Chloroquine Terminates Stretch-Induced Atrial Fibrillation More Effectively Than Flecainide in the Sheep Heart

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Background—Blockade of inward-rectifier K⁺ channels by chloroquine terminates reentry in cholinergic atrial fibrillation (AF). However, it is unknown whether inward-rectifier K⁺ channels and reentry are also important in maintaining stretch-induced AF (SAF). We surmised that reentry underlies SAF, and that abolishing reentry with chloroquine terminates SAF more effectively than traditional Na⁺-channel blockade by flecainide.

Methods and Results—Thirty Langendorff-perfused sheep hearts were exposed to acute and continuous atrial stretch, and mapped optically and electrically. AF dynamics were studied under control and during perfusion of either chloroquine (4 µmol/L, n=7) or flecainide (2–4 µmol/L, n=5). Chloroquine increased rotor core size and decreased reentry frequency from 10.6±7 Hz in control to 6.3±0.7 Hz (P<0.005) just before restoring sinus rhythm (7/7). Flecainide had lesser effects on core size and reentry frequency than chloroquine and did not restore sinus rhythm (0/5). Specific Iₖₛ blockade by E-4031 (n=7) did not terminate AF when frequency values were >8 Hz. During pacing (n=11), flecainide reversibly reduced conduction velocity (=30% at cycle length 300, 250, and 200 ms; P<0.05) to a larger extent than chloroquine (11% to 19%; cycle length, 300, 250, and 200 ms; P<0.05). Significant action potential duration prolongation was demonstrable only for chloroquine at cycle length 300 (12%) and cycle length 250 ms (9%) (P<0.05).

Conclusions—Chloroquine is more effective than flecainide in terminating SAF in isolated sheep hearts by significantly increasing core size and decreasing reentry frequency. Chloroquine’s effectiveness may be explained by its inward-rectifier K⁺ channel blockade profile and suggest that reentry is important to maintain acute SAF. (Circ Arrhythm Electrophysiol. 2012;5:561-570.)

Key Words: arrhythmia mechanisms ■ atrial fibrillation ■ drug therapy ■ electrophysiology mapping

Atrial fibrillation (AF) is the most common sustained arrhythmia in clinical practice. Although the mechanisms that sustain AF are incompletely understood, strong evidence in humans and in animal models supports the hypothesis that high-frequency reentrant sources (rotors) are essential to maintain AF. It is possible to demonstrate discrete sites of high frequency periodic activity during AF, along with frequency gradients between left and right atria. Occasionally, a long-lasting rotor is identified within the area of maximum dominant frequency (DFmax). 5

Clinical Perspective on p 570

In the human heart, atrial dilatation and stretch predisposes to AF with DF values directly related to left atrial (LA) pressure. Similarly, in a sheep model of stretch-induced AF (SAF), the posterior left atrium (PLA) plays a major role in the maintenance of the arrhythmia. 4

Less clear is the role of rotors in SAF, where focal discharges (FDs) and reentrant sources interact in such a way that FDs can destabilize and terminate rotors but also can give rise to new wave breaks and rotor formation. 5 The mechanism underlying FDs is uncertain with either triggered activity or reentry being possible; a more depolarized resting membrane potential (RMP) and the activation of stretch-activated nonselective cationic channels enable the generation of afterdepolarizations that might explain FDs (breakthroughs) in the optically-mapped areas. However, FDs might also be the surface reflection of intramural reentrant sources. 8

Inward-rectifier K⁺ currents (Iₖ₁, Iₖ₃₆, and Iₖ_ATP) play important roles in controlling rotor dynamics. 5,8,10 Recently, Noujaim et al have demonstrated that chloroquine blocks the pore-forming subunits Kir2.1, Kir3.1, and Kir6.2 responsible for the inward-rectifier K⁺ current (Iₖ₁), the acetylcholine-sensitive K⁺ current (Iₖ₃₆), and the ATP-sensitive K⁺ current (Iₖ_ATP), respectively. Interestingly, chloroquine depolarizes the RMP and increases automaticity, which can be explained by its blocking

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effects on I_K1. The latter might increase FDs underlying a triggered activity mechanism.

We hypothesized that if reentry plays a critical role sustaining SAF, then chloroquine should effectively restore sinus rhythm (SR), based on its ability to preferentially block inward-rectifier K+ currents. We therefore compared the effects of chloroquine with those of flecainide on SAF and excitatory properties using optical mapping in Langendorff-perfused sheep hearts. The rationale for such a comparison was based on the fact that, similar to amiodarone11 and the most recently introduced drugs dronedarone14 and vernakalant,15 flecainide16 does not block I_K1 within the therapeutic range of concentrations. Flecainide is more effective than amiodarone in early reversion of recent-onset AF17 (<48 hours), and it is highly recommended in patients with no underlying structural heart disease.18 However, our data demonstrate that chloroquine is more effective than flecainide in restoring SR in this model. Taken together, our results support the hypothesis that rotor activity underlies SAF and that blockade of inward-rectifier K+ currents may be a viable approach for its termination.

Methods
Experimental Setup
All procedures were approved by the University of Michigan Committee on Use and Care of Animals (UCUCA) and complied with National Institutes of Health guidelines. Thirty, 6-month-old sheep (weight, 35 kg) were included in the study. Anesthesia was induced with 4 to 6 mg/kg of propofol and 60 to 100 mg/kg of sodium pentobarbital. Hearts were removed via thoracotomy and connected to a Langendorff perfusion system with recirculating oxygenated (95% O2, 5% CO2) Tyrode solution at constant flow rate of 240 to 270 mL/min, pH 7.4 and 35.5 to 37.5°C. The Tyrode composition (in mmol/L) was: NaCl 130, KCl 4.0, MgCl2 1, CaCl2 1.8, NaHCO3 24, NaH2PO4 1.2, glucose 5.6, and albumin 0.04 g/L. Blebbistatin 10 µmol/L was used to reduce the contractile force.

After atrial transseptal puncture, the intra-atrial pressure was increased to 14 cm H2O to induce continuous atrial stretch. Bipolar electrograms from each of the pulmonary veins (PVs), top and roof of the left atrial appendage (LAA) and right atrial appendage (RAA) were obtained (sampling rate, 1.0 kHz). All vein orifices were sealed except for the inferior vena cava, which was cannulated and used for controlling the level of the intra-atrial pressure.

Optical mapping movies (5 seconds) were obtained using a Little Joe CCD camera (80x80 pixels, 500–1000 frames per second). After a bolus injection of 5 to 10 mL Di-4-ANEPPS (10 mg/mL) (Sigma-Aldrich), voltage-sensitive fluorescence was acquired from the RAA and LAA (area, 14 cm²). In 7 experiments, epicardial mapping from the LAA was complemented with endocardial mapping of the PLA (area, 3.7 cm²), using a dual-channel rigid borescope (see online-only Data Supplement Methods and online-only Data Supplement Figure I).

Experimental Protocols
Baseline action potential duration at 70% repolarization (APD70) was optically measured on both atrial appendages (n=5) at progressively shorter pacing cycle lengths (CLs; 300, 250, 200 ms). The pacing electrode was placed on the top of the LAA. Mean APD70 was obtained for RAA and LAA surfaces by averaging APD70 from all pixels. AF was then induced via burst pacing (12 Hz) and allowed to continue for 15 minutes of baseline control. Thereafter, the antifibrillatory effects of either 4 µmol/L chloroquine diphosphate (n=7) or 2 µmol/L flecainide (n=5) (Sigma-Aldrich) dissolved in Tyrode solution were investigated. The concentrations were based on the highest therapeutic level for flecainide19 and chloroquine.20 Drug perfusion was maintained for 20 minutes, after which the concentration was doubled and maintained for 30 additional minutes, as long as SR was not already restored. AF was terminated by DC shock in those hearts in which AF persisted for 50 minutes. Burst pacing was subsequently applied after AF termination to reinduce AF. Finally, AF reinduction was attempted again after a washout period of 30 and 45 minutes for flecainide and chloroquine, respectively.

To better delineate the specific roles of I_K1 versus I_Ca, we conducted experiments (n=7) using the same stretch-induced AF model to study the antifibrillatory effects of specific I_Ca-blockade. After 15 minutes of baseline AF, 0.5 to 1 µmol/L E-4031 was perfused for 20 and 30 minutes as above.21

An additional set of experiments was carried out to investigate the electrophysiological effects of 4 µmol/L chloroquine (n=5) and 4 µmol/L flecainide (n=6). Pacing protocols were completed under basal conditions, after 15 minutes of either chloroquine or flecainide, and after washout. No AF was induced in these hearts. Activation times were determined at 50% of action potential amplitude and conduction velocity (CV) was calculated.22 Online-only Data Supplement Figure II, A and B, illustrates the time line of all experimental protocols.

Frequency Analysis
Dominant frequency (DF) maps were obtained for each optical movie in AF after applying a fast Fourier transform (FFT) of the fluorescence signal recorded at each pixel.23 Bipolar electrograms recorded during AF were high-pass–filtered at 3 Hz and low-pass–filtered at 35 Hz. FFT was also applied to the 5-second bipolar signals synchronized with the optical movies.

AF Dynamics
Phase movies were constructed using the Hilbert transformation.24 In each movie, a rotor was defined as a point of phase convergence (singularity point; SP) lasting more than 1 rotation. A breakthrough was defined as a wave appearing inside the field of view and propagating outward (online-only Data Supplement Figure III). During AF, 5-second movies were acquired and analyzed frame by frame. Rotor analysis was carried out on the PLA and LAA by measuring the total number of rotations in 5 seconds, regardless of the lifespan of individual rotors but considering their frequency of rotation. The number of breakthroughs in 5 seconds was also quantified on the PLA, since PVs represent the region where FDs are identified in some cases of AF.25 Reentry meandering around SPs was analyzed further to quantify the respective effects of chloroquine and flecainide on the unexcited core size. A detailed description of the method is described in Figures IV, V, and VI in the online-only Data Supplement.

Statistical Analyses
Results are reported as mean±SEM. DF data were verified (Shapiro-Wilk) and other continuous measurements were assumed to distribute normally. One-way or 2-way repeated-measures ANOVA or a mixed-model ANOVA was used for continuous measurements as appropriate. Fisher exact test was used to demonstrate significant differences on AF termination and reinducibility. Post hoc comparisons were used after Bonferroni correction. P<0.05 was considered statistically significant.

Results
SAF in the Control
Figure 1A (left) shows examples of simultaneously obtained single pixel recordings from the LAA (red) and RAA (black) at 300-ms CL. As summarized graphically on the right, APD70 was significantly shorter in LAA than RAA (n=5) at 3 different CLs (300, 149.3±9.3 versus 175.7±8.2 ms; 250, 138.7±6.0 versus 153.3±4.5 ms; 200, 123.5±5.0 versus 132.8±3.6 ms, P<0.05).
**Figure 1.**

A, **left**, Single-pixel action potential (AP) from the left atrial appendage (LAA, red) and right atrial appendage (RAA, black). **Right,** Action potential duration (APD)\(_{70}\) at varying pacing cycle lengths (CLs) is shorter in LAA than RAA. APD\(_{70}\) significantly shortens in both appendages from 300 to 200 ms CL.

B, **left column,** Dominant frequency (DF) maps of the posterior left atrium (PLA) and LAA and electrode location on the RAA. **Center column,** Single-pixel activations (PLA, LAA) and bipolar electrograms (RAA). **Right column,** Power spectra showing DF\(_{\text{max}}\) at each location. **C, top**, LAA, RAA, and PLA/pulmonary veins (PVs) DF\(_{\text{max}}\) during 15 minutes of control AF. **Bottom,** Bar graph of means±SEM. DF\(_{\text{max}}\) during control AF shows significant DF gradient between PLA and LAA/RAA.
Once SAF stabilized, the DF<sub>max</sub> locations were identified across the mapped regions. Figure 1B shows DF maps, sample local activations, and corresponding power spectra from the PLA, LAA, and RAA after 14 minutes of control AF in a representative heart. The DF<sub>max</sub> was localized at the right PVs with a frequency gradient from PLA (13.2 Hz) to LAA (12.0 Hz), and RAA (7.1 Hz).

The regional DF<sub>max</sub> was consistently localized either at one of the PVs or somewhere on the PLA (Figure 1C, top). As expected, a statistically significant frequency gradient was observed from PLA to LAA and RAA (Figure 1C, bottom) (10.8±0.3 Hz, 8.5±0.2 Hz, and 7.0±0.1 Hz, respectively; n=12, P<0.001).

Chloroquine Terminates SAF
To test the hypothesis that reentry is essential to maintain SAF, we determined the effect of 4 µmol/L chloroquine in terminating AF, based on its ability to block the inward-rectifier K<sup>+</sup> currents. As shown in Figure 2, 4 µmol/L chloroquine reduced AF frequency and restored SR after 4 to 19 minutes (n=7). Figure 2A shows representative PLA DF maps in control AF (DF<sub>max</sub> 9.6 Hz) and just before AF termination (DF<sub>max</sub> 6 Hz). Single-pixel optical activations from the DF<sub>max</sub> region and electrograms from the left superior PV (LSPV) are shown below the maps. Figure 2B shows the time course of the DF<sub>max</sub> location in control AF and after chloroquine. The DF<sub>max</sub> sharply decreased from 10.6±0.7 Hz to 6.3±0.2 Hz (n=7) in the first few min after chloroquine onset.

Five additional experiments were conducted, using 1 to 2 µmol/L chloroquine to increase the selectivity of I<sub>K,ATP</sub> blockade. At 1 µmol/L, chloroquine terminated AF in 4 of 5 AF episodes after 3.5 to 9.5 minutes. Increasing the concentration to 2 µmol/L restored SR in the fifth AF episode after 14 minutes. The DF<sub>max</sub> decreased from 10.5±0.8 Hz in control to 6.7±0.2 Hz before resumption of SR (online-only Data Supplement Figure VIII).

Flecainide Does Not Terminate SAF
Na<sup>+</sup>-channel blockade may affect reentrant activity and rotor dynamics leading to AF termination. As illustrated in Figure 3A, after 15 minutes of control AF, perfusion with 2 µmol/L flecainide for 20 minutes and 4 µmol/L for 30 additional minutes significantly decreased the DF<sub>max</sub> from 11.1±1.3 Hz to 8.1±1.3 Hz (P=0.0014) but did not restore SR (n=5). As shown in Figure 3B, flecainide was significantly inferior to chloroquine in its ability to restore SR (0/5 versus 7/7, respectively, P=0.0013). In 2 hearts, the initial DF<sub>max</sub> was slower than in the other 3 cases (8.0±0.1 Hz versus 13.1±1.8 Hz), and AF converted to atrial tachycardia (AT). Figure 3C shows DF maps and bipolar recordings from the PLA during control AF and after 4 µmol/L flecainide. Interestingly, after 40 minutes flecainide, AF converted to AT, which was sustained by a long meandering rotor located in the roof/PLA-LAA junction (online-only Data Supplement Movie I).

Sustained AF Is Inducible in the Presence of Flecainide But Not Chloroquine
As shown in Figure 4A, only nonsustained AF or AT was induced in the presence of chloroquine (n=7). Conversely, on cardioversion, AF (n=4) or AT (n=1) was reinduced and sustained for 15 minutes in the presence of flecainide. After the washout period, AF (n=6, chloroquine; n=5, flecainide) or AT (n=1, chloroquine) was reinduced and sustained in both groups. In Figure 4B, the bipolar electrograms illustrate how initial rapid pacing in the presence of chloroquine was followed by a very brief AF episode, in contrast to sustained AF under flecainide.

Chloroquine Terminates AF Where Flecainide Does Not
We determined the effects of chloroquine in the 5 experiments in which flecainide was unable to restore SR. Thus, AF was reinduced after 30 minutes of flecainide washout. Fifteen minutes of control AF was registered and optically mapped. Figure 4C shows the evolution of the DF<sub>max</sub> on the PLA. DF<sub>max</sub> values were significantly higher than at the beginning of the protocol (16.9±2.0 Hz versus 11.1±1.3 Hz, P=0.018), which might be due to a decrease in optimal conditions of the heart after 3 hours of artificial perfusion. Higher DF<sub>max</sub> may also imply a role of other inward-rectifiers K<sup>+</sup> currents, possibly I<sub>K,ATP</sub>. Nevertheless, 4 µmol/L chloroquine effectively terminated AF (n=5). Time to AF termination was longer (10–29 minutes) relative to the first application of chloroquine (4–19 minutes, Figure 2B).
Chloroquine Effects on Reentry and Breakthroughs

To determine the basis for chloroquine superiority, we studied the effects of each drug on AF patterns of excitation. In Figure 5A, the number of identifiable rotations was quantified on the PLA and LAA every 2 minutes in control AF and every minute after chloroquine. Data were normalized as number of rotations per centimeter squared. In control AF, the PLA (N=2; n=16; where N is the number of animals and n is the number of episodes analyzed) harbored twice as many identifiable rotations per centimeter squared compared with LAA (N=7; n=56) (3.2±0.3 versus 1.6±0.1). On Figure 3. A, Time course of dominant frequency (DF)max before and during flecainide. B, Flecainide is significantly inferior to chloroquine in resuming sinus rhythm (SR). C, DF maps of the posterior left atrium (PLA) and bipolar electrograms from the left inferior PV (LIPV) show control atrial fibrillation (AF) and conversion to atrial tachycardia (AT) during flecainide.

Figure 4. A, left, Sustained atrial fibrillation/atrial tachycardia (AF/AT) is reinducible during flecainide but not during chloroquine perfusion. Right, After washout, sustained AF/AT is reinducible in both groups. B, Representative traces; rapid pacing at 12 Hz did not capture or reinduce nonsustained AF during chloroquine (top); at the same pacing frequency, sustained AF was readily induced during flecainide (bottom). C, Time course of dominant frequency (DF)max; sinus rhythm (SR) is restored when chloroquine was perfused after flecainide washout. LAA indicates left atrial appendage.
chloroquine, the last 2 minutes before termination showed a drastic reduction in the number of rotations in both PLA (3.2 ± 0.3 to 0.7 ± 0.3, P = 0.003) and LAA (1.6 ± 0.1 to 0.6 ± 0.2, P = 0.0002) (see also online-only Data Supplement Figure VII, A). Online-only Data Supplement Movies II and III show, respectively, PLA movies of short-lasting rotors (1–2 rotations) in control AF and during a short time before termination. Quantification of the core width using the new method illustrated in online-only Data Supplement Figures IV to VI, showed a significant increase before AF termination under chloroquine (Figure 5B). A larger core due to preferential I_k1 blockade may reduce the probability of stable rotor initiation as shown in Figure 5C, where a rotor around a SP is observed after a wave break and curling of the wave front. However, the rotating front is annihilated by a new wave front coming from the PLA.

In the stretched atrium, chloroquine’s effects on I_k1 might increase automaticity and FDS, which may underlie breakthroughs in the field of view. However, the number of breakthroughs per centimeter squared decreased (12.3 ± 0.8 to 6.8 ± 0.7, P < 0.05; Figure 6A and 6D). The core size showed no significant increase after 4 µmol/L flecainide (Figure 6C).

Electrophysiological Effects of Chloroquine and Flecainide
As summarized on the left side of Figure 7A and 7B; 4 µmol/L chloroquine significantly increased the APD_70 at 300 and 250-ms CL (155 ± 3.1 to 176 ± 6.0 and 144 ± 3.4 to 158 ± 5.0 ms, respectively, P < 0.05) compared with 4 µmol/L flecainide, which had no significant effect at any pacing CL (online-only Data Supplement Figure IX). On the right side of Figure 7A and 7B, both chloroquine and flecainide decreased the CV at all CLs. However, flecainide’s effects were substantially greater than chloroquine: 33% reduction at 250-ms CL compared with 12% reduction, respectively.

Role of I_kr in Acute SAF
Chloroquine, while nominally an I_k1 blocker, also blocks other currents, including I_kr. An additional series of experiments (n=7) using the same AF model showed that I_kr-blockade after 0.5 to 1 µmol/L E-4031 restored SR in 4 of 7 animals (Figure 8A). When we classified those experiments based on DF_{max} values at baseline (Figure 8B, left); E-4031 restored SR in those cases in which the AF DF_{max} was <8 Hz (4/4) but not in cases with DF_{max} >8 Hz (0/3) (7.1 ± 0.4 Hz versus 9.2 ± 0.5 Hz, respectively, P = 0.029). Unlike E-4031, chloroquine effectively terminated SAF when DF_{max} was >8 Hz (7/7 versus 0/3, P = 0.008. Figure 8B, right).

Discussion
The present study demonstrates that chloroquine but not flecainide effectively converted SAF to SR in a sheep heart model that approximates AF in the human heart with pressure overload and atrial dilatation. SAF termination by chloroquine...
is preceded by a significant increase in rotor core size, which correlates with a reduction in AF frequency and number of breakthroughs. Altogether, these data support the idea that reentry and fibrillatory conduction underlie the overall dynamics of SAF in this model. The Na⁺-channel blocker flecainide converted AF to AT but did not restore SR. Preferential blockade of the inward-rectifier K⁺ currents by chloroquine terminated fast reentrant sources persisting after flecainide washout. In addition, unlike E-4031, chloroquine terminated AF episodes whose DF_max was >8 Hz. Thus, even though E-4031 terminated some of the episodes, the above results demonstrate the superiority of chloroquine over E-4031 in terminating SAF. Nevertheless, chloroquine being a “dirty drug,” its antifibrillatory effect probably is not due solely to I_K1 blockade but to some combination of I_K1 and I_KC blockade, which would vary, depending on the dose being used.

Because I_K-ATP plays a prominent role in ischemia and I_K-ACh underlies vagally mediated AF, it seems reasonable to consider the role of I_K1 in the present SAF model. Rotor acceleration and stabilization of the reentry have been associated with I_K1 overexpression. In humans, gain of function of I_K1 due to mutation in KCNJ2 is also associated with familial AF.

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**Figure 6.** Comparing chloroquine with flecainide. A and B, Although flecainide reduces the number of rotations per centimeter squared at the posterior left atrium (PLA) and left atrial appendage (LAA) (5-second movies), only chloroquine significantly decrease the number of rotations per centimeter squared at the PLA. C, Core size does not significantly increase during flecainide. D, Similarly, only chloroquine significantly decreases the number breakthroughs per centimeter squared at the PLA.

**Figure 7.** A, left, Mean action potential duration (APD)70 for control, chloroquine, and washout at 300-, 250-, and 200-ms cycle length (CL); right, mean conduction velocity for control, chloroquine, and washout; 300-, 250-, and 200-ms CL. B, left, Flecainide does not significantly affect the APD70 at any CL; right, flecainide strongly reduces velocity in a reversible manner.
The greater effectiveness of chloroquine over flecainide in terminating SAF in this model may be related to the fact that atrial dilation decreases the CV and increases the degree of spatial heterogeneities in conduction.\textsuperscript{29} Therefore, under conditions of stretch, anatomic, and functional obstacles may become prominent and facilitate initiation and maintenance of reentrant activity. Flecainide further decreases CV, which increases core size and promotes attachment to and rotation around an obstacle. It therefore results in slower rotating activity. This would explain the decrease in DF values and might subsequently increase the propensity for triggered arrhythmias.\textsuperscript{32} However, such a fear may be reduced by considering that heterozygous Andersen syndrome patients, who lack 1 allele of the KCNJ2 gene with loss-of-function of Kir2.1 channels, have mild QTc prolongation and seldom undergo sudden cardiac death.\textsuperscript{33} In addition, chloroquine does not induce early afterdepolarizations in single myocytes under current-clamp conditions, even at 10 $\mu$mol/L.\textsuperscript{12} Finally, Noutjain et al\textsuperscript{11} demonstrated that rather than being proarrhythmic, I$\textsubscript{K1}$ blockade is antiarrhythmic, as evidenced by chloroquine’s ability to terminate reentry in cholinergic-induced AF. In the present study, we have used a well-established model,\textsuperscript{4} in which if at all present, the proarrhythmic effect of blocking I$\textsubscript{K1}$ should be more evident.\textsuperscript{5,7} Clinical AF cases reported in the 1950s also demonstrated high rates of AF termination after high doses of chloroquine.\textsuperscript{34}

Based on the expression profile of the Kir2.X subfamily, Kir2.3 transcripts are more concentrated in the human atria than in the ventricles.\textsuperscript{35} Protein crystallization, molecular modeling, and interactions with modulators (eg, PIP2)\textsuperscript{36} might lead to new pharmacological strategies focusing on selective blockade of Kir2.3 subunits. The latter becomes especially relevant in the setting of congestive heart failure, in which I$\textsubscript{K1}$ downregulation may be associated with increased susceptibility to ventricular arrhythmias.\textsuperscript{37}

Limitations
Although the chloroquine concentrations we have used preferentially block the inward-rectifier K$^+$ channels, termination of AF might also be mediated in part through its less prominent effects on I$\textsubscript{Ks}$, I$\textsubscript{Na}$, and I$\textsubscript{C,L}$. Additional experiments at the endocardial side of the PLA. However, after either chloroquine or flecainide, the number of breakthroughs decreased concomitantly with reentry, suggesting the possibility that the breakthroughs represent intramural reentry. More interesting is the role of chloroquine, which, on preferential blockade of the inward-rectifier K$^+$ channels, strongly affects reentry and terminates AF. Patterns of activation on the PLA before AF termination also show that the number of breakthroughs is higher than the number of rotors in control AF but the arrhythmia terminates. The latter suggests that reentrant activity is essential to sustain SAF and that intramural reentry in the PLA was responsible for the majority of those breakthroughs.

Clinical Perspective
Atrial dilatation and stretch predispose to AF, with DF values directly related to LA pressure.\textsuperscript{6} Similar to the human heart,\textsuperscript{2} SAF in the sheep heart results in DF gradients, where DF at PLA>LA>RAA. We demonstrate that a reentry-based strategy of blocking the inward-rectifier K$^+$ channels effectively terminates SAF. Conversely, Na$^+$-channel blockade by flecainide was unable to restore SR during atrial stretch, similar to the loss of antifibrillatory effect that is observed also in patients with atrial dilatation.\textsuperscript{31}

Although attractive, I$\textsubscript{K1}$-blockade is currently not a clinical strategy to treat AF because of the lack of antiarrhythmic drugs having this property at therapeutic concentrations, along with the fear that reducing I$\textsubscript{K1}$ would cause diastolic depolarization and might subsequently increase the propensity for triggered arrhythmias.\textsuperscript{32} However, such a fear may be reduced by considering that heterozygous Andersen syndrome patients, who lack 1 allele of the KCNJ2 gene with loss-of-function of Kir2.1 channels, have mild QTc prolongation and seldom undergo sudden cardiac death.\textsuperscript{33} In addition, chloroquine does not induce early afterdepolarizations in single myocytes under current-clamp conditions, even at 10 $\mu$mol/L.\textsuperscript{12} Finally, Noutjain et al\textsuperscript{11} demonstrated that rather than being proarrhythmic, I$\textsubscript{K1}$ blockade is antiarrhythmic, as evidenced by chloroquine’s ability to terminate reentry in cholinergic-induced AF. In the present study, we have used a well-established model,\textsuperscript{4} in which if at all present, the proarrhythmic effect of blocking I$\textsubscript{K1}$ should be more evident.\textsuperscript{5,7} Clinical AF cases reported in the 1950s also demonstrated high rates of AF termination after high doses of chloroquine.\textsuperscript{34}

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with 1 μmol/L chloroquine (I_{Kr}-blockade, 74%; I_{K1},38%; I_{K1,4},14%) and E-4031 support the role of preferential I_{Kr}-blockade to terminate SAF. Further experiments would be required to rule out the possibility that the effectiveness of chloroquine in terminating SAF depends at least in part on a drug action on stretch-activated nonselective cationic channels. Stretch-activated K+ channels (TREK-1) might also be involved in the absence of APD prolongation after flecainide.

The antiarrhythmic effects of chloroquine must be tested in persistent AF, in which I_{K1} upregulation might be involved. In addition, the role of the autonomic system is difficult to mimic in isolated hearts. However, in previous experiments using the same model and adrenergoclinic stimulation, reentrant activity played a major role in the sustainability of the arrhythmia after abolishing triggered activity.

Conclusions
Chloroquine effectively terminates SAF in the sheep heart and is more effective than flecainide in restoring SR, which may be explained by a preferential blockade of the inward-rectifier K+ channels and reentry termination. The results may open novel therapeutic strategies for clinical AF.

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Disclosures
None.

References


CLINICAL PERSPECTIVE

Atrial dilatation and stretch are thought to predispose to atrial fibrillation (AF), but the mechanism is not well understood. A stretch-related mechanism has been proposed in the pathogenesis of AF in various diseases, including mitral valve disease, heart failure, and sleep apnea. We have proposed that electrical rotors are the high-frequency drivers that maintain stretch-induced AF (SAF) and that are responsible for the direct relationship that exists between the dominant frequency of AF and the left atrial pressure in both human patients and animal models. Because inward-rectifier K+ currents (IK1, IK-Ach, and IK-ATP) play important roles in enabling and controlling rotor dynamics, then blocking those channels should effectively terminate SAF. We compared the effects of chloroquine, which preferentially blocks IK1, with those of flecainide, which preferentially blocks IKr, on acute SAF excitation properties and termination in Langendorff-perfused sheep hearts that approximate AF in the human heart with pressure overload and atrial dilatation. The results show that both chloroquine and flecainide slow activation frequency, but chloroquine is more effective than flecainide in converting SAF to sinus rhythm. As a further confirmation for the important role of the inward-rectifier K+ currents in SAF, we observed that chloroquine terminated all AF episodes, in contrast with the IKr blocker E-4031 that did not terminate the fastest SAF episodes. The demonstration that chloroquine treatment eliminates SAF in a clinically relevant animal model suggests that novel pharmacological strategies focusing on selective blockade of inward rectifier K+ currents may be an effective approach to treat AF.
Chloroquine Terminates Stretch-Induced Atrial Fibrillation More Effectively Than Flecainide in the Sheep Heart

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SUPPLEMENTAL MATERIAL

Chloroquine Terminates Stretch-Induced Atrial Fibrillation in the Sheep Heart More Effectively than Flecainide.

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Supplemental Methods.

Experimental Setup.

Stretch-induced atrial fibrillation in the Langendorff-perfused sheep heart.

The isolated, coronary perfused heart underwent an atrial trans-septal puncture to enable equalize intracavitary pressure in both atria. Tetrapolar electrode catheters (Torq®, Medtronic Inc./Minneapolis/MN/USA) were placed into each of the pulmonary veins (PVs) to record bipolar signals from the two distal electrodes (sampling rate, 1.0 kHz) using a Biopac Systems amplifier (DA100C; Biopac Systems, Inc., Goleta, CA, USA). Additional bipolar recordings were obtained from the top and roof of the left atrial appendage (LAA) and right atrial appendage (RAA). All vein orifices were then sealed, except the inferior vena cava, which was cannulated and connected to a digital sensor (Biopac Systems transducer-TSD104A; Biopac Systems, Inc., Goleta, CA, USA) and to an outflow cannula whose open-ended height above the atria controlled the intra-atrial pressure. The pressure was then
increased to 14 cm H2O, which led to an increase in atrial volume and dilatation. The pressure was maintained stable throughout the experiment.¹

**Epicardial mapping of the LAA and RAA.**

A bolus injection of 5 to 10 ml Di-4-ANEPPS (10 mg/mL) (Sigma-Aldrich, St. Louis, MO, USA) and a loading period of 10 min are needed to obtained voltage-sensitive fluorescence upon laser excitation (532 nm) of the epicardial surface. The emitted fluorescence is then transmitted through a 600 nm long pass filter and projected onto LittleJoe CCD video camera (80x80 pixels, SciMeasure Analytical Systems, Inc. Decatur, GA, USA) and acquired at a rate between 500-1000 frames per second. Five-second movies were obtained at 2 min intervals during control AF. The area of the mapped epicardial surface was ~14 cm².

**Endocardial optical mapping of the posterior left atrium (PLA) of the intact heart.**

A second LittleJoe CCD camera (80x80 pixels) was synchronized with the epicardial camera. A 10 mm diameter dual-channel rigid borescope (Everest VIT, Inc. Flanders, NJ, USA) with a 90-degree field of view was introduced through the anterior wall of the left ventricle, across the mitral valve and focused on the endocardial surface of the PLA (supplemental Figure 1). The optically mapped area on the PLA was ~3.7 cm², which allowed visualizing the four PVs and the atrial septo-pulmonary bundle. The borescope was c-mounted to the CCD camera through a custom-made eyepiece adapter. Laser excitation (532 nm) was delivered to the endocardium through a liquid light-guide (0.2 in core).¹ In 7 experiments the endocardial surface of the PLA was optically mapped.
**Experimental protocols.**

As shown in Supplemental Figure 2A, the intra-atrial pressure was adjusted to 14 cm H₂O at the beginning of the protocol. Baseline pacing at 300, 250 and 200 ms was applied from the LAA. We used burst pacing at 12 Hz to induce AF, which was allowed to continue for a control period of 15 min, after which either chloroquine (N=7; 4 µM) or flecainide (N=5; 2-4 µM) was added to the Tyrode’s solution. The drug concentration was doubled if AF persisted after 20 min under chloroquine or flecainide. A period of 50 min was allowed for AF termination to occur. AF was then terminated by DC shock (DFib) in those cases in which it persisted. AF re-induction was then attempted via burst pacing at 12 Hz, and was considered to be persistent when it lasted 15 min or longer after re-induction. Before washout a new DC shock (DFib) was used to restore the SR, if necessary. The washout period was 45 and 30 min for chloroquine and flecainide, respectively. Finally, after the washout, AF was re-induced by burst pacing and allowed to continue for 15 more min before the end of the protocol.

Supplemental Figure 2B illustrates the protocol of an additional set of optical mapping experiments (N=11) in which we determined the effects of chloroquine and flecainide on action potential duration and conduction velocity during pacing. Mapping and pacing were conducted on the epicardial surface of the LAA. The pacing protocol (300, 250 and 200 ms CL) was carried out at baseline, after 15 min of chloroquine (N=5) or flecainide (N=6), and after the washout. Representative electrograms are shown below the time-course of both protocols.
Atrial fibrillation dynamics.

Analysis of AF dynamics takes advantage of phase movies generated via Hilbert transformation. Patterns of activation allow identifying rotors and breakthroughs.

• A rotor was identified by the presence of all phases converging on a singularity point (SP) lasting more than one rotation. Supplemental Figure 3A shows snapshots of the PLA after generation of phase maps: a rotor pattern is identified lasting for 3 rotations; from frame 0 to frame 114.

• A breakthrough was defined as a wavefront appearing in the field of view and propagating outward in a target-like pattern. Supplemental Figure 3B shows a breakthrough pattern in the PLA after generation of phase maps.

Quantification of the core size.

During functional reentry, the perimeter of the center of rotation (the “core”) is inscribed by the trajectory of the rotating SP that is formed after a wavebreak. As it completes full rotations, the SP in fact becomes the rotor that organizes the overall reentrant activity. The core size and shape reflect critical parameters of the excitable medium that control the frequency and dynamics of a stationary rotor responsible for generating spiral waves (SW). Typically during AF in the sheep left atrium, the cores of identifiable stationary rotors are ellipsoidal and have an area of ~4 mm². However, most rotors that are observed during AF are non-stationary. In addition, particularly in the area with the highest frequency, multiple drifting rotors may form locally and then become extinct after highly variable lifetimes, which
makes it difficult to accurately quantify their properties. In addition, as they drift through the atrial muscle their cores no longer appear elliptical. Instead, their fingerprint in an amplitude map (see supplemental Figure 4)) is an extended dark band (i.e., a “line of block”) whose dimensions (width and length) depend on the velocity of impulse propagation, the speed of the core drift, and the lifespan of the individual rotor, and whose width provides an accurate measure of the core diameter. Here, we present a technique that allowed us to estimate the core widths of non-stationary rotors during control AF and during AF in the presence of chloroquine or flecainide (see Figure 5B and 6C in main manuscript). Our approach took advantage of the fact that during optical mapping of reentry the amplitude of the fluorescence signal at the core is appreciably lower than outside the core. Thus, as the rotor drifts into a camera pixel location, its core leaves a low fluorescence mark at that location. Supplemental Figures 4-6 illustrate this new method.

**Supplemental Results**

Supplemental Figure 7 shows the time-course of the $DF_{\text{max}}$, number of rotations/cm$^2$ (PLA/LAA) and number of breakthroughs/cm$^2$ during control AF and after the administration of either chloroquine (Panel A) or flecainide (Panel B). Two representative cases are shown; one under chloroquine and one under flecainide. In panel A, the $DF_{\text{max}}$ sharply decreases after chloroquine, along with the number of rotations/cm$^2$ and breakthroughs/cm$^2$. Few number of identifiable rotations/cm$^2$ are present before AF termination under chloroquine (Figure 7A, central panel). In panel B, $DF_{\text{max}}$ slightly decreases after flecainide. The number
of identifiable rotations/cm² and breakthroughs/cm² also decrease, however the effect is not as strong as the chloroquine effect and the AF does not terminates.

Additional experiments (N=5) were performed at 1 µM chloroquine. Such a concentration has less blocking effects on I_{Kr} (38%) and I_{Na} (14%), but I_{K1} is still highly blocked (74%). Supplemental Figure 8A shows how low doses of chloroquine (1 µM) were able to terminate AF in 4 out of 5 AF episodes. In one AF episode chloroquine concentration was increased to 2 µM to terminate the arrhythmia. In panel B, representative electrograms from the left superior PV (LSPV) show the restoration of SR under 1 µM chloroquine. In panel C, control DF_{max} was similar in all chloroquine groups, with a significant decrease in DF_{max} before restoration of SR. The DF_{max} decreased from 10.6±0.7 to 6.3±0.2 Hz in the first few minutes after 4 µM chloroquine, and from 10.5±0.8 Hz to 6.7±0.2 Hz after 1-2 µM chloroquine and before the resumption of the SR.
Supplemental References.

Supplemental Figure legends.

**Figure 1.** Diagrammatic representation of the experimental setup used to map optically and electrically from the endocardial and epicardial surfaces of the left atrium. Epicardial bipolar electrograms were obtained from the PVs in all experiments.

**Figure 2.** Time-course of the experimental protocols. A, protocol to study AF termination after flecainide and chloroquine. B, protocol to study the effects of chloroquine and flecainide on action potential duration and conduction velocity during pacing. AF: atrial fibrillation. CL: cycle length. DFib: Defibrillation. SR: sinus rhythm.

**Figure 3.** A, snapshots of the PLA after generation of phase maps: a rotor pattern is identified lasting for 3 rotations; from frame 0 to frame 114. B, a breakthrough pattern in the PLA; Wavefront appearing in the field of view and propagating outward in a target-like pattern.

**Figure 4.** Panel A shows a single CCD camera pixel recording to illustrate the first step needed to build the amplitude maps used to measure core width. The peak fluorescence recorded by each pixel during each excitation (black) is counted as a fluorescence step (red) whose magnitude is measured from the zero fluorescence line, after correction of the baseline shift (blue). Panel B shows representative sequential amplitude maps obtained by the 80x80 pixel camera during propagation of a fibrillatory wavefront around a line of block. The frame time is indicated on the upper right corner of each frame. At time 0, the wavefront started from the top right corner of the field of view (red star), with the red arrows showing the wavefront location. Between frames 0 and 64, the same wavefront circumnavigated the line
of block, almost completing a full rotation before the next wave appeared at frame 95 (blue arrows).

**Figure 5.** The figure was taken from the same AF episode as figure 4. Panel **A** shows on the right a set of single pixel recordings for 4 sequential excitations recorded along the vertical red line on the amplitude map of panel **B**. In **A**, the upper recordings marked in red correspond to a wavefront that moved downward and from left to right during frames 150-250 in the map; the lower recordings are marked blue. On the left of panel **A** is the consolidated 3D amplitude profile across the dark band for the blue signals at frames 150 and the red signals at frame 200. Note that in each case the amplitude of the recordings gradually decreased toward the center of the map forming a clearly visible dark band. This dark band closely associated with singularity points (SPs). In panel **C** the red spots represent all the SPs obtained during propagation of the red wave. All the SPs were located inside the dark band, which clearly demonstrates that such a band is the fingerprint of a drifting core.

**Figure 6.** **A and B,** to measure the width of a dark band (panel **A**) we first draw an isoamplitude line (IAL) at about 30% of the maximal wave amplitude (panel **B**) to mark the dark band perimeter. Then lines are drawn perpendicular to the IAL across the width of the dark band at each relevant pixel location. The length of each perpendicular is a measure of the local core width. **C and D,** lines that do not encounter an IAL on both sides of the dark band are automatically excluded (green in panel **C**), as are lines whose angle was significantly different from 90 degrees (blue in panels **C** and expanded inset in **D**). In panel **E,** the obtained local core band width is plotted versus the distance along the IAL, where point 0 corresponds to the tip of the band (circle on panel **B**) and the shape of the plot is relatively
symmetrical at either side of point 0. Thereafter, all the obtained values for any particular band are averaged and the result is considered to be the width of that particular core.

**Figure 7.** Two representative cases are shown. **A,** the $DF_{\text{max}}$ sharply decreases after chloroquine, along with the number of rotations/cm$^2$ and breakthroughs/cm$^2$. **B,** $DF_{\text{max}}$, the number of identifiable rotations/cm$^2$ and breakthroughs/cm$^2$ slightly decrease after flecainide (5-sec long movies were analyzed).

**Figure 8.** **A,** low doses of chloroquine (1 µM) were able to terminate AF in 4 out of 5 AF episodes. **B,** representative electrograms from the left superior PV (LSPV) show the restoration of SR under 1 µM chloroquine. **C,** control $DF_{\text{max}}$ was similar in all chloroquine groups, with a significant decrease in $DF_{\text{max}}$ before restoration of SR.

**Figure 9.** **A,** $B,$ single pixel action potential from the LAA at baseline stretch (black), after chloroquine 4µM (red) and flecainide 4 µM (blue). $APD_{70}$ significantly prolongs after chloroquine. Conversely, non-significant changes are present after flecainide.
Supplemental Movie legends.

**Movie 1.** Phase movie showing a long meandering rotor anchored to the roof/PLA-LAA junction after 40 min of flecainide.

**Movie 2.** Phase movie of the posterior left atrium; short-lasting rotors (1-2 rotations) are present in control AF (chloroquine protocol).

**Movie 3.** Same AF episode as in Movie 2 after chloroquine; very few rotors are identified during the last minute before AF termination.

**Movie 4.** Phase movie of the posterior left atrium during control AF (flecainide protocol).

**Movie 5.** Same AF episode as in Movie 4; although reentrant activity decreases still persists after 50 min under flecainide.
Supplemental Figures.

Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 6.
Figure 7.
Figure 8.

A

![Graph showing AF Termination rates with Chloroquine concentrations](image)

- **Percentage** of AF Termination:
  - 1 μM: 4/5
  - 4 μM: 7/7

B

![Electrocardiogram graph showing LSPV, AF, and SR with 1 μM chloroquine](image)

C

![Bar graph comparing DFmax before and after termination with Chloroquine](image)

- Control: Black column
- Before termination: Gray column
- Chloroquine concentrations: 1-2 μM, 4 μM

* *p < 0.05*
Figure 9.