Intracellular calcium modulation of voltage-gated sodium channels in ventricular myocytes

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1. Introduction

Voltage-gated sodium (Na⁺) channels control excitability in the heart and other excitable tissues by generating the action potential (AP) upstroke and cell excitability. Intracellular calcium (Ca²⁺) regulates AP properties by modulating various ion channels. Whether Ca²⁺ modulates sodium channels in ventricular myocytes is unresolved. We studied whether Ca²⁺ modulates sodium channels in ventricular myocytes at Ca²⁺ concentrations ([Ca²⁺]) present during the cardiac AP (0–500 nM), and how this modulation affects sodium channel properties in heart failure (HF), a condition in which Ca²⁺ homeostasis is disturbed.

Methods and results

Sodium current (Iₕ) and maximal AP upstroke velocity (dV/dtₘₐₓ), a measure of Iₕ, were studied at 20 and 37°C, respectively, in freshly isolated left ventricular myocytes of control and HF rabbits, using whole-cell patch-clamp methodology. [Ca²⁺] was varied using different pipette solutions, the Ca²⁺ buffer BAPTA, and caffeine administration. Elevated [Ca²⁺] reduced Iₕ, density and dV/dtₘₐₓ, but caused no Iₕ gating changes. Reductions in Iₕ density occurred simultaneously with increase in [Ca²⁺], suggesting that these effects were due to permeation block. Accordingly, unitary sodium current amplitudes were reduced at higher [Ca²⁺]. While Iₕ density and gating at fixed [Ca²⁺] were not different between HF and control, reductions in dV/dtₘₐₓ upon increases in stimulation rate were larger in HF than in control; these differences were abolished by BAPTA.

Conclusion

Ca²⁺ exerts acute modulation of Iₕ density in ventricular myocytes, but does not modify Iₕ gating. These effects, occurring rapidly and in the [Ca²⁺] range observed physiologically, may contribute to beat-to-beat regulation of cardiac excitability in health and disease.

KEYWORDS

Ion channels; Na-channel; Arrhythmia

Aims

Cardiac voltage-gated sodium channels control action potential (AP) upstroke and cell excitability. Intracellular calcium (Ca²⁺) regulates AP properties by modulating various ion channels. Whether Ca²⁺ modulates sodium channels in ventricular myocytes is unresolved. We studied whether Ca²⁺ modulates sodium channels in ventricular myocytes at Ca²⁺ concentrations ([Ca²⁺]) present during the cardiac AP (0–500 nM), and how this modulation affects sodium channel properties in heart failure (HF), a condition in which Ca²⁺ homeostasis is disturbed.

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underlies beat-to-beat variations in \( I_{\text{Na}} \) magnitude, e.g. those that occur upon changes in heart rate. We aimed to establish how \( Ca^{2+} \) modulates \( Na^{+} \) channels under physiological conditions and during HF.

2. Methods

2.1 Cell preparation

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication 85–23, revised 1996) and was approved by the institutional animal experiments committee. HF was induced by combined volume overload (aortic regurgitation) and pressure overload (aortic banding) in New Zealand White rabbits. Mid-myocardial left ventricular myocytes were isolated from healthy (control) and HF animals.

CpDNA of SCNSA, the gene encoding the \( \alpha \)-subunit (Na,1.5) of the human cardiac \( Na^{+} \) channel, was prepared in the pCIG vector for bicistronic expression of Na,1.5 and green fluorescent protein. Human embryonic kidney (HEK293) cells were transiently transfected with 1 \( \mu g \) SCNSA cDNA and 1 \( \mu g \) \( \alpha \)-subunit cDNA using lipofectamine (Gibco BRL, Life Technologies). Transfected HEK293 cells were cultured in minimum essential medium (Earlis salts and HEPES 10, glucose 11, CsCl, 1.8, MgCl2 1.2, pH 7.4 (CsOH)). Pipettes were filled with (mM): NaCl 3, BAPTA 10, Mg-ATP 2, Na,1.5 cDNA and 1 \( \mu g \) h\( \alpha \)-subunit cDNA using lipofectamine (Gibco BRL, Life Technologies). Transfected HEK293 cells were cultured in minimum essential medium (Earlis salts and HEPES 10, glucose 11, CsCl, 1.8, MgCl2 1.2, pH 7.4 (CsOH)).

2.2 Electrophysiology

2.2.1 Data acquisition

Membrane currents and potentials were recorded in the whole-cell configuration of the patch-clamp technique using patch pipettes with a tip resistance of 1.5–2 MΩ, unless mentioned otherwise. \( I_{\text{Na}} \) signals were low-pass filtered (cut-off frequency 5 kHz) and digitized (20 kHz). Series resistance (Rs) was compensated by > 80%. AP measurements were filtered and digitized at 10 and 40–50 kHz, respectively. Voltage control, data acquisition, and analysis of \( I_{\text{Na}} \) and AP recordings were performed with pClamp8.0/Clampfit (Axon Instruments) and custom-made software, respectively.

2.2.2 Sodium current properties

In myocytes, \( I_{\text{Na}} \) was analysed at 20 °C; at 37 °C, AP upstroke velocity was analysed as a measure of \( I_{\text{Na}} \) (see below). At 20 °C, activation, inactivation, recovery from inactivation, and slow inactivation parameters were determined using protocols as indicated in Figure 1 (cycle time of 2 s for activation, inactivation, and slow inactivation, and 4 s for recovery from inactivation). Data were fitted as described in the Supplementary material online. \( I_{\text{Na}} \) was recorded using intracellular solutions to which CaCl2 was added to obtain a free \( [Ca^{2+}]_{\text{i}} \) of 0, 100, and 500 nM as calculated using WEBMAX Standard software (http://www.stanford.edu/~cpatton/webmaxS5.htm). Pipettes were filled with (mM): NaCl 3, BAPTA 10, Mg-ATP 2, CsOH 140, HEPES 10, pH 7.2 (HCl). The bath solution contained (mM): NaCl 7, CaCl2 1.8, MgCl2 1.2, HEPES 10, glucose 11, CsCl 125, nifedipine 0.005, pH 7.3 (CsOH). BAPTA was used as \( Ca^{2+} \) buffer, because it provides stringent control over \([Ca^{2+}]_{\text{i}}\), thanks to its fast \( Ca^{2+} \) buffering kinetics. \( I_{\text{Na}} \) was defined as the difference between peak current and the current at the end of the depolarizing voltage step. \( I_{\text{Na}} \) density (Table 1) was calculated by dividing maximal \( I_{\text{Na}} \) by cell membrane capacitance (CM). The mean values for CM were 161.6 ± 7.8 pF (control, \( n = 27 \)) vs. 213.0 ± 14.1 pF (HF, \( n = 28 \), \( P = 0.007 \)). Access resistance (Ra) values were 5.1 ± 0.3 MΩ (control, \( n = 27 \)) vs. 6.1 ± 0.4 MΩ (HF, \( n = 28 \)).

In HEK293 cells, experiments were conducted at 20 °C and \( I_{\text{Na}} \) was elicited by depolarizing steps from a holding potential of −140 mV (cycle time 5 s). Cm and Ra were 11.9 ± 0.9 pF and 5.8 ± 0.7 MΩ, respectively (\( n = 10 \)). Acute effects of \( Ca^{2+} \) on \( I_{\text{Na}} \) were studied with a modified dialyzable pipette (Supplementary material online and Tang et al.14). The pipette solutions were identical to those used for \( I_{\text{Na}} \) measurements in myocytes at 20 °C (described above, Ca0 and Ca500), while the bath solution (Solution 1) contained (mM): NaCl 10, Na-glucuronate 130, HEPES 10, glucose 5.5, CaCl2 1.8, MgCl2 1.2, pH 7.4 (CsOH). To study possible effects of caffeine on \( I_{\text{Na}} \) 10 mM caffeine was washed into the bath which contained (mM): NaCl 140, KCl 4.7, CaCl2 1.8, MgCl2 2.0, NaHCO3 4.3, Na2HPO4 1.4, glucose 11.0, HEPES 16.8, pH 7.4 (NaOH). Pipettes were filled with (mM): CsIf 110, CsCl 1.0, NaATP 2.0, HEPES 10, pH 7.3 (CsOH).

2.2.3 Upstroke velocity measurements

To investigate \( I_{\text{Na}} \) characteristics at 37 °C and physiological Na+ concentrations, we recorded AP upstroke velocities and expressed them as the maximum value of the first derivative of AP upstroke (dV/dtmax). dV/dtmax was a convenient index for \( I_{\text{Na}} \). Although it slightly overestimates Na+ channel availability, the discrepancies between \( I_{\text{Na}} \) and dV/dtmax are reduced at high temperatures (26–27 °C).17 dV/dtmax was measured using an alternate voltage/current-clamp mode with a custom-made amplifier.18 Using dV/dtmax as a measure of \( Ca^{2+} \) channel availability, steady-state inactivation, recovery from inactivation, and slow inactivation were analysed with the protocols indicated in Figure 2. As a measure of peak current magnitude (Table 2), the maximum dV/dtmax of the Na+ channel availability curve was given. Bath solution (Solution 2) contained (mM): NaCl 140, KCl 5.4, CaCl2 1.8, MgCl2 1.0, glucose 5.5, and HEPES 5.0, pH 7.4 (NaOH). Pipette solution contained (mM): NMDG-OH 140, BAPTA 10, K-glucuronate 125, KCl 20, NaCl 5, Mg-ATP 5, and HEPES 10, pH 7.2 (HCl). CaCl2 was added to obtain free \([Ca^{2+}]_{\text{i}}\)=0 or 500 nM. Data for voltage-dependence of recovery and inactivation from inactivation were fitted as described in the Supplementary material online.

To investigate \( I_{\text{Na}} \) availability at various stimulus frequencies and, consequently, at various \([Ca^{2+}]_{\text{i}}\), dV/dtmax were measured in control and HF myocytes at 0.2–4 Hz in the presence or absence of 10 mM intracellular BAPTA. Experiments were conducted at 37 °C. The use of BAPTA eliminates calcium (\( Ca^{2+} \)) transients and \( Ca^{2+} \)-dependent L-type \( Ca^{2+} \) channel inactivation, causing modification of several parameters in the cell, including the normal regulation of the cardiac AP. Consequently, AP duration is prolonged, especially in HF.19,20 To control the duration of diastolic intervals, we used the combined voltage/current-clamp method. Bath solution was Solution 2. Pipette solution without BAPTA (Solution 3) contained (mM): K-glucuronate 125, KCl 20, NaCl 5, Mg-ATP 5, and HEPES 10, pH 7.2 (KOH). Pipette solution with BAPTA contained (mM) BAPTA 10, KOH 40, gluconic acid 20, NaCl 5, Mg-ATP 5, HEPES 10, K-glucuronate 85, KCl 20, pH 7.2 (KOH).

To study acute effects of changing \([Ca^{2+}]_{\text{i}}\) on Na+ channel availability in the range of \([Ca^{2+}]_{\text{i}}\) as observed during the cardiac cycle, we created acute increases in \( Ca^{2+} \) by inducing \( Ca^{2+} \) release from the SR with 10 mM caffeine. \([Ca^{2+}]_{\text{SR}}\) and dV/dtmax were simultaneously measured at 37 °C using the alternate voltage/current-clamp mode. For each cell, dV/dtmax represents the average of 10 consecutive values recorded at steady-state. Bath solution was Solution 2, pipette solution was Solution 3.

2.2.4 \( Ca^{2+} \) measurements

\([Ca^{2+}]_{\text{SR}}\) was measured in Indo1-loaded myocytes (1 Hz stimulation). Dye-loaded myocytes were excited through a 100× oil-immersion objective at 340 nm (75W Xenon arc lamp). Intensities of the emitted light at 405 nm (\( I_{405} \)) and 505 nm (\( I_{505} \)) were recorded, subsequently digitized at 1 kHz, and filtered at 100 Hz. A rectangular adjustable slit was used to select a single myocyte and reduce background fluorescence, which was subtracted offline before the ratio values were calculated (\( I_{505}/I_{405} \)). Fluorescent ratio was
translated as $\left[\text{Ca}^{2+}\right] = \beta K_d (R - R_{\text{min}})/(R_{\text{max}} - R)$. After calibration of the setup, $\beta$ (ratio of maximum to minimum $I_{\text{Na}}$) was 2.2, maximal ratio ($R_{\text{max}}$) $2.21 \pm 0.24$, minimal ratio ($R_{\text{min}}$) $0.31 \pm 0.04$, and $K_d$ 250 nM (Data sheet Indo-1, Molecular Probes, Eugene OR, USA).

2.2.5 Single-channel measurements

Single Na$^+$ channel currents were recorded at 20°C from inside-out patches of ventricular myocytes isolated from control rabbit hearts, in the presence of veratridine ($30 \mu\text{M}$). Veratridine reduces peak $I_{\text{Na}}$, but also induces a non-inactivating current due to long-lasting channel openings.$^{21}$ Therefore, the use of veratridine provides a more reliable analysis of unitary current amplitude compared to unmodified Na$^+$ channels.$^{22}$ Voltage steps (900 ms) were applied from $-80$ to $-40$ mV (holding potential $-140$ mV, cycle time 5 s). Pipettes were fire polished and had a tip resistance of 2.5–3.5 MΩ. Signals were filtered and digitized at 1 and 10 kHz, respectively. Bath solutions were identical to those used as pipette solutions for $I_{\text{Na}}$ measurements in myocytes at 20°C (Ca$^0$ and Ca$^{500}$). Pipette solution was Solution 1 with the addition of nifedipine ($0.005$ mM). Unitary current amplitudes were estimated from sweeps exhibiting well-resolved closings as determined by fitting Gaussian distributions.

Figure 1 Sodium current densities and gating properties in control myocytes at various $\left[\text{Ca}^{2+}\right]$. (A) Typical examples of whole-cell sodium current ($I_{\text{Na}}$) traces recorded at 20°C from control myocytes at $\left[\text{Ca}^{2+}\right] = 0$ nM (Ca$^0$), 100 nM (Ca$^{100}$), and 500 nM (Ca$^{500}$) in response to depolarizing voltage steps (protocol in C, right part). (B) Average $I_{\text{Na}}$ densities at Ca$^0$, Ca$^{100}$, and Ca$^{500}$ obtained by dividing maximal $I_{\text{Na}}$ by cell membrane capacitance. (C) On the right part, average voltage dependence of activation. Normalized current was plotted as a function of the membrane potential. On the left part, steady-state inactivation. Peak sodium currents were normalized to maximum values in each cell and plotted as a function of the voltage of the conditioning step. (D) Average fast ($\tau_f$) and slow ($\tau_s$) time constants of $I_{\text{Na}}$ decay plotted as a function of membrane potential. (E) Average time course of recovery from inactivation. Peak sodium currents elicited by $P_2$ were normalized ($P_2/P_1$) and plotted as a function of the recovery interval ($\Delta t$). (F) Average development of slow inactivation. $I_{\text{Na}}$ elicited by $P_2$ were normalized ($P_2/P_1$) and plotted as a function of the duration of $P_1$. Insets: voltage protocols.
2.2.6 Statistical analysis

Results are expressed as mean ± SEM. Unpaired Student’s t-test was used to study the effects of Ca²⁺ on Na⁺ channel properties, and to compare i_{Na} gating, current densities, Cm and Ra between control and HF. When data were not normally distributed, a Mann-Whitney test was performed. Paired Student’s t-test was performed to compare [Ca²⁺] and dV/dt_{max} in control myocytes before/after addition of caffeine, and i_{Na} amplitudes in HEK293 cells before/after addition of caffeine or increases in [Ca²⁺]. To analyse i_{Na} decay, the development of slow inactivation and to

![Figure 2](image_url)

**Figure 2** Action potential upstroke velocity as a measure of sodium current magnitude and gating properties in control myocytes at various [Ca²⁺]. (A) Typical examples of action potential (AP) upstroke velocities recorded at 37°C from control myocytes at [Ca²⁺]=0 nM (Ca0) and 500 nM (Ca500). (B) Average maximum AP upstroke velocities (dV/dt_{max}) of the sodium current (i_{Na}) availability curve, as a measure of i_{Na} magnitude. (C) Average voltage dependence of inactivation. dV/dt_{max} elicited by P₂ were normalized (P₂/P₁) and plotted as a function of the voltage of the conditioning step. (D) Average time course of recovery from inactivation. dV/dt_{max} elicited by P₂ were normalized (P₂/P₁) and plotted as a function of the duration of P₁. Insets: combined voltage/current-clamp protocols. CC, current-clamp.

<table>
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<th>Table 1</th>
<th>Intracellular calcium modulation of sodium channel properties at 20°C</th>
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<td><strong>Control myocytes</strong></td>
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<td>Ca0</td>
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<tr>
<td>Peak current</td>
<td>i_{Na} (pA/pF)</td>
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<td>−50.4 ± 3.8</td>
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<td>Activation</td>
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<td>−36.8 ± 1.1</td>
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<td>k (mV)</td>
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<td>4.9 ± 0.2</td>
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<td>Inactivation</td>
<td>V_{1/2} (mV)</td>
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<td>−78.0 ± 1.4</td>
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<td>k (mV)</td>
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<td>−5.1 ± 0.1</td>
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<td>Recovery</td>
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<td>τ₅ (ms)</td>
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<td>Slow inactivation</td>
<td>A at 1000 ms</td>
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A at 1000 ms, fraction of channels that enter the slow inactivated state at the end of a 1000 ms step, calculated as 1 − (P₂/P₁); Ca0, [Ca²⁺]=0 nM; Ca100, [Ca²⁺]=100 nM; Ca500, [Ca²⁺]=500 nM; i_{Na}, sodium current density; k, slope factor of voltage-dependence of (in)activation; τ₁, slow time constant of recovery from inactivation; τ₅, slow time constant of recovery from inactivation; V_{1/2}, voltage of half-maximal (in)activation.

⁵P < 0.05 vs. control Ca0.
⁶P < 0.05 vs. Ca100.
⁷P < 0.05 vs. Ca500.
3. Results

3.1 Effects of Ca\textsuperscript{2+} on Na\textsuperscript{+} channel properties

We first studied the effects of Ca\textsuperscript{2+} at 20°C on I\textsubscript{Na} properties in myocytes isolated from control rabbits (Table 1 and Figure 1). We used pipette solutions buffered at 0, 100, or 500 nM free [Ca\textsuperscript{2+}] (Ca\textsubscript{0}, Ca\textsubscript{100}, and Ca\textsubscript{500}) to cover the [Ca\textsuperscript{2+}] range found in myocytes of control and HF rabbits\textsuperscript{3} during the cardiac cycle. Figure 1A shows representative examples of I\textsubscript{Na} traces recorded from control myocytes at different [Ca\textsuperscript{2+}]. I\textsubscript{Na} density was significantly smaller at Ca\textsubscript{500} than at Ca\textsubscript{0}, while values at Ca\textsubscript{100} were intermediate (Table 1 and Figure 1B). This reduction was not due to changes in gating properties. \(V_{1/2}\) and \(k\) of activation and inactivation were not different between Ca\textsubscript{0}, Ca\textsubscript{100}, and Ca\textsubscript{500} (Table 1 and Figure 1C), as well as the rates of current decay (Figure 1D), recovery from inactivation (Figure 1E), and slow inactivation, a conformational state that partly determines I\textsubscript{Na} availability at fast heart rates because of its slow kinetics\textsuperscript{23} (Table 1 and Figure 1F).

To study whether these findings also apply to physiological temperatures, we repeated these experiments at 37°C, using \(dV/dt_{\text{max}}\) as an index of I\textsubscript{Na} magnitude.\textsuperscript{18} Results were similar to the voltage-clamp studies at 20°C, with \(dV/dt_{\text{max}}\) being smaller at Ca\textsubscript{500} than at Ca\textsubscript{0} and gating properties similar at Ca\textsubscript{0} and Ca\textsubscript{500} (Table 2 and Figure 2). However, the percentage of \(dV/dt_{\text{max}}\) reduction observed at high [Ca\textsuperscript{2+}] was smaller than the I\textsubscript{Na} reduction observed at 20°C. While differences in recording temperatures may cause this discrepancy, future studies must resolve the underlying mechanisms.

As we found no evidence that Ca\textsuperscript{2+} modulates Na\textsuperscript{+} channel gating, we hypothesized that its effect to reduce I\textsubscript{Na} magnitude results from impediment of Na\textsuperscript{+} channel permeation. To test this hypothesis, we conducted paired experiments on human cardiac Na\textsuperscript{+} channels expressed in HEK293 cells. We measured I\textsubscript{Na} at different [Ca\textsuperscript{2+}] in the same cell by varying [Ca\textsuperscript{2+}] with a dialyzable pipette.\textsuperscript{16} We observed a similar reduction (~30%) in peak I\textsubscript{Na} amplitude as in myocytes at 20°C when [Ca\textsuperscript{2+}] was increased from 0 to 500 nM (Figure 3B). To verify whether current amplitudes were stable up to the change in intracapillary solution within the same cell, I\textsubscript{Na} amplitudes were plotted against time (Figure 3A, bottom). This analysis indicated that I\textsubscript{Na} amplitudes were stable during recording at [Ca\textsuperscript{2+}]=0 nM (Ca\textsubscript{0}) and during/after wash-in of 500 nM Ca\textsuperscript{2+} (Ca\textsubscript{500}). (B) Average peak I\textsubscript{Na} (n = 4) normalized in each cell to the highest I\textsubscript{Na} value obtained with Ca\textsubscript{0}.

3.2 Effects of Ca\textsuperscript{2+} on unitary Na\textsuperscript{+} current amplitude

To provide direct evidence for permeation block, we next performed inside-out patch-clamp studies in control myocytes to test the effects of Ca\textsuperscript{2+} on veratridine-modified single Na\textsuperscript{+} current amplitudes. The effect of veratridine was first tested on whole-cell I\textsubscript{Na} (Figure 4A). Consistent with previous findings,\textsuperscript{21} veratridine reduced peak I\textsubscript{Na} amplitudes and...
induced a non-inactivating current due to long-lasting channel openings (Figure 4A, left panel). Moreover, activation of veratridine-modified Na\(^+\) channels occurred already at relatively negative potentials (Figure 4A, right panel). In inside-out patches, resolvability of unitary channel events was only possible in a minority of current traces. This may have been due to long-lasting openings of multiple channels simultaneously, as a consequence of the relatively slow channel inactivation in the presence of veratradine. Nevertheless, two distinct single-channel current amplitudes were observed (Figure 4B), in accordance with a previous study which showed that veratradine induces sub-conductance states of the cardiac Na\(^+\) channel.\(^{21}\) Figure 4C, left panel, shows typical examples of single channel currents from two Na\(^+\) channel sub-conductance states with 0 and 500 nM Ca\(^{2+}\). When Ca\(^{2+}\) was increased from 0 to 500 nM in paired experiments, the single channel current amplitude decreased to 82.2 ± 2.5% (n = 6 patches). Fitting the pooled data by linear regression yielded a decrease from 38.5 to 32.2 pS for the ‘larger’ channel conductance and from 24.3 to 20.6 pS for the ‘smaller’ channel conductance (Figure 4C, right).

3.3 Effects of Ca\(^{2+}\) on AP upstroke velocity
Having established that steady-state increases in [Ca\(^{2+}\)] reduce \(I_{\text{Na}}\) density in freshly isolated myocytes by impeding ion permeation, we next investigated whether this interaction may be relevant to beat-to-beat modulation of the cardiac AP.
To study how changes in $[\text{Ca}^{2+}]$, which occurred acutely and in the range of $[\text{Ca}^{2+}]$ as observed during the cardiac cycle in this model, impacted on $dV/dt_{\text{max}}$ at 37°C, we conducted simultaneous recordings of $dV/dt_{\text{max}}$ and $[\text{Ca}^{2+}]$. Adding 10 mM caffeine to the bath solution caused $\text{Ca}^{2+}$ release from the SR, and resulted in an increase in $[\text{Ca}^{2+}]$. This was associated with a reciprocal reduction in $dV/dt_{\text{max}}$, which occurred instantaneously upon changes in $[\text{Ca}^{2+}]$ (Figure 5A). Of note, $dV/dt_{\text{max}}$ reduction occurred over the whole range of $[\text{Ca}^{2+}]$, following depletion of the SR, indicating that $[\text{Ca}^{2+}]$ acutely reduces $I_{\text{Na}}$ density in a dose-dependent fashion (Figure 5B). Average $dV/dt_{\text{max}}$ before and after 30 s of caffeine exposure is shown in Figure 5C, and corresponding average $[\text{Ca}^{2+}]$ in Figure 5D.

3.4 Effects of $\text{Ca}^{2+}$ on $I_{\text{Na}}$ channel properties in heart failure

To further explore whether this acute modulation of $I_{\text{Na}}$ properties by $\text{Ca}^{2+}$ may have pathophysiological relevance, we studied the effects of $\text{Ca}^{2+}$ on $I_{\text{Na}}$ properties in a rabbit model of HF. As reported in earlier studies from our laboratory, $\text{Ca}^{2+}$ handling is disturbed in this model. In particular, diastolic $[\text{Ca}^{2+}]$ is significantly increased.
First, to study whether the nature of \( \text{I}_{\text{Na}} \) modulation by Ca\(^{2+} \), as observed in control, is also present in HF, we conducted experiments in myocytes isolated from HF rabbits using pipette solutions with the same steady-state \([\text{Ca}^{2+}]\) as in control (0 and 500 nM) at 20 °C (\( \text{I}_{\text{Na}} \)) and 37 °C (d\( V/dt_{\text{max}} \)). \( \text{I}_{\text{Na}} \) density and gating were similar in control and HF at both temperatures (Tables 1 and 2). Ca\(^{2+} \) exerted similar acute modifications of \( \text{I}_{\text{Na}} \) density in HF as in control. Thus, also in HF, \( \text{I}_{\text{Na}} \) density and d\( V/dt_{\text{max}} \) were significantly smaller at \([\text{Ca}^{2+}]\)=500 nM than at \([\text{Ca}^{2+}]\)=0 nM, while gating properties were unaltered (Tables 1 and 2).

To study whether acute \([\text{Ca}^{2+}]\)–mediated changes in \( \text{I}_{\text{Na}} \) density may translate into differences in excitability between control and HF under physiological conditions, we studied d\( V/dt_{\text{max}} \) at different stimulation rates. We made use of previous findings from our laboratory,\(^8\) that in our model, diastolic \([\text{Ca}^{2+}]\) increases in parallel with the stimulation rate, and that this increase is larger in HF than in control. For instance, when pacing rate is increased from 0.2 to 3 Hz, diastolic \([\text{Ca}^{2+}]\) increases by 40 nM (from 50 to 90 nM) in control, but by 80 nM (from 145 to 225 nM) in HF. Thus, d\( V/dt_{\text{max}} \) was expected to decline at fast heart rates, and this reduction was expected to be more prominent in HF than in control. To better appreciate the changes in d\( V/dt_{\text{max}} \) in response to different stimulation rates, values were normalized to the highest d\( V/dt_{\text{max}} \) in each myocyte. When stimulation rates were increased in the absence of Ca\(^{2+} \) chelators in the pipette solution, d\( V/dt_{\text{max}} \) decreased more in HF than in control. Thus, normalized d\( V/dt_{\text{max}} \) was significantly smaller in HF than control at 3 and 4 Hz. Yet, when BAPTA was added to the pipette solution to maintain \([\text{Ca}^{2+}]\) at virtually zero levels,\(^11\) the differences between control and HF in the d\( V/dt_{\text{max}} \)-heart rate relationships were abolished (Figure 6).

4. Discussion

We demonstrated that Ca\(^{2+} \) modulates Na\(^+ \) channel density without changing its kinetic properties in freshly isolated ventricular myocytes. \([\text{Ca}^{2+}]\)–mediated changes in \( \text{I}_{\text{Na}} \) density were acute and occurred in the range of \([\text{Ca}^{2+}]\) levels found in control and HF. \( \text{I}_{\text{Na}} \) reduction was due to decreased Na\(^+ \) channel conductance. Ca\(^{2+} \)–mediated permeation block of the Na\(^+ \) channel may contribute to beat-to-beat modulation of \( \text{I}_{\text{Na}} \) properties as it provides a mechanism by which diastolic \([\text{Ca}^{2+}]\) controls \( \text{I}_{\text{Na}} \) density. We observed this regulatory mechanism not only in the physiological state, but also in a disease state in which diastolic \([\text{Ca}^{2+}]\) is increased, i.e. HF.

4.1 Ca\(^{2+} \)–dependent modulation of Na\(^+ \) channel

Previous single-channel experiments in cardiac myocytes have shown that extracellular Ca\(^{2+} \) reduces Na\(^+ \) channel unitary current amplitude.\(^{21,22,25}\) Reduction of single-channel conductance was explained by a very fast open channel block, due to a rapid movement of Ca\(^{2+} \) ions into and out of a binding site within the Na\(^+ \) channel, rather than to changes in the surface charges.\(^{25}\) In contrast, the effects of Ca\(^{2+} \) on Na\(^+ \) channels were not resolved.

It was proposed that Ca\(^{2+} \) regulates Na\(^+ \) channel biosynthesis in rat cardiac myocytes.\(^7\) Accordingly, the decrease in \( \text{I}_{\text{Na}} \) density observed in cultured rat neonatal ventricular myocytes exposed for 24 h to culture medium containing 10 mM Ca\(^{2+} \) and 10 mM K\(^+ \) (in order to raise \([\text{Ca}^{2+}]\)) was attributed to reduced sarcolemmal Na\(^+ \) channel expression rather than to changes in single-channel conductance or gating.\(^8\)

While these studies revealed long-term modulation of Na\(^+ \) channel expression and current density by Ca\(^{2+} \), the findings that single Na\(^+ \) channel conductance in cells exposed to high \([\text{Ca}^{2+}]\) was not different from that in control cells\(^6\) contrasted with a study by French et al.,\(^9\) in which rat brain-type Na\(^+ \) channels were inserted into lipid bilayers. In these experiments, either external or internal application of 10 mM Ca\(^{2+} \) reduced single-channel conductance. These experimental findings were reproduced in a computer model of Na\(^+ \) channels.\(^{26} \) Using the same lipid bilayer system as French et al., Zamponi et al.\(^{10} \) showed that, in the skeletal-muscle isoform of the rat Na\(^+ \) channel, internally applied millimolar Ca\(^{2+} \) concentrations caused a dose-dependent reduction of single-channel \( \text{I}_{\text{Na}} \). A block of the pore involving multiple sites together with a surface charge effect was suggested as mechanisms.

Wingo et al.\(^{11} \) showed that in tsA201 cells transiently transfected with the cardiac Na\(^+ \) channel, an increase in \([\text{Ca}^{2+}]\) from 0 to 10 \(\mu\)M (with the effect saturating at 1 \(\mu\)M) caused a positive shift of the \( \text{I}_{\text{Na}} \) availability curve. A putative EF hand motif located in the C-terminus of the channel was proposed as a Ca\(^{2+} \) binding site and regulator of the Ca\(^{2+} \)-dependent effects.\(^{11} \) However, a separate study failed to detect any Ca\(^{2+} \) binding to that region.\(^{27}\)

To complement these previous studies, we now provide evidence for a direct modulation of \( \text{I}_{\text{Na}} \) by physiological \([\text{Ca}^{2+}]\) in freshly isolated cardiomyocytes. We showed that Ca\(^{2+} \) modulates \( \text{I}_{\text{Na}} \) density without affecting Na\(^+ \) channel gating properties. The latter finding contrasts with the report of Wingo et al. This discrepancy may be explained by the differences in the cell types studied. Moreover, it emphasizes that findings obtained in heterologous expression systems may not always be applicable to native myocytes. Further evidence that it is relevant to consider the cell type studied comes from the observation that increases in \([\text{Ca}^{2+}]\) from 100 to 500 nM, did cause Na\(^+ \) channel gating changes in rabbit ventricular myocytes which were cultured for 24 h.\(^{12}\)

Our findings that changes in \( \text{I}_{\text{Na}} \) magnitude and d\( V/dt_{\text{max}} \) in response to changes in \([\text{Ca}^{2+}]\) (following Ca\(^{2+} \) dialysis with a perfused pipette, and caffeine administration and its cessation, respectively) occurred instantaneously, argued against a Ca\(^{2+} \) effect on expression and subsequent sarcolemmal insertion of Na\(^+ \) channels. It is believed that the expression of plasma membrane proteins that must traverse the Golgi apparatus before membrane insertion would require a significantly longer time. However, our single-channel experiments indicated that Ca\(^{2+} \) acts on \( \text{I}_{\text{Na}} \) density by modulating Na\(^+ \) channel permeation. Indeed, Na\(^+ \) channel conductance was reduced in the presence of Ca500.

4.2 Ca\(^{2+} \)–dependent modulation of Na\(^+ \) channel through Ca\(^{2+} \)–dependent proteins

While we aimed to establish the direct effect of physiological \([\text{Ca}^{2+}]\) on \( \text{I}_{\text{Na}} \) properties under control and HF conditions, it must be noted that, besides a direct modulation, Ca\(^{2+} \) can regulate the cardiac Na\(^+ \) channel through Ca\(^{2+} \)-activated...
proteins. Ca\(^{2+}\)-dependent interaction of the Ca\(^{2+}\)-binding protein calmodulin (CaM) with an IQ-like motif in the Na\(^+\) channel C-terminus may modulate Na\(^+\) channel gating in heterologous expression systems\(^{27-29}\) and isolated cardiomyocytes.\(^{30}\) However, the nature of the reported gating changes was not consistent\(^{27-30}\) and binding between CaM and the cardiac Na\(^+\) channel was not confirmed in all studies.\(^{31}\) A role for the Ca\(^{2+}\)/CaM-dependent protein kinase II (CaMklII) has also emerged. Adenovirus-mediated CaMKlIIic overexpression in cultured rabbit myocytes induced a hyperpolarizing shift in voltage-dependent inactivation, increased slow inactivation, and slowed recovery from inactivation in a Ca\(^{2+}\)-dependent manner. Moreover, INa decay was slower, and the late component of INa was increased, the latter effect being confirmed in a recent study.\(^{32}\) All these effects were also seen in transgenic mice overexpressing CaMKlIIic and they could be reversed with the use of CaMKlII inhibitors.\(^{12}\) Of note, none of these studies, which were performed either in heterologous expression systems or isolated cardiac myocytes, have reported some effects of CaM or CaMKlII on INa density. On the other hand, altered Ca\(^{2+}\)-, homeostasis leading to protein kinase C (PKC) activation was considered the probable cause of reductions in INa and dV/dt\(_{max}\) in transgenic mice overexpressing a constitutively active form of calcinurin.\(^{32}\) These results were in line with previous studies showing a decrease following PKC activation.\(^{33,34}\) Although our use of the inside-out configuration of the patch-clamp technique allowed INa recording from patches detached from myocyte cell membranes, it cannot be excluded that INa reduction was (partly) mediated by the activation of the Ca\(^{2+}\)-, dependent PKC.\(^{32-34}\)

### 4.3 Na\(^+\) channel modulation in heart failure

In HF, disturbed Ca\(^{2+}\)- handling (in particular, increased diastolic [Ca\(^{2+}\)]\(_i\)), ranks among the most consistent changes, found in ventricular myocytes isolated from various animal HF models, and in myocytes from failing human hearts. Previous studies have addressed the chronic effects of HF on INa density, but these results were not consistent. Downregulation of INa was found in different canine HF models\(^{35-37}\) and in human ventricular cells.\(^{36}\) Decrease in INa density may reduce excitability, thereby slowing myocardial conduction and contributing to re-entrant arrhythmias. However, Wiegerinck et al.\(^{38}\) provided no evidence for reduced INa in the volume/pressure overload HF rabbit model as used by us, in line with the present study and other findings which did not reveal changes in INa in dogs with pacing-induced HF.\(^{39}\) The contrasting results might be due to species/model differences.

To complement these studies, we demonstrated that acute reduction in INa density following increases in Ca\(^{2+}\)\(_i\), as observed at fast heart rates, is more prominent in HF. This finding agrees with observations that HF patients are particularly susceptible to reductions in cardiac excitability at fast heart rates.\(^{40}\) Several studies have shown that increased electrocardiographic QRS width, a clinically useful marker of cardiac excitability, is an independent predictor of sudden (arrhythmia-induced) death in HF patients.\(^{41,42}\) Future studies aimed at preventing arrhythmias and sudden death in HF may target the regulation mechanism revealed here.

**Supplementary material**

Supplementary material is available at *Cardiovascular Research* online.

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