Intracellular Chloride Accumulation and Subcellular Elemental Distribution During Atrial Fibrillation

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**Background**—Ion channel remodeling occurs during atrial fibrillation (AF); however, the extent of alteration in the subcellular distribution of elements (Na, K, Cl, Ca, Mg, P) is unknown. Electron probe microanalysis was used to determine the total (free+bound) in vivo subcellular concentration of these elements during AF.

**Methods and Results**—The left atrial appendage (LAA) was snap-frozen in situ after pacing (640 bpm) for 3 minutes (n=5 dogs), 30 minutes (n=3), or 48 hours (n=5). Dogs in sinus rhythm (n=3) served as controls. Whole-cell, cytosolic, and mitochondrial elemental concentrations were measured in cryosections. LAA effective refractory period (ERP) was measured before and after pacing. LAA ERP decreased significantly after 48 hours (116±3 to 88±10 ms, P=0.02). Whole-cell Cl increased by 9.0 mmol/L and 17 mmol/L after 3 and 30 minutes of pacing, respectively (P<0.0001), without a concomitant increase in Na. However, at 48 hours, whole-cell Na was reduced by 51% (P<0.01). Cytosolic Ca increased by 1.1 mmol/kg dry wt after 3 minutes (P<0.005), but mitochondrial Ca remained low and unchanged. Cell size measured in transverse cryosections increased after 3 minutes of pacing (75±5 to 109±11 μm^2, P=0.007) but returned to baseline by 30 minutes (66±5 μm^2).

**Conclusions**—Intracellular Cl accumulation induced by rapid pacing is a novel finding and may play a role in AF pathogenesis by causing resting membrane depolarization and ERP reduction. There was no evidence of cellular or mitochondrial Ca overload despite the development of electrical remodeling and transient increase in cytoplasmic Ca. ([Circulation. 2003;107:1810-1815.])

**Key Words:** fibrillation ■ electron probe microanalysis ■ chloride ■ calcium ■ pacing

Electrical remodeling of the atria occurs early in the course of atrial fibrillation (AF) and is manifested by shortened effective refractory periods (ERPs).^1^-^3^ Although the changes in ionic currents leading to electrical remodeling have been well studied, the cellular and subcellular concentrations and distribution of elements during AF remain unknown. It is not clear how AF alters the normal homeostasis of biological elements such as sodium (Na), potassium (K), chloride (Cl), phosphorus (P), magnesium (Mg), and calcium (Ca). Previous studies examining changes in ionic current density and/or ion fluxes have used isolated hearts or single myocytes; however, the cellular elemental composition as it exists in vivo during AF has not been determined. Electron probe microanalysis (EPMA) can directly quantify whole-cell, cytosolic, and mitochondrial concentrations of biological elements independently of their chemical environment and was used in conjunction with in situ freezing and cryoultramicrotomy to determine the in vivo subcellular distribution of elements during AF.  

Another goal of the present study was to address the role of intracellular Ca overload in the development of electrical remodeling. Calcium overload has been suggested to be involved in electrical remodeling largely on the basis of indirect evidence using L-type Ca-channel antagonists. Mitochondrial Ca accumulation has been implicated in the process of electrical remodeling because mitochondria can buffer large increases in intracellular Ca when cytoplasmic free Ca reaches pathologically high (micromolar) levels. However, under physiological conditions, total mitochondrial Ca is low (<1 mmol/kg dry wt) and does not increase significantly during systole, despite a Ca transient of approximately 500 to 600 nmol/L. Whether cytoplasmic Ca is sufficiently elevated during AF to result in mitochondrial Ca overload has not been directly assessed. Therefore, we used EPMA to determine the effect of AF on whole-cell, cytosolic, and mitochondrial Ca distribution during the development of atrial electrical remodeling.

**Methods**

**Animal Preparation**

All protocols conformed to the guidelines published in the “Position of the American Heart Association on Research Animal Use” and

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were approved by the Animal Research Committee at the University of Virginia Health Sciences Center.

Sixteen female mongrel dogs were induced with sodium pentothal (0.25 mg/kg) and intubated. Isoflurane (0.5% to 1.5%) and fentanyl were used for maintenance anesthesia. In 3 dogs, the left atrial tissue was harvested for EPMA without their undergoing rapid pacing (control group). The femoral vessels were accessed in the other 13 dogs, and a transseptal catheterization was performed under fluoroscopic guidance with a Brockenbrough needle and a 9.5F 60-cm sheath.

Electrophysiological Testing and Pacing Protocols

The left atrial appendage (LAA) ERP was determined with a standard ablation catheter (EP Technologies) by pacing at twice diastolic threshold with 8-beat drive trains at cycle lengths of 250, 300, and 350 ms, followed by a single extrastimulus. Subsequently, the dogs underwent rapid atrial pacing. In the short-term pacing groups, the dogs underwent pacing for 3 minutes (n=5 dogs) or 30 minutes (n=3 dogs) via a quadripolar catheter (EP Technologies) placed in the right atrial appendage. In the 48-hour pacing group (n=5 dogs), an active fixation atrial “J” permanent pacemaker lead (Medtronic Inc) was introduced into the right atrial appendage under fluoroscopic guidance, connected to a modified implantable pulse generator (St. Jude Corp or Medtronics Inc), and programmed to a rate of 640 bpm and an output of 2 to 3 times atrial diastolic threshold.

On cessation of pacing, a repeat electrophysiological study lasting <60 sec was performed in the 30-minute and 48-hour pacing groups. This was followed by resumption of pacing and subsequent LAA harvest for EPMA (see below). Dogs found to be in AF on cessation of pacing were allowed to remain in AF and proceeded directly to tissue harvest.

Tissue Acquisition

A left lateral thoracotomy was performed, and the LAA was exposed and frozen in situ during sinus rhythm (control group) or on completion of the pacing protocols while the atria were fibrillating (paced groups). A liquid nitrogen–cooled gun with a polished copper anvil tip (PS1000, Delaware Diamond Knives, Inc) was used for in situ snap-freezing. The specimens were immediately stored in liquid nitrogen.

Cryosectioning

Frozen tissue was sectioned at 70- to 80-nm thickness at −130°C with a Reichert Ultracut S with an FCS Cryokit (Leica). The specimen was oriented for transverse sectioning by mounting on the microscope chuck with a mixture of ethanol and 2-propanol (1:3), which serves as a cryogluce. Unstained sections were transferred to 10-μm-thick Pioloform support film generously donated by Wacher Co, Adrian, Mich, on 200-mesh copper thin bar grids, freeze-dried at 2×10−6 mm Hg, and lightly coated with carbon.7

Electron Probe Microanalysis

EPMA is based on the generation of x-ray spectra by atomic core shell excitation of elements in a given cellular microvolume, along with the simultaneous measurement of the mass of this microvolume by detection of continuum (background) x-rays. The ratio between the characteristic x-ray count of a certain element and the continuum x-ray count is thus proportional to the concentration of that element in the microvolume being analyzed.8,11 The accuracy of this technique in measuring subcellular element concentrations has been well documented.8

EPMA was performed with a Phillips CM12 electron microscope operated at 120 keV in transmission mode and fitted with a LaB6 filament. The microscope was equipped with an ultrathin window energy-dispersive x-ray detector and an XPS pulse processor (Oxford Link) interfaced to a 4 pi Spectral Engine. X-ray spectra were collected and displayed at 2.5 eV per channel. Specimens were viewed and analyzed at −100°C to reduce contamination and radiation damage.

Potassium and Ca concentrations were measured by use of a program that directly fits the unfiltered spectra to known standards. This method reduces the fitting uncertainty and optimizes the precision of Ca measurement.11 Elements other than K and Ca were quantified by traditional filtered fit methods.7,8 Because of the requirement for sensitive Ca concentration measurements, spectra were collected until the SD of an individual Ca concentration measurement was <1.4 mmol/kg dry weight. Typical spectral acquisition time was ~45 minutes.

Electrophysiological Changes

Figure 1 shows changes in LAA ERPs after 30 minutes and 48 hours of rapid pacing. Although mean ERP decreased from 93±6 to 80±10 ms after 30 minutes, this did not reach statistical significance because of the small number of dogs. However, after 48 hours of pacing, the mean ERP decreased from 116±3 to 88±10 ms (P=0.02), consistent with the development of electrical remodeling.

Measurement of Cell Size

Electron micrographs of cryosections from the different groups were obtained in transmission mode at 3000× magnification. The cross-sectional surface area of transversely sectioned myocytes was measured. Longitudinally oriented cells were excluded.

Data Analysis

Statistical analysis and comparisons among groups were performed with ANOVA and paired 2-tailed Student’s t tests. Results were expressed as the mean±SEM. Statistical significance was defined as P<0.05.

Results

Electrophysiological Changes

Figure 1 shows changes in LAA ERPs after 30 minutes and 48 hours of rapid pacing. Although mean ERP decreased from 93±6 to 80±10 ms after 30 minutes, this did not reach statistical significance because of the small number of dogs. However, after 48 hours of pacing, the mean ERP decreased from 116±3 to 88±10 ms (P=0.02), consistent with the development of electrical remodeling.

Electron Probe Microanalysis

EPMA was performed on multiple cells from the sinus rhythm (n=28), 3-minute pacing (n=53), 30-minute pacing (n=32), and the 48-hour pacing (n=21) groups. Figure 2 shows typical longitudinal (left) and transverse (middle) cryosections of myocytes used for EPMA. Within each cell, the beam was focused over the whole-cell (excluding nuclei), cytosol (excluding mitochondria and nuclei), or individual mitochondria. An average of 1 to 2 whole-cell, 1 to 2 cytosolic, and 2 to 3 mitochondrial measurements were made per cell. An example of a typical EPMA spectrum and
changes in Cl counts after 3 minutes of pacing are shown in Figure 2.

Changes in Subcellular Na and Cl Concentrations
Changes in the subcellular Cl concentrations (in mmol/kg dry wt) with pacing are shown in Figure 3 (top). Cytosolic Cl concentrations increased from 89 ± 8 during sinus rhythm to 143 ± 10 after 3-minute pacing (P = 0.0001) and 151 ± 19 after 30-minute pacing (P = 0.005). After 48-hour pacing, cytosolic Cl remained elevated (129 ± 9, P = 0.002) compared with controls. On the basis of 80% cell hydration, the increase in cytosolic Cl concentration is equivalent to 14 mmol/L after 3-minute and 16 mmol/L after 30-minute pacing. Whole-cell Cl followed a similar pattern, rising by 9.0 mmol/L after 3-minute (P = 0.0001) and 17 mmol/L after 30-minute (P < 0.0001) pacing.

The changes in Na concentrations are shown in Figure 3, bottom. A trend toward increased cytosolic Na after 3- and 30-minute pacing did not reach statistical significance. However, after 48 hours of pacing, whole-cell, cytosolic, and mitochondrial Na concentrations were reduced by 51% (P < 0.001), 34% (P < 0.02), and 60% (P < 0.005), respectively, compared with sinus rhythm.

Potassium, Phosphorus, and Magnesium Concentrations
There was a trend toward decreased cytosolic K after 3 and 30 minutes that did not reach statistical significance and paralleled the increase in cytosolic Na (Figure 4, top). Otherwise, there was no change in whole-cell or mitochondrial K with pacing. Similarly, Mg concentrations did not differ among the pacing groups (Figure 5, top). Phosphorus concentrations also remained stable with pacing (Figure 4, bottom), although mitochondrial P content was significantly higher than whole-cell and cytosolic levels in all groups, reflecting the phospholipid-rich composition of mitochondrial membranes and the abundance of organophosphates.

Changes in Subcellular Ca Distribution
EPMA provides an accurate measure of total (free + bound) Ca. Figure 5, bottom, depicts the subcellular total Ca distribution in the different groups. In the control group, the mean mitochondrial Ca level (in mmol/kg dry wt) was low (0.6 ± 0.2) and significantly less than mean whole-cell

Figure 2. EPMA was performed on whole-cell, cytosol (Cyto), and individual mitochondria (magnification, 20,000–25,000×). Left, Longitudinal cryosection of a myocyte, with a central large oval nucleus, Z-bands, and multiple dark mitochondria. Middle, Cross sections of 2 myocytes, including some extracellular matrix in lower right quadrant. Right, Example of a typical EPMA spectrum. inset shows an example of changes in Cl counts after 3 minutes of pacing.

Figure 3. Top, Intracellular Cl accumulation after rapid pacing. Whole-cell and cytosolic (Cyto) Cl concentrations (mmol/kg dry wt ± SEM) increased significantly after 3-minute pacing and remained elevated after 48 hours (P < 0.003 vs sinus rhythm). Assuming 80% cytosolic hydration, increase in whole-cell Cl is equivalent to 9.0 mmol/L after 3 minutes (P = 0.0001) and 17 mmol/L after 30 minutes of pacing (P < 0.0001). Bottom, Changes in subcellular Na concentrations after rapid pacing. A trend toward increased cytosolic Na after 3- and 30-minute pacing did not reach statistical significance. However, after 48 hours of pacing, whole-cell, cytosolic, and mitochondrial (Mito) Na were significantly reduced (P < 0.02 vs sinus rhythm).
Potassium

Phosphorous

Calcium

Magnesium

![Graphs showing subcellular distribution of K, Mg, P, and Ca after rapid pacing.](http://circ.ahajournals.org/)

**Figure 4.** Top, Subcellular distribution of K after rapid pacing. No significant change in whole-cell or mitochondrial (Mito) concentrations was observed among different groups. A trend toward decreased cytosolic (Cyto) K after 3- and 30-minute pacing did not reach statistical significance. Bottom, Subcellular distribution of P after rapid pacing. No significant difference among different pacing groups was observed. Mitochondrial P concentrations were higher than whole-cell and cytosolic P levels in all groups (*P*<0.0001) because of abundance of mitochondrial phospholipid membranes and organophosphates.

**Figure 5.** Top, Subcellular distribution of Mg after rapid pacing. No significant change in whole-cell, cytosolic (Cyto), or mitochondrial (Mito) Mg concentrations was observed among different groups. Bottom, Changes in Ca after rapid pacing. Ca concentrations (mmol/kg dry wt±SEM) represent total Ca (free+bound). Mitochondrial Ca was significantly lower than whole-cell and cytosolic Ca levels in all groups (*P*<0.01). Cytosolic Ca increased significantly after 3-minute pacing (*P*<0.005); however, mitochondrial Ca remained low and did not change with pacing (*P*=NS among all comparisons).

Changes in Myocyte Cross-Sectional Area

The mean cross-sectional myocyte area during sinus rhythm was 75.0±5.1 μm² (n=68 cells from 10 cryosections), increased to 108.7±11 μm² after 3-minute pacing (n=27 cells from 7 cryosections, *P*<0.008 compared with sinus rhythm), and subsequently decreased to 66.3±4.5 μm² after 30-minute pacing (n=59 cells from 10 cryosections, *P*=NS compared with sinus rhythm) (Figure 6).

Discussion

Although alterations in ion currents associated with electrical remodeling have been well studied,4,5 this is the first report of changes in the in vivo intracellular and subcellular distribution of elements during AF. EPMA, combined with in situ snap-freezing and cryoultramicrotomy, is a powerful approach that allows the accurate quantification of elements as they exist in vivo during a given pathophysiological condition.7-9,11 in this case AF.

Intracellular Chloride Accumulation

Early reports of 36Cl exchange in isolated atria suggested that 36Cl influx is greater than 36Cl efflux during AF.6 On the basis of 80% cytosolic hydration, we found that cytosolic Cl...
increased by 14 and 16 mmol/L after 3 and 30 minutes of rapid pacing, respectively, and whole-cell Cl content increased by 9.0 and 17 mmol/L, respectively. The slightly lower value of whole-cell compared with cytosolic Cl reflects the low Cl content of mitochondria included in the whole-cell volume measured. Although multiple cardiac Cl channels exist (reviewed in References 14 and 15 and references therein), only 2 are known to be present in the canine atrium: (1) the calcium-activated Cl channel ($I_{\text{Cl,Ca}}$) and (2) the swelling-activated Cl channel ($I_{\text{Cl,swell}}$). Therefore, it is likely, and may be of pathophysiological significance, that activation of one or both of these channels leads to Cl accumulation during AF. The side effects and low specificity of available Cl channel blockers unfortunately preclude a pharmacological identification of the specific Cl channel involved, especially when applied to intact organs.

$I_{\text{Cl,Ca}}$ amplitude increases with membrane depolarization and follows the time course of Ca transients. It promotes repolarization and contributes to APD shortening while predisposing to delayed early depolarizations and triggered activity. It may contribute to arrhythmogenesis by promoting reentry and reduction in conduction velocity. Although $I_{\text{Cl,Ca}}$ density was not significantly altered with rapid atrial pacing, it may contribute significantly to Cl accumulation during AF, given the 10-fold increase in atrial activation rate.

Another potential mechanism of Cl accumulation involves activation of $I_{\text{Cl,swell}}$, a current that is larger in the atrium than in the ventricle and that has been detected in cardiac tissue of all species examined, including canine and human. Activation of $I_{\text{Cl,swell}}$ causes resting membrane depolarization and APD shortening, and persistent $I_{\text{Cl,swell}}$ activation has been described in isolated canine ventricular myocytes after rapid pacing. The reversal potential ($E_{\text{Cl}}$) of $I_{\text{Cl,swell}}$ is -30 to -40 mV; thus, inward or outward currents may be generated depending on the membrane potential. On average, the membrane potential during AF is more depolarized (positive depending on the membrane potential. On average, the membrane potential during AF is more depolarized (positive

Subcellular Changes in Total Ca

Although Ca overload has been suggested to play a role in atrial electrical remodeling largely on the basis of indirect evidence, there is no direct evidence that long-term pacing causes cellular Ca overload. The present study is the first to quantify in vivo subcellular Ca distribution during AF. After 3-minute pacing, mean total cytoplasmic Ca increased by 1.1 mmol/kg dry wt, which, given a total free Ca ratio of $\sim 500:1$, represents an increase of $\sim 550$ nmol/L of free Ca. This is consistent with reports showing elevations in free Ca after short-term stimulation and also with the atria maintaining pulsatile contractility during AF without going into contracture. Physiological elevation of cytoplasmic Ca does not reach levels known to cause mitochondrial Ca loading, and we found no evidence of mitochondrial Ca overload after pacing.

Our findings also support the hypothesis that $I_{\text{Ca,L}}$ reduction and electrical remodeling develop as a protective mechanism against rapid activation-induced calcium overload and show that normalization of total intracellular Ca begins within 30 minutes of pacing and is complete by 48 hours. This time course is significantly shorter than has previously been reported and parallels that of electrical remodeling.

Role of Stretch and Neurohormonal Factors

Increased atrial pressure and dilation probably predispose to the development of AF via multiple different mechanisms, including cytoskeletal-dependent modulation of ionic currents. Direct transmission of transatrial force via stretch-activated cation channels may play an important role in promoting AF, and inhibition of these channels reduces AF vulnerability. Furthermore, increased atrial size and pressure may modulate myocyte volume and cellular elemental concentrations via neurohormonal mechanisms. Atrial natriuretic factor reduces atrial myocyte volume by inhibiting Na/K/Cl cotransport and inhibits Na-H exchange in vascular smooth muscle cells. Inhibition of Na-H exchange has been shown to prevent pacing-induced atrial electrical remodeling.
However, atrial natriuretic factor secretion measured at 24 to 48 hours of AF is not increased, and left atrial volume, pressure, and wall stress are not significantly altered. Thus, it is unlikely that stretch-activated channels or alterations in atrial natriuretic factor secretion played a significant role in the elemental changes observed in this study.

Conclusions
Previously unrecognized accumulation of intracellular Cl, probably a result of activation of $I_{Cl,p}$ and $I_{Cl,swell}$, occurs during AF and may play a role in its pathogenesis by causing resting membrane depolarization and ERP reduction. The exact mechanism of pacing-induced Cl accumulation warrants further evaluation and may lead to the development of novel therapeutic agents for the treatment of AF.

Despite the transient increase in cytosolic Ca levels, mitochondrial total (free + bound) Ca remained low during AF. Intrapcellular Ca removal mechanisms leading to electrical remodeling operate with a time course significantly shorter than previously reported, thereby maintaining Ca homeostasis and preventing overload.

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