Chronic Atrial Fibrillation Increases MicroRNA-21 in Human Atrial Myocytes Decreasing L-Type Calcium Current

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Background—Atrial fibrillation is characterized by progressive atrial structural and electrical changes (atrial remodeling) that favor arrhythmia recurrence and maintenance. Reduction of L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)) density is a hallmark of the electrical remodeling. Alterations in atrial microRNAs could contribute to the protein changes underlying atrial fibrillation–induced atrial electrical remodeling. This study was undertaken to compare miR-21 levels in isolated myocytes from atrial appendages obtained from patients in sinus rhythm and with chronic atrial fibrillation (CAF) and to determine whether L-type Ca\(^{2+}\) channel subunits are targets for miR-21.

Methods and Results—Quantitative polymerase chain reaction analysis showed that miR-21 was expressed in human atrial myocytes from patients in sinus rhythm and that its expression was significantly greater in CAF myocytes. There was an inverse correlation between miR-21 and the mRNA of the \(\alpha_{1c}\) subunit of the calcium channel (CACNA1C) expression and \(I_{\text{Ca,L}}\) density. Computational analyses predicted that CACNA1C and the mRNA of the \(\beta_2\) subunit of the calcium channel (CACNB2) could be potential targets for miR-21. Luciferase reporter assays demonstrated that miR-21 produced a concentration-dependent decrease in the luciferase activity in Chinese Hamster Ovary cells transfected with CACNA1C and CACNB2 3′ untranslated region regions. miR-21 transfection in HL-1 cells produced changes in \(I_{\text{Ca,L}}\) properties qualitatively similar to those produced by CAF (ie, a marked reduction of \(I_{\text{Ca,L}}\) density and shift of the inactivation curves to more depolarized potentials).

Conclusions—Our results demonstrated that CAF increases miR-21 expression in enzymatically isolated human atrial myocytes. Moreover, it decreases \(I_{\text{Ca,L}}\) density by downregulating Ca\(^{2+}\) channel subunits expression. These results suggested that this microRNA could participate in the CAF-induced \(I_{\text{Ca,L}}\) downregulation and in the action potential duration shortening that maintains the arrhythmia. (Circ Arrhythm Electrophysiol. 2014;7:861-868.)

Key Words: atrial fibrillation ■ calcium channel ■ human atrial myocytes ■ microRNA ■ miR-21

Atrial fibrillation (AF) is the most prevalent arrhythmia and the main risk factor associated with myocardial-related cerebrovascular events.\(^1,2\) Extremely fast atrial rates lead to progressive atrial structural and electrical changes (remodeling) that favor arrhythmia recurrence and maintenance. Fibrosis is one of the most important structural changes associated with atrial remodeling.\(^1,2\) Electrical remodeling is characterized by a marked shortening of the atrial action potential duration (APD) and refractoriness because of changes in Ca\(^{2+}\) and K\(^+\) channel densities.\(^1,5\) Reduction of the density of the L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)), which is generated by channels composed of \(\alpha_{1c}\) or Cav1.2 (encoded by CACNA1C), \(\beta_2\) (CACNB2), and \(\alpha_2\delta\) (CACNA2D) subunits, is a hallmark of the electrical remodeling.\(^3,3\) Importantly, the molecular mechanisms underlying \(I_{\text{Ca,L}}\) downregulation have not been completely elucidated yet. Some authors proposed that it is because of a reduction of CACNA1C and CACNB2 mRNA expression,\(^4,6\) whereas others suggested post-transcriptional mechanisms, such as protein dephosphorylation.\(^7\)

Clinical Perspective on p 868

MicroRNAs (miRNA) are a class of small noncoding RNAs (21–25 nucleotides) that regulate gene expression at the post-transcriptional level by binding to 3′ untranslated regions (3′UTR) of the target mRNA promoting its degradation or blocking translation.\(^8\) Recent evidence pointed to

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the involvement of miRNAs in the AF-induced $I_{\text{Ca,L}}$ down-regulation. In this line, miR-1 and miR-328 bind 3′ UTR of CACNA1C and decrease $I_{\text{Ca,L}}$. Expression of miR-21 is increased in plasma samples from patients with chronic AF (CAF) compared with patients in sinus rhythm (SR). Moreover, Adam et al., showed a 2.5-fold upregulation of miR-21 expression in left atrial appendages from patients with CAF relative to patients in SR. They proposed that miR-21 could participate in atrial fibrosis formation and contribute to the AF-induced structural remodeling. Indeed, miR-21 is highly expressed in cardiac fibroblasts, where it is implicated in the activation of profibrotic pathways and atrial profibrillatory remodeling in experimental models of heart failure. However, expression of miR-21 in cardiac myocytes has not been consistently demonstrated, and its putative effects on the electrical remodeling are currently unknown. We hypothesized that miR-21 is expressed in human atrial myocytes and upregulated in myocytes from patients with CAF compared with those in SR, playing a role in the CAF-induced $I_{\text{Ca,L}}$ decrease. Therefore, this study was undertaken to compare the expression of miR-21 in isolated myocytes from atrial appendages obtained from patients in SR and with CAF and to analyze whether Ca++ channel subunits are targets for miR-21.

Methods

The study was approved by the Investigation Committee of the Hospital Universitario Gregorio Marañón (CNIC-13) and conforms to the principles outlined in the Declaration of Helsinki. Each patient gave written informed consent. Clinical data of the patients are included in Table I in the Data Supplement.

Analysis of the mRNA Expression in Human Atrial Myocytes

Real-Time Quantitative Polymerase Chain Reaction

RNA was extracted with miRNeasy Macro Kit (Qiagen, UK) from human atrial myocytes enzymatically isolated from right atrial appendages obtained from patients in SR (n=10) and with CAF (n=10) as described. From each sample, 2 RNA fractions were obtained: a large RNA (>200 nucleotides) fraction and a separate miRNA-enriched fraction. Quantitative polymerase chain reaction (qPCR) was performed using TaqMan gene and miRNA expression assays (Life Technologies, USA). The cycle to threshold (Ct) values were normalized to 18S rRNA.

miRNA Target Prediction

Computational prediction of putative targets for miR-21 was performed by using DIANA-microT3.1, TargetMiner, PicTar, mIRANDA, TargetScan2.6, EIMMo, and RNA22-HSA miRNA target prediction algorithms.

Luciferase Gene Expression Reporter Assay

Luciferase activity assays were performed in Chinese Hamster Ovary (CHO) cells transfected with the 3′ UTR regions of CACNA1C, CACNB2, and CACNA2D cloned into pMirTarget vector (BlueHeron, USA) and contransfected or not with miR-21 mimic (hsa-miR-21-5p; MC10206), miRNA mimic negative control No. 1, or miR-21 inhibitor (MH10206; Life Technologies).

Western Blot Analysis

To measure Cav1.2 protein expression, a Western blot analysis in HL-1 cells transfected or not with miR-21 mimic was performed by using anti-Cav1.2 antibody (1:1000; Neuromab, USA).

Calcium Current Recordings

Currents were recorded in human atrial myocytes and HL-1 cells at room temperature using the whole-cell patch-clamp technique.

Results

miR-21 Expression Levels Are Increased in Myocytes Isolated From Patients With CAF

We first compared the expression of miR-21 in myocytes enzymatically isolated from atrial appendages obtained from patients in SR (n=10) and with CAF (n=10) by qPCR. Figure 1A shows the ΔCt values corresponding to miR-21 levels measured in myocytes from patients in SR and with CAF. Importantly, miR-21 was expressed in human atrial myocytes from patients in SR, and as demonstrated by the comparison of the ΔCt values, miR-21 expression was significantly greater in CAF myocytes. Transformation of ΔCt to fold differences demonstrated that miR-21 expression was ∼3.8× greater in CAF than in SR myocytes (Figure 1D). Because decreased $I_{\text{Ca,L}}$ density is a hallmark of the CAF-induced electrical remodeling, we measured CACNA1C and CACNB2 mRNA expression in isolated atrial myocytes from patients in SR and with CAF by qPCR. mRNA expression of the target genes was measured in the fractions of RNA >200 nucleotides obtained from the same human atrial myocyte samples used for miR-21 expression assay. The results demonstrated that CACNA1C and CACNB2 mRNA expression was ≥60% and >40%, respectively, in SR than in CAF myocytes (Figure 1B–1D). Interestingly, association studies demonstrated an inverse correlation of miR-21 expression with that of CACNA1C in patients in SR and with CAF (Figure 1E) and with $I_{\text{Ca,L}}$ density at +10 mV (Figure 1F), in such a way that the greater miR-21 expression, the lower CACNA1C expression and $I_{\text{Ca,L}}$ density. Therefore, we were interested in determining whether miR-21 could modulate the expression of Ca++ channel subunits. A computational analysis using several algorithms revealed CACNA1C and CACNB2 as potential targets for miR-21. Indeed, CACNA1C and CACNB2 3′ UTR regions contain a sequence, which is complementary to the seed site of miR-21 (nucleotides 2–8; Figure I in the Data Supplement). On the contrary, 3′ UTR region of CACNA2D did not exhibit any complementary sequence to the seed site of miR-21.

miR-21 Directly Binds to the 3′ UTR of CACNA1C and CACNB2

To analyze whether miR-21 binds to the 3′ UTR regions of the subunits forming the cardiac Ca++ channel, CACNA1C, CACNB2, and CACNA2D 3′ UTR regions were cloned into pMirTarget vector, which contains firefly luciferase as a
reporter. Cotransfection of miR-21 mimic (15–60 nmol/L) resulted in a concentration-dependent decrease of the luciferase activity in CHO cells transfected with CACNA1C and CACNB2 3′UTR regions compared with that measured in sham-transfected cells (Figure 2A and 2B). In both cases, miR-21 mimic at 30 nmol/L concentration reduced luciferase activity by >60%, and thus, this was the concentration selected for the rest of the experiments. The specificity of the miR-21 effect was supported by the absence of changes produced by the negative control miRNA (30 nmol/L). Furthermore, transfection of miR-21 inhibitor (antimiR-21; 30 nmol/L) significantly increased luciferase activity, demonstrating the relief of tonic repression by endogenous miR-21 in these cells.17 As shown in Figure 2C, transfection of miR-21 mimic did not significantly modify luciferase activity in cells transfected with the CACNA2D 3′UTR region in accordance with the absence of any complementary sequence to the seed site of miR-21.

miR-21 Reduces the Density of L-Type Calcium Currents

qPCR experiments demonstrated that CAF myocytes displayed greater miR-21 expression than SR myocytes and that there was a correlation between \( I_{\text{Ca,L}} \) density and miR-21 expression. We next compared properties of \( I_{\text{Ca,L}} \) between SR and CAF by pooling recordings from 52 and 31 cells, respectively. Cell capacitance of CAF myocytes was greater than that of SR myocytes (77.9±5.6 versus 58.7±3.8 pF; \( P=0.005 \)). Currents were recorded by applying 500-ms pulses from −80 mV to potentials between −40 and +50 mV in 5 mV increments, with a prepulse to −30 mV to inactivate the sodium current. Figure 3A and 3B show traces for \( I_{\text{Ca,L}} \) recorded at +10 mV in SR and CAF myocytes and current density–voltage curves. As expected, \( I_{\text{Ca,L}} \) density was significantly lower in CAF than in SR myocytes (−2.3±0.3 versus −3.3±0.3 pA/pF at +10 mV; \( P=0.016 \)), whereas no differences in the activation...
and inactivation kinetics and voltage-dependent activation were observed. However, the midpoint of the inactivation curve was significantly shifted to more positive potentials (Figure 3C and 3D; Table).

We next analyzed whether miR-21 transfection in HL-1 cells reproduced the effects on the $I_{Ca,T}$ induced by electrical remodeling. HL-1 cells exhibit functional L- and T-type Ca$^{2+}$ channels, and thus, both $I_{Ca,L}$ and $I_{Ca,T}$ can be recorded. HL-1 cells randomly patched were divided into 3 groups depending on the predominant voltage-gated Ca$^{2+}$ current recorded (Figure II in the Data Supplement). In control conditions (n=54), around 24% of cells did not exhibit any Ca$^{2+}$ current and ≈39% exhibited a large Ca$^{2+}$ current, identified as $I_{Ca,T}$ because it was completely abolished by NiCl (50 μmol/L) and reached its maximum density at −30 mV ($I_{Ca,T}$-predominant cells; Figure II in the Data Supplement). The rest of the cells (≈37%) exhibited a large $I_{Ca,L}$ that was abolished by nifedipine (1 μmol/L) and reached its maximum density at +20 mV (Figure II in the Data Supplement) or cell capacitance (22.9±3.2 pF; $P<0.05$).

Transfection of miR-21 mimic did not modify the proportion of cell phenotypes ($P>0.05$, n=60; Figure II in the Data Supplement) or cell capacitance (22.9±3.2 pF; $P<0.05$). Currents through L-type Ca$^{2+}$ channels were recorded in $I_{Ca,L}$-predominant cells using Ba$^{2+}$ as the charge carrier ($I_{Ba}$). Figure 4A shows $I_{Ba}$ traces recorded by applying the protocol at the top transfected or not with miR-21 mimic (30 nmol/L). As can be observed in Figure 4B, current density reached its maximum value at +20 mV (n=30) and transfection of miR-21 mimic significantly reduced $I_{Ba}$ density (n=33; $P=0.02$) and slowed the time course of inactivation ($P=0.005$), without modifying activation kinetics (Figure 4A–4C; Table). This $I_{Ba}$ density decrease could be attributed to the reduction of Cav1.2 expression produced by miR-21 as demonstrated by Western blot analysis (Figure 5A and 5B). $I_{Ba}$ density was not modified by the negative control miRNA (30 nmol/L; n=15), but was significantly increased by antimiR-21 (30 nmol/L, n=15; Figure 4C), demonstrating a tonic inhibition of $I_{Ba}$ by endogenous miR-21 in HL-1 cells. miR-21 did not modify activation curves (Figure 4D) and shifted the midpoint of the inactivation curve to more positive potentials, without modifying the slope factor (Figure 4D).

### Table. Time- and Voltage-Dependent Properties of $I_{Ca,L}$ and $I_{Ba}$ Recorded in Human Atrial and HL-1 Cells, Respectively

<table>
<thead>
<tr>
<th></th>
<th>Maximum Density, pA/pF</th>
<th>$E_{rev}$, mV</th>
<th>Time Course of Activation* ($\tau$, ms)</th>
<th>Time Course of Current Decay† ($\tau$, ms)</th>
<th>Voltage Dependence of Activation</th>
<th>Voltage Dependence of Inactivation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\tau_{act}$</td>
<td>$\tau_{act}$</td>
<td>$V_{1/2}$, mV</td>
<td>$K$</td>
</tr>
<tr>
<td>$I_{Ca,L}$</td>
<td></td>
<td></td>
<td>$-3.3±0.3$</td>
<td>$3.1±0.3$</td>
<td>$21.3±6.5$</td>
<td>$189±20$</td>
</tr>
<tr>
<td>SR (n=52)</td>
<td></td>
<td></td>
<td>$56.1±1.5$</td>
<td>$1.1±0.1$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAf (n=31)</td>
<td></td>
<td></td>
<td>$54.8±2.1$</td>
<td>$2.0±0.1$</td>
<td>$165±12$</td>
<td>$5.7±0.5$</td>
</tr>
<tr>
<td>Control (n=30)</td>
<td></td>
<td></td>
<td>$54.8±2.1$</td>
<td>$1.2±0.1$</td>
<td>$21.8±2.3$</td>
<td>$163±11$</td>
</tr>
<tr>
<td>+miR-21 (n=33)</td>
<td></td>
<td></td>
<td>$74.8±4.0$</td>
<td>$1.9±0.1$</td>
<td>$300±48$</td>
<td>$7.5±1.9$</td>
</tr>
</tbody>
</table>

$E_{rev}$ indicates reversal potential; $\tau_{act}$, time constant of activation; $\tau_{act}$ and $\tau_{sust}$ fast and slow time constants, respectively, of current decay; $V_{1/2}$ and $k$, midpoint and slope, respectively, of the conductance voltage and availability curves. CAf, chronic atrial fibrillation; and SR, sinus rhythm.

*Activation kinetics was measured by fitting monoeponential functions to current traces recorded at +10 mV (human atrial myocytes) and +20 mV (HL-1).
†Inactivation kinetics were measured by fitting biexponential and monoexponential functions to current traces recorded in human atrial myocytes (+10 mV) and HL-1 (+20 mV), respectively. † and ‡ $P<0.05$ vs SR and control values, respectively. Comparison between $I_{Ba}$ recorded from SR and CAf myocytes were made using multilevel mixed-effects models.
Moreover, miR-21 did not significantly modify reactivation kinetics (Figure III in the Data Supplement).

miR-21 Does Not Modify the Density of T-Type Calcium Currents

To determine whether the effects of miR-21 are specific to L-type Ca\(^{2+}\) channel subunits, the consequences of miR-21 mimic transfection on \(I_{\text{Ca,T}}\) were determined. In this group of experiments, only \(I_{\text{Ca,T}}\)-predominant HL-1 cells were included for the analysis. As can be observed, miR-21 did not significantly modify \(I_{\text{Ca,T}}\) density and time- and voltage-dependent properties (Figure 6A and 6B).

**Discussion**

The results presented here demonstrated that CAF upregulates miR-21 in human atrial myocytes, which, in turn, decreases \(I_{\text{Ca,L}}\), but not \(I_{\text{Ca,T}}\) density.

miR-21 is Expressed in Human Atrial Myocytes and Is Upregulated by CAF

Our qPCR results demonstrated for the first time that miR-21 was expressed in enzymatically isolated atrial myocytes obtained from patients in SR. Some authors suggested that miR-21 was expressed in mouse and rat cardiac myocytes, where it could play a role in cardiac hypertrophy and in the protective effect of ischemic preconditioning against ischemia-induced cardiac myocyte damage. On the contrary, other authors described that miR-21 was not expressed in mouse cardiac cells, and thus, its role in cardiac remodeling produced in a pressure overload–induced mouse model was attributed to its presence in fibroblasts that affects cardiac myocytes in a paracrine fashion. In human atria, miR-21 was found in whole atrial biopsies. However, it is generally accepted that the presence of fibroblasts and nonmyocyte cells in whole biopsies complicates gene expression analysis. From
our results, we suggest that, besides in fibroblasts, miR-21 is expressed in human atrial myocytes.

Our results also demonstrated that miR-21 expression was increased by 3.8-fold in CAF relative to SR myocytes. Expression of several miRNAs is reduced (eg, miR-1, miR-26, miR-29, miR-30, miR-133, miR-208, and miR-590) or increased (eg, miR-21, miR-328, miR-499, and miR-155) in atrial tissue collected from patients with AF relative to patients in SR.２,２１ Interestingly, CAF-induced increase of miR-21 expression has been a consistent finding in several studies.２,２１,２２ being one of the most upregulated (3- to 4-fold) miRNAs.２１,２２ Adam et al１２ demonstrated that miR-21 upregulation was correlated positively with atrial collagen content and increased expression of profibrotic mediators. Therefore, miR-21 was considered to play a critical role in the structural remodeling associated with AF by means of an increased production of fibrosis. Indeed, atrial miR-21 knockdown suppressed atrial fibrosis and AF promotion in a rat model of myocardial infarction.２,２１ Several mechanisms could underlie AF-induced miR-21 expression increase involving post-transcriptional, nontranscriptional, and mainly transcriptional mechanisms.２１

In this regard, miR-21 gene is located on chromosome 17, in the 10th intron of the TMEM49 gene, although miR-21 is independently transcribed by its own promoter regions.２３ Nuclear factor κB expression increase in response to several stimuli (hypertrophy, oxidative stress, tumor necrosis factor-α, transforming growth factor-β, and the renin–angiotensin system) involved in structural and electrical AF-induced remodeling.２４ Moreover, an increase in nuclear factor κB expression/activity under oxidative stress correlates with an increase in miR-21 expression, whose promoter region exhibits 5 nuclear factor κB binding sites.２５ Therefore, it can be speculated that increase in the nuclear factor κB expression/activity could be responsible, at least partially, of the increase in miR-21 expression observed in CAF patients.

miR-21 Regulates Calcium Channel Subunits Expression and Decreases $I_{\text{Ca,L}}$

Decrease in the expression of the channels generating $I_{\text{Ca,L}}$ critically contributes to the abbreviation of APD and atrial refractory period.２３ Our results demonstrated that there is an inverse correlation between miR-21 and CACNA1C expression and $I_{\text{Ca,L}}$ density, suggesting that miR-21 could target Ca$^{2+}$ channel subunits. This hypothesis was supported by the results yielded by the bioinformatic analyses. Alignment of CACNA1C and CACNB2 3’UTR sequences with miR-21 demonstrated a highly conserved 7mer interaction with the seed site of miR-21 and additional Watson–Crick base pairing matches near the seed site. It has been described that CACNA1C is a validated target for miR-1 and miR-328.９,２１ Importantly, both miRNAs are predicted to bind to 3’UTR sequence of CACNA1C with complementarity scores lower than those of miR-21. These predictions were confirmed by the concentration-dependent decrease in luciferase activity measured in cells transfected with the 3’UTR region of CACNA1C and CACNB2 produced by miR-21. The functional consequences of the interaction were demonstrated by the decreased expression of Cav1.2 protein in HL-1 cells transfected with miR-21. Importantly, patch-clamp studies demonstrated that miR-21 produced changes in $I_{\text{Ca,L}}$ properties qualitatively identical to those produced by CAF (ie, a marked reduction of $I_{\text{Ca,L}}$ density and shifts of the inactivation curves to more depolarized potentials). Current density reduction may be attributed to miR-21 effects on CACNA1C expression. However, voltage-dependent effects would not be expected from the decrease in the expression of the α-subunit and could be attributed to the repression of CACNB2. Our results confirmed previous data showing that miR-21 decreased luciferase activity generated by cells transfected with 3’UTR of CACNB2.２６ Thus, considering that coexpression of Cav1.2 subunits leads to a substantial increase in $I_{\text{calc}}$ density and a shift of the inactivation curves to more hyperpolarized voltages,２７ it is reasonable to propose that the voltage-dependent effects produced by miR-21 could be mediated by the decrease in CACNB2 expression. However, it cannot be ruled out that the presence of ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid in the internal solution can be partially responsible of these voltage-dependent effects, as was proposed previously,２８ even when miR-21 effects on $I_{\text{calc}}$ were similar under ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid-free conditions (Figure IV in the Data Supplement). To determine the specificity of the interaction, HL-1 cells were transfected with an miRNA mimic negative control, which is a random sequence miRNA mimic molecule with no predicted targets or with an miR-21 inhibitor that specifically binds to and inhibits endogenous miR-21. The absence of effects of miRNA mimic negative control confirmed that miR-21 effects were caused by its interaction with the 3’UTR of the channel subunits. On the contrary, miR-21 inhibitor increased $I_{\text{calc}}$ density, suggesting that endogenous miR-21 was producing a tonic inhibition
of the current that was relieved by the inhibitor. Further support came from the fact that miR-21 did not modify $I_{\text{Ca,L}}$ in accordance with the absence of complementarity between miR-21 and the 3’UTR of CACNA1G, CACNA1H, and CACNA1I, which encode the 3 types of α-subunits responsible for $I_{\text{Ca,L}}$ (Cav3.1, Cav3.2, and Cav3.3).

Reduction of $I_{\text{Ca,L}}$ density by CAF has been consistently described in patients, as well as in AF experimental models, and it has been proposed to be triggered by Ca2+ overload secondary to the rapid atrial rates. The molecular mechanisms underlying $I_{\text{Ca,L}}$ downregulation are complex and can include impaired Cav1.2 protein trafficking induced by a zinc-binding protein, activation of the Ca2+/calmodulin/calcineurin/nuclear factor of activated T cells system causing transcriptional downregulation of the Cav1.2 α-subunit, Ca2+ channel diphosphorylation because of serine/threonine protein phosphatase activation, enhanced Cav1.2 α-subunit S-nitrosylation, activation of calpains, ankyrin-B dysfunction, or impaired src kinase activity.

Besides all these mechanisms, it has been recently proposed that miRNAs could be involved in AF-induced $I_{\text{Ca,L}}$ downregulation. Among all the miRNAs that are upregulated in patients with CAF, only miR-328 has been described to regulate Ca2+ channel α1c and β subunits expression. miR-328 is increased in right atrial appendages from patients with CAF relative to those in SR. Moreover, overexpression of miR-328 through adenoviral infection in canine atrium and transgenic murine models induced Ca2+ channel downregulation in mice enhanced AF susceptibility, decreased $I_{\text{Ca,L}}$ density, and shortened atrial APD as a result of the repression of CACNA1C expression. It has been described that other miRNAs, such as miR-1 or miR-26 whose expression is decreased in AF, play a role in the AF-induced electrical remodeling, mainly by targeting Kir2.1 channels. Both miRNAs are involved in the AF-induced increase of the inward rectifier current and, thus, in the shortening of the APD. Therefore, it can be proposed that all these miRNAs (miR-1, miR-21, miR-26, and miR-328) may contribute to AF-induced electrical remodeling and eventually to the perpetuation of the arrhythmia.

Our study provides the first description of a putative involvement of miR-21 in the modulation of $I_{\text{Ca,L}}$ and a potential mechanism linking structural and electrical remodeling processes. Recent evidence suggests that structural and electrical remodeling processes may not be independent and would share some common pathways. In fact, it has been demonstrated that activation of fibroblast signaling pathways via soluble cytokines, such as TGF-β1 and platelet-derived growth factor, modulate Na+, Ca2+, and K+ currents involved in the control of atrial APD.

**Study Limitations**

All samples came from right atrial appendages, which could not be representative of the rest of the atria. Furthermore, miR-21 and Ca2+ channel subunits expression could be influenced by age, pharmacological treatment, and underlying cardiac diseases of the patients. The proportion of patients with ischemic heart disease alone and combined with valvular cardiopathy or under statin therapy were equally distributed in both groups, indicating that the changes described here could be attributed to CAF itself.

**Conclusions**

Our results demonstrated that miR-21 expression increases in myocytes isolated from patients with CAF. Moreover, it decreases $I_{\text{Ca,L}}$ density by downregulatting Ca2+ channel subunits expression. These results suggested that miR-21 could participate in the CAF-induced $I_{\text{Ca,L}}$ downregulation and in the APD shortening that maintains the arrhythmia.

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**Disclosures**

None.

**References**


**CLINICAL PERSPECTIVE**

Chronic atrial fibrillation is associated with atrial structural and electrical changes (atrial remodeling) that favor arrhythmia recurrence and maintenance. Electrical remodeling is characterized by a marked shortening of the atrial action potential duration mainly because of the reduction of L-type Ca2+ current (I(Ca,L)) density. Importantly, molecular mechanisms underlying I(Ca,L) downregulation are not completely understood. MicroRNAs are small noncoding RNAs that regulate expression of the target mRNA, promoting its degradation or blocking translation. MiR-21 is highly expressed in cardiac fibroblasts, where it is implicated in the activation of profibrotic pathways. For this reason, it was proposed to participate in atrial fibrosis formation and contribute to the AF-induced structural remodeling. However, expression of miR-21 in human atrial myocytes has not been explored, and its putative involvement in the electrical remodeling is currently unknown. Our results demonstrated that miR-21 was expressed in human atrial myocytes from patients in sinus rhythm, and its expression was 3.8-fold greater in chronic atrial fibrillation myocytes. Interestingly, miR-21 expression and I(Ca,L) density in human atrial myocytes were inversely correlated. Furthermore, miR-21 transfection in cultured atrial cells produced changes in I(Ca,L) profiles qualitatively similar to those produced by chronic atrial fibrillation (i.e., a marked reduction of I(Ca,L) density and shift of the inactivation curves to more depolarized potentials). These results demonstrate that, besides in fibroblasts, miR-21 is expressed in human atrial myocytes and would imply that miR-21 participates in the chronic atrial fibrillation–induced electrical remodeling by contributing to I(Ca,L) downregulation.
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SUPPLEMENTAL MATERIAL

Supplemental Methods

1. Human atrial myocyte isolation

The study was approved by the Investigation Committee of the Hospital Universitario Gregorio Marañón (CNIC-13) and conforms to the principles outlined in the Declaration of Helsinki. Each patient gave written informed consent. Data regarding age, sex, type of surgery, and pharmacological treatment of the patients are included in Supplemental Table 1. Human right atrial samples were obtained from patients in sinus rhythm (SR) (n=10) and with chronic atrial fibrillation (CAF) (n=10) that underwent cardiac surgery. Atrial myocytes were enzymatically isolated following previously described methods.\textsuperscript{1,2} Just after excision, atrial appendages were submerged in RNA later (Qiagen, United Kingdom) to stabilize RNA and immediately placed into chilled Ca\textsuperscript{2+}-free Tyrode’s solution containing (mmol/L): NaCl 100, KCl 10, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 5, taurine 50, MOPS 5, 2,3-butanedione monoxime (BDM, 30 mmol/L), and glucose 20 (pH 7.0 with NaOH), chopped into small pieces (\(\approx\)1 mm\textsuperscript{3}), and washed 3 times for 3 minutes with Ca\textsuperscript{2+}-free Tyrode’s solution. Tissue pieces were then changed to Ca\textsuperscript{2+}-free solution containing 254 U/mL collagenase type I (Worthington, USA) and 0.5 mg/mL protease type XXIV (Sigma Chemical Co., United Kingdom) and gently stirred for 15 minutes. Afterwards, the Ca\textsuperscript{2+} concentration was raised to 0.2 mmol/L, and the tissue was stirred for 30 minutes more. Stirring was continued with Tyrode’s solution (0.2 mmol/L Ca\textsuperscript{2+}) containing only collagenase until rod-shaped striated myocytes were seen (\(\approx\)35 minutes). During all these steps, the solutions were continuously oxygenated with 100% O\textsubscript{2} at 37°C. Myocytes were kept in a storage solution containing (mmol/L): KCl 20, KH\textsubscript{2}PO\textsubscript{4} 10, glucose 10, K-glutamate 70, \(\beta\)-hydroxybutyrate 10, taurine 10, EGTA 10, and albumin 1% (pH 7.4 with KOH)
supplemented with RNAprotect Cell Reagent (Qiagen) to avoid RNA degradation in isolated cells until use for mRNA/microRNA expression assays or electrophysiological experiments.

2. Analysis of the mRNA expression

Tissue and RNA preparation

The suspension of isolated human atrial myocytes obtained was centrifuged at 500 g for 30 sec to remove non-myocytes (including fibroblasts) and dead or hypercontracted myocytes. Supernatant was discarded and the pellet with the isolated myocytes was used for subsequent RNA extraction with miRNeasy Micro Kit (Qiagen) according to the manufacturer’s instructions. This kit enabled to obtain two fractions of RNA from each sample: a large RNA (>200 nucleotides) fraction and a separate miRNA-enriched fraction. Large RNA was quantified by spectrophotometry (λ = 260 nm) and purity of the samples was verified by the 260/280 ratio with a Nano-Drop 2000 (Thermo Scientific, USA).

Real-time quantitative polymerase chain reaction

RT-PCR was performed using the High Capacity cDNA Reverse Transcription kit and the TaqMan MicroRNA Reverse Transcription Kit for large RNA and miRNA-enriched fractions, respectively, following manufacturer instructions (Life Technologies, USA). In both cases, the resulting cDNA template was subjected to real-time quantitative PCR (qPCR) using Taqman-based gene and microRNA expression assays, TaqMan Fast Universal PCR Master Mix (Life Technologies) and the 7900HT Fast Real-Time PCR System (Life Technologies). Gene expression analysis was performed using the TaqMan Gene Expression Assays Hs00167681_m1 and Hs00167861_m1 for target genes α1c (CACNA1C) and β2 (CACNB2) subunits of the L-type Ca^{2+} channel,
respectively and the TaqMan MicroRNA Assay hsa-miR-21-5p for target miR-21 (Life Technologies). The eukaryotic 18S rRNA endogenous control was used as the normalization gene. Each sample was run in triplicates and non-template control to test for contamination of assay reagents was also included in the plate. Moreover, three different controls aimed at detecting genomic DNA contamination in the RNA sample or during the RT or qPCR reactions were always included: a RT mixture without reverse transcriptase, a RT mixture including the enzyme but no RNA, negative control (reaction mixture without cDNA template). The data were collected and analyzed using One-Step Software (Life Technologies). The obtained cycle to threshold (Ct) values were normalized to the 18S rRNA. The Ct values are based on a log scale and were transformed to delta Ct (ΔCt) values by subtracting the value corresponding to the gene of interest from that of 18S. To compare CAF vs SR expression differences, the respective data were transformed from ΔCt values to equivalent fold differences using the following equation, previously used for the same purposes: Fold Difference (mean $\Delta Ct_{SR} – \text{mean } \Delta Ct_{CAF}$) = $2^{(\text{mean } \Delta Ct_{SR} – \text{mean } \Delta Ct_{CAF})}$. The standard errors were omitted due to transformation of the data and the subsequent loss of meaning.2

3. MicroRNA target prediction

Computational prediction of putative targets for miR-21 was performed by using seven established miRNA target prediction algorithms: DIANA-microT3.1, TargetMiner, PicTar, miRANDA, TargetScan6.2, EIMMo, and RNA22-HSA. Most of the algorithms (6 out 7) predicted CACNA1C and CACNB2 as putative targets for miR-21.

4. Luciferase gene expression reporter assay

For luciferase reporter assays, CHO cells were seeded in 96-well plates and cultured in Ham-F12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin,
and 100 μg/mL streptomycin at 37°C and 5% CO₂. Cells were transfected with the 3’ UTR regions of the subunits forming the human cardiac L-type Ca²⁺ channel, CACNA1C, CACNB2, and CACNA2D cloned into pMirTarget vector (BlueHeron, USA), which contains firefly luciferase as a reporter. Since 3’ UTR of CACNA1C is extremely large (6750 nt), a 1500-nt fragment comprising the region predicted to bind miR-21 was cloned. Cotransfections were performed with 15–60 nmol/L miR-21 mimic (hsa-miR-21-5p; MC10206; Life Technologies), 30 nmol/L miRNA mimic negative control #1 (Life Technologies) or miR-21 inhibitor (MH10206; Life Technologies). These mimics are chemically modified to avoid off-target effects induced by the passenger strand. All transfections were performed by using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions. Luciferase activity assays were performed 48 hours after transfection using the Steady-Glo Luciferase Assay System (Promega, USA) and a Fluoroskan Ascent Microplate Fluorometer (Thermo Scientific). Luciferase activity was normalized to sample protein concentration. All reporter assays were performed in triplicate.

5. HL-1 cell culture and transfection

HL-1 cells were cultured in gelatin/fibronectin-coated dishes at 37°C in an atmosphere of 5% in air, with humidity of ≈95%, as previously described. Cells were grown in Claycomb medium (Sigma) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 μmol/L norepinephrine, 100 U/mL penicillin, and 100 μg/mL streptomycin. Medium was changed every 24-48 h and when cells reached high confluency at 3-4 days, the cultures were split 1 to 3 using a brief trypsin treatment. HL-1 cells were transfected with miR-21 mimic, miRNA mimic negative control #1 or miR-21 inhibitor (all at 30 nmol/L) by using Lipofectamine 2000, according to manufacturer instructions. In a different group of experiments FAM dye-labeled synthetic miRNA
mimic (Life Technologies) was transfected to allow evaluation of the transfection efficiency. In all cases, transfection efficiencies were greater than 85%. For culture and transfections, 60 mm culture dishes were used. Preliminary qPCR experiments demonstrated that transfection of miR-21 mimic increased more than 10-fold miR-21 levels in HL-1 cells. Twenty four-forty eight h after transfection, cells were removed from the dish by trypsinization (1%, 37°C for 5 min).

6. Western-blot analysis

Detection of Cav1.2 proteins was performed in HL-1 cells transfected or not with miR-21 mimic (30 nmol/L) by Western blot following previously described procedures.\(^6\) HL-1 cells were homogenized in a non-denaturing solution (in mmoL/L): Tris-HCl 50, NaCl 500, and 1% NP-40, 0.5% sodium-deoxicolate and protease inhibitor cocktail (Sigma). Nuclei and cell debris were removed by centrifugation at 10000 \(g\) for 20 min at 4°C. The total protein amount of the extracts was calculated with the bicinchoninic acid method (BCA, Pierce, USA) and each extract was then adjusted to 0.6 mg/mL of protein. Afterwards, samples were separated on denaturing SDS polyacrylamide 8% gels, transferred to nitrocellulose membranes, blocked with 5% nonfat dried milk in PBS-Tween, and incubated with anti-Cav1.2 antibody (1:1000; Neuromab, USA) overnight at 4°C and then for 1 h with a peroxidase-conjugated goat anti-mouse secondary antibody (1:5000; Jackson Immuno Research, USA). Membranes were washed three times with PBS-Tween before adding primary and secondary antibodies. Protein expression was detected by chemiluminescence (ECL, General Electric Healthcare, USA). To ensure equal protein loading, GADPH expression was determined by using GAPDH antibody (1:1000; Sigma).

7. Calcium current recordings
Recording techniques

A small aliquot of a suspension containing human atrial myocytes or HL-1 cells was placed in a 0.5 mL chamber mounted on the stage of an inverted microscope (Nikon TMS, Nikon Co., Japan). After settling to the bottom of the chamber, cells were perfused at 1 mL/min with external solution (see composition below). Currents were recorded at room temperature (21-23ºC) using the whole cell patch-clamp technique using an Axopatch-200B patch clamp amplifier (Molecular Devices, USA).\textsuperscript{1,2,4} Recording pipettes were pulled from 1.0 mm o.d. borosilicate capillary tubes (GD1, Narishige Co., Ltd, Japan) using a programmable patch micropipette puller (Model P-2000 Brown-Flaming, Sutter Instruments Co., USA) and were heat-polished with a microforge (Model MF-83, Narishige). Micropipette resistance was kept below 3.5 MΩ when filled with the internal solution and immersed in the external solution. The capacitive transients elicited by symmetrical 10 mV steps from 0 mV were recorded at 50 kHz (filtered at 10 kHz) for subsequent calculation of capacitative surface area, access resistance and input impedance. In all the experiments, series resistance was compensated manually by using the series resistance compensation unit of the Axopatch amplifier, and ≥80% compensation was achieved. In myocytes from SR patients, mean maximum Ca\textsuperscript{2+} current (I_Ca,L) amplitude at +10 mV, uncompensated access resistance, and capacitance averaged -190±17 pA, 3.3±0.4 MΩ, and 58.7±3.8 pF (n=52), respectively. In HL-1 cells, mean maximum I_Ca,L amplitude at +20 mV, uncompensated access resistance, and capacitance averaged -88.2±18.7 pA, 3.1±0.7 MΩ, and 20.6±1.8 pF (n=20), respectively. Thus, under our experimental conditions no significant voltage errors (<5 mV) due to series resistance were expected with the micropipettes used. In all cases currents were filtered at half the sampling frequency and stored on the hard disk of a computer for subsequent analysis. To record I_Ca,L in human atrial myocytes and T-
type Ca\(^{2+}\) current (I\(_{\text{Ca,T}}\)) and I\(_{\text{Ca,L}}\) in HL-1 cells, the external solution contained (mmol/L): Tetraethylammonium (TEA)-Cl 137, CaCl\(_2\) 1, MgCl\(_2\) 0.5, HEPES 10, and glucose 10, (pH 7.4 with CsOH). Recording pipettes were filled with an internal solution containing (mmol/L): CsCl 125, TEA-Cl 20, MgATP 5, phosphocreatine 3.6, HEPES 10, and EGTA 10 (pH 7.2 with CsOH).\(^2\) To increase current amplitude and eliminate Ca\(^{2+}\)-induced Ca\(^{2+}\) inactivation, Ba\(^{2+}\) was used as charge carrier (I\(_{\text{Ba}}\)). Therefore, for these experiments the external solution contained (mmol/L): N-Methyl-D-glucamin (NMDG) 137, CsCl 12, BaCl\(_2\) 20, MgCl\(_2\) 1, HEPES 10, and glucose 10 (pH 7.4 with HCl).\(^6\) Under these conditions current amplitudes were stable during the time of recordings. In some experiments, I\(_{\text{Ba}}\) was recorded in the absence of EGTA in the internal solution.

**Pulse protocols and analysis**

To record I\(_{\text{Ca,L}}\) in human atrial myocytes, the holding potential was maintained at -80 mV and the cycle time for any protocol was 10 s. The protocol to obtain current-voltage relationships consisted of 500-ms pulses that were imposed in 5 mV increments between -40 and +50 mV. The I\(_{\text{Na}}\) was inactivated by the application of a 50-ms prepulse to -30 mV. The protocol to obtain current-voltage relationships for I\(_{\text{Ca,T}}\) and I\(_{\text{Ca,L}}\) consisted of 500-ms pulses in 10 mV increments from -80 mV to potentials ranging -70 and +70 mV. However, the protocol to obtain current-voltage relationships for I\(_{\text{Ba}}\) consisted of 500-ms pulses in 10 mV increments from -30 mV to potentials ranging -40 and +70 mV.

In each experiment, current amplitude (measured as the difference between peak current and zero current level) was normalized to membrane capacitance to obtain current density. Activation curves for I\(_{\text{Ca,L}}\) and I\(_{\text{Ba}}\) were constructed plotting the normalized
conductance as a function of the membrane potential. The conductance was estimated by the equation:

\[ G = \frac{I}{(V_m - E_{\text{rev}})} \]

where \( G \) is the conductance at the test potential \( V_m \), \( I \) represents the current amplitude at \( V_m \), and \( E_{\text{rev}} \) is the reversal potential. To determine the \( E_{\text{rev}} \), the current density-voltage relationships were fitted to a function of the form:

\[ I = (V_m - E_{\text{rev}}) \cdot G_{\text{max}} \cdot (1 + \exp \left[ \frac{V_m - V_h}{k} \right])^{-1} \]

where \( I \) is the current amplitude elicited at the test potential \( V_m \), \( G_{\text{max}} \) is the maximum conductance, and \( k \) is the slope factor. The fit of a Boltzmann function to the data yielded the midpoint (\( V_h \)) and the slope (\( k \)) of the activation curve.

To obtain the inactivation curves for human atrial \( I_{\text{Ca,L}} \) and \( I_{\text{Ba}} \) recorded in HL-1 cells, a two-step protocol was used. For \( I_{\text{Ca,L}} \) the first 500-ms conditioning pulse from \(-80\) mV to potentials between \(-90\) and \(+50\) mV was followed by a 500-ms test pulse to \(+10\) mV. For \( I_{\text{Ba}} \) recorded in HL-1 cells the first 500-ms conditioning pulse from \(-30\) mV to potentials between \(-50\) and \(+20\) mV was followed by a 500-ms test pulse to \(+20\) mV. In both cases, inactivation curves were constructed by plotting the current amplitude obtained with the test pulse normalized to the largest current, as a function of the voltage command of the conditioning pulse. Thereafter, a Boltzmann function was fitted to the data to obtain \( V_h \) and \( k \) values of the inactivation curve. In order to describe the time course of current activation and decay, an exponential analysis was used as an operational approach, fitting current traces with an equation of the form:

\[ y = C + A \cdot \exp(-t/\tau) \]

where \( \tau \) is the time constant, whereas \( A \) is the amplitude of the exponential, and \( C \) is the baseline value. To analyze the recovery from inactivation for \( I_{\text{Ba}} \) two 500-ms pulses (P1 and P2) from \(-30\) to \(+20\) mV were applied at increasing coupling intervals (5-4000 ms).
Afterwards, a monoexponential function was fitted to the data to obtain the time constant of recovery from inactivation.

8. Statistical analysis

Results are expressed as mean±SEM. Unpaired \( t \)-test or one-way ANOVA followed by Newman-Keuls test were used where appropriate. In small-size samples, statistical significance was confirmed by using nonparametric tests. Comparisons between categorical variables were done using Fisher’s exact test. To take into account correlations between multiple levels of within-patient measurements, data were analyzed with multilevel mixed-effects models. A value of \( P<0.05 \) was considered significant.
**Supplemental Table 1.** Characteristics of the patients whose samples were used for qPCR experiments and I<sub>Ca,L</sub> recordings.

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<td>56.4±2.4</td>
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ACE, angiotensin converting enzyme; ARBs, angiotensin II type 1 receptor blockers; CABG, coronary artery bypass grafting. * P<0.05 vs SR patients.
Expanded results

Supplemental Figures

Supplemental Figure 1. Prediction of miR-21 binding to 3’UTR of human CACNA1C (top) or CACNB2 (bottom) performed by using miRANDA prediction algorithm.

Computational analysis using several algorithms revealed CACNA1C and CACNB2 as potential targets for miR-21. Indeed, CACNA1C and CACNB2 3’UTR regions contain a sequence, which is complementary to the seed site of miR-21 (nucleotides 2-8). Perfect Watson-Crick-base pair complementarity between nucleotides 2673 to 2678 of CACNA1C and 1178 to 1183 CACNB2 and nucleotides 2 to 7 of miR-21 were established together with G•U wobbles with nucleotide 8 in miR-21 (7mer binding).

Furthermore, in both cases additional Watson-Crick-base pair matches elsewhere were also established that reinforce complementarity. In addition, eight 6mer interaction sites between CACNA1C 3’UTR and miR-21 were also found. On the contrary, 3’UTR region of CACNA2D did not exhibit any complementary sequence to the seed site of miR-21.
Human CACNA1C 3’UTR

CACNA1C 3’UTR  2659:
5’ ggCACAUA-UAUAAGUAAGCUa  3’

miR-21

Human CACNB2 3’UTR

CACNB2 3’UTR  1157:
5’ ugAGCAGUGCAGGGAAUGUGAGUAAGCUu  3’

miR-21

Supplemental Figure 1
Supplemental Figure 2. (A and B) I_{Ca,T} (A) and I_{Ca,L} (B) traces obtained by applying the protocol shown at the top in HL-1 cells perfused or not with 50 μmol/L NiCl (A) or 1 μmol/L nifedipine (B). (C) Percentage of HL-1 cells that exhibited I_{Ca,T}- or I_{Ca,L}-predominant patterns and that of cells that did not exhibit any Ca^{2+} current in sham-(black) or in miR-21-transfected cells (white). (D) Current density-voltage relationships for I_{Ca,T} and I_{Ca,L}. In D, each point represents mean±SEM of ≥20 experiments.

Currents were recorded in randomly selected HL-1 cells by applying 500-ms pulses from -80 mV to potentials ranging -70 and +70 mV. In control conditions (n=54), around 24% of cells did not exhibit any Ca^{2+} current and ≈39% exhibited a large Ca^{2+} current, which was identified as I_{Ca,T} since it was completely abolished by NiCl (50 μmol/L) and reached its maximum density (-7.9±1.5 pA/pF) at -30 mV (I_{Ca,T}-predominant cells) (Panels A and D). The rest of the cells (≈37%) exhibited a large I_{Ca,L} that was completely inhibited by nifedipine (1 μmol/L) and reached its maximum density (-4.6±1.2 pA/pF) at +20 mV (Panels B and D) and small or absent I_{Ca,T} (I_{Ca,L}-predominant cells). These percentages are in reasonable agreement with those previously described for these cells and transfection of miR-21 mimic did not modify them (P>0.05, n=60) (Panel C).
A

Control

NiCl 50 μmol/L

200 pA

100 ms

B

Control

Nifedipine 1 μmol/L

100 ms

C

Cells (%)

ICa,L

ICa,T

None

Supplemental Figure 2

Membrane potential (mV)

ICa density (pA/pF)

Supplemental Figure 2
Supplemental Figure 3. Recovery from inactivation data for $I_{Ba}$ recorded by applying the protocol shown in the inset in sham- or in miR-21 transfected HL-1 cells. The solid lines represent the best fit of a monoexponential function to the data points. Each point represents the mean±SEM of >30 experiments.

In this group of experiments, the effects of miR-21 transfection on the $I_{Ba}$ reactivation were analyzed. As can be observed, the time course of current reactivation in the presence of miR-21 was not different from that measured in control conditions ($\tau_{re}$=450.1±20.6 vs. 436.6±51.6 ms; $P>0.05$).
Supplemental Figure 3
Supplemental Figure 4. (A) Current density-voltage relationships for \( I_{Ba} \) measured under EGTA free conditions in the absence and presence of miR-21. (B) Normalized steady-state \( I_{Ba} \) inactivation curves. In this panel, continuous lines represent the fit of a Boltzmann equation to the data. Each point represents mean±SEM of >8 experiments. *P<0.05 vs Control.

This group of experiments was conducted to determine the effects of miR-21 transfection on \( I_{Ba} \) recorded in the absence of EGTA in the internal solution. Under these conditions, current-density reached its maximum value at +20 mV (-3.7±0.6 pA/pF, n=10) and transfection of miR-21 mimic significantly reduced \( I_{Ba} \) density (-1.7±0.4 pA/pF at +20 mV) (Panel A) and slowed time course of current decay (\( \tau_{inact} \) MIR21=143.8±8.6 ms vs. \( \tau_{inact} \) CON=81.5±18.8 ms) (P<0.05, n=8). The analysis of the voltage dependence of activation and inactivation (Panel B) revealed that miR-21 did not modify conductance curves (\( V_h=10.3±1.8 \) mV and \( k=5.4±0.5 \)). However, it shifted the midpoint of the inactivation curves from -20.1±2.8 to -11.6±3.2 mV, without modifying the slope of the curves (11.3±1.0). These results indicated that under EGTA-free conditions, miR-21 effects on \( I_{Ca,L} \) are identical to those observed in the presence of EGTA.
Suplemental references


