Thrombin Facilitation of Voltage-Gated Sodium Channel Activation in Human Cardiomyocytes
Implications for Ischemic Sodium Loading

Caroline Pinet, MSc; Bruno Le Grand, PhD; Gareth W. John, PhD; Alain Coulombe, PhD

Background—Thrombin plays a role in mediating ischemic injury and cardiac arrhythmias, but the mechanisms involved are poorly understood. Because voltage-gated sodium channels (VGSCs) have not previously been considered, putative effects of thrombin on VGSC function were investigated in human isolated cardiomyocytes.

Methods and Results—Sodium current (I\textsubscript{Na}) was recorded by the whole-cell patch-clamp method. Thrombin increased peak \( I_{\text{Na}} \) amplitude in an activity-dependent manner, from 1 to 100 U/mL, with an apparent EC\textsubscript{50} of 91±16 U/mL. When tested at 32 U/mL, thrombin-increased \( I_{\text{Na}} \) was abolished by tetrodotoxin (50 \( \mu \)mol/L). Thrombin effects on \( I_{\text{Na}} \) were reversible and repeatable, and 100 U/mL doubled peak \( I_{\text{Na}} \) amplitude. Thrombin (32 U/mL) shifted \( I_{\text{Na}} \) activation to hyperpolarized potentials without affecting steady-state inactivation, producing unusually large increases in window current. Hirudin (320 U/mL) or haloenol lactone suicide substrate (10 \( \mu \)mol/L) failed to significantly affect these effects of thrombin. In current-clamped cardiomyocytes, thrombin (32 U/mL) depolarized resting membrane potential by 10 mV.

Conclusions—Facilitation of VGSC activation causing large increases in window current is a major mechanism by which thrombin may promote ischemic sodium loading and injury. (Circulation. 2002;106:2098-2103.)

Key Words: ion channels • sodium • thrombosis • ischemia • myocytes

Besides its well-known role in the circulation during coagulation in cleaving fibrinogen, thrombin also exerts direct actions on cardiomyocytes.\textsuperscript{1} It has been postulated that the serine protease plays a significant role in mediating ischemic injury, malignant ventricular arrhythmias, and sudden death during acute thrombotic occlusion.\textsuperscript{2–6} However, the molecular mechanisms underlying these deleterious effects of thrombin are poorly understood.

In cardiomyocytes, thrombin elevates [Na\textsuperscript{+}], by Na\textsuperscript{+}/H\textsuperscript{+} exchange activation\textsuperscript{7} or via production of membrane-derived lysophosphatidylcholine.\textsuperscript{4,5,8} A possible involvement of voltage-gated sodium channels (VGSCs) in mediating thrombin-induced increases in [Na\textsuperscript{+}], has not, however, been previously considered. Increases in [Na\textsuperscript{+}], in ischemic cardiomyocytes generate Ca\textsuperscript{2+} loading via reverse Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange, leading to contractile dysfunction and ultimately cell death.\textsuperscript{9–11} Na\textsuperscript{+}/H\textsuperscript{+} exchange activity during ischemia is inactive or weak\textsuperscript{12} because the exchanger is subject to inhibition by extracellular acidosis,\textsuperscript{13} which suggests a more significant role for VGSCs as mediators of ischemic Na\textsuperscript{+} loading.

A large fraction of VGSCs become rapidly nonrecruitable in ischemic tissues after resting membrane potential depolarization, and action potentials (APs) initially shorten and subsequently cease with exhaustion of cellular ATP. Na\textsuperscript{+} influx continues, however, through noninactivated VGSCs, giving rise to persistent window currents\textsuperscript{14,15} and further cardiomyocyte depolarization. Moreover, the slowly inactivating component of Na\textsuperscript{+} current also increases substantially during ischemia, amplifying Na\textsuperscript{+} influx.\textsuperscript{15–18}

The putative effects of thrombin on VGSCs in human atrial myocytes were studied by the patch-clamp technique. The data demonstrate, for the first time, that thrombin acts as a powerful activator of human cardiac VGSCs, constituting a major mechanism by which the protease increases [Na\textsuperscript{+}], in ischemic cardiomyocytes.

Methods

Heart Tissue Samples

Protocols for obtaining human cardiac tissue were approved by the Hôpital Bicêtre, Université Paris XI Ethics Committee. Specimens of human right atrial appendages were obtained from hearts of patients (54 to 77 years of age) undergoing heart surgery for coronary artery bypass graft or valve replacement. Of the patients involved, 8% had received \( \beta \)-blockers, 38% calcium antagonists, 44% antilucr drugs, 55% analgesics, 10% diuretics, and 22% antithrombotics. Treat-
ments were usually stopped 24 hours before operation. Patients with atrial dilation were avoided, and none had a history of supraventricular arrhythmias.

Cell Isolation
Human atrial myocytes were isolated enzymatically as previously described. Only quiescent rod-shaped myocytes with clear cross-striations, sharp edges, and well-delineated cell membranes were chosen for experiments. Small myocytes were preferred to optimize spacial voltage-clamp.

Solutions and Drugs
For whole-cell current recordings, the intracellular pipette solution contained (in mmol/L): NaCl 5, CsF 130, MgCl2 2, CaCl2 1, EGTA 15, HEPES 10, and MgATP 4, pH 7.2 with CsOH. The external solution contained: NaCl 25, CsCl 105.8, CaCl2 2.5, MgCl2 2.5, 4-AP 5, HEPES 10, and glucose 10, pH 7.4 with CsOH. To record APs, myocytes were bathed in Tyrode’s solution (in mmol/L): NaCl 135, KCl 15, MgCl2 2, CaCl2 1.5, HEPES 10, NaH2PO4 0.5, Na-pyruvate 2.5, and glucose 20, pH 7.4 with NaOH. The pipette solution contained (in mmol/L): K-aspartate 115, NaCl 5, KCl 15, MgCl2 2, HEPES 10, glucose 10, MgATP 3, tris-phosphocreatine 5, and EGTA 5, pH 7.2 with KOH. Hirudin (leech, 2000 U/mg) was obtained from Boehringer Mannheim. Haloenol lactone suicide substrate (HELSS) from TEBU France was dissolved in dimethyl sulfoxide (DMSO) that did not exceeded 0.05% and thrombin (human plasma ~1000 U/mg). Other chemicals were purchased from Sigma.

Current and AP Recordings
Ionic currents were recorded with the whole-cell patch-clamp technique with a patch-clamp amplifier (Axopatch 200B, Axon Instruments). Patch pipettes (Corning Kovar Sealing code 7052, WPI) had resistances of 0.5 to 1 MΩ. Currents were filtered at 20 kHz (~3 dB, 8-pole low-pass Bessel filter) and digitized at 50 kHz (Digidata 1200, Axon Instruments).

Cell membrane capacitance was determined according to Montaz et al. Cell membrane capacitance was 47.9±2.4 pF (n=113 cells, from 68 donors). Series resistance was compensated at 80% to 95%, resulting in voltage errors of <3 mV, whereas neither cell membrane capacitive current nor leakage current was compensated. In sodium current (INa) recording media, the average holding current densities at a holding potential (HP) of −100 mV were −1.8±0.5 pA/pF in control, −1.9±0.4 pA/pF under 32 U/mL thrombin, −2.0±0.4 pA/pF under tetrodotoxin, and −1.9±0.4 pA/pF after washout (not significant respective to control [n=17]). Peak INa amplitude was measured according to a steady-state pulse protocol: a 300-ms depolarizing pulse to −30 mV from a HP of −100 mV at 0.2 Hz. An equilibration period was allowed until peak INa reached steady state and remained stable without evidence of a leftward shift of the availability-voltage relationship (hNa-VNa). This protocol was designed to detect any such shifts. After each sequence of 5 depolarizing pulses, HP was set to −140 mV. Consequently, during the stabilization period when peak current amplitude was higher after a HP at −140 mV compared with −100 mV, the recording was discarded. Similarly, because many cells showed no effect of thrombin on the hNa-VNa relationship, recordings showing an irreversible hyperpolarizing shift of hNa-VNa after thrombin application were also discarded.

The steady-state pulse protocol was applied before activation-VNa availability-VNa, and reactivation protocols in control, thrombin and washout. For the activation-VNa protocol, currents were elicited by 100-ms depolarizing pulses applied at 0.2 Hz from HP = −140 mV, in 5- or 10-mV increments between −100 and +50 mV. The steady-state availability-VNa protocol was from a HP of −100 mV; a 2-s conditioning prepulse was applied in 5- or 10-mV increments between −140 and −30 mV followed by a 300-ms test pulse to −20 mV at 0.2 Hz.

The recovery from inactivation protocol was from a HP of −100 mV: a 500-ms pulse followed by a 30-ms pulse was applied to −10 mV. The interval between the two pulses varied from 3 to 1000 ms.

APs were recorded in the whole-cell current-clamp configuration. The recording pipette had a resistance of 1 to 3 MΩ. Under control conditions, cells were current-clamped to a resting potential of −80 mV. APs were then elicited by a 2-ms hyperpolarizing current pulse (to reach a Vm of −100 mV), followed by a 2-ms depolarizing pulse of twice diastolic threshold at 0.2 Hz.

Experiments were carried out at room temperature (22°C to 25°C).

Statistics and Data Analysis
Data are expressed as mean±SEM of n determinations or myocytes, and when relevant, the number (N) of preparations was specified. Statistical significance was estimated by paired or unpaired Student’s t test or ANOVA, as appropriate. P<0.05 was considered significant.

Data for activation-VNa and steady-state availability-VNa relationships of INa were fitted to the Boltzmann equation:

\[ Y = \frac{1}{1 + \exp \left( -\frac{V_{Na} - V_{Na,0}}{k} \right) } \]

where \(V_{Na}\) is the membrane potential, \(V_{Na,0}\) is the half-activation or half-availability potential, and \(k\) is the inverse slope factor. For activation-VNa curves, \(Y\) represents the relative conductance and \(k\) is >0. For availability-VNa curves, \(Y\) represents the relative current (INa/max) and \(k\) is <0.

The same algorithm was applied to the Hill form (see legend to Figure 2) of the thrombin activity-response relationship and to the

![Figure 1](http://circ.ahajournals.org/figure.png)

Figure 1. Thrombin increased peak INa amplitude in a concentration-dependent manner. A, Effects of cumulative thrombin (thr) applications, reversible on washout. B, tetrodotoxin block of thrombin-induced INa. C, Successive thrombin applications showing the reproducibility and reversibility of the effect of a high concentration. Upper panels: whole-cell current recordings at the times indicated. Lower panels: time course of peak INa density, under conditions indicated by horizontal bars. Currents were elicited according to the steady-state pulse protocol. Peak INa amplitude was measured with respect to current at the end of the test pulse.
Figure 2. Activity-response curve for thrombin-induced increases in peak I$_{Na}$. Mean relative increase in peak I$_{Na}$ amplitude was calculated as follows: (peak I$_{Na}$ thrombin/peak I$_{Na}$ control) – 1. Currents were evoked as in Figure 1. Each point represents the mean of n measurements indicated in parentheses. Data points were fitted by the equation $y_{max}/[1+(EC_{50}/$thrombin activity$)^{nH}$]. $y_{max}$ (maximum mean relative increase), $EC_{50}$ (activity inducing half-maximal effect), and Hill parameter $nH$ were respectively 1.9±0.2, 91±16 U/mL, and 0.75±0.03.

Results

Thrombin Increases Peak I$_{Na}$

Figure 1 shows representative recordings of I$_{Na}$ obtained in the absence and presence of thrombin. At 0.1 U/mL, thrombin had no detectable effect on I$_{Na}$ (data not shown). Figure 1A shows that thrombin (1 or 10 U/mL) induced a reversible increase of mean peak I$_{Na}$ amplitude normalized to cell capacitance, from $-141.7±37.2$ to $-148.1±37.3$ pA/pF (9 myocytes; N=6; P<0.05) and from $-143.6±12.8$ to $-187.2±18.8$ pA/pF (n=25; N=14; P<0.01), respectively. Similarly, Figure 1B shows that thrombin (32 U/mL) induced a marked increase of peak I$_{Na}$, from $-118.9±12.6$ to $-191.7±20.8$ pA/pF (mean of 23 myocytes; N=17; P<0.001), which was abolished by 50 µmol/L tetrodotoxin, a specific voltage-gated sodium channel blocker. Current activation by thrombin and its blockade by tetrodotoxin were fully reversible during washout (Figure 1B). Thrombin (100 U/mL) exerted more marked and reversible increases in peak I$_{Na}$ ($-117.9±34.6$ versus $-245.2±61.1$ pA/pF, n=7; N=5; $P<0.001$) (Figure 1C) after repeated applications. These increases occurred despite a reversible hyperpolarizing shift of steady-state availability-V$_{m}$. A clear indication of this shift is given by the I$_{Na}$ points elicited from a HP of $-140$ mV (Figure 1C). In addition, thrombin (100 U/mL) induced a marked increase of the slow component of I$_{Na}$ measured at the end of the depolarizing test pulse ($-1.9±0.3$ versus $-3.1±0.5$ pA/pF, n=7; N=5; $P<0.01$). Time to peak I$_{Na}$ remained unchanged in the presence of thrombin (0.1 to 10 U/mL), whereas with 32 and 100 U/mL, it was significantly decreased from $0.88±0.18$ to $0.75±0.17$ ms (n=17; N=17; $P<0.001$) and from $0.93±0.13$ to $0.62±0.14$ ms (n=9; N=5; $P<0.001$), respectively. Application of heat-inactivated thrombin, at a nominal activity of 32 U/mL, failed to increase peak I$_{Na}$ ($-67.9±14.6$ in control versus $-77.1±17.8$ pA/pF under heat-inactivated thrombin; n=7; N=4; NS).

Activity-Response Relationship

Thrombin increased peak I$_{Na}$ in an activity-dependent manner as shown in Figure 2. The maximal effect was reached at 100 U/mL thrombin because higher concentrations elicited myocyte hypercontracture. Thrombin (100 U/mL) doubled peak I$_{Na}$ compared with controls, indicating an unusually large augmentation of I$_{Na}$.

Current Density-Voltage Relationships

Figure 3A shows typical examples of I$_{Na}$ recordings obtained in the absence and presence of 32 U/mL of thrombin with incremental test pulses of the activation-V$_{m}$ protocol. The current density-voltage relationships obtained are presented in Figure 3B. The peak I$_{Na}$ density obtained with a depolarizing test pulse to $-40$ mV was significantly increased from $-64.7±6.6$ pA/pF in controls to $-132.4±12.2$ pA/pF ($P<0.001$) by thrombin. Membrane potential values (V$_{m}$) associated with maximal amplitude of peak current were $-20$ mV in controls and $-40$ mV with thrombin. The activation-V$_{m}$ relationship was displaced toward more negative membrane potentials. The V$_{0.5}$ (V$_{m}$ at which half-activation occurs) was significantly shifted by $-9.2$ mV (Table and Figure 4B). This effect was reversible on thrombin washout (Table and Figure 4B). Finally, thrombin (32 U/mL) was devoid of significant effect on the I$_{Na}$ reversal membrane potential (+34 versus +36 mV in control, NS; Figure 3B) indicating that sodium channel selectivity was unaffected.
Steady-State Availability-Voltage Relationships

Figure 4A shows typical examples of I Na recordings obtained in absence and presence of thrombin (32 U/mL) according to the steady-state availability-V m protocol. Figure 4B and the Table demonstrate that thrombin (32 U/mL) was devoid of significant effect on steady-state availability relationships (Table).

I Na Window

The overlap of the steady-state availability-V m curve and the activation-V m curve in the potential range −85 to −40 mV determines the sodium window current amplitude as depicted in Figure 4B and 4C. Because sodium channels are not totally inactivated, a small fraction of these channels remain open and mediate window current. In the presence of thrombin, the I Na activation curve was displaced toward hyperpolarizing potentials, which gave rise to large increases in window current amplitude in the −95 to −40 mV range of potentials (Figure 4C).

Reactivation

The time course of I Na reactivation was assessed at a HP of −100 mV by the conventional double-pulse method (Figure 5). Time courses of relative current in control and thrombin (32 U/mL) were fitted by a double exponential function (Methods). The data in the Table confirm that there was no difference between the two constants of reactivation in controls versus thrombin.

Effects of Hirudin and HELSS

Figure 6 shows a typical recording of a relatively high concentration of hirudin (320 U/mL), a direct thrombin inhibitor, on thrombin-increased I Na elicited according to the steady-state pulse protocol (see Methods). Hirudin (320 U/mL) per se failed to significantly affect peak I Na (−58.7±13.4 pA/pF in controls versus −60.1±17.3 pA/pF with hirudin; n=6, NS). Even at this high concentration, hirudin failed to affect thrombin-induced increases in peak I Na (−122.5±22.4 pA/pF under thrombin, −100.4±21.6 pA/pF after thrombin plus hirudin, n=7, NS). HELSS (10 μmol/L), an inhibitor of calcium-independent phospholipase A 2 , was unable to inhibit the effects of thrombin on peak I Na (−79.1±16.3 pA/pF after a 30-minute preincubation with HELSS versus −120.6±20.3 pA/pF under thrombin plus HELSS, n=6; N=6; P<0.01).

Single-Cell AP

As thrombin markedly increased I Na window, sarcolemmal depolarization is an expected consequence. Figure 7 shows the effect of thrombin (32 U/mL) on averaged APs recorded from current-clamped single myocytes. Resting membrane potential was significantly shifted to depolarized potentials, and maximum upstroke velocity and AP amplitude were significantly and reversibly decreased by thrombin (Table).

### Activation and Availability Parameters, Reactivation Time Constants, and AP Parameters

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<th>Reactivation</th>
<th>AP Parameters</th>
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<td>k, mV</td>
<td>V 0.5, mV</td>
<td>k, mV</td>
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<td>Thrombin (32 U/mL)</td>
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<td>−81.5±1.9</td>
<td>−7.4±0.2</td>
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<td>n</td>
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<tr>
<td>Washout</td>
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<td>6.1±0.6</td>
<td>−81.8±1.8</td>
<td>−7.6±0.2</td>
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Data are mean±SEM of the number (n) of myocytes tested. V 0.5 indicates the activation or availability curve midpoint potential; k, the slope factor; τ a and τ s, fast and slow time constants of reactivation; APA, AP amplitude; E r, resting membrane potential; and dV/dt max, maximum upstroke velocity.

For availability, N=9, and for AP parameters, N=8.

*P<0.001 and †P<0.01 vs control.
I\textsubscript{Na} Facilitation by Thrombin

Thrombin is considered to play a role in mediating ischemic injury and ventricular arrhythmias during coronary occlusion.\textsuperscript{2-5} However, the mechanisms underlying these deleterious effects are not well understood. In cardiomyocytes, thrombin increases $[\text{Na}^+]_i$ by $\text{Na}^+-\text{H}^+$ exchange activation\textsuperscript{7} or via lysophosphatidylcholine production,\textsuperscript{3,5} but no consideration has been given to putative activation of VGSCs by the serine protease. The present study therefore attempted to determine whether VGSC activation by thrombin occurred in human cardiomyocytes.

Thrombin induced an increase in peak $I_{\text{Na}}$ amplitude caused by a marked hyperpolarizing shift of the current activation curve. Consequently, thrombin potentiates the $\text{Na}^+$ window current, generated by overlap of activation- and steady-state voltage-inactivation $I_{\text{Na}}$ curves.\textsuperscript{18} In the presence of thrombin, the marked activation shift to more negative potentials produced abnormally large increases in window current amplitude. This generates a large, sustained depolarizing current and potentially massive $\text{Na}^+$ influx at the resting membrane potential during ischemia. The consequences are a depolarization of resting membrane potential associated with a decrease in AP maximum upstroke velocity. Persistent $\text{Na}^+$ current is caused by sustained VGSC openings.\textsuperscript{15} These late channel reopenings and bursting behavior occur with $I_{\text{Na}}$ in animal\textsuperscript{22,23} and human\textsuperscript{24} ventricular cardiomyocytes. This increase in steady-state $\text{Na}^+$ window current resulting from a prolongation of VGSC opening directly increases free $[\text{Na}^+]_i$.\textsuperscript{9} The present results suggest that thrombin, by facilitating $\text{Na}^+$ channel activation causing abnormally large increases in window current, may amplify $\text{Na}^+$ accumulation during ischemia, leading to elevated $[\text{Ca}^{2+}]_i$, through reverse $\text{Na}^+-\text{Ca}^{2+}$ exchange.

Because the large, sustained sodium window current increase elicited by thrombin occurred in the voltage range $-95$ to $-40$ mV, this inward current will be further augmented by sarcolemmal depolarization during ischemia.\textsuperscript{15,17,24} Thus, the current-clamp data demonstrate that thrombin depolarized resting membrane potential by 10 mV, which could be a direct consequence of sodium window current amplification. These results corroborate those reported\textsuperscript{2} in depolarized ventricular fibers, in which thrombin increased automaticity. Depolarized fibers are thought to provide a substrate for arrhythmogenesis resulting from abnormal automaticity in myocardial infarction.\textsuperscript{18,2} Human atrial cardiomyocytes express $\text{Na}^-$ window current\textsuperscript{25} with operational characteristics similar to their ventricular counterparts,\textsuperscript{24} although we acknowledge that possible electrophysiological differences between atrial and ventricular cardiomyocytes may exist. The mechanism for thrombin-evoked automaticity could

Discussion

The effects of thrombin on VGSC function were studied in human isolated atrial cardiomyocytes. For the first time, it is demonstrated that thrombin markedly increased peak $I_{\text{Na}}$ and window current amplitude. These effects were reversible and repeatable, and they are attributable to a shift in the $I_{\text{Na}}$ activation-voltage relationship to hyperpolarized potentials without affecting steady-state voltage-inactivation characteristics. Moreover, thrombin depolarized cardiomyocytes. Because the direct thrombin inhibitor, hirudin, failed to attenuate VGSC activation by thrombin, enzymatic peptide cleavage is unlikely to be involved. The data suggest that thrombin could promote ischemic sodium loading and arrhythmogenesis by facilitating VGSC activation.

![Figure 5. Recovery of $I_{\text{Na}}$ from inactivation. A, Typical $I_{\text{Na}}$ recordings in control and in presence of thrombin (32 U/mL), elicited by recovery from the inactivation protocol shown in inset. B, $I_{\text{Na}}$ amplitude elicited by the second pulse was normalized to current amplitude elicited by the first pulse and was plotted against intervals between the two pulses. Data are mean±SEM (n: control, 9; thrombin, 7; N=5). Data were fitted by a double exponential function. Fast and slow time constants of recovery (Table) show no significant difference between control and thrombin.]()

![Figure 6. Representative time courses of peak $I_{\text{Na}}$ showing that hirudin (320 U/mL) had no effect on thrombin (32 U/mL)-induced increases in $I_{\text{Na}}$.]()

![Figure 7. Comparison of averaged APs obtained from isolated cardiomyocytes in presence of control (n=11), thrombin 32 U/mL (n=11) and after washout (n=10).]()}
Thrombin Access to VGSCs

Thrombin concentrations that activated VGSCs in the present study correspond to those that are found during clotting and thrombosis. Nevertheless, in order to activate VGSCs, the enzyme must access cardiomyocytes across the capillary wall of the coronary microcirculation. Available evidence would indicate this to be highly likely to occur.

Mechanism of Action

Because the direct thrombin inhibitor, hirudin, failed to affect thrombin activation of VGSCs, proteolytic cleavage was not required. Consequently, an involvement of protease-activated receptors can be excluded. Thrombin may also increase [Na+]i by calcium-independent phospholipase A2-dependent production of lysophosphatidylcholine. However, HELSS, an inhibitor of cardiomyocyte phospholipase A2, failed to significantly affect thrombin increases in peak INa in the present study, which is suggestive of another mechanism. This issue merits further study, because the phospholipase enzyme must access cardiomyocytes across the capillary wall and may well occur concomitantly. The precise molecular mechanism of thrombin increases in peak INa therefore remains to be elucidated. A direct nonproteolytic effect on VGSC α or β subunits or unidentified G protein–coupled receptors cannot be excluded at present.

In conclusion, thrombin has been shown for the first time to be a powerful activator of cardiac VGSCs by selectively shifting the INa voltage-activation relationship to hyperpolarized potentials. The consequences of such an effect are 2-fold: amplified ischemic sodium loading and arrhythmogenesis. These effects of thrombin are not dependent on proteolytic activity. Facilitation of VGSC activation may therefore be considered as a key molecular mechanism by which thrombin mediates ischemic injury.

Acknowledgments

Part of this work was supported by a grant from the Bonus Qualité Recherche (Université Paris-Sud XI) and from the Fondation de France. We thank Jean-Michel Talmant for technical support.

References

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Circulation. 2002;106:2098-2103
doi: 10.1161/01.CIR.0000034510.64828.96
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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