Molecular Mechanisms Underlying Ionic Remodeling in a Dog Model of Atrial Fibrillation

Lixia Yue, Peter Melnyk, Rania Gaspo, Zhiguo Wang, Stanley Nattel

Abstract—The rapid atrial rate during atrial fibrillation (AF) decreases the ionic current density of transient outward \( K^+ \) current, L-type \( Ca^{2+} \) current, and \( Na^+ \) current, thereby altering cardiac electrophysiology and promoting arrhythmia maintenance. To assess possible underlying changes in cardiac gene expression, we applied competitive reverse transcriptase–polymerase chain reaction to quantify mRNA concentrations in dogs subjected to 7 (group P7 dogs) or 42 (group P42 dogs) days of atrial pacing at 400 bpm and in sham controls. Rapid pacing reduced mRNA concentrations of Kv4.3 (putative gene encoding transient outward \( K^+ \) current; by 60% in P7 and 74% in P42 dogs; \( P<0.01 \) and \( P<0.001 \), respectively, versus shams), the \( \alpha_1c \) subunit of L-type \( Ca^{2+} \) channels (by 57% in P7 and 72% in P42 dogs; \( P<0.01 \) versus shams for each) and the \( \alpha \) subunit of cardiac \( Na^+ \) channels (by 18% in P7 and 42% in P42; \( P=NS \) and \( P<0.01 \), respectively, versus shams) genes. The observed changes in ion channel mRNA concentrations paralleled previously measured changes in corresponding atrial ionic current densities. Atrial tachycardia did not affect mRNA concentrations of genes encoding delayed or Kir2.1 inward rectifier \( K^+ \) currents (of which the densities are unchanged by atrial tachycardia) or of the \( Na^+,Ca^{2+} \) exchanger. Western blot techniques were used to quantify protein expression for Kv4.3 and \( Na^+ \) channel \( \alpha \) subunits, which were decreased by 72% and 47%, respectively, in P42 dogs (\( P<0.001 \) versus control for each), in a manner quantitatively similar to measured changes in mRNA and currents, whereas \( Na^+,Ca^{2+} \) exchanger protein concentration was unchanged. We conclude that chronic atrial tachycardia alters atrial ion channel gene expression, thereby altering ionic currents in a fashion that promotes the occurrence of AF. These observations provide a potential molecular basis for the self-perpetuating nature of AF. (Circ Res. 1999;84:776-784.)

Key Words: arrhythmia, cardiac ▶ molecular biology ▶ channels, ion ▶ remodeling, atrial ▶ \( Ca^{2+} \) ▶ \( K^+ \)

A iterations in gene expression are important in a wide variety of disease processes. 1 Pathological perturbations of physiological function can cause changes in gene expression that lead to secondary abnormalities that contribute to the phenotypic manifestations and progression of disease.2–4 Atrial fibrillation (AF) is the most common cardiac arrhythmia in clinical practice and can lead to potentially serious clinical consequences, including thromboembolism, impaired physical capacity, reduced left ventricular function, and stroke.5 The treatment of AF remains suboptimal because of limited efficacy and potential side effects of drug therapy.6 Recent research indicates that one important potential element in the resistance of AF to treatment is the tendency of the arrhythmia to alter the properties of the heart in a way that promotes AF maintenance.7,8 The rapid atrial rate appears to be the central factor in the electrophysiological changes produced by AF.9 Chronic atrial tachycardia at a rate (400 bpm) in the range of that of AF produces important alterations in ion channel function (reduced densities of transient outward \( K^+ \) current \( [I_n] \), L-type \( Ca^{2+} \) current \( [I_Ca] \), and \( Na^+ \) current \( [I_Na] \)) that result in a functional substrate that supports the maintenance of AF.7,10–13 The molecular mechanisms that cause these changes in ion channel function are unknown. Over the past several years, considerable progress has been made in identifying the molecular composition of the principal subunits of cardiac ion channels. The sequences encoding pore-containing subunits of cardiac \( I_n \)14,15 \( I_{Ca} \)16 and \( I_{Na} \)17,18 in mammalian systems have been described, as have sequences encoding components of 2 currents, the inward rectifier19 and rapid delayed rectifier20,21 \( K^+ \) currents \( \bar{I}_K \) and \( I_K \), respectively), that are not altered by atrial tachycardia.10 The present studies were designed to quantify mRNA concentrations corresponding to pore-containing subunits of 5 cardiac ion channels \( (I_n, I_{Na}, I_{Ca}, I_{Kr}, and I_K) \) in atrial tissue from dogs subjected to 7 and 42 days of rapid atrial pacing (P7 and P42 dogs, respectively), and to compare the results with measurements obtained in sham-instrumented dogs (P0 dogs) and previously determined alterations in ion current density. In addition, Western blot studies were performed to quantify changes in membrane protein content of 2 molecular species...
(I_{Ca} and I_{Na}) for which significant changes in mRNA concentration were detected.

Materials and Methods
Preparation of the Dog Model
We used a previously described approach to implant pacemakers in subcutaneous pockets in the necks of 15 mongrel dogs. After 24 hours of recovery, 10 dogs were subjected to continuous right atrial pacing at 400 bpm for 7 or 42 days (n=5 for each group). Five dogs in which the pacemaker was not activated served as the P0 (sham control) group. On the study day, dogs were anesthetized with morphine 2 mg/kg and α-chloralose 120 mg/kg and a right thoracotomy was performed. AF was induced by burst atrial pacing at a 4× threshold 4-ms pulses at 10 Hz. Mean±SEM AF duration was 8.4±3.0 s for P0 dogs, 1522±563 s for P7 dogs (P<0.05 versus P0 dogs) and 2700±0 s (AF sustained in all) for P42 dogs (P<0.001 versus P0 dogs), which confirmed the arrhythmogenic alterations caused by rapid atrial pacing. After AF duration measurement, the right atrium was removed, frozen in liquid nitrogen and stored at −80°C.

RNA Preparation
Total RNA was isolated from frozen pectinate muscle tissue of right atria. In brief, 1 g of tissue was homogenized in 10 mL of Trizol reagent (Gibco BRL) extracted with chloroform and precipitated in isopropyl alcohol. Total RNA was incubated in DNase I (0.1 U/μL; Ambion) for 15 minutes, extracted by use of phenol-chloroform, precipitated in isopropyl alcohol, and subsequently dissolved in diethylpyrocarbonate-treated water. The amount of total RNA was determined spectrophotometrically (Spectronic Genesys) at a wavelength of 260 nm, and the RNA was stored at −80°C for later analysis. mRNA was purified with the Poly(A) Quick mRNA isolation system (Stratagene). The integrity of each sample was confirmed by analysis on a denaturing agarose gel.

Cloning of cDNA Fragments of Ion Channels From Dog Atrium and Designing of Gene-Specific Primers
The principle of competitive polymerase chain reaction (PCR) involves amplification in a truly competitive fashion, because the internal standard (mimic) and the target sequences compete for the same primer and, therefore, for amplification. It is thus important to design gene-specific rather than degenerate primers. We therefore cloned partial cDNA sequences of the α1 subunit of the cardiac Ca^{2+} channel, the α subunit of the Na^{+} channel, the Kv4.3 α subunit, canine Kir2.1 (DIRK), the canine counterpart ofHERG (DERG), and the Na^{+}/Ca^{2+} exchanger (Ncx) from canine atrial tissue samples. Degenerate primers for reverse transcription (RT)–PCR were designed on the basis of published sequences.11–22 The specificity of the primers was confirmed with the basic local alignment search tool (BLAST).23 PCR products were size-separated on 1.5% agarose gels. The primers was confirmed with the basic local alignment search tool (BLAST).23 PCR products were size-separated on 1.5% agarose gels.

Synthesis of RNA Internal Standards
Synthetic RNA internal standards (mimics) were manufactured with the procedure shown in Figure 1. Gene-specific primers for RT-PCR (Table) were designed without degeneracy according to the obtained sequences and their specificity confirmed by BLAST and the FASTA program for rapid comparison of nucleotide sequences. A 392-bp fragment of α-actin was synthesized from the region of 111 to 502 using the primers shown in the Table. The 392-bp PCR product was purified on an agarose gel and its sequence confirmed. The defined product was used to construct an internal standard for the α1 subunit of the L-type Ca^{2+} channel, DERG, and the Na^{+} channel α subunit. Similar methods were used to construct internal standards for the Kv4.3 potassium channel α subunit, NCX, and DIRK with the use of a 460-bp fragment of human α-actin.

First-strand cDNA was synthesized by RT with canine atrial mRNA and random primers. Chimeric primer pairs were constructed by appending gene-specific primers at the 5'-end of α- (β-actin primers) and an 8-nucleotide (GGCCGGCGG) linker homologous to the 3'-end of the T7 promoter sequence was conjugated to the 5'-end of each gene-specific sense primer. The chimeric primers were used in a PCR reaction (Tag polymerase; annealing temperature, 54°C) with the first-strand DNA to generate an actin cDNA sequence flanked by gene-specific primers, with the short T7 promoter sequence at the 5'-end. The product of this PCR was diluted 50-fold, and 1 μL was used as a template in a second PCR. Primers used in the second PCR included a T7 promoter primer (sense) and a gene-specific anti-sense primer, and PCR was performed at annealing temperature of 60°C. The resulting product, carrying the T7 promoter, gene-specific primers, and an internal α- or β-actin fragment was gel-purified (Glassmax DNA isolation Spin Cartridge System, Gibco BRL) and used as a template for in vitro transcription. In vitro transcription was conducted with mMESSAGE mMACHINE (Ambion) at 37°C for 3 hours. A 1 μL of 2 μL solution was added to a 20-μL reaction mixture, which was then incubated at 37°C for 15 minutes. The RNA formed was extracted with phenol-chloroform and precipitated with ethanol. The RNA pellet was dried and dissolved in RNase-free water, the quantity of RNA was determined by spectrophotometry and gel analysis, and the sample was stored at −80°C for later use.

Competitive RT-PCR
Serial dilutions of the RNA internal standards were added to 100 ng of sample mRNA in a series of reaction mixtures. Sample mRNA and RNA internal standards were denatured at 65°C for 15 minutes and chilled on ice for 5 minutes before being added to the reaction mixture. RT was conducted at 25°C for 10 minutes and 42°C for 60 minutes with a 20-μL first-strand cDNA synthesis mixture (3.2 μg of random hexamers, 1 mmol/L deoxyxynucleotide mixture (dNTP), 50 U of RNase inhibitor, 20 U of AMV reverse transcriptase). Aliquots of first-strand cDNA (10 μL) were amplified by PCR in a 50-μL solution containing (mmol/L) Tris-HCl 10 (pH 8.3), KCl 50, dNTP 0.8, and MgCl2 1.5 as well as 2.6 U Taq polymerase and 0.2 μmol/L gene-specific primers. The reaction mixture was denatured (94°C for 3 minutes), run for 30 cycles of 94°C denaturing for 30 seconds, annealed at the appropriate temperature (Table) for 45 seconds, and
**Conditions Used for Competitive PCR**

<table>
<thead>
<tr>
<th>Sense/Antisense Primer</th>
<th>Bases Spanned, bp</th>
<th>Product Size, bp</th>
<th>Tm, °C</th>
<th>Reference No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>h&lt;sub&gt;0&lt;/sub&gt;</td>
<td>4237–4550</td>
<td>314</td>
<td>54</td>
<td>16</td>
</tr>
<tr>
<td>b&lt;sub&gt;0&lt;/sub&gt;</td>
<td>761–1041</td>
<td>281</td>
<td>54</td>
<td>14, 15</td>
</tr>
<tr>
<td>Kv4.3</td>
<td>1533–1744</td>
<td>212</td>
<td>54</td>
<td>17</td>
</tr>
<tr>
<td>DIRK</td>
<td>910–1302</td>
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<td>52</td>
<td>19</td>
</tr>
<tr>
<td>NCX</td>
<td>2020–2330</td>
<td>311</td>
<td>54</td>
<td>20</td>
</tr>
<tr>
<td>β-actin</td>
<td>1889–2100</td>
<td>212</td>
<td>52</td>
<td>21</td>
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<td>392</td>
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<tr>
<td>T7 promoter</td>
<td>329–1538†</td>
<td>460</td>
<td>54</td>
<td>. . . *</td>
</tr>
</tbody>
</table>

*Sequences obtained from GenBank; accession numbers Z70044 (β-actin) and J00070 (α-actin).
†The product is generated from exons in the 329- to 1583-bp region.

The NCX clone we obtained was identical to the sequence (Reference 21) previously published for dog atrium.

The absence of genomic contamination in the mRNA samples and the absence of cDNA contamination in the RNA internal standards were confirmed by the absence of a signal for reverse transcriptase–negative controls. To assure equal amplification of sample mRNA and RNA internal standards, known quantities of target and mimic sequences were coamplified in single-reaction tubes. In all cases, the target sequence and its corresponding mimics were amplified with similar efficiencies (see Figure 2). The equivalence of sample mRNA input in each experiment was established by spectrophotometric and noncompetitive PCR for the actin internal standard.

**Western Blot Studies**

Membrane preparations were obtained as previously described<sup>24</sup> by a modification of the protease inhibitors. Specifically, pepstatin 1 µg/mL, leupeptin 1 µg/mL, aprotinin 2 µg/mL, benzamidine 0.1 mg/mL, calpain inhibitors I and II (8 µg/mL each), and 4-(2-aminoethyl)-benzenesulfonyl-fluoride hydrochloride (Pefabloc SC) (0.5 mmol/L) were included in the Tris/EDTA solution (10 mmol/L Tris-base, 1 mmol/L EDTA) that contained the tissue samples. All protease inhibitors were obtained from Sigma Chemical Co except for Pefabloc SC (Boehringer Mannheim). All procedures were performed on ice, the centrifuge rotor was precooled, and centrifugation was performed at 4°C.

The solubilized membrane proteins (45 µg) were fractionated on 8% SDS-polyacrylamide gels. The proteins were then transferred electrophoretically to Immobilon-P polyvinylidene fluoride membrane (Millipore) in 25 mmol/L Tris-base, 192 mmol/L glycine, and 5% methanol at 0.07A for 18 hours. The membranes were blocked using 5% nonfat dry milk (Bio-Rad) in TBS (Tris-HCl 50 mmol/L, NaCl 500 mmol/L; pH 7.5) containing 0.05% Tween-20 (TTBS) for 2 hours at room temperature. Membranes were washed 3 times with TTBS and reblocked in 1% nonfat dry milk in TTBS for 1 hour. The membranes were then incubated overnight in primary antibody solutions in 1% nonfat dry milk in TTBS. The primary antibody against the α<sub>c</sub> subunit of the L-type Ca<sup>2+</sup> channel with an antibody from Alomone Labs; however, because of a low signal-to-noise ratio, these attempts were unsuccessful. After overnight incubation, the membranes were washed 3 times in TTBS and reblocked in 1% nonfat dry milk in TTBS for 10 minutes. They were incubated with horseradish peroxidase–conjugated anti-rabbit IgG (1:5000) in 5% nonfat dry milk in TTBS for 30 minutes and washed in TTBS 4 more times. Antibody detection was performed with Western blot chemiluminescence reagents (NEN Life Science Products).

The density of bands on Western blots was quantified by use of a scanner (PD1 420oe) and Quantity One software (PD1), which included a background subtraction algorithm. To confirm equal protein loading, the densities of nonspecific bands in the one reporter (circles) were plotted as a function of the number of cycles (lower panel). The relative intensities of the bands corresponding to internal standard and DERG products were quantified by computer imaging. The intensities of specific amplified products (circles) were plotted as a function of the number of cycles (lower panel). Similar studies were performed for all constructs to confirm equal amplification of internal standards and targets.

**Figure 2.** Kinetics of amplification of DERG internal standard and DERG fragments. Constant amounts of RNA internal standard and DERG mRNA (2 pg each) were amplified in a single reaction tube with DERG primers. After 24 amplification cycles and after each of 5 additional cycles, a 10-µL aliquot of reaction mixture was removed and the products resolved on agarose gel (upper panel). The relative intensities of the bands corresponding to internal standard and DERG products were quantified by computer imaging. The intensities of specific amplified products (circles) were plotted as a function of the number of cycles (lower panel). Similar studies were performed for all constructs to confirm equal amplification of internal standards and targets.
region (for the Na\textsuperscript{+} channel) and the 45 kDa region (for Kv4.3) were compared. All gels contained membrane preparations for each of several P42 and several P0 hearts to exclude artifacts due to intergel differences in density and background.

Statistical Analysis

Group data are expressed as mean ± SEM. Group comparisons were performed with ANOVA. If significant differences were indicated by ANOVA, a t test with Bonferroni’s correction was used to evaluate differences between individual mean values. A 2-tailed P, 0.05 was taken to indicate statistical significance.

Results

Canine cDNA Clones

Partial cDNA sequences were cloned from dog atrium for the \(\alpha_{1c}\) subunit of the L-type Ca\textsuperscript{2+} channel (281 bp, GenBank accession No. AF048752); Kv4.3 (212 bp, No. AF049887); the \(\alpha\) subunit of the Na\textsuperscript{+} channel (314 bp, No. AF017428); canine atrial Kir2.1, DIRK (378 bp, No. AF017428); and DERG (378 bp, No. AF017429). The sequences show substantial homology with analogous clones from other species14–20 (87% to 94% at the nucleotide level and 94% to 100% at the amino acid level).

Expression of mRNA for the \(\alpha_{1c}\) Subunit of the L-Type Ca\textsuperscript{2+} Channel

Figure 3A shows representative gels from the competitive RT-PCR of the \(\alpha_{1c}\) subunit of \(I_{Ca}\) in tissue from P0 and P42 hearts. Lane 0 shows DNA mass marker; lanes 1 to 5, results obtained with serial dilutions of RNA internal standard (lane 1, 300 pg; lane 2, 30 pg; lane 3, 3 pg; lane 4, 0.3 pg; and lane 5, 0.03 pg); lane 6, reverse transcriptase-negative control. Lower bands correspond to channel mRNA; upper bands are internal standards. B, Abundance of \(\alpha\) subunit of Ca\textsuperscript{2+} channel mRNA was determined from a graph of the logarithmic ratio of optical intensity of amplified target DNA/mimic DNA versus logarithm of mimic RNA concentration. C, Mean±SEM abundance of \(I_{Ca}\) \(\alpha\) subunit mRNA from P0, P7, and P42 dogs (n=5 analyses from 1 heart each per group; **P<0.01 vs P0 dogs).

Expression of Kv4.3 Subunit mRNA

Figure 4A shows gels obtained by competitive PCR for the Kv4.3 K\textsuperscript{+} channel subunit in tissue from sham (A) and P42 (B) hearts. Lane 0 shows DNA mass ladder; lanes 1 to 6, results obtained with serial dilutions of RNA internal standard (lane 1, 20 pg; lane 2, 2 pg; lane 3, 0.2 pg; lane 4, 0.02 pg; lane 5, 0.002 pg; and lane 6, 0.0002 pg); lane 7, reverse transcriptase-negative control. Lower bands correspond to channel mRNA; upper bands are internal standards. C, Mean±SEM Kv4.3 mRNA concentration in right atria from P0, P7, and P42 dogs (n=5 analyses, 1 analysis per heart per group; **P<0.01, ***P<0.001 vs P0 dogs).
and significantly in the rapid-pacing AF model. To evaluate mRNA vs P0 dogs).

Mean mRNA abundance of Na

5 channels that we have found to be altered significantly in the rapid-pacing AF model. To evaluate mRNA expression in sham (P0) and paced (P42) dogs. A, Lane 0 shows DNA mass ladder; lanes 1 to 6, serial dilutions of DIRK RNA internal standard (lane 1, 100 pg; lane 2, 10 pg; lane 3, 2 pg; lane 4, 0.4 pg; lane 5, 0.08 pg; and lane 6, 0.016 pg); lane 7, reverse transcriptase–negative control. B, Lane 0 shows DNA mass ladder; lanes 1 to 6, serial dilutions of DERG RNA internal standard (lane 1, 200 pg; lane 2, 20 pg; lane 3, 2 pg; lane 4, 0.2 pg; lane 5, 0.02 pg; and lane 6, 0.002 pg); lane 7, reverse transcriptase–negative control. C, Lane 0 shows DNA mass ladder; lanes 1 to 6, serial dilutions of NCX RNA internal standard (lane 1, 300 pg; lane 2, 30 pg; lane 3, 3 pg; lane 4, 0.3 pg; lane 5, 0.03 pg; and lane 6, 0.003 pg); lane 7, reverse transcriptase–negative control. Lower bands correspond to channel mRNA products; upper bands to internal standards. C, Mean±SEM abundance of Na

3 channels that we have found to be altered significantly in the rapid-pacing AF model. To evaluate mRNA expression in sham (P0) and paced (P42) dogs. A, Lane 0 shows DNA mass ladder; lanes 1 to 6, serial dilutions of DIRK RNA internal standard (lane 1, 100 pg; lane 2, 10 pg; lane 3, 2 pg; lane 4, 0.4 pg; lane 5, 0.08 pg; and lane 6, 0.016 pg); lane 7, reverse transcriptase–negative control. B, Lane 0 shows DNA mass ladder; lanes 1 to 6, serial dilutions of DERG RNA internal standard (lane 1, 200 pg; lane 2, 20 pg; lane 3, 2 pg; lane 4, 0.2 pg; lane 5, 0.02 pg; and lane 6, 0.002 pg); lane 7, reverse transcriptase–negative control. C, Lane 0 shows DNA mass ladder; lanes 1 to 6, serial dilutions of NCX RNA internal standard (lane 1, 300 pg; lane 2, 30 pg; lane 3, 3 pg; lane 4, 0.3 pg; lane 5, 0.03 pg; and lane 6, 0.003 pg); lane 7, reverse transcriptase–negative control. Lower bands correspond to channel mRNA products; upper bands to internal standards. The results shown were from 1 control and 1 P42 heart per group. Similar results were obtained in 5 P0 and 5 P42 dogs per construct.

Expression of Cardiac Na\(^{+}\) Channel α Subunit mRNA

Figure 5 shows results for the cardiac Na\(^{+}\) channel α subunit from a P0 (Figure 5A) and a P42 (Figure 5B) dog. RNA internal standard quantities in the reaction mixture for lanes 1, 2, 3, 4, 5, and 6 of Figure 4A were 100, 10, 2, 0.4, 0.08, and 0.016 pg, respectively. The equivalent point is shifted to the right in the paced dog, which indicates decreased mRNA concentrations. Figure 5C shows mean mRNA concentrations from 5 hearts in each group. A slight, nonsignificant reduction was noted after 7 days of pacing, and a significant reduction was noted after 42 days.

Expression of DIRK, DERG, and NCX Transcripts

The above data are for clones believed to play a role in \(I_{\text{Kr}}\), \(I_{\text{K1}}\), and \(I_{\text{Na}}\). 3 channels that we have found to be altered significantly in the rapid-pacing AF model. To evaluate mRNA concentrations corresponding to other ion transport mechanisms, we measured the concentration of mRNA for DIRK, DERG, and NCX, clones that correspond to the inward rectifier \(I_{\text{Kr}}\), the rapid delayed rectifier \(I_{\text{K1}}\), and NCX, respectively (Figure 6). There were no changes in the point of equivalence between gene-specific signals and internal standards in paced dogs. Overall, mRNA concentrations averaged 64.5±6.7 and 61.5±5.7 amol per 100 ng mRNA in 5 P0 and 5 P42 hearts, respectively (\(P=NS\)), for DIRK; 30.0±7.1 and 30.8±8.2 amol per 100 ng mRNA in 5 P0 and 5 P42 hearts, respectively (\(P=NS\)), for DERG; and 128.8±40.2 and 137.2±31.2 amol per 100 ng mRNA in 5 P0 and 5 P42 hearts, respectively (\(P=NS\)), for NCX.

Western Blot Analysis of Changes in Kv4.3 and Na\(^{+}\) Channel Membrane Protein Expression

Figure 7 (top) shows representative bands from a gel on which Na\(^{+}\) channel protein was studied. The bands were less intense in the P42 dog hearts. Incubation with the Na\(^{+}\) channel antigen against which the antibody was raised (supplied by Alomone) resulted in the disappearance of the 220-kDa \(I_{\text{Na}}\) band (last lane), without altering the lower-molecular-weight marker band lower on the gel. Similar results were obtained in 5 P42 and 7 P0 hearts, with an overall 46.5% reduction in \(I_{\text{Na}}\) protein in P42 hearts (\(P<0.0001\)). Figure 7 (middle) shows bands of the expected molecular weight (72 kDa) identified by the anti-Kv4.3 antibodies in a typical gel. Two lanes show Kv4.3 bands from 2 control dog hearts and 2 lanes that were obtained with tissue from P42 hearts. The bands in P42 dogs were consistently of lesser intensity than those in the control hearts.
density compared with bands from sham (P0) dogs, and showed an average 71.6% reduction in Kv4.3 band intensity compared with P0 hearts (n=5 for each, P<0.001). Figure 7 (bottom) shows bands corresponding to NCX (MW, 120 kDa), which had the same intensity in P0 and P42 hearts. For 5 hearts in each group, the density in P42 hearts averaged 103% of that in control hearts (P=NS). The marker bands used to confirm equal loading were of very similar density (eg, in the Kv4.3 experiments, their density in P42 hearts averaged 99.8% of the density in P0 hearts; in INa experiments their density in P42 hearts averaged 100.8% of P0 hearts).

Discussion

We have found that atrial tachycardia reduces the mRNA concentrations of several cardiac ion channels while leaving others unaffected. The ion channels for which mRNA concentrations are altered by rapid atrial pacing are the same channels (ICa, IKr, and INa) that show reduced function in rapidly paced dogs. In addition, Western blot studies confirmed reductions for P42 dogs in the membrane protein concentrations of Kv4.3 (by 71.6%) and INa (46.5%) that were quite similar to the reductions in corresponding mRNA concentration (by 74% and 42% in Kv4.3 and INa, respectively) in P42 dogs. These findings suggest that reduced message levels are responsible for the changes in ion current density that characterize the electrophysiological alterations by which AF leads to its own perpetuation.

Relations Between mRNA Concentrations and Changes in Current Density

The results described above show qualitatively that decreases in mRNA concentration are observed only for currents with a density that changes in paced dogs; for currents (IK1 and INa) for which function remains unaltered by atrial tachycardia, mRNA concentrations remain stable. To relate quantitatively the mRNA concentrations we measured to physiological changes in current density, we compared changes in mRNA concentration measured in the present study with alterations in current density that we previously recorded in each group of dogs.10,11 Both functional and molecular data were obtained from tissues in the same right atrial region, the pectinate muscles. Figure 8 shows mean values of mRNA concentration (measured separately for 5 hearts for each group or construct) and mean densities of the corresponding ionic current in each group of dogs. Currents were measured in 25 cells from 5 hearts for each group of dogs for ICa and INa (Reference 10), and 28 cells (5 hearts), 59 cells (6 hearts), and 43 cells (6 hearts) for INa of P0, P7, and P42 dogs, respectively (Reference 11). Results are mean±SEM for both mRNA concentration and current density.

Figure 7. Western blots of membrane protein with an INa (top), a Kv4.3 (middle), and an NCX (bottom) antibody. Results are shown for 2 hearts per group for each protein.

Figure 8. Changes in mRNA concentrations and densities of the corresponding ionic current in each group of dogs. Currents were measured in 25 cells from 5 hearts for each group of dogs for ICa and INa (Reference 10), and 28 cells (5 hearts), 59 cells (6 hearts), and 43 cells (6 hearts) for INa of P0, P7, and P42 dogs, respectively (Reference 11). Results are mean±SEM for both mRNA concentration and current density.
Alterations in Ion Channel Gene Expression in Experimental Models of Heart Disease

A variety of changes in ion channel gene expression have been observed in animal models of heart disease, primarily at the ventricular level. Matsubara et al.25 noted decreased Kv1.5 mRNA expression and increased Kv1.4 mRNA in rat models of ventricular hypertrophy, with a normalization of gene expression after treatment. Gidh-Jain et al.26 showed a reemergence of fetal pattern L-type Ca\(^{2+}\) current mRNA in noninfarcted zones of rats 21 days after myocardial infarction. Subsequent work from the same group showed decreases in mRNA content of 3 K\(^+\) channel genes, Kv1.4, Kv2.1, and Kv4.2, without changes in Kv1.2 or Kv1.5 in the same model.27 Downregulation of cardiac mRNA encoding the a subunit of I\(_{Ca}\) has been noted in patients with end-stage heart failure.28

Our observations constitute the first detailed study of changes in ion channel gene expression in experimental models of atrial electrical remodeling due to atrial tachycardia. Because the changes that we observed parallel ion channel abnormalities that account for action potential changes that underlie refractoriness alterations caused by remodeling,10 they are likely to be of pathophysiological significance.7

The regulatory mechanisms that cause the changes in ion channel mRNA concentration that we observed are a subject of great potential interest. We attempted to perform nuclear runoff assays to evaluate changes in transcription rate; however, canine atrial tissue has a relatively small mass, and the largest number of nuclei we were able to isolate (<10\(^6\)) was insufficient to perform runoff studies. A variety of hormones and neurotransmitter transducers, including thyroid hormone, glucocorticoids, and cAMP, can regulate ion channel expression.29-32 Autonomic neurotransmitters do not appear to be involved in the phenotypic changes caused by atrial tachycardia,9 but the potential importance of other endogenous bioactive substances has not been assessed. Cellular Ca\(^{2+}\) loading that results from increased action potential frequencies is an attractive possibility because I\(_{Ca}\) downregulation could serve an important protective function against Ca\(^{2+}\) overload. Evidence for Ca\(^{2+}\) overload has been shown early in the rapid-pacing AF model.33 Ca\(^{2+}\) antagonists appear to prevent AF-induced remodeling,34 and increased cytosolic Ca\(^{2+}\) can downregulate mRNA encoding cardiac Na\(^+\) channels.35 On the other hand, incubation of quiescent rat ventricular myocytes in increased extracellular [Ca\(^{2+}\)] solutions increases I\(_{Ca}\),36 and varying results have been obtained in studies of I\(_{Ca}\) in ventricles from dogs subjected to rapid ventricular pacing: both a significant decrease37 and no change38 have been reported.

Comparison With Previous Observations of Molecular Changes Related to Electrophysiological Abnormalities in AF

We applied competitive RT-PCR, the most sensitive and quantitative method presently available for studying genes expressed at a low level,39,40 to quantify changes in ion channel expression in a dog model of AF. There is relatively little information available regarding the molecular changes occurring in AF. Van Wagoner et al.41 have reported that atrial myocytes from patients with chronic AF have a reduced density of sustained outward current at the end of a depolarizing pulse, which suggests a reduced density of the ultrarapid delayed rectifier, I\(_{Kur}\),42 and have shown that protein levels of Kv1.5, the a subunit that carries I\(_{Kur}\),12,43 are reduced. Expression of Kv2.1 was not altered, and expression of other K\(^+\), Na\(^+\), and Ca\(^{2+}\) channels was not determined. Preliminary data suggest that the density of I\(_{Ca}\) in atrial cells from patients with AF is reduced,44 in agreement with our findings, but biochemical analyses have not been reported. Contradictory findings have been published regarding changes in gap junction protein expression during AF.45,46 A preliminary communication points to a 36% reduction of mRNA levels of CIR, a component of I\(_{KCa}\), in patients with AF,47 although it is unclear whether there is a corresponding functional change. An intriguing recent report identifies a genetic locus for a familial form of AF.48 More precise identification of the gene and its protein product promises to provide exciting insights into the molecular pathophysiology of AF.

Potential Relevance to AF Mechanisms

Changes in atrial refractoriness are ubiquitous in tachycardia-related animal models of AF,7,9,13 and resemble abnormalities in patients susceptible to AF.50,51 Action potential duration changes caused by I\(_{Ca}\) downregulation account largely for refractoriness changes associated with AF.50 I\(_{Ca}\) reductions could be due to a variety of mechanisms, including decreased production of the channel, changed functional properties of the channel, and altered regulation by guanine nucleotide binding proteins coupled to receptors for endogenous ligands. The voltage and time dependence of I\(_{Ca}\) is unaltered, which argues against changes in functional channel properties.10 In this article, we show that the development of a substrate for sustained AF is associated with decreased levels of mRNA encoding the a subunit of I\(_{Ca}\), and that changes in mRNA levels are strongly correlated with alterations in I\(_{Ca}\) density. These observations suggest that decreased levels of mRNA encoding the pore-containing a subunit of I\(_{Ca}\) lead to decreased channel production and thereby reduce macroscopic current. Several groups have noted prolongations in atrial conduction time, implying slowed atrial conduction, in experimental animals13,52 and patients33,54 with AF. Downregulation of I\(_{Na}\) caused by a reduction in mRNA levels encoding the I\(_{Na}\) a subunit could explain these findings. Together, the changes in atrial refractoriness and conduction are potentially important for explaining how rapid atrial activation promotes the perpetuation of AF.12

Potential Limitations

Whereas transcriptional regulation is an important controller of phenotypic expression, the concentration of ion channel mRNA does not always correspond to the expression of the protein or functional channel for which the gene encodes.24,55,56 It is significant that mRNA concentration changes for I\(_{Kur}\), I\(_{Na}\), and I\(_{Ca}\) in the present study correspond closely to changes in macroscopic current measured previously in our laboratory from identical experimental prepara-
tions. Furthermore, we were able to confirm with the Western blot method that membrane protein concentrations of Kv4.3 and \( I_{\text{Na}} \) were altered by rapid pacing in a fashion quantitatively very similar to those of corresponding mRNA and macroscopic current. Because of technical limitations, we were unable to assay \( \text{Ca}^{2+} \) channel proteins by Western blot; however, in a recent study of radioligand binding to dihydropyridine and adrenergic receptors, we found a significant decrease in dihydropyridine receptors in dogs subjected to rapid atrial pacing.\(^7\) This observation suggests that, as for \( I_{\text{Na}} \) and \( I_{\text{Ca}} \), atrial electrical remodeling reduces \( I_{\text{Ca}} \) by decreasing the number of L-type \( \text{Ca}^{2+} \) channels. Although our findings point to transcriptional regulation as the mechanism of changes in ionic current expression, they do not exclude other types of changes (eg, in protein turnover, posttranscriptional modification and functional regulation).

**Conclusions**

Previous work has shown that alterations in the density of \( I_{\text{Ca} \text{L}} \), \( I_{\text{Ca} \text{N}} \), and \( I_{\text{Na}} \) are important in a dog model of sustained AF that shows many electrophysiological features similar to those described in patients with AF. We have found downregulation of messenger RNA concentrations for these channels that parallels quantitatively changes in current density and (for \( I_{\text{Ca} \text{L}} \) and \( I_{\text{Na}} \)) changes in membrane concentration of the channel \( \alpha \) subunit protein. These findings provide a potential molecular mechanism for the electrical remodeling that is both caused by and promotes the persistence of AF and provides an example of how a physiological perturbation can cause changes in gene expression that promote its own perpetuation.

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**References**


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