Calpain inhibition prevents pacing-induced cellular remodeling in a HL-1 myocyte model for atrial fibrillation

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Abstract

Objective: Atrial fibrillation (AF) is a progressive disease. Previously, clinical and animal experimental studies in AF revealed a variety of myocyte remodeling processes including L-type Ca\(^{2+}\) channel reduction and structural changes, which finally result in electrical remodeling and contractile dysfunction. There are indications that myocyte remodeling is mediated by Ca\(^{2+}\) overload induced calpain activation. To study in more detail the mechanisms underlying myocyte remodeling and to develop strategies for drug-interference, we utilised a paced cell model for AF.

Methods and results: HL-1 atrial myocytes were subjected to a 10 times increase in rate over basal values by electrical field stimulation at 5 Hz. It was found that 24-h pacing reduced plasmalemmal levels of L-type Ca\(^{2+}\) channel \(\alpha_{1C}\) subunit by \(\sim 72\%\) compared to controls. No changes in amount of the potassium channel subunits Kv4.3 and Kv1.5 were found. Pacing also induced marked structural changes; myolysis and nuclear condensation, paralleled by a 14-fold increase in calpain activity. The pacing-induced reduction of L-type Ca\(^{2+}\) channel protein was fully prevented by treatment with verapamil, the active stereoisomer of methoxyverapamil D600, the calpain inhibitors PD150606 and E64d, and LaCl\(_3\). Interestingly, PD150606, E64d and LaCl\(_3\), but not (methoxy)verapamil, prevented structural changes.

Conclusions: Paced HL-1 atrial myocytes undergo myocyte remodeling similar to that found in myocytes from patients with AF. Calcium influx independent of the L-type Ca\(^{2+}\) channel and subsequent activation of calpain represent key features in the progression towards overt structural changes. Calpain inhibition may therefore represent a useful lead for therapeutic intervention in AF.

Keywords: Atrial fibrillation; Calpain; Myolysis; L-type Ca\(^{2+}\) channel; Verapamil; PD150606; La\(^{3+}\), pacing

1. Introduction

Atrial fibrillation (AF) is currently the most common cardiac arrhythmia and is responsible for a substantial proportion of hospital costs incurred in the treatment of cardiac rhythm disorders [1]. AF has the tendency to become more persistent over time. This is illustrated by the fact that about 30% of patients with paroxysmal AF (PAF) eventually will develop persistent or even permanent, chronic AF (CAF) [2].

AF induces myocyte remodeling which leads to a heterogeneity in the electrical activation pattern [3–7] and the loss of contractile function of atrial tissue [8–10]. It was demonstrated that AF-induced changes at the structural level (myolysis and myocyte degeneration) are of prime importance for the vulnerability to AF [11–13]. Calcium overload via the L-type Ca\(^{2+}\) channel is thought to play a key role in the underlying mechanism for myocyte remodeling [14,15]. Since Ca\(^{2+}\) overload is a threat to cell viability, cytoprotective mechanisms are triggered that cause a Ca\(^{2+}\) concentration dependent inactivation of the L-type Ca\(^{2+}\) current [16], resulting in a decrease of the action potential duration, in turn enhancing the likelihood of AF [17]. If the arrhythmia persists, this might lead to the reduction of L-type Ca\(^{2+}\) channel protein levels [17–21], possibly via the activation of Ca\(^{2+}\) overload induced proteases like calpain [22],

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followed by a decrease in the L-type Ca\(^{2+}\) channel mRNA levels [18,19,21,23]. Finally, the persistent Ca\(^{2+}\) overload-induced activation of calpain and pro-apoptotic pathways [24,25] might result in structural remodeling (myolysis, nuclear condensation) of the myocyte [22,26,27].

To get a more conclusive insight in the mechanism of AF-induced myocyte remodeling, we used a paced atrial myocyte cell model. So far, isolated atrial myocytes from canine [14] were subjected to electrical field stimulation and revealed pacing induced Ca\(^{2+}\) overload and contractile dysfunction as found in human and experimental AF [8–10,15]. Unfortunately, cellular models from primary cultures do not allow long-term culturing and hence (genetic) manipulation. Therefore, we used the HL-1 atrial myocyte cell line, since these myocytes are particularly attractive for manipulation by their unique ability to proliferate and to be repeatedly passaged with preservation of genes coding for adult protein isoforms [28]. The atrial myocytes were subjected to a 10 times increase in rate over basal values by electrical field stimulation at 5 Hz, comparable to the overstimulation observed in human AF. In this study, we investigated the appropriateness of this model for AF by measuring pacing induced activation of calpain and two important myocyte remodeling processes, i.e. a specific decrease in L-type Ca\(^{2+}\) channel protein amounts and structural changes (myolysis and degeneration). Furthermore, the significance of calcium overload and activation of calpain for the myocyte remodeling was investigated by assessment of the effects of Ca\(^{2+}\) entry blockers and calpain inhibitors.

### 2. Methods and materials

#### 2.1. HL-1 cell culture conditions

HL-1 atrial myocytes, a cell line derived from adult mouse atria [28] were obtained from Dr. William Claycomb (Louisiana State University, New Orleans, LA, USA). The myocytes were maintained in Complete Claycomb Medium (JRH, UK) supplemented with 100 μM norepinephrine stock (consisting of 10 mM norepinephrine (Sigma, The Netherlands) dissolved in 0.3 mM L-ascorbic acid (Sigma)), 4 mM L-glutamine (Gibco, The Netherlands) and 10% FBS (Life Technologies, Gaithersburg, MD). The myocytes were cultured in flasks coated with 12.5 μg/ml fibronectin (Sigma) and 0.02% gelatin (Sigma), in a 5% CO\(_2\) atmosphere at 37 °C.

#### 2.2. Induction tachycardia

Under regular cell culture conditions, the basal activation frequency of the HL-1 myocytes is about 0.5 Hz (data not shown). For the tachycardia, HL-1 myocytes (≥1 × 10\(^6\) myocytes) were cultured on coverslips and subjected to a 10-fold rate increase (rapid pacing) by electrical field stimulation (5 Hz, 1.5 V/cm field strength; Grass S88 stimulator). Verapamil (1 and 20 μM, Sigma), (-)-D600 (1 μM, Sigma), (+)-D600 (1 μM, Sigma), PD150606 (20 μM, Calbiochem, The Netherlands), E64d (10 μM, Roche, The Netherlands) or LaCl\(_3\) (50 μM, Sigma) were added 2 h before start of the rapid pacing.

Fig. 1. The effect of pacing on the protein expression of different plasma membrane ion channels. Western blot showing that atrial myocytes subjected to pacing for 12 and 24 h reveal a significant decrease in L-type Ca\(^{2+}\) channel protein expression compared to control myocytes (Con) (A). Western blot showing that protein levels of Kv1.5 (B) and Kv4.3 (C) are unaffected by pacing. GAPDH was used as loading control. Protein ratios (%) for L-type Ca\(^{2+}\) channel (D), Kv1.5 (E) and Kv4.3 (F) of myocytes subjected to pacing for 0, 12 and 24 h.
2.3. Determination cysteine protease activity

Calpain activity, as described previously [22], was measured with Suc-Leu-Leu-Val-Tyr-7-amino-4-methyl-coumarin (AMC, Sigma) as substrate for calpain.

2.4. Protein extraction and Western blot analysis

For the isolation of proteins from HL-1 myocytes, the cells were lysed by the addition of SDS-PAGE sample buffer followed by sonication before separation on 10% PAA-SDS

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Fig. 2. The effect of pacing on structural remodeling and L-type Ca\(^{2+}\) channel protein staining. (A) Immunofluorescent staining of L-type Ca\(^{2+}\) channel (green) is confined to the cell membrane in normal HL-1 myocytes (control), and strongly reduced after 24 h pacing (B, paced). Red staining is myosin and blue staining are nuclei (DAPI). HL-1 myocytes were paced for 0 (control) and 24 h and stained for myosin (green) and its nucleus (blue, DAPI, panels C, D, E). Electron microscopic details are shown in F, G and H. In the control situation, myosin is diffusely distributed in the cytoplasm (C, F). After 24 h of pacing, myolysis appears (D, G; arrows). Some myocytes show blebbing of the cell membrane, a hallmark for degeneration (E, H: arrowhead). (I) Quantification of the amount of myolysis (% of positive cells; paced myocytes ○, non-paced controls □) and nuclear condensation (% of positive cells; paced myocytes ■, non-paced controls □) in time. (J) Atrial myocytes subjected to pacing (p) for 12 h reveal a significant increase in activation of calpain compared with normal HL-1 myocytes (c). Calpain activity is expressed as arbitrary units. **p<0.001.
gels (5.10^4 cells/slot). After transfer to nitrocellulose membranes (Stratagene, The Netherlands), membranes were incubated with primary antibodies against GAPDH (Affinity Reagents, USA), L-type Ca^{2+} channel α1C subunit, Kv1.5 or Kv4.3 (all Alomone Labs, Israel). Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology, The Netherlands) were used as secondary antibodies. Signals were detected by the ECL-detection method (Amersham, The Netherlands) and quantified by densitometry. The amount of protein chosen was in the linear immunoreactive signal range and expressed relative to GAPDH.

2.5. Immunofluorescent staining, quantification and confocal analysis

After subjecting HL-1 myocytes to rapid pacing, the cells were fixed for 10 min in 100% methanol (−20 °C), dried and blocked in 5% BSA (20 min room temperature). Antibodies against myosin heavy chain (MF-20, Developmental Studies Hybridoma Bank, Baltimore, MD, USA) or L-type Ca^{2+} channel α1C subunit (Alomone Labs) were used as primary antibody. Fluorescein-labeled isothiocyanate (FITC) anti-mouse (Jackson ImmunoResearch, The Netherlands) or anti-rabbit (Dako, The Netherlands) or N,N-(dipropyl)-tetramethyl-indocarbocyanine Cy3 anti-mouse (Amersham) was used as secondary antibody. Nuclei were visualized by 4′,6-diamidino-2-phenylindole (DAPI) staining. Images of FITC, Cy3 and DAPI fluorescence were obtained by using a Leica confocal laser-scanning microscope (Leica TCS SP2).

For the quantification of the amount of myocyte degeneration and myolysis, at least five fields were examined to a total amount of 250–500 myocytes, and intense DAPI staining (nuclear condensation, marker for cell degeneration [25]) and myosin disruption (characteristic for myolysis [11]) were scored by three independent observers blinded for the experimental groups.

2.6. Morphological evaluation

For morphological evaluation by electron microscopy, myocytes were fixed for at least 2 h at 4 °C in 2% glutaraldehyde (in 0.1 M cacodylate buffer, pH 7.4). Post-fixation was performed for 2 h in 1% osmium tetroxide (supplemented with 1.5% K4Fe(CN)6 in cacodylate buffer, pH 7.4) at 4 °C. After dehydration in ethanol, myocytes were cut in ultrathin sections (60 nm) and stained with uranylacetate and lead citrate and examined in a Philips 201 electron microscope operating at 60 kV.

2.7. Statistical analysis

Results are expressed as mean ± S.D. All Western blot procedures, calpain measurements and morphological quantifications were performed in duplo series of at least n = 6 wells per series, and mean values were used for statistical analysis. The Mann–Whitney U-test was performed for group to group comparisons. All p-values were two-sided, a p-value of < 0.05 was considered statistically significant. SPSS version 8.0 was used for all statistical evaluations.

3. Results

3.1. Pacing induces myocyte remodeling

HL-1 atrial myocytes were subjected to a 10-fold rate increase, which induced in time a significant decrease in L-type Ca^{2+} channel protein amounts (−63% ± 8%, after 12 h and −72% ± 10%, after 24 h pacing, both p < 0.001, Fig. 1A,D). The reduction in protein level corresponded with a strong decrease of L-type Ca^{2+} channel in the cell membrane (Fig. 2A,B). To test if the decrease in L-type Ca^{2+} channel amount is a specific or a general response to pacing, protein amounts of the potassium channel subunits Kv1.5 and Kv4.3 were measured. No differences in Kv1.5 (Fig. 1B,E) and Kv4.3 (Fig. 1C,F) protein levels were observed between paced myocytes and normal myocytes.

Furthermore, pacing of the myocytes induced marked structural changes in the form of myolysis and degeneration.
Myosin disappeared from the center of some myocytes (Fig. 2D,G) and pacing resulted in atrophy and membrane blebbing (Fig. 2E,H). The induction of myolysis and degeneration (nuclear condensation) of the myocyte was quantified in time (Fig. 2I). Pacing induced a significant increase in the amount of myolysis in about 30% of the myocytes after 8-h pacing. Furthermore, a significant increase in nuclear condensation was observed in about 11% of the myocytes after 24-h pacing. In parallel to structural changes, 12 h of pacing also lead to a 14-fold increase in calpain activity (Fig. 2J).

To exclude that the observed effects were due to electrolysis at the electrodes rather than to pacing, we analyzed the above features in myocytes that were placed outside the electrical field. In those myocytes, none of the above changes were found (data not shown).

3.2. Ca\(^{2+}\) overload and calpain activation as underlying mechanism for myocyte remodeling

To test whether Ca\(^{2+}\) overload via the L-type Ca\(^{2+}\) channel and/or the activation of calpain are involved in the pacing induced myocyte remodeling, myocytes were pre-incubated with the L-type Ca\(^{2+}\) channel antagonists verapamil (1 and 20 μM), the active (−) and inactive (+) stereoisomers of the L-type Ca\(^{2+}\) channel antagonist D600 (1 μM) or the calpain inhibitors PD150606 (20 μM) and E64d (10 μM). Furthermore, to test whether Ca\(^{2+}\) overload occurred independent of the L-type Ca\(^{2+}\) channel, myocytes were incubated with LaCl\(_3\) (50 μM). Pre-incubation with verapamil, (−)D600, PD150606 or E64d (blot not shown) did not affect the amount of L-type Ca\(^{2+}\) channel in control cells, but fully prevented the reduction of L-type Ca\(^{2+}\) channel protein amounts following 24 h of rapid pacing (Fig. 3A,B).

To investigate if verapamil, D600, LaCl\(_3\) or PD150606 prevent formation of structural changes, the amount of myolysis and nuclear condensation was determined after pacing in control myocytes and myocytes pre-incubated with the drugs. Pacing induced a significant increase in both myolysis and nuclear condensation after 8, 16 and 24 h, which was unaffected by preincubation with the L-type Ca\(^{2+}\) channel blockers verapamil (1 μM, Fig. 4C and D and 20 μM, Fig. 4A and B) and both stereoisomers of D600.

![Fig. 4](https://academic.oup.com/cardiovascres/article-abstract/62/3/521/319624)

Fig. 4. Quantification of percentage of myolysis (A) and nuclear condensation (B) in time in normal myocytes (non-paced control = ○ and paced = ●) and myocytes pre-incubated with 20 μM verapamil (non-paced control = △ and paced = ▲) or 20 μM PD150606 (non-paced control = □ and paced = ■). Quantification of percentage of myolysis (C) and nuclear condensation (D) after 16-h pacing in combination with 1 and 20 μM verapamil (∨), 20 μM PD150606 (pd), 1 μM (−)D600 ((−)D), or (++)D600 ((++)D), LaCl\(_3\) (La), or D150606 (PD). *Significant increase compared to non-paced control myocytes (p<0.05); #Significant reduction compared to paced control myocytes (p<0.01).
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enantiomers may block the L-type Ca\(^{2+}\) channel, while their
regarded as a pure L-type Ca\(^{2+}\) channel [29], it is conceivable that La\(^{3+}\) interferes with calcium overload
yverapamil (D600), the calpain inhibitors PD150606 and
treatment with verapamil, the active stereoisomer of methox-
structural changes in paced myocytes, we investigated the
prevented pacing-induced reduction of the L-type Ca\(^{2+}\) channel and initiation of
Ca\(^{2+}\) influx through the L-type Ca\(^{2+}\) channel. Consequently, the most straightforward explanation of the differences between enantiomers on L-type Ca\(^{2+}\) channel reduction would be the selective blockade of the L-type Ca\(^{2+}\) channel by the (−) enantiomer. Alternatively, both enantiomers may block the L-type Ca\(^{2+}\) channel, while their beneficial effect is counteracted in case of the (+) enantiomer because of additional blockade of \(I_{\text{To}}\).

In contrast to the dihydropyridines, the aspecific blocker of calcium entry, La\(^{3+}\), prevented both reduction in L-type Ca\(^{2+}\) channel protein levels and structural changes. Whereas it is conceivable that La\(^{3+}\) interferes with calcium overload of the myocytes, the precise mechanism involved is not
disclosed by our experiments. The obvious explanation would be that La\(^{3+}\) prevents Ca\(^{2+}\) influx in the myocytes [31]. However, La\(^{3+}\) may also be transported into the myocytes [32]. As La\(^{3+}\) blocks both ryanodine [33] and IP\(_3\) receptors [34], cellular uptake may represent an alternative way for La\(^{3+}\) to reduce intracellular calcium levels and hence limit pacing-induced myocyte remodeling. In such way, La\(^{3+}\) would be able to attenuate increased Ca\(^{2+}\) levels either dependent on the L-type Ca\(^{2+}\) channel or alternative routes previously identified in AF such as activation of the T-type Ca\(^{2+}\) channel [35], angiotensin receptors [36], endothelin receptors [37], and stretch-activated ion channels [38]. Activation of the latter routes in presence of dihydropyridines may still result in a ‘low-grade’ Ca\(^{2+}\) overload, which would be capable of activation of the calpain most sensitive to Ca\(^{2+}\), calpain I [24]. Notably, activation and up-regulation of calpain I have been found in atrial tissue of patients with AF [22].

Our data provide the first indication that calpain activation is indeed a key molecular switch in the AF related myocyte remodeling processes. Previously, in patients with paroxysmal AF and chronic AF, a significant increase in calpain activity has been observed, which correlated negatively with the amount of L-type Ca\(^{2+}\) channel protein and positively with the amount of structural changes [22]. In turn, calpain is known to activate the downstream protease caspase 3 [24] whose activity has been reported to be increased in patients with chronic AF [39]. The activation of cysteine proteases is widely known to initiate and execute programmed cell death [25]. Initiation of programmed cell death routes was found in our study, as demonstrated by activation of calpain. However, particularly in cardiac myocytes, programmed cell death is not always completed. Rather, it results in myolysis defined by the ability of the myocytes to turn into a non-functional phenotype, which leads to contractile dysfunction to maintain cell viability and tissue integrity for a prolonged period of time [26,27,40]. As myolysis is found specifically in patients with chronic AF, but not in patients with paroxysmal AF [22], continuous in vitro pacing might be used to disclose molecular mechanisms which are also activated in patients with chronic AF.

The lack of an effect of pacing on the protein levels of K\(^{+}\) channels, such as the ultra rapid component of the delayed rectifier \(I_{\text{Kur}}\) and calcium-independent transient outward current \(I_{\text{To1}}\), demonstrates that the pacing-induced reduction of the L-type Ca\(^{2+}\) channel protein amounts is not due to a general reduction of proteins expressed at the cell membrane level. The observation suggests that L-type Ca\(^{2+}\) channel reduction is an early myocyte remodeling process as found in the rapid atrial pacing model in rabbits [21] and underscores the importance of the L-type Ca\(^{2+}\) channel in early myocyte remodeling [41].

In summary, we found rapidly paced HL-1 atrial myocytes to display characteristics of molecular remodeling as found in atrial tissue from patients with chronic AF. Furthermore, our data underline the importance of calpain

4. Discussion

The present study shows that rapidly paced HL-1 atrial myocytes display the key characteristics of myocyte remodeling as observed in human and experimental AF. Thus, we found rapid pacing in HL-1 myocytes to reduce L-type Ca\(^{2+}\) channel protein levels, to cause myolysis and degeneration and to induce calpain activity. The pacing induced reduction of the L-type Ca\(^{2+}\) channel protein was fully prevented by treatment with verapamil, the active stereoisomer of methoxyverapamil (D600), the calpain inhibitors PD150606 and E64d, and La\(^{3+}\). Interestingly, PD150606, E64d and La\(^{3+}\), but not (methoxy)verapamil, prevented myolysis and myocyte degradation. These results suggest that calcium influx independent of the L-type Ca\(^{2+}\) channel and subsequent activation of calpain represent key features in the progression towards overt structural changes.

In the present study, the most distinct features of atrial remodeling, i.e. reduction of L-type Ca\(^{2+}\) channel protein as well as structural changes, were attenuated by inhibition of calpains, a class of Ca\(^{2+}\)-activated cysteine proteases [24]. To further substantiate the role of Ca\(^{2+}\) entry in the reduction of the L-type Ca\(^{2+}\) channel and initiation of structural changes in paced myocytes, we investigated the action of the stereoisomers of the dihydropyridine methoxyverapamil and of the nonspecific blocker of Ca\(^{2+}\) entry, La\(^{3+}\). Of the enantiomers, (−)methoxyverapamil, which is regarded as a pure L-type Ca\(^{2+}\) channel blocker [29], prevented pacing-induced reduction of the L-type Ca\(^{2+}\) channel, whereas (+)methoxyverapamil was without effect. Besides their action on the L-type Ca\(^{2+}\) channel, recent data suggest dihydropyridines to also affect conductance of K\(^{+}\) channels, such as the ultra rapid component of the delayed

In contrast, paced myocytes pre-incubated with PD150606 were protected against induction of myolysis and nuclear condensation (Fig. 4A and B), as were myocytes preincubated with LaCl\(_3\) (Fig. 4C and D).
activation as a molecular switch in the remodeling process. Inhibition of calpain protease activity may represent a lead for therapeutic intervention in AF.

4.1. Clinical relevance of the results

Various experimental animal models and human AF studies reveal electrical, structural and myocyte remodeling processes associated with the disease [11,23,41,42]. Our current cellular model shows many of these characteristics of AF. In contrast to formerly used cellular models from primary cultures [14], our current model employs immortalised atrial myocytes allowing for long-term culturing and hence providing an excellent tool to dissect underlying mechanisms for myocyte remodeling, to genetically manipulate key factors that may be involved in the myocyte response to AF, and to perform screens for therapeutic (drug) interventions.

Our data suggest that inhibition of calpain activity may be superior over verapamil treatment to prevent the myocyte remodeling processes in AF. Thus, therapeutic intervention directed at development of clinically adequate calpain inhibitors might be useful.

4.2. Limitations of the study

The focus of the present study was to investigate the pacing-induced molecular mechanisms involved in myocyte remodeling (myolysis and L-type Ca2+ ion-channel reduction). While pacing of cultured atrial myocytes clearly induces similar changes as observed in experimental and human AF, it cannot be ruled out that additional factors like stretch and hormonal influences are involved in the pathophysiology of AF in the whole organism.

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