Inhibitory Effects of Carvedilol on Calcium Channels in Vascular Smooth Muscle Cells

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SUMMARY

Carvedilol has hypotensive effects and inhibits agonist-induced cell proliferation of vascular smooth muscle and then prevents vascular remodeling. However, the basic mechanisms have not been clarified. We examined the effects of carvedilol on [Ca2+]i mobilization and voltage-dependent L-type Ca2+ current (I_{Ca,L}) in vascular smooth muscle cells, and compared them with metoprolol. [Ca2+]i, was measured using fura-2 AM and patch clamp techniques in rat embryonic aortic smooth muscle cells (A7r5). In the presence of extracellular Ca2+, vasopressin and endothelin-1 increased [Ca2+]i due first to the Ca2+ release from store sites, and subsequently Ca2+ entry. Carvedilol did not inhibit the Ca2+ release, but significantly suppressed the sustained rise due to Ca2+ entry concentration-dependently. Nifedipine and nicardipine (10 µM) partly inhibited the sustained rise, but carvedilol inhibited it more effectively than the Ca2+ channel blockers. Under voltage clamp conditions, carvedilol (0.2-10 µM) reversibly inhibited the I_{Ca,L} concentration-dependently without any changes in the current-voltage relationships of I_{Ca,L}. Carvedilol shifted the steady-state inactivation for I_{Ca,L} to more negative potentials and inhibited I_{Ca,L} in a voltage-dependent manner. In addition, carvedilol did not inhibit Ca2+ release from store sites induced by thapsigargin, but significantly inhibited the sustained rise due to capacitative Ca2+ entry unrelated to I_{Ca,L}. In contrast, metoprolol did not mimic these effects of carvedilol. These results provide evidence that carvedilol inhibits I_{Ca,L} and may also inhibit the channels for agonist (vasopressin and endothelin-1)-induced Ca2+ entry in vascular smooth muscle cells, which might contribute to the vasorelaxing and antiproliferative effects of carvedilol. (Jpn Heart J 2003; 44: 963-978)

Key words: Carvedilol, Metoprolol, Vascular smooth muscle, Voltage-dependent L-type Ca2+ current, Vasopressin, Endothelin-1

CARVEDILOL is a unique cardiovascular drug with a wide spectrum of pharmacological activities used for the treatment of hypertension, angina pectoris, and heart failure.1,2) It is a potent nonselective β-adrenoceptor antagonist3,4) with...
antioxidant activities\textsuperscript{5} and also possesses selective $\alpha_1$-adrenoceptor blocking effects,\textsuperscript{6} which contribute to the vasorelaxing action. Furthermore, carvedilol reduces the contraction induced by membrane depolarization in a high K\textsuperscript{+} medium in isolated rabbit coronary artery and rat aortic preparations.\textsuperscript{3,7,8} It also antagonizes the Bay K\textsuperscript{+}-induced contraction in vascular smooth muscle.\textsuperscript{8} These observations suggest that carvedilol may inhibit voltage-dependent L-type Ca\textsuperscript{2+} channels (I\textsubscript{Ca,L}) in vascular smooth muscle cells (VSMCs). In addition, in the mesenteric artery of spontaneously hypertensive rats, chronic carvedilol treatment has been shown to hyperpolarize the membrane potential.\textsuperscript{9} However, little is known about the effects of carvedilol on ionic currents including I\textsubscript{Ca,L} in VSMCs.

Carvedilol inhibits cell proliferation and migration of VSMCs elicited by various vasoactive agents such as endothelin-1 and platelet-derived growth factor,\textsuperscript{10,11} which lead to a pronounced protection of myointimal proliferation \textit{in vivo} following vascular injury by balloon angioplasty.\textsuperscript{10} It also prevents cardiac hypertrophy induced by aortic banding or in spontaneously hypertensive rats.\textsuperscript{12,13} These effects may be also involved in preventing progression of cardiovascular remodeling observed in physiological conditions such as myocardial infarction.\textsuperscript{14} The basic mechanisms of carvedilol have not been clarified, but Ca\textsuperscript{2+} is considered to be an intracellular signal implicated in proliferation as well as vasoconstriction.\textsuperscript{15} In fact, DNA synthesis and cell proliferation are inhibited by Ca\textsuperscript{2+}-blockers such as nifedipine and verapamil in VSMCs.\textsuperscript{16} Agonists such as endothelin-1 induce an initial release of Ca\textsuperscript{2+} from intracellular stores, followed by a sustained Ca\textsuperscript{2+} entry through the plasma membrane.\textsuperscript{17,18} The voltage-dependent Ca\textsuperscript{2+} channel (VDCC) is an important Ca\textsuperscript{2+} entry pathway activated by agonists, but non-VDCC pathways are also involved. The mechanism by which Ca\textsuperscript{2+} entry is activated by the depletion of intracellular Ca\textsuperscript{2+} stores (capacitative Ca\textsuperscript{2+} entry) is considered to involve agonist-induced Ca\textsuperscript{2+} entry via non-VDCC.\textsuperscript{19-22} Carvedilol has been reported to inhibit Ca\textsuperscript{2+} influx induced by acetylcholine in adrenal chromaffin cells,\textsuperscript{23} and veratridine-induced increase in intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) in cerebellar granule cells,\textsuperscript{24} but until now, the effects of carvedilol on [Ca\textsuperscript{2+}], and the Ca\textsuperscript{2+} entry pathways have not been investigated in VSMCs.

In this paper, to clarify the direct effects of carvedilol on VSMCs, we examined the effects of carvedilol on VDCC and agonists (vasopressin and endothelin-1)-induced Ca\textsuperscript{2+} mobilization, and compared them with those of metoprolol, a potent $\beta_1$-selective antagonist.\textsuperscript{25}
### METHODS

**Cell preparation:** A7r5 cells (ATCC-7), a well-established VSMC line obtained from embryonic rat aorta,²⁶,²⁷ were purchased from the American Type Culture Collection through Dainippon Seiyaku (Kyoto, Japan). Cells were grown in Dulbecco’s modified Eagle’s Medium (DMEM, Nissui Seiyaku, Tokyo) supplemented with 10% fetal bovine serum (FBS, M.A. Bioproducts, Walkersville, MD, USA), 50 units mL⁻¹ penicillin and 50 µML⁻¹ streptomycin at 37°C in a fully humidified atmosphere of 5% CO₂ in air. Cells subcultured in passage number 10-20 were grown as monolayers on glass slides, and confluent cell layers were serum deprived by incubating them in DMEM containing 0.3% FBS for 24 hours. Each cell was isolated by an enzymatic procedure using trypsin and used for the experiments. All experiments were performed at 35-37°C.

**Solutions and drugs:** The composition of the control (Tyrode) extracellular solution was as follows (in mM): NaCl 136.5, KCl 5.4, CaCl₂ 1.8, MgCl₂ 0.53, glucose 5.5, and HEPES-NaOH buffer 5.5 (pH 7.4). The Ca²⁺-free solution was the same as normal Tyrode solution with the exception that CaCl₂ was omitted and EGTA (0.5 mM) was added. The high K⁺-bathing solution was as follows (in mM): KCl 140, CaCl₂ 1.8, MgCl₂ 0.53, glucose 5.5, and HEPES-KOH buffer 5.5 (pH 7.4). The K⁺-internal solution contained (mM): KCl 140, EGTA 0.1, MgCl₂ 2, Na₂ATP 1, and HEPES-KOH buffer 5 (pH 7.2). To block K⁺ currents, the patch pipette contained the Cs⁺-internal solution (mM): CsCl 140, EGTA 5, MgCl₂ 2, Na₂ATP 1, guanosine-5’-triphosphate (Na⁺ salt) 0.1, and HEPES-CsOH buffer 5 (pH 7.2). The Ba²⁺-containing bathing solution was the same as the control Tyrode solution, with the exception that CaCl₂ was replaced with BaCl₂ (5 mM), which was used to record the voltage-dependent Ca²⁺ channels (I_{Ba}). Carvedilol ((±)-1-(carbazol-4-yloxy)-3-[[2-(o-methoxyphenoxy) ethyl] amino]-2-propanol) was provided by Daiichi Seiyaku Co. (Tokyo). Nifedipine, nicardipine, tetraethylammonium, glibenclamide, and metoprolol were purchased from Sigma Chemical Co. (St Louis, MO). Carvedilol was prepared in dimethylsulfoxide (DMSO) to make 10 mM stock solution. Nicardipine and nifedipine were dissolved in ethanol to make 10 mM stock solutions. All drugs were diluted in superfusates to the desired concentrations, and the final concentration of DMSO or ethanol was less than 0.1%.

**Determinations of [Ca²⁺]i:** [Ca²⁺]i was determined using the fluorescence method as described previously.²⁸,²⁹ Fura-2 acetoxymethyl ester (fura-2) was obtained from Dojin Chemicals (Tokyo, Japan). A7r5 cells were trypsinized, washed twice in the standard solution, adjusted to a cell density of 10⁶ cells mL⁻¹ and loaded with 1 µM fura-2 AM for 60 minutes in a 20°C -shaking water bath. After incubation, the medium containing fura-2 AM was removed and the fluorescent cells in suspensions were measured at 37°C while stirring continuously in a cuvette.
placed in a spectrofluorometer (CAF-110, JASCO Co., Ltd., Tokyo). The excitation wavelengths were 340 and 380 nm, and the emission wavelength was 500 nm. In the evaluation of the Ca\(^{2+}\) response, the amplitude of Ca\(^{2+}\) elevation in response to each stimulant (vasopressin, endothelin-1 or thapsigargin) was calculated using the percentage of an increase in F\(_{340}/F\(_{380}\) with reference to the value at the resting state. The actual data for F\(_{340}\) and F\(_{380}\) are not shown, and the ratio of F\(_{340}/F\(_{380}\) is indicated in each Figure.

**Recording technique and data analysis:** The membrane potential and currents were recorded using the tight-seal whole cell clamp technique.\(^{30,31}\) The heat-polished patch electrode, filled with the artificial internal solution (for composition, see above), had a tip resistance of 3-5 M\(\Omega\). The series resistance was compensated. The membrane potential and currents were continuously monitored with a high-gain storage oscilloscope (COS 5020-ST, Kikusui Electronic, Tokyo). The data were stored on videotapes using a PCM converter system (RP-880, NF Electronic Circuit Design, Tokyo). The data were reproduced, low-passed filtered at 1 kHz (-3dB) with a Bessel filter (FV-625, NF, 48 dB/octave slope attenuation), sampled at 5 kHz, and analyzed off-line on a computer using p-Clamp software (Axon Instruments, California).

The quasi-steady state inactivation through VDCC at various membrane potentials was estimated using a double-pulse protocol. Conditioning voltage pulses (3 seconds in duration) to various membrane potentials between -80 and +10 mV were applied from a holding potential of -80 mV. At 10 ms after the end of each conditioning pulse, a test pulse to + 10 mV (200 ms in duration) was applied to elicit the Ca\(^{2+}\) currents (I\(_{Ba}\)). The ratio of the amplitude of I\(_{Ba}\) with and without the conditioning pulse was plotted against the membrane potential of each conditioning pulse (inactivation curve). The interval between the sets of double pulses was 60 seconds. Statistical data are expressed as the mean ± SEM. Student’s \(t\)-test was used for statistical analysis of the data, and a value of \(P < 0.05\) was considered significant.

**RESULTS**

**Effects of carvedilol on Ca\(^{2+}\) mobilization elicited by vasopressin or endothelin-1:** Figures 1 and 2 show the effects of carvedilol on Ca\(^{2+}\) mobilization elicited by vasopressin and endothelin-1. In the presence of extracellular Ca\(^{2+}\), vasopressin (100 nM, Figure 1Aa) and endothelin-1 (100 nM, Figure 1Ab) induced a biphasic increase in [Ca\(^{2+}\)]. The first transient increase in [Ca\(^{2+}\)], elicited by these agonists resulted mainly from Ca\(^{2+}\) release from intracellular store sites, and the persistent elevation of [Ca\(^{2+}\)], resulted from the entry of extracellular Ca\(^{2+}\). Carvedilol (10 \(\mu\)M) did not alter basal [Ca\(^{2+}\)], (data not shown). Alternatively, after the [Ca\(^{2+}\)],
rise elicited by these agonists reached a steady-state, carvedilol (10 µM) significantly decreased the sustained phase of [Ca$^{2+}$]$_i$ by 61 ± 11% ($n = 8$) in vasopressin and 65 ± 12% ($n = 6$) in endothelin-1, but DMSO (0.1%) by itself had no effect. In the absence of extracellular Ca$^{2+}$, vasopressin (Figure 2A and B) or endothelin-1 transiently increased [Ca$^{2+}$]$_i$ due to Ca$^{2+}$ release from the store sites, but the addition of Ca$^{2+}$ into the bath solution rapidly increased [Ca$^{2+}$]$_i$ levels by promoting Ca$^{2+}$ entry (Figure 2A and B). The transient [Ca$^{2+}$]$_i$ rise elicited by vasopressin due to the Ca$^{2+}$ release was not significantly affected by carvedilol (10 µM) ($n = 6$, Figure 2A and C). On the other hand, [Ca$^{2+}$]$_i$ rise elicited by Ca$^{2+}$ entry was significantly reduced to 46 ± 13% ($n = 5, P < 0.05$) in carvedilol-treated cells (Figure 2A and C). In contrast, metoprolol (10 µM) did not significantly affect either the transient increase in [Ca$^{2+}$]$_i$ or the sustained rise elicited by vasopressin (Figure 2B and D, $n = 6$).

Figure 1B shows the concentration-dependent effects of carvedilol on vasopressin- (Figure 1Ba) and endothelin-1 (Figure 1Bb)-induced sustained [Ca$^{2+}$]$_i$ rise. Metoprolol (10 µM, Figure 1Ba and b) failed to affect the sustained rise of [Ca$^{2+}$]$_i$. The protocols of the drug are shown in the upper part of each trace. A: Effects of carvedilol (10 µM) on [Ca$^{2+}$]$_i$ responses elicited by vasopressin (a) or endothelin-1 (b). B: Concentration-dependent effects of carvedilol (0.2-10 µM) on the sustained rise induced by vasopressin (100 nM, a) and endothelin-1 (100 nM, b). Note that metoprolol (10 µM) did not affect [Ca$^{2+}$]$_i$, but carvedilol (0.2-10 µM) and Ni$^{2+}$ (1 mM) inhibited the sustained phase. C: Effects of metoprolol, carvedilol, nicardipine, and nifedipine on [Ca$^{2+}$]$_i$ responses elicited by vasopressin.

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**Figure 1.** Effects of carvedilol and metoprolol on vasopressin- or endothelin-1-induced [Ca$^{2+}$]$_i$ rise in A7r5 cells. The bath contained 1.8 mM Ca$^{2+}$. A: Effects of carvedilol (10 µM) on [Ca$^{2+}$]$_i$ responses elicited by vasopressin (a) or endothelin-1 (b). The protocols of the drug are shown in the upper part of each trace. B: Concentration-dependent effects of carvedilol (0.2-10 µM) on the sustained rise induced by vasopressin (100 nM, a) and endothelin-1 (100 nM, b). Note that metoprolol (10 µM) did not affect [Ca$^{2+}$]$_i$, but carvedilol (0.2-10 µM) and Ni$^{2+}$ (1 mM) inhibited the sustained phase. C: Effects of metoprolol, carvedilol, nicardipine, and nifedipine on [Ca$^{2+}$]$_i$ responses elicited by vasopressin.
[Ca\textsuperscript{2+}]\textsubscript{i} elicited by vasopressin and endothelin-1. On the other hand, carvedilol (0.2-10 \mu M) decreased it concentration-dependently.

**Carvedilol inhibits receptor (endothelin-1 and vasopressin)-activated Ca\textsuperscript{2+} entry unrelated to I\textsubscript{Ca,L}**: Figure 1C compares the effects of carvedilol and the dihydropyridine Ca\textsuperscript{2+} blockers nicardipine and nifedipine on Ca\textsuperscript{2+} mobilization elicited by vasopressin and endothelin-1. After the [Ca\textsuperscript{2+}]\textsubscript{i} rise elicited by vasopressin and endothelin-1. On the other hand, carvedilol (0.2-10 \mu M) decreased it concentration-dependently.

Figure 2. Comparative effects of carvedilol and metoprolol on [Ca\textsuperscript{2+}]\textsubscript{i} responses elicited by vasopressin. Cells were perfused without (Aa and Ba) or with carvedilol (10 \mu M, Ab) and metoprolol (10 \mu M, Bb) in the absence of extracellular Ca\textsuperscript{2+}. After the transient [Ca\textsuperscript{2+}]\textsubscript{i} rise was elicited by vasopressin (100 nM), Ca\textsuperscript{2+} (1.8 mM) was added to the bathing solution. (C and D) Effects of carvedilol (10 \mu M, C) and metoprolol (10 \mu M, D) on the transient [Ca\textsuperscript{2+}]\textsubscript{i} rise elicited by vasopressin in the absence of extracellular Ca\textsuperscript{2+} and the sustained [Ca\textsuperscript{2+}]\textsubscript{i} rise after the addition of extracellular Ca\textsuperscript{2+}. An increase in F\textsubscript{340}/F\textsubscript{380}, which was measured at the transient and sustained [Ca\textsuperscript{2+}]\textsubscript{i} rise, was obtained by subtracting the basal level in the absence or presence of drugs from the value of these phases. The value obtained in the absence of drugs is considered as 100%. The data in C and D represent the relative increased value of these phases in the presence of carvedilol (C) and metoprolol (D), compared with the value obtained in the absence of drugs. Each datum represents the mean ± SEM of paired 6 different experiments. *P < 0.05 vs controls.
(100 nM, Figure 1Ca and b) reached a steady-state, application of metoprolol did not affect the sustained phase of [Ca\(^{2+}\)], significantly. But, nicardipine (Figure 1Ca, 10 \(\mu\)M) and nifedipine (Figure 1Cb, 10 \(\mu\)M), which completely eliminated \(I_{Ca,L}\), partly decreased it by 16 ± 7% (\(n = 5\)) and 15 ± 6% (\(n = 5\)) respectively. Carvedilol (10 \(\mu\)M) significantly decreased the sustained phase of [Ca\(^{2+}\)], elicited by vasopressin in the presence of nicardipine (Figure 1Ca) or nifedipine (Figure 1Cb). Similar results were obtained from 5 different cells.

Figure 3 shows the effects of carvedilol on thapsigargin-induced Ca\(^{2+}\) entry in the presence of nicardipine (10 \(\mu\)M). In the absence of extracellular Ca\(^{2+}\), thapsigargin (1 \(\mu\)M) transiently increased [Ca\(^{2+}\)], due to Ca\(^{2+}\) release from Ca\(^{2+}\) store sites (Figure 3Aa and Ba). The addition of Ca\(^{2+}\) into the bathing solution rapidly increased [Ca\(^{2+}\)], due to Ca\(^{2+}\) entry. Carvedilol suppressed the [Ca\(^{2+}\)], rise by 50 ± 14% (Figure 3C, \(n = 5\), \(p < 0.05\)), in contrast to metoprolol (10 \(\mu\)M, Figure 3D), where both the transient increase in [Ca\(^{2+}\)] and sustained [Ca\(^{2+}\)] rise induced by thapsigargin (Figure 3B and D) were not altered significantly, compared with the control (\(n = 5\), \(p = NS\)).

**Effects of carvedilol and metoprolol on \(I_{Ca,L}\):**

VDCCs also play an important role in agonist (vasopressin or endothelin-1) -induced Ca\(^{2+}\) entry in VSMCs. In A7r5 cells, it has been shown that VDCCs are mainly composed of a dihydropyridine-sensitive high-threshold Ca\(^{2+}\) current, which can be classified as L-type.\(^{32}\) Figures 4 and 5 show the effects of nicardipine, carvedilol and metoprolol on \(I_{Ca,L}\). To eliminate K\(^{+}\) currents, the patch pipette was filled with the Cs\(^{+}\)-internal solution, and the bathing solution contained 5 mM Ba\(^{2+}\) in place of Ca\(^{2+}\). During the depolarizing steps of +0 mV from a holding potential of -40 mV, the inward current (\(I_{Ba}\)) was elicited at 0.2 Hz, and completely inhibited by nicardipine (Figure 4A, 1 \(\mu\)M) or nifedipine (1 \(\mu\)M), and La\(^{3+}\) (1 mM, Figure 4B). DMSO at concentrations lower than 0.1% did not affect \(I_{Ba}\) significantly (data not shown), and \(I_{Ba}\) moreover, to eliminate the DMSO effects, we usually added 0.1% DMSO into the bathing solution. Carvedilol (10 \(\mu\)M) reduced the amplitude of \(I_{Ba}\) by 68 ± 9% (\(n = 6\)) within 2 minutes (Figure 4B). The inhibitory effect of carvedilol on \(I_{Ba}\) was reversible.

Figures 4C and D show the effects of carvedilol on the current-voltage relationships of \(I_{Ba}\). The cells were held at -40 mV, and command voltage steps to various membrane potentials were applied at 0.2 Hz. The current-voltage relationships of \(I_{Ba}\) measured at the peak inward current are shown in Figure 4D. Carvedilol reduced the current amplitude of \(I_{Ba}\) at all command voltages without affecting the voltage dependency of \(I_{Ba}\). On average, carvedilol (10 \(\mu\)M) decreased peak \(I_{Ba}\) at +0 mV by 62 ± 8% (\(n = 6\)). The effects of various concen-
trations of carvedilol on the amplitude of $I_{Ba}$ are shown in Figure 5A. The cells were held at -40 mV, and command pulses to +0 mV were applied at 0.2 Hz. Carvedilol at concentrations higher than 0.2 µM decreased $I_{Ba}$, and the half maximal inhibitory concentration (IC$_{50}$) of carvedilol on $I_{Ba}$ was 3 µM (Figure 5B). In contrast, metoprolol (10 µM) failed to inhibit $I_{Ba}$ as shown in Figure 5A and B.

To characterize the mode of action of carvedilol on $I_{Ba}$, the influence of the holding potential on the inhibitory effects of carvedilol was investigated as shown
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in Figure 6A. In these experiments, command voltage steps (320 ms in duration) to +10 mV from a holding potential of -40 or -80 mV were applied. The peak amplitude of I\(_{Ba}\) in the absence of carvedilol was normalized to 100. The percent inhibition induced by carvedilol (10 \(\mu\)M) is shown in Figure 6B. Carvedilol reduced the amplitude of I\(_{Ba}\) by 66 \(\pm\) 7\% \((n = 6)\) at -40 mV, whereas it inhibited it by only 18 \(\pm\) 7\% \((n = 6)\) at -80 mV. Figure 6C illustrates the quasi-steady state inactivation (f\(_{\infty}\)) curve of I\(_{Ba}\) in the absence and presence of carvedilol (10 \(\mu\)M). The current-voltage relationships of I\(_{Ca,L}\) peak in controls and presence of carvedilol are shown in D.

Figure 4. (A and B) Effects of nicardipine (A, 1 \(\mu\)M) and carvedilol (B, 10 \(\mu\)M) on I\(_{Ca,L}\). The cells were held at -40 mV, and command voltage pulses (320 ms in duration) to +0 mV were applied at 0.2 Hz. The patch pipette was filled with Cs\(^+\)-internal solution. The time courses of the alterations in I\(_{Ca,L}\) amplitude are indicated in the right part. Original current traces (B) are indicated in the absence (a) and in presence of carvedilol (10 \(\mu\)M) (b), after washout (c) and La\(^{3+}\) (1mM, d). Zero current level is shown as lines. Current traces obtained at times indicated by a through d in B in the right part are shown in B (a-d). (C and D) Effects of carvedilol on I\(_{Ca,L}\). In C, original current traces are shown in controls and in the presence of carvedilol (10 \(\mu\)M). The current-voltage relationships of I\(_{Ca,L}\) peak in controls and presence of carvedilol are shown in D.
The effects of the drug were fitted by the following Boltzmann equation using the least-squares method:

$$f(V) = f_{\infty \text{MAX}} / \{1 + \exp[(V-a)/b]\},$$

where $f_{\infty \text{MAX}}$ is the maximal value of $f_\infty$ (in control conditions, the value of $f_\infty = 1$), $V$ is membrane potential in mV, $a$ is membrane potential at $1/2 f_{\infty \text{MAX}}$, and $b$ is the slope factor. In the absence of the drug, $f_{\infty \text{MAX}} = 1$, $a = -26$ mV, and $b = 4.77$ mV. In the presence of carvedilol (10 µM), $f_{\infty \text{MAX}} = 0.73$, $a = -35.8$ mV, and $b = 5.9$ mV (Figure 6C). Thus, carvedilol decreased the maximal conductance of $I_{\text{Ba}}$ (0.77 ± 0.07, $n = 6$) with a significant shift of the voltage-dependent inactivation curve (-23.4 ± 5.4 mV ($n = 6$) in the control versus -36.8 ± 4.8 mV ($n = 6, p < 0.05$) in the presence of carvedilol).

**Effects of carvedilol on membrane potentials:** Chronic carvedilol treatment has been reported to hyperpolarize the membrane potential in the mesenteric artery of
spontaneously hypertensive rats. Therefore, the effects of carvedilol on membrane potential were investigated in Figure 7. Under the current clamp conditions with a K⁺-internal solution, the membrane potential was about -45 ± 7 mV (n = 15). As shown in Figure 7A, B and C, carvedilol, metoprolol and glibenclamide (10 µM) did not significantly affect the membrane potential. However, application of high K⁺ solution (Figure 7B) and tetraethylammonium (10 mM, Figure 7C) depolarized the potential.
DISCUSSION

The major findings of the present study are: (1) carvedilol inhibited Ca^{2+} entry elicited by vasopressin or endothelin-1 in VSMCs, (2) carvedilol inhibited $I_{Ca,L}$ voltage-dependently, and (3) carvedilol inhibited [Ca^{2+}]_i rise unrelated to $I_{Ca,L}$ due to Ca^{2+} entry induced by thapsigargin. These results suggest that carvedilol inhibits $I_{Ca,L}$, and may also inhibit capacitative Ca^{2+} entry unrelated to $I_{Ca,L}$ in VSMCs, which are different from metoprolol and the classical dihydropyridine Ca^{2+} antagonists. These effects may be responsible for the unique antihypertensive and antiproliferative effects of carvedilol.

This study provides direct evidence showing that carvedilol inhibits $I_{Ca,L}$ in VSMCs. These results are consistent with the previous findings that carvedilol inhibited high K^{+}-induced contractions in VSMCs^{3,7,8} and antagonized Bay K+-induced contractions and hypertensive effects.^{8} The IC_{50} value of carvedilol required for the inhibition of $I_{Ca,L}$ was approximately 3 $\mu$M in the present study,

Figure 7. Effects of carvedilol on membrane potential. Effects of carvedilol (10 $\mu$M, A and B) and metoprolol (10 $\mu$M, A) on membrane potential. High K⁺ solution contained 140 mM KCl, and the patch pipette contained K⁺-internal solution.
and the inhibitory effect was observed at concentrations higher than 0.2 µM. Carvedilol has been reported to inhibit cardiac I_{Ca,L} with an IC_{50} of about 3.59 µM in rabbit ventricular myocytes, which is compatible to the present study using VSMCs. In addition, the inhibitory effect of carvedilol on I_{Ca,L} was voltage-dependent, and carvedilol shifted the steady-state inactivation curve to the left along the voltage axis, suggesting that the inhibitory action of carvedilol on I_{Ca,L} may be more pronounced as the holding potential is more depolarized. Thus, the voltage-dependent effects of carvedilol on I_{Ca,L} may relate to the smaller effect of carvedilol on the heart compared to that of other Ca^{2+} antagonists such as verapamil, rather than inhibit I_{Ca,L} in VSMCs. Metoprolol, a β1-selective antagonist, did not affect I_{Ca,L}. These results are compatible with the previous clinical findings showing that carvedilol decreases vascular resistance, while metoprolol does not.

It has been reported that carvedilol inhibits prostaglandin F_{2α}-induced contraction in VSM. The present study also showed that carvedilol inhibits [Ca^{2+}]_i rise through Ca^{2+} entry elicited by vasopressin and endothelin-1. Since carvedilol is a competitive antagonist at α_{1}-adrenoceptor sites, it is likely that the inhibitory effects of carvedilol may be located on the receptors (vasopressin and endothelin-1). However, in the absence of extracellular Ca^{2+}, carvedilol did not significantly inhibit the [Ca^{2+}]_i rise elicited by Ca^{2+} release from storage sites, suggesting that carvedilol cannot inhibit signaling pathways between receptors (vasopressin and endothelin-1) and IP3 production. Alternatively, it preferentially inhibited Ca^{2+} entry elicited by vasoactive agents, possibly via a direct inhibitory effect on the Ca^{2+} channels. Nicardipine or nifedipine, potent dihydropyridine Ca^{2+} channel antagonists, partly inhibited the vasopressin and endothelin-1-induced sustained rise in [Ca^{2+}]_i, suggesting that carvedilol may inhibit the agonists-induced sustained rise in [Ca^{2+}]_i by inhibiting I_{Ca,L}. However, as shown in Figure 1C, carvedilol further inhibited the sustained rise in [Ca^{2+}]_i elicited by vasopressin even in the presence of nicardipine or nifedipine. Thus, it is likely that vasopressin and endothelin-1 induce Ca^{2+} entry via a dihydropyridine-insensitive pathway as well as a dihydropyridine-sensitive pathway, and carvedilol may inhibit it. The dihydropyridine-insensitive Ca^{2+} entry pathways have been thought to be mediated through receptor-mediated Ca^{2+} channels such as capacitative Ca^{2+} entry (CRAC). Several studies have shown that CRAC contributes to the sustained rise in [Ca^{2+}]_i induced by agonists such as endothelin-1 and vasopressin in VSMCs including A7r5 cells. In the present study, depletion of intracellular Ca^{2+} stores with thapsigargin induced a transient [Ca^{2+}]_i rise due to Ca^{2+} release from the store sites in the absence of extracellular Ca^{2+}, and the subsequent application of Ca^{2+} to the bath solution increased [Ca^{2+}]_i due to Ca^{2+} entry via CRAC. Carvedilol did not modify the [Ca^{2+}]_i release from internal stores by
thapsigargin, but it significantly inhibited the \([\text{Ca}^{2+}]_i\) rise induced by \(\text{Ca}^{2+}\) entry due to CRAC unrelated to \(I_{\text{Ca,L}}\).

The inhibitory effects of carvedilol on agonist (vasopressin and endothelin-1)-induced \([\text{Ca}^{2+}]_i\) mobilization and \(I_{\text{Ca,L}}\) were observed at concentrations higher than 0.2 \(\mu\)M. Since the plasma peak concentration of carvedilol in humans is approximately 0.1-0.6 \(\mu\)M after an oral dose of 12.5-50 mg/day,\(^{36,37}\) carvedilol at therapeutic concentrations may inhibit \([\text{Ca}^{2+}]_i\) and \(I_{\text{Ca,L}}\) in clinical settings. In addition, carvedilol is a highly lipophilic drug with an octane / pH 7.4 buffer partition coefficient of 3.3.\(^{38}\) Consistent with its lipophilic nature, carvedilol can readily distribute into extravascular tissues, and may accumulate in cell membranes, then reaching concentrations higher than plasma concentrations. Thus, these observations suggest that an effective level of carvedilol for inhibiting \([\text{Ca}^{2+}]_i\) and \(I_{\text{Ca,L}}\) may be attainable, especially during chronic administration of carvedilol in clinical settings.

Proliferation and migration of VSMCs in response to mitogenic stimuli such as endothelin-1 play fundamental roles in advancing lesions of atherosclerosis, and may also play important roles in the vascular restructuring associated with hypertension and restenosis of a coronary artery after percutaneous transluminal coronary angioplasty. Carvedilol has been reported to produce antimitogenic effects against a diverse group of potent vascular smooth muscle mitogens such as endothelin-1.\(^{10,11}\) The mechanism of the antimitogenic effects does not appear to be mediated solely through \(\beta\)-adrenoceptor antagonists. Alternatively, elevation of \([\text{Ca}^{2+}]_i\) may be an important intracellular signal stimulating mitogenesis.\(^{15}\) The antimitogenic effects have been reported to be observed at concentrations higher than 1 \(\mu\)M,\(^{11}\) which is similar to the inhibitory effects of carvedilol on agonist-induced \(\text{Ca}^{2+}\) entry in the present study. Thus, the inhibitory effects of carvedilol on agonist-mediated \(\text{Ca}^{2+}\) entry may play a role in the antimitogenic effects of carvedilol.

Chronic carvedilol treatment has been reported to hyperpolarize the membrane in VSMCs,\(^9\) which may contribute to the antihypertensive effects of carvedilol. In addition, in rabbit ventricular myocytes,\(^{33}\) it has been shown that carvedilol inhibits several repolarizing \(K^+\) currents as well as \(I_{\text{Ca,L}}\). However, acute application of carvedilol did not affect the membrane potential at all. Therefore, it is unlikely that the effects of carvedilol on \([\text{Ca}^{2+}]_i\) observed in the present study are secondarily induced by the alterations of membrane potential.

In summary, carvedilol inhibits \(I_{\text{Ca,L}}\) and may also inhibit agonist (endothelin-1 and vasopressin)-induced \(\text{Ca}^{2+}\) entry unrelated to \(I_{\text{Ca,L}}\) in VSMCs. These inhibitory effects of carvedilol on \([\text{Ca}^{2+}]_i\) mobilization could not be observed in cases of metoprolol, a \(\beta_1\)-selective blocker, and classical dihydropyridine \(\text{Ca}^{2+}\) antagonists (nifedipine and nicardipine). These unique actions of carvedilol may
contribute to the antihypertensive and antimitogenic effects of carvedilol in cardiovascular systems.

REFERENCES


