Contribution of L-type Ca\(^{2+}\) current to electrical activity in sinoatrial nodal myocytes of rabbits

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DIASTOLIC DEPOLARIZATION underlies automatcity of the sinoatrial (SA) node (3) and starts when net ionic membrane current changes from outward to inward. Membrane current is composed of an outward current, mainly carried by the delayed rectifier current (I\(_{K}\)), and several inward currents: the hyperpolarization-activated current (I\(_{Na}\)), the T-type calcium current (I\(_{Ca,T}\)), and the L-type calcium current (I\(_{Ca,L}\)); and a background current (I\(_{b}\)), which is inward during diastole (18). Recently, a sustained inward current (I\(_{K}\)) has been described, which could provide an inward current during early diastolic depolarization (14). The relative contribution of these currents to diastolic depolarization is still a matter of debate. At present, there are three views on the ionic mechanism of pacemaking. In the first view, the time course of decay of I\(_{K}\) together with an inward I\(_{Na}\) are the dominant factors for the rate of diastolic depolarization (4, 34). In the second view, I\(_{Na}\) is activated at a relatively negative membrane potential, plays a dominant role (8, 11). In the third view, it is proposed that I\(_{Ca,L}\) is active during early diastolic depolarization (12, 15) and that the latter part of the diastole is governed by I\(_{Ca,L}\), initiating the next action potential (12, 15, 26, 38).

The role of L-type calcium current (I\(_{Ca,L}\)) in impulse generation was studied in single sinoatrial nodal myocytes of the rabbit, with the use of the amphotericin-perforated patch-clamp technique. Nifedipine, at a concentration of 5 \(\mu\)M, was used to block I\(_{Ca,L}\). At this concentration, nifedipine selectively blocked I\(_{Ca,L}\) for 81% without affecting the T-type calcium current (I\(_{Ca,T}\)), the fast sodium current, the delayed rectifier current (I\(_{K}\)), and the hyperpolarization-activated inward current. Furthermore, we did not observe the sustained inward current. The selective action of nifedipine on I\(_{Ca,L}\) enabled us to determine the activation threshold of I\(_{Ca,L}\), which was around -60 mV. As nifedipine (5 \(\mu\)M) abolished spontaneous activity, we used a combined voltage- and current-clamp protocol to study the effects of I\(_{Ca,L}\) blockade on repolarization and diastolic depolarization. This protocol mimics the action potential such that the repolarization and subsequent diastolic depolarization are studied in current-clamp conditions. Nifedipine significantly decreased activation potential duration at 50% repolarization and reduced diastolic depolarization rate over the entire diastole. Evidence was found that recovery from inactivation of I\(_{Ca,L}\) occurs during repolarization, which makes I\(_{Ca,L}\) available already early in diastole. We conclude that I\(_{Ca,L}\) contributes significantly to the net inward current during diastole and can modulate the entire diastolic depolarization.

nifedipine; delayed rectifier current; hyperpolarization-activated current; T-type calcium current; fast sodium current; sustained inward current

METHODS

Cell Isolation

Single SA nodal myocytes were isolated according to the method of DiFrancesco et al. (9) with some modifications as previously described in detail (34). Briefly, New Zealand White rabbits of ether sex weighing 1.8–2.5 kg were anesthetized with 1 ml/kg Hypnorm (0.32 mg/ml fentanyl citrate im and 10 mg/ml fluanisone im; Janssen Pharmaceuticals, Tilburg, The Netherlands) under artificial ventilation. The thorax was opened, and 0.1 ml of heparin sodium (5,000 IU/ml) was injected into the left ventricle. The heart was excised and mounted on a Langendorf perfusion system. Blood was washed out for 5 min with oxygenated HEPES-buffered solution containing (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 1.0 MgCl\(_2\), 5.0 HEPES, and 5.5 glucose (“normal Tyrode solution”). The solution was kept at 37°C, and pH was

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adjusted to 7.4 with NaOH. The SA node region was excised and cut into small strips (0.5 to 1-mm width, ~2-mm length) perpendicular to the crista terminalis. The strips were allowed to equilibrate for 15 min in normal Tyrode solution at room temperature. Thereafter, they were placed in a test tube with an oxygenated "calcium-free" Tyrode solution at room temperature containing (in mM) 140 NaCl, 5.4 KCl, 0.5 MgCl₂, 1.2 KH₂PO₄, 5.0 HEPES, and 5.5 glucose. The pH was adjusted to 6.9 with NaOH. The solution was refreshed three times. Next, the strips were transferred to a calcium-free Tyrode solution to which collagenase B (0.28 U/ml, Boehringer Mannheim, Mannheim, Germany), pronase E (0.92 U/ml, Serva, Heidelberg, Germany), elastase (12.4 U/ml, Serva), and 0.1% bovine serum albumin were added ("dissociation solution"). In this solution, strips were incubated at 37°C for 10–14 min and were gently triturated through a pipette with a tip diameter of 2.0 mm. At regular intervals, the solution was microscopically examined for the presence of dissociated myocytes. When single cells appeared, dissociation was stopped, and the strips were transferred into a modified Kraftbrühe (KB) solution (19) containing (in mM) 85 KCl, 30 K₂HPO₄, 5.0 MgSO₄, 20 glucose, 5.0 pyruvic acid, 5.0 creatine, 30 tauurine, 0.5 EGTA, 5.0 β-hydroxybutyric acid, 5.0 succinic acid, 2.0 Na₃ATP, and 50 g/l polyvinylpyrrolidone (pH adjusted with KOH to 6.9) and gently shaken. The KB solution was refreshed three times to remove the dissociation solution. Thereafter, the strips were again triturated in KB solution through a pipette (tip diameter of 0.8–1.2 mm) for 5–10 min. Samples of the resulting cell suspension (0.4 ml) were placed in a recording chamber on the stage of an inverted microscope (Nikon Diaphot) and superfused (0.6 ml/min) with normal Tyrode solution. For our experiments, we selected spontaneously active spindle and elongated spindle-like cells (7, 35). All experiments were performed at 35 ± 0.5°C. Temperature was maintained by a translucent heating plate underneath the bottom of the recording chamber (31) and continuously monitored. Animal care was in accordance with institutional guidelines.

**Electrophysiological Recording**

Membrane potentials and membrane currents, except I₉, were recorded using the amphotericin-perforated patch technique (17, 33) to prevent rundown of membrane currents by dilution of intracellular components. To minimize series resistance, I₉ was recorded in the whole cell mode with low-resistance electrodes of 1–2 MΩ. We only used cells that did not display rundown of membrane currents studied during the first 5 min. Electrodes were pulled from borosilicate glass (1-mm outer diameter, with a glass fiber inside the lumen) using a vertical one-stage patch-electrode puller and were heat polished. When using the amphotericin-perforated patch technique, we dissolved 6 mg of amphotericin B (Sigma Chemical, St. Louis, MO) in 100 µl of dimethyl sulfoxide shortly before the experiment of which 10 µl of the solution were added to 3 ml of the electrode solution. Measurements of action potentials and I₉, Iₓ, and I₇ were performed in normal Tyrode solution and an electrode solution containing (in mM) 120 potassium gluconate, 20 KCl, 5 HEPES, 5 MgCl₂, 0.6 CaCl₂, 5 Na₂ATP, 0.1 cAMP, and 5 EGTA (pH adjusted with KOH to 7.2). For the measurement of I₉ and the measurements of I₉, the pipette solution was diluted by 50% with equimolar Tris-HCl. For the measurement of I₉ and I₇, the pipette solution contained (in mM) 110 CsOH, 20 CsCl, 5 EGTA, 1 MgCl₂, 4 MgATP, and 5 HEPES (pH adjusted with aspartic acid to 7.2). In these experiments, 5 mM CsCl was added to the normal Tyrode solution in which the amount of CaCl₂ was lowered to 0.1 M.

For the amphotericin-perforated patch technique, electrodes tips were immersed in normal electrode solution for 1 s and backfilled with the electrode solution to which amphotericin was added. Electrode resistance ranged between 3 and 5 MΩ. After being sealed to the membrane, series resistance dropped quickly to 8–12 MΩ and became stable within 10 min for a period of at least 1 h. Series resistance was compensated for ~75%, resulting in a residual series resistance of ~2–3 MΩ. For the I₈, measurements, series resistance after compensation was ~0.5–2 MΩ. Membrane potential and membrane current were recorded with a custom-built voltage-damp amplifier. Command potentials for voltage-damp and stimulus pulses were obtained from a custom programmable pulse generator. All potentials were corrected by subtracting the pipette-to-bath liquid junction potential as calculated using the JPCalc software package (1). Table 1 summarizes the ionic composition of pipette and bath solutions and the subsequent liquid junction potential, which amounted to 14 mV for the perforated patch recording of action potentials, I₉, Iₓ, and I₇; 10 mV for the perforated patch recording of I₉; and 15 mV for the whole cell recording of I₉ and Iₗ. Data were stored on videotape (Sony Betamax) using a pulse code modulation system (Sony PCM501) modified to enable DC recordings. For off-line processing a Macintosh Quadra 650 personal computer (Apple Computer, Cupertino, CA), a custom-written data acquisition and analysis program was used.

**Table 1. Ionic composition of pipette and bath solutions and resulting liquid junction potential**

<table>
<thead>
<tr>
<th>Ions</th>
<th>I₉, Iₓ, I₇</th>
<th>I₉</th>
<th>Whole Cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cpip</td>
<td>Cbath</td>
<td>Cpip</td>
<td>Cbath</td>
</tr>
<tr>
<td>Aspartate</td>
<td>0.6</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>31.2</td>
<td>151</td>
<td>142</td>
</tr>
<tr>
<td>Cs⁺</td>
<td>140</td>
<td>0</td>
<td>130</td>
</tr>
<tr>
<td>Glutamate</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>K⁺</td>
<td>140</td>
<td>5.4</td>
<td>0</td>
</tr>
<tr>
<td>Mg₂⁺</td>
<td>5</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Na⁺</td>
<td>10</td>
<td>140</td>
<td>0</td>
</tr>
<tr>
<td>Tris⁺</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquid junction potential,*</td>
<td>14.3</td>
<td>9.8</td>
<td>15.5</td>
</tr>
</tbody>
</table>

*Liquid junction potential is defined as the potential of the bath solution minus the potential of the pipette solution and was calculated using the JPCalc software package (1).
pulses were applied every 15 s to the cell both 2 min before the application of the drug and during drug administration. Only cells without detectable rundown of membrane currents were used. The protocol was as follows: after a conditioning prepulse of 0.5 s to −40 mV, a test pulse to 0 mV of 0.5 s was applied, after which the voltage was clamped back to −40 mV for 0.5 s. Then the current-clamp mode was switched on again. After 15 s, a similar pulse protocol was used with a hyperpolarizing test pulse of 1 s to −90 mV instead of the depolarizing pulse. To study drug effects in more detail, another protocol in which depolarizing and hyperpolarizing voltage-clamp steps from a holding potential of −40 mV to various potentials were applied intermittent this protocol. Steady-state currents and inward peak currents during the test pulse as well as tail currents after the test pulse were recorded and examined off-line. Currents are expressed as absolute values, unless stated otherwise. Voltage-clamp protocols are described in more detail in RESULTS.

Membrane capacitance was determined from the initial slope of the transmembrane voltage in response to the hyperpolarizing current pulses of 40 pA. Membrane capacitance was 53 ± 6 pF (mean ± SE, n = 23). For normalization, currents were expressed relative to membrane capacitance (pA/pF).

Because spontaneous activity was arrested by nifedipine even at a low concentration, a combined voltage- and current-clamp protocol was necessary to study drug effects on diastolic depolarization (see Figs. 9 and 10). Under control conditions, a depolarizing voltage-clamp step of ~50-ms duration to +10 mV mimicked the action potential, after which the amplifier was switched back to the current-clamp mode, leaving the repolarization and diastolic depolarization to occur naturally. Voltage-clamp pulses were applied at fixed intervals of 200 or 250 ms. The interval of pacing was chosen such that it was as close as possible and therefore only slightly shorter than the intrinsic interval.

To characterize action potentials, several action potential parameters were determined: action potential amplitude, action potential duration between 50% depolarization and 50% repolarization (APD50), action potential duration between 50% depolarization and 100% repolarization (APD100), diastolic depolarization rate (DDR), maximum upstroke velocity (dV/dtmax), and maximum diastolic potential (MDP). Diastolic depolarization rate was measured by fitting a straight line over the 50- or 75-ms time interval starting at the MDP + 1 mV (DDR50 and DDR75, respectively). MDP + 1 mV was used rather than MDP because the time at which the MDP + 1 mV was reached could more reliably be determined than the time at which the MDP was reached.

Statistics

For statistical analysis we used the means values of the action potential parameters of 10 subsequent action potentials. All results are presented as means ± SE. Statistical significance was determined by a Student’s t-test for paired or unpaired observations, where appropriate. A probability of P < 0.05 was considered significant.

Drugs

Nifedipine (Sigma Chemical) was freshly dissolved in 97% ethanol, after which the solution was 1,000 times diluted in normal Tyrode solution. Because of the photosensitivity of the drug, the room was darkened during the experiment. E-4031 [1-(6-methyl-2-pyridyl)ethyl]-4-(4-methylsulfonyl)aminobenzyl)piperidine was a kind gift from Eisai. The agent was dissolved in distilled water at 1,000 times the concentration used. Batches of both stock solutions were stored at −20°C until use.

RESULTS

Specificity of Nifedipine as Inh Blocker

We first performed a series of voltage-clamp experiments to study the effects of nifedipine on six membrane ionic currents possibly involved in pacemaking: I_{CaL}, I_{CaT}, I_{Na}, I_{K}, I_{K,1}, and I_{f}.

Effect of nifedipine on I_{CaL}.

I_{CaL} was studied using depolarizing voltage-clamp pulses (P1) of 500-ms duration that were applied from a holding potential of −40 mV (Fig. 1A, top). On depolarization, I_{CaL} activates rapidly (15), which results in a net inward current (Fig. 1A, bottom left). Inactivation of I_{CaL} coincides with the activation of I_{K} resulting in a less negative net membrane current, eventually becoming outward. Nifedipine (5 µM) drastically reduces the inward current (Fig. 1A, bottom right) and unmarks an instantaneous current step together with a slowly activating outward current.

Figure 1B shows the reduction of peak current amplitude at different test potentials during the administration of 5 µM nifedipine. Averaged current-voltage (I−V) relations, obtained after administration of nifedipine, clearly demonstrate the reduction of peak inward current (n = 8). Subtracting the I−V relation measured in the presence of nifedipine (Fig. 1B, closed circles) from that measured under control conditions (open circles) would result in a new I−V relation, which represents the I−V relation of the nifedipine-sensitive Ca^{2+} current. However, the I−V relation measured in the presence of nifedipine has a maximum near 0 mV, which is the same potential at which the control I−V relation has its maximum, indicating that I_{CaL} is not completely blocked. Further evidence for an incomplete block of I_{CaL} is given by the third I−V relation in Fig. 1B (open squares), which was made in the presence of 5 µM nifedipine to block I_{CaL} and 10 µM E-4031, which fully blocked I_{K} (34). To remove all I_{CaL} not blocked by nifedipine, depolarizing steps to +20 mV for 500 ms were used to completely inactivate I_{CaL}. Thereafter, steps were made to various potentials, and currents were measured immediately after these steps. In this way an I−V relation could be obtained that contained no I_{CaL} and no I_{K}. In contrast to the I−V relation obtained in the presence of nifedipine, this “background” I−V relation was linear. It had a slope of 39.5 ± 5.6 pS and reversed at −32 ± 5.4 mV (n = 8). This I_{b} most likely consists of various time-independent currents, e.g., Na−K pump (27), Na\(^{+}/Ca^{2+}\) exchanger, background Cl− current, background Na+ current, and a small leakage current flowing through the seal resistance [see Verheijck et al. (34)]. For each individual experiment, the slope conductance and reversal potential of I_{b} were determined. For each experiment new I−V relations were made by subtracting the calculated I_{b} from the peak current. Figure 1C shows the average “corrected” I−V relations in the absence and presence of nifedipine (n = 8). From these corrected I−V relations it
is apparent that they have the same voltage dependence. The similar voltage dependence can be appreciated even more when the corrected I-V relation in the presence of nifedipine is scaled up by a constant factor of 5.3 (Fig. 1D). From these experiments it can be concluded that 5 µM nifedipine blocks 81% of I_{Ca,L}.

Effect of nifedipine on I_{Ca,T}. To explore the effect of nifedipine on I_{Ca,T}, we first had to isolate the current from other membrane currents. Activation of I_{Na} was prevented by replacing the extracellular NaCl with an equimolar concentration of Tris·HCl. Furthermore, I_{K} was blocked by replacing potassium with cesium in the intracellular solution. The removal of extracellular Na\(^+\) and intracellular K\(^+\) also strongly reduces I_{f}. Under these conditions, we employed the method of Bean (2) to dissect the two types of Ca\(^{2+}\) currents, i.e., to use two different holding potentials. We used holding potentials of −90 and −50 mV similar to those used by Hagiwara et al. (15). After appropriate correction for liquid junction potential, their holding potentials amount to −93 and −53 mV, respectively. In the experiment shown in Fig. 2A, a depolarizing voltage-clamp pulse (P\(_1\)) of 150-ms duration was applied from alternate holding potentials of −90 and −50 mV to minimize the effect of possible “rundown” during the time course of the experiment. During steps from −50 mV to more positive test potentials, I_{Ca,L} activates rapidly. Also, I_{Ca,T} may activate to a small extent. When stepping from the more negative holding potential of −90 mV, the test pulse still elicited an inward current. The difference in currents generated at the two holding potentials represents I_{Ca,T} and appears to be insensitive to nifedipine (Fig. 2A, bottom traces, and
Fig. 2B). Notice that the different current traces under the normal and nifedipine conditions follow a similar time course. The absence of an effect of nifedipine on $I_{Ca,T}$ was confirmed in the three other cells.

Effect of nifedipine on $I_{Na}$. We then questioned whether the nodal cells contained $I_{Na}$ and if so, whether $I_{Na}$ was sensitive to nifedipine in these cells. Therefore, $I_{Na}$ was isolated from other transmembrane currents by 1) reducing $I_{Ca,L}$ by lowering the extracellular Ca$^{2+}$ concentration to 0.1 mM (14), 2) blockade of $I_f$ by adding 5 mM CsCl to the external solution, and 3) blockade of $I_K$ by replacing potassium with cesium in the intracellular solution (cf. Table 1). Only two of eight cells tested showed $I_{Na}$. Directly after seal breakthrough, we were able to record action potentials for 1 min after which spontaneous electrical activity ceased. For these two cells, the first recorded action potentials showed a high maximum rate of rise (16 and 19 V/s). These two cells were used to study the effect of nifedipine on $I_{Na}$. Figure 3 shows the results of an experiment in which a depolarizing voltage-clamp pulse of $-50$ mV ($P_1$) of 100-ms duration was applied from a holding potential of $-95$ mV. The bottom trace of Fig. 3A shows the current recording in response to the test pulse. Activation of $I_{Na}$ produces a large inward current that inactivates rapidly and is followed by a steady-state current during the rest of the test pulse. Nifedipine neither affected the inward current nor the steady-state current during $P_1$ (Fig. 3A, bottom right). Figure 3B shows the absence of an effect of nifedipine on $I_{Na}$ to different test potentials. The absence of an effect of nifedipine on $I_{Ca,T}$ was confirmed in the other cell that showed $I_{Na}$.

Effect of nifedipine on $I_{st}$. The experimental protocol used to study $I_{Na}$ could also be used to investigate
whether the nodal cells contained $I_{st}$ and if so, whether $I_{st}$ was sensitive to nifedipine in these cells. According to Guo et al. (14), $I_{Ca}$ could only be separated from $I_{Ca,L}$ by reducing the extracellular Ca$^{2+}$ concentration, as we did, thereby substantially decreasing the amplitude of $I_{Ca,L}$ but enhancing $I_{st}$. The much slower activation and inactivation of $I_{st}$ compared with $I_{Na}$ enables discrimination of $I_{st}$ from $I_{Na}$. In the experiment of Fig. 3 we applied a voltage-clamp step from a holding potential of $-95$ to $-50$ mV, a voltage at which $I_{st}$ should be clearly visible as a slowly inactivating current (14). However, directly after the inactivation of $I_{Na}$, a steady-state current was reached (Fig. 3A, bottom left trace), which was not altered by the administration of 5 µM nifedipine (Fig. 3A, bottom right trace), demonstrating the absence of $I_{st}$ in our preparation. Similar observations were made at the other potentials tested as illustrated in Fig. 3C, which shows the steady-state current measured after 15 ms ($I_{ss,15}$, cf. Fig. 3A), in both the presence and absence of nifedipine. Similar results were obtained for the other cell that showed $I_{Na}$.

The six experiments in which $I_{Na}$ was not present were also used to investigate the presence of $I_{st}$. Figure 4A shows the results for one such cell, under similar experimental conditions as used in the experiment of Fig. 3, in which a series of depolarizing voltage-clamp pulses ($P_1$) of 100-ms duration were applied from a holding potential of $-95$ mV. In response to each depolarizing voltage-clamp pulse, the membrane current reached a steady-state level immediately after the capacitative current transient. Thus this experiment clearly demonstrates the absence of both $I_{Na}$ and $I_{st}$. Similar results were obtained in the five other cells.

![Fig. 3. Absence of effect of 5 µM nifedipine on fast sodium current ($I_{Na}$). A: membrane potential (top trace) and membrane current (bottom trace) during a voltage-clamp step from a $V_{hold}$ of $-95$ mV to a test potential ($P_1$) of $-50$ mV, to activate the $I_{Na}$. Under control conditions (left) the membrane current reaches a steady-state level during $P_1$ directly after inactivation of $I_{Na}$, demonstrating the absence of sustained inward current ($I_{st}$). Nifedipine does not alter amplitude of $I_{Na}$ or steady-state level of the current during $P_1$ (right). B: peak I-V relation for $I_{Na}$ obtained by plotting peak current amplitude vs. test potential ($P_1$) in presence and absence of nifedipine. C: steady-state I-V relation obtained by plotting current amplitude after 15 ms ($I_{ss,15}$, cf. A) vs. test potential ($P_1$) in presence and absence of nifedipine. Membrane capacitance of this cell was 50 pF.](http://ajpheart.physiology.org/)

![Fig. 4. Absence of $I_{st}$. A: membrane potential (top trace) and membrane current (bottom trace) during voltage-clamp steps from a $V_{hold}$ of $-95$ mV to various test potentials ($P_1$) in the range at which $I_{st}$ should be activated. Experimental conditions are similar to those for Fig. 3. Note absence of $I_{Na}$ in this cell. Membrane capacitance of this cell was 45 pF. B: mean steady-state I-V relation obtained by plotting $I_{ss,15}$ (cf. A) vs. test potential ($P_1$). Current amplitude is expressed as pA/pF. Bars indicate means ± SE.](http://ajpheart.physiology.org/)
Figure 4B illustrates the average I-V relation of the $I_{ss,15}$ (cf. Fig. 4A) and clearly shows a linear I-V relation in the voltage range of $-90$ to $0$ mV. Such a linear relationship would not be expected if $I_{st}$ were present (14).

Effect of nifedipine on $I_K$. The effect of nifedipine on $I_K$ was investigated using depolarizing voltage-clamp pulses ($P_1$) of 500-ms duration that were applied from a holding potential of $-40$ mV (Fig. 5A, top trace). The bottom traces of Fig. 5A show current recordings in response to these depolarizing test pulses ($P_1$). Activation of $I_K$ produces, in conjunction with an outward $I_b$ (34), an outwardly directed current. We previously demonstrated that in the preparation we used, $I_K$ only consists of the rapid component of $I_K$ ($I_{K,r}$) (34). Tail currents following depolarizing pulses ($I_{tail}$) are predominantly due to deactivation of $I_K$ (10, 32, 34). In this experiment, 5 µM nifedipine blocked the inward transient current completely and produced an outward shift of the holding current at $-40$ mV of $12$ pA. It did not affect the currents at the end of the depolarizing voltage-clamp step or their time course. $I_{tail}$ were reduced slightly without a change in their time course.

Figure 5B summarizes the effect of nifedipine (5 µM) on the quasi-steady-state outward current during the depolarizing steps ($I_{SSD}$) to different test potentials ($n = 11$). Nifedipine shifted the quasi-steady-state I-V relation slightly outward between $-40$ and $-10$ mV, but this shift was not significant at any of the potentials tested.

Figure 5C summarizes the effect of nifedipine (5 µM) on $I_{tail}$ to different test potentials ($n = 11$) and shows the absence of an effect of nifedipine on $I_{tail}$ at any potential measured.

From the data presented in Fig. 5 it cannot be excluded that nifedipine affects $I_K$ at voltages at which diastolic depolarization occurs (from about $-60$ to $-40$ mV). Therefore, the effect of nifedipine on $I_K$ was studied in more detail in an additional series of experiments. In those experiments we used a protocol in which $I_K$ was fully activated, whereas other voltage-dependent currents were absent because they were either fully inactivated ($I_{Ca,T}$ and $I_{Ca,L}$) or not activated ($I_f$). From a holding potential of $-40$ mV, a conditioning voltage-clamp step to $+20$ mV was used to fully activate $I_K$ (Fig. 6A, top trace). Thereafter, repolarizing
steps to various test potentials ($P_1$) were made. Currents were measured directly after the surge of the capacitative transient on the test potential. In this way, an instantaneous I-V relation was obtained in which $I_K$ was fully present. Figure 6A (bottom trace) shows two representative current traces recorded under normal conditions and after the administration of 5 mM nifedipine. Nifedipine reduces the peak inward current during the conditioning voltage-clamp step to +20 mV but does not alter the steady-state level at the end of the pulse. During $P_1$ the control and nifedipine current traces completely overlap and have a similar time course. Figure 6B shows the lack of effect of 5 mM nifedipine on the instantaneous I-V relationship ($n = 8$). Because no effect of nifedipine was observed on the instantaneous I-V relationship of $I_K$ (Fig. 6B), we conclude that nifedipine does not affect $I_K$.

Effect of nifedipine on $I_f$. Next, the effect of nifedipine on the $I_f$ was investigated. Figure 7A (bottom trace) shows representative current recordings in response to 2-s hyperpolarizing voltage-clamp steps ($P_1$) from a holding potential of $-40$ mV to $-50$ and $-70$ mV (Fig. 7A). On hyperpolarization an inward current is activated that predominantly consists of $I_f$ (11) and deactivates after return to the holding potential ($I_f$ tail). In this experiment and eight others, 5 mM nifedipine did not alter the quasi-steady-state current ($I_f$ steady state) or $I_f$ tail (Fig. 7B). Therefore, we conclude that nifedipine does not affect $I_f$.

**Activation Threshold of $I_{Ca,L}$**

From the above experiments, we conclude that 5 mM nifedipine blocks $I_{Ca,L}$ without affecting $I_{Ca,T}$, $I_{Na}$, $I_K$, and $I_f$. Furthermore, we demonstrate the absence of $I_{st}$ in our cells. The highly selective action of nifedipine on $I_{Ca,L}$ enabled us to determine the activation threshold for $I_{Ca,L}$ from the data obtained in the experiments in which the effect of nifedipine on $I_{Ca,T}$ was evaluated (Fig. 2). In these experiments, given the selective
blockade of $I_{\text{Ca,L}}$ by nifedipine, the time-dependent difference current (control $- n$ifedipine) in response to voltage-clamp steps from a holding potential of $-90$ mV should be $I_{\text{Ca,L}}$. Possible errors caused by a change in passive membrane properties between the control and nifedipine recordings were avoided by capacitive current subtraction performed on individual membrane current recordings. The capacitive transient elicited on stepping back from the test potentials to the holding potential was added to the capacitive transient during the test pulse. This resulted in a complete removal of the capacitive transient during the test step and thus in an uncontaminated recording of the activation of $I_{\text{Ca,L}}$ (Fig. 8A). This procedure is allowed, because stepping back from the test potential to the holding potential does not activate time-dependent currents under our experimental conditions: 1) activation of $I_{\text{Na}}$ is prevented by replacement of the extracellular NaCl with an equimolar concentration of Tris·HCl; 2) $I_{\text{K}}$ is blocked by replacement of potassium with cesium in the intracellular solution, and 3) $I_{\text{f}}$ is blocked by the removal of extracellular Na$^{+}$ and intracellular K$^{+}$.

Figure 8A shows current recordings in response to a series of depolarizing test pulses ($P_1$) from a holding potential of $-90$ mV, under control conditions (Fig. 8A, middle left), and in the presence of 5 µM nifedipine (Fig. 8A, middle right). The bottom panel of Fig. 8A shows the nifedipine-sensitive difference current (control $- n$ifedipine) reflecting $I_{\text{Ca,L}}$. From this figure it becomes clear that a depolarizing voltage-clamp step to $-60$ mV can already activate some $I_{\text{Ca,L}}$. Figure 8B shows the average I-V relation of the difference current reflecting $I_{\text{Ca,L}}$ ($n = 4$). The I-V relation reaches its most negative value of $-12.2 \pm 0.8$ pA/pF at $-10$ mV.

To our knowledge this is the first time that direct evidence is presented that $I_{\text{Ca,L}}$ can be activated at
potentials as negative as $-60$ mV and thus may be able to serve as an inward current during the entire diastolic depolarization.

### Effect of $I_{Ca,L}$ Blockade on Electrical Activity of SA Nodal Myocytes

From the results presented above, we conclude that $I_e$ is absent in our preparation and that $5 \mu$M nifedipine blocks $I_{Ca,L}$ in nodal myocytes for $81\%$, without affecting $I_{Ca,T}$, $I_K$, and $I_f$ as well as $I_{Na}$, when present. In higher concentrations, nifedipine slightly blocked $I_f$ (data not shown). Therefore, it was justified to use $5 \mu$M nifedipine as a tool in studying the role of $I_{Ca,L}$ in diastolic depolarization. To study the effect of $I_{Ca,L}$ blockade on electrical activity, we had to use a combined voltage- and current-clamp protocol, because spontaneous electrical activity was arrested at $5 \mu$M nifedipine. A voltage-clamp pulse ($P_1$, Fig. 9A) was applied to depolarize the cell to such an extent that after the release of the voltage-clamp pulse, repolarization and the subsequent diastolic depolarization followed a time course as under free running conditions (see also Table 2). It should be noted that the cycle length of the spontaneously beating cell is always somewhat (on the average $17$ ms, Table 2) longer than the cycle of the paced cell. This is due to the protocol being used: when a SA node cell is paced, one is obliged to use a slightly shorter pacing interval than the intrinsic interval. This explains why the upstroke of the second paced action potential starts earlier than the upstroke of the second spontaneous action potential (Fig. 9A). Careful examination of Fig. 9A further shows that the repolarization phases of the spontaneously initiated second action potential and the driven second action potential are also very similar. Table 2 shows that, under control conditions, the combined voltage- and current-clamp protocol does not alter any of the action potential parameters compared with spontaneous electrical activity ($n = 11$).

Figure 9B shows that nifedipine (5 $\mu$M) predominately reduces the early phase of repolarization and therefore reduces APD$_{50}$. The drug also slows diastolic depolarization from the start of diastole, while APD$_{100}$ and MDP remain unaltered. Table 2 summarizes the effect of 5 $\mu$M nifedipine on action potential parameters studied with the combined voltage- and current-clamp protocol. Data are obtained by averaging (for each individual experiment) 10 subsequent action potentials; MDP, maximum diastolic potential; APD$_{50}$, action potential duration between 50% depolarization and 50% repolarization; APD$_{100}$, action potential duration between 50% depolarization and maximum diastolic potential; DDR$_{50}$ and DDR$_{75}$, diastolic depolarization rate measured over the first 50 and 75 ms, respectively, starting at the MDP + 1 mV; $dv/dt_{max}$, maximum upstroke velocity; APA, action potential amplitude. *Significant change to driven control ($P < 0.05$).

### Availability of Inward Current During Diastole

In the previous section it was demonstrated that selective but partial blockade of $I_{Ca,L}$ reduced diastolic depolarization rate during the entire diastole (Fig. 9B). To study the “dynamic” availability of the inward current during repolarization and diastolic depolarization, we performed a combined current- and voltage-clamp protocol comparable to that described in the

![Fig. 9. Effects of 5 $\mu$M nifedipine on electrical activity of a sinoatrial nodal myocyte. A: control action potentials were mimicked by a voltage-clamp pulse ($P_1$) of $-50$ ms to $+10$ mV. Between consecutive voltage-clamp pulses, voltage-clamp amplifier was set in current-clamp mode, allowing free repolarization and diastolic depolarization. B: effect of nifedipine on electrical activity was recorded after 10 min of drug administration. Membrane capacitance of this cell was 51 pF.](http://ajpheart.physiology.org/)

<table>
<thead>
<tr>
<th>Control</th>
<th>Nifedipine (5 $\mu$M)</th>
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<tr>
<td>Interval, ms</td>
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<td>230±5</td>
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<td>MDP, mV</td>
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<td>APD$_{50}$, ms</td>
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<td>DDR$_{75}$, mV/s</td>
<td>142±11</td>
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<td>$dv/dt_{max}$, V/s</td>
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<tr>
<td>APA, mV</td>
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Table 2. Effect of nifedipine on action potential parameters of sinoatrial nodal myocytes under spontaneous and voltage-current clamp conditions (means±SE; $n = 11$). Interval, interval between subsequent action potentials; MDP, maximum diastolic potential; APD$_{50}$, action potential duration between 50% depolarization and 50% repolarization; APD$_{100}$, action potential duration between 50% depolarization and maximum diastolic potential; DDR$_{50}$ and DDR$_{75}$, diastolic depolarization rate measured over the first 50 and 75 ms, respectively, starting at the MDP + 1 mV; $dv/dt_{max}$, maximum upstroke velocity; APA, action potential amplitude. *Significant change to driven control ($P < 0.05$).
previous section. During repolarization and diastole, extra voltage-clamp steps to 0 mV of 10 ms duration (P2) were applied to fully activate I_Ca,L at different times after the onset of P1 (Fig. 10A). The bottom panel shows current traces in response to the combined voltage- and current-clamp protocol as described in the top panel of Fig. 10A. Under control conditions, an inwardly directed peak current (superimposed on a larger outward current) can already be elicited after ~40 ms, which becomes net inward ~10 ms before the MDP is reached. After the administration of 5 µM nifedipine, which only affects I_Ca,L, the amount of inward current is drastically reduced and no net inward current could be elicited during either repolarization or diastolic depolarization. Figure 10B summarizes the effect of nifedipine on the inward peak current at different times after the onset of P1 (n = 5). Under control conditions, inwardly directed peak currents can be elicited after 40 ms, which is considerably earlier than the time at which the MDP is reached (67 ± 4 ms). Nifedipine (5 µM) blocked most of the inwardly directed current throughout repolarization and diastolic depolarization. These experiments demonstrate that I_Ca,L is available to serve as an inward current during the entire diastolic depolarization.

DISCUSSION

Selective Effect of Nifedipine on I_Ca,L

In this study we investigated the role of I_Ca,L by blockade with nifedipine in the generation of electrical activity in rabbit SA nodal myocytes. Binding studies revealed that among the available calcium channel blockers, dihydropyridines like nifedipine have the highest affinity for calcium channels (23, 24, 29), suggestive for a high selectivity. Although the potency of nifedipine as a I_Ca,L blocker is widely accepted, much less is known about its selectivity. Concentrations of 2–10 µM are commonly used to block I_Ca,L completely (12, 15, 16). Hagiwara et al. (15) demonstrated a complete blockade of I_Ca,L by 2 µM nifedipine in rabbit SA nodal myocytes. They also showed that concentrations as high as 2 µM did not affect I_Ca,T. We studied the effects of 5 µM nifedipine on I_Ca,L, I_Ca,T, I_Na, I_K, and I, and demonstrated that 5 µM nifedipine reduces I_Ca,L by 81% without affecting I_Ca,T, I_Na, I_K, and I. The lower blocking effect of nifedipine on I_Ca,L in our study compared with Hagiwara et al. (15) can at least in part be explained by the correction we made for the presence of a time-independent I, which results in a more reliable estimate of the blocking effect of nifedipine (Fig. 1, C and D). Our results further show that nifedipine blocked I_Ca,L at all test potentials to an equal extent.

Contribution of I_Ca,L to Impulse Generation

Effect of nifedipine on diastolic depolarization. Doerr et al. (12) used an “action potential clamp" method to measure the contribution of I_Ca,L to the action potential in rabbit SA nodal myocytes. This technique can be more easily used in cells with low membrane resistance like ventricular and atrial cells. In SA node cells, membrane resistance is high. Thus small offsets in command voltage may introduce considerable errors in measurements of small currents during diastole and make this method not reliable in measuring the contribution of I_Ca,L to diastolic depolarization. This problem is avoided in the method we used in this study. With the combined voltage- and current-clamp method, the cell is only depolarized with a voltage-clamp pulse and the

Fig. 10. Availability of inward current during diastole. A: an extension of the protocol as described in Fig. 9 was used. During current-clamp mode a second voltage-clamp pulse of 20 ms to 0 mV (P2) was applied to fully activate I_Ca,L at different times after the onset of P1. P2 was applied every 10th action potential and shifted with steps of 10 ms throughout diastole. Bottom panel of A shows, on the same time scale, a composite plot of membrane currents in response to P1 and P2 before and during administration of 5 µM nifedipine. Vertical dashed line represents moment at which maximum diastolic potential is reached. Membrane capacitance of this cell was 33 pF. B: availability of inward current during repolarization and diastole (n = 5) measured at different time intervals between P1 and P2 in absence and presence of nifedipine. Current amplitudes are expressed as pA/pF. Bars indicate means ± SE.
subsequent repolarization and diastolic depolarization are in the current-clamp mode. It can be argued that the depolarizing voltage-clamp step will slightly alter the activation process of $I_{\text{Ca,L}}$. However, the duration of the voltage-clamp step used is such that $I_{\text{Ca,L}}$ will reach a level at the end of the step similar to that reached during the spontaneously pacing action potential. This is likely to occur because the repolarization after the voltage-clamp step is similar to the repolarization during spontaneous activity (Fig. 10A).

Another argument against our method might be that alterations in the repolarization process induced by nifedipine also affect the time course of activation of other currents, which could influence the rate of diastolic depolarization. From Fig. 9B, it is clear that nifedipine predominantly affects the initial repolarization rate and hardly affects the remaining repolarization rate. It is therefore not likely that the deactivation process of $I_K$, the dominant current during repolarization, has been altered. Because the last phase of repolarization is not affected, activation of $I_K$, which occurs at negative potentials, is also not likely to be altered. Therefore, the method used seems to be justified and seems to be the best way to directly measure the contribution of $I_{\text{Ca,L}}$ to diastolic depolarization.

The combined voltage- and current-clamp experiments show that 5 μM nifedipine reduced the rate of the diastolic depolarization during the entire diastole. This finding is in good agreement with the findings of Doerr et al. (12) who showed that the D-600-sensitive current is present during entire diastolic depolarization. Recently, however, Zaza et al. (39) also used the action potential clamp method and showed that the net effect of nifedipine is not a reduction in inward current but a reduction in outward current. They provide evidence that the reduction in outward current is indirectly caused by a reduction in the intracellular calcium level which in turn reduces a calcium-dependent potassium conductance. Their results thus suggest that nifedipine would not reduce but enhance diastolic depolarization rate. However, we directly show that nifedipine does reduce diastolic depolarization rate, which is to be expected from a blockade of an inward current. It should be noted that the experiments of Zaza et al. (39) do not exclude this action of $I_{\text{Ca,L}}$ during diastolic depolarization because they show the net effect of a reduction in both inward and outward currents.

Activation threshold of $I_{\text{Ca,L}}$. The effect of nifedipine on diastolic depolarization can be explained either by a nonselective blockade of the drug, an indirect effect on $I_{\text{NaCa}}$ caused by a reduction in intracellular Ca$^{2+}$ (30), or by activation of $I_{\text{Ca,L}}$ earlier in diastole and at more negative potentials than generally is assumed. The activation threshold of $I_{\text{Ca,L}}$ is thought to be around −40 mV (18), although a more negative threshold of −50 mV has been reported as well (4, 22). However, in the latter experiments (4), a contribution of $I_{\text{Ca,T}}$ could not be excluded.

The exact “threshold” for activation of $I_{\text{Ca,L}}$ is difficult to obtain, because at a holding potential more negative than −40 mV, $I_K$ will activate and on depolarization, $I_{\text{Ca,T}}$ will be activated as well. In most studies, therefore, a holding potential of approximately −40 mV is used, thus fixing the threshold for activation to that potential. The selective action of nifedipine on $I_{\text{Ca,L}}$ enabled us to use a more negative holding potential (−90 mV) to evaluate the threshold for activation of $I_{\text{Ca,L}}$ (Fig. 8). We clearly show that $I_{\text{Ca,L}}$ can indeed be activated at potentials between −60 and −40 mV, i.e., the voltage range in which diastolic depolarization takes place.

In the experiments in which we determined the threshold for activation of $I_{\text{Ca,L}}$, a more negative holding potential (−90 mV, Fig. 8) was used in comparison with the experiments in which we tested the effect of nifedipine on $I_{\text{Ca,L}}$ (−40 mV, Fig. 1). At the holding potential of −90 mV, the most negative value of the normalized I-V relation was 12.2 ± 0.8 pA/pF (n = 4), which is slightly more negative (not significant) than the value of −11.8 ± 1.8 pA/pF (n = 8) obtained at a holding potential of −40 mV. This slightly more negative value is most likely due to a larger number of available $I_{\text{Ca,L}}$ channels at more negative holding potentials. This may also explain that a holding potential of −90 mV induces a 10-mV voltage shift of the most negative current value of the I-V relationship. Direct evidence of the holding potential-dependent voltage shift was obtained in the same cells because two different holding potentials were used (−90 and −50 mV). At a holding potential of −90 mV used to determine the activation threshold of $I_{\text{Ca,L}}$ (Fig. 8), the most negative current value of the I-V relationship was observed at a test potential of −10 mV. At a holding potential of −50 mV, the most negative current value of the I-V relationship was observed at a test potential of 0 mV (data not shown). This demonstrates that the voltage at which the most negative current value of the $I_{\text{Ca,L}}$-V relationship occurs depends on the holding potential used.

Contribution of $I_{\text{Ca,L}}$ to diastolic depolarization. In the SA node model of Wilders et al. (37), in which the activation and inactivation curves of $I_{\text{Ca,L}}$ reported by Hagiwara et al. (15) are used, $I_{\text{Ca,L}}$ amounts to ∼0.5 pA or 0.0147 pA/pF at the MDP level, i.e., one or a few $I_{\text{Ca,L}}$ channels are in the conducting state at the MDP level (36). This indicates that also on the basis of previously measured properties of $I_{\text{Ca,L}}$, $I_{\text{Ca,L}}$ contributes to the first part of diastolic depolarization.

The rapid availability of $I_{\text{Ca,L}}$ during repolarization and diastole is demonstrated in Fig. 10. During P1 the inward peak current will mainly consist of $I_{\text{Ca,L}}$ because $P_1$ starts at potentials between −45 and −40 mV. Inward currents elicited by P2 during the last phase of repolarization and the onset of diastole could, due to the more negative membrane potential, also consist of a small fraction of $I_{\text{Ca,T}}$ (15). However, from the steady-state inactivation relations of $I_{\text{Ca,T}}$ and $I_{\text{Ca,L}}$ found by Hagiwara et al. (15), it can be derived that even in steady state at the average MDP level (−59 mV, see Table 2), only 7% of $I_{\text{Ca,T}}$ is available as opposed to $I_{\text{Ca,L}}$, which is only modestly inactivated (1%) at this potential. This means that only a very small part of inward-
going peak currents elicited on P2 can be attributed to activation of I_{Ca,T}.

The inward currents elicited by P2 during the last phase of repolarization and the onset of diastole may also consist of a small fraction of I_{Na}. We found I_{Na}, in only two of eight cells that we used to study the effect of nifedipine on I_{Na}. The two cells containing I_{Na} showed a high dV/dt_{max} (16 and 19 V/s), which is much higher than the average dV/dt_{max} of 6.1 V/s observed in the cells in which the availability of I_{Ca,L} was studied. This indicates that the cells used to study the availability of I_{Ca,L} presumably do not contain I_{Na}. Moreover, it is demonstrated that nifedipine does not affect I_{Na} or I_{Ca,T}. Therefore, the nifedipine-sensitive current in Fig. 10 can be fully attributed to I_{Ca,L}. The envelope of peak currents elicited on P2 shows the dynamic recovery from inactivation of I_{Ca,L}. Under control conditions, inwardly directed peak current could be activated 40 ms after P2, which is considerably earlier than the time at which the MDF is reached (67 ms after P2). Experiments like the one shown in Fig. 10 demonstrate that recovery from inactivation occurs already during diastole, which supports the idea that I_{Ca,L}, if activated, can contribute a significant inward current during the entire diastolic depolarization.

Attributing the reduction in diastolic depolarization rate after the administration of nifedipine to a reduction of I_{Ca,L}, we now can make a back-of-the-envelope estimate of its contribution to diastole. Administration of 5 µM nifedipine decreased DDR75 by 26%. At this concentration I_{Ca,L} is reduced by 81%. Thus we may conclude that I_{Ca,L} contributes to 32% of the net inward current during diastole. In the SA node model of Wilders et al. (37), the average contribution of I_{Ca,L} to the net membrane current calculated also over the first 75 ms of diastolic depolarization was also 32%. A similar contribution of I_{Ca,L} of ~30% to this early phase of diastolic depolarization can be observed in the SA node model of Demir et al. (6).

It must be noted, however, that because of the slowing of the diastolic depolarization rate when nifedipine is present, membrane potential will remain more negative for a longer time. This will result in a smaller I_{K} because of a decrease in driving force, a more pronounced activation of I_{K}, and a larger inward current due to I_{b}. These effects will increase net inward current during diastole, thereby counteracting to some extent the effect of the reduction of I_{Ca,L}. Therefore, it is likely that the 32% contribution of I_{Ca,L} to the net inward current during diastole is an underestimate. On the other hand, blockade of I_{Ca,L} will indirectly lower the Na^+/Ca^{2+} exchange current (I_{NaCa}). Thus the reduction in inward current during diastole will in part also be due to a reduction in I_{NaCa}.

We now can also compare the contribution of several inward currents to diastolic depolarization. Van Ginneken and Giles (31) calculated that the amplitude of I_{f} during diastolic depolarization is similar to that of the net inward current. For I_{b} we recently measured a conductance of 39.5 pS/pF and a reversal potential of −32 mV (34). During diastolic depolarization from −60 to −40 mV, I_{b} will thus generate an average inward current of 0.71 pA/pF, which is about five times the net inward current during diastolic depolarization. The summed inward currents I_{f} and I_{b}, together with I_{Ca,L} and I_{Ca,T}, necessitate a large repolarizing outward current, most likely I_{K}, counterbalancing all but a small portion of total inward current. It is this resulting net inward current that underlies diastolic depolarization.

The above considerations demonstrate that it is not possible to dissect the process of diastolic depolarization into fixed contributions of several distinct ionic membrane currents. Rather, they demonstrate that all currents are important. Inhibition or stimulation of a single pacemaker current inevitably influences the other pacemaker currents.

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