Independent Modulation of L-type Ca\textsuperscript{2+} Channel in Guinea Pig Ventricular Cells by Nitrendipine and Isoproterenol

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SUMMARY

Dihydropyridine (DHP) Ca\textsuperscript{2+} channel blockers decrease L-type Ca\textsuperscript{2+} channel current (I_{Ca,L}) by enhancing steady-state inactivation, whereas β-adrenergic stimulation increases I_{Ca,L} with small changes in the kinetics. We studied the effects of DHP Ca\textsuperscript{2+} channel blockers on cardiac I_{Ca,L} augmented by β-adrenergic stimulation. We recorded I_{Ca,L} as Ba\textsuperscript{2+} currents (I_{Ba}) from guinea pig ventricular myocytes using the whole-cell patch clamp technique, and compared the effects of nitrendipine (NIT) in the absence and presence of isoproterenol (1 µM, ISO) or forskolin (10 µM, FSK). Maximal I_{Ba} elicited from a holding potential of -80 mV were diminished to 69.4\textpm 13.5% (mean and SE, n=5) of control by NIT (100 nM) and the diminished I_{Ba} were increased to 180.3\textpm 23.2% of control by ISO in the presence of NIT, which was similar to the enhancement seen in the absence of NIT. NIT shifted the V_{1/2} of the I_{Ba} inactivation curve from -34.6\textpm 1.9 mV (n=5) to -48.7\textpm 1.2 mV, enhancing I_{Ba} decay with shortening T_{1/2} at -10 mV from 164.6\textpm 24.2 ms (n=7) to 105.4\textpm 15.2 ms. ISO elicited a small additional shift in the V_{1/2} of I_{Ba} inactivation in the same direction. ISO and FSK each slowed I_{Ba} decay in the absence of NIT, but not in its presence. Thus, β-adrenergic agonists increase and DHP Ca\textsuperscript{2+} channel blockers decrease the amplitude of cardiac I_{Ca,L} independently and the kinetics of I_{Ca,L} is determined mainly by the latter when these drugs coexist. (Jpn Heart J 2001; 42: 771-780)

Key words: Guinea pig, Calcium channel, Myocardium, cAMP, Dihydropyridine Ca\textsuperscript{2+} channel blocker

DIHYDROPYRIDINE Ca\textsuperscript{2+} channel blockers are widely used to treat such cardiovascular disorders as hypertension, angina pectoris and arrhythmia.\textsuperscript{1,2}) They act by depressing L-type Ca\textsuperscript{2+} channel currents (I_{Ca,L}), which play key roles in excitation-contraction coupling in cardiac and vascular smooth muscle cells (VSM), thereby exhibiting negative inotropic and vasodilatory actions. DHP Ca\textsuperscript{2+} channel blockers inhibit I_{Ca,L} in VSM more readily than in cardiac muscle as a consequence of differences in the resting membrane potentials, which are more

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negative in normal cardiac myocytes, and in the molecular structure of the α1 subunit.3) On the other hand, β-adrenergic agonists increase cardiac L-type Ca\(^{2+}\) channel activity due to cAMP-mediated phosphorylation of the channels. Pheochromocytoma is characterized by a massive release of noradrenaline and adrenaline. It is believed that the released catecholamines increase I\(_{\text{CaL}}\) in cardiac myocytes via β-adrenoceptors and increase VSM contractility via α-adrenoceptors, resulting in the development of hypertension. Administration of nifedipine, a DHP Ca\(^{2+}\) channel blocker, to pheochromocytoma patients effectively suppresses the severe rise in blood pressure by decreasing systemic vascular resistance.4,5) Anesthetic management for surgical removal of pheochromocytomas remains a clinical challenge.6) However, nicardipine, another DHP Ca\(^{2+}\) channel blocker, is useful during this surgery to control preoperative and intraoperative hypertension.7)

Patch clamp studies have established that blockade of I\(_{\text{CaL}}\) by a DHP Ca\(^{2+}\) channel blocker is characterized by a negative shift in the steady-state inactivation and acceleration of I\(_{\text{CaL}}\) decay during depolarization of cardiac muscle 9-11) and VSM.12,13) On the other hand, β-agonist-induced increases in I\(_{\text{CaL}}\) are associated with small, negative shifts in the current-voltage (I-V) relationship14) and the slowing of voltage-dependent inactivation during depolarization in cardiac muscle.15) In the present study, we examined whether a DHP Ca\(^{2+}\) channel blocker accelerates the voltage-dependent inactivation also in the presence of a β-agonist.

**METHODS**

All protocols followed the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences issued by the Council of the Physiological Society of Japan and were approved by the Institutional Animal Experimentation Committee of Juntendo University School of Medicine.

**Cell isolation:** The methods used have been described previously.11,15) Briefly, guinea pigs (250-350 g) were anesthetized with pentobarbital sodium (50 mg/kg) and the ascending aorta was cannulated under artificial ventilation. The heart was then excised and sequentially perfused by the Langendorff method with normal Tyrode solution (5 min), Ca\(^{2+}\)-free Tyrode solution (5 min), collagenase solution (10-15 min) and high K\(^+\), Ca\(^{2+}\)-free (Kraftbrühe, KB) solution (5 min). All perfusates were oxygenated and pre-warmed to 37°C. The digested heart was then minced with scissors, shaken gently for several minutes and filtered through a coarse metal filter. Filtered myocytes were stored in KB solution at 4°C. For experimentation, myocytes were dispersed in a chamber mounted on an inverted microscope and superfused with normal Tyrode solution for more than 10 min.
Whole-cell patch clamp was then carried out at room temperature (25°C).

**Experimental protocols:** Once gigaseals were formed and the whole-cell configuration was obtained, the cells were allowed to equilibrate with the pipette solution for about 10 minutes. The holding potential (HP) was -80 mV. After making several control recordings, nitrendipine (NIT), isoproterenol (ISO) and/or forskolin (FSK) were superfused for 90 seconds before the application of command pulses. Three command pulse protocols were applied to analyze the gating properties of L-type channels. To characterize the I-V relationship, 300 ms depolarization steps were applied from the HP to 30 mV in 10 mV increments; to obtain steady-state inactivation curves, 2 second conditioning depolarization pulses were applied from the HP to -10 mV in 10 mV increments, and after each conditioning pulse a 500 ms test pulse to -10 mV was applied; and to determine the time course of inactivation, 1 second depolarization steps to -10 mV were applied.

**Solutions and drugs:** Normal Tyrode solution contained (mM): NaCl 135, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, HEPES 5, glucose 10 at pH 7.4 (adjusted with NaOH). The pipette solution contained (mM): CsCl 140, MgCl₂ 1, EGTA 5, ATP·Mg 5, HEPES 5 at pH 7.3 (adjusted with Tris). Barium currents (I_{Ba}) were recorded in solution containing (mM): choline Cl 135, CsCl 5.4, BaCl₂ 1.8, MgCl₂ 1.0, HEPES 5 at pH 7.4 (adjusted with Tris). KB solution contained (mM): KOH 110; taurine 10; oxalic acid 10; glutamic acid 70; KCl 25; KH₂PO₄ 10; EGTA-Tris 5; Heps-Tris 5; glucose 10; the pH was adjusted to 7.40 with KOH. Nitrendipine was a kind gift from Dr. Jun Inui of Yoshitomi Pharmaceutical Co. (Osaka, Japan). Isoproterenol and forskolin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA)

**Electrical recordings:** Membrane currents were recorded using an EPC 7 patch clamp amplifier (List, Darmstadt, Germany). Data acquisition and analysis were carried out using pClamp software (Axon Instruments Inc., Foster City, CA, USA). Current signals were filtered at 3 kHz and sampled at a frequency of 10 kHz. Peak I_{Ba} amplitudes were estimated as the difference between the maximal inward current and zero-current. The time to half inactivation (T_{1/2}) was measured as the time required for I_{Ba} to decay from its peak to half amplitude.

**Statistics:** The data are expressed as the mean±SE. Statistical analysis was performed using paired t-tests to compare sequentially obtained data for two groups and Bonferroni’s multiple comparison test to compare data for three groups. Values of $p<0.05$ were considered significant.
RESULTS

Effects of nitrendipine and isoproterenol on the \( I_{Ba} \)-V relationship: The effects of 100 nM NIT and 1 \( \mu \)M ISO on the amplitude of \( I_{Ba} \) were examined by generating \( I_{Ba} \)-V curves while consecutively applying NIT and NIT+ISO (Figure 1). Under control conditions, \( I_{Ba} \) were first detected at -30 mV and became maximal at -10 mV (1.4±0.4 nA, \( n=5 \)). The maximal amplitude was diminished to 69.4±10.5% of control in the presence of 100 nM NIT, with no appreciable shift in the voltage dependence. Subsequent application of ISO increased \( I_{Ba} \) amplitude to 180.3±23.2% of that obtained in the continued presence of NIT and produced a small negative shift in the voltage dependence. In the absence of NIT, 1 \( \mu \)M ISO increased \( I_{Ba} \) to 188.3±9.4% (\( n=5 \)) of control (not shown).

Effects of nitrendipine and isoproterenol on the steady-state inactivation of \( I_{Ba} \): Figure 2 shows the effects of NIT and ISO on the \( I_{Ba} \) steady state inactivation curves. The results were well fitted by the Boltzmann equation \( I/I_{max} = 1/(1+exp(V-V_{1/2})/k) \), here \( I/I_{max} \) is the ratio of \( I_{Ba} \) amplitude to its maximal value, \( V_{1/2} \) is the voltage at which the normalized \( I_{Ba} \) amplitude is 0.5, and \( k \) is the slope factor. NIT (100 nM) caused a marked shift of the inactivation curve toward more negative potentials, shifting \( V_{1/2} \) from -34.6±1.9 mV (\( n=5 \) control) to -48.7±1.2 mV with little change in \( k \) (6.1∼7.4) (Figure 2A). Subsequent application of ISO in the continued presence of NIT elicited an additional small, leftward shift in \( V_{1/2} \) to -54.0±1.6 mV.

Figure 1. Effects of nitrendipine and isoproterenol on \( I_{Ba} \)-V curves. Step pulses were applied in 10 mV increments from a HP of -80 mV to 30 mV. After recording \( I_{Ba} \) under control conditions, 100 nM NIT was superfused for 90 seconds before recording a second series of \( I_{Ba} \). Finally, 100 nM NIT+1 \( \mu \)M ISO were applied for 90 seconds and \( I_{Ba} \) were recorded.
Figure 2. Effects of nitrendipine and isoproterenol on steady-state inactivation curves. Conditioning step pulses (2 seconds) were applied in 10 mV increments from a HP -80 mV to -10 mV, and after a 500 ms test pulse to -10 mV was applied. 

A: Effect of NIT (100 nM) and subsequent application of NIT+ISO (1 µM). Curves were obtained by simulating the voltage dependent changes in $I/I_{\text{max}}$ using the Boltzmann equation: $I/I_{\text{max}} = 1/(1 + \exp(V-V_{1/2})/k)$. $V_{1/2}$ was -34.6 mV for control, -48.7 mV in the presence of NIT, and -54.0 mV in the presence of NIT+ISO. $k$ was 6.1, 7.1 and 7.4 in control, NIT and NIT+ISO, respectively.

B: First 1 µM ISO and then ISO+NIT were superfused. $V_{1/2}$ was -35.2 mV in control, -40.9 mV in the presence of ISO, and -55.0 in the presence of ISO+NIT. $k$ was 6.7, 6.3 and 7.8 in control, ISO and ISO+NIT, respectively.
In another series of experiments, NIT and ISO were applied in the reverse order (Figure 2B). In this case, ISO applied in the absence of NIT again caused a small negative shift in the inactivation curve, with V_{1/2} going from -35.2±3.3 mV (n=5) to -40.2±2.2 mV. Subsequent application of NIT in the continued presence of ISO again elicited a marked negative shift in the inactivation curve (V_{1/2}=-55.0±2.1 mV (n=5)). Thus, NIT elicited similar negative shifts in the steady-state inactivation of I_{CaL} both in the presence and absence of ISO.

**Effects of nitrendipine, isoproterenol and forskolin on the time course of I_{Ba} inactivation:** Modulation by NIT (10 nM) and ISO (1 μM) of the time course of I_{Ba} was analyzed by applying 1 second test potentials from the HP to -20, -10, 0, 10 or 20 mV and comparing the currents elicited in the absence and presence of the drugs (Figure 3). We found that NIT accelerated current decay at each depolarization and consistently depressed peak I_{Ba} amplitude. Subsequent addition of ISO in the continued presence of NIT increased peak I_{Ba} amplitude, but did not prolong the time course of I_{Ba} inactivation.

![Figure 3](image-url)

*Figure 3.* Effect of nitrendipine and nitrendipine plus isoproterenol on the decay of I_{Ba}. One second depolarizing pulses to -20 mV (a), -10 mV (b), 0 mV (c), 10 mV (d) or 20 mV (e) were applied from a holding potential of -80 mV; control in the absence of any drugs (m), in the presence of 100 nM NIT (l), or in the presence of NIT+1 μM-ISO (Δ). NIT accelerated the decay of I_{Ba} both in the absence and presence of ISO.
Figure 4. Nitrendipine-, isoproterenol- and forskolin-induced changes in the time course of inactivation of I_{BA}. I_{BA} were elicited by depolarization to -10 mV in the absence of drugs (Cont) or in the presence of NIT, ISO or FSK, and the T_{1/2} were compared. A, 100 nM NIT and NIT+1 µM ISO, n=7; B, 10 nM NIT and NIT+ISO, n=5; C, 1 µM ISO, n=6; D, 1 µM ISO and ISO+100 nM NIT, n=5; E, 100 nM NIT and NIT+10 µM FSK, n=6; F, FSK, n=5. 100 nM NIT significantly decreased T_{1/2} both in the absence and presence of ISO or FSK, while ISO and FSK increased T_{1/2} in the absence of NIT. Data are presented as means±SD for four to seven independent experiments; *p<0.05, **p<0.01 vs control.
Figure 4 summarizes the effects of NIT, ISO and FSK on the time course of \( I_{Ba} \) decay during depolarization steps to -10 mV. NIT (100 nM) elicited significant shortening of \( T_{1/2} \) from a control time of 164.6±24.2 ms (\( n=7 \)) to 105.4±15.2 ms (A). In the absence of NIT, 1 \( \mu \)M ISO increased \( T_{1/2} \) to 218.5±48.7 ms (\( n=6 \)) (C); however in the presence of NIT, application of ISO decreased it to 86.8±11.8 ms (A). Similar inhibition of ISO-induced prolongation of \( T_{1/2} \) was also elicited by 10 nM NIT, a concentration at which NIT did not significantly affect \( T_{1/2} \) (B). Inhibition by NIT of ISO-induced increases in \( T_{1/2} \) was also apparent when the order of ISO and NIT application was reversed. \( T_{1/2} \) declined from 223.0±55.4 ms in the presence of ISO to 88.9±23.5 ms (\( n=5 \)) in the presence of ISO plus 100 nM NIT (D) in accordance with \( T_{1/2} \) in A. Moreover, addition of 10 \( \mu \)M FSK to 100 nM NIT did not significantly affect \( T_{1/2} \) (E), although in the absence of NIT, FSK increased \( T_{1/2} \) from 194.2±44.7 ms to 252.6±56.8 ms (\( n=5 \)) (F).

**DISCUSSION**

Our findings demonstrate that NIT produced similar voltage-dependent blockade of \( I_{CaL} \) both in the absence and presence of a \( \beta \)-adrenergic agonist (Figure 2), i.e., ISO increases cardiac muscle \( I_{CaL} \) in the presence of NIT without affecting the NIT-induced blockade of \( I_{CaL} \).

\( \beta \)-Agonist-evoked increases in cardiac L-type \( Ca^{2+} \) channel activity are due to cAMP-mediated phosphorylation of the channels by protein kinase A (PKA) \(^{16} \), similar effects are elicited by FSK, which directly activates adenylate cyclase. We found that the \( \beta \)-agonist ISO increases the amplitude of \( I_{CaL} \) to a similar degree in the presence and absence of NIT, and that ISO-induced increases in \( I_{CaL} \) were associated with small negative shifts in the current-voltage relationship, which is consistent with earlier reports. \(^{14} \) Blockade of \( I_{CaL} \) by a DHP \( Ca^{2+} \) channel blocker is characterized by a shift of its steady-state inactivation curve towards more negative potentials. \(^{10} \) Accordingly, more extensive L-type channel blockade is obtained at less negative resting potentials. This enhanced depression of \( I_{CaL} \) through depolarization of resting potential is explained by the preferential binding of the drug to the channels in the inactivated state. \(^{10,17} \) Indeed, we previously used single channel recording to show that sustained blanks (unavailable state) appear more often at depolarized holding potentials. \(^{11} \)

During surgery to remove pheochromocytomas, application of a DHP \( Ca^{2+} \) channel blocker normalizes blood pressure by decreasing systemic vascular resistance, while heart rate and cardiac index are augmented by catecholamines released as a consequence of tumor manipulation. \(^{7,8} \) The decrease in systemic vascular resistance is explained by the binding of the DHP \( Ca^{2+} \) channel blocker
to the \( \alpha_1 \) subunit of the L-type channel in vascular smooth muscle: the resting membrane potential of the vascular smooth muscle is more positive than that of cardiac muscle, making the \( \alpha_1 \) subunit more susceptible to DHP Ca\(^{2+} \) channel blocker binding.\(^3 \) On the other hand, cardiac \( I_{\text{CaL}} \) is expected to be augmented considerably by the released catecholamines, even in the presence of a DHP Ca\(^{2+} \) channel blocker, resulting in the increased cardiac index. Our study has provided clear evidence that guinea pig cardiac \( I_{\text{CaL}} \) are augmented by catecholamines in the presence of a DHP Ca\(^{2+} \) channel blocker to the extent that is not much different from control augmentation obtained in the absence of the blocker. The augmentation of \( I_{\text{CaL}} \) results in an increased cardiac index, a necessary condition for the therapeutic use of a DHP Ca\(^{2+} \) channel blocker in pheochromocytoma patients. However, caution is necessary when administering DHP Ca\(^{2+} \) channel blocker to patients with a damaged myocardium that may contain cells with a low resting potential; their L-type channels would be expected to be more effectively blocked by the DHP Ca\(^{2+} \) channel blocker, even in the presence of a \( \beta \)-agonist.

NIT accelerated \( I_{\text{CaL}} \) decay during depolarization, as would be expected for a drug binding to opened channels.\(^\text{11} \) In contrast, both ISO and FSK prolonged \( I_{\text{CaL}} \) decay. When NIT and ISO or FSK were applied together, the ISO- or FSK-induced prolongation of \( I_{\text{Ba}} \) decay was not observed (Figures 3 and 4). Similar suppression of ISO-induced \( I_{\text{Ba}} \) prolongation by DHP Ca\(^{2+} \) channel blockers has been reported in rat ventricular myocytes using (+)-Bay K 8644, a pure Ca\(^{2+} \) channel antagonist.\(^\text{18} \) These actions may be a consequence of the fact that they bind to PKA-dependently phosphorylated channels as avidly as to unphosphorylated channels, which would immediately close those channels, blocking the ability of ISO to prolong \( I_{\text{CaL}} \). The overall effect of these drugs on the cardiac L-type channel activity would thus be the sum of the independent effects of the DHP Ca\(^{2+} \) channel blocker and \( \beta \)-agonists on \( I_{\text{CaL}} \): Such independence may originate from the difference in the molecular domains involved in the modulation of the \( \alpha_{1C} \) subunit by each. The L-type Ca\(^{2+} \) channel is constructed from \( \alpha_{1C}, \beta \) and \( \alpha_2/\delta \) subunits.\(^\text{19} \) The \( \alpha_{1C} \) subunit contains the DHP binding sites, which are assigned to domains III\( S_5 \) and IV\( S_6 \), and is also a substrate for PKA,\(^\text{22} \) which phosphorylates the Ser1928 residue located near the C-terminal.\(^\text{22,23} \)

In conclusion, ISO- and FSK-induced Ca\(^{2+} \) channel phosphorylation does not modulate the effect of a DHP Ca\(^{2+} \) channel blocker on cardiac L-type Ca\(^{2+} \) channel inactivation. Furthermore, the applicability of a DHP Ca\(^{2+} \) channel blocker during the excision of a pheochromocytoma apparently hinges on the fact that when the resting potential is within the normal range, blockade of cardiac L-type Ca\(^{2+} \) channels by a DHP Ca\(^{2+} \) channel blocker is minimal, regardless of phosphorylation.
REFERENCES