Ca\textsubscript{V}1.3 L-type Ca\textsuperscript{2+} channel contributes to the heartbeat by generating a dihydropyridine-sensitive persistent Na\textsuperscript{+} current

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The spontaneous activity of sinoatrial node (SAN) pacemaker cells is generated by a functional interplay between the activity of ionic currents of the plasma membrane and intracellular Ca\textsuperscript{2+} dynamics. The molecular correlate of a dihydropyridine (DHP)-sensitive sustained inward Na\textsuperscript{+} current (\(I_{\text{Ca,L}}\)) share Ca\textsubscript{V}1.3 as a common molecular determinant. Patch-clamp recordings of mouse SAN cells showed that \(I_{\text{Ca,L}}\) is activated in the diastolic depolarization range, and displays Na\textsuperscript{+} permeability and minimal inactivation and sensitivity to \(I_{\text{Ca,L}}\) activators and blockers. Both Ca\textsubscript{V}1.3-mediated \(I_{\text{Ca,L}}\) and \(I_{\text{Ca,T}}\) were abolished in Ca\textsubscript{V}1.3-deficient (Ca\textsubscript{V}1.3\textsuperscript{-/-}) SAN cells but the Ca\textsubscript{V}1.2-mediated \(I_{\text{Ca,t}}\) current component was preserved. In SAN cells isolated from mice expressing DHP-insensitive Ca\textsubscript{V}1.2 channels (Ca\textsubscript{V}1.2\textsuperscript{DHP/-/-}), \(I_{\text{Ca,T}}\) displayed overlapping sensitivity and concentration-response relationships to the DHP blocker nifedipine. Consistent with the hypothesis that Ca\textsubscript{V}1.3 rather than Ca\textsubscript{V}1.2 underlies \(I_{\text{Ca,T}}\) a considerable fraction of \(I_{\text{Ca,T}}\) was resistant to nifedipine inhibition in Ca\textsubscript{V}1.2\textsuperscript{DHP/-/-} SAN cells. These findings identify Ca\textsubscript{V}1.3 channels as essential molecular components of the voltage-dependent, DHP-sensitive \(I_{\text{Ca,L}}\) Na\textsuperscript{+} current in the SAN.

Heart automaticity is generated by the spontaneous excitation of sinoatrial node (SAN) pacemaker cells. Spontaneous activity is due to the presence of the diastolic depolarization, which leads the membrane voltage from the end of the repolarization phase to the threshold of the following action potential. There has long been considerable debate regarding the ionic mechanisms underlying diastolic depolarization, reflecting the complex nature of this physiological process. Diastolic depolarization requires a net inward current, which results from the relative balance between the decaying outward delayed rectifier K\textsuperscript{+} currents (\(I_{\text{K,L}}\) and \(I_{\text{K,S}}\)) and the growing inward currents (see Mangoni and Nargeot for review\textsuperscript{1}). Previous studies have identified several voltage-gated inward currents activated in the diastolic depolarization range, including the hyperpolarization-activated inward current (\(I_{\text{f}}\)), the L- and T-type Ca\textsuperscript{2+} currents (\(I_{\text{Ca,L}}\) and \(I_{\text{Ca,T}}\))\textsuperscript{3,4} and the sustained inward Na\textsuperscript{+} current (\(I_{\text{Na}}\))\textsuperscript{5}. Additionally, recent experimental evidence has supported an alternative mechanism to promote pacemaker activity, in which spontaneous local Ca\textsuperscript{2+} release from intracellular Ca\textsuperscript{2+} stores stimulates electrogenic Na\textsuperscript{+}–Ca\textsuperscript{2+} exchanger (\(I_{\text{NCX}}\)) activity to depolarize the membrane voltage during diastolic depolarization\textsuperscript{6}. Thus, multiple inward current systems rather than a single pacemaker current are responsible for the spontaneous activity in the SAN.

Selective pharmacological block or genetic ablation of ion channels has been extensively used to describe the contribution of ionic currents to pacemaker activity. The molecular correlates of most cardiac ionic currents have been identified allowing the development of genetically modified mouse models targeting specific ion channels including HCN4\textsuperscript{7,8}, HCN2\textsuperscript{-/-}, \(I_{\text{Ca,L}}\)\textsuperscript{10}, Ca\textsubscript{V}1.3-\(I_{\text{Ca,L}}\)\textsuperscript{11,12}, Ca\textsubscript{V}3.1-\(I_{\text{Ca,T}}\) and Ncx1-\(I_{\text{NCX}}\)\textsuperscript{14}. By contrast, the complete lack

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of knowledge about the molecular determinants of $I_{st}$ has so far prevented the evaluation of the physiological role of this important ionic current.

$I_{st}$ was reported as a novel inward current in SAN cells of several mammalian species including rabbits, guinea-pigs, rats and mice. $I_{st}$ is activated at low membrane voltages and supplies persistent inward current flowing over the full diastolic depolarization range. Therefore, it has been proposed that this current is a physiologically important contributor to diastolic depolarization. However, two decades after the first description of $I_{st}$ by Guo et al., there remains little progress in the identification of the molecular determinant of $I_{st}$. Furthermore, no specific blocker for $I_{st}$ is available, limiting the understanding of its physiological role in SAN pacemaker activity. Although $I_{st}$ is carried by Na$^+$, its pharmacological features closely resemble those of $I_{Ca,L}$: $I_{st}$ is not affected by the voltage-gated Na$^+$ current ($I_{Na}$) blocker tetrodotoxin (TTX), but is inhibited by various chemical classes of organic Ca$^{2+}$ channel blockers, e.g. dihydropyridines (DHPs), and enhanced by the $I_{Ca,L}$ channel activator Bay-K8644. Moreover, like $I_{Ca,L}$, $I_{st}$ is also stimulated by $\beta$-adrenergic activation. These pharmacological properties are highly specific for $I_{Ca,L}$ and suggest the possibility that the pore-forming $\alpha_1$-subunit of $L$-type Ca$^{2+}$ channels which carry the drug-binding domains for organic Ca$^{2+}$-channel blockers and activators are also essential for $I_{st}$ activity. Cardiac $L$-type Ca$^{2+}$ channels are heteromultimers in which the pore-forming $\alpha_1$-subunit associates with auxiliary subunits (in particular $\beta$, $\delta$ and $\gamma$ subunits). In SAN cells, two different $\alpha_1$-subunits, Ca$\alpha_{1.2}$ and Ca$\alpha_{1.3}$, are expressed. Ca$\alpha_{1.2}$ is uniformly expressed in heart tissue, whereas Ca$\alpha_{1.3}$ is nearly absent in ventricles but is abundant in the conduction system including the SAN 12, 23, 24. While Ca$\alpha_{1.3}$ channels activate in the diastolic depolarization range, Ca$\alpha_{1.2}$ channels are activated in the upstroke phase of the action potential. In addition to forming distinct types of $I_{Ca,L}$ with different voltage dependencies of activation and inactivation, Ca$\alpha_{1.3}$ and Ca$\alpha_{1.2}$ channels are also differentially localized in SAN cell membranes.

Here we tested the hypothesis that Ca$\alpha_{1.2}$ and/or/or Ca$\alpha_{1.3}$ channels are required for generating $I_{st}$. Using two genetically modified mouse strains we demonstrate that the Ca$\alpha_{1.3}$ $L$-type Ca$^{2+}$-channel isoform is essential for functional expression of $I_{st}$ in mouse SAN cells. Although the exact molecular mechanism linking Ca$\alpha_{1.3}$ activity to $I_{st}$ remains to be elucidated, our data show that Ca$\alpha_{1.3}$ channels participate in the formation of a DHP-sensitive, voltage-dependent Na$^+$ conductance in SAN cells.

**Results**

**Identification of $I_{st}$ in mouse SAN cells.** The magnitude of $I_{st}$ varies depending on SAN cell types with distinct morphologies. Mouse SAN cells used for $I_{st}$ recordings were typically spindle- or spider-shaped with no obvious striations. These cells were small ($C_m = 34.8 \pm 1.2 \text{ pF}$; $n = 42$) compared to rod-shaped atrial-like cells and were spontaneously beating when superfused with normal Tyrode solution. To confirm the presence of $I_{st}$ in these cells, the late currents elicited by 1-s depolarizing voltage-clamp steps to various test potentials from a holding potential of $-90 \text{ mV}$ were examined for the characteristics of $I_{st}$ (Fig. 1). In order to avoid contamination of recordings by K$^+$ currents, we employed a Cs$^+$-rich internal solution. $I_{st}$ was removed by substituting K$^+$ with Cs$^+$ in the external Tyrode solution, which contained 1.8 mM Ca$^{2+}$. To confirm the sensitivity of the sustained current to DHPs, the typical hallmark of $I_{Ca,L}$, we tested the sensitivity of the current to the potent DHP L-type Ca$^{2+}$-channel blocker isradipine, which has not previously been tested. In Fig. 1Aa, membrane currents recorded under control conditions (black trace), after lowering [Ca$^{2+}$-$\text{in}$], from 1.8 to 0.1 mM (blue trace) and during subsequent application of 1 $\mu$M isradipine (red trace) are superimposed at individual test potentials. In the control bathing solution, membrane depolarization positive to $-60 \text{ mV}$ evoked a large transient inward current attributable to the activation of $I_{Na}$ and $I_{Ca,L}$ (note that peaks are not to scale in the figure), followed by a late inward current sustained during the entire period of 1-s depolarizing pulses. An inward current with a slow current decay was observed at test potentials of $>-40 \text{ mV}$, as expected for $I_{Ca,L}$ activation. The current-to-voltage ($I$–$V$) relationship obtained by plotting the current amplitude measured near the end of test pulses indicated that the late current level becomes more inward with increasing depolarization between $-70$ and $-50 \text{ mV}$ (black circles, Fig. 1Ab), generating a negative slope conductance in the range of the diastolic depolarization. Lowering external Ca$^{2+}$ reduced a considerable fraction of $I_{Ca,L}$ at membrane voltages positive to $-30 \text{ mV}$ (inset, Fig. 1Aa), whereas the sustained inward current was not reduced. It is thus unlikely that the sustained inward current was generated by a window component of $I_{Ca,L}$. However, bath application of isradipine readily inhibited the sustained inward current and unmasked an almost linear background conductance (Fig. 1Aa,b). Under conditions of low [Ca$^{2+}$-$\text{in}$], the DHP-sensitive sustained inward current peaked at $-50 \text{ mV}$ and the current direction was reversed at $+26 \text{ mV}$ (Fig. 1Ac).

Since $I_{st}$ has been shown to be carried by Na$^+$, we tested the permeability of the sustained inward current component for Na$^+$. The external Na$^+$ was replaced with an equimolar amount of N-methyl-D-glucamine (NMDG) in the presence of 1.8 mM Ca$^{2+}$ (Fig. 1B). Perfusion of SAN cells with Na$^+$-free NMDG solution readily suppressed the sustained inward current as well as Na$^+$-dependent background conductance (Fig. 1Ba and b). Subsequent application of 1 $\mu$M nifedipine did not affect the late inward current component, indicating that Na$^+$ was the predominant ion carrying the DHP-sensitive sustained inward current. At voltages positive to $-20 \text{ mV}$ the outward current component was partially reduced by nifedipine (average current density of the DHP-sensitive outward current, 0.34 \pm 0.08 pA/pF at $+20 \text{ mV}$; $n = 4$, two independent experiments: N = 2), suggesting that Ca$^{2+}$ was carrying the DHP-sensitive current component.

We next evaluated the kinetics of inactivation of the sustained inward current in mouse SAN cells in further detail (Fig. 1C and D). In the experiment shown in Fig. 1C, the inactivation time course was determined by measuring the fractional change of the sustained current elicited by a depolarizing step to $-50 \text{ mV}$ from a holding potential of $-80 \text{ mV}$ in 0.1 mM [Ca$^{2+}$-$\text{in}$] immediately (0.05 s) before ($I_{st}$) and after ($I_{st}$) conditioning pulses to 0 mV of variable duration (0.5–4.5 s). Nifedipine was then applied to acquire the background current (red trace) at $-50 \text{ mV}$, which was used to evaluate the net amplitude of the DHP-sensitive inward current. In Fig. 1C the ratio of $I_{st}/I_{net}$ is plotted as a function of the conditioning pulse duration, indicating that while $I_{st}$ displayed
Figure 1. Presence of $I_{Ca}$ in mouse SAN cells. (A) (a) Superimposed whole-cell membrane currents recorded from the same cell in 1.8 mM $[Ca^{2+}]_{o}$ (control, black), in 0.1 mM $[Ca^{2+}]_{o}$ (blue) and after exposure to 1 µM isradipine (red). Individual SAN cells were voltage clamped at a holding potential of −90 mV and depolarized for 1 s to test potentials in 10-mV increments. Peaks of transient inward currents at the beginning of test pulses are not to scale. The inset shows close-up views of the initial part (red shaded area) of current traces. (b) Corresponding isochronal $I–V$ relationships of the late current measured at time points marked with arrows in a. (c) $I–V$ relationship of the isradipine-sensitive current obtained by subtraction of the current recorded after application of isradipine from that recorded in 0.1 mM $[Ca^{2+}]_{o}$. (B) (a) Superimposed whole-cell membrane currents recorded in the same cell in 1.8 mM $[Ca^{2+}]_{o}$ (control, black), in NMDG-substituted, Na$^{+}$-free solution (blue) and after exposure to 1 µM nifedipine (red) using the same pulse protocol as in (A). The inset shows close-up views of the initial part (red shaded area) of current traces. (b) Corresponding $I–V$ relationships of the late currents from the recording depicted in a. (c) $I–V$ relationship of the nifedipine-sensitive current obtained by subtraction of currents after application of nifedipine from recordings in the NMDG-substituted, Na$^{+}$-free solution. (C) Time dependency of the sustained inward current inactivation measured using a protocol (upper panel) consisting of a reference test pulse ($V_{test}$), a conditioning prepulse of various durations, and a subsequent test pulse ($V_{ref}$). Sample traces of $I_{test}$ (black) and $I_{ref}$ (blue) recorded in response to $V_{ref}$ and $V_{test}$, respectively, in 0.1 mM $[Ca^{2+}]_{o}$ were superimposed. The red trace (Nife) was recorded in the presence of 1 µM nifedipine and indicates the background level at −50 mV (red dash line). The dotted line (black) indicates the zero-current level. The bottom panel shows a plot of the average ratio of $I_{test}/I_{ref}$ as a function of the conditioning pulse duration. The continuous line represents a single exponential fit. (D) Time dependency of the recovery from inactivation of the sustained inward current measured using a double-pulse protocol (upper panel) with varying recovery intervals (0.05–8.05 s) at −80 mV between a 5-s conditioning prepulse to 0 mV and a test pulse ($V_{test}$) to −50 mV. Current amplitudes are normalized to the largest current obtained with a recovery interval of 8.05 s. Sample traces and panels are labelled as in (C).
The inset shows expanded traces at the beginning of the voltage-command pulse. (c) I–V relationship of $I_{st}$ isolated by subtracting current recordings before and after application of nifedipine in the presence of TTX. (B) Effects of Na+ replacement with Li+ on $I_{st}$. The I–V relationships were constructed on current recordings before (black) and after (blue) total replacement of Na+ with Li+ in the 0.1 mM [Ca2+]o solution. Currents were elicited by 1-s depolarizing pulses (10 mV increment) to various test potentials from a holding potential of −90 mV. The inset shows original current traces at −50 mV. (C) Effects of autonomic agonists on $I_{st}$. The I–V relationships were obtained from current recordings in the same cell during the descending limb of a voltage ramp (similar to (A)) in the control 0.1 mM [Ca2+]o solution (black), during exposure to 100 nM Iso (blue) and Iso plus 1 µM ACh (purple), and after addition of 1 µM diltiazem (Dil, red). (D) Effects of a non-DHP $I_{Ca,L}$ agonist on $I_{st}$. Superimposed I–V relationships were obtained during the descending limb of a voltage ramp (similar to (A)) in the control 0.1 mM [Ca2+]o solution (black), during exposure to 1 µM FPL-64176 (Fpl, blue) and after subsequent addition of 1 µM verapamil (Ver, red).

**Figure 2.** Pharmacological properties of $I_{st}$ in mouse SAN cells. (A) Effects of TTX on $I_{st}$. (a) Voltage ramp pulse protocol and original current traces recorded from the same cell in 0.1 mM [Ca2+]o, (control, black), during exposure to 10 µM TTX (blue) and after subsequent addition of 1 µM nifedipine (red). The inset shows expanded traces at the beginning of the voltage-command pulse. (b) Corresponding I–V relationship obtained from current recordings during the descending ramp from +40 to −110 mV in a. (c) I–V relationship of $I_{st}$ isolated by subtracting current recordings before and after application of nifedipine in the presence of TTX. (B) Effects of Na+ replacement with Li+ on $I_{st}$. The I–V relationships were constructed on current recordings before (black) and after (blue) total replacement of Na+ with Li+ in the 0.1 mM [Ca2+]o solution. Currents were elicited by 1-s depolarizing pulses (10 mV increment) to various test potentials from a holding potential of −90 mV. The inset shows original current traces at −50 mV. (C) Effects of autonomic agonists on $I_{st}$. The I–V relationships were obtained from current recordings in the same cell during the descending limb of a voltage ramp (similar to (A)) in the control 0.1 mM [Ca2+]o solution (black), during exposure to 100 nM Iso (blue) and Iso plus 1 µM ACh (purple), and after addition of 1 µM diltiazem (Dil, red). (D) Effects of a non-DHP $I_{Ca,L}$ agonist on $I_{st}$. Superimposed I–V relationships were obtained during the descending limb of a voltage ramp (similar to (A)) in the control 0.1 mM [Ca2+]o solution (black), during exposure to 1 µM FPL-64176 (Fpl, blue) and after subsequent addition of 1 µM verapamil (Ver, red).

**slow inactivation (τ = 1.94 ± 0.57 s, n = 3, N = 1), a considerable current fraction remained available even after a 4.5-s conditioning pulse (0.48 ± 0.04, n = 3, N = 1). In addition, recovery from inactivation was assessed by applying a 5-s conditioning prepulse followed by test pulses to −50 mV after varying intervals of recovery (0.05–8.05 s) at −80 mV (Fig. 1D). Recovery of the sustained current proceeded exponentially with a time constant of 2.66 ± 0.63 s (n = 3, N = 1).

These properties (low voltage for activation, DHP sensitivity, Na+ permeability and slow inactivation), clearly identified the sustained inward current in our mouse SAN cell preparations as $I_{st}$ 15–18, 26. We only failed to record the sustained current in five of 24 experiments (~20%), which is likely to indicate inhomogeneous expression of $I_{st}$ in SAN cells 16. Four of five $I_{st}$-deficient cells were nearly indistinguishable from clear striated atrial-like myocytes.

$I_{Na}$ and $I_{NCX}$ do not contribute to $I_{st}$ in mouse SAN. To assess whether voltage-gated Na+ currents could interfere with $I_{st}$ recordings in mouse SAN cells, we investigated the effect of the $I_{Na}$ blocker TTX on the membrane current (Fig. 2A). Since $I_{st}$ exhibited little inactivation during the 1-s square pulse, the I–V relationship was measured using a slow (150 mV/s) voltage-ramp protocol in 0.1 mM [Ca2+]o. Under these conditions, the contributions of $I_{Na}$ and $I_{NCX}$ to the total membrane current were minimized 15–18. Figure 2Aa shows a superimposition of the original current traces in response to the voltage ramp in the control (black trace), during 10 µM TTX application (blue trace) and after nifedipine application (red trace). Figure 2Ab displays the corresponding...
I–V relationships obtained from the descending limb of the voltage ramp. As evidenced in the current recordings and in the corresponding I–V curve, bath application of TTX readily inhibited the transient inward \( I_{\text{Na}} \) at the beginning of the pulse (see expanded traces in the inset), but did not affect the subsequent current. Application of nifedipine (1 µM) then revealed that the TTX-insensitive and DHP-sensitive current component could be attributed to \( I_{\text{Ca,L}} \) (Fig. 2Ac). It should be noted that partial inhibition of the late inward current by TTX was observed in six of 23 cells (26%) (average current density of TTX-sensitive current, 0.82 ± 0.17 pA/pF at −50 mV; \( n = 6 \), \( N = 4 \)), suggesting that some mouse SAN cells also express a TTX-sensitive persistent Na\(^+\) current\(^27\).

The involvement of \( I_{\text{SCX}} \) was also investigated (Fig. 2B). \( I_{\text{Na}} \) was hardly affected by total replacement of external Na\(^+\) with an equimolar concentration of Li\(^+\) to abolish \( I_{\text{SCX}} \). This result is consistent with the previously characterized selectivity of \( I_{\text{Na}} \) to monovalent cations\(^28\). In conclusion, \( I_{\text{SCX}} \) did not contaminate our recordings of \( I_{\text{Na}} \).

**Sensitivity of \( I_{\text{Na}} \) to \( I_{\text{Ca,L}} \) modulators in mouse SAN cells.** We then characterized the pharmacological properties of \( I_{\text{Na}} \) by testing its sensitivity to various \( I_{\text{Ca,L}} \) modulators in the presence of 0.1 mM [Ca\(^{2+}\)]\(_{o}\) solution. In the experiment shown in Fig. 2C, 0.1 µM isoprenaline (Iso) strongly increased \( I_{\text{Na}} \) (116.1 ± 16.8%, \( n = 8 \), \( N = 2 \), \( p = 0.0003 \)). This stimulatory effect was almost reversed by addition of 1 µM acetylcholine (ACh) in the presence of Iso (84.3 ± 2.7%, \( n = 4 \), \( N = 2 \), \( p = 0.0029 \)). Finally, the non-DHP \( I_{\text{Ca,L}} \) blocker diltiazem (1 µM) completely abolished \( I_{\text{Na}} \). By contrast, the \( I_{\text{Ca,L}} \) agonist FPL-64176 (1 µM) potentiated the amplitude of \( I_{\text{Na}} \) nearly twofold, whereas application of 1 µM verapamil totally abolished \( I_{\text{Na}} \) (Fig. 2D). Our observations, in addition to the findings of previous studies\(^5,15,16\), indicate that the pharmacological properties of \( I_{\text{Na}} \) are undistinguishable from those of \( I_{\text{Ca,L}} \) (Table 1).

### Table 1. Pharmacological similarities between \( I_{\text{Na}} \) and \( I_{\text{Ca,L}} \)

| Drugs                  | Concentration [µM] | \( I_{\text{Na}} \) | \( I_{\text{Ca,L}} \) | References |
|------------------------|-------------------|---------------------|---------------------|------------|
| Dihydropyridines       |                   |                     |                     |            |
| Nicardipine            | 0.25–0.5          | block               | block               | Guo et al\(^2\) |
| Nifedipine             | 0.03–1.0          | block               | block               | this study |
| Isradipine             | 1.0               | block               | block               | this study |
| Bay-K8644 (agonist)    | 1.0               | increase            | increase            | Guo et al\(^15\) |
| Benzothiazepines       |                   |                     |                     |            |
| Diltiazem              | 1.0               | block               | block               | this study |
| Phenylalkylamines      |                   |                     |                     |            |
| D600                   | 0.1               | block               | block               | Guo et al\(^2\) |
| Verapamil              | 1.0               | block               | block               | this study |
| FPL-64176 (agonist)    | 1.0               | increase            | increase            | this study |
| Tetrodotoxin           | 10–30             | no effect           | no effect           | this study |
| Isoprenaline           | ~0.1              | increase            | increase            | Toyoda et al\(^27\); this study |

\( I_{\text{Na}} \) is absent in Ca\(^{2+}\)-conducting SAN cells.** The undistinguishable pharmacological properties of \( I_{\text{Na}} \) and \( I_{\text{Ca,L}} \) (Table 1) provided a strong rationale for testing the hypothesis that these currents share common molecular determinants. It is now generally accepted that \( I_{\text{Ca,L}} \) in SAN cells is composed of two separate current components mediated by distinct pore-forming alpha subunits, Ca\(_{v}1.2 \) and Ca\(_{v}1.3\(^11,12\). To directly examine the possibility of a functional link between \( I_{\text{Na}} \) and Ca\(_{v}1.3 \), we recorded \( I_{\text{Ca,L}} \) and \( I_{\text{Na}} \) in SAN cells from mice lacking Ca\(_{v}1.3 \) channels (Ca\(_{v}1.3\)-/- mice, Fig. 3). Since most SAN cells obtained from Ca\(_{v}1.3\)-/- mice were quiescent, we selected single cells for recordings based on morphological criteria rather than spontaneous activity. After the control recording in Ca\(^{2+}\)-substituted, K\(^{-}\)-free Tyrode solution with 1.8 mM [Ca\(^{2+}\)]\(_{o}\) (black traces), \( I_{\text{Na}} \) was separated from \( I_{\text{Ca,L}} \) by switching the bath solution to 0.1 mM [Ca\(^{2+}\)]\(_{o}\) containing 10 µM TTX (blue traces). \( I_{\text{Na}} \) was identified as a current component inhibited by subsequent application of 1 µM nifedipine (red traces). Consistent with previous studies, genetic ablation of Ca\(_{v}1.3 \) channels resulted in considerable reduction of \( I_{\text{Ca,L}} \)\(^12\) as well as a shift in the current half-activation voltage\(^12\) (Fig. 3A–C). Indeed, the peak density of \( I_{\text{Ca,L}} \) was significantly reduced from −6.97 ± 0.85 pA/pF in wild-type SAN cells (\( n = 19 \), \( N = 6 \)) to −4.81 ± 0.45 pA/pF in Ca\(_{v}1.3\)-/- cells (\( n = 18 \), \( N = 6 \), \( p = 0.0336 \)), and was accompanied by a positive shift in the peak of the \( I–V \) relationship by ~20 mV (Fig. 3C). The calculated half-maximal activation voltage (\( V_{0.5\text{act}} \)) was shifted from −29.3 mV in wild-type cells to −12.8 mV in Ca\(_{v}1.3\)-/- SAN cells.

\( I_{\text{Ca,L}} \) was evident in wild-type SAN cells after \( I_{\text{Ca,L}} \) removal by lowering [Ca\(^{2+}\)]\(_{o}\) as manifested by the increase in the sustained inward current with depolarization between −70 and −50 mV that was finally blocked by nifedipine (Fig. 3A and B). By contrast, the late current obtained from Ca\(_{v}1.3\)-/- SAN cells changed linearly with command voltage in the 0.1 mM [Ca\(^{2+}\)]\(_{o}\) solution with 10 µM TTX, and there remained no detectable DHP-sensitive current. As shown in Fig. 3D, the average peak density of \( I_{\text{Na}} \) was −0.98 ± 0.09 pA/pF (\( n = 19 \), \( N = 6 \)) in wild-type cells, while it was reduced below detectable levels in Ca\(_{v}1.3\)-/- cells (\( n = 18 \), \( N = 6 \)). Thus, we concluded that \( I_{\text{Na}} \) was virtually absent in SAN cells from Ca\(_{v}1.3\)-/- mice.

The above results suggested that Ca\(_{v}1.3 \) mediated two different currents in SAN cells, i.e. Ca\(^{2+}\)-conducting \( I_{\text{Ca,L}} \) and Na\(^{-}\)-conducting \( I_{\text{Na}} \). To support this hypothesis and estimate the contribution of Ca\(_{v}1.3 \)-mediated \( I_{\text{Ca,L}} \) and \( I_{\text{Na}} \) to the diastolic depolarization, \( I_{\text{Ca,L}} \) and \( I_{\text{Na}} \) were alternately recorded in the same cell under distinct external ionic conditions and activation in the diastolic depolarization range was evaluated in wild-type and Ca\(_{v}1.3\)-/- SAN
cells (Fig. 4). A slow ascending ramp (−65 to −35 mV, 100 mV/s) voltage command was employed to mimic the diastolic depolarization. We first recorded $I_{Ca,L}$ using the 0 Na, 1.8 mM Ca$^{2+}$ external solution (Fig. 4A). Under these recording conditions, the voltage ramp gradually activated an inward current yielding negative slope conductance in wild-type SAN cells. This current was strongly augmented by Iso and inhibited by subsequent perfusion of nifedipine (red). Under these recording conditions, the voltage ramp gradually activated an inward current yielding negative slope conductance in wild-type SAN cells. This current was strongly augmented by Iso and inhibited by subsequent perfusion of nifedipine (red).

**Figure 3.** Absence of $I_{Ca,L}$ in SAN cells from CaV1.3−/− mice. (A) Representative examples of current recordings in SAN cells obtained from wild-type (upper panel) and CaV1.3−/− (lower panel) mice. Currents were elicited by voltage steps to test potentials between −80 and +40 mV (10 mV increments) preceded by a conditioning pulse to −50 mV from a holding potential of −90 mV in 1.8 mM [Ca$^{2+}$]o (control, black), in 0.1 mM [Ca$^{2+}$]o solution containing 10 µM TTX (blue) and after applying 1 µM nifedipine (red). (B) Average I-V relationships of current densities measured at the time points indicated in (A). Data represent the mean ± S.E.M. of wild-type (left, n = 19) and CaV1.3−/− (right, n = 18) SAN cells. (C) I-V relationships of $I_{Ca,L}$ in wild-type (closed symbols) and CaV1.3−/− (open symbols) SAN cells, obtained by subtraction of currents after lowering [Ca$^{2+}$]o, from recordings in the control solution. (D) I-V relationships of $I_{Ca,L}$ in wild-type (closed symbols) and CaV1.3−/− (open symbols) SAN cells, measured as the nifedipine-sensitive current in 0.1 mM [Ca$^{2+}$]o.
We did not observe either $I_{st}$ or $I_{Ca,L}$ in atrial-like myocytes isolated from wild-type SAN ($n=4$, $N=3$, data not shown). Thus, the presence of $I_{st}$ was always coupled to the low voltage-activated $I_{Ca,L}$, which is consistent with the view that $CaV_{1.3}$ mediates both $I_{Ca,L}$ and $I_{st}$. $CaV_{1.2}$ channels are not involved in the generation of $I_{st}$. The absence of $I_{st}$ in $CaV_{1.3}^{-/-}$ SAN cells does not exclude the possibility that $CaV_{1.2}$ also contributes to the generation of $I_{st}$. To examine the involvement of $CaV_{1.2}$ in $I_{st}$, we employed knock-in mice in which a point mutation (T1066Y) abolishes the sensitivity of $CaV_{1.2}$ to DHPs without changing channel function and expression ($CaV_{1.2}^{DHP^{-/-}}$ mice, Fig. 5)\(^2\). In this mouse model, selective blockade of $CaV_{1.3}$ by DHPs enables the functional contributions of $CaV_{1.2}$ and $CaV_{1.3}$ to the generation of $I_{Ca,L}$ to be distinguished. We first tested the effect of nifedipine on $I_{Ca,L}$ in $CaV_{1.2}^{DHP^{-/-}}$ mice (Fig. 5A). $I_{Ca,L}$ was recorded after elimination of $I_{st}$ and $I_{Na}$ by Na\(^+\) removal from the external recording solution. Bath application of nifedipine (0.03–1 µM) reduced the peak amplitude of $I_{Ca,L}$ in a concentration-dependent manner to a maximum of ~64% even at a saturating concentration of DHP (1 µM). This residual DHP-resistant $I_{Ca,L}$ was completely blocked by application of verapamil (3 µM), in line with previous data showing that the T1066Y mutation preserves the high sensitivity of $CaV_{1.2}$ to phenylalkylamines\(^2\). Peak inward current of the nifedipine-resistant component activated more slowly (Fig. 5A), as expected for $CaV_{1.2}$-mediated

**Figure 4.** $CaV_{1.3}$ mediates both $I_{Ca,L}$ and $I_{st}$ in the SAN diastolic depolarization range. (A,C) Representative whole-cell membrane currents recorded from the same wild-type SAN cell using two different external solutions: TEA\(^+\)-substituted, Na\(^+\)-free external solution containing 1.8 mM Ca\(^{2+}\) (A) to record $I_{Ca,L}$ and 140 mM [Na\(^+\)]\(_o\) solution containing 0.1 mM Ca\(^{2+}\) plus 10 µM TTX (C) to record $I_{st}$. The cell was first held at −75 mV. Then, a slow ascending ramp (100 mV/s) voltage command was used to elicit $I_{Ca,L}$ or $I_{st}$ (top panel), from −65 to −35 mV, followed by depolarization to +10 mV for 50 ms. Under distinct external conditions, currents were recorded in the absence (black trace, left panel) and presence (blue trace, left panel) of 100 nM Iso, and after addition of 1 µM nifedipine (red trace, left panel). Nifedipine-sensitive net $I_{Ca,L}$ and $I_{st}$ in the absence (black trace, right panel) and presence (blue trace, right panel) of Iso were obtained by digital subtraction of current traces before and after application of nifedipine (right panel). (B,D) Representative $I_{Ca,L}$ (B) and $I_{st}$ (D) in $CaV_{1.3}^{-/-}$ SAN cells, recorded using the same protocol as in (A,C), respectively. (E) Sample current recordings in a wild-type SAN cell during gradual replacement of the external solution for $I_{Ca,L}$ recording with that for $I_{st}$ recording in the presence of Iso. Arrows indicate the peak of $I_{Ca,L}$. 

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**CaV\(_{1.2}\) channels are not involved in the generation of $I_{st}$.** The absence of $I_{st}$ in $CaV_{1.3}^{-/-}$ SAN cells does not exclude the possibility that $CaV_{1.2}$ also contributes to the generation of $I_{st}$. To examine the involvement of $CaV_{1.2}$ in $I_{st}$, we employed knock-in mice in which a point mutation (T1066Y) abolishes the sensitivity of $CaV_{1.2}$ to DHPs without changing channel function and expression ($CaV_{1.2}^{DHP^{-/-}}$ mice, Fig. 5)\(^2\). In this mouse model, selective blockade of $CaV_{1.3}$ by DHPs enables the functional contributions of $CaV_{1.2}$ and $CaV_{1.3}$ to the generation of $I_{Ca,L}$ to be distinguished. We first tested the effect of nifedipine on $I_{Ca,L}$ in SAN cells from $CaV_{1.2}^{DHP^{-/-}}$ mice (Fig. 5A). $I_{Ca,L}$ was recorded after elimination of $I_{Na}$ and $I_{st}$ by Na\(^+\) removal from the external recording solution. Bath application of nifedipine (0.03–1 µM) reduced the peak amplitude of $I_{Ca,L}$ in a concentration-dependent manner to a maximum of ~64% even at a saturating concentration of DHP (1 µM). This residual DHP-resistant $I_{Ca,L}$ was completely blocked by application of verapamil (3 µM), in line with previous data showing that the T1066Y mutation preserves the high sensitivity of $CaV_{1.2}$ to phenylalkylamines\(^2\). Peak inward current of the nifedipine-resistant component activated more slowly (Fig. 5A), as expected for $CaV_{1.2}$-mediated
Figure 5. Sensitivity of $I_{\text{Ca,L}}$ and $I_\text{Ca,L}$ to nifedipine in SAN cells from CaV1.2DHP−/− mice. (A) $I_{\text{Ca,L}}$ inhibition by nifedipine in CaV1.2DHP−/− SAN cells. (a) Superimposed sample traces of $I_{\text{Ca,L}}$ elicited by depolarization to −10 mV from a holding potential of −50 mV in TEA−-substituted, Na+−free Tyrode solution (control, black) and during nifedipine application at various concentrations (0.03–1 µM, red), and after subsequent addition of 3 µM verapamil (blue). (b) Concentration-dependent inhibition of $I_{\text{Ca,L}}$ by nifedipine. Inhibition is expressed as the percentage of residual $I_{\text{Ca,L}}$ inhibited by nifedipine relative to control $I_{\text{Ca,L}}$. Data are mean ± S.E.M. of four experiments.

We then examined whether altered sensitivity of CaV1.2 to DHP also affected the response of $I_\text{Ca,L}$ to nifedipine. The concentration–dependence inhibition of $I_\text{Ca,L}$ by nifedipine was investigated in SAN cells isolated from wild-type and CaV1.2DHP−/− mice (Fig. 5B). After suppressing $I_{\text{Ca,L}}$ by lowering [Ca2+]o to 0.1 mM, $I_\text{Ca,L}$ was elicited by the voltage ramp in the presence of nifedipine at various concentrations (0.03–1 µM). $I_\text{Ca,L}$ in CaV1.2DHP−/− SAN cells was reduced by nifedipine to levels similar to those observed in wild-type cells. Indeed, we failed to detect a nifedipine-resistant $I_\text{Ca,L}$ component at 1 µM. Fitting the concentration–response relationship to the Hill equation gave a half-maximal inhibitory concentration (IC50) value of 18.5 ± 2.8 nM (n = 4, N = 4) in CaV1.2DHP−/− SAN cells, similar to the value of 12.9 ± 2.8 nM (n = 4, N = 4) in wild-type cells. Thus, it is unlikely that CaV1.2 confers the structural basis for $I_\text{Ca,L}$ sensitivity to DHPs. Of interest, the IC50 for $I_\text{Ca,L}$ was close to that for $I_{\text{Ca,L}}$ in CaV1.2DHP−/− mice (10.2 ± 2.6 nM, n = 4, N = 4, Fig. 5A), suggesting that CaV1.3 is responsible for the DHP sensitivity of $I_\text{Ca,L}$.

Discussion

Here we have demonstrated, for the first time, that voltage-gated L-type CaV1.3 Ca2+ channels are essential for the expression of a DHP-sensitive, voltage-dependent Na+ conductance, previously described as $I_\text{Ca,L}$. Our finding is based on the observations that (1) $I_\text{Ca,L}$ is consistently identified in wild-type SAN cells but not in CaV1.3-deficient cells; (2) block of $I_\text{Ca,L}$ by nifedipine was unaffected upon ablation of CaV1.2 DHP sensitivity in CaV1.2DHP−/− SAN cells; (3) DHP sensitivity of $I_\text{Ca,L}$ overlapped that of CaV1.3-mediated $I_{\text{Ca,L}}$ in CaV1.2DHP−/− SAN cells; and (4) $I_\text{Ca,L}$ could not be attributed to late $I_\text{Na}$ or $I_{\text{NCX}}$. Sensitivity to Ca2+−channel blockers such as DHPs, verapamil, and diltiazem, as well as activators such as Bay-K8644 and FPL-64176, is based on highly specialized structural motifs conserved in CaV1.3 α1-subunits of L-type Ca2+ channels. Our genetic and pharmacological evidence showing overlapping properties between $I_\text{Ca,L}$ and CaV1.3-mediated $I_{\text{Ca,L}}$ indicates a close functional relationship between these currents in SAN cells (Table 1). The demonstration of CaV1.3 α1-subunits as essential molecular determinants of
a voltage-dependent DHP-sensitive Na\(^+\) conductance is a novel and unexpected finding and constitutes a fundamental step in elucidating the molecular nature of \(I_{\text{Ca}}\).

Our patch-clamp recordings clearly show that under physiological conditions \(I_{\text{Ca}}\) is predominantly carried by Na\(^+\) rather than Ca\(^{2+}\) ions. Indeed, while lowering external Ca\(^{2+}\) did not affect \(I_{\text{Ca}}\), removal of extracellular Na\(^+\) abolished the current even in the presence of a physiological concentration of Ca\(^{2+}\) (Fig. 1). L-type Ca\(^{2+}\) channels are permeable to Na\(^+\) in the absence of extracellular divalent cations\(^{32,33}\). However, L-type Ca\(^{2+}\) channels are highly selective for Ca\(^{2+}\) over Na\(^+\) with a permeation ratio \((P_{\text{Ca}}/P_{\text{Na}})\) of ~1000 under physiological conditions\(^{32}\). Therefore, Na\(^+\) influx through L-type Ca\(^{2+}\) channels is blocked by extracellular Ca\(^{2+}\) in the submicromolar range\(^{32,33}\). It is thus unlikely that the "classical" permeation pathway of L-type Ca\(^{2+}\) channels mediates \(I_{\text{Ca}}\). Indeed, currently available recombinant Ca\(^{1.3}\) channels with canonical channel pore sequence are Ca\(^{2+}\)-selective \(I_{\text{Ca,L}}\), with poor permeability to Na\(^+\) at least in the experimental solutions used for the \(I_{\text{Ca}}\) recording in the present study (Toyoda et al., unpublished observation). Our results therefore suggest that Ca\(^{1.3}\) \(\alpha\)-subunits in the SAN cell not only form Ca\(^{1.3}\) L-type channels but also contribute to the formation of voltage-gated Na\(^+\) conductance through an unknown mechanism.

However, revealing the molecular mechanism allowing Ca\(^{1.3}\) \(\alpha\)-subunits to form \(I_{\text{Ca}}\) is challenging. We favour the hypothesis that Ca\(^{1.3}\) \(\alpha\)-subunits themselves form the \(I_{\text{Ca}}\) pore due to the observation that \(I_{\text{Ca}}\) and Ca\(^{1.3}\) possess an essentially indistinguishable pharmacological profile and Ca\(^{2+}\)-channel blockers have been shown to exert their pharmacological modulation exclusively by binding to \(\alpha\)-subunits\(^{40}\). In this case the different ion selectivity of \(I_{\text{Ca}}\) in SAN cells would require a modification of the ion permeation pathway. Substitution of negatively charged residues forming the ion selectivity filter of voltage-gated Ca\(^{2+}\) channels by lysine can indeed induce persistent Na\(^+\) currents similar to \(I_{\text{Ca,L}}\)\(^{35,36}\), suggesting that increased Na\(^+\) permeability per se could reproduce \(I_{\text{Ca}}\) properties. To date, analysis of Ca\(^{1.3}\) transcripts has not identified alternatively spliced Ca\(^{1.3}\) variants with a modified selectivity filter\(^{37-39}\). Since Na\(^+\) conductance through such modified channels may be larger than for Ca\(^{2+}\)\(^{36,40}\), \(I_{\text{Ca}}\) transcripts may be present at low levels. This would make their detection particularly difficult in tissues with low cell numbers such as the SAN. On the other hand, the possibility that \(I_{\text{Ca}}\) could be generated by alternative splicing of Ca\(^{2+}\) channels is also suggested by a recent report that T-type (Ca\(^{3}\)) Ca\(^{2+}\) channels of the snail heart have high permeability to Na\(^+\) due to unique splicing in the outer pore region\(^{41}\). Although this possibility cannot be excluded for mammalian SAN, splicing of T-type \(\alpha\)-subunits appears an unlikely explanation for \(I_{\text{Ca}}\) because of the L-type channel-specific pharmacology. Another possible explanation for Na\(^+\) selectivity of Ca\(^{1.3}\) \(\alpha\)-subunits could be structural modifications of the ion conducting pathway through RNA editing, which so far has only been detected in the brain and in the cytoplasmic C-terminal tail of the channel\(^{39,42}\).

Alternatively, a cationic channel functionally coupled to Ca\(^{1.3}\) activity could mediate \(I_{\text{Ca}}\). In mouse SAN cells Ca\(^{1.3}\) is co-localized with sarcoplasmic reticulum ryanodine receptors (RyRs) and controls diastolic RyR-dependent Ca\(^{2+}\) release\(^{25-45}\). RyR-dependent Ca\(^{2+}\) release could then activate an inward Na\(^+\) current. However, the possibility that \(I_{\text{Ca}}\) is mediated by Ca\(^{2+}\)-dependent opening of a cationic channel appears unlikely, because \(I_{\text{Ca}}\) density did not decrease upon lowering extracellular Ca\(^{2+}\) (Fig. 1), as one would expect for Ca\(^{2+}\)-dependent opening of a Na\(^+\)-selective channel associated with Ca\(^{1.3}\). Finally, the possibility that \(I_{\text{Ca}}\) could be generated by direct opening of a Na\(^+\) channel physically coupled to Ca\(^{1.3}\) channel gating is also unlikely, because \(I_{\text{Ca}}\) activates negative to Ca\(^{1.3}\)-mediated \(I_{\text{Ca,L}}\) (Fig. 1).

Ca\(^{1.3}\) loss-of-function in mice or humans results in SAN dysfunction, which indicates that Ca\(^{1.3}\) channels play a major role in pacemaker activity\(^{11,12,44-46}\). Consequently, the present findings also suggest that the loss of \(I_{\text{Ca}}\) could contribute to the SAN dysfunction induced by Ca\(^{1.3}\) gene inactivation. In addition, our results indicate that the heart rate reducing effect of Ca\(^{2+}\) channel antagonists can be explained by drug binding to Ca\(^{1.3}\) channels and reduction of Ca\(^{1.3}\)-mediated \(I_{\text{Ca,L}}\) and \(I_{\text{Ca}}\). Consistent with previous observations\(^{11,12}\), our recordings in Ca\(^{1.3}\)-/- SAN cells show that Ca\(^{1.3}\) underlies a low-threshold \(I_{\text{Ca,L}}\) activated at voltages spanning the diastolic depolarization range. \(I_{\text{Ca}}\) differs from Ca\(^{1.3}\)-mediated \(I_{\text{Ca,L}}\) in the charge carrier and shows a more negative voltage for half-activation. \(I_{\text{Ca}}\) and Ca\(^{1.3}\)-mediated \(I_{\text{Ca,L}}\) could thus differentially contribute to the generation of the diastolic depolarization. For example, \(I_{\text{Ca}}\) could generate a persistent Na\(^+\) influx in the diastolic depolarization, while Ca\(^{1.3}\)-mediated \(I_{\text{Ca,L}}\) could generate inward Ca\(^{2+}\) current\(^{45}\) and control RyR-dependent Ca\(^{2+}\) release\(^{45}\). Notably, both \(I_{\text{Ca}}\) and Ca\(^{1.3}\)-mediated \(I_{\text{Ca,L}}\) are strongly potentiated by \(\beta\)-adrenergic activation, which suggests a dual role of Ca\(^{1.3}\) in the sympathetic control of heart rate via \(I_{\text{Ca,L}}\) and \(I_{\text{Ca}}\).

In conclusion, we provide novel evidence supporting the involvement of Ca\(^{1.3}\) in the generation of \(I_{\text{Ca}}\) in SAN cells. Our work provides valuable new insights into the molecular basis of \(I_{\text{Ca}}\) as well as the diverse functional significance of Ca\(^{1.3}\) in cardiac pacemaker activity.

Methods

Ethics. The investigation conforms to the Guide for the Care and Use of Laboratory Animals (8th edition, 2011), published by the US National Institutes of Health and European directives (2010/63/EU). The experimental protocol was approved by the Institutional Animal Care and Use Committee of Shiga University of Medical Science (Nos 2009-5-11, 2012-1-10 and 2014-12-3), the University of Montpellier and the University of Innsbruck.

**Ca\(^{1.3}\)–/-** and **Ca\(^{1.2}\)DHP–/-** mice. Ca\(^{1.3}\)–/- and Ca\(^{1.2}\)DHP–/- mice were obtained by crossing mice from the original mutant colonies\(^{28,44}\) with mice with a C57B6/J genetic background from Charles River in the animal facility, free of specific pathogenic organisms, of the Réseau d'Animalerie de Montpellier (RAM) at the Institut de Génétique Humaine (Montpellier, France). We next backcrossed the offspring for 10 generations with C57B6/J mice before starting the study. Animals were given ad libitum access to food and drinking water and were maintained in a 12-h light–dark cycle (light, 8:30 a.m. to 8:30 p.m.). Only homozygous Ca\(^{1.3}\)–/- and Ca\(^{1.2}\)DHP–/- mice were used for the experiments.
SAN cell preparations. Isolation of single SAN cells from mouse hearts was performed according to the methods of Mangoni and Nargeot(1). Wild-type (N = 18), Ca_v.1.3_−/− (N = 10) and Ca_v.1.2DHP_−/− (N = 5) mice were anaesthetized with ketamine (100 mg/kg) combined with xylazine (10 mg/kg), and anticoagulated with heparin (450 units in 0.2 ml/mouse). Beating hearts were quickly removed and the SAN region was excised and cut into small strips in warm (35 °C) Tyrode solution containing (in mM): 140.0 NaCl, 5.4 KCl, 1.8 CaCl_2, 1.0 MgCl_2, 5.0 HEPES-NaOH and 5.5 D-glucose (adjusted to pH 7.4 with NaOH). The SAN tissue strips were then transferred to a low-Ca^2+, low-Mg^2+ solution containing (in mM): 140.0 NaCl, 5.4 KCl, 0.5 MgCl_2, 0.2 CaCl_2, 1.2 KH_2PO_4, 50.0 taurine, 5.5 D-glucose and 5.0 HEPES-NaOH with 1.0 mg/ml bovine serum albumin (BSA) (adjusted to pH 6.9 with NaOH), and then subjected to digestion by adding Liberase TH (0.1 mg/ml, Roche Diagnostics GmbH) and elastase (1.9 U/ml, Worthington Biochem. Co.) at 35 °C for a variable time of ~9–14 min. Tissue strips were then transferred and washed in a Kraft-Bruhe (KB) solution containing (in mM): 70.0 L-glutamic acid, 20.0 KCl, 80.0 KOH, 10.0 (±) D-β-OH-butyric acid, 10.0 KH_2PO_4, 10.0 taurine and 10.0 HEPES-KOH, with 1 mg/ml BSA (pH adjusted to 7.4 with KOH). SAN cells were manually dissociated by agitation using a flame-forged Pasteur pipette in KB solution at 35 °C for ~5 min. Cellular automaticity was recovered by readapting the cells to physiological extracellular Na^+ and Ca^2+ concentrations by adding aliquots of solutions containing (in mM): 10.0 NaCl, 18 CaCl_2 and, subsequently, normal Tyrode solution containing 1 mg/ml BSA. The final storage solution contained (in mM): 100.0 NaCl, 35.0 KCl, 1.3 CaCl_2, 0.7 MgCl_2, 14.0 L-glutamic acid, 2.0 (±) D-β-OH-butyric acid, 2.0 KH_2PO_4 and 2.0 taurine, with 1.0 mg/ml BSA (pH 7.4). Cells were harvested in custom-made recording Plexiglass chambers with glass bottoms for proper cell attachment and rinsed with normal Tyrode solution warmed to 36 °C just before patch-clamp recording.

Whole-cell patch-clamp technique and data analysis. Isolated SAN cells were voltage-clamped using the whole-cell configuration of the patch-clamp technique with an EPC-8 patch-clamp amplifier equipped with an LIH-1600 AD/DA interface (HEKA) controlled by PatchMaster software or an Axon MultiClamp 700A amplifier equipped with Digidata 1332 A interface-controlled PC clamp software. Patch electrodes had a resistance of 2.5–4.0 MΩ when filled with the Cs^+-rich intracellular solution containing (in mM): 125 CsOH, 20 tetrathy- ammonium chloride (TEA-Cl), 1.2 CaCl_2, 5 Mg-ATP, 0.1 Li_2-GTP, 5.0 EGTA and 10.0 HEPES (pH adjusted to 7.2 with aspartate). The concentration of free Ca^2+ in the pipette solutions was calculated to be approximately 4.8 × 10^{-4} M (pCa = 7.3). The Cs^+-substituted, K^+-free external Tyrode solution contained (in mM): 140.0 NaCl, 5.4 CsCl, 1.8 CaCl_2, 0.5 MgCl_2, 0.33 NaH_2PO_4, 5.5 glucose and 5.0 HEPES (pH adjusted to 7.4 with NaOH). The concentration of CaCl_2 in the external solution was reduced from 1.8 to 0.1 mM to separate I_{Ca,L} from I_{Ca,T}. In some experiments, NaCl was totally substituted with NMDG-Cl, TEA-Cl or LiCl. All experiments were performed at 34–36 °C.

Chemicals. Isradipine, nifedipine, verapamil, diltiazem, FPL-64176, Iso and ACh were purchased from Sigma-Aldrich. Drugs were prepared as 10 mM stock solutions in DMSO and then diluted in the external solution. TTX (Wako Chemical Co.) was dissolved in distilled water at a concentration of 10 mM and then diluted to the final concentration of 10 μM in the experimental solution.

Statistical analysis. The results are expressed as mean ± S.E.M. Statistical comparison among the different groups was performed by one-way ANOVA followed by Tukey’s post-hoc HSD test. Statistical comparison between two groups was evaluated using Student’s t-test. N indicates the number of hearts and n indicates the number of cells used in experiments. A p value < 0.05 was considered statistically significant.

Data availability. The datasets generated and/or analysed during the current study are available from the corresponding author upon request.

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Author Contributions
The experiments presented in this study were performed at the Shiga University of Medical Science and the Institut de Génomique Fonctionnelle, CNRS, UMR-5203, Inserm U 1191 in Montpellier. Specific contributions are as follows: conception and design of experiments: F.T., M.E.M. and H.M.; collection, analysis and interpretation of data: F.T., P.M., S.D., W.-G.D., M.E.M. and H.M.; drafting the manuscript and revising it critically for important intellectual content: F.T., J.S., M.E.M. and H.M. All authors critically revised the manuscript for technical and important contents.

Additional Information
Competing Interests: The authors declare that they have no competing interests.

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