Pharmacological effects of carvedilol on T-type calcium current in murine HL-1 cells

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Abstract

Carvedilol was originally used as an antihypertensive agent mainly because of its blocking activity of β- and α1-adrenoceptors (McTavish et al., 1993; Dunn et al., 1997). Subsequent investigations showed that the cardiovascular protection induced by carvedilol could be additionally due to its antiproliferative (Ohlstein et al., 1993; Patel et al., 1995), antioxidant/antiapoptotic (Schwarz et al., 2003; Kawai et al., 2004), and anti-inflammatory mechanisms (Yuan et al., 2004). Recent studies indicated that carvedilol possessed an anti-arrhythmic efficacy, which resulted from its blockade of adrenoceptors and/or its direct interaction with the voltage-gated ion channels in the heart. Several evidences have demonstrated that carvedilol directly inhibited the L-type Ca2+ current (ICa,L), delayed rectifier K+ currents (IKr and IKs), 4-aminopyridine-sensitive transient outward K+ current (Ito), ultra-rapid delayed rectifier K+ current (IKur) and hyperpolarization-activated inward current (Ih) (Cheng et al., 1999; Karle et al., 2001; Deng et al., 2007; Katritsis, 2008; Yokoyama et al., 2007). T-type Ca2+ channel is characterized by low voltage activation range, rapid inactivation, and slow deactivation (Ferron et al., 2002). It is not present in all cardiac tissues but appears to be significant in sinoatrial node, Purkinje, and atrial cells. It may be involved in cardiac pacemaker activity under physiological conditions (Kino-Oka et al., 2005). T-type Ca2+ channel blockade may attenuate the functional remodeling caused by atrial fibrillation (Fareh et al., 1999). It seems to play a particularly important role in pathophysiological or remodeled cardiovascular tissue. Therefore, T-type Ca2+ channel inhibition may be beneficial for preventing electrical remodeling caused by atrial tachycardia. However, effects of carvedilol on T-type Ca2+ current (ICa,T) have not been fully examined.

Molecular and Cellular Pharmacology

1. Introduction

Carvedilol was originally used as an antihypertensive agent mainly because of its blocking activity of β- and α1-adrenoceptors (McTavish et al., 1993; Dunn et al., 1997). Subsequent investigations showed that the cardiovascular protection induced by carvedilol could be additionally due to its antiproliferative (Ohlstein et al., 1993; Patel et al., 1995), antioxidant/antiapoptotic (Schwarz et al., 2003; Kawai et al., 2004), and anti-inflammatory mechanisms (Yuan et al., 2004). Recent studies indicated that carvedilol possessed an anti-arrhythmic efficacy, which resulted from its blockade of adrenoceptors and/or its direct interaction with the voltage-gated ion channels in the heart. Several evidences have demonstrated that carvedilol directly inhibited the L-type Ca2+ current (ICa,L), delayed rectifier K+ currents (IKr and IKs), 4-aminopyridine-sensitive transient outward K+ current (Ito), ultra-rapid delayed rectifier K+ current (IKur) and hyperpolarization-activated inward current (Ih) (Cheng et al., 1999; Karle et al., 2001; Deng et al., 2007; Katritsis, 2008; Yokoyama et al., 2007). T-type Ca2+ channel is characterized by low voltage activation range, rapid inactivation, and slow deactivation (Ferron et al., 2002). It is not present in all cardiac tissues but appears to be significant in sinoatrial node, Purkinje, and atrial cells. It may be involved in cardiac pacemaker activity under physiological conditions (Kino-Oka et al., 2005). T-type Ca2+ channel blockade may attenuate the functional remodeling caused by atrial fibrillation (Fareh et al., 1999). It seems to play a particularly important role in pathophysiological or remodeled cardiovascular tissue. Therefore, T-type Ca2+ channel inhibition may be beneficial for preventing electrical remodeling caused by atrial tachycardia. However, effects of carvedilol on T-type Ca2+ current (ICa,T) have not been fully examined.

Murine HL-1 cells are derived from atrial tumor cells of transgenic mice expressing the SV40 large T antigen under the control of the atrial natriuretic factor promoter (Claycomb et al., 1998). The HL-1 cells are a unique in vitro model system for the study of mammalian atrial myocytes Ca2+ channel regulation and functional expression, and can
proliferate indefinitely and maintain a differentiated cardiac phenotype. In the present study, we investigated the effects of carvedilol on $I_{Ca,T}$ using whole-cell patch–clamp recording technique in HL-1 cells.

2. Materials and methods

2.1. HL-1 cell culture

HL-1 cell line was obtained from the laboratory of Dr. William Claycomb (Louisiana State University Health Science Center, New Orleans, LA). Cells were cultured using Claycomb medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin on flasks pre-coated with fibronectin and gelatin, then incubated at 37 °C, 5% CO₂/95% O₂. The medium was changed every 24–48 h. Single HL-1 cells were isolated from the culture dishes using a 2 min enzymatic dissociation with 0.05% trypsin-EDTA. Digestion was stopped by adding 0.025% trypsin inhibitor and medium, and the sediment cells were used for electrophysiological experiments within 6 h.

2.2. Electrophysiology

Culture dishes mounted on the stage of an inverted microscope (Olympus IX70, Japan). Cells were perfused with solution containing (mM): TEA-Cl 140, CaCl₂ 5, MgCl₂ 2, HEPES 10, and Glucose 10 (pH was adjusted to 7.4 with CsOH). $I_{Ca,T}$ was recorded by the whole-cell patch–clamp technique. Patch pipettes were fabricated from borosilicate glass capillaries (Corning 7740, 1.2 mm OD). Tip resistances were 2–3 ΩM when filled with the internal solution of the following composition (mM): CsCl 100, TEA-Cl 20, Na₂ATP 5, Na₂GTP 0.4, EGTA 10, HEPES 10 (pH was adjusted to 7.2 with Tris). Tip potentials were compensated before the pipette touched the cell. After a gigaseal was obtained, the cell membrane was ruptured by gentle suction to establish the whole-cell configuration. Current signals were recorded with an Axopatch 200B amplifier using the Digidata 1322A-pClamp 9.0 data-acquisition system (Axon Instruments, USA). The signals were filtered at 5 kHz and stored on the hard disk of a computer. The series resistance ($R_s$) was 3–5 ΩM and was electrically compensated by 70–80% to minimize the capacitive surge on the current recording and voltage drop across the clamped membrane and was kept at a constant value during the current recording. To account for differences in cell size, all mean data are expressed as current density. All experiments were performed at 25 ± 1°C.

2.3. Fluo-4/AM dye loading and confocal laser scanning microscopy (CLSM) for recordings of intracellular calcium

Spontaneous calcium transient was monitored in HL-1 cells using the fluorescent dye Fluo 4-AM. Cells were loaded for 25 min in DMEM with 5 µM Fluo 4-AM, which were dissolved in dimethyl sulfoxide (final concentration 0.1%) and pluronic F-127 (final concentration <0.025%). Then, the culture dishes were rinsed on the hard disk of a computer. The scanning line was placed as approximately equidistant between the cell membrane and the nucleus/nuclei, to ensure the nuclear area was not included in the scanning line. Processing of images was carried out using the time-software facilities of the confocal setup. The time-dependent change of mean fluorescence along the scanning line was used to generate the calcium transient. The calcium level was reported as $F/F_0$, where $F_0$ is the resting or diastolic Fluo-4 fluorescence. The measurements were made on six serial steady-state calcium transients in each cell, and those obtained in 5 cells were averaged in each preparation.

Fig. 1. Identification of $I_{Ca,T}$ in HL-1 cells. A, The representative $I_{Ca,T}$ was recorded in 300-ms voltage steps to between −80 and +50 mV from the holding potential of −80 mV as shown in the inset in the absence and presence of 10 µM mibefradil, and from the holding potential of −40 mV during control condition. B, Current–voltage relations of mean values of $I_{Ca,T}$ density in the absence (control; open circles) and presence of 10 µM mibefradil (filled triangle down), and from the holding potential of −40 mV during control condition (filled circles).
2.4. Chemicals and applications

Carvedilol\(((\pm)-1-\text{carbazol-4-yloxy})-3-[[2-(o-methoxyphenoxy)ethyl]amino]-2-propanol\) was obtained from Qilu Pharmaceutical Company (Jinan, Shandong, China). Most of the chemicals, including mibefradil, nimodipine, norepinephrine, isoproterenol, L-glutamine, fibronectin and gelatin, Tetraethyl-ammonium chloride (TEA-Cl), N-2-hydroxyethyl-piperazine-N-ethane-sulphonic acid (HEPES), Ethylene glycol bis (2-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), Na2ATP, and Na2GTP were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Drugs were added to the superfusate applied to the cell by a fast perfusion system (Y-tube), which allows a complete solution change within 40 ms.

2.5. Data analysis

Non-linear curve fitting was performed using Sigmaplot (SPSS, Chicago, IL, USA). Concentration–response curves for carvedilol were fit to the Hill equation: 

\[ E = E_{\max}/\left(1 + \left(\frac{IC_{50}}{C}\right)^b\right) \]

where \( E \) is the effect at concentration \( C \), \( E_{\max} \) is the maximal effect, \( IC_{50} \) is the concentration giving 50% inhibition and \( b \) is the Hill coefficient. Curves for steady-state activation \((d)\) and inactivation \((f)\) of \( I_{Ca,T} \) were fit to Boltzmann relations for activation and inactivation as follows:

\[ d \text{ (or } f) = 1/(1 + \exp \left(\frac{V_{0.5} - V}{K}\right)) \]

where \( V \) is the membrane potential, \( V_{0.5} \) is a midpoint of potential for activation or inactivation and \( K \) is a slope factor. Results are presented as the mean±SEM. Results were analysed using paired or unpaired Student’s t-tests and one-way ANOVA. Values of \( P<0.05 \) were considered to indicate statistical significance.

3. Results

3.1. Identification of T-type calcium currents in HL-1 cells

HL-1 cells have both \( I_{Ca,L} \) and \( I_{Ca,T} \). \( Ca^{2+} \) currents were considered to be L-type if they were activated at a holding potential of −40 mV and inhibited by nimodipine, a selective inhibitor of L-type \( Ca^{2+} \) channels (but insensitive to mibefradil, a selective inhibitor of T-type \( Ca^{2+} \) channels). \( I_{Ca,T} \) was defined as those activated at a holding potential of −80 mV, but not −40 mV, that were also sensitive to block by mibefradil (but not nimodipine). In cells \((n=50)\) that were examined for the presence of both \( I_{Ca,L} \) and \( I_{Ca,T} \), \( I_{Ca,T} \) was expressed exclusively in ~15% of HL-1 cells (i.e. \( I_{Ca,T} \) was not evident at detectable levels), while \( I_{Ca,L} \) was expressed exclusively in ~39% of the cells. Both \( I_{Ca,L} \) and \( I_{Ca,T} \) were significant in ~44% of the cells. In the remaining ~4% of cells, neither \( I_{Ca,L} \) nor \( I_{Ca,T} \) were expressed at detectable levels.
It is so difficult to separate ICa,T from ICa,L when a HL-1 cell contains simultaneously ICa,L and ICa,T. Therefore, the only expression of ICa,T of the cells were chosen to investigate the effect of carvedilol. Fig. 1A displays the representative ICa,T recorded in 300-ms voltage steps to between −80 and +50 mV from the holding potential of −80 mV, from the holding potential of −40 mV, and from the holding potential of −80 mV in the presence of 10 µM mibefradil. Fig. 1B shows current–voltage relations of mean values of ICa,T density. ICa,T was not recorded from the holding potential of −40 mV. ICa,T was completely blocked by the application of 10 µM mibefradil.

3.2. Effect of carvedilol on ICa,T in HL-1 cells

Fig. 2A displays the representative ICa,T recorded in 300-ms voltage steps to between −40 and +50 mV from −80 mV in the absence and presence of 3 µM carvedilol.

Fig. 2B illustrates the time-dependent effect of carvedilol at 3 µM on ICa,T activated by a 300-ms voltage step from −80 mV to −20 mV. Carvedilol gradually inhibited the ICa,T density, and this effect reached a steady-state level within 1 min. The reduced current largely restored upon 3 min washout of drug in four cells.

Fig. 2C illustrates the concentration–response relation for the carvedilol-induced reduction in ICa,T. The IC50 was 2.1 µM, the 95% confidence interval of the IC50 was from 1.3 µM to 2.9 µM, the Hill coefficient was −0.68 (n = 5).

Fig. 2D shows current–voltage relations of mean values of ICa,T density in the absence and presence of 3 µM carvedilol. The density of ICa,T at −20 mV corresponding to peak current was decreased by 54.58% [from 20.1 ± 1.8 pA/pF to 9.9 ± 2.1 pA/pF; n = 7, P < 0.05].

3.3. Effect of carvedilol on kinetics of activation and inactivation of ICa,T in HL-1 cells

The voltage dependence of ICa,T activation can be determined from the ratio of g/gmax, where g is the conductance and gmax is full-scale value of g, by using the I–V relation of ICa,T. The relationship between g/gmax and membrane potential was fit to Boltzmann equation as follows: d = 1/[1 + exp ([V0.5 − V]/K)], where V is the membrane potential, V0.5 is a midpoint of potential for activation and K is a slope factor. Mean values of V0.5 and S were −29.1 ± 0.4 mV and −28.3 ± 0.3 mV for activation. In the presence of 3 µM carvedilol, corresponding V0.5 and S values were 6.5 ± 0.7 mV and 5.6 ± 0.5 mV for activation (n = 5, P > 0.05) (Fig. 3B). The voltage dependence of ICa,T activation was not affected by carvedilol.

The voltage protocol and representative recordings used to assess ICa,T inactivation are illustrated in Fig. 3A. The voltage dependence of the steady-state inactivation relationship was examined by using a standard two-pulse protocol, 1000-ms preconditioning pulses between −100 and −20 mV from the holding potential of −80 mV,
followed by a 300-ms test pulse to $-20\text{ mV}$. The inactivation variability ($I_{\text{max}}/I_{\text{max}}$) was determined as $I_{\text{Ca,T}}$ at a given prepulse potential divided by the maximum $I_{\text{Ca,T}}$ in the absence of a prepulse. The inactivation curve was fit by a Boltzmann function. 3 µM carvedilol significantly shifted the steady-state inactivation curve of $I_{\text{Ca,T}}$ towards more negative potential by 12.8 mV, the voltage at half-inactivation changing from $-47.2 \pm 3.4\text{ mV}$ in control to $-60.0 \pm 4.2\text{ mV}$ in the presence of carvedilol ($n = 5$, $P < 0.05$) (Fig. 3B).

### 3.4. Effect of carvedilol on recovery of $I_{\text{Ca,T}}$ from inactivation in HL-1 cells

Time-dependent Recovery of $I_{\text{Ca,T}}$ from inactivation was studied with a paired-pulse protocol ($P_1$, $P_2$). The current during $P_2$ relative to the current during $P_1$ was plotted as function of the $P_1$-$P_2$ interval (Fig. 4B). The curves were well fitted to an exponential function, Time constant ($\tau$) was $112.4 \pm 3.5\text{ ms}$ in control and $270.1 \pm 4.7\text{ ms}$ in the presence of 3 µM carvedilol. Carvedilol delayed recovery from inactivation of $I_{\text{Ca,T}}$ ($n = 5$, $P > 0.05$) (Fig. 4).

### 3.5. Frequency-dependent effect of carvedilol on $I_{\text{Ca,T}}$ in HL-1 cells

The frequency-dependent effect of 3 µM carvedilol was studied by a series of 10 depolarizing pulses (300 ms duration) from a holding potential of $-80\text{ mV}$ to $-20\text{ mV}$ at two different stimulation frequencies (0.2 and 2 Hz). 3 µM carvedilol-induced inhibition in $I_{\text{Ca,T}}$ was enhanced with an increase in stimuli frequency ($n = 6$, $P < 0.05$).

### 3.6. Effect of carvedilol on $I_{\text{Ca,T}}$ in the presence of H-89 in HL-1 cells

We further tested the inhibitory effects of carvedilol on $I_{\text{Ca,T}}$ in the presence of H-89 (PKA inhibitor) in Fig. 6. A myocyte was first exposed to control solution, $I_{\text{Ca,T}}$ was recorded (a), the perfusion solution was then changed to a solution containing 10 µM H-89, which had no effect on $I_{\text{Ca,T}}$ (b), carvedilol still produced the significant decrease in the amplitude of $I_{\text{Ca,T}}$ in the presence of (c) H-89. It recovered completely after washout (d).

### 3.7. Effect of carvedilol on calcium transient in HL-1 cells

The representative recordings of calcium transients were shown for the control cells and cells added with different concentration of carvedilol in Fig. 7A. Carvedilol significantly inhibited the amplitude of the calcium transient in a concentration-dependent manner in Fig. 7B.

### 4. Discussion

The present study shows that carvedilol reversibly inhibited $I_{\text{Ca,T}}$ in a concentration- and frequency-dependent manner, the steady-state inactivation curve of $I_{\text{Ca,T}}$ was shifted to more negative potentials, the recovery from inactivation of $I_{\text{Ca,T}}$ was delayed. Carvedilol-induced inhibition rate was enhanced with an increase in stimuli frequency. Carvedilol significantly inhibited the increase in intracellular calcium concentration.

Murine HL-1 cells are a unique adult cardiomyocyte cell line derived from proliferating atrial cardiomyocytes of transgenic mice, which express the SV40 large T antigen in the atria of the heart. These cells express protein markers of cardiac muscle as well as several currents normally found in cardiac tissues, such as the rapidly activating delayed rectifier K+ current and several specific Na+ and Ca2+ currents. They retain a highly differentiated cardiac morphological, biochemical and electrophysiological phenotype reflective of atrial cardiomyocytes. But there was clear cell-to-cell heterogeneity of expression of the channels in the HL-1 cells. In our studies, not all cells had both types of Ca2+ currents. $I_{\text{Ca,L}}$ was recorded in the majority of HL-1 cells examined, while $I_{\text{Ca,L}}$ was observed less frequently. $I_{\text{Ca,T}}$ was expressed exclusively in ~39% of the cells, while $I_{\text{Ca,L}}$ was expressed exclusively in ~15% of HL-1 cells. Both $I_{\text{Ca,L}}$ and $I_{\text{Ca,T}}$ were significant in ~44% of the cells. In the remaining ~4% of cells, neither $I_{\text{Ca,L}}$ nor $I_{\text{Ca,T}}$ was expressed at detectable levels. $I_{\text{Ca,T}}$ and $I_{\text{Ca,L}}$ expression levels and
current densities of HL-1 cells are in agreement with other previous reports. (Xia et al., 2004; Yang et al., 2005).

Changes in T-type Ca\(^{2+}\) channel occur during development, so that while it is recorded in all embryonic and neonatal cells investigated, T-type Ca\(^{2+}\) channel has been reported in normal adult cardiomyocytes of only few species. However, \(I_{Ca,T}\) is often recorded at some phases of remodeling under various pathological conditions. The three T-type Ca\(^{2+}\) channel proteins, Cav3.1–3.3, are clearly re-expressed under the influence of IGF-1, endothelin, and angiotensin II. For example, re-expression of \(I_{Ca,T}\) has been observed in hypertrophied heart, its contribution to the remodeling of excitation–contraction coupling in the cardiac myocyte. Mibefradil, a selective inhibitor of T-type Ca\(^{2+}\) channels, can improve deleterious left ventricular remodeling (Villame et al., 2001; Ferron et al., 2003; Vassort et al., 2006; Suzuki et al., 2007). In previous studies, atrial \(I_{Ca,T}\) was downregulated by sustained rapid atrial pacing in dogs, whereas \(I_{Ca,T}\) was not reduced. Mibefradil prevents AF-promoting electrophysiological remodeling by atrial tachycardia (Fareh et al., 1999). Therefore, \(I_{Ca,T}\) contributes to the control of electrical activity including pacemaker and arrhythmia.

Carvedilol is known to exert an anti-arrhythmic effect (Karle et al., 2001). The electrophysiologic properties of carvedilol include moderate prolongation of action potential duration and effective refractory period; slowing of atrioventricular conduction; and reducing the dispersion of refractoriness. Although the mechanism is still unclear, it is conceivable that carvedilol interacts with voltage-gated ion channels and therefore modifies the electrophysiological properties of excitable cells. It has been reported that carvedilol interacts with voltage-gated ion channels in various types of cells. However, carvedilol inhibited \(I_{Ca,L}\), \(I_{Ca,T}\), and \(I_{Ks}\) in rat ventricular myocytes (Cheng et al., 1999). Carvedilol inhibited HERG current in a concentration-dependent manner, \(I_{C50}\) is 0.51 µM (Kawakami et al., 2006). In the present study, our findings provide the first evidence that carvedilol inhibited \(I_{Ca,T}\) in atrial myocytes in a dose-dependent manner, \(I_{C50}\) is 2.1 µM.

In our studies, acute addition of carvedilol to HL-1 cells reversibly inhibited \(I_{Ca,T}\) peak in a concentration-dependent manner. Effects of carvedilol may directly or indirectly act on T-type calcium channel. Carvedirol is a β- and α1-adrenoceptors blocker, while HL-1 cells need the presence of norepinephrine in the culture medium to maintain the phenotype. Therefore, firstly, we observed the effect of isoproterenol on \(I_{Ca,T}\) in our experiments, the result showed that \(I_{Ca,T}\) was not affected by β-adrenergic stimulation (data not shown) in agreement with a previous report by Hagiwara in rabbit SAN cells (Hagiwara et al., 1988). However, \(I_{Ca,T}\) was increased by isoproterenol in frog atrial myocytes and guinea-pig ventricular cells (Alvarez et al., 1996; Zhang et al., 2002). In rat ventricular cells, endothelin increased \(I_{Ca,T}\) via a protein kinase C-dependent mechanism (Furukawa et al., 1992). Secondly, if effects of carvedilol are related to β-adrenoceptor, cyclic-AMP dependent protein kinase A (PKA) may be involved. We determined the effect of carvedilol on \(I_{Ca,T}\) in the presence of H-89 (a PKA inhibitor), but carvedilol still reduced the significant decrease in the amplitude of \(I_{Ca,T}\). So, PKA doesn’t participate in effect of carvedilol. Therefore, carvedilol may exert mainly an inhibitory efficacy through direct ionic channel modulating effects.

The characteristics of carvedilol-inhibited inhibition of \(I_{Ca,T}\) were further investigated. Carvedilol did not affect the activation curve of \(I_{Ca,T}\), but shifted the steady-state inactivation curve towards more hyperpolarized potential, suggesting that carvedilol may preferentially interact with the inactivated state of T-type calcium channel. The frequency-dependent block of cardiac T-type calcium channels can be described by a periodical ligand binding process between drug molecules and channel binding sites. The carvedilol-induced block of \(I_{Ca,T}\) enhanced with increasing stimulus frequency, reflecting a complex binding kinetics between carvedilol and T-type calcium channels.

HL-1 cells are derived from adult mouse atrial myocytes, and retain many properties of cardiac cells, including rhythmic transient of cytosolic Ca\(^{2+}\) and spontaneous contractions. We also observed rhythmic spontaneous Ca\(^{2+}\) transient in HL-1 cells. We monitored that carvedilol significantly inhibited the amplitude of the calcium...
transient in dose-dependent manner. T-type channels were most prevalent and largest in amplitude in HL-1 cells, and, therefore, were the dominant population responsible for the Ca^{2+} influx active under the conditions of the Ca^{2+}-influx studies (Xia et al., 2004). So, carvedilol inhibiting cytosolic [Ca^{2+}] resulted principally from its blocking T-type channel. Carvedilol can reduce the Ca^{2+}-influx and prevent from calcium overload.

In conclusion, the present study suggests that carvedilol inhibits \( \text{I}_{\text{Ca,T}} \) through calcium channel by mechanisms involving preferential interaction with the inactivated state. These findings are potentially important for understanding the mechanisms and prevention of clinically-relevant atrial tachycardia-induced remodeling.

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**References**


