3A shows raw data of an experiment in which conduction in an untreated MFB–CMC strand is compared to an MFB–CMC strand exposed for 24 hours to 100 nmol/L CytoD. Typically, the control strand displayed phenotype switch observed initially in cell culture. In addition to the reduction and reorganization of α-SMA, actin-targeting drugs induced characteristic changes in MFB morphology as revealed by time-lapse microscopy (movies in Online Data Supplement). During the first hour of exposure to 100 nmol/L CytoD, MFBs tended to lose their normally flat and outspread morphology and became spherical while remaining physically anchored to the substrate by thin cytoplasmic extensions. This change was transient as cells recovered a flattened shape over the remainder of the observation period (~15 hours). At identical concentrations, LatB caused similar morphological changes. However, the onset was acute and the loss of the flat and outspread cell morphology became increasingly pronounced during the initial approximately 7 hours of drug exposure, after which time cells regained a flat and outspread morphology. In contrast to these transient effects of CytoD and LatB on MFB morphology, Jasp caused the flat and outspread MFBs to adopt an increasingly dendritic morphology that showed no tendency for reversal in the presence of the drug.

Because the rigidity of the culture substrate is an important determinant of the transformation of fibroblasts to MFBs, we investigated whether actin-targeting drugs were equally effective when applied to fibroblasts cultured on top of “soft” monolayers of CMCs. Similar to glass substrates, fibroblasts adhering to CMCs underwent a rapid phenotype switch to MFBs and formed a uniform cell coat after 48 hours in culture (Figure 2, control). Treating the preparations with CytoD or LatB (100 nmol/L each for 24 hours) caused α-SMA-SFs to disappear almost completely, whereas Jasp (1 \( \mu \)mol/L for 24 hours) again induced the formation of α-SMA aggresomes (Figure 2). All three drugs reduced the number of cells displaying α-SMA positive stress fibers by >95%, suggesting that they were more effective in inducing a phenotype reversal in the setting of soft as opposed to rigid substrates. Irrespective of the type of treatment, the density of vimentin-positive fibroblastic cells remained largely unchanged, which indicates that the decline of cells with an MFB phenotype was not attributable to a drug-induced cell loss but rather reflected the presence of “back-transformation” of MFBs to fibroblasts.
slow overall $\theta$ (202 mm/s) and slow $dV/dt_{\text{max}}$ (39.5±7.1%APA/ms). By contrast, the CytoD-treated MFB–CMC strand exhibited a substantial increase of $\theta$ (370 mm/s) and $dV/dt_{\text{max}}$ (77.8±1.7%APA/ms). The dose–response curve for CytoD-induced restoration of $\theta$ and $dV/dt_{\text{max}}$ revealed an EC$_{50}$ of approximately 3 nmol/L, with maximal effects reached at approximately 100 nmol/L (Figure 3B). At this concentration, $\theta$ (379±6 mm/s; n=48) and $dV/dt_{\text{max}}$ (78±1%APA/ms; n=48) became virtually indistinguishable from CMC-only control strands ($\theta$=365±20 mm/s; $dV/dt_{\text{max}}$=79±2%APA/ms; n=72), indicating that CytoD caused complete elimination of MFB-induced slow conduction. This effect was most likely attributable to a specific action of CytoD on MFBs because exposure of CMC-only control strands to 100 nmol/L CytoD for 24 hours failed to induce significant changes in action potential propagation.

Similar to CytoD, LatB completely eliminated adverse effects of MFBs on impulse conduction in MFB–CMC strands (Figure 3C). The EC$_{50}$ of LatB for increasing $\theta$ and $dV/dt_{\text{max}}$ was approximately 10 nmol/L, with maximal effects reached at approximately 100 nmol/L. Normalization of conduction by LatB was attributable to its specific effects on MFBs because it failed to induce alterations in action potential propagation characteristics in CMC-only preparations. At dosages above the maximal effective concentration (1 and 10 µmol/L), LatB caused an increasing reduction of $\theta$, whereas $dV/dt_{\text{max}}$ was only minimally affected, suggesting that LatB possibly decreased gap junctional coupling among CMCs at these high concentrations.

In contrast to CytoD and LatB, which destabilize F-actin, Jasp is thought to stabilize F-actin. Accordingly, this compound was expected to have opposite or no impact on impulse conduction characteristics in MFB-coated CMC strands. Surprisingly, however, Jasp acted like CytoD and LatB and caused a restoration of impulse conduction in MFB-coated CMC strands with an EC$_{50}$ of approximately 300 nmol/L. Maximal effects were observed at 1 µmol/L, where $\theta$ (366±16 mm/s; n=48) was slightly higher and $dV/dt_{\text{max}}$ (70±2%APA/ms; n=48) was slightly lower than values recorded in the corresponding control CMC strands ($\theta$=328±22 mm/s; $dV/dt_{\text{max}}$=76±3%APA/ms; n=84). At maximal effective concentrations (1 µmol/L), Jasp also affected impulse propagation in CMC-only strands, where it induced a significant increase of $\theta$ (379±18 mm/s vs 328±22 mm/s in controls) that was accompanied by a slight increase in $dV/dt_{\text{max}}$ (78±2%APA/ms vs 76±3%APA/ms in controls; not significant).

Suppression of Ectopic Activity by Actin-Targeting Drugs
It was shown before that MFBs cultured at sufficient densities on top of dense monolayers of ventricular CMCs induce ectopic activity. As for MFB-induced slowing of conduction, induction of ectopic activity by MFBs is based on partial depolarization of CMCs after establishment of heterocellular
electrotonic coupling, which leads to precipitation of spontaneous activity because of “depolarization induced automaticity.”

Given these mechanistic similarities, actin-targeting drugs were expected to suppress ectopic activity at concentrations similar to those found to be maximally effective in rescuing slow conduction. This hypothesis was tested with disc-shape monolayer preparations (10-mm diameter) consisting of CMCs only and of CMCs coated with MFBs in which we recorded synchronized spontaneous contractile activity continuously for 28 hours under stable incubating conditions using a custom-made videomicroscopy recording system.

With the exception of a few sporadic activations, CMC-only preparations remained completely quiescent during the entire 28-hour recording period irrespective of the addition of drugs (Figure 4A–C, left panels). By contrast, MFB–CMC preparations exhibited regular spontaneous activity during the control recording period with frequencies ranging from approximately 50 to 80 beats per minute. After exchange of the culture medium, control MFB–CMC preparations receiving no drugs showed a transient increase of beats per minute to approximately 80 to 100, which displayed multiple phases: a fast initial increase in beats per minute peaking approximately 1.5 hours after the medium exchange was followed by a slight decline and a subsequent slower increase, with maximal frequencies attained at approximately 6 hours. Thereafter, beats per minute declined monotonically to values comparable to those rec-
ordered before the medium exchange. This characteristic evolution of frequencies in control preparations was substantially altered by the addition of CytoD, LatB, and Jasp. All three drugs caused a reduction in beats per minute to zero (CytoD and LatB) or close to zero (Jasp), albeit with different time courses (Figure 4A–C, right panels). When normalized to average beats per minute values recorded before addition of the drugs, CytoD progressively reduced spontaneous activity with complete cessation occurring after 18 hours (time constant, $\tau \approx 9.7$ hours). Equimolar concentrations of LatB were more effective because the drug caused acute decrease of activity (25%) that was accentuated over time following a sigmoidal time course until complete cessation of spontaneous activity occurred after approximately 10 hours ($\tau \approx 3.2$ hours). In contrast to both CytoD and LatB, Jasp displayed a substantial latency in the onset of decrease of ectopic activity ($\approx 4$ hours). Of note, beats per minute during the initial 2 hours after addition of drug were consistently higher in Jasp-treated preparations than in controls. After this phase, spontaneous activity progressively decayed with near-complete loss of activity being reached after 24 hours ($\tau \approx 13.7$ hours).

Mechanisms of Antiarrhythmic Activity of Actin-Targeting Drugs

Given that both the extent of conduction slowing and the likelihood of occurrence of ectopic activity induced by MFBs is a function of the amount of depolarizing current flow from MFBs to CMCs, suppression of these effects by actin-targeting drugs may depend on a reduction in MFB density, a loss of gap junctional coupling between MFBs and CMCs, or a change in MFB electrophysiology (or a combination of these).

To investigate whether CytoD, LatB, and Jasp caused a loss of MFBs secondary to their effects on cell morphology, counts of 4',6-diamidino-2-phenylindole stained nuclei of the preparations used for the experiments depicted in Figure 3 were used as an index of cell densities present during the different interventions. Whereas 4',6-diamidino-2-phenylindole staining is an indirect measure of cell number (no differentiation between mononucleated and multinucleated cells), the usefulness of the index is illustrated by the finding that the excess number of nuclei in MFB–CMC versus CMC-only preparations (+46%) approximately mirrors the number of fibroblasts seeded on top of CMC.
monolayers to obtain MFB–CMC preparations (+38% cells). As shown in Figure 5, CytoD had no effect on nuclear counts at concentrations as high as 10 μmol/L. By contrast, both LatB and Jasp led to a slight decrease of the number of nuclei at concentrations being maximally effective in suppressing arrhythmogenic slow conduction and ectopic activity (LatB 100 nmol/L: 9%; Jasp 1 μmol/L: 12%; not significant). Given the only modest reductions in nuclei counts for LatB and Jasp, it is unlikely that either of these compounds led to a sufficiently large decrease in the number of MFBs to explain the observed antiarrhythmic effects.

The question of whether actin-targeting drugs induced a loss of gap junctional communication between MFBs and CMCs was investigated by assessing intercellular diffusion of lucifer yellow in MFB–CMC preparations under control conditions (Figure 6A) and after exposition to CytoD (100 nmol/L; Figure 6B), LatB (100 nmol/L; Figure 6C), and Jasp (1 μmol/L; Figure 6D) for 24 hours. Under control conditions, intercellular diffusion was present in 91% of MFB–CMC preparations (n=23; Figure 6A). Similarly, lucifer yellow diffusion was found in 80% of preparations exposed to CytoD (n=10) and in 82% of preparations exposed to LatB (n=11). In contrast, dye diffusion was completely blocked after treatment with Jasp (n=8). These findings suggest that disruption of heterocellular gap junctional communication is likely responsible for the antiarrhythmic activity of Jasp, whereas CytoD and LatB exerted their effects by a different mechanism.

The possibility that actin-targeting drugs reduced MFB arrhythmogeneity by affecting membrane polarization of
CMCs or MFBs or both was investigated using whole-cell patch clamp recordings in low-density cell cultures exposed to actin-targeting drugs for 24 hours. As shown in Figure 7A, exposure of CMCs to CytoD, LatB, and Jasp (1 μmol/L each) had no significant effects on CMC resting potentials (control: 74.6 ± 3.3 mV; CytoD: 73.3 ± 5.9 mV; LatB: 73.2 ± 4.6 mV; Jasp: 73.5 ± 6.0 mV; n=5 each). By contrast, actin-targeting drugs induced a significant hyperpolarization of MFBs (control: 26.6 ± 7.2 mV; CytoD: 44.5 ± 12.3 mV; LatB: 40.5 ± 9.8 mV; Jasp: 42.0 ± 9.1 mV; n=5 each). As could be expected, MFB hyperpolarization caused a corresponding hyperpolarization of MFB–CMC cell pairs (control: −63.0 ± 12.0 mV; CytoD: −72.5 ± 10.3 mV; LatB: −73.0 ± 6.7 mV; Jasp: −73.4 ± 9.8 mV; n=7 each; for an extended discussion of electrotonic CMC–MFB interactions, see Online Supplement). Given that cell pairs exposed to Jasp were not likely coupled by gap junctions any more, the value shown for this particular drug most likely reflects the membrane polarization of the patched CMCs alone. As shown by typical I–V curves obtained from single MFBs (Figure 7B), actin-targeting drug-induced MFB hyperpolarization was based on a reduction of net inward currents at negative membrane potentials. When tested at holding potentials of −60 and −75 mV, these reductions amounted to −66/−68% for CytoD, −57/−65% for LatB, and −40/−52% for Jasp, respectively (Figure 7C). Determinations of membrane capacities of the cells in Figure 7C indicated that drug treatments did not significantly affect average cell sizes (control: 60.1 ± 15.5 pF; CytoD: 57.5 ± 21.4 pF; LatB: 50.1 ± 27.5 pF; Jasp: 65.8 ± 23.8 pF). In contrast to MFBs, exposing CMCs to actin-targeting drugs had no effect on CMC net membrane currents recorded under identical conditions.

**Discussion**

The results of this study demonstrate that reverting the phenotype of cardiac MFBs to fibroblasts by pharmacological ablation of α-SMA-SFs abolishes their arrhythmogenic interaction with CMCs. This effect depends primarily on the hyperpolarization of cells undergoing depletion of α-SMA-
SFs, which reveals a novel function of this cytoskeletal component in MFBs. Cardiac fibroblasts emerging from back-transformation of MFBs exert no adverse effects on CMC electrophysiology, even though remaining electrically coupled. Apart from conveying increased contractility, the presence of α-SMA appears instrumental for determining MFB arrhythmogeneity.

Pharmacological Transformation of MFBs to Fibroblasts
Under physiological conditions, connective tissue fibroblasts in organs as different as the skin, the liver, and the lungs are shielded from mechanical stress by the surrounding extracellular matrix. As a consequence, they generally lack stress fibers and display a mostly dendritic morphology. These features are retained when fibroblasts are cultured at moderate densities in three-dimensional collagen matrices where they form an in vivo-like network of dendritically shaped cells that are metabolically coupled. The phenotype of fibroblasts changes drastically when cells are exposed to mechanical stress as typically present in vivo during tissue repair and in vitro when cultured on rigid substrates or on top of CMCs. These conditions present fibroblasts with mechanical cues that initiate a rapid phenotype switch to MFBs that express abundant α-SMA–positive stress fibers. As found in the present study, exposing MFBs to actin-targeting drugs reverses the phenotype switch by reducing the abundance of α-SMA and its targeting to stress fibers. Although the reduction of α-SMA-SFs was the expected result for the F-actin destabilizing drugs CytoD and LatB, Jasp exerted similar effects despite its reported function as an F-actin stabilizer. With Jasp, we observed a loss of stress fibers and the appearance of actin-aggregates that were scattered throughout the cytoplasm. This observation is consistent with earlier findings in rat embryonic fibroblasts and Vero cells, where treatment with Jasp for 24 hours caused disappearance of stress fibers and formation of actin “aggresomes.” Mechanistically, this effect has been explained by a Jasp-induced increase in actin nucleation frequency that causes a critical reduction in G-actin and results in the formation of disordered polymeric actin that is stored in F-actin–enriched pathological inclusion bodies. Apart from reducing α-SMA expression and targeting to stress fibers, actin-targeting drugs caused MFBs to adopt a fibroblast-like dendritic morphology either transiently (CytoD, LatB) or permanently (Jasp) during the 24-hour exposure period. Such morphological changes are not specific for MFBs but have previously been reported for a wide range of other adherent cell types. Whereas the transient nature of the effect of LatB on cell morphology was proposed to depend on progressive serum inactivation of the drug, it remains to be shown why morphological effects of CytoD were similarly transient.

Pharmacological Turnover With F-Actin Turnover Reduces MFB Arrhythmogeneity
In the past, the direct demonstration that MFBs, but not fibroblasts, exert arrhythmogenic effects on CMCs was hampered by the fact that cardiac fibroblasts in standard primary cell culture have a pronounced tendency to undergo a phenotype switch to MFBs. Given the possibility to pharmacologically ablate α-SMA-SFs and, as a result, back-transform MFBs to fibroblasts, we investigated to which extent this intervention might affect MFB-dependent slow conduction and ectopic activity. Irrespective of the type of actin-targeting drug used, ablation of α-SMA-SFs consistently led to complete suppression of MFB arrhythmogeneity. Specifically, all three drugs caused normalization of θ and dV/dtmax in hybrid MFB–CMC preparations to values that were indistinguishable from CMC-only control strands. Restoration of impulse conduction by actin-targeting drugs was dose-dependent, with drugs exhibiting an EC50/maximal effective concentration of 3/100 nmol/L (CytoD), 10/100 nmol/L (LatB), and 300/1000 nmol/L (Jasp). The finding that none of the drugs affected impulse conduction in CMC-only preparations to any major extent at maximally effective concentrations demonstrates that amelioration of conduction by actin-targeting drugs was predominantly achieved through their action on MFBs. A similar scenario was observed in regard to MFB-induced ectopic activity. When used at maximally effective concentrations, all three drugs led to increasing depression and finally complete cessation of ectopic activity. As revealed by a newly developed recording device that permits combined video time-lapse recordings and long-term assessments of spontaneous contractile activity under stable incubating conditions, the time course of depression of ectopic activity showed characteristic differences for the three actin-targeting drugs used in regard to the onset of action (LatB, acute; CytoD delayed by 0.8 hours; Jasp delayed by 2 hours) and the time constant of depression of the frequency of ectopic activity (LatB, 3.2 hours; CytoD, 9.7 hours; Jasp, 13.7 hours). As demonstrated by time-lapse video recordings, acute depression of ectopic activity by LatB was paralleled by an equally acute change of MFB morphology, with cells transiently losing their outspread morphology. Whether and by which mechanisms the acute change in morphology can explain the acute change in ectopic activity remains to be shown. Overall, even though all three actin-targeting drugs share a common end point in regard to suppressing MFB arrhythmogeneity, their different modes of action are partially reflected by differences in maximally effective concentrations (Jasp > CytoD, LatB) and the time constant of suppression of ectopic activity (Jasp > CytoD > LatB). Of note, Jasp, which might initially have stabilized F-actin, was the only drug that transiently increased ectopic activity during the first 2 hours above control levels, suggesting that stabilization of stress fibers might be proarrhythmogenic. Whether this effect was related to Jasp-induced acute changes in gap junctional coupling remains to be shown. In summary, the findings suggest that α-SMA-SFs are conducive for myofibroblasts arrhythmogeneity. This conclusion is supported by a recent study in which cultured cardiac fibroblasts that did not express α-SMA-SFs showed only little adverse effects on the electrophysiology of cocultured cardiomyocytes.
Mechanisms Underlying Actin-Targeting Drug-Induced Reduction of MFB Arrhythmogeneity

In principle, the antiarrhythmic effects of actin-targeting drugs can be explained by an action on CMCs, MFBs, or a combination thereof. Regarding CMCs, the finding that actin-targeting drugs at maximally effective concentrations had little if any effect on resting membrane potentials, impulse conduction parameters, and spontaneous activity in CMC-only preparations, suggests that this cellular compartment was not involved. This leaves MFBs as cellular targets in which antiarrhythmic actin-targeting drug activity can be explained by a reduction of the number of MFBs, a reduction of heterocellular gap junctional coupling, or a hyperpolarization of MFBs (or a combination of these). Even though, in principle, all three actin-targeting drugs are capable of inducing apoptosis when used at sufficiently high concentrations, cell numbers as indirectly inferred from the count of 4′,6-diamidino-2-phenylindole–stained nuclei were not significantly altered at maximally effective concentrations. Similarly, actin-targeting drug treatment for 24 hours did not substantially change the density of vimentin-positive cells in hybrid preparations. Accordingly, it seems safe to conclude that actin-targeting drugs did not act via a reduction of MFB cell number. As qualitatively illustrated by persistence of dye diffusion after exposure to CytoD and LatB, gap junctional coupling was not affected to any major degree by these two drugs. In contrast, Jasp caused complete disruption of MFB–CMC communication while leaving gap junctional coupling among CMCs unaffected as evidenced by a lack of depression of ϑ. The discrepancy between CytoD/LatB and Jasp may be explained by the Jasp-specific induction of a highly dendritic cell type that possibly disrupted gap junctional communication with CMCs. In contrast to their lack of effect on CMC resting membrane potentials, actin-targeting drugs induced a significant hyperpolarization of MFBs from approximately −25 mV to approximately −40 mV that was accompanied by a substantial reduction of net inward currents at negative potentials, with Jasp being slightly less effective than CytoD/LatB (range, −41% to −66%). In principle, these findings can be explained by a reduction of depolarizing inward currents or an increase in hyperpolarizing outward currents secondary to the disruption of the cytoskeleton by actin-targeting drugs (or a combination of these). Among the different cytoskeletal components, actin filaments seem particularly important in this context because their breakdown has been shown to affect ion channels and exchangers in a way that could explain observed effects: BKca (upregulation);26,27 L-type Ca2+ channels (downregulation);28,29 Na+/Ca2+ exchanger (upregulation of reverse-mode activity);29,30 and TRP channels (complex effects including downregulation).31,32

In an attempt to understand which ion channels, exchangers, and pumps might be involved in the effects of α-SMA-SF disruption on myofibroblast electrophysiology under our experimental conditions, we performed a reverse-transcription quantitative polymerase chain reaction study in which we screened expression of 70 genes in myofibroblasts under control conditions and after incubation with 1 μmol/L CytoD (for data and discussion, see Online Supplement). With the exception of a single gene coding for K2P3.1 that was significantly upregulated, the gene expression profile showed no consistent changes between control and treated preparations that would have permitted unequivocally identification of specific channels, exchangers, or pumps as being responsible for the hyperpolarizing effect of CytoD on myofibroblast electrophysiology. Similar to the reverse-transcription quantitative polymerase chain reaction results, Western blots of a selection of channels (TRPC3, TRPM4, TRPV2, TRPV4, Kv2.1, Kir2.1, and Kir2.3; for data and discussion, see Online Supplement) suggested that expression of channel proteins was not significantly altered by treating myofibroblasts with CytoD. As an exception, CytoD treatment resulted in a decrease of the complex glycosylated form of TRPC3, which was reported before to affect channel activity and channel targeting.33–35 Even though the combination of an increase in K2P3.1 currents and a decrease of TRPC3-mediated currents may contribute to myofibroblast hyperpolarization after CytoD treatment, understanding in full which ion channels and exchangers are ultimately responsible for actin-targeting drug-induced MFB hyperpolarization requires future experiments that, given that α-SMA conveys increased contractility to MFBs, will also have to take into account the possible involvement of mechanosensitive channels. In this context, a further mechanism possibly contributing to the effects of actin-targeting drugs on impulse conduction and ectopic activity may involve a reduction of mechanical stress exerted by myofibroblasts on cardiomyocytes after loss of their α-SMA-SFs. Reducing contractile forces of myofibroblasts was recently shown to restore impulse conduction velocities in cardiomyocyte–myofibroblast preparations in a Cx43-independent manner, suggesting that myofibroblasts can also cause slow conduction by activating mechanosensitive channels in cardiomyocytes.48 Future studies will have to show whether and to what extent this mechanism might contribute to actin-targeting drug-induced restoration of impulse conduction and suppression of ectopic activity. Finally, given that previous studies showed paracrine interactions between CMCs and fibroblastic cells with adverse consequences for CMC electrophysiology, it furthermore may be warranted to investigate whether actin-targeting drugs might possibly also interfere with these signaling pathways.37

Limitations of the Study

The main limitation of the present study relates to the question of to what extent the findings presented can be extrapolated to intact diseased cardiac tissue. Apart from possible differences in the phenotype of cultured cells versus cells in vivo, it is still unclear whether stromal cells establish heterocellular electric coupling with parenchymal cells in the intact working myocardium. Such coupling has been suggested to be present between fibroblasts and CMCs in healthy myocardia based on connexin immunocytochemistry.38 The finding of this study showing that cells devoid of α-SMA-SFs, ie, fibroblasts, neither slow impulse conduction nor elicit ectopic activity in the continued presence of heterocellular gap junctional coupling lends support to the possibility that such heterocellular coupling might exist in vivo. Neverthe-

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less, definite extrapolation of the results will have to await equally definite proof of heterocellular gap junctional coupling in vivo.

Conclusions

The findings of this study demonstrate that the hallmark of MFBS, \( \alpha \)-SMA-SFs, are instrumental for establishing a membrane potential depolarized enough to permit arrhythmogenic “injury current flow” to electrotonically coupled CMCs. Vice versa, in the absence of \( \alpha \)-SMA-SFs, neither conduction nor excitability of cardiac tissue is compromised, even in the sustained presence of heterocellular gap junctional coupling. Pending direct proof that similar functional differences between fibroblasts and MFBS exist in intact hearts in vivo, the findings suggest that targeted suppression of the formation of \( \alpha \)-SMA-SFs by using pharmacological or genetic approaches might form a valid strategy to counteract MFB arrhythmogeneity.

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Disclosures

None.

References

Novelty and Significance

What Is Known?

- After insults to the heart like mechanical overload and infarction, normal resident fibroblasts can undergo a phenotype switch to myofibroblasts ("activated fibroblasts"), which is characterized by de novo expression of α-smooth muscle actin containing stress fibers (α-SMA-SFs).
- In addition to their role in cardiac fibrotic remodeling, in vitro data show that myofibroblasts induce arrhythmogenic slow conduction and ectopic activity in cardiac tissue.
- Myofibroblast arrhythmogenicity stems from depolarizing current flow ("injury current flow") from moderately polarized myofibroblasts to well-polarized cardiac myocytes after establishment of heterocellular gap junctional coupling.

What New Information Does This Article Contribute?

- Pharmacological ablation of α-SMA-SFs in myofibroblasts with actin-targeting drugs (actin-targeting drugs: Cytochalasin D, Latrunculin B, Jasplakinolide) abolishes their arrhythmogenic interactions with cardiac myocytes in vitro.
- Suppression of myofibroblast arrhythmogenicity is likely attributable to a hyperpolarization of cells undergoing disruption of α-SMA-SFs, which, in turn, causes a reduction in arrhythmogenic "injury current flow."

The formation of α-SMA-SFs in myofibroblasts exerts adverse arrhythmogenic effects on cardiac tissue. Although myofibroblasts are not present in the healthy myocardium, they appear on myocardial injury and contribute to fibrotic remodeling by excessive production of extracellular matrix proteins. In vitro, myofibroblasts form heterocellular gap junctions with cardiac myocytes and induce arrhythmogenic slow conduction and ectopic activity. Given that myofibroblasts differ from nonarrhythmogenic resident fibroblasts by expressing α-SMA-SFs, we hypothesized that pharmacological ablation of these fibers with actin-targeting drugs might abolish myofibroblast arrhythmogenicity. Exposure of hybrid myofibroblast–cardiac myocyte preparations to actin-targeting drugs led to a dose-dependent normalization of conduction velocities and complete suppression of ectopic activity. We found that these antiarrhythmic effects were attributable to an actin-targeting drug-induced membrane hyperpolarization (Cytochalasin D, Latrunculin B) and a loss of heterocellular gap junctional communication (Jasplakinolide), both of which reduce arrhythmogenic "injury current flow" from myofibroblasts to cardiomyocytes. These findings reveal for the first time that the formation of α-SMA-SFs in myofibroblasts promotes arrhythmogenic slow conduction and ectopic activity in electrotonically coupled cardiomyocytes. Assuming that myofibroblasts in diseased hearts in vivo exert similar arrhythmogenic effects, our results suggest that pharmacological ablation of α-SMA-SFs might represent a novel therapeutic strategy for preventing arrhythmogenesis in hearts undergoing fibrotic remodeling.
Abolishing Myofibroblast Arrhythmogeneity by Pharmacological Ablation of \( \alpha \)-Smooth Muscle Actin Containing Stress Fibers

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SUPPLEMENT MATERIAL

Detailed Methods

**Cell culture**

**Cardiomyocyte preparations:** Primary cultures of neonatal rat ventricular cardiomyocytes were prepared using previously described techniques. Procedures used were approved by the State Veterinary Department. In short, hearts from neonatal rats (Wistar, 1 day) were excised, minced with scissors and the resulting tissue pieces were dissociated in Hank’s balanced salt solution (HBSS without Ca^{2+} and Mg^{2+}; Bioconcept) containing trypsin (0.12 %, Roche Diagnostics) and pancreatin (120 μg/ml, Sigma). The dispersed cells were centrifuged and re-suspended in culture medium consisting of M199 with Hanks' salts (Sigma), penicillin (20’000 U/L; Biochrom AG), streptomycin (34 μmol/L; Biochrom AG), vitamin B12 (1.5 μmol/L; Sigma) and 10% neonatal calf serum (Bioconcept). The resulting cell suspension was pre-plated for 2.2 h in large cell culture flasks under vibration-free conditions to reduce the content of fibroblasts that adhered more readily to the substrate than cardiomyocytes. Non-adherent cells (mostly cardiomyocytes) were pooled, passed through a cell strainer and vitamin C (18 μmol/L; Sigma), epinephrine (10 μmol/L; Sigma) and bromodeoxyuridine (100 μmol/L; Sigma) were added. Cardiomyocytes were seeded at a density of 1.6 x 10^{3} cells/mm^2 on patterned growth substrates fabricated using techniques described in detail elsewhere. Patterns used consisted either of linear strands (0.6 x 4.5 mm, 24 per individual substrate) or discs (10 mm diameter; 1 per substrate). The cultures were kept in an incubator at 35 °C in a humidified atmosphere containing 1.2% CO2. Medium exchanges were performed 16 h after seeding with supplemented medium M199 (cf. above) containing a reduced amount of neonatal calf serum (5%).

**Low density cultures:** For patch clamp experiments, freshly dissociated cells not undergoing pre-plating were seeded at a density of ~60 cells/mm^2 on collagen coated coverslips which resulted in suitable numbers of single cardiomyocytes, single myofibroblasts and cardiomyocyte-myofibroblast cell pairs. The preparations were grown in supplemented medium M199 (cf. above) containing vitamin C (18 μmol/L; Sigma) and epinephrine (10 μmol/L; Sigma). After 1 day, the medium was exchanged and the serum content was reduced to 5%.

**Hybrid cell preparations:** Cardiac fibroblasts obtained during the pre-plating step described above were kept in the incubator for 8 days during which time they formed a dense cell monolayer that consisted mostly of myofibroblasts. Following dissociation of these monolayers using trypsin-containing solution (identical composition as that used for dissociation of cardiac tissue), cells were counted and seeded at a density of 5.1 x 10^{2} cells/mm^2 on 1-d old patterned cardiomyocyte preparations. After 2 h, cultures were washed with supplemented culture medium in order to remove non-adherent cells. Culture media composition was the same as that used for pure cardiomyocyte cultures except that bromodeoxyuridine was omitted.

**Drugs**

Actin targeting drugs were added to the cultures for 20 - 28 h. Drugs used included Cytochalasin D (CytoD; 2 mmol/L stock solution in DMSO; Sigma), Latrunculin B (LatB; 1 mmol/L stock solution in DMSO; Alexis), and Jasplakinolide (Jasp; 1 mmol/L stock solution in DMSO; Alexis).

**Immunocytochemistry**

Preparations were washed with phosphate buffered saline (PBS, Invitrogen) followed by fixation with methanol at -20 °C (vimentin staining) or 2% paraformaldehyde at room temperature (α-smooth muscle actin staining) for 5 min. Thereafter, they were incubated for 20 min at room temperature (RT) with
blocking buffer (PBS containing 20% goat serum) before being exposed for 2 h to anti-vimentin (mouse monoclonal, Sigma) or anti-α-smooth muscle actin (mouse monoclonal, Thermo Fisher) dissolved in PBS containing 1% goat serum and 0.15% triton X-100. After washing, preparations were incubated for 20 min at RT with secondary antibodies (Alexa Fluor 488 and 546, goat anti-mouse, Molecular Probes) containing DAPI (Molecular Probes). This step was followed by washing and mounting. The preparations were imaged on an inverted microscope equipped for epifluorescence (Zeiss, Axiovert 200) using a slow-scan camera (Spot RT, Diagnostic Instruments). DAPI stained nuclei were counted using appropriate routines of an image analysis program (ImagePro, Media Cybernetics, USA).

Optical measurement of impulse conduction characteristics

Impulse propagation characteristics were assessed optically after staining the preparations for 5 min with the voltage sensitive dye di-8-ANEPPS (135 μmol/L; Biotium). Stained preparations were mounted in a temperature controlled chamber placed on the stage of an inverted microscope (Zeiss 135M) and were superfused at 36°C with Hanks’ balanced salt solution (HBSS, Sigma) containing 1% neonatal calf serum and (mmol/L) NaCl 137, KCl 5.4, CaCl₂ 1.3, MgSO₄ 0.8, NaHCO₃ 4.2, KH₂PO₄ 0.5, NaH₂PO₄ 0.3, and HEPES 10 titrated to pH 7.40 with 1 mol/L NaOH. Individual cell strands were stimulated with a bipolar electrode consisting of a glass micropipette filled with HBSS and a silver wire coiled around the shank. The electrode was placed ≥1 mm from measurement site in order to permit propagation to reach steady-state conditions at the recording site. Individual strands were pre-stimulated for 10s by a stimulator (SD9, Grass Instruments Co., Quincy, MA) at a basic cycle length of 500 ms before recording a single propagating action potential using a custom made fiberoptic recording system described in detail elsewhere. Recordings were made at 20x magnification (Fluar 20x, 0.75 N.A., Zeiss, Switzerland) resulting in a spatial resolution of 50 μm. The amplitude of optically recorded action potential upstrokes were scaled to 100% (%APA). Maximal upstroke velocities (dV/dt max) were calculated from %APA values and are given in %APA/ms. With an assumed action potential amplitude of 100 mV, %APA/ms values correspond to V/s. Activation profiles of the preparations were assessed from the time when depolarization reached 50% of the entire action potential amplitude.

Longterm assessment of ectopic activity

Because extracellular multielectrode arrays failed to record spontaneous electrical activity in myofibroblast coated cardiomyocyte cultures due to the partially depolarized state of the preparations, we developed a prototype measurement system that permitted to record ectopy-induced contractile activity instead. Continuous recordings of contractions were obtained with a video camera (Sony XCD-V60; resolution: 640x480 pixels; 90 frames per second) coupled to an inverted microscope (Zeiss, Axiovert S100) that was equipped with phase contrast optics. The entire system was placed in an incubator to ensure stable environmental conditions during the entire recording period (up to 48 h). Preparations placed in multiwell dishes were imaged at 10x magnification and positions were changed by means of a motorized x-y table. Contraction signals were calculated from the video data by realtime subtraction of consecutive images followed by thresholding and by summing intensity differences as recorded by each pixel. This algorithm permitted unequivocal identification of synchronized contractions of the cell monolayers with high signal-to-noise ratios. A semi-automatic detection algorithm identified the contractions and, after assessing beat-to-beat periods, calculated average beat rates as shown in Figure 4 of the manuscript. At the end of each recording of a given preparation, a picture was taken to permit offline construction of time-lapse movies.

Patch clamp recordings

Electrophysiological parameters of cardiomyocytes and myofibroblasts were measured using a HEKA patch clamp system (EPC-10 amplifier, HEKA) with experimental protocols controlled by PatchMaster software (v2.15, HEKA). The pipette filling solution contained (in mmol/L): K-aspartate 120, NaCl 10, MgATP 3, CaCl₂ 1, EGTA 10, Hepes 5 (pH 7.2). Pipette resistances ranged from 4 to 6 MΩ and pipette
potentials were zeroed before cell contact. Digitized signals were stored for off-line analysis using PatchMaster or FitMaster (v2.53, HEKA). Membrane voltages were corrected offline for liquid junction potentials as calculated by pCLAMP software (Axon Instruments). During measurements, low density cell cultures were mounted in a custom-made chamber 3 that was placed on the stage of an inverted microscope (Nikon eclipse, TE2000-E). Preparations were superfused with Hank’s balanced salt solution containing 1% neonatal calf serum (pH 7.40, buffered with 10 mmol/L Hepes) at 2-3 ml/min at RT. Resting membrane potentials were recorded in single CMCs, single MFBs or in CMC-MFB hybrid cell pairs in current clamp mode. Voltage clamp experiments using both step and ramp protocols were used to measure net membrane currents in single CMCs or MFBs. During step protocols, cells were clamped for 1000 ms from -100 to 60 mV in 10 mV increments starting from a holding potential of -80 mV. For obtaining I-V relationships of MFBs, 2000 ms long voltage ramps (-110 to 90 mV) were applied from a holding potential of -70 mV. The amplitude of whole cell net membrane currents where normalized to cell capacitance and are reported as pA/pF.

Dye coupling
The presence of functional intercellular coupling between CMCs and MFBs was probed with the gap junction permeable dye Lucifer yellow (concentration in pipette solution: 2 mmol/L). Dye diffusion was assessed 8 min after establishing successful patches on CMCs coupled to MFBs at which time a fluorescence image was taken with a CCD camera (Macrofire, Optronics). After each experiment, preparations were usually subjected to immunocytochemistry to verify that the cells coupled to CMCs were indeed MFBs (positive staining for α-smooth muscle actin containing stress fibers).

RT-qPCR
Expression levels of ion channel genes in cardiac myofibroblasts were determined using the 7900HT Micro Fluidic Card system from Applied Biosystems. TaqMan® Low Density Arrays pre-loaded with a customized selection of standardized primer pairs permitted the quantitative determination of the expression levels of 70 genes of interest (reference genes: GAPDH, Hprt1, 18S).

Total RNA was isolated from 6 independent cultures of myofibroblasts that had been grown according to the protocols used for generating hybrid cell preparations, i.e., adherent cells obtained during the preplating step were kept for 8 days in culture before being split and subjected to a second preplating procedure in order to efficiently remove 'contaminating' cardiomyocytes. Thereafter, cells were kept for another 24 h in culture before undergoing a medium exchange with control medium or with medium containing 1 µmol/L Cytochalasin D. Then, cell lysis was performed and the quality and quantity of the total extracted RNA was determined photometrically (NanoDrop Technologies). Corresponding cDNA was obtained using a high capacity RNA-to-cDNA Kit (Applied Biosystems). Finally, a two-step RT-PCR was performed using a TaqMan® Universal Master Mix II. Analysis of the data was performed using RT2 software (SABiosciences).

Western blotting
Total cell lysate was obtained from cardiac myofibroblasts cultured according to the protocol used for RT-qPCR. After the final 24 h of incubation in control medium or medium containing 1 µmol/L Cytochalasin D, cells were harvested on ice, rinsed twice with cold PBS and incubated on ice with lysis buffer for 10 min (NP40 Lysis Buffer, Invitrogen, CA, USA) supplemented with protease inhibitors (Complete mini, Roche, Germany). Cells were scraped and collected into a microcentrifuge tube, vortexed and centrifuged at 13,000 rpm for 5 min. The cell supernatant was kept and the sediment containing cell membranes was discarded. Protein concentration in the supernatant was quantified using a protein assay quantification kit (BCA Protein Kit Assay, Thermo Scientific, IL, USA). From each sample, 20µg was loaded and run on an 8% SDS-PAGE gel and then transferred to PVDF membranes. Membranes were incubated overnight at 4°C with the following primary antibodies at a dilution of 1/200 in TBS-blocking solution: rabbit polyclonal antibodies against GAPDH, TRPM4, TRPV4 (Abcam,
Cambridge, UK), TRPC3, TRPV2 and Kv2.1 (Alomone Labs, Jerusalem); rabbit monoclonal antibodies against Kir2.1 (Abcam) and mouse monoclonal antibodies against Kir2.3 (Abcam). The membranes were then rinsed with TBS-Tween 0.1% and incubated for 1 hour at room temperature with secondary antibodies coupled to an infrared chromophore (anti-mouse 700DX and anti-rabbit 800nm, Rockland, PA, USA) at a dilution of 1/10,000 in TBS-blocking solution. After being rinsed, the membranes were assessed using an infrared imager (Odyssey, Li-Cor Biosciences, NE, USA) and analysed using ImageJ software.

**Online Data, Figures and Movies**

**Timecourse of changes in myofibroblast morphology during exposure to actin targeting drugs**

Gradual changes in cellular morphology of myofibroblasts subjected to acting targeting drugs were assessed using time-lapse recordings of myofibroblast-only monolayer cultures grown on collagen-coated coverslips. As shown in Online Movie 1 and the first row of still-images from this movie in Online Figure I, cell morphology of myofibroblast cultures undergoing a control medium exchange remained essentially unchanged over the entire observation period (~ 20 h) despite the rather dynamical behavior of myofibroblasts that showed substantial locomotion and cell division (dividing myofibroblasts can be easily identified because they transiently lose their flattened shape and become spherical before division occurs). When exposed to CytoD (100 nmol/L), myofibroblasts tended to become spherical (cf. Online Movie 2 and red arrows in the second row of panels in Online Figure I) before regaining a flattened morphology in the continued presence of the drug. Highly similar morphological changes were observed during exposure to LatB (100 nmol/L). However, this particular compound acted faster (cells started to become spherical a few minutes after drug addition) and concerned nearly all cells (cf. Online Movie 3 and red arrows in the third row of panels in Online Figure I). Again, changes in cellular morphology were transient as cells tended to regain their flattened morphology in the continued presence of the drug towards the end of the observation period. In contrast to CytoD and LatB, Jasp showed no acute effects on cell morphology but caused increasingly severe morphological changes with progressive retraction of cells and induction of complete disarray of their cytoplasmic extension. These changes showed no tendency to reverse with increasing exposition time (cf. Online Movie 4 and the last row of images in Online Figure I).

Overall, morphological changes induced by actin targeting drugs in myofibroblast monolayers cultured on glass substrates were highly similar to those observed in hybrid cell cultures. Interestingly, the timecourse with which actin targeting drugs induced changes in myofibroblast morphology paralleled the timecourse of their suppressive effect on ectopic activity: LatB, that exhibited acute effects on myofibroblast morphology, similarly led to an acute depression of the frequency of ectopic activations whereas CytoD and even more so Jasp, whose effects on myofibroblast morphology were delayed, also showed a delay in suppressing ectopic activity (CytoD < Jasp). This suggests that changes in myofibroblast morphology were related to changes in emergent electrophysiological properties of hybrid cardiomyocyte-myofibroblast preparations. A full understanding of this interrelation will require future studies into the mechanisms that determine structure-function relationships at the molecular level in myofibroblasts.

**Membrane potentials in hybrid cell pairs: Theoretically predicted and measured values**

Upon establishment of functional gap junctional coupling between myofibroblasts and cardiomyocytes, current flow between the two cells exhibiting different resting potentials will cause membrane potentials to converge. The degree to which this happens is dependent, at first approximation, on the relative sizes of the input resistances of the two cell types ($r_{i\text{CMC}}$, $r_{i\text{MFB}}$) and the resistance of gap junctional coupling ($r_{GJ}$, for extended formulations cf. 4). We determined input resistances in cultured cardiomyocytes and myofibroblasts to be 240 ± 70 MOhm and 1200 ± 440 MOhm, respectively (all data from preparations of
Figure 7 of the manuscript). Average gap junctional resistance in 6 hybrid cell pairs measured with dual whole cell patch clamp protocols amounted to 125 MOhm. The voltage gradient between CMCs and MFBs under control conditions was 48 mV (V_{m,CMC} = -74.6 ± 3.3 mV, V_{m,MFB} = -26.6 ± 7.2 mV). Upon establishment of gap junctional coupling, this voltage will drop across the serially arranged resistors (r_{MFB} - r_{GJ} - r_{CMC}) according to their relative sizes. Based on these considerations, CMCs are predicted to undergo a depolarization by 7.4 mV to -67.2 while myofibroblasts will undergo a hyperpolarization by 36.8 mV to -63.4 mV. For CMCs, this value is less depolarized than the actually measured value (-63.0 mV) but is well within the standard deviation of these determinations (±12 mV, n = 17). For the case of CytoD treated preparations (V_{m,CMC} = -73.3 ± 3.0 mV, V_{m,MFB} = -44.3 ± 12.3 mV; r_{MFB} = 3610 ± 670 MOhm) and under the assumption that r_{CMC} and r_{GJ} remained unchanged, the resting potential of CMCs following gap junctional coupling is predicted to decrease by 1.7 mV to -71.6 mV which is in agreement with measured values (-72.5 ± 10.3 mV).

**Ion channel gene expression**

The expression of ion channel genes potentially involved in myofibroblast electrophysiology with a specific focus on genes potentially associated with hyperpolarization following exposure to CytoD, is depicted in Online Table I (list of gene targets investigated), Online Figure II (22 Trp channel genes), Online Figure III (25 potassium channel related genes), Online Figure IV (11 chloride channel related genes), Online Figure V (9 pumps and exchanger related genes) and Online Figure VI (connexins, α-SMA). Among the 70 genes tested, expression was either non-detectable (10 genes), remained unchanged (5 genes), showed a trend towards a decrease (41 genes) or was increased (14 genes) by CytoD treatment. Changes were moderate and reached significance only for K2P3.1 (increase). Variability in expression was generally larger in treated preparations than in controls which may point to culture-to-culture variations in responsivity to CytoD treatment. Among the 47 Trp and K+ channels tested which are the most likely candidates for CytoD related changes in myofibroblast electrophysiology, 2 genes were highly expressed (C_t < 25), 9 were moderately expressed (C_t < 25-30) and 31 showed low expression (C_t 30-35). For 5 genes, no expression could be detected. The group of highly/moderately expressed genes consisted of Trpm7, Trpc6, Trpv2, Trpc1, Trpm4, Trpc2, Trpv4 and Kv4.3, K2P2.1, Kv2.1 and Kir2.1. Among the Trp channels, CytoD induced a reduction of expression in 6 out of seven channel genes that, even though being non-significant, may contribute to the CytoD induced myofibroblast hyperpolarization by reducing non-specific cation inward currents. In parallel, however, CytoD also reduced expression of the highly/moderately expressed potassium channels which would be expected to counteract the suppression of Trp channel expression. On the other hand, CytoD caused an upregulation of expression of K2P3.1 (significant), Kir1.1, Kv1.2, Kir3.1, Kir3.3 to C values <30 (moderate expressed channels) which may tip the balance again towards myofibroblasts hyperpolarization. Expression of chloride transporters, chloride channels, sodium-potassium pump subunits and sodium-calcium exchangers tended to be slightly decreased by CytoD treatment. This was also the case for α-SMA and Cx43 and 45 expression. Overall, the RT-qPCR findings suggest that CytoD, apart from inducing a slight reduction of gene expression in the majority of genes tested, had little consistent effect that would unambiguously explain the changes in myofibroblast electrophysiology following exposure to CytoD. Nevertheless, the wide screen of ion channels and transporters expressed in cultured myofibroblasts of cardiac origin may serve as a basis for future investigations into the molecular basis of the electrophysiology exhibited by this cell type important in cardiac disease. In this respect, it is worth noting that the profile of Trp channel gene expression found in the present study is highly similar to the profile reported recently for human atrial fibroblasts (robust expression of Trpc1, Trpc 6, Trpv2, Trpv4 and Trpm7) with Trpm7, as in our study, showing the highest level of expression that was further upregulated in fibroblasts from AF patients. These similarities suggest that cultured cardiac myofibroblasts may constitute a useful model for the investigation of molecular mechanisms governing adverse crosstalk with cardiomyocytes in diseased hearts.
Western blot results

In order to identify ion channels possibly responsible for the observed changes in myofibroblast electrophysiology following exposure to CytoD (1 µmol/L for 24 h), we performed western blots. The channels chosen, TRPC3, TRPM4, TRPV2, TRPV4, Kv2.1, Kir2.1 and Kir2.3, were shown in previous studies to be expressed by fibroblastic cells of cardiac origin and to be involved in setting the membrane potential.\textsuperscript{5,12} Similar to the RT-qPCR results and as shown in Online Figure VII, we were unable to detect significant differences of expression between control and CytoD treated preparations for TRPM4, TRPV2, TRPV4, Kv2.1, Kir2.1 and Kir2.3. RT-qPCR and western blot results were qualitatively similar for TRPC3, TRPV2, Kv2.1 (all slightly reduced), TRPV4 (unchanged) and Kir2.3 (slight upregulation). TRPM4 and Kir2.1 showed a discordant behavior.

In contrast to all other channels, TRPC3 showed a migration profile that was dependent on CytoD treatment. The expected size of this protein is \(~ 97\) kDa, thus corresponding to the band just below the \(100\) kDa protein size marker. The upper band (slightly above \(100\) kDa) represents the complex glycosylated form of the TRPC3 channel protein which importantly determines channel activity and cellular targeting as reported by others before.\textsuperscript{13-15} The significant reduction of this band following treatment suggests that CytoD affected glycosylation and hence channel function at the post translational level. Being a nonselective \(\text{Ca}^{2+}\) permeable cation channel, a reduction of TRPC3 activity might contribute to a hyperpolarizing shift of the membrane potential observed in myofibroblasts following CytoD exposure. Clearly, definite proof of such a mechanism requires additional investigations that elucidate quantitative aspects of a change in TRPC3 glycosylation on the myofibroblast membrane potential. Also, such investigations would have to show whether and by which mechanisms the CytoD-induced disruption of \(\alpha\)-SMA-SFs relate to a change in TRPC3 glycosylation.

In summary and similar to the RT-qPCR results, western blots of a subset of ion channels present in cardiac fibroblastic cells failed to give definite answers as to the molecular identity of ion channel(s) involved in the CytoD induced hyperpolarization that accompanied the phenotype reversal from myofibroblasts to fibroblasts. The results suggest that in addition to the possibility of modifications of ion channel trafficking and targeting, post translational modifications might contribute to changes in cellular electrophysiology accompanying the phenotype reversal.

Online References

7. Chilton L, Ohya S, Freed D, George E, Drobic V, Shibukawa Y, MacCannell KA, Imaizumi Y, Clark RB, Dixon IMC, Giles WR. K+ currents regulate the resting membrane potential, proliferation, and contractile

Online Movie Legends

Online Movie 1: Time-lapse movie showing changes in cellular morphology of a control myofibroblast monolayer culture during an observation period of 20 h. The first frame after the control medium exchange is taken at 26:13:55 (~1.5 h after start of the recording). The imaged area measures 460 x 340 µm.

Online Movie 2: Time-lapse movie of changes in cellular morphology after exposure of myofibroblast monolayer cultures to 100 nmol/L CytoD. The first frame after the control medium exchange is taken at 26:24:33 (~1.5 h after start of the recording). The imaged area measures 460 x 340 µm.

Online Movie 3: Time-lapse movie of changes in cellular morphology after exposure of myofibroblast monolayer cultures to 100 nmol/L LatB. The first frame after the control medium exchange is taken at 26:23:47 (~1.5 h after start of the recording). The imaged area measures 460 x 340 µm.

Online Movie 4: Time-lapse movie of changes in cellular morphology after exposure of myofibroblast monolayer cultures to 1 µmol/L Jasp. The first frame after the control medium exchange is taken at 26:25:18 (~1.5 h after start of the recording). The imaged area measures 460 x 340 µm.
Online Figure I: Still images from time-lapse movies 1-4 that show the morphology of myofibroblast monolayer cultures 1.5 h before and 1 and 18 h after a medium exchange containing either no drugs (control), 100 nmol/L CytoD, 100 nmol/L LatB, or 1 µmol/L Jasp. Red arrows point to myofibroblasts that lost their flattened morphology and became spherical during drug treatment either transiently (CytoD and LatB) or permanently (Jasp).
Online Table I: List of ion channel genes investigated with RT-qPCR in control myofibroblasts and myofibroblasts exposed for 24 h to 1 µmol/L Cytochalasin D.

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Acta2-Rn01759928_g1           | a-SMA       | NM_031004.2   |
Online Figure II: RT-qPCR results of TRP channel gene expression in control myofibroblasts (black columns) and myofibroblasts treated with 1µmol/L Cytochalasin D for 24 h (grey columns; mean ± S.D of 5 separate cultures). Empty columns: no gene expression detected.
Online Figure III: RT-qPCR results of potassium channel gene expression in control myofibroblasts (black columns) and myofibroblasts treated with 1µmol/L Cytochalasin D for 24 h (grey columns; mean ± S.D of 5 separate cultures). Empty columns: no gene expression detected.
Online Figure IV: RT-qPCR results of chloride channel gene expression in control myofibroblasts (black columns) and myofibroblasts treated with 1µmol/L Cytochalasin D for 24 h (grey columns; mean ± S.D of 5 separate cultures). Empty columns: no gene expression detected.
**Online Figure V:** RT-qPCR results sodium-potassium ATPase and sodium-calcium exchanger gene expression in control myofibroblasts (black columns) and myofibroblasts treated with 1µmol/L Cytochalasin D for 24 h (grey columns; mean ± S.D of 5 separate cultures). Empty columns: no gene expression detected.

**Online Figure VI:** RT-qPCR results of connexin43/45 and α-SMA gene expression in control myofibroblasts (black columns) and myofibroblasts treated with 1µmol/L Cytochalasin D for 24 h (grey columns; mean ± S.D of 5 separate cultures).
Online Figure VII: Western-blot analysis of the differential expression of 7 ion channel proteins in myofibroblasts undergoing treatment with Cytochalasin D. **Migration patterns**: left, control myofibroblasts; right: Cytochalasin D treated myofibroblasts (1 µmol/L for 24 h). **Graphs**: protein expression levels (normalized to GAPDH) of each protein in CytoD treated condition (Cyto-D) normalized to controls (Ctrl). Each column represents the mean ± S.D. of 3 different experiments. **TRPC3**: upper bands correspond to glycosylated form of the channel protein. *P<0.05 = significantly different from control.