T-type calcium channels are regulated by hypoxia/reoxygenation in ventricular myocytes

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Pluteanu F, Cribbs LL. T-type calcium channels are regulated by hypoxia/reoxygenation in ventricular myocytes. Am J Physiol Heart Circ Physiol 297: H1304–H1313, 2009. —Low-voltage-activated calcium channels are reexpressed in ventricular myocytes in pathological conditions associated with hypoxic episodes, but a direct relation between oxidative stress and T-type channel function and regulation in cardiomyocytes has not been established. We aimed to investigate low-voltage-activated channel regulation under oxidative stress in neonatal rat ventricular myocytes. RT-PCR measurements of voltage-gated Ca\(^{2+}\) (Cav)3.1 and Cav3.2 mRNA levels in oxidative stress were compared with whole cell patch-clamp recordings of T-type calcium current. The results indicate that hypoxia reduces T-type current density at \(-30\) mV (the hallmark of this channel) based on the shift of the voltage dependence of activation to more depolarized values and downregulation of Cav3.1 at the mRNA level. Upon reoxygenation, both Cav3.1 mRNA levels and the voltage dependence of total T-type current are restored, although differently for activation and inactivation. Using Ni\(^{2+}\), we distinguished different effects of hypoxia/reoxygenation on the two current components. Long-term incubation in the presence of 100 \(\mu\)M CoCl\(_2\) reproduced the effects of hypoxia on T-type current activation and inactivation, indicating that the chemically induced oxidative state is sufficient to alter T-type calcium current activity, and that hypoxia-inducible factor-1\(\alpha\) is involved in Cav3.1 downregulation. Our results demonstrate that Cav3.1 and Cav3.2 T-type calcium channels are differentially regulated by hypoxia/reoxygenation injury, and, therefore, they may serve different functions in the myocyte in response to hypoxic injury.

Low-voltage-activated calcium channel; oxidative stress; cardiomyocytes

Since the initial biophysical (34) and molecular characterization (5, 27, 36) of low-voltage-activated T-type calcium currents in heart and neurons, these channels have been studied in various systems and pathological conditions. T-type currents are reexpressed in the adult hypertrophied ventricle and in postmyocardial infarction (18), in the monocrotaline model of pulmonary hypertension and heart failure (24; 44), and in vitro in adult ventricular myocytes exposed to 10% FBS (9) and endothelin-1 (19), relating the T-type current to the pathology rather than to normal physiological function of the myocytes. The mechanisms for regulation of the T-type channels are incompletely characterized, largely due to the lack of specific inhibitors and the presence of the more robust L-type and store-operated calcium channels, which make it difficult to isolate T-type Ca\(^{2+}\) channel functions, such as secretion (29), excitation (52), or transcription regulation (4).

The lesions at the site of ischemia-reperfusion injury can trigger structural and functional cardiac remodeling. Due to the complex paracrine and autocrine regulation of cells as a response to injury (30), there are many potential mediators at the injury site. Both low-oxygen conditions and the subsequent reoxygenation phase perturb normal function in cardiac myocytes. Hypoxia/reoxygenation (H/R) injury alters the activity of ionic transport systems by inhibiting K\(^{+}\) channels (38), voltage-dependent L-type Ca\(^{2+}\) channels (10), Na\(^{+}/Ca\(^{2+}\) exchanger (47), and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump (40), and by stimulating store-operated channels (32) and ryanodine receptors (22).

Multiple lines of evidence suggest that factors secreted at the site of injury can modulate the T-type calcium channel, such as growth hormone secreted by atrial tumor cells (50), endothelin-1 and IGF-I in ventricular myocytes (45), and PDGF in fibroblasts (48). Of the two cardiovascular \(\alpha\)-subunits of the T-type channel [voltage-gated Ca\(^{2+}\) (Cav)3.1 and Cav3.2], Cav3.2 regulation has been observed more often than Cav3.1, since its expression is increased in response to hypoxia (7), high glucose (31), aldosterone (26), and angiotensin II (12). Transcription factors proposed to regulate Cav3.2 include hypoxia-inducible factor (HIF)-1\(\alpha\) (7), neuron restrictive silencer factor (25, 51), and Csx/Nkx2.5 (46). The effects of hypoxia on T-type calcium channels in renal proximal tubule (1), neurons (20, 21), PC12 cells (7), and in a heterologous expression system (11) implicate Cav3.2 as the primary hypoxia-sensitive \(\alpha\)-subunit.

Less is known about the pathways involved in Cav3.1 \(\alpha\)-subunit regulation, although there is evidence that Cav3.1 mRNA is increased in adult ventricular myocytes exposed to endothelin-1 (19). Also, heterologously expressed Cav3.1 membrane currents can be increased when coexpressed with the L-type calcium channel auxiliary \(\alpha_{2}\delta\)-subunit (13) or decreased by the \(\gamma_{5}\)-subunit (16).

The present study is based on the hypothesis that oxidative stress that is present in different forms under pathological conditions induces the reexpression of T-type current. We have investigated the effects of H/R conditions on T-type channels in neonatal rat ventricular myocytes (NRVM), and we show that both Cav3.1 and Cav3.2 \(\alpha\)-subunits are regulated in oxidative stress conditions, possibly involving different mechanisms. Our results show that cardiomyocytes downregulate Cav3.1 in hypoxic conditions, and the window current is shifted toward more depolarized intervals, which might explain the decrease in spontaneous activity of neonatal myocytes in hypoxic conditions by decreasing the influx of calcium for diastolic depolarization. The changes induced in vitro by H/R conditions suggest that Cav3.2 can increase via a negative shift in activation, which may explain the reappearance of the

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and experiments were initiated 24 h later. Hypoxic conditions were modified Eagle’s medium (Mediatech, Herndon, VA) plus antibiotic,ersville, MD). The PC1 medium was changed to serum-free Dulbecco’s were isolated from newborn rats (1–3 days old), as described else-}

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nifedipine sensitive, nifedipine resistant, and 50

hypoxia was induced for 24 h with 100

normoxia for the same length of time as control cells. Chemical

MO). Chemical reoxygenation was induced with 100

barium current recordings, pipette solution contains the following (in

of pClamp8 software (Axon Instruments, Union City, CA). For

logne, Germany), and digitized with a Digidata 1200 under the control

capillaries (Kimble Glass, Vineland, NJ) of 2- to 3-M

old cycle (7

relative changes in gene expression were quantified using the thresh-

50°C, 10 min at 95°C, and 50 cycles of 15 s at 95°C and 1 min at

(fermentas, Glen Burnie, MD). Cycling conditions were 2 min at

Hercules, CA) using Platinum Taq Quantitative Super Mix with ROX

Hypoxy conditions were induced using an anaerobic microbiological system with indicator (1% oxygen, Becton Dickinson, Sparks, MD) for 18–24 h. After this time interval, cells were returned to normal oxygen levels in a 5% CO2 incubator for 1–6 days. In parallel, NRVM were cultured in normoxia for the same length of time as control cells. Chemical

hypoxia was induced for 24 h with 100 μM CoCl2 (Sigma, St. Louis, MO). Chemical reoxygenation was induced with 100 μM H2O2 (Sigma) for 24 h.

Quantitative RT-PCR. NRVM were harvested, and RNA was purified using RNasey Plus Mini kit (Qiagen, Valencia, CA), accord-

Electrophysiology. Whole cell recordings using borosilicate glass capillaries (Kimble Glass, Vineland, NJ) of 2- to 3-MΩ resistance

were used to record barium currents. Data are sampled at 10 kHz, filtered at 3 kHz with an EPC-7 amplifier (HEKA Electronics, Col-

of pClamp8 software (Axon Instruments, Union City, CA). For

for the following (in μM): 120 CsCl, 1 MgCl2, 10 EGTA, and 10 HEPES, pH 7.2. The bath solution contains the following (in μM): 130 N-methyl-

30% ) plots (ΔI/ΔV)

from the linear part of the current-voltage (I-V) plots (+43 ± 1.3

and −30 mV in 10-mV increments. For L-type channels, the prepulse

voltage ranges from −100 to +10 mV, followed by a pulse to 0 mV. The currents are normalized to maximum (Ii2.max) and fitted with Boltzmann equation: Ii2.max = Ii2/1+ exp[(Vi2/2 − V)/k].

Statistics. Data are expressed as means ± SE. Statistical analyses

were performed using Student’s t-test and considered significant at

RESULTS

Recording of T-type channels in NRVM. In NRVM, calcium currents are dominated by the robust high-voltage-activated L-type calcium current. The most conventional method used to measure low-voltage-activated T-type currents is by subtracting the voltage protocols from two different holding potentials; −100 mV (both T- and L-type currents) and −50 mV (L-type current). In NRVM, traces derived after the subtraction of the voltage protocols recorded in 10 mM BaCl2 indicated that, at −30 mV, the inward barium current is predominantly carried by a fast inactivating channel, with a significant fraction of L-type channels already inactivated at −50 mV (Fig. 1A). The I-V plots (Fig. 1B) show considerable overlap of the T- and L-type currents between −20 and 0 mV, which affects the accurate assessment of T-type current in NRVM. Therefore, we have chosen to record T-type current by voltage subtraction method of the protocols from −100 and −50 mV applied in the presence of 5 μM nifedipine. Figure 1C shows typical current traces obtained after voltage protocol subtraction in the presence of 5 μM nifedipine, with a peak current density between −30 and −20 mV. Because further experiments required extended culture times, we investigated whether T-type current density changes with time in culture. Figure 1D shows that the peak current density for T-type current did not change with time in culture (up to 7 days). However, there was a reproducible positive shift in the peak current from −20 mV to −10 mV over time. Because the currents from days 2 and 3 in culture were not different from each other, the results were pooled together and considered as “early” time in culture, while the results from days 5–7 are referred to as “late” cultured cells.

An earlier report of T-type current in NRVM (37) charac-

terized a nifedipine-resistant component of the high-voltage-activated L-type current, and, therefore, we sought to establish that the nifedipine-resistant current recorded in our experiment-

conditions passes through low-voltage-activated T-type channels, by analyzing in more detail the current properties. Figure 2A shows that the voltage dependence of activation and inactivation in the early time are typical for T-type current. At a later time in culture, there is an average 5-mV shift of the voltage dependence of activation and inactivation to more depolarized values. The parameters are summarized in Table 1. In Fig. 2B, the voltage dependence of the inactivation kinetic was faster with depolarization at both early and late times in culture, which is a hallmark of T-type channels (early vs. late: 26 ± 2.1 vs. 32.6 ± 6.7 ms at −50 mV, and 18.7 ± 2.4 vs. 26.3 ± 3.5 ms, P < 0.05, at −20 mV).

Pharmacological characterization of T-type channel α-sub-

units in heterologous expression systems has shown that 50 μM NiCl2 blocks 66% of Ca3.2 and 10–15% of Ca3.1, while 250 μM NiCl2 completely blocks Ca3.2 and blocks 50% of Ca3.1 (28). Figure 2C shows representative traces of the nifedipine-resistant current in the presence of NiCl2, showing
the sensitivity of the current to 50 μM NiCl₂ and further to 250 μM NiCl₂, in a partially reversible manner, all characteristics of the T-type channel. When 50 μM NiCl₂ was applied, the residual current represented 43 ± 3.5% of total T-type current (n = 10) at early times, and 52.9 ± 9.6% (n = 5) at later times in culture. These results indicate that the nifedipine-resistant current corresponds to the T-type Ca²⁺ channel and comprises both Ca₃.1 and Ca₃.2 α-subunits at both early and late culture times.

Effect of H/R conditions on calcium currents in cardiomyocytes. We next sought to test the effect of H/R injury on T-type channels in NRVM. Myocytes were exposed to hypoxic conditions for 18 h, followed by 24 h of normoxia, representing the reoxygenation phase. Currents were recorded 1–4 h after the hypoxic episode [acute reoxygenation (AR)], after 24 h of reoxygenation (T24R), and at 4–6 days of reoxygenation [chronic reoxygenation (CR)] and compared with time-matched control myocytes (Fig. 3). The I-V plots for early cultures (Fig. 3A) indicate that the peak current density was shifted in AR from −20 to 0 mV and not changed at T24R compared with control conditions. Notably, the reduction of T-type current density during hypoxia was significant at lower voltages, where these channels are typically active (−30 mV and below), without significantly altering the current density at higher voltages (∼0 mV and above).

In the context of the reversibility of the injury, we addressed the changes in T-type current properties at a later time in culture after reoxygenation. In Fig. 3B, there was no significant difference in the current density between treated and untreated myocytes and no noticeable shift in peak current density.

Next we analyzed in more detail the effect of H/R conditions on the voltage dependence of activation and inactivation of T-type current, as suggested from the shift in the peak current.
membrane potentials, similar to total T-type current (Fig. 5A). Activation and inactivation were shifted to more depolarized potentials during AR, indicating increased probability of opening the T-type channel at lower voltages. For the Ni\(^{2+}\)-resistant component, only the activation was sensitive to hypoxic conditions and shifted to more depolarized membrane potentials, while inactivation was unchanged; reoxygenation reversed the effect of hypoxia on activation and shifted the inactivation to more hyperpolarized values (Fig. 5C), therefore decreasing the probability of opening the channels at lower voltages. At a later time in culture, the Ni\(^{2+}\) residual current displayed robust shifts in both activation and inactivation compared with time-matched control myocytes (Fig. 5D), indicating more availability of the channels at resting membrane potential, but requiring higher depolarization to open. The parameters of activation and inactivation are summarized in Table 2. Overall, these data suggest that Ca\(_{\text{a},3.1}\) and Ca\(_{\text{a},3.2}\) have different sensitivities to oxidative stress, and the total T-type Ca\(^{2+}\) current properties can be altered, depending on the balance between these two components.

**Effect of H/R conditions on T-type calcium channel mRNA levels in cardiomyocytes.** To determine whether H/R injury regulates expression of Ca\(_{\text{a},3.1}\) and/or Ca\(_{\text{a},3.2}\) at the transcriptional level, we measured mRNA expression levels in NRVM subjected to the same H/R injury conditions. Figure 6A shows that, in hypoxic conditions, the Ca\(_{\text{a},3.1}\) α-subunit mRNA level was decreased nearly fivefold [relative quantification (RQ): 0.21 ± 0.03, n = 20, P < 0.001], while Ca\(_{\text{a},3.2}\) was not significantly increased (RQ: 1.69 ± 0.23, n = 17). After T24R, mRNA level returned to normal for Ca\(_{\text{a},3.1}\) (RQ: 1.24 ± 0.22, n = 12) and remained unchanged for Ca\(_{\text{a},3.2}\) (RQ: 1.54 ± 0.27, n = 12). To compare mRNA expression and T-type current levels, Figure 6B summarizes changes in the Ni\(^{2+}\) residual current, as a measure of Ca\(_{\text{a},3.1}\) current at the same time points, showing a corresponding decrease in the Ca\(_{\text{a},3.1}\) current following hypoxia (from 43 ± 3.5%, n = 10, to 30.3 ± 4.3%, n = 8, P < 0.05) with recovery in the first T24R (53 ± 3.9%, n = 7, P < 0.001 compared with AR). The mRNA data correlate well with the regulation of Ni\(^{2+}\)-sensitive T-type currents, corroborating downregulation of Ca\(_{\text{a},3.1}\) during the hypoxic phase and its upregulation during the reoxygenation phase. Taken together, molecular and electrophysiological measurements of T-type Ca\(^{2+}\) channel expression and function show that both Ca\(_{\text{a},3.1}\) and Ca\(_{\text{a},3.2}\) are differentially regulated in conditions of hypoxia and reoxygenation in cultured cardiac myocytes.

**Effect of chemical H/R conditions on T-type calcium channels in cardiomyocytes.** The regulation of Ca\(_{\text{a},3.1}\) mRNA during H/R injury led us to study the possible effectors involved in T-type channel transcriptional regulation. To test whether Ca\(_{\text{a},3.1}\) regulation is mediated by HIF-1α, we induced conditions of chemical hypoxia by incubating NRVM with 100 μM CoCl\(_{2}\) for 48 h, which increases the levels of HIF-1α [relative quantification (RQ): 53.0 ± 3.9%, n = 7, P < 0.001 compared with AR]. The mRNA data correlate well with the regulation of Ni\(^{2+}\)-sensitive T-type currents, corroborating downregulation of Ca\(_{\text{a},3.1}\) during hypoxia and its upregulation during the reoxygenation phase. Taken together, molecular and electrophysiological measurements of T-type Ca\(^{2+}\) channel expression and function show that both Ca\(_{\text{a},3.1}\) and Ca\(_{\text{a},3.2}\) are differentially regulated in conditions of hypoxia and reoxygenation in cultured cardiac myocytes.

Fig. 2. Electrophysiological characterization of nifedipine-resistant current in NRVM. A: voltage dependence of activation and inactivation at early (days 2–3, n = 17) and late (days 5–7, n = 11) time in culture, in normal conditions. Values are means ± SE fitted with Boltzmann equation. B: voltage dependence of the inactivation kinetic at early and late time of culturing. Values are means ± SE. C: representative traces showing sensitivity to 50 and 250 μM NiCl\(_{2}\) of the T-type current recorded at −20 mV pulse from −100 mV and the partial reversibility of NiCl\(_{2}\) block (wash). Scale bars represent 25 pA and 10 ms.

The results show that, during AR, both activation and inactivation were shifted to more depolarized membrane potentials, similar to total T-type current (Fig. 5A). After T24R, inactivation is completely reversed, while activation remains shifted to more depolarized values (Fig. 5A). At a later time in culture, there is no difference in the inactivation, but the activation is shifted to more hyperpolarized potentials (Fig. 5B), indicating increased probability of opening the channel at lower voltages. For the Ni\(^{2+}\)-resistant component, only the activation was sensitive to hypoxic conditions and shifted to more depolarized membrane potentials, while inactivation was unchanged; reoxygenation reversed the effect of hypoxia on activation and shifted the inactivation to more hyperpolarized values (Fig. 5C), therefore decreasing the probability of opening the channels at lower voltages. At a later time in culture, the Ni\(^{2+}\) residual current displayed robust shifts in both activation and inactivation compared with time-matched control myocytes (Fig. 5D), indicating more availability of the channels at resting membrane potential, but requiring higher depolarization to open. The parameters of activation and inactivation are summarized in Table 2. Overall, these data suggest that Ca\(_{\text{a},3.1}\) and Ca\(_{\text{a},3.2}\) have different sensitivities to oxidative stress, and the total T-type Ca\(^{2+}\) current properties can be altered, depending on the balance between these two components.
In the cardiovascular system, T-type Ca\textsuperscript{2+} channels are formed by either Ca\textsubscript{3.1} or Ca\textsubscript{3.2} \(\alpha\)-subunits. When expressed (15 and 12 mV, respectively) (Fig. 7D), with a similar trend to that seen in hypoxic conditions.

While many cellular signaling pathways are affected during H/R injury, both hypoxia and reoxygenation share a common effector that is generated from different sources: reactive oxygen species (ROS). We attempted to simulate the reoxygenation phase by incubating NRVM in 100 \(\mu\)M H\textsubscript{2}O\textsubscript{2} for 24 h, to test whether the upregulation of T-type channels observed during the reoxygenation phase might be ROS dependent. Similar real-time PCR measurements of mRNA levels for Ca\textsubscript{3.1} and Ca\textsubscript{3.2} indicated that, in the presence of 100 \(\mu\)M H\textsubscript{2}O\textsubscript{2}, these genes were downregulated compared with control conditions (0.61 \(\pm\) 0.31 for Ca\textsubscript{3.1}, 0.55 \(\pm\) 0.07, \(P < 0.05\) for Ca\textsubscript{3.2}), confirming results reported previously (35). These results suggest that increased levels of ROS alone do not induce the upregulation of T-type channels recorded during reoxygenation, and, therefore, other pathways activated during injury might be responsible. Another explanation might be that the sources of ROS as well as the active pathway at the time of ROS generation are important for the trend of T-type channel regulation.

**DISCUSSION**

In the cardiovascular system, T-type Ca\textsuperscript{2+} channels are formed by either Ca\textsubscript{3.1} or Ca\textsubscript{3.2} \(\alpha\)-subunits. When expressed
in heterologous cells, each α-subunit cDNA can form individual functional low-voltage-activated channels with similar electrophysiological properties and differential sensitivity to nickel (5, 28, 36). Although T-type Ca\(^{2+}\) channels have been reexpressed in pathology associated with hypoxic conditions, such as heart failure (17), little is known about the effect of hypoxia and reoxygenation on the regulation of these channels.

In this study, we investigated the effects of H/R injury on T-type Ca\(^{2+}\) channels at the mRNA and current levels. Patch clamp electrophysiology in the presence of NiCl\(_2\) was used to distinguish the two subtypes, which allowed us to discern differential effects of H/R on the two T-type channels at the functional level. Hypoxic conditions led to decreased expression of Ca\(_{3.1}\) mRNA and reproducible shifts in the voltage dependence of activation and inactivation of the T-type Ca\(^{2+}\) current. The effects of CoCl\(_2\)-induced hypoxic injury on Ca\(_{3.1}\) mRNA and the T-type Ca\(^{2+}\) current resembled the effects of physical hypoxia, suggesting the involvement of a HIF-1α-dependent mechanism in the negative regulation of Ca\(_{3.1}\). Upon reoxygenation, the Ca\(_{3.1}\) mRNA and current voltage dependence were restored to control, which could not be explained by the direct actions of ROS on the channel. Therefore, these experiments implicate distinct regulation of Ca\(_{3.1}\) and Ca\(_{3.2}\) T-type channel expression and function during H/R injury in cardiomyocytes.

A decrease in the amplitude of the T-type calcium current with time in culture has been observed previously (15). Because our study required prolonged culture times up to 10 days, we characterized the expression of T-type currents during this time interval, and we show that NRVM cultured in serum-free medium maintain T-type calcium currents up to 7 days in culture, with no change in the peak current density. However, we noted changes in the voltage dependence of T-type currents in early vs. late time of culturing. The observed increase in the residual Ni\(^{2+}\)-resistant current component in late vs. early cultures suggests that the Ca\(_{3.1}\) current becomes more prominent with prolonged culture of NRVM. These observations are consistent with isoform switching previously reported in developing cardiomyocytes (33). Furthermore, our unpublished observations in mouse embryonic stem cells differentiating to cardiomyocytes suggest that, while Ca\(_{3.2}\) may be necessary during the early differentiation phase, the expression of Ca\(_{3.1}\) becomes more predominant as the cardiomyocytes mature, supporting the idea that the Ca\(_{3.1}\) and Ca\(_{3.2}\) channels are differentially regulated and have distinct functions.

The effect of hypoxia on T-type channel regulation has been investigated in PC12 cells (7) and adrenal chromaffin cells (3), but not in cardiomyocytes. In our experimental conditions, both physical and chemical oxidative stress (CoCl\(_2\)) induced similar shifts in the voltage dependence of T-type currents in cardiomyocytes, suggesting that different forms of oxidative stress can induce changes in T-type channel expression and function. The analyses of voltage dependence indicate that activation gating of both Ca\(_{3.1}\) and Ca\(_{3.2}\) is sensitive to hypoxic conditions, whereas, for inactivation, only Ca\(_{3.2}\) is affected by hypoxia. The effect of hypoxia on the activation (positive shift) might be related to the loss of spontaneous activity with hypoxia. Since T-type calcium channels are involved in late diastolic depolarization in pacemaker cells (52), this relation can be established in particular for NRVM. For example, when hypoxia shifts the activation of T-type channels, neonatal myocytes require more time to initiate the action potential, therefore decreasing the spontaneous activity. Also, our results suggest that T-type current is not completely blocked in hypoxic conditions, but the window current is shifted to higher voltages, based on the positive shift of both activation and inactivation. Without this shift, Ca\(_{3.2}\) would be completely inactive at hypoxic resting membrane potentials and would have no contribution.

While relatively little is known about the posttranslational modulation of T-type channels, it is possible that such modifications could alter the gating properties of these channels. For example, calmodulin-regulated protein kinase II shifts Ca\(_{3.2}\) activation to more hyperpolarized values without altering the inactivation in human embryonic kidney (HEK)-293 cells, increasing the T-type current at lower voltages (2). It is also conceivable that, in pathological conditions such as H/R injury, T-type calcium channel α-subunits interact with auxiliary subunits to regulate their function, as in the modulation of high-voltage-activated P/Q-type Ca\(^{2+}\) channels by Ca\(^{2+}\) channel β\(_{4}\)-subunits (14). Also, recent studies revealed selective up-regulation of the β\(_{3}\) auxiliary subunit in atrial myocytes isolated from a rat model of pulmonary hypertension-induced heart failure (24). These observations warrant further investi-
gation of possible modulation of T-type calcium channels, specifically in pathological conditions.

Our observations in NRVM differ from those obtained for T-type channels heterologously expressed in HEK-293 cells exposed to acute hypoxia (20 mmHg) (11), which showed no significant effects on the current amplitude and no change in the voltage dependence of activation and inactivation. This might be explained by different experimental models of H/R injury, the use of different mechanisms by NRVM vs. HEK-293 cells in response to H/R injury, and/or possible modulatory effects on the T-type α-subunits that might depend on the cell background.

Our data show that, in NRVM, T-type Ca^{2+} channels are regulated at the transcriptional level during H/R injury. Hypoxic conditions had a robust negative effect on Cav3.1 mRNA expression in NRVM via HIF-1α (as suggested by CoCl2 stimulation), an effect that was not previously reported in other systems. The hypoxia effect on Cav3.2 is in agreement neither with the HIF-1α-dependent upregulation reported in PC12 and chromaffin cells, nor with the robust upregulation of Cav3.2 mRNA that we have observed in resistance pulmonary artery smooth muscle cells in the same conditions (data not published), indicating possible tissue or developmental stage-specific mechanisms for regulation of Cav3.2.

<table>
<thead>
<tr>
<th>Voltage Dependence</th>
<th>50 μM Ni^{2+} Sensitive</th>
<th>50 μM Ni^{2+} Resistant</th>
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<tbody>
<tr>
<td></td>
<td>Activation</td>
<td>Inactivation</td>
</tr>
<tr>
<td>Control “Early”</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V_{1/2}</td>
<td>-30.5±3.7 (8)</td>
<td>-66.6±2.6 (6)</td>
</tr>
<tr>
<td>k</td>
<td>10.4±1.4</td>
<td>-4±1</td>
</tr>
<tr>
<td>AR</td>
<td>-10.5±2.9 (8)</td>
<td>-61.6±1.6 (7)</td>
</tr>
<tr>
<td>T24R</td>
<td>8.9±0.8</td>
<td>-5.7±0.6</td>
</tr>
<tr>
<td>Control “Late”</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V_{1/2}</td>
<td>-20.9±3.4 (5)</td>
<td>-66.6±4.4 (4)</td>
</tr>
<tr>
<td>k</td>
<td>10.9±1.1</td>
<td>-7.5±2.5</td>
</tr>
<tr>
<td>CR</td>
<td>-17.5±5.1 (3)</td>
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</tr>
<tr>
<td></td>
<td>8.8±1.1</td>
<td>-5.5±1.7</td>
</tr>
</tbody>
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Values are means ± SE for (n) recordings. V_{1/2} and k values are derived from Boltzmann fit. *P < 0.05, †P < 0.01, ‡P < 0.001. Statistical significance is calculated vs. “early” control myocytes.
Compared with its decrease in the hypoxic phase, Cav3.1 mRNA was significantly upregulated in the first T24R. Notably, our experimental culture conditions of chronic (up to 4 days) reoxygenation failed to reproduce the long-term upregulation of either Cav3.1 or Cav3.2 previously observed in vivo in pathological conditions associated with ischemia (18, 43, 44). Future studies are needed to address additional factors induced during in vivo cardiac ischemia-reperfusion, but not present in cardiomyocyte cultures, that may influence T-type Ca²⁺ channel expression and function.

These studies were conducted in ventricular myocytes isolated from newborn rats, which are a suitable system for studying T-type channel regulation and modulation for two main reasons: 1) NRVM express endogenous T-type currents due to their developmental stage; and 2) they can be maintained in culture for up to 2 wk, creating an in vitro system to study the regulation of T-type channels in response to cumulative pathological stimuli. While it is likely that T-type Ca²⁺ channels are reexpressed in adult ventricular myocytes in H/R injury, these cells are less amenable to longer term culture that is required for mechanistic gene regulation studies. Nevertheless, most of the pathways active in NRVM are reactivated in injured adult myocytes, and, furthermore, our data derived from immature cardiomyocytes may still apply to cardiovascular complications associated with hypoxia in early development.

In hypoxic conditions, the metabolism of the cell shifts to glycolysis, and the ATP-dependent processes are slowed down with a direct impact on intracellular calcium regulation. In these conditions, the downregulation of calcium channels can be considered a protective mechanism to counteract the calcium overload. Our observed shift of the T-type window current to more depolarized values may represent a compensatory mechanism for the impaired function of L-type channels, but it could also be detrimental by providing a sustained source of calcium influx in myocytes with impaired ability to pump it out, due to the reduction of ATP. In this context, the observed functional regulation of T-type Ca²⁺ channel subunits in NRVM subjected to H/R injury suggests that they may play an increasing role in calcium homeostasis in pathological conditions.

From these studies, we conclude that T-type channels can be differentially modulated by changes in the redox state of the cardiomyocyte. Elucidating the mechanisms involved will address the open question of the interactions and function of this class of Ca²⁺ channels in cardiomyocytes.

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Fig. 6. Effects of H/R injury on mRNA levels of T-type channel α-subunits in NRVM. A: bar graphs representing the relative expression of mRNA for Cav3.1 and Cav3.2 after 18–24 h of incubation in hypoxia followed by 24 h of reoxygenation. Relative quantification (RQ) was estimated based on the threshold cycle (ΔΔCt) method, using 18S rRNA as endogenous control vs. NRVM maintained in normoxic conditions for the same length of time. Values are means ± SE for n = 10, 8, and 7 myocytes. *P < 0.05 and ***P < 0.001 calculated vs. control. ###P < 0.001 calculated vs. AR.

Fig. 7. Effect of 100 µM CoCl₂ on T-type current in NRVM. Bar graphs represent the changes in T-type current mRNA (A; n = 4), current density at peak (B), and the percentage of 50 µM Ni²⁺ residual current (C) after 48 h of 100 µM CoCl₂. D: analysis of voltage dependence of activation and inactivation of T-type current in the presence of CoCl₂, Iₚ, barium current. Values are means ± SE for 5 myocytes. *P < 0.05.
REFERENCES


36. Rowe GT, Manson NH, Caplan M, Hess ML. Hydrogen peroxide and hydrogen radical mediation of activated leukocyte depression of cardiac


