Long-Term Amiodarone Administration Remodels Expression of Ion Channel Transcripts in the Mouse Heart

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Background—The basis for the unique effectiveness of long-term amiodarone treatment on cardiac arrhythmias is incompletely understood. The present study investigated the pharmacogenomic profile of amiodarone on genes encoding ion-channel subunits.

Methods and Results—Adult male mice were treated for 6 weeks with vehicle or oral amiodarone at 30, 90, or 180 mg · kg⁻¹ · d⁻¹. Plasma and myocardial levels of amiodarone and N-desethylamiodarone increased dose-dependently, reaching therapeutic ranges observed in human. Plasma triiodothyronine levels decreased, whereas reverse triiodothyronine levels increased in amiodarone-treated animals. In ECG recordings, amiodarone dose-dependently prolonged the RR, PR, QRS, and corrected QT intervals. Specific microarrays containing probes for the complete ion-channel repertoire (IonChips) and real-time reverse transcription–polymerase chain reaction experiments demonstrated that amiodarone induced a dose-dependent remodeling in multiple ion-channel subunits. Genes encoding Na⁺ (SCN4A, SCN5A, SCN1B), connexin (GJA1), Ca²⁺ (CaCNA1C), and K⁺ channels (KCNA5, KCNB1, KCND2) were downregulated. In patch-clamp experiments, lower expression of K⁺ and Na⁺ channel genes was associated with decreased Iₙ, Kv1.4, Iₛ, and Iₙa currents. Inversely, other K⁺ channel α- and β-subunits, such as KCNA4, KCNK1, KCNB1, and KCNE3, were upregulated.

Conclusions—Long-term amiodarone treatment induces a dose-dependent remodeling of ion-channel expression that is correlated with the cardiac electrophysiologic effects of the drug. This profile cannot be attributed solely to the amiodarone-induced cardiac hypothyroidism syndrome. Thus, in addition to the direct effect of the drug on membrane proteins, part of the therapeutic action of long-term amiodarone treatment is likely related to its effect on ion-channel transcripts. (Circulation. 2004;110:3028-3035.)

Key Words: antiarrhythmic agents ▪ ion channels ▪ molecular biology ▪ electrophysiology

A

miodarone, a widely used antiarrhythmic drug, has remarkable efficacy for the treatment of ventricular tachyarrhythmias and atrial fibrillation. However, the basis for its effectiveness is still poorly understood. The pharmacologic profile of this drug is complex, and much remains to be clarified about both short- and long-term actions. Amiodarone has been referred to as a class III antiarrhythmic agent, but it also possesses electrophysiologic characteristics of class I and IV agents and minor class II effects. The drug is also known to modify thyroid function extensively because of its iodinated nature.

The question arose as to whether the long-term effects of amiodarone might stem from its molecular interaction with thyroid hormone receptors or other mechanisms. In particular, it has been hypothesized that the effects of amiodarone could depend on modulation of transcript expression in addition to its direct effect on cell membrane channels. Genomic techniques now bring gene expression studies to a genome scale, allowing investigators to examine simultaneous changes in the expression of the complete gene repertoire. We have developed a specific cDNA microarray (IonChip) containing probes for virtually all known mouse and human ion-channel genes (α- and β-subunits). With this new tool, our previous investigation explored ion-channel remodeling as produced by altered thyroid status in the mouse heart. We now evaluate the effects of long-term amiodarone...
treatment on global ion-channel expression. Our data show that long-term amiodarone treatment dose-dependently remodels ion-channel expression. Remodeling, which includes downexpression of K⁺ channel genes and of genes involved in conduction, coincides with the dose-dependent effects of amiodarone on mouse cardiac conduction velocity and repolarization. We propose that in addition to the well-known direct effect of the drug on membrane proteins, part of the action of amiodarone on the heart is related to its pharmacogenomic profile.

Methods
An expanded Methods section containing details for microarray, relative quantitative reverse transcription-polimerase chain reaction (RT-PCR), and functional studies and statistical analysis is available in the online-only Data Supplement.

Animal experiments were performed in accordance with institutional guidelines for animal use in research. Ten-week-old male C57BL/6 mice (Ifa Credo, F’Arbresle, France) were used. Mice were treated for 6 weeks with 3 doses of amiodarone (a gift from Dr Patrick Gautier, Sanofi-Synthelabo, Montpellier, France): 30, 90, and 180 mg · kg⁻¹ · d⁻¹. Sham animals received the same food without amiodarone. The study included 54 sham mice and 18 mice in each amiodarone group. IonChip contained 208 probes representing most mouse ion-channel subunits and connexins cloned so far. The complete probe list can be found at http://www.nantes.inserm.fr/u533.

Results
Effects of Long-Term Amiodarone Treatment on Functional Parameters
Plasma and myocardial levels of amiodarone and N-des-ethylamiodarone (DEA) were measured in sham and treated animals. As illustrated in Figure 1, there was a clear dose-dependent increase in plasma and tissue amiodarone levels. The 180 mg · kg⁻¹ · d⁻¹ amiodarone group was significantly different from the 90 mg · kg⁻¹ · d⁻¹ group, which in turn was significantly different from the 30 mg · kg⁻¹ · d⁻¹ group. With DEA, 180 mg · kg⁻¹ · d⁻¹ amiodarone was required to reach detectable levels in the plasma (Figure 1A). DEA accumulated dose-dependently in cardiac tissue (Figure 1B).

As shown in Table 1, mice treated with amiodarone at 90 and 180 mg · kg⁻¹ · d⁻¹ had decreased body and heart weights, although their heart weight-to-body weight ratios were not significantly different from sham. As expected, 6-week amiodarone treatment induced a decrease in plasma triiodothyronine and an increase in reverse triiodothyronine. This effect reached significance for the 90 and 180 but not for the 30 mg · kg⁻¹ · d⁻¹ dose groups.

Representative ECG recordings from sham and amiodarone-treated mice are illustrated in Figure 2A. As is evident, the 180 mg · kg⁻¹ · d⁻¹ dose prolonged the P-wave duration and the RR, PR, QRS, and QT intervals. Average data reported in Table 2 show that the heart rate and conduction velocity (PR and QRS intervals) were significantly affected by the 90 mg · kg⁻¹ · d⁻¹ dose. In contrast, 90 mg · kg⁻¹ · d⁻¹ amiodarone did not prolong the corrected QT. Thus, conduction was slowed by both the 90 and 180 mg · kg⁻¹ · d⁻¹ doses of amiodarone, whereas repolarization was prolonged by the 180 mg · kg⁻¹ · d⁻¹ dose only. The lowest dose of 30 mg · kg⁻¹ · d⁻¹ had no significant effect on ECG parameters. Intraventricular recordings as illustrated in Figure 2B showed that the prolonged PR interval observed with the 90 and 180 mg · kg⁻¹ · d⁻¹ doses was related to an intranodal conduction defect (AH [atrial-His bundle interval]=46±2 and 55±4 ms, respectively, vs 36±2 ms in sham; P<0.005; n=6 animals per group), as well as a His bundle conduction defect (HV [His bundle-ventricular interval]=14±1 and 21±2 ms, respectively, vs 10±1 ms in sham; P<0.005). Atrioventricular Wenckebach cycle length was increased from 96±9 ms in sham to 146±9 ms or 181±17 ms (P<0.005) in mice receiving 90 or 180 mg · kg⁻¹ · d⁻¹ amiodarone, respectively.

Effects of Long-Term Amiodarone Treatment on Cardiac Ion-Channel Transcripts
cDNA microarrays were used to screen cardiac RNA samples from sham and amiodarone-treated (180 mg · kg⁻¹ · d⁻¹) mice. Figure 3 represents the percentage variation of a list of control genes in the 180 mg · kg⁻¹ · d⁻¹ group versus sham. Downregulation of cardiac thyroid hormone α1-receptor (THRA1) mRNA was previously reported in mice6 and rats.7 THRA2 and/or THRA3 mRNA was downregulated, whereas THRβ was unaffected. Downregulation of phospholipase A2 coincided with its decreased activity under amiodarone treatment.8 Upregulation of nitric oxide synthase occurred in accordance with its activation by amiodarone in human veins,9 whereas downregulation of sphingomyelinase (SMPD1) coincided with its decreased activity in mouse pulmonary endothelial cells.10 CD4, CD8A, interleukin-1, and interleukin-6 were used as negative controls.
Figure 4 illustrates the effects of long-term amiodarone treatment on ion-channel expression. For clarity, only transcripts expressed in the mouse heart are shown. Among the differentially expressed ion-channel subunits were genes involved in cardiac conduction, such as sodium channels α- and β-subunits (SCN4A, SCN5A, and SCN1B) and connexin 43 (GJA1), which were downregulated. Western blot experiments showed that these modifications were correlated with decreased protein amounts of Nav1.5 (SCN5A) and connexin 43 (data not shown). Calcium homeostasis was affected by

<table>
<thead>
<tr>
<th>BW, g</th>
<th>Before Treatment</th>
<th>After Treatment</th>
<th>HW, mg</th>
<th>HW:BW, mg/g</th>
<th>Plasma T3, ng/ml</th>
<th>Plasma rT3, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>24.2±0.2</td>
<td>27.9±0.3</td>
<td>115.7±3.8</td>
<td>4.2±0.1</td>
<td>1.32±0.06</td>
<td>0.84±0.05</td>
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<tr>
<td>30 mg·kg⁻¹·d⁻¹</td>
<td>23.8±0.2</td>
<td>26.9±0.3</td>
<td>114.2±2.4</td>
<td>4.3±0.2</td>
<td>1.20±0.09</td>
<td>0.96±0.06</td>
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<td>90 mg·kg⁻¹·d⁻¹</td>
<td>24.5±0.2</td>
<td>25.2±0.3*</td>
<td>111.9±4.3</td>
<td>4.4±0.1</td>
<td>1.01±0.03†</td>
<td>1.47±0.12‡</td>
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<tr>
<td>120 mg·kg⁻¹·d⁻¹</td>
<td>24.4±0.3</td>
<td>21.8±0.4*</td>
<td>101.0±3.8‡</td>
<td>4.5±0.1</td>
<td>0.73±0.01†</td>
<td>1.64±0.10‡</td>
</tr>
</tbody>
</table>

Data expressed as mean±SEM. n=18 for BW and HW; n=6 for T3 and rT3 in each group. BW indicates body weight; HW, heart weight; rT3, reverse plasma T3.
*P<0.0001 vs sham.
†P<0.02 vs sham.
‡P<0.001 vs sham.

Figure 2. Typical ECGs (lead I) recordings (A) and in vivo intracardiac electrogram recordings of atrioventricular conduction parameters (B) in sham and amiodarone-treated mice. A indicates atrial; H, His bundle; and V, ventricular deflections. Other abbreviations are as defined in text.
amiodarone, with downregulation of Cav1.2 channels (CACNA1C) and Cavβ1- and Cavβ2-subunits (CACNB1 and CACNB2). In addition, the type I sodium-calcium exchanger (SLC8A1), type II calsequestrin (CASQ2), and type III calmodulin (CALM3) were downregulated. The voltage-gated potassium channel genes involved in mouse cardiac repolarization, Kv1.5 (KCNA5), and Kv4.2 (KCND2), were downregulated, whereas Kv1.4 (KCNA4) and regulatory subunits such as Kvβ1 (KCNB1), MiRP1 (KCNE2), and MiRP2 (KCNE3) were upregulated. Two-pore potassium channels, including TWIK-1 (KCNK1), and TASK-2 (KCNK5), and the intermediate/small-conductance calcium-activated potassium channel (KCNN2) were upregulated. It should be noted, however, that these latter genes are expressed at a low level in the mouse heart.

Inversely, mRNA expression for Kir6.2 (KCNJ11) and SUR2 (ABCC9) forming ATP-sensitive K⁺ channels were downregulated. This was also observed for the two-pore K⁺ channel gene TASK (KCNK3).

We used real-time PCR to confirm differential expression of 18 selected genes and also to explore dose dependence (Figure 5). The results show excellent qualitative correlation between microarray and PCR data. As previously observed, however, comparison of the obtained fold changes with microarray analysis versus real-time PCR revealed larger differences with the latter approach. For example, SCN5A transcripts decreased by ~40% (180 mg·kg⁻¹·d⁻¹ dose) in real-time PCR experiments, whereas changes detected with the microarrays, though significant, were ~20%. Most important, real-time PCR data showed that long-term amiodarone treatment dose-dependently regulated the expression of a large set of ion channels. Interestingly, the 30 mg·kg⁻¹·d⁻¹ dose, which did not affect the surface ECG, produced no effect on ion-channel expression. Finally, genes targeted as nonvariant by microarray analysis, such as GJA5, KCNE1, and KCNQ1, were also nonvariant in real-time PCR analysis.

## Functional Correlation With Molecular Data

Whole-cell patch-clamp recordings were conducted in ventricular myocytes isolated from sham and 180 mg·kg⁻¹·d⁻¹ amiodarone–treated hearts. As illustrated in Figure 6A, the amplitude of the transient outward current Iₒ was reduced significantly in the amiodarone-treated group. This was correlated with the observed downregulation of Kv4.2 (KCND2) expression. Similarly, the density of the current remaining at the end of 400-ms pulses, which reflects Iₛslow, was also significantly decreased, in accordance with the reduced expression of Kv1.5 (KCNA5) and Kv2.1 (KCNB1). By contrast, the density of the steady-state current remaining at the end of 4-second pulses, Iₛ, was not modified by long-term amiodarone treatment (2.94±0.47 vs 2.52±0.32 pA/pF at +50 mV) in sham and amiodarone-treated myocytes, respectively; not illustrated). The amplitude of the inwardly rectifying background K⁺ current Iᵢᵣ was not affected (Figure 6B), which was correlated with the molecular data, because neither Kir2.1 (KCNJ2) nor Kir2.2 (KCNJ12) mRNA was affected in amiodarone-treated mice. Voltage-clamp experiments further revealed that the peak inward sodium current (Iᵢᵥ) decreased by 33±6 pA/pF (P<0.001) in amiodarone-treated myocytes (Figure 6C). These data are also correlated with the reduced expression of SCNA5 mRNA in microarrays. Surprisingly, the L-type calcium current density was unmodified in treated mice (data not shown), contrasting with the 50% reduced expression of CACNA1C in real-time RT-PCR experiments.

## Discussion

The present study shows that amiodarone dose-dependently remodels cardiac ion-channel expression and

### TABLE 2. Comparison of ECG Parameters From Sham and Amiodarone-Treated Mice

<table>
<thead>
<tr>
<th>RR</th>
<th>P</th>
<th>PR</th>
<th>QRS</th>
<th>QT</th>
<th>QTc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>213±11</td>
<td>20±0.6</td>
<td>45±1</td>
<td>15±0.3</td>
<td>71±2</td>
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<tr>
<td>30 mg · kg⁻¹·d⁻¹</td>
<td>220±10</td>
<td>20±0.7</td>
<td>48±1</td>
<td>16±0.3</td>
<td>70±1</td>
</tr>
<tr>
<td>90 mg · kg⁻¹·d⁻¹</td>
<td>230±9*</td>
<td>21±0.6</td>
<td>55±2†</td>
<td>18±0.3‡</td>
<td>75±2*</td>
</tr>
<tr>
<td>120 mg · kg⁻¹·d⁻¹</td>
<td>274±13‡</td>
<td>22±0.9*</td>
<td>57±1†</td>
<td>19±0.5‡</td>
<td>85±3‡</td>
</tr>
</tbody>
</table>

Data expressed as mean±SEM. n=18 in each group. QTc=QT/2√(RR/100).

*P<0.05 vs sham.
†P<0.001 vs sham.
‡P<0.01 vs sham.
that remodeling is correlated with the cardiac electrophysiologic effects of the drug, ie, slowed conduction (decreased SCN5A, SCN4A, and GJA1 expression) and prolonged repolarization (decreased KCND2, KCNB1, and KCNA5 expression). These gene expression modifications impact the corresponding current density and/or protein level. Importantly, the prolongation of cardiac repolarization induced by 180 mg · kg⁻¹ · d⁻¹ amiodarone was associated with a complex rearrangement of several repolarizing K⁺ channel genes. These include not only decreased expression of the major K⁺ channels involved in mouse cardiac repolarization but also increased expression of other K⁺ channel subunits (KCNA4, KCNAB1, KCNE2, and KCNE3). We previously observed a comparable rearrangement in the same mouse model under the effects of hypothyroism. To our knowledge, this is the first large-scale study showing pharmacogenomic effects of an antiarrhythmic drug, which at least partly account for the control of mouse cardiac electrical function by amiodarone and potentially for its antiarrhythmic action, in addition to the known direct effect of the drug on membrane proteins.

Comparison With Previous Studies

Despite the high doses of amiodarone administered to mice, plasma and myocardial concentrations remained within the therapeutic range observed in patients. One difference with the clinical situation is the low level of DEA plasma and myocardial concentrations, which never exceeded 0.3 times the parent compound, whereas in humans, concentrations of DEA can be as high as 3 times those of amiodarone. This is consistent with previous observations in animal studies and is probably related to species differences in liver metabolism.

In our model, long-term amiodarone treatment induced a dose-dependent bradycardia and a decrease in conduction velocity, in agreement with previous clinical and experimental findings. The small but significant increase in corrected QT at the highest dose of amiodarone is also consistent with the 5% to 18% increase obtained in different animal models treated orally for the same period of time. In humans, the prolongation of repolarization rarely exceeds 10% to 15%, as assessed with surface ECG or monophasic action potential (MAP) recording.

Early on, Drvota and coworkers performed in vitro experiments suggesting that the effects of DEA on cardiac electrical activity could depend on gene expression. In their extensive 1997 review, Kodama et al also reported earlier results from their group, showing a decreased
expression of Kv1.5 (KCNA5) in rats treated with amiodarone. Our results show that remodeling affects every gene family represented on our microarrays. As previously mentioned, amiodarone induced a prolongation of ventricular repolarization that can be explained by the decrease in Ito and IK,slow current densities. A decrease in Ito density without alteration in its voltage dependence has already been described in different models and can be explained partly by the decreased expression of Kv4.2 (KCND2). Similarly, the decreased expressions of Kv2.1 (KCNB1) and to a lesser extent of Kv1.5 (KCNA5) are consistent with the reduction of IK,slow. In the rat, Isus,a current similar to IK,slow, is also decreased by long-term amiodarone, probably because of the decreased expression in Kv1.5 (KCNA5). Finally, a previous study in rabbits has shown that long-term amiodarone administration decreases the Ik, current density without affecting KCNQ1 or KCNE1 mRNA. We observed that Ca2+ channel subunits, including Cav1.2 that forms the pore of the myocardial L-type Ca2+ channel (IK,calc), are downregulated in treated mice, consistent with previous binding studies. At the functional level, however, previous results are conflicting, because IK,calc has been shown to be either decreased or increased by long-term amiodarone administration. In our model, the IK,calc current density was unaffected by amiodarone. This finding opposes the decreased expression of CACNA1C, suggesting either post-transcriptional or translational mechanisms or compensatory adaptation linked to the expression of KCNB1 and KCNB2 regulatory subunits. In fact, we found that the expression of the regulatory subunit was largely altered by amiodarone. Because these regulatory proteins can exert close control of current amplitude, the correlation between changes in gene expression and function may not be straightforward.

Potential Mechanisms for Amiodarone-Induced Ionic Remodeling

The mechanism of the relation between oral amiodarone dose and gene expression is principally unknown. Some genes (ie, SCN5A, GJA1, etc) show progressive remodeling in relation to dose, whereas others (ie, CACNA1C, KCNA4, etc) do not. Different nonexclusive hypotheses can explain ionic remodeling, including (1) the hypothyroidism syndrome induced by amiodarone, (2) a direct effect of amiodarone/DEA on gene promoters, (3) an effect of amiodarone/DEA on transcription factors, and (4) physiologic alterations induced by direct effects of the drug on membrane proteins.

Amiodarone is a diiodinated benzofuran derivative that presents structural similarities to thyroid hormones. Cardiac electrophysiologic changes induced by long-term amiodarone closely resemble those induced by hypothyroidism.
roidism (for a review, see Kodama et al\textsuperscript{15}). However, hypothyroidism does not mimic all amiodarone long-term effects.\textsuperscript{29,30} Recently, Bosh et al\textsuperscript{30} have shown that hypothyroidism and long-term amiodarone have different effects on guinea pig cardiac \(K^+\) currents and that their combination prolongs repolarization to a greater extent than either alone. Our study also suggests that amiodarone-induced ionic remodeling cannot be explained by hypothyroidism alone. Indeed, although some of the variations in transcripts induced by long-term amiodarone treatment are similar to those induced by hypothyroidism in the same mouse model,\textsuperscript{5} eg, the decreased expression of KCNA5, KCNB1, or KCND2, the expression of several genes such as GJA1, KCNA4, KCNAB1, and KCNJ11 that are not regulated by thyroid function appear to be regulated by amiodarone. In addition, genes such as CACNA1C, SLC8A1, KCNE1, and KCNQ1 were upregulated by hypothyroidism, whereas others were downregulated (CACNA1C, SLC8A1) or unmodified (KCNE1, KCNQ1) by amiodarone. Finally, nongenomic effects of thyroid hormones are not inhibited by amiodarone.\textsuperscript{29,30} Thus, it is not surprising that long-term amiodarone treatment causes remodeling of ion channels different from those associated with systemic hypothyroidism.

One can also speculate that amiodarone can directly modify gene expression. Amiodarone, or most probably DEA, may interfere with nuclear receptor/coactivator interaction.\textsuperscript{31,32} It reduces basal LDL receptor promoter activity. It also disrupts the interaction between the glucocorticoid receptor interacting protein-1 and the thyroid hormone receptor \(\beta_1\).

Alternatively, ionic remodeling could be a long-term consequence of the effects of amiodarone on ionic-channel proteins and could result from an adaptation of the myocardes to a new physiologic steady state. This hypothesis seems least plausible. If true, one would expect, for instance, compensatory overexpression of \(Na^+\) and \(Ca^{2+}\) channel subunits that are major targets of short-term amiodarone treatment, rather than downexpression, as observed in our model.

**Clinical and Physiologic Implications**

Our results contribute to understanding of the unique profile of amiodarone in the treatment of arrhythmias and may account for the long-term effects of the drug, which differ from its short-term effects. However, their clinical relevance remains unclear for at least 2 reasons: (1) the choice of species dictated by the availability of extensive genomic information and (2) the absence of primary cardiac pathology with or without arrhythmias. It is therefore essential to investigate whether remodeling is also observed in humans and how it interferes with the remodeling resulting from cardiac pathologies.

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Title: **Chronic amiodarone dose-dependently remolds expression of ion channel transcripts in the mouse heart**

Corresponding author: Sophie Demolombe

**Experimental animals**

Animal experiments were performed in accordance with institutional guidelines for animal use in research. Ten-week male C57BL/6 mice (IFFA CREDO, l’Arbresle, France) were used. Mice were treated with three different doses of amiodarone (a gift from Dr. Patrick Gautier, Sanofi-Synthelabo, Montpellier, France) mixed in food powder: 30, 90 and 180 mg/kg/day for 6 weeks. Sham animals received the same food without amiodarone. The study included 54 mice in sham groups and 18 mice in each amiodarone group.

Levels of free T3 and reverse T3 were determined in the serum from amiodarone and sham mice by using competitive radioimmunoassay (Clinical Assays™ GammaCoat™ T3 125I RIA kit, Diasorin, Stillwater, USA; Reverse T3 Kit, Biochem Immunosystems, Bologna, Italy) according to the manufacturer’s specifications. Serum samples from 3 mice were pooled. Six pools were processed in each amiodarone and sham group.

Plasma and myocardial levels of amiodarone and its metabolite n-desethylamiodarone (DEA) were determined. Compounds were quantified by HPLC and spectrometric detection. Serum and cardiac samples from 3 mice were pooled. Six pools were processed in each group.
**Functional studies**

Surface numerically recorded ECGs (EMKA technologies, Paris, France) were obtained as previously reported, except for the anesthetic protocol (30 mg/kg etomidate in this study versus 15 mg/kg in the previous study). All measurements done independently by two experienced examiners were averaged from 3 consecutive PQRST complexes in lead I. The QT interval was corrected for heart rate using the formula, QTc=QT/(RR/100)^{1/2} established for mice with QT and RR expressed in ms.

For intracardiac electrogram recordings, animals were anesthetized with intraperitoneal injection of etomidate 8 mg/kg and pentobarbital 30 mg/kg. A warming light was used to prevent hypothermia. Surface ECGs were obtained with 25-gauge subcutaneous electrodes placed in each limb. ECG channels were filtered between 0.5 and 250 Hz. Under sterile conditions, an octopolar 2F electrode catheter with an electrode spacing of 0.5 mm (Cordis Webster®) was introduced into the right atrium and ventricle through the right internal jugular vein. Using this catheter, simultaneous atrial and ventricular pacing and recording were performed. The His-bundle activity was also recorded. Intracardiac electrograms were filtered between 30 and 500 Hz. Surface ECG and intracardiac electrograms were recorded to computer through an analog to digital converter (IOX 1.585, EMKA Technologies) for monitoring and later analysis and measurement. Intracardiac pacing was performed with a Biotronik® UHS20 stimulator (Berlin, Germany), modified by the manufacturer to pace a short coupling intervals. Atrio-ventricular nodal conduction was evaluated by atrial incremental pacing until Wenckebach and 2:1 atrio-ventricular block. AH and HV intervals were measured.
Our methods to isolate cardiac cells from the mouse heart and to perform whole-cell recordings have appeared elsewhere. To record the transient outward and sustained voltage-gated K⁺ currents (\(I_{to}\) and \(I_{K,slow}\)), cells were superfused at room temperature with an extracellular solution containing (in mmol/L): N-Methyl-D-Glucamine, 130; KCl, 5.4; MgSO₄, 1.2; HEPES, 10; glucose, 10; mannitol, 10; pH adjusted to 7.4 with HCl. Substitution of NaCl by N-Methyl-D-Glucamine suppressed the Na⁺ current. The addition of 2 mmol/L CoCl₂ blocked the L-type Ca²⁺ current. Patch pipettes had tip resistances of 2.0-3.0 MΩ. They were filled with a solution containing (in mmol/L): KCl, 20; K-aspartate, 110; HEPES, 5; MgCl₂, 2 (free-Mg²⁺: 0.6); K₂ATP, 5; CaCl₂, 1; pH 7.2 with KOH. The transient (\(I_{to}\)) and sustained voltage-gated outward currents were elicited by applying at 15-s intervals, 400-ms or 4-s voltage steps to potentials between -40 mV and +50 mV in 10 mV increments from a holding potential of -70 mV. For the inward rectifier K⁺ current \(I_{K1}\), the external solution was identical to that used for \(I_{to}\) and \(I_{K,slow}\). The addition of 3 µmol/L nifedipine blocked the L-type Ca²⁺ current. Patch pipettes were filled with a solution containing (in mmol/L): KCl, 20; K-aspartate, 110; HEPES, 5; EGTA, 5; MgCl₂, 2 (free-Mg²⁺: 0.6); K₂ATP, 5; Na₂phosphocreatine, 5; CaCl₂, 1; pH 7.2 with KOH. \(I_{K1}\) was elicited by applying at 3-s intervals, 500-ms voltage steps to potentials between -110 mV and -20 mV in 10 mV increments from a holding potential of -70 mV. All current measurements were normalized using the cell capacitance. The whole-cell configuration of the patch-clamp technique was used to record \(I_{Na}\). Pipettes (tip resistance: 1 to 2 MΩ) were filled with a solution containing (in mmol/L): CsCl, 60; aspartic acid, 50; CaCl₂, 1; MgCl₂, 1; HEPES, 10; EGTA, 11 and Na₂ATP, 5 (pH 7.3 with CsOH). Myocytes were bathed with a solution containing (in mmol/L): NaCl, 136.9; KCl, 5.4; CaCl₂, 2; glucose, 10; MgCl₂, 1.06; and HEPES, 10 (pH 7.4 with
NaOH). For current recordings, external Na$^+$ was reduced to 10 mM using N-methyl-
glucamine as a Na$^+$ substitute, and KCl was replaced by an equal amount of cesium 
chloride. Membrane potential values were corrected offline for the measured junction 
potential of +16 mV. Experiments were performed at room temperature (22°C to 
24°C).

Microarrays

cDNA microarrays were produced and hybridized as previously described.$^4$ Our “IonChip” contained 208 and 286 probes representing most mouse and human 
voltage-gated Na$, Ca^{2+}$, Cl$^-$, and K$^+$ channel subunits, inward rectifier and 2-pore 
domains K$^+$ channels, epithelial Na$^+$ channels, and connexins cloned so far. It 
contained also genes encoding proteins involved in Ca$^{2+}$ homeostasis and thyroid 
hormone receptors. The complete probe list can be found at 
http://www.nantes.inserm.fr/u533. Clones were obtained by PCR amplification of 
genomic DNA. Because ion channels possess strong structural similarities, the 3' 
untranslated region (3'UTR) sequences specific for each gene were used as probes. 
Because of the species specificity of the 3' UTR, human probes generated low non-
differential signals that were useful for the normalization of the data during analysis. 
PCR products were precipitated, resuspended in 3xSSC and Betaine 5M and 
spotted on amino-silane coated glass slides GAPSII (Corning Life Science, Schipol-
Rijk, Netherlands) with the Eurogentec SDDC/C200 robot (Eurogentec, Seraing, 
Belgium). All probes were spotted in triplicate.

Total RNA were extracted from mouse heart and then treated with DNase I 
(Sigma). mRNA were isolated by polyA selection using Oligotex resin (Oligotex 
mRNA midi Kit, Qiagen, Valencia, USA). The purified mRNA were quantified with
Agilent 2100 (Bioanalyseur Agilent technologies, Palo Alto, USA). Hybridizations were designed as follows. One mouse selected at random in the 180 mg/kg/day treated group was hybridized against one out of 3 pools of sham hearts (each from 6 mice). Eight microarrays were so hybridized.

Aminoallyl-dUTP were incorporated during reverse transcription of 1 µg mRNA with Cyscript reverse transcriptase (Amersham Biosciences, Buckinghamshire, England) and oligo(dT)/random primer mix. Quality and quantity of each cDNA were checked with Agilent 2100 Bioanalyseur. Then, cDNA were post-labelled using Cy3/Cy5-dye-NHS ester (Amersham Biosciences). Before hybridization, Yeast tRNA (Invitrogen), poly[A] (Sigma), Formamide and Denhardt’s solution (Sigma) were added to the combined Cy3- and Cy5-dUTP-AA labeled targets. Each labeled cDNA from the amiodarone group was mixed with an equal amount of labeled cDNA from sham. Hybridized arrays were scanned using confocal microscopy (ScanArray 3000, GSI Lumonics, Northville, USA). Measurements were obtained for each fluorochrome at 5 µm/pixel resolution. Fluorescence intensity values and ratios for each cDNA array were analyzed using GenepixPro4.0 software (Axon Instruments, Inc., CA, USA). All clones with a defective signal (spot deformation, dust…) were flagged and not further analyzed. A normalization procedure was performed to compensate for moderate differences between Cy3 and Cy5 signals.

To avoid confusion, genes are named using the HUGO nomenclature throughout the manuscript (http://www.gene.ucl.ac.uk/hugo/).
**Relative quantitative RT-PCR**

This technique was used as previously reported using 2x SYBR Green Master Mix (Applied Biosystems, Foster City, USA). Briefly, 20 ng of cardiac mRNA samples were reverse transcribed into cDNA by the M-MLV transcriptase (Invitrogen). We checked that PCR efficiency was identical between the genes of interest and GAPDH used as the reference house-keeping gene. For data analysis, the fluorescence signals were normalized to acidic ribosomal phosphoprotein P0 (Arbp) mRNA ($Rh$), yielding a normalized value $\Delta Rh$. Relative quantification, following the $\Delta\Delta Ct$-method, was applied to compare the amounts of mRNA in sham and each treated groups.

**Statistical Analysis**

All data are given as mean ± SEM. Statistical analysis of dosages and functional studies was performed with the Student t-test. Statistical analysis of real-time RT-PCR and patch-clamp experiments was performed with 2-way ANOVA completed by a Tukey t-test. A value of $p < 0.05$ was considered significant.

SAM (Significance of Microarrays analysis) software was used for statistical analysis of microarrays. SAM uses pairwise comparisons of gene expression after each gene is assigned a score on the basis of its change in expression relative to the standard deviation of repeated measurements for that gene (www-stat-class.stanford.edu/SAM/SAMservlet). The delta threshold corresponding to the lowest median false discovery rate (FDR <0.5%) was used.
REFERENCES


