T-Type and Tetrodotoxin-Sensitive Ca\textsuperscript{2+} Currents Coexist in Guinea Pig Ventricular Myocytes and Are Both Blocked by Mibefradil

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**Abstract**—Under Na\textsuperscript{+}-free conditions, low-voltage–activated Ca\textsuperscript{2+} currents in cardiomyocytes from various species have been described either as Ni\textsuperscript{2+}-sensitive T-type Ca\textsuperscript{2+} current (\(I_{\text{Ca(T)}}\)) or as tetrodotoxin (TTX)-sensitive Ca\textsuperscript{2+} current (\(I_{\text{Ca(TTX)}}\)). So far, coexistence of the 2 currents within the same type of myocyte has never been reported. We describe experimental conditions under which \(I_{\text{Ca(T)}}\) and \(I_{\text{Ca(TTX)}}\) can be separated and studied in the same cell. Rat and guinea pig ventricular myocytes were investigated with the whole-cell voltage-clamp technique in Na\textsuperscript{+}-free solutions. Whereas rat myocytes lack \(I_{\text{Ca(T)}}\) and exhibit \(I_{\text{Ca(TTX)}}\) only, guinea pig myocytes possess both of these low-voltage–activated Ca\textsuperscript{2+} currents, which are separated pharmacologically by superfusion with TTX or Ni\textsuperscript{2+}. \(I_{\text{Ca(T)}}\) and \(I_{\text{Ca(TTX)}}\) were of similar amplitude but significantly differed in their electrophysiological properties: \(I_{\text{Ca(TTX)}}\) activated at more negative potentials than did \(I_{\text{Ca(T)}}\), the potential for half-maximum steady-state inactivation was more negative, and current deactivation and recovery from inactivation were faster. \(I_{\text{Ca(TTX)}}\) but not \(I_{\text{Ca(T)}}\) increased after membrane rupture (“run-up”). Isolation of \(I_{\text{Ca(TTX)}}\) by application of the bivalent cation Ni\textsuperscript{2+} is critical because of possible shifts in voltage dependence. Therefore, we investigated whether the T-type Ca\textsuperscript{2+} channel blocker mibefradil (10 \(\mu\)mol/L) is a suitable tool for the study of \(I_{\text{Ca(TTX)}}\). However, mibefradil not only blocked \(I_{\text{Ca(T)}}\) by 85±2% but also decreased \(I_{\text{Ca(TTX)}}\) by 48±8%. We conclude that under Na\textsuperscript{+}-free conditions \(I_{\text{Ca(T)}}\) and \(I_{\text{Ca(TTX)}}\) coexist in guinea pig ventricular myocytes and that both currents are sensitive to mibefradil. Future investigations of \(I_{\text{Ca(T)}}\) will have to consider the TTX-sensitive current component to avoid possible interference. (Circ Res. 2000;86:628-635.)

**Key Words:** T-type Ca\textsuperscript{2+} currents ■ tetrodotoxin-sensitive Ca\textsuperscript{2+} currents ■ guinea pig ventricular myocytes ■ mibefradil

Low-voltage–activated inward currents of myocardial cells are critical for the generation and conduction of physiological excitation. Well-characterized currents include the T-type Ca\textsuperscript{2+} current (\(I_{\text{Ca(T)}}\))\textsuperscript{1,2} and the tetrodotoxin (TTX)-sensitive Na\textsuperscript{+} current (\(I_{\text{Na}}\))\textsuperscript{3} \(I_{\text{Ca(T)}}\) has been reported to contribute to pacemaking in sinoatrial cells\textsuperscript{4} in conjunction with the hyperpolarization-activated current \(I_{f}\).\textsuperscript{5,6} Very recently, 3 T-type Ca\textsuperscript{2+} channel–forming α subunits have been cloned and expressed, i.e., α1G, α1H, and α1I.\textsuperscript{7-9} Significant mRNA levels of the former 2 were detected in cardiac tissue.\textsuperscript{7,8} The expression of T-type Ca\textsuperscript{2+} channels in myocytes from the working myocardium declines with maturation\textsuperscript{10} but is reactivated in certain models of disease.\textsuperscript{11-13}

\(I_{\text{Na}}\) on the other hand, determines excitation of the working myocardium. Under physiological conditions, this current is much larger in amplitude than \(I_{\text{Ca(T)}}\).\textsuperscript{14} In the past, ion selectivity of the Na\textsuperscript{+} channel has been considered an exclusive channel property linked to distinct amino acid sequences within the pore-forming loop of the channel protein,\textsuperscript{15} but recent reports suggest that cardiac Na\textsuperscript{+} channels may in fact allow Ca\textsuperscript{2+} to pass when the channels become modified by phosphorylation or by the presence of cardiotonic steroids like ouabain or digoxin (“slip-mode conductance”).\textsuperscript{16}

Recently, another low-voltage–activated inward current was observed under Na\textsuperscript{+}-free experimental conditions in human atrial cells\textsuperscript{17} as well as in rat\textsuperscript{18} and guinea pig\textsuperscript{19} ventricular myocytes. This current was found to be carried by Ca\textsuperscript{2+} passing through a subpopulation of distinct TTX-sensitive Na\textsuperscript{+} channels and was therefore referred to as TTX-sensitive Ca\textsuperscript{2+} current (\(I_{\text{Ca(TTX)}}\)). The relation between \(I_{\text{Ca(TTX)}}\) and the slip-mode conductance of Na\textsuperscript{+} channels is still a matter of debate.\textsuperscript{20}

Rat ventricular myocytes lack a macroscopic \(I_{\text{Ca(T)}}\)\textsuperscript{21} and \(I_{\text{Ca(TTX)}}\) is the only Ca\textsuperscript{2+} current that is activated in the low potential range.\textsuperscript{15} With ventricular myocytes from guinea pig heart, results from various studies are more controversial. Both \(I_{\text{Ca(T)}}\) and \(I_{\text{Ca(TTX)}}\) have been demonstrated individually in separate studies; however, to the best of our knowledge, evidence for coexistence of the 2 currents in the same...
myocytes has never been reported. One reason may be that when the focus was on $I_{\text{Ca(T)\text{TTX}}}$, TTX was often applied in addition to Na-free superfusion solutions to ensure complete elimination of $I_{\text{Na}}$. Remarkably, in the only study so far involving $I_{\text{Ca(T)\text{TTX}}}$ in guinea pig ventricular myocytes, $I_{\text{Ca(T)}}$ has not been detected.\(^{19}\)

In the present study, we analyzed the low-voltage–activated Ca\(^{2+}\) current ($LVACC$) of guinea pig ventricular myocytes for a possible coexistence of $I_{\text{Ca(T)}}$ and $I_{\text{Ca(TTTX)}}$. Identification of $I_{\text{Ca(TTTX)}}$ was supported by a comparison with rat myocytes, in which $I_{\text{Ca(TTTX)}}$ is the only LVACC.

**Materials and Methods**

**Cell Isolation**

All studies complied with the German Home Office Regulations Governing the Care and Use of Laboratory Animals. Ventricular myocytes were isolated by standard enzymatic dissociation of hearts from adult male Wistar rats (360±22 g, n=11) and Dunkin-Hartley guinea pigs (305±11 g, n=34), respectively. For rat hearts, 300 U/mL collagenase (Worthington type 1, 254 U/mg) was used, and for guinea pig hearts, an additional 0.034 mg/mL pronase E (Merck, 4000 proteolytic units per milligram) was used. The preparation buffer contained 150 mmol/L NaCl. Single myocytes were harvested into Na'-free storage buffer and washed 3 times before use.

**Current Measurements**

Whole-cell voltage-clamp details have been reported previously.\(^{22}\) Average values for membrane capacitance measured with 5-millisecond depolarizing ramp pulses (from −55 to −50 mV) were 195±6 pF (rat, n=29) and 138±4 pF (guinea pig, n=75; mean±SEM). Series resistance was routinely compensated by 50% to 70%. Currents were low-pass–filtered at 2 kHz.

In general, experiments were performed at 36±1°C with the following Na'-free superfusion solution (mmol/L): tetraethylammonium chloride 120, CsCl 10, HEPES 10, CaCl\(_2\) 2, MgCl\(_2\) 1, and glucose 10, pH 7.4 (adjusted with CsOH). Tail currents (Figure 6) were recorded at 21±1°C and with 10 mmol/L [Ca\(^{2+}\)]\(_{\text{o}}\). The pipette solution (pH 7.2) included (mmol/L) cesium methanesulfonate 90, CsCl 20, HEPES 10, Mg-ATP 4, Tris-GTP 0.4, EGTA 10, and CaCl\(_2\) 3, with calculated free Ca\(^{2+}\) and Mg\(^{2+}\) concentrations of 60 nmol/L and 450 μmol/L, respectively.\(^{23}\) After experimentation, all membrane potentials were corrected for the calculated liquid junction potential of −15 mV (JPCalc version 2.2 by Barry\(^{24}\)).

Current amplitude was determined as the difference between peak inward current and current at the end of the depolarizing step. LVACCs were separated from high-voltage–activated L-type Ca\(^{2+}\) current ($I_{\text{Ca(L)}}$) by 2 protocols: (1) with test steps (3-second interval) from −115 to −50 mV, ie, negative to activation threshold for $I_{\text{Ca(L)}}$, and (2) as difference current between the traces from the holding potential, which was −115 mV (both LVACC and $I_{\text{Ca(L)}}$ available), and from −65 mV (only $I_{\text{Ca(L)}}$ current available). The latter method was used for determining maximum LVACC amplitude and the respective potential ($V_{\text{peak}}$).

**Chemicals**

Mibebradil (ASTA Medica AWD) and TTX citrate (Tocris) were dissolved in H\(_2\)O as stock solutions (10 mmol/L). Nifedipine (RBI) was dissolved in dimethyl sulfoxide (10 mmol/L). Aliquots were stored at −20°C until use. All chemicals were purchased from commercial suppliers and were of analytical grade.

**Data Analysis**

Absolute current amplitudes (in pA) were corrected for cell size and expressed in pA/pF; pClamp software (Clampfit) or Prism (Graph-
50% of the current, whereas in rat myocytes (test potential 55 mV), the entire LVACC was sensitive to TTX. There were 3 to 6 values per concentration (see text for IC50 and nH values).

Figure 2. Block of LVACCs by Ni2+ and TTX. In panels A through C, I-Vm relations of a representative guinea pig myocyte are shown. A, Control (same data are indicated by lines in panels B and C). Vh indicates holding potential. B, In the presence of Ni2+ and TTX (10 μmol/L). C, In the presence of Ni2+ and TTX (10 μmol/L) and 40 μmol/L Ni2+ (Vh = -115 mV). D, I-Vm relations in a representative rat myocyte in the presence and absence of TTX (30 μmol/L). E, Concentration-response curves for block of LVACCs. In guinea pig myocytes (test potential ~50 mV), Ni2+ and TTX each blocked ~50% of the current, whereas in rat myocytes (test potential ~55 mV), the entire LVACC was sensitive to TTX. There were 3 to 6 values per concentration (see text for IC50 and nH values).

Results

LVACCs of Rat and Guinea Pig Ventricular Myocytes

Current-voltage relations for rat and guinea pig ventricular myocytes clearly indicated LVACC (Figure 1). In rat cells, the LVACC peaked at ~53.0±1.1 mV and had a maximum amplitude of ~1.07±0.18 pA/pF (n = 11). The LVACC of guinea pig myocytes was of similar amplitude (~1.18±0.12 pA/pF, n = 11) but peaked at a more positive potential (~43.2±1.0 mV, P < 0.0001). Typical current tracings for LVACC and ICaL are shown in Figure 1 (bottom) for rat and guinea pig myocytes.

In rat myocytes, current-voltage relations exhibited clear separation of LVACC and ICaL. LVACC was completely blocked by 30 μmol/L TTX and therefore identified as ICaL (Figure 2D). In guinea pig myocytes, however, LVACC partially overlapped ICaL because of activation over a broader potential range (Figure 2A), suggesting that LVACC might consist of >1 component. The contribution of ICaL was tested with Ni2+, which clearly decreased the LVACC at potentials positive to ~50 mV (Figure 2B). Ni2+-sensitive current peaked at ~40.9±0.6 mV and had a maximum amplitude of ~0.88±0.06 pA/pF (n = 11), whereas Ni2+-resistant current was similar in amplitude (~0.72±0.07 pA/pF) but peaked at a more negative potential (~49.1±0.6 mV, P < 0.0001). The current-voltage relation of Ni2+-resistant LVACC remarkably resembled that of ICaL found in rat myocytes (Figure 2D). Indeed, Ni2+-resistant current was blocked by TTX and hence identified as ICaL (Figure 2C). Concentration-response curves (Figure 2E) indicated that the entire LVACC in rat myocytes was sensitive to TTX (IC50 1.7 μmol/L, Hill coefficient [nH] ~0.96). In guinea pig cells, TTX (IC50 1.2 μmol/L, nH ~1.3) and Ni2+ (IC50 16 μmol/L, nH ~2.1) each blocked ~50% of the total current.

Because both LVACC components of guinea pig myocytes could be activated by clamp steps from ~115 to ~50 mV without interference from ICaL, the time courses of the effects of TTX and Ni2+ were studied at this potential (Figure 3). The amplitude of LVACC increased substantially during the initial few minutes after membrane rupture. Therefore, at least 6 minutes of equilibration was always allowed before starting further experimental protocols. Successive application of TTX or Ni2+ blocked ~50% of the current (Figure 3A). Total LVACC and the residual currents ICaL and ICaL (indicated as a, b, and c, respectively, in Figure 3A) had similar kinetics of inactivation. The time constants from monoexponential curve fitting were 6.7±0.2 milliseconds (n = 45), 6.6±0.3 milliseconds (n = 20), and 6.3±0.3 milliseconds (n = 22), respectively. Cumulative application of TTX and Ni2+ almost completely blocked the total LVACC (Figure 3B).
Although the cells were superfused with nominally Na\(^+\)-free solutions, Na\(^+\) contamination originating from cell isolation procedures could still be present and could provide the charge for current passing through TTX-sensitive Na\(^+\) channels. However, such Na\(^+\) should be washed out rapidly, as simulated in the experiment shown in Figure 3C, in which the current increase that was due to 1 mmol/L Na\(^+\) was reversed on washout within <1 minute. The remaining amplitude was completely abolished when all extracellular Ca\(^{2+}\) was replaced with Mg\(^{2+}\).

Development of LVACCs After Rupture of Membrane

LVACCs increased within the first minutes after access to the cell (Figure 3). The contribution of \(I_{\text{Ca(T)}}\) and \(I_{\text{Ca(TTX)}}\) to the total current increase in guinea pig myocytes was studied in the presence of TTX and Ni\(^{2+}\), respectively; \(I_{\text{Ca(TTX)}}\) of rat myocytes was recorded without blockers. Figure 4 shows that \(I_{\text{Ca(T)}}\) could be measured immediately after series resistance compensation and that it remained relatively constant. In contrast, \(I_{\text{Ca(TTX)}}\) of guinea pig and rat myocytes was often undetectable during the first 2 minutes before it started to increase (“run-up”).

Steady-State Inactivation of \(I_{\text{Ca(T)}}\) and \(I_{\text{Ca(TTX)}}\)

Steady-state inactivation curves for \(I_{\text{Ca(T)}}\) and \(I_{\text{Ca(TTX)}}\) were obtained by using a conventional protocol (see inset in Figure 5B), in which \(I_{\text{Ca(T)}}\) and \(I_{\text{Ca(TTX)}}\) were dissected pharmacologically by adding TTX and Ni\(^{2+}\), respectively. In this set of experiments, \(I_{\text{Ca(T)}}\) had a higher amplitude than \(I_{\text{Ca(TTX)}}\) (Figure 5A) and therefore dominated steady-state inactivation of the total LVACC. In the absence of any blocker (ie, \(I_{\text{Ca(T)}} + I_{\text{Ca(TTX)}}\)), the potential for half-maximum steady-state inactivation (\(V_{0.5}\)) and the slope factor were \(-69.0 \pm 1.0\) mV and \(-3.9 \pm 0.3\) mV (\(n=9\), respectively). However, after separation of the 2 current components and normalizing to unity (Figure 5B), it became evident that \(I_{\text{Ca(TTX)}}\) inactivated at more negative potentials than \(I_{\text{Ca(T)}}\) (\(-73.9 \pm 0.8\) versus \(-69.5 \pm 0.4\) mV, respectively; \(P<0.001; n=8\)). The slope factors were not significantly different (\(-3.9 \pm 1.1\) mV for \(I_{\text{Ca(TTX)}}\) and \(-3.2 \pm 0.3\) mV for \(I_{\text{Ca(T)}}\)).

Recovery From Inactivation

The kinetics of recovery from inactivation are characteristic properties that may also help to distinguish between current

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**Figure 3.** Time course (top) and individual current traces (bottom) of LVACC of guinea pig ventricular myocytes, with small letters indicating time point of current traces below (arrowheads indicate zero current). A, Successive superfusion with TTX and Ni\(^{2+}\). B, Cumulative application of Ni\(^{2+}\) and TTX. C, Effects of various extracellular Na\(^+\) concentrations and removal of extracellular Ca\(^{2+}\) on TTX-sensitive current, \(I_{\text{Ca(T)}}\), blocked with Ni\(^{2+}\). Note fast washout of 1000 \(\mu\)mol/L Na\(^+\) and absence of current during superfusion with Ca\(^{2+}\)-free solution (CaCl\(_2\) replaced with MgCl\(_2\)).

**Figure 4.** Representative examples of current amplitude after rupture of cell membrane. \(I_{\text{Ca(T)}}\) and \(I_{\text{Ca(TTX)}}\) from guinea pig ventricular myocytes were measured in the presence of TTX (30 \(\mu\)mol/L) and Ni\(^{2+}\) (40 \(\mu\)mol/L), respectively; \(I_{\text{Ca(T)}}\) in rat myocytes was recorded without any blockers. Test potentials were \(-55\) mV in guinea pig cells and \(-50\) mV in rat cells.
components (Figures 5C and 5D). As shown by the original recordings (insets in Figure 5D), \( I_{\text{Ca(TTX)}} \) recovered faster from inactivation than did \( I_{\text{Ca(T)}} \). For a more detailed analysis, the currents of individual cells were fitted with double exponential functions, which described the data with the highest accuracy for all 3 conditions (total LVACC, \( I_{\text{Ca(T)}} \), and \( I_{\text{Ca(TTX)}} \)). The curve fits provided time constants \( t_f \) and \( t_s \) and amplitudes \( A_f \) and \( A_s \) for fast and slowly recovering current fractions, respectively. The values are summarized in the Table. The time constant \( t_f \) for the fast recovering current fraction of \( I_{\text{Ca(TTX)}} \) was significantly smaller than \( t_f \) of \( I_{\text{Ca(T)}} \). The time constants \( t_f \) and \( t_s \) of total LVACCs had intermediate values.

**Deactivation Kinetics of \( I_{\text{Ca(L)}} \), \( I_{\text{Ca(T)}} \), and \( I_{\text{Ca(TTX)}} \)**

Deactivation of \( I_{\text{Ca(L)}} \) occurs significantly faster than deactivation of \( I_{\text{Ca(T)}} \).\(^{25}\) Therefore, we investigated whether this current characteristic is also suitable to distinguish between \( I_{\text{Ca(T)}} \) and \( I_{\text{Ca(TTX)}} \). Tail currents of all 3 Ca\(^{2+}\) currents were recorded at \(-260\) mV (Figure 6). To slow the pace of deactivation for more accurate recordings, these experiments were performed at \( 21\pm1^\circ\text{C} \). The time constant for \( I_{\text{Ca(T)}} \) deactivation (\( 4.6\pm0.5 \) milliseconds, \( n=7; P<0.001 \)) was significantly larger than the time constants for \( I_{\text{Ca(L)}} \) (\( 1.3\pm0.2 \) milliseconds, \( n=8 \)) and for \( I_{\text{Ca(TTX)}} \) (\( 0.7\pm0.1 \) milliseconds, \( n=8 \)). There was no significant difference between the latter values.

**Mibefradil Block of \( I_{\text{Ca(T)}} \) and \( I_{\text{Ca(TTX)}} \)**

Separation of the 2 LVACCs by application of Ni\(^{2+}\) might be critical because of the possible shifts in voltage dependence of the remaining currents. Therefore, we aimed to suppress \( I_{\text{Ca(T)}} \) with the T-type Ca\(^{2+}\) channel blocker mibefradil. However, mibefradil may be used as a tool to separate the 2 LVACCs only if it is highly selective for \( I_{\text{Ca(T)}} \) and does not affect \( I_{\text{Ca(TTX)}} \). This was investigated by applying mibefradil (10 \( \mu\text{mol/L} \)) in the presence of TTX or Ni\(^{2+}\). Mibefradil reversibly reduced not only \( I_{\text{Ca(T)}} \) (Figure 7A) but also \( I_{\text{Ca(TTX)}} \) (Figure 7B). At test steps from \(-115\) to \(-50\) mV, the mean block of \( I_{\text{Ca(T)}} \) by mibefradil was \( 85\pm2\% \) (\( n=6 \)), whereas the mean block of \( I_{\text{Ca(TTX)}} \) was \( 48\pm8\% \) (\( n=5 \)). Mibefradil blocked \( I_{\text{Ca(TTX)}} \) over the whole potential range for activation, as illustrated by the current-voltage relations in the absence and presence of the drug (Figure 7C).

**Discussion**

**Characterization of the Two LVACCs**

In guinea pig ventricular myocytes, 2 LVACCs can be distinguished under nominally Na\(^{+}\)-free conditions on the basis of their sensitivity to the blockers Ni\(^{2+}\) and TTX as well
as by their distinct electrophysiological properties. The Ni²⁺-sensitive current shares many characteristics with I_{Ca(T)} previously identified under similar conditions. When studying I_{Ca(T)} of guinea pig ventricular myocytes, almost all investigators eliminated I_{Na} by using Na¹⁻free superfusion solutions. Most investigators even added Na¹⁻ channel blockers like TTX to the external solutions to block any residual current due to Na¹⁻ contamination or Ca²⁺ permeation. The presence of TTX certainly excluded the characterization of I_{Ca(TTX)} described here.

The I_{Ca(TTX)} characterized in the present study corresponds well to the I_{Ca(TTX)} described previously in human atrial and rat ventricular myocytes. An I_{Ca(TTX)} was also detected in a study involving guinea pig ventricular myocytes; the IC₅₀ value of 2.4 μmol/L resembled the value reported in the present study, although for reasons unknown, I_{Ca(T)} was not characterized.

Figure 6. Deactivation of Ca²⁺ current tails in guinea pig myocytes. A, Clamp protocol. B, Original current traces. C, Difference current obtained by subtraction of traces in panel B. Tail currents were recorded at −60 mV after brief current activation for 4 to 8 milliseconds from V_h −115 mV (●). Capacitive transients during the step to −60 mV were measured after a 200-millisecond Ca²⁺ current inactivating step to 0 mV (○) and subtracted from the current obtained with the activating protocol. Time constants of current deactivation were deduced from monoexponential curve fits to the tail currents (difference currents). All tail currents were recorded at 21±1°C and 10 mmol/L [Ca²⁺]₀. Under these conditions, voltage dependence was shifted by −10 mV toward more positive potentials, compared with the conditions at 36°C and 2 mmol/L [Ca²⁺]₀. Separation of individual current components was as follows: for I_{Ca(L)}, Ni²⁺ (100 μmol/L) and TTX (30 μmol/L); for I_{Ca(T)}, TTX (30 μmol/L) and nifedipine (5 μmol/L); and for I_{Ca(TTX)}, Ni²⁺ (100 μmol/L) and nifedipine (5 μmol/L).

Figure 7. A and B, Effects of mibefradil on peak I_{Ca(T)} (A) and I_{Ca(TTX)} (B) in guinea pig ventricular myocytes. LVACC components were dissected with TTX and Ni²⁺ as indicated. C, I-V_m relations of LVACC and part of I_{Ca(L)} under control conditions (left), in the presence of Ni²⁺ (middle), and with Ni²⁺ and mibefradil (right, n=4). V_m values were −115 mV (filled symbols) and −65 mV (open symbols). The dashed lines represent control data at V_m −115 mV. Mibefradil blocked I_{Ca(TTX)} over the whole potential range for activation (right).
The lack of \( I_{\text{Ca(T)}} \) could have been caused by significant differences in cell isolation procedures, which are often critical for detection of a specific ionic current.\(^{28}\) In contrast to that previous work,\(^{19}\) we found the coexistence of \( I_{\text{Ca(TTX)}} \) and \( I_{\text{Ca(T)}} \) under comparable (21°C, 10 mmol/L \([\text{Ca}^{2+}]_o\)) conditions. \(^{28}\) We consider \( \text{Ca}^{2+} \) to be the charge carrier for TTX-sensitive current, because amplitude was abolished on removal of extracellular \( \text{Ca}^{2+} \). Certainly, \( \text{Na}^+ \) contamination originating from cell isolation procedures could have a large impact because of the enormous size of regular \( \text{Na}^+ \) current amplitude in relation to the currents investigated in the present study. However, Figure 3C shows that 1 mmol/L \( \text{Na}^+ \), which simulates \( \text{Na}^+ \) contamination, could be washed out rapidly. Nevertheless, traces of \( \text{Na}^+ \) have to be assumed in our nominally \( \text{Na}^+ \)-free solutions. This \( \text{Na}^+ \) contamination could affect current amplitude in 2 ways, depending on the true, yet unsettled, nature of the \( I_{\text{Ca(TTX)}} \)-conducting channel: (1) \( I_{\text{Ca(TTX)}} \) might be conducted by classic \( \text{Na}^+ \) channels that reversibly alter their properties under nominally \( \text{Na}^+ \)-free conditions.\(^{19}\) This hypothesis was based on the finding that \( \text{Na}^+ \) concentrations in the micromolar range reduced \( I_{\text{Ca(TTX)}} \) by a competitive interaction between \( \text{Ca}^{2+} \) and \( \text{Na}^+ \) at the channel pore, leading to block of \( \text{Ca}^{2+} \) conduction and switch from \( \text{Ca}^{2+} \) to \( \text{Na}^+ \) permeation as the \( \text{Na}^+ \) concentration is increased.\(^{19}\) (2) \( I_{\text{Ca(TTX)}} \) is conducted by a distinct TTX-sensitive channel that is different from the classic \( \text{Na}^+ \) channel.\(^{18}\) In this case, \( \text{Na}^+ \) contamination should not decrease \( I_{\text{Ca(TTX)}} \) but add an additional current (ie, \( I_{\text{Na}} \)) with increasing \( \text{Na}^+ \) concentration. In support of the latter hypothesis, we did not observe \( I_{\text{Ca(TTX)}} \) reduction after the addition of low \( \text{Na}^+ \) concentrations (Figure 3C), although this was not studied in detail.

**Separation of \( I_{\text{Ca(T)}} \) and \( I_{\text{Ca(TTX)}} \)**

Superfusion of the myocytes with TTX isolates \( I_{\text{Ca(T)}} \), whereas \( \text{Ni}^{2+} \) preserves \( I_{\text{Ca(TTX)}} \). \( V_{\text{peak}} \) and \( V_{0.5} \) of \( I_{\text{Ca(TTX)}} \) were more negative than \( V_{\text{peak}} \) and \( V_{0.5} \) of \( I_{\text{Ca(T)}} \). These differences may be taken as evidence for 2 independent currents, but only if the respective separating procedure by itself does not affect the voltage dependence of residual currents. In the case of \( I_{\text{Ca(T)}} \), there is general agreement that current characteristics are not modified by TTX.\(^{27}\) Whereas in the case of \( I_{\text{Ca(TTX)}} \), it cannot be excluded that \( \text{Ni}^{2+} \) influences current properties. Application of 50 \( \mu \text{mol/L} \) \( \text{Ni}^{2+} \) was found to alter the inactivation of current elicited at \( \sim 25 \text{ mV} \). However, at this potential, \( I_{\text{Ca(TTX)}} \) is contaminated by \( I_{\text{Ca(L)}} \) (see Figure 1B),\(^{29}\) which limits interpretation. In rat ventricular myocytes, the same \( \text{Ni}^{2+} \) concentration affected neither current amplitude nor the current-voltage relation of \( I_{\text{Ca(TTX)}} \).\(^{18}\) Theoretically, \( \text{Ni}^{2+} \) would shift activation and steady-state inactivation curves toward more positive potentials by screening off fixed membrane charges because of its nature as a bivalent cation.\(^{29}\) Therefore, the more negative values for \( V_{\text{peak}} \) and \( V_{0.5} \) of \( I_{\text{Ca(TTX)}} \) cannot be caused by \( \text{Ni}^{2+} \), which is expected to produce a shift into the opposite direction. If anything, the presence of \( \text{Ni}^{2+} \) would lead to an underestimation of the true differences between the 2 LVACCs.

\( I_{\text{Ca(T)}} \) and \( I_{\text{Ca(TTX)}} \) differ not only in their voltage dependence but also in current development after breaking the membrane (Figure 4). The reason for run-up of \( I_{\text{Ca(TTX)}} \) is presently unclear; its time course suggests some relation to cell dialysis.

In addition, the kinetics of recovery from inactivation and current deactivation are significantly different between \( I_{\text{Ca(TTX)}} \) and \( I_{\text{Ca(T)}} \). As shown previously,\(^{25}\) tail currents of \( I_{\text{Ca(T)}} \) decayed with a relatively slow time constant.

**Selectivity of Mibefradil**

Only few blockers of \( \text{Ca}^{2+} \) channels are sufficiently selective for a distinct \( \text{Ca}^{2+} \) channel subtype in order to be exploited as pharmacological tools. The nondihydropyridine mibefradil was introduced as a T-type \( \text{Ca}^{2+} \) channel blocker\(^{30}\) because of its selectivity for T-type over L-type \( \text{Ca}^{2+} \) channels. Because separation of \( I_{\text{Ca(TTX)}} \) by application of \( \text{Ni}^{2+} \) is critical for the reasons discussed, we aimed to block \( I_{\text{Ca(T)}} \) with mibefradil. We found that mibefradil not only blocked \( I_{\text{Ca(T)}} \) but also decreased \( I_{\text{Ca(TTX)}} \). Thus, the compound is not a suitable tool for the study of \( I_{\text{Ca(TTX)}} \) in guinea pig ventricular myocytes. Block of \( I_{\text{Ca(TTX)}} \) provides another example of the limited selectivity of mibefradil, which also impairs currents other than \( I_{\text{Ca(T)}} \) at moderately higher concentrations.

**Relation Between \( I_{\text{Ca(TTX)}} \) and Slip-Mode Conductance**

A detailed study of \( I_{\text{Ca(TTX)}} \) suggested that the current is conducted by \( \text{Na}^+ \) channels that are functionally distinct from those conducting the classic \( I_{\text{Ca}} \).\(^{18}\) Another study involving rat ventricular myocytes suggested that classic \( \text{Na}^+ \) channels become “promiscuous” by phosphorylation or by the presence of cardiotonic steroids (slip-mode conductance).\(^{16}\) Under these conditions, the modified \( \text{Na}^+ \) channels conduct \( I_{\text{Ca(TTX)}} \), which triggers \( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \) release, which in turn leads to intracellular \( \text{Ca}^{2+} \) transients. At present, the relation between \( I_{\text{Ca(TTX)}} \) and slip-mode conductance is the subject of controversy.\(^{20}\)

In our view, the \( \text{Ca}^{2+} \) current due to slip-mode conductance and \( I_{\text{Ca(TTX)}} \) are not identical. One reason is that activation of protein kinase A or the presence of cardiotonic steroids was not a prerequisite for the detection of \( I_{\text{Ca(TTX)}} \) in the present or any previous study.\(^{17-20}\) Certainly, any basal activity of protein kinase A could modify a small fraction of \( \text{Na}^+ \) channels to conduct \( I_{\text{Ca(TTX)}} \) even in the absence of protein kinase A activators. However, the \( I_{\text{Ca}(0)} \) values for the TTX block of \( I_{\text{Ca(TTX)}} \) of 1.2 and 1.7 \( \mu \text{mol/L} \) in guinea pig and rat myocytes, respectively, reported in the present study, argue against this possibility for the following reasons: All experiments involving slip-mode conductance were performed in the presence of extracellular \( \text{Na}^+ \). Thus, the \( \text{Ca}^{2+} \) current through modified \( \text{Na}^+ \) channels was always measured in the simultaneous presence of \( I_{\text{Ca}} \) and was therefore not assessed directly. Instead, the intracellular \( \text{Ca}^{2+} \) transient assumed to be triggered by this \( \text{Ca}^{2+} \) current was used as an indicator. These transients were blocked by TTX with an \( I_{\text{Ca}(0)} \) value of 0.1 \( \mu \text{mol/L} \). In contrast, the \( I_{\text{Ca}(0)} \) value for the block of the current conducted by all \( \text{Na}^+ \) channels (modified and unmodified) was 1 \( \mu \text{mol/L} \).\(^{16}\) The authors concluded that modified \( \text{Na}^+ \) channels are more sensitive to TTX than unmodified \( \text{Na}^+ \).
channels. In the present study, \( I_{\text{Ca(TX)}} \) was measured under Na\(^+\)-free conditions, i.e., without interference of \( I_{\text{sc}} \). Provided that \( I_{\text{Ca(TX)}} \) was conducted by these modified Na\(^+\) channels, an IC\(_{50}\) in the range of 0.1 \( \mu \text{mol/L} \) would have been expected. However, the 12-fold and 17-fold higher values measured in the present study argue against the conductance of \( I_{\text{Ca(TX)}} \) by modified Na\(^+\) channels. We conclude that \( I_{\text{Ca(TX)}} \) and Ca\(^{2+}\) current via slip-mode conductance are 2 separate currents.

In summary, the present study demonstrates that \( I_{\text{Ca(TX)}} \) and \( I_{\text{Ca(TX)}} \) coexist in guinea pig ventricular myocytes under Na\(^+\)-free conditions. Both LVACCs are sensitive to mibe-fradil. Future physiological and pharmacological investigations of \( I_{\text{Ca(TX)}} \) will have to consider the TTX-sensitive Ca\(^{2+}\) current to avoid possible interference.

Acknowledgments
The excellent technical assistance of Manja Schöne and Margarete Stieck is gratefully acknowledged.

References
T-Type and Tetrodotoxin-Sensitive Ca\textsuperscript{2+} Currents Coexist in Guinea Pig Ventricular Myocytes and Are Both Blocked by Mibefradil

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doi: 10.1161/01.RES.86.6.628

\textit{Circulation Research} is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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