Mechanisms by Which Adenosine Restores Conduction in Dormant Canine Pulmonary Veins

Tomás Datino, MD, PhD; Laurent Macle, MD; Xiao-Yan Qi, PhD; Ange Maguy, PhD; Philippe Comtois, PhD; Denis Chartier, BSc; Peter G. Guerra, MD; Angel Arenal, MD; Francisco Fernández-Avilés, MD, PhD; Stanley Nattel, MD

Background—Adenosine acutely reconnects pulmonary veins (PVs) after radiofrequency application, revealing “dormant conduction” and identifying PVs at risk of reconnection, but the underlying mechanisms are unknown.

Methods and Results—Canine PV and left-atrial (LA) action potentials were recorded with standard microelectrodes and ionic currents with whole-cell patch clamp before and after adenosine perfusion. PVs were isolated with radiofrequency current application in coronary-perfused LA-PV preparations. Adenosine abbreviated action potential duration similarly in PV and LA but significantly hyperpolarized resting potential (by 3.9±0.5%; P<0.05) and increased dV/dt max (by 34±10%) only in PV. Increased dV/dt max was not due to direct effects on I Na, which was reduced similarly by adenosine in LA and PV but correlated with resting-potential hyperpolarization (r=0.80). Adenosine induced larger inward rectifier K+ current (I KAdo) in PV (eg, −2.28±0.04 pA/pF; −100 mV) versus LA (−1.28±0.16 pA/pF). Radiofrequency ablation isolated PVs by depolarizing resting potential to voltages positive to −60 mV. Adenosine restored conduction in 5 dormant PVs, which had significantly more negative resting potentials (−57±6 mV) versus nondormant (−46±5 mV, n=6; P<0.001) before adenosine. Adenosine hyperpolarized both, but more negative resting-potential values after adenosine in dormant PVs (−66±6 mV versus −56±6 mV in nondormant; P<0.001) were sufficient to restore excitability. Adenosine effects on resting potential and conduction reversed on washout. Spontaneous recovery of conduction occurring in dormant PVs after 30 to 60 minutes was predicted by the adenosine response.

Conclusions—Adenosine selectively hyperpolarizes canine PVs by increasing I KAdo. PVs with dormant conduction show less radiofrequency-induced depolarization than nondormant veins, allowing adenosine-induced hyperpolarization to restore excitability by removing voltage-dependent I Na inactivation and explaining the restoration of conduction in dormant PVs. (Circulation. 2010;121:963-972.)

Key Words: arrhythmia ablation ■ adenosine ■ atrium ■ conduction ■ electrophysiology ■ ion channels

Pulmonary vein isolation (PVI) is an effective treatment for atrial fibrillation (AF). Nevertheless, many patients require repeated ablation procedures because of AF recurrence, which in most cases are associated with reconnection of previously isolated PVs. It has recently been noted that intravenous purinergic agonists such as adenosine can transiently restore conduction through a previously isolated PV, a phenomenon called “dormant conduction.” The demonstration of dormant conduction has predictive value for eventual reconnection, and additional radiofrequency (RF) applications to veins showing dormant conduction at the time of initial PVI may prevent reconnection and AF recurrence. The mechanisms by which adenosine restores conduction to dormant PVs are unknown. The objectives of this study were to (1) explore the effects of adenosine on ionic currents and action potentials (APs) in canine left-atrial (LA) and PV cardiomyocytes, and (2) relate these effects to changes in conduction between the PV and LA after RF ablation in an in vitro model.

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Materials and Methods
See the online-only Data Supplement for the complete Materials and Methods section. The following text summarizes the complete section.

Animals and Tissues
Forty-seven adult mongrel dogs were anesthetized with pentobarbital (30 mg kg−1 intravenously) and artificially ventilated. Hearts were excised and immersed in oxygenated Tyrode solution.
For standard microelectrode experiments, intact tissue preparations, including LA and PVs, were perfused via the left-circumflex coronary artery with oxygenated Krebs solution at 35°±0.5°C, and APs were recorded as previously described.9 Measurements included resting membrane potential (RMP), AP amplitude, and AP duration (APD) at 90% of repolarization (APD90). Conduction time was measured during LA pacing between peaks of differentiated (dV/dt) signals for APs recorded with 2 stable microelectrodes 1 cm apart, one in a PV and the other in the closest adjacent LA region. Cell isolation was performed as previously described.10,11 After isolation, cells were stored (4°C) and studied within 12 hours.

RF-Induced PVI
PVI was conducted in LA-PV preparations subjected to microelectrode AP recordings. One PV was isolated in each dog by ablating in the antral region as close as possible to the PV-LA junction. Bipolar electrodes were attached to the epicardial surface of the LA and target PV to evaluate PV-LA conduction. RF energy was delivered epicardially in the unipolar mode between the standard 4-mm tip of a 7F quadrupolar ablation catheter and an indifferent peripheral electrode, with a power limit of 25 to 35 W, for only 10 seconds at each site to control damage. The endpoint of PVI was a bidirectional conduction block (both from LA to PV and from the PV at 4 different quadrants to LA, with pacing stimuli up to maximum possible stimulation strength).

When PVI was achieved, APs were recorded in PV sleeves immediately above the ablation line for 15 minutes after the final RF application. If conduction recovered, then additional RF was delivered to complete PVI. Adenosine was then added, and APs were recorded for an additional 15 minutes in 11 preparations (6 left superior and 5 left inferior PVs). In preparations that recovered conduction with adenosine, APs were recorded during a final 15-minute adenosine washout period. In 6 preparations (5 left superior and 1 left inferior PVs) studied to establish the time course of spontaneous changes after PVI, the same PVI protocol was conducted without subsequently adding adenosine, and preparations were monitored for up to 4 hours (average monitoring time 3.4±0.3 hours). In 9 other preparations (6 left superior and 3 left inferior PVs), prolonged monitoring was obtained after adenosine washout to evaluate whether adenosine reconnection predicts subsequent spontaneous reconnection.

Adenosine Receptor Protein Expression
Protein-enriched fractions were obtained from cardiomyocytes isolated as described above, with paired PV and LA samples from each of 5 dogs studied. Antiadenosine A1- and A2A-receptor antibodies were obtained from ABCAM (ab3460 and ab3461); antiadenosine A3R- and A7-receptor antibodies (A2bR23 and A3R32) were from Alpha Diagnostic (San Antonio, TX). Donkey anti-rabbit secondary antibodies conjugated to horseradish peroxidase were used for detection. Signals for APs recorded with 2 stable microelectrodes were averaged to obtain single representative values for that dog for statistical comparison of distribution (Kolmogrov-Smirnov) except for conduction time. The effect of drug on conduction time was studied with Wilcoxon signed rank test because it was not normally distributed. Pearson correlation coefficients were used to study the relation between RMP and dV/dtmax. Fisher exact test was used to compare dormant-conduction prevalence in different PVs. Whenever more than 1 cell was obtained per region for a dog, all of the cell values in each region were averaged to obtain single representative values for that dog for statistical comparison to avoid weighting values unevenly for different dogs. A 2-tailed P<0.05 was considered statistically significant. The authors had full access to and take responsibility for the integrity of the data. All of the authors have read and agree to the article as written.

Results

AP and Conduction-Time Changes
AP effects of adenosine were explored in 6 dogs. AP recordings before and after adenosine are illustrated in Figure 1 (Top). Adenosine shortened APD in both PV (Figure 1A) and LA (Figure 1B) cells but significantly hyperpolarized RMP and increased dV/dtmax only in PV cells. Complete results and statistics are provided in Table 1, and mean data are illustrated in Figure 1 (bottom). In PV sleeves but not in LA, adenosine significantly increased RMP (Figure 1C) and dV/dtmax (Figure 1D). Adenosine abbreviated APD similarly in PV and LA (Figure 1E). AP amplitude was increased only in PV (Figure 1F). RMP and dV/dtmax correlated (Figure 2A), with similar and strong correlation for PV data alone (Figure 2B) and LA data alone (Figure 2C), consistent with known voltage dependence of INa availability. Adenosine also reduced conduction time between LA and PV electrodes from 17.4±3.2 to 14.9±3.1 ms (P=0.031).

Adenosine-Induced Ion-Current Changes
IKAdo recordings from a PV and LA cardiomyocyte are illustrated in Figure 3A. Larger adenosine-induced K+ currents were consistently seen in PVs. Overall results are shown in Figure 3B. The reversal potential was approximately −70 mV (when corrected for the 15-mV mean junction potential). Location was a highly significant determinant of IKAdo, which was larger in PV than LA cardiomyocytes. For example, at −100 mV, IKAdo averaged −2.28±0.04 pA/pF in PV cells versus −1.28±0.16 pA/pF in LA cells. IKAdo recordings from LA and PV cardiomyocytes are shown in Figure 4A and 4B. Panels labeled “a” show control currents, panels labeled “b” show currents from the same cells in the presence of adenosine, and panels labeled “c” show corresponding current-voltage relations. Overall, aden-
Adenosine reduced peak $I_{\text{Na}}$ by 26±4% in PV cells versus 26±8% in LA cells ($P=\text{NS}$). Washout resulted in up to 99.8% reversal of effect, indicating that the response was due to adenosine. These results indicate that the adenosine-induced $\frac{dV}{dt}$ increases in PV cardiomyocytes are not due to direct $I_{\text{Na}}$-enhancing effects.

### Recordings After PVI

Figure 5A shows a typical LA-PV preparation, with the left superior PV attached, before PVI. Figure 5B shows the same preparation after PVI. Figure 5C shows PV and LA electrograms during PV pacing before PVI (before ablation) and recordings from the same sites after PVI (after ablation). Adenosine led to restoration of 1:1 PV-LA conduction. Adenosine washout was followed initially by loss of 1:1 conduction and then by complete PV-LA conduction block. Figure 5D shows corresponding recordings from a vein without dormant conduction. In contrast to the dormant vein, adenosine did not restore PV-LA conduction. Adenosine revealed dormant conduction in 5 of 11 PVs (46%). Reconnection with adenosine occurred after 1 minute of perfusion initiation in 2 cases and after 2, 4, and 8 minutes in the other 3 dormant PVs. Reconnection with adenosine was neither related to the type of PV (3 of 5 left inferior PVs recovered conduction versus 2 of 6 left superior; $P=0.567$), nor to the total RF time required to achieve PVI (57±21 seconds in dormant and 54±21 seconds in nondormant PVs; $P=0.925$).

Microelectrode recordings just above the ablation line in a PV without dormant conduction are illustrated in Figure 6A and in a PV with dormant conduction in Figure 6B. RF-induced PVI depolarized RMP, causing inexcitability. Adenosine hyperpolarized RMP comparably (by $\approx 10 \text{ mV}$) in PVs without (Figure 6C) or with (Figure 6D) dormant conduction. However, PVs with dormant conduction had significantly

### Table 1. Effects of Adenosine on APs in 6 Preparations

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<tr>
<td></td>
<td>Control</td>
<td>Adenosine</td>
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<tr>
<td>$APD_{90}$</td>
<td>155.5±22.1</td>
<td>125.5±26.7***</td>
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<tr>
<td>APA</td>
<td>90.3±4.0</td>
<td>96.3±5.1*</td>
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<td>RMP</td>
<td>−70.2±2.1</td>
<td>−72.9±2.2***</td>
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<td>$\frac{dV}{dt}_{\text{max}}$</td>
<td>71.9±17.3</td>
<td>95.8±22.1**</td>
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$n=6$ for all comparisons. *$P<0.05$, **$P<0.01$, ***$P<0.001$, for adenosine (1 mmol/L) versus drug-free control. †Significant interaction indicates location-dependent effect of adenosine. APA=AP amplitude (in mV); $APD_{90}$=APD at 90% repolarization (in ms); NS=not significant; RMP=resting membrane potential (in mV); $\frac{dV}{dt}_{\text{max}}$=maximum rate of change of voltage during phase 0 (in V/s).
more negative RMPs before adenosine exposure (−57±6 versus −46±5 mV in nondormant veins; *P*<0.001), so that after adenosine-induced hyperpolarization, the RMP in dormant veins became negative to −60 mV (mean −66±6 mV), whereas in nondormant veins the RMP remained more depolarized (−56±6 mV; *P*<0.001). Adenosine effects in dormant PVs disappeared on washout, with RMP depolarization and return of block in 4 of the 5 PVs with dormant conduction (80%).

To examine another agent sometimes used to reveal dormant conduction, we administered isoproterenol (1 μmol/L) to 5 veins subjected to PVI. Figure I in the online-only Data Supplement compares spontaneous RMP changes after PVI (panel A), changes caused by adenosine (panel B), and those occurring with isoproterenol (panel C). Although hyperpolarization occurred with isoproterenol, its magnitude was significantly smaller than with adenosine (panel D), and no cases of isoproterenol-induced reconnection occurred.

**Time Course of RMP Changes After PVI**

To assess the time course of spontaneous RMP changes after PVI (in the absence of adenosine), RF ablation was applied in 6 preparations, and APs were then recorded over time during continued perfusion. In these PVs, RMP averaged −73±2 mV before ablation versus −49±4 mV after ablation (*P*<0.001).

No statistically significant hyperpolarization was seen from 15 to 25 minutes after ablation (Figure 7A), in contrast to the clear hyperpolarization after adenosine administration during the same interval (Figure 7B). Statistically significant spontaneous hyperpolarization began after 30 minutes and progressed slowly thereafter (Figure 7C). Spontaneous reconnection occurred in 2 of 6 PVs (33%), 1 at 34 minutes, and the other at 51 minutes after PVI. These data suggest that spontaneous recovery of RMP occurs gradually after PVI and can lead to reconnection. However, the time course of such changes in the absence of adenosine is slow compared with the rapid hyperpolarization and reconnection of dormant veins seen after adenosine administration.

We then assessed whether the response to adenosine could predict spontaneous reconnection. Adenosine reconnection occurred in 6 of 9 additional PVI preparations monitored for 90 minutes after adenosine perfusion and washout (within 1 minute of adenosine perfusion in 2, 2 minutes in 1, 5 minutes in 2, and 10 minutes in 1). Adenosine hyperpolarized RMP in these veins (Figure 7D) with reversal of hyperpolarization, and return of block in 5 of 6 PVs, on adenosine washout. Thereafter, slow spontaneous hyperpolarization followed, and 4 of 5 veins reconnected after 10 to 40 minutes. In all 3 PVs that failed to reconnect with adenosine, no spontaneous late reconnection occurred. Thus, 5 of 6 PVs (83%) with adenosine-exposed dormant conduction showed reconnection, versus none of 3 PVs (0%) without adenosine-exposed dormant conduction (*P*=0.048).

**Adenosine-Receptor Expression**

Figure 8 shows expression data for adenosine receptor types 1, 2A, 2B, and 3. No PV-LA differences were seen.

**Discussion**

In the present study, we assessed the mechanisms by which adenosine restores conduction to PVs that are isolated by RF ablation. We found that adenosine selectively hyperpolarizes PV cardiomyocytes and elicits larger *I*$_{K_{Ado}}$ in PV cells versus LA cells. Furthermore, we found that RF-induced PVI polarizes PV cells to voltages positive to −60 mV, at which Na+ channels are known to be inactivated, thereby inducing inexcitability. By hyperpolarizing cells to voltages negative to −60 mV, adenosine restores excitability to dormant PVs.
Ionic Mechanisms of Adenosine Action on PVs

The majority of the principal electrophysiological effects of adenosine, including hyperpolarization and repolarization acceleration in atrial cells and conduction slowing and refractoriness prolongation in AV nodal tissues, are attributable to the activation of a G protein-coupled K⁺ current, $I_{K,\text{Ado}}$. This current is mediated by the same G protein system and coupled K⁺ channels (Kir3.1/3.4) as acetylcholine-regulated K⁺ current.

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Figure 4. Typical $I_{Na}$ recordings from an LA cell (A) and a PV cell (B), with results shown for one cell each before (a) and after (b) adenosine in the same cell and corresponding current-voltage relations (c). Similar results were obtained in 6 and 4 cells in PV and LA, respectively, from 4 dogs.

Figure 5. Photographs of a typical LA preparation used for pulmonary vein isolation and action potential recording with standard microelectrode techniques (A) before and (B) after isolation of the left superior pulmonary vein (LSPV). The ablated area in the antral region is indicated in B. C. Representative bipolar recordings from LA and PV regions during pacing from the PV in a preparation with dormant conduction. After ablation, there was complete PV-LA block. During adenosine perfusion, conduction to the LA reappeared and was maintained throughout adenosine perfusion, disappearing again with washout. D, Representative bipolar recordings in a preparation without dormant conduction, showing no recovery of conduction with adenosine. LAA, LA appendage; LIPV, left inferior PV; LSPV, left superior PV; LA-PE, LA pacing electrode; PV-PE, PV pacing electrode.
We found that adenosine reduced LA and PV APD but significantly hyperpolarized RMP only in PV cells. This differential action on RMP may have been due to 2 factors: (1) $I_{KAdo}$ was larger in PV than in LA cardiomyocytes, and (2) PV cells have smaller $I_{K1}$ (and therefore less negative RMPs) than LA cells, which would increase the membrane input resistance and increase the effect on transmembrane potential of a given amount of change in ionic current. A similar phenomenon may also account for the larger degree of adenosine-induced hyperpolarization in PVs after PVI ($\Delta$H1015 $\approx$ 3 mV), compared with the effect in PVs not subjected to PVI ($\approx$ 3 mV), because PVI causes substantial PV cell depolarization. The mechanistic basis for larger $I_{KAdo}$ in PVs is unclear, but interestingly constitutive $I_{KACH}$, which shares similar Kir3.1/3.4 ion-channel subunits and regulatory G protein pathways with $I_{KAdo}$, is also larger in PV than LA. These differences are not due to differential Kir3.x or inhibitory G protein expression, which are similar in PV and LA cells, nor is LA-PV differential adenosine receptor expression involved (Figure 8). Further work is needed to clarify the molecular basis of differential constitutive $I_{KACH}$ and $I_{KAdo}$ in PV versus LA cardiomyocytes.

Two mechanisms could account for the adenosine-induced $\Delta V/\Delta t_{max}$ increase in PV cells. The first possibility is removal of voltage-dependent $I_{Na}$ inactivation by membrane hyperpolarization, which is expected to be particularly important at the relatively depolarized RMP of PV cells. This idea is consistent with the correlation between RMP and $\Delta V/\Delta t_{max}$ shown in Figure 2. Another possibility would be direct adenosine-induced increases in $I_{Na}$. However, on direct measurement, adenosine reduced rather than increased $I_{Na}$. Acetylcholine inhibits $I_{Na}$ via inhibitory G protein–dependent mechanisms in the presence of adenylate-cyclase activation by isoproterenol or forskolin, an action that could presumably occur with adenosine because it activates the same inhibitory G proteins as cholinergic agonists.

**Clinical Significance of PV Reconnection**

The effectiveness of PVI to treat AF is well established. Considerable evidence supports the relationship between electric isolation of PVs and the success of catheter ablation procedures. The majority of the patients who fail an initial ablation procedure have resumption of PV conduction. The most convincing evidence for the crucial role of successful PV-LA disconnection in curing AF comes from reports that describe a dramatic difference in the PV reconnection rate between patients cured of AF (few of whom show recurrent PV conduction) and those with recurrences (most showing PV reconnection). Repeated procedures to ensure PVI significantly improve long-term outcomes in patients who have recurrent AF after an initial procedure.

Early spontaneous recurrence of PV conduction has been observed in 24% to 50% of isolated PVs after a waiting period of 9 months. This phenomenon suggests that the electrical isolation may not be permanent and that the PVs may reestablish conduction. The mechanisms underlying PV reconnection are not well understood, but several explanations have been proposed. One possibility is the development of new channels or changes in existing channels that allow for conduction to resume. Another possibility is the presence of gaps or discontinuities in the isolated PVs, which could allow for conduction to occur. Finally, the reconnection could be due to the presence of circulating circulating cells or molecules that facilitate conduction.

**Figure 6.** Left, Representative standard fine-tipped microelectrode recordings before and after pulmonary vein isolation in a PV without dormant conduction (A) and in a PV in which dormant conduction was observed (B). S, stimulus artifacts with no AP response. Right, Effects of adenosine on mean $\pm$ SEM RMP in PVs without (C) and with (D) dormant conduction ($n$ = 6 dogs without and 5 dogs with dormant conduction). **$P$ $<$ 0.01, ***$P$ $<$ 0.001, for individual group mean differences by Bonferroni-adjusted comparisons in the presence of significant group-by-region interaction.
period of 30 to 60 minutes. Additional RF lesions to ensure isolation of PVs showing acutely recovered conduction provides similar long-term AF control to that seen in patients without early reconnection and better AF control than in cases in which early reconnection was not explored. Based on these observations, some authors recommend a 60-minute waiting period after initial PVI to detect early recurrences of conduction. Similar to clinical observations, we noted that 33% of PVs reconnected spontaneously during the 30- to 60-minute window after PVI. Adenosine has been used to assess rapidly the ability of PVs to reconnect, with 25% to 36% of PVs showing acute reconnection on adenosine infusion and repeated RF delivery to adenosine-reconnected veins apparently improving long-term outcome.

Figure 7. Mean±SEM RMP recorded during 30 minutes after PVI without (A) and with (B) adenosine (ADO) perfusion and during 2 hours after PVI under control conditions (C) and in separate experiments (D) with 15-minute adenosine perfusion, followed by 90-minute follow-up after adenosine washout. *P<0.05, **P<0.001 versus 0 to 15 minutes for A–C, D; †, P<0.001 for adenosine versus 0 to 15 minutes by Bonferroni-adjusted t tests; §, P<0.001 for adenosine versus washout period 30 to 45 minutes by Bonferroni-adjusted t tests; ¥, P<0.001 for washout period 30 to 45 versus washout period 60 to 120 minutes by Bonferroni-adjusted t tests.

Figure 8. (A) A₁, (B) A₂A, (C) A₂B, and (D) A₃ adenosine receptor expression levels in LA versus PV cardiomyocytes. Top, Representative immunoblots obtained from paired LA and PV cardiomyocyte samples from 1 dog, hybridized with respective antibodies, and GAPDH protein-loading control. Bottom, Mean±SEM. GAPDH-normalized expression values (n=5 per group). No significant LA-PV differences were found. A₁R, A₂AR, A₂BR, A₃R=adenosine A₁, A₂A, A₂B, and A₃ receptors, respectively.
present study, we observed adenosine-induced reconnection in 46% of PVs. We noted that RF lesions caused PV conduction block in association with extreme membrane depolarization that produced cellular inexcitability. We also observed recovery of membrane potential rapidly on adenosine administration and more slowly in its absence to the point that excitability returned in some veins. Our study raises 2 potential explanations for the relationship between adenosine-induced restoration of excitability and long-term success of PVI. One is that adenosine simply mimics the hyperpolarizing effect that occurs spontaneously with time, allowing for more rapid identification of PVs in which spontaneous reconnection would be observed if they were followed long enough in the hours after the initial procedure. The second possible explanation is that PVs that are more strongly depolarized (and therefore fail to reconnect with adenosine) are more severely damaged and less likely eventually to recover conduction. Further work is indicated to define more clearly the relationship between acute and long-term PV reconnection and to understand better the significance of the extent of PV depolarization.

**Novelty and Potential Clinical Relevance**

Our study is the first to examine systematically the effects of adenosine on PV cellular electrophysiology, ion currents, conduction, and the response to PVI. Previously, authors have suggested that membrane hyperpolarization and APD shortening by adenosine may facilitate excitatory conduction.6,7 Adenosine-induced changes in autonomic tone have also been implicated2-25 but were clearly not involved in the present study because adenosine-induced reconnection was observed in the absence of autonomic innervation in our in vitro preparations. In humans, dormant conduction demonstrated during sinus rhythm could have been indirectly promoted by adenosine-induced sinus cycle-length prolongation if disconnection was due to rate-dependent LA-PV conduction block.8 However, in the present study, all preparations were continuously paced at 2 Hz, indicating that heart rate slowing is not necessary for adenosine-induced reconnection.

Tissue injury by RF ablation is recognized to be thermally mediated.26 Hyperthermia significantly changes cardiomyocyte electrophysiological properties, producing potentially reversible (but irreversible when more severe) membrane depolarization and loss of cellular excitability.27 This observation is consistent with our finding of a key role for membrane depolarization in the determination of conduction block and reconnection.

Our observations may have relevance for the design of improved approaches to identify PVs at risk of reconnection. The primary mechanism for adenosine effects on dormant conduction appears to be $I_{K\text{Ado}}$, activation in PV cardiomyocytes, which occurs via stimulation of adenosine type-1 (A1) receptors. Adenosine also stimulates A2A, A2B, and A3 receptors, which may not contribute to the drug’s effect on PV excitability and conduction, but rather to the drug’s adverse-effect profile.28 Novel A1 selective receptor agonists are presently under development. Some, such as tecadenoson, selodenoson, and PJ-875, are being developed for the treatment of supraventricular tachyarrhythmias.29,30 Theoretically, these drugs may have several advantages over adenosine, including more sustained and selective cardiac A1 receptor effect and fewer adverse effects (eg, hypotension, flushing, bronchostenosis, chest discomfort) in the assessment of PVs at risk of reconnection after PVI.

Our findings may also have relevance for understanding adenosine-induced proarrhythmia. Paroxysmal atrial fibrillation is associated with adenosine since they are well recognized to induce AF episodes in some patients after intravenous administration.31 One potential mechanism is acceleration and stabilization of atrial reentrant rotors by increased inward-rectifier current.32 However, another possibility, based on our studies, would be improved conduction through potential PV drivers that fail to induce AF in the absence of adenosine because of poor PV-LA coupling.

**Potential Limitations**

PVI was achieved with RF delivered epicardially to multicellular preparations. This technique is different from clinical practice, in which RF is delivered by endocardially positioned transvenous catheters. Because pulmonary veins are smaller in dogs versus humans, and because we applied RF energy directly to the epicardial surface, we limited RF applications to 25 to 35 W, for only 10 seconds at each site, to control damage. Although in humans RF applications are longer and with higher power, the end point is similar: complete electrical isolation of PVs from LA. In addition, we isolated single PVs with epicardial antral lesions adjacent to the PV-LA junction, different from the circumferential endocardial antral lesions most commonly created in humans. Nevertheless, we did observe conduction block between PV and LA and recovery with adenosine, with properties similar to those noted after PVI in humans.

Although we observed that adenosine acutely restores PV-LA conduction by hyperpolarizing PV cells and thereby enhancing Na$^+$-current availability, other potential mechanisms that we did not study could also be involved. RF-induced tissue edema or inflammation could be reversible and may not be well tracked in all cases by RMP changes. Reversible edema or inflammation could take longer to reverse than RMP, with eventual gaps developing in the RF-induced scar that allows recovery of conduction. Such recurrences may not be well predicted by the acute response to adenosine and might account for cases in which late reconnection occurs despite the absence of an acute adenosine response. Long-term in vivo studies of recurrent PV conduction in experimental models to assess mechanisms of restored PV conduction and the relationship to the acute adenosine response would be of interest.

Ionic currents are sensitive to cell-isolation technique. Great care was therefore taken to record the same ionic currents from similar numbers of PV and LA cells from each dog so that any influence of the isolation procedure would be equally distributed. When recordings could be obtained from cells of only one of PV or LA, they were rejected for analysis. Furthermore, ionic current recordings can change over time due to rundown. Therefore, all of the currents were recorded.
after the same time intervals and with protocols applied in the same order for both cell types.

There is wide species variability in adenosine sensitivity, and the dog is relatively insensitive,12 so we had to use a relatively high adenosine concentration (1 mmol/L) to reproducibly achieve significant effects. We chose to work with dogs because of the similarity of their PV cardiomyocytes to humans and because of the appropriateness of their PVs for RF isolation and conduction assessment. The electrophysiological effects and IKAdo properties we observed for adenosine were typical for the compound across a range of species.12,13,28,32 Nevertheless, the differential adenosine sensitivity of canine versus human atrium could affect the applicability of our results.

Conclusions

Adenosine selectively hyperpolarizes canine PV cardiomyocytes compared with LA cells, apparently by selectively increasing $I_{KAdo}$. Adenosine-induced hyperpolarization increases $dV/dt_{max}$ by removing voltage-dependent $I_{Na}$ inactivation. RF energy isolation of PVs is associated with severe depolarization, and adenosine significantly hyperpolarizes PV cells after PVI. RMP is less depolarized by RF application in PVs with dormant conduction versus PVs with fixed conduction block, allowing adenosine-induced hyperpolarization to voltages negative to $-60 \mathrm{mV}$ to restore excitability and PV-LA conduction by removing voltage-dependent $I_{Na}$ inactivation. The effects observed in our animal model could explain the restoration of conduction in damaged but viable PVs, thereby potentially accounting for the clinical phenomenon of adenosine-revealed dormant conduction.

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Disclosures

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References


**CLINICAL PERSPECTIVE**

Ablation procedures are becoming an increasingly important therapeutic option for atrial fibrillation, particularly the paroxysmal form. A major limitation to such procedures is arrhythmia recurrence, often attributable to recurrent pulmonary-vein conduction, or so-called “reconnection.” Intravenous adenosine administration at the time of an ablation procedure can cause acute pulmonary-vein reconnection, revealing “dormant” conduction, and is sometimes used as a test of the ability of veins to undergo later reconnection to guide additional ablation lesions. This experimental study aimed to clarify the unknown mechanism by which adenosine reveals dormant conduction. Arterially perfused canine left atrial/pulmonary vein preparations were exposed to adenosine and radiofrequency ablation during action-potential and extracellular-electrode monitoring. Adenosine alone selectively hyperpolarized pulmonary-vein cardiomyocytes sleeves, increased their phase 0 upstroke velocity, and accelerated pulmonary vein/left atrial conduction. Radiofrequency ablation to the antral region adjacent to the pulmonary vein/left atrial junction produced pulmonary vein/left atrial conduction block and depolarized pulmonary-vein cardiomyocytes near the ablation line. Adenosine caused acute reconnection by hyperpolarizing damaged pulmonary-vein cells sufficiently to restore excitability. Similar phenomena occurred spontaneously in some veins during a prolonged (mean 3-hour) observation period. Adenosine effects were reversible on washout, and adenosine-exposed dormant conduction was a good predictor of subsequent spontaneous reconnection in the same vein. Voltage-clamp experiments suggested that the primary ionic-current mediator of adenosine’s actions was adenosine-induced inward-rectifier potassium current. These studies provide insights into the mechanisms by which adenosine acutely restores pulmonary vein/left atrial conduction after radiofrequency ablation, with potential implications for our understanding of mechanisms of pulmonary-vein disconnection and reconnection.
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SUPPLEMENTAL MATERIAL
Expanded On-line Materials and Methods Section

Animals and Tissues

A total of 47 adult mongrel dogs of either sex weighing 18-37 kg were anaesthetized with pentobarbital (30 mg kg$^{-1}$ I.V.) and artificially ventilated. Hearts and adjacent lung tissues were excised via a left thoracotomy and immersed in oxygenated 2-mmol/L Ca$^{2+}$-containing Tyrode solution. Animal care procedures followed Canadian Council on Animal Care guidelines and were approved by the Animal Research Ethics Committee of the Montreal Heart Institute.

For standard microelectrode experiments, intact tissue preparations including the LA and adjacent PVs were mounted in a perfusion chamber and perfused via the left-circumflex coronary-artery with oxygenated Kreb’s solution at 35±0.5°C. Fine-tipped microelectrodes (resistance 15-20 MΩ when filled with 3-mol/L KCl) coupled to a high input-impedance amplifier were used to record action potentials (APs) as previously described.$^1$ A platinum bipolar electrode was used to stimulate the tissue at 2 Hz, with a pulse duration of 2 ms and twice diastolic threshold current-intensity. Measurements made from AP-recordings included: resting membrane potential (RMP, transmembrane potential at phase-0 onset or at steady-state in quiescent preparations), AP-amplitude and AP-duration (APD) at 90% of repolarization (APD$_{90}$). Conduction-time was measured during LA-pacing between the peaks of differentiated (dV/dt) signals for APs recorded with 2 stable microelectrodes ~1 cm apart, one in a PV and the other in the closest adjacent LA region.

For cell isolation, the circumflex coronary artery was cannulated and LA and distal ends of PV myocardial sleeves were marked with silk thread to facilitate
identification after enzymatic digestion, prior to ~60-minute enzyme perfusion with Ca\textsuperscript{2+}-free Tyrode solution containing collagenase (~0.5 mg/mL, CLSII, Worthington) and 0.1% bovine serum albumin (Sigma). Cell isolation was performed as previously described.\textsuperscript{2} PVs were well-perfused and single cardiomyocytes could be isolated from all PVs and from the LA. PV-cells were isolated as far distally from the LA-PV junction as possible. After isolation, cells were stored at 4°C and studied within 12 hours.

**RF-induced Isolation of PVs**

PVI was conducted in LA-PV preparations subjected to microelectrode AP-recordings. One PV was isolated in each dog, by ablating in the antral region as close as possible to the PV-LA junction. Bipolar electrodes were attached to the epicardial surface of the LA and target PV to evaluate continuously PV-LA conduction. RF-energy was delivered epicardially at the PV-LA junction in the unipolar mode between the standard 4-mm tip of a 7F quadripolar ablation catheter (Cordis-Webster) and an indifferent electrode in the periphery of the preparation, with a power limit of 25-35 W, for only 10 seconds at each site to control damage. The endpoint of PVI was the establishment of bidirectional conduction block (block both from LA to PV and from the PV at 4 different quadrants to LA, with pacing-stimuli up to maximum possible stimulation-strength).

When PVI had been achieved, APs were recorded in PV sleeves immediately above the ablation line for 15 minutes following the final RF-application. If conduction recovered, additional RF was delivered to complete PVI. Adenosine was then added and APs recorded for an additional 15 minutes in 11 preparations (6 left superior and 5 left inferior PVs). In preparations that recovered conduction with adenosine, APs were
recorded during a final 15-minute adenosine-washout period. In 6 preparations (5 left superior and 1 left inferior PVs) studied to establish the time-course of spontaneous changes post-PVI, the same PVI-protocol was conducted without subsequently adding adenosine and preparations were monitored for up to 4 hours (average monitoring time 3.4±0.3 hours). In 9 other preparations (6 left superior and 3 left inferior PVs), prolonged monitored was obtained following adenosine-washout to evaluate whether adenosine reconnection predicts subsequent spontaneous reconnection.

**Determination of adenosine-receptor protein-expression**

Protein-enriched fractions were obtained from cardiomyocytes isolated as described above, with paired PV and LA samples from each of 5 dogs studied. Snap-frozen cardiomyocyte-samples were homogenized in an extraction buffer containing (mmol/L): Tris 25, NaCl 150, EDTA 5, EGTA 5, NaF 20, Na₃VO₄ 0.2, Glycerol-2-phosphate 20, AEBSF 0.1; Microcystin-LR 1 μmol/L, leupeptin 25 μg/ml, aprotinin 10 μg/ml, pepstatin 1 μg/ml and Triton-X-100 1%. Homogenized samples were incubated 30 minutes on ice and centrifuged at 1000×g (10 minutes, 4°C) to pellet debris and nuclei. Supernatant corresponding to the protein-enriched fraction was collected and protein-concentration determined by Bradford assay (Biorad). Protein-samples (70-μg) were separated with sodium-dodecyl-sulfate/polyacrylamide-gel electrophoresis and transferred electrophoretically to polyvinylidene-fluoride transfer membranes. Membranes were blocked 1 hour with TTBS (Tris-HCl 50-mmol/L/NaCl 500-mmol/L, pH 7.5, 0.1%-Tween) containing 5% non-fat dried milk and incubated overnight with primary antibodies in TTBS/5% non-fat dried milk. Rabbit polyclonal anti-adenosine A₁ and A₂A-
receptor antibodies were obtained from ABCAM (ab3460 and ab3461). Rabbit polyclonal anti-adenosine A2B and A3-receptor antibodies were obtained from Alpha-Diagnostics (A2bR23 and A3R32). Primary antibodies were used at a dilution of 1/2000. A donkey anti-rabbit secondary antibody (minimal cross reactions; Jackson-Immunoresearch) conjugated to horseradish-peroxidase (HRP) was used for detection. Staining was revealed with chemiluminescence (Western Lightning Chemiluminescence Reagent Plus, Perkin-Elmer) and quantified with Quantity-One software (Biorad). All expression-data are provided relative to GAPDH-staining for the same samples on the same gels.

Solutions

The solution for cell storage contained (mmol/L): KCl 20, KH2PO4 10, dextrose 10, mannitol 40, L-glutamic acid 70, β-hydroxybutyrate 10, taurine 20, EGTA 10 and 0.1 % BSA (pH 7.3, KOH). Tyrode (extracellular) solution contained (mmol/L): NaCl 136, KCl 5.4, MgCl2 1, CaCl2 1, NaH2PO4 0.33, HEPES 5 and dextrose 10 (pH 7.35, NaOH). For K+-current recording CdCl2 (200-µmol/L), 4-aminopyridine (4AP, 2-mmol/L) and atropine (200-nmol/L) were added to suppress Ca2+-current, transient-outward and 4AP-dependent muscarinic K+-currents.11 The internal solution for K+-current recording contained (mmol/L): K-aspartate 110, KCl 20, MgCl2 1, MgATP 5, GTP (lithium salt) 0.1, HEPES 10, Na-phosphocreatine 5 and EGTA 10 (pH 7.3, KOH). Inward-rectifier K+-current was recorded upon stepping from -40 mV to voltages between -120 and +40 mV. Na+-current (INa) contamination was avoided by using a holding potential of -50 mV. Adenosine-dependent K+-current (IKado) was measured as the inward-rectifier
difference-current between recordings obtained before and after adenosine in each cell. For $I_{Na}$-recording, the external solution contained (mmol/L): NaCl 10, TEA-Cl 126, MgCl$_2$ 3.0, CsCl 5.4, HEPES 10, glucose 5.5 (pH 7.35, CsOH), 4AP 2. The internal solution contained (mmol/L): CsCl 120, TEA-Cl 20, MgCl$_2$ 1.0, HEPES 10, EGTA 10, MgATP 5, Li-GTP 0.1 (pH 7.2, CsOH). For standard microelectrode experiments, a solution containing (mmol/L): NaCl 120, KCl 4, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, NaHCO$_3$ 25, CaCl$_2$ 1.25 and dextrose 5 (95% O$_2$–5% CO$_2$, pH 7.4), was used to perfuse the tissue. Adenosine (Sigma Chemicals) was freshly prepared as a 1-mmol/L solution before each experiment.

**Electrophysiology Data Acquisition**

The whole-cell patch-clamp technique was used to record currents. Borosilicate glass electrodes (Sutter Instrument) filled with pipette solution were connected to a patch-clamp amplifier (Axopatch 200A, Axon). Electrodes had tip resistances of 1 to 4 MΩ, with pipettes of 1 to 2 MΩ used for $I_{Na}$-recording. Cell capacitance averaged 97±9 pF for PV and 99 ±7 pF for LA cardiomyocytes. Rs averaged 5.6±0.5 MΩ and the capacitive time-constant 552±56 µs before, and 2.9±0.3 MΩ and 279±18 µs after, compensation in LA; with corresponding values in PV of 5.8±0.5 MΩ and 575±59 µs before and 2.6±0.2 MΩ and 242±13 µs after compensation. Leakage-compensation was not used. Currents are expressed as densities (pA/pF). Junction potentials between bath and pipette solution averaged 15.0±0.7 mV and were not compensated. K$^+$-current recording was performed at 35±0.5°C. $I_{Na}$ was recorded at room temperature. The maximum phase-0 upstroke-
velocity (dV/dt<sub>max</sub>) of standard microelectrode recordings was determined by electronic differentiation.

**Statistical Analysis**

Data are expressed as mean±SEM. Repeated-measures 1-way ANOVA was used to compare RMP at different times after PVI. Repeated-measures 2-way ANOVA was used to study the interaction between location (PV vs LA) and adenosine for RMP, APD<sub>90</sub>, AP amplitude and dV/dt<sub>max</sub>, the interaction between test potential and treatment (adenosine versus no adenosine) for K<sup>+</sup>-current and I<sub>Na</sub>, and the interaction between test potential and location (PV vs LA) for I<sub>Kado</sub>. Bonferroni-adjusted comparisons were used to compared group means when ANOVA was significant. Two-way repeated-measures analysis was used to study interactions between dormant/non-dormant state and adenosine effect on RMP after PVI. An unpaired Student t-test was used to compare ablation time between dormant and non-dormant PVs and between control and adenosine-treated PVs. All data satisfied criteria for normality of distribution (Kolmogrov-Smirnov) except for conduction time. The effect of drug on conduction time was studied with Wilcoxon Signed Rank test since it was not normally distributed. Pearson correlation-coefficients were used to study the relation between RMP and dV/dt<sub>max</sub>. Fisher’s exact test was used to compare dormant-conduction prevalence in different PVs. Whenever values for more than one cell were obtained per region for a dog, all cell-values in each region were averaged to obtain single representative values for that dog for statistical comparison, to avoid weighting values unevenly for different dogs. For regression analyses between resting potential and V<sub>max</sub>, for which we were interested to know whether resting
potential values were a determinant of $V_{\text{max}}$ values for each individual cell, irrespective of the dog of origin, we did not average values on a per-dog basis. A two-tailed $P<0.05$ was considered statistically-significant. The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
References


**On-line Figure Legend**

**Figure I:** Mean±SEM resting membrane potential (RMP) recorded during 30 minutes after pulmonary vein isolation (PVI) without (A) and with (B) adenosine (ADO) perfusion, and with isoproterenol perfusion (C). *P<0.05 and ***P<0.001 vs 0-15 minutes, for individual group mean differences by Bonferroni-adjusted comparisons. D, Mean±SEM difference (RMPd) between mean RMP recorded during the 15 to 30 minute period (RMP 15-30) and RMP recorded during the 0 to 15 minute period (RMP 0-15). ***P<0.001 for individual group-mean differences by Bonferroni-adjusted t-tests.
**A. CONTROL (n=6)**

![Graph showing RMP changes over time with no drug and with RMP 15-30 and RMP 0-15 subtracted.]

**B. ADENOSINE (n=11)**

![Graph showing RMP changes over time with no drug and with RMP 15-30 and RMP 0-15 subtracted.]

**C. ISOPROTERENOL (n=5)**

![Graph showing RMP changes over time with no drug and with isoproterenol.]

**D. RMPd=(RMP 15-30)-(RMP 0-15)**

![Graph showing the difference in RMP between 15-30 and 0-15 with control, isoproterenol, and adenosine.]

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*On-line Figure I*