Inhibition of hyperpolarization-activated cyclic nucleotide-gated channels by beta-blocker carvedilol

Running title: Inhibition of HCN channels by carvedilol

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Author contributions
Y.C., S.L. and P.Z. designed the research; Y.C., S.C., Y.L., T.W. and J.P. performed the experiments; Y.C., S.L. and P.Z. analyzed the data and wrote the paper.

Conflict of interest
The authors state no conflict of interest.
Abstract

Background and Purpose
Carvedilol is a clinically broadly used and effective beta-blocker for treatment of congestive heart failure (CHF) and several clinical trials have demonstrated that carvedilol shows a favourable effect compared to other beta-blocking agents in patients with CHF. The mechanism underlying the advantage of carvedilol over other beta-blocking agents is not clearly understood. In addition to beta-blockers, inhibitors of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, which play a critical role in spontaneous rhythmic activity in the heart, have also been proposed to be suitable in reducing the heart rate and therefore beneficial for treatment of CHF. In the present study, we investigated the effect of carvedilol on HCN channels.

Experimental Approach
Whole cell patch clamp recordings were used to assess the effect of carvedilol on currents from wild-type (WT) and mutant HCN1, 2 and 4 channel expressed in Chinese hamster ovary (CHO) cells.

Key Results
Carvedilol is the only beta-blocker tested that showed inhibitory effects on the major sinoatrial isoform HCN4 channel in the present study. Carvedilol inhibited HCN4 in a concentration-dependent manner with an EC_{50} value of 4.4 µM. In addition, carvedilol also inhibited HCN1 and HCN2 channels. Carvedilol blocked HCN channels by decelerating the channel activation and hyperpolarizing shift the voltage-dependent of activation. Our data also showed carvedilol is not an ‘open channel’ inhibitor of HCN4, which is different from previously reported inhibitors, e.g., ivabradine or ZD7288.

Conclusion and Implications
Carvedilol is a negative gating modulator of HCN channels. It represents a novel structure for future drug design of HCN channel inhibitors.

Keywords
HCN, Carvedilol, inhibitor, heart failure, gating, pain
Abbreviations

CHF, chronic heart failure; CNBD, cyclic-nucleotide binding domain; HCN, hyperpolarization-activated cyclic nucleotide-gated channel; WT, wild type
Hyperpolarization-activated cyclic nucleotide-gated (HCN) channels play a central role for spontaneous rhythmic activity in both the heart and nervous system (Biel et al., 2009). The current mediated by HCN channels is called \( I_f \) or \( I_h \) in the sinoatrial node and neuronal cells, respectively. In the heart, \( I_f \) is a major determinant of cardiac diastolic depolarization and is crucial in controlling the heart rate. Four isoforms of HCN channels (HCN1-4) exist in mammals with different biophysical properties and diverse tissue expression (Gauss et al., 1998; Ludwig et al., 1998; Biel et al., 2009). Among them, HCN4 is the principle isoform of \( I_f \) while the expression of other isoforms is significantly weaker and species dependent (Shi et al., 1999; Fürst et al., 2015). In the nervous system, widely expressed HCN channels are involved in neuronal excitability and network activity been reported to be involved in several physio-pathological processes, including dendritic integration, synaptic transmission, spatial working memory, hydroelectrolyte homeostasis, epilepsy, inflammatory and neuropathic pain (Wang et al., 2007; Benarroch et al., 2013; Pires et al., 2016). Discovery of modulators targeting HCN channels may be an important strategy for treatment of CHF, arrhythmias, epilepsy and pain (Postea et al., 2011). One HCN channel blocker, ivabradine, has been approved by FDA to treat chronic heart failure in 2015 (U.S. Food and Drug Administration, 2015).

Topologically, HCN channels belong to the superfamily of voltage-gated potassium (Kv) and cyclic nucleotide-regulated (CNG) channels. Each subunit contains 6 transmembrane domains (S1–S6), a re-entrant loop between the S5–S6 helices that forms the selectivity filter and pore, and a cytosolic cyclic-nucleotide binding domain (CNBD) attached to S6 via an 80 amino acid C-linker (Zagotta et al., 2003; Xu et al., 2010; Cao et al., 2016). Characteristic electrophysiological properties of HCN channel include activation on hyperpolarization, conduction of both \( \text{Na}^+ \) and \( \text{K}^+ \), and inhibition by millimolar concentrations of external \( \text{Cs}^+ \) (Robinson et al., 2003; Biel et al., 2009). Another property of HCN channels is their direct modulation by cAMP / cGMP, which shifts the channels open probability towards more depolarized voltages and accelerates their activation speed (Gauss et al., 1998; Ludwig et al., 1999; Santoro et al., 1999).

Carvedilol (chemical structure in Figure 1A), a third generation beta-adrenoceptor blocker, is one of the most extensively studied and effective beta-blockers for preventing CHF. Several multi-centre, placebo-controlled clinical trials have shown that carvedilol could reduce the
risk of death and hospitalization from cardiovascular causes in patients with mild to moderate and severe CHF (Michael et al., 1996; Milton et al., 1996; Weir et al., 2005). Furthermore, another large randomized clinical trial, COMET, demonstrated carvedilol shows favourable benefits compared to metoprolol for CHF patients (Poole-Wilson et al., 2003; DiNicolantonio et al., 2012). The mechanism underlying the advantage of carvedilol over other beta-blockers is still not completely clear. In addition to blocking beta receptors, carvedilol also shows a broad range of activities, such as alpha-blockage and antioxidant properties (Stroe et al., 2004; Weir et al., 2005). It has been suggested that carvedilol’s non-selectivity and additional properties maybe important for treatment of CHF. Since HCN channels play a critical role in spontaneous rhythmic activity in the heart, the effect of carvedilol on HCN channels was investigated in our present study.

The effect of several broadly used beta-blockers (carvedilol, metoprolol, bisoprolol and labetalol) on HCN4 channel was examined and only carvedilol demonstrated an inhibitory effect on HCN4 currents. The action mechanism of action of carvedilol on HCN channels was further characterized in the present study.
Methods

Group size

The size (n) for each experimental group and the total number (N) of cells are provided in the figures and tables. The n refers to different transfections and at least two cells were recorded for each transfection. The N refers to the total number of cells for each experimental group. Data subjected to statistical analysis had an n of at least five for each group.

Randomization

Randomization was not performed as this is not a standard procedure in electrophysiologial recordings.

Blinding

Blinding of experiments is not applicable. However, when comparisons were made between WT and mutant channels, the transfection and electrophysiological recordings were conducted by different person. The electrophysiological recordings and data collection were accomplished by an operator who was blinded to the transfection.

Cell culture and Transfection

CHO cells were cultured in 50 / 50 Dulbecco’s modified Eagle’s medium / Ham’s F-12 (Invitrogen) supplemented with 10% foetal bovine serum (FBS) in 35 mm dishes and incubated at 37°C with 5% CO₂. At 80–90% confluence, plasmids expressing EGFP as a marker and the channel cDNAs of HCN1 (from human), HCN2 (from mouse and human) or HCN4 (from mouse and human) were cotransfected into the CHO cells using 10 µL Lipofectamine 2000 (Life Technologies) with a ratio of 1:9. Then, 24 hours after transfection, the cells were split by trypsin-EDTA and replated onto 10 mm coverslips coated with 0.1 mg ml⁻¹ poly-L-lysine (Sigma-Aldrich, St. Louis, MO) for electrophysiology experiments.

Mutagenesis

QuikChange (Agilent) was used to introduce mutations into mHCN2 or mHCN4. To delete the CNBD and extreme C terminus of mHCN4 (HCN4△CNBD), a stop codon was introduced after residue F603 (V604stop) as described earlier (Wainger et al., 2001). Eight mutants including four mHCN2 mutants (A425G, Y428A, F431A and I432A) and four mHCN4 mutants (Y506A, F509A, I510A and △CNBD) were constructed. All clones were
verified by further DNA sequencing of the complete ORF.

Electrophysiological recordings

HCN currents were recorded 24–48 hours after transfection by the whole-cell patch-clamp technique. Currents were measured with a MultiClamp 700B patch-clamp amplifier / Digidata 1550B digitizer and pClamp 10 software (Molecular Devices, Sunnyvale, CA). The sampling rate was 20 kHz and digitally filtered at 2 kHz. Series resistance compensation was set to 60%. The electrodes were pulled from borosilicate glass capillaries (BF150-110-10, Sutter Instruments) and had a resistance of 2–5 MΩ when filled with intracellular solution. The intracellular solution contained 145 mM KCl, 1 mM MgCl₂, 10 mM HEPES and 5 mM EGTA (pH adjusted to 7.2 with KOH). To evaluate the effect of cAMP on the WT and mutant HCN channels, 100 μM 8-Br-cAMP (Sigma-Aldrich, St. Louis, MO) was added to the intracellular solution. The extracellular solution was composed of 140 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1.5 mM MgCl₂, 10 mM HEPES and 10 mM glucose (pH adjusted to 7.4 with NaOH).

Carvedilol, ivabradine, metoprolol, bisoprolol and labetalol (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in dimethylsulfoxide (DMSO) to make 100 mM stock solutions that were stored at −20°C. On the day of the experiments, the stock solutions were diluted in extracellular solution to the final desired concentrations. During the experiments, a constant perfusion of bath solution (control or test solution) was delivered by a homemade perfusion device allowing rapid solution switches with a flow velocity approximately 2 ml min⁻¹.

Data analysis and statistics

The data and statistical analysis comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2015). Electrophysiological data were processed using Clampfit 10.6 (Molecular Devices, Sunnyvale, CA) and further analysed in GraphPad Prism 5 (GraphPad Software, San Diego, CA).

The activation (at −120 mV) and deactivation (at +50 mV) kinetics were fitted with single exponential equation using Clampfit 10.6. Voltage-dependent activation curves for HCN channels were fitted with the Boltzmann equation, \( G = G_{\text{max}} \times (G_{\text{max}} - G_{\text{min}}) / [1 + \exp (V - V_{0.5}) / S] \), where \( G_{\text{max}} \) is the maximum conductance, \( G_{\text{min}} \) is the minimum conductance, \( V_{0.5} \) is the half-activation voltage and \( S \) is the slope factor. In the fit, the top and the bottom of the...
curve was allowed to vary. Dose-response curves were fitted to the Hill equation, \( \Delta V_{0.5} = \Delta V_{0.5}^{max} + \Delta V_{0.5}^{max} / [1 + (C / EC_{50})^H] \), where \( \Delta V_{0.5} = V_{0.5}^{drug} - V_{0.5}^{control} \), \( EC_{50} \) corresponds to a drug concentration producing half of the maximum shift in \( V_{0.5} \), \( C \) corresponds to the drug concentration, and \( H \) is the Hill coefficient. Data are presented as the mean ± SEM of \( N \) (total number of cells). Differences between groups were compared by Student’s t-tests. Comparisons between three groups were performed by using one-way ANOVA. A \( P \) value < 0.05 was considered as statistically significant.

Nomenclature of Targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Harding et al., 2018), and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18 (Alexander et al., 2017).
Results

Inhibition of HCN4 channel by carvedilol

Given that HCN4 is the most abundant subunit expressed in the heart, we first investigated the action of several clinically broadly used and structurally diverse beta-blockers (chemical structures in Figure 1A) for their effects on mHCN4 channel. Among the beta-blockers tested in our system, only carvedilol displayed significant modulation of the mHCN4 currents at 10 µM (Figure 1B&C) or 30 µM (Figure S1A&B). Perfusion of 10 µM carvedilol reversibly suppressed the current by 44.6 ± 2.2% at −120 mV (Figure 1C). Figure 1D compared the mHCN4 currents elicited from −50 to −140 mV in the absence or presence of 10 µM carvedilol. Carvedilol demonstrated inhibitory effects on mHCN4 in three aspects. First, 10 µM carvedilol strongly left-shifted the voltage dependence of activation by roughly 27 mV (Figure 1E, Table 1), similar effect was observed for hHCN4 (Figure S2A&B). Second, the observed onset of blockage by carvedilol was rapid, reversible and not accompanied by any significant change in membrane capacitance (Figure 1F). Third, the compound decelerated the activation and accelerated the deactivation process dramatically. Channel activation is much slower in the presence of 10 µM carvedilol (Tau_{act} increased from 766.0 ± 58.9 ms to 2350.2 ± 397.1 ms with carvedilol) (Figure 1G). On the other hand, 10 µM carvedilol decreased the deactivation time constant of mHCN4 from 168.0 ± 15.9 ms to 58.3 ± 10.2 ms (Figure 1H). Furthermore, the dose response relationship showed that the EC_{50} of carvedilol was 4.4 ± 1.2 µM on the mHCN4 channel with a Hill coefficient of 3.2 ± 1.6 (Figure 1I, Table 2).

Effect of carvedilol on HCN4 channel is not dependent on either cAMP or the CNBD region

As described before, intracellular cAMP shifts the G-V curve of HCN channels to the depolarized direction and accelerates the activation of HCN channels, thereby promoting the activation of HCN channels. To further investigate whether the inhibition caused by carvedilol is relevant to alterations in endogenous cAMP concentrations or the CNBD region, we assessed the effect of carvedilol on the cAMP-insensitive HCN4 mutant, HCN4ΔCNBD channel (Wainger et al., 2001). Application of 100 µM 8-Br-cAMP via the patch pipette shifted the voltage-dependent activation curve of mHCN4 towards a more positive potential, which is similar to earlier reports (ΔV_{0.5} = 10.4 ± 1.8 mV, Figure 2A&B) (Ludwig et al., 1998). In contrast to the WT channel, no significant change was observed for
mHCN4ΔCNBD under the same condition ($\Delta V_{0.5} = 2.0 \text{ mV} \pm 1.5 \text{ mV}$, Figure 2A&B) (Wainger et al., 2001). Figure 2C compared HCN4ΔCNBD-mediated currents elicited from −50 to −140 mV in the absence or presence of 10 µM carvedilol. The 10 µM carvedilol strongly left-shifted the voltage dependence of the activation by approximately 40 mV on mHCN4ΔCNBD (Figure 2D). In addition, the drug also increased the activation time constant by 5-fold and decreased the deactivation time constant by 2.5-fold on mHCN4ΔCNBD (Figure 2E). We also examined the blockage effect of carvedilol on the mHCN4 WT channel when 100 µM 8-Br-cAMP was added into the intracellular solution. The $\Delta V_{0.5}$ was −28.2 mV (Figure 2F, S3) by 10 µM carvedilol when 100 µM 8-Br-cAMP was added intracellularly. The shift of $V_{0.5}$ was similar regardless of whether 8-Br-cAMP was included in the intracellular solution.

The above results demonstrated that the blockage by carvedilol on mHCN channels in the CHO cells was not relating to cellular cAMP concentration or the CNBD region of the channel and thus is not the result of its beta-blocking activity.

**Blockade of HCN1 and HCN2 by carvedilol**

To assess the potency and selectivity of carvedilol towards other HCN isoforms, we individually expressed hHCN1 and mHCN2 channel in CHO cells to evaluate their sensitivity to carvedilol. Figure 3A&D show the representative current traces of hHCN1 or mHCN2 in the absence and presence of 10 µM carvedilol. For hHCN1 channel, carvedilol left shifted the voltage dependence of activation by 24.4 mV (Figure 3B&G, Table 1). Besides, 10 µM carvedilol left shifted the voltage dependence of activation by 17.7 mV in mHCN2 (Figure 3E&G, Table 1), and a similar effect was observed in the hHCN2 (Figure S2C&D). Further dose-response curves analysis determined that the EC$_{50}$ of carvedilol was 7.9 ± 1.2 µM for hHCN1 (H = 2.1 ± 1.0) and 7.6 ± 1.1 µM for mHCN2 (H = 2.4 ± 0.7), respectively (Figure 3C&F, Table 2). Moreover, carvedilol caused longer activation and shorter deactivation processes on the hHCN1 and mHCN2 channel (Figure 3H&I), which were similar to the effect of carvedilol on mHCN4.

**Mechanistic characterization of carvedilol modulation on HCN4 channel**

Previous studies have shown that ivabradine is an ‘open-channel’ blocker of HCN4, which indicates that both the blockade by ivabradine and block removal requires channel opening (Bucchi et al., 2002; Bucchi et al., 2013). Next we examined whether or not carvedilol was
an ‘open-channel’ inhibitor of HCN4 channel. Figure 4A shows an experiment to test whether carvedilol could bind to mHCN4 channel when the channel was closed. When carvedilol was applied, the repetitive activation / deactivation protocol (−120 / +50 mV) was interrupted and the cell was held under −30 mV during which the channels were predominantly closed. The pulsing protocol was then resumed after 150 s perfusion of carvedilol. We found 10 µM carvedilol in the absence of mHCN4 activation produced a similar blockage extent (46.8 ± 4.5%) to that when the channels were activated every 15s throughout the carvedilol application shown in Figure 1 (44.6 ± 2.2 %).

On the other hand, we also examined whether the blockade effect of carvedilol could be removed when the channel was closed (Figure 4B). Following the steady suppression by carvedilol on mHCN4, the membrane voltage was held at −30 mV and the drug was washed off for 150 s. Then, the train stimulation was resumed and still in the absence of carvedilol. The current was almost immediately fully recovered under this condition, showing that block removal could occur under the closed-state channel conformation. In addition, we used a prolonged 50 s activation protocol and applied carvedilol under the steady-state condition, and the results indicated the blockade effect could not occur during steady state activation (Figure 4C). In contrast to carvedilol demonstrated an obvious inhibitory effect within 50 s of perfusion using the repetitive activation / deactivation protocol (Figure 1F), the current ratio before and after the perfusion of 10 µM carvedilol was nearly 1 as shown in Figure 4C (n=5). Overall, the above results indicate carvedilol does not preferentially bind to the open state of HCN channel, which is different from that of ivabradine (Bucchi et al., 2006).

Distinct interaction sites of carvedilol compared to ivabradine

We next set out to characterize the molecular mechanism by which carvedilol blocks HCN channels. We investigated whether carvedilol and ivabradine are competing for the same sites to suppress HCN currents.

Previous studies have identified several residues important for conferring sensitivity to ivabradine or ZD7288 in HCN4 and HCN2 subtypes (Cheng et al., 2007; Bucchi et al., 2013). The identified amino acids for ivabradine or ZD7288 sensitivity are mainly located in the pore region (S5-P-S6 region): Y506, F509 and I510 in mHCN4 for ivabradine and A425 and I432 in mHCN2 for ZD7288 (Figure 5A). Given that none of the constructs produced a large enough currents when mutated in mHCN4 channel in our experiment system, we systematically mutated the corresponding residues in mHCN2 channel (A425G, Y428A,
F431A and I432A) and compared the sensitivity of carvedilol on WT and mutant channels. Among the four mutant channels, Y428A and I432A could not generate a large enough currents to evaluate the inhibitory effect of carvedilol. Therefore, we compared carvedilol’s action on WT, A425G, and F431A mHCN2 channels. The mutation of mHCN4 F509A (corresponding to mHCN2 F431A) could dramatically reduce the effect of ivabradine compared to the WT channel (Cheng et al., 2007). Another study showed that the sensitivity of ZD7288 on HCN2 was much less than A425G compared to the WT channel (Bucchi et al., 2013). In contrast to these results, our study showed carvedilol exerted similar inhibitory effects on the WT and mutant channels by current reduction at −120 mV (Figure 5B&C) and strongly left-shifted the voltage dependence of activation by approximately 20 mV on the mutant channels (Figure 5E&F), a similar extent to that observed for WT channel (Figure 5D&G). These observations indicate that carvedilol may act by a different molecular mechanism compared to ivabradine / ZD7288 on HCN channels.
Discussion

Carvedilol blocks HCN channels

We showed here that carvedilol blocks HCN channels in a concentration-dependent manner with an EC$_{50}$ value of 4.4 µM on the major sinoatrial isoform HCN4. In addition, carvedilol also inhibited HCN1 and HCN2 currents. The effect of carvedilol on $I_f$ in the rabbit sinoatrial node was investigated previously and an inhibition of $I_f$ was noted at micromolar levels of carvedilol (Yokoyama et al., 2007), but the characteristics and biophysical mechanism of action were not studied there. We analysed the biophysical mechanism of HCN inhibition by carvedilol in detail in our study. A rapid onset and reversible block supported the hypothesis that carvedilol is a direct channel inhibitor rather than acting via modulating protein trafficking or intracellular signalling pathways. Major effects caused by carvedilol include strong modulation of channel voltage-dependence and activation kinetics. For HCN4 channel, 10 µM carvedilol decelerated the activation speed by 3-fold at $-120$ mV and left shifted the $V_{0.5}$ approximately 27 mV. Our study demonstrated that blockage of carvedilol on HCN channels in CHO cells is irrelevant of its beta-receptor blocking property given that carvedilol still exerted dramatic inhibitory effects on cAMP-insensitive HCN4 mutant channels. In addition, blockage by carvedilol’s blockage of HCN4 was also not affected by a saturated concentration of intracellular cAMP.

Recent progress of HCN channels structure-function study

As mentioned before, the unique biophysical properties of HCN channels have strongly attracted the interest of researchers. In most Kv channels, the voltage sensors are “swapped”, which indicates each voltage sensor contacts the pore through amino acids from a neighbouring subunit (Catterall et al., 2010; Bezanilla et al., 2000). The domain-swapped voltage sensors and the long α-helical S4–S5 linker in most Kv channels likely functions as a mechanical lever through which voltage-sensor conformational changes impart a force to open or close the pore’s gate. The cryo-EM structures of human HCN1 revealed that the voltage sensors are “non-swapped” and the S4–S5 linker is not α-helical and much shorter in HCN1 (Lee et al., 2017). Thus, instead of using the S4–S5 linker as a mechanical lever, it is proposed that HCN channels utilize the long S4 helix which is unusual, for coupling to the pore to mediate the reversed polarity gating. HCN channels share the glycine-tyrosine-glycine (GYG) motif, which is a signature selectivity filter for K$^+$ channels.
However, HCN channels are non-selective monovalent cation channels, permitting K\(^+\) over Na\(^+\) at a ratio of ~4:1 (Ludwig et al., 1998). High potassium selectivity in K\(^+\) channels comes from the precise geometry of the selectivity with four ion binding sites (Zhou et al., 2001; Hille., 2018). Interestingly, the first two ion binding sites are absent in HCN1 as revealed by the cryo-EM structure which leads to the non-selectivity property of the channel (Lee et al., 2017). Four isoforms of HCN channels (HCN1–4) differ in their voltage-dependent gating properties (Biel et al., 2009; Cao et al., 2016). Among them, HCN1 channel has the most positive \(V_{0.5}\) for activation and the fastest activation kinetics, while HCN4 channel is the slowest voltage-gated isoform (Ludwig et al., 1998; Biel et al., 2009). In addition, HCN2 and HCN3 subunits have intermediate biophysical properties compared to other subtypes (Ludwig et al., 1998; Biel et al., 2009). Differences in the extent of cAMP modulation between the HCN1 and HCN2 isoforms result largely from differences in the efficacy of CNBD inhibition from the C-terminus (Wainger et al., 2001). In addition, regions in S1, S1–S2 loop and S2 have been reported to play key roles in different channel activation kinetics (Ishii et al., 2001; Stieber et al., 2003; Ishii et al., 2007). The cryo-EM structure and extensive biophysical studies have provided a framework to investigate the molecular mechanism underlying HCN channels and inhibitors interaction in the future.

**Carvedilol and previously reported HCN channel blockers**

Two widely studied HCN blockers are ivabradine and ZD7288 (Sartiani et al., 2017). Ivabradine was approved by FDA for the treatment of CHF in 2015 (U.S. Food and Drug Administration, 2015). It is used in combination with beta-blockers in people with CHF with a left ventricular ejection fraction lower than 35 percent inadequately controlled by beta-blockers alone and whose heart rate exceeds 70 beats per minute (Müller-Werdan et al., 2016). In patients not sufficiently managed with beta-blockers for their heart failure adding ivabradine decreases the risk of hospitalization due to worsening CHF (McMurray et al., 2012; Tse et al., 2015). In addition to ivabradine, several other ‘bradine’ compounds also demonstrate inhibitory effects on HCN channels, including cilobradine and zatebradine (Postea et al., 2011). Neither ivabradine nor ZD7288 shows subtype selectivity among HCN1–4 channels (Stieber et al., 2006; Sartiani et al., 2017). Furthermore, several blockers with high subtype selectivity have been found based on structure modification of known nonselectivity inhibitors or high throughput screening (Melchiorre et al., 2010; Del et al., 2012). Extensive studies have demonstrated that ivabradine blocks HCN channels from the...
intracellular side, and it is an ‘open-channel’ inhibitor since it can only occur after channel opening (Bucchi et al., 2002; Bucchi et al., 2013). For ZD7288, it has been shown that ZD7288 blocks HCN1 channel only after they were opened by hyperpolarization; however, further hyperpolarization reduced the blockade sharply (Shin et al., 2001). As mentioned before, several residues near the intracellular activation gate were then identified to be critical for the action of ivabradine / ZD7288 on HCN channels (Bucchi et al., 2013; Cheng et al., 2007).

Our study shows that the action of carvedilol on HCN channels is distinct from ivabradine or ZD7288. The similar sensitivity of WT and mutants (A425G and F431A) HCN2 channel by carvedilol indicates carvedilol interacts with distinct regions compared to ivabradine / ZD7288. Based on the dramatic modulation of voltage-dependent gating and activation / deactivation kinetics by carvedilol, we propose here that the compound is a negative gating modulator rather than a simple pore blocker of HCN channels. In addition, as a gating modifier, carvedilol also reduces the maximal open probability of HCN channels to different extents depending on the subtypes. Niflumic acid has also been reported to act as a negative gating modifier of HCN channels (Accili et al., 1996; Satoh et al., 2001). Cheng L et al. (Cheng et al., 2009) found that niflumic acid inhibited HCN2 channel by interaction with the outer region of the S4 voltage sensing domain. Taken together, future thorough mutational analysis throughout the voltage sensor domains (mainly S1–S2 and S4) is needed to identify the putative binding sites of carvedilol to HCN channels.

**Clinical implications**

CHF is a major public health concern associated with a high prevalence and poor clinical outcomes (Yancy et al., 2013). In the US, CHF is the leading cause of hospitalization among adults older than 65 years of age (Desai et al., 2012). Heart rate increasing is a risk factor for heart failure and a reduction of the increased basal heart rate can benefit patients and prolong their lifespan (Benetos et al., 1999; Perret-Guillaume et al., 2009). Beta-blockers can obviously reduce heart rate, but their use is limited by adverse effects, including negative inotropic and blood pressure lowering effects (Doesch et al., 2007). In addition to carvedilol, we tested 3 other known beta-blockers (metoprolol, bisoprolol and labetalol) on HCN4 channel, and found only carvedilol effectively inhibited HCN4 channel. During therapeutic application of carvedilol, plasma concentrations of the drug are between 0.1 and 0.6 µM (Karle et al., 2001), while the concentrations of carvedilol in the heart are five to seven-fold
higher than in the plasma due to its high degree of lipophilicity and the large volume of distribution of the drug (Fujimaki et al., 1992; Stahl et al., 1993; Caron et al., 1999; Karle et al., 2001). This suggests a possible therapeutic relevance of HCN currents blockade by carvedilol.

In conclusion, our study demonstrated carvedilol is a negative gating modulator of HCN channels. It may represent a novel structure for future drug design of HCN channel inhibitors.

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**Figure 1.** The beta-blocker carvedilol inhibits HCN4. (A) Structures of carvedilol, metoprolol, bisoprolol and labetalol. (B) Examples of the mHCN4 currents in the absence or presence of the tested compound (10 µM). Currents were elicited by a 2 s hyperpolarizing step to −120 mV from a holding potential of −40 mV, tail currents were measured at +50 mV and the protocol was applied every 15 s. The activation (at −120 mV) and deactivation (at +50 mV) kinetics were fitted with single exponential equations and labeled in red. (C) Inhibitory effects of tested beta-blockers (carvedilol, metoprolol, bisoprolol and labetalol) at −120 mV on mHCN4 channel. (D) Representative current traces of mHCN4 in the absence (upper panel) or presence (bottom panel) of 10 µM carvedilol. Currents were evoked by a series of hyperpolarizing steps ranging from −50 mV to −140 mV from a holding potential of...
−40 mV. Voltage was then returned to +50 mV to measure the tail current. (E) Voltage-dependent activation curves of mHCN4 determined in control or 10 µM carvedilol conditions. (F) Time course of mHCN4 relative current at −120 mV with the perfusion of 10 µM carvedilol is indicated by the bar above. (G&H) Activation time constant ($\tau_{\text{act}}$) at −120 mV (G) and deactivation time constant ($\tau_{\text{deact}}$) at +50 mV (H) for mHCN4 in the absence or presence of 10 µM carvedilol as indicated (*$P<0.05$ vs. control). (I) Dose response curve of carvedilol on mHCN4 channel. The dose response curve was obtained by the Hill equation, $\Delta V_{0.5} = \Delta V_{0.5 \text{ max}} + \Delta V_{0.5 \text{ max}} / [1 + (C / E\text{C}_{50})^h]$, where $\Delta V_{0.5}$ values represent shifts of the activation curve with different concentrations of carvedilol (1, 3, 5, 10, 30 and 50 µM). The number of independent experiments / total number of cells (n/N) are indicated in the parentheses at the top of the graphs.
Figure 2. Effect of carvedilol on HCN4 does not involve with the CNBD region or cAMP.

(A) Voltage activation curves of mHCN4 (left) and mHCN4ΔCNBD (right) in the absence or presence of 100 µM 8-Br-cAMP. (B) V_{0.5} of mHCN4 and mHCN4ΔCNBD in the absence or presence of 100 µM 8-Br-cAMP. (C) Representative current traces of mHCN4ΔCNBD before (upper panel) or after (bottom panel) 10 µM carvedilol application. (D) Voltage activation curves of mHCN4ΔCNBD in the absence or presence of 10 µM carvedilol as indicated. (E) Summaries of the activation time constant (τ_{act}) at −120 mV and deactivation time constant (τ_{deact}) at +50 mV for mHCN4ΔCNBD in the absence or presence of 10 µM carvedilol.
carvedilol as indicated. (F) ΔV0.5 of mHCN4 in the presence of 10 µM carvedilol without or with 100 µM 8-Br-cAMP in intracellular solution as indicated. The number of independent experiments / total number of cells (n/N) are indicated in parentheses at the top of graphs. *P<0.05 vs. control.
Figure 3. Subtype selectivity of carvedilol. (A&D) Whole cell currents of hHCN1 (A) and mHCN2 (D) were recorded in the absence (upper panel) or presence (bottom panel) of 10 µM carvedilol. (B&E) Voltage dependent activation curves of hHCN1 (B) and mHCN2 (E) in the absence or presence of 10 µM carvedilol as indicated. (C&F) Dose response curves of carvedilol on the hHCN1 (C) and mHCN2 (F) channel obtained as in Figure 1I. (G) Bar graphs plotting the V_0.5 of hHCN1 and mHCN2 in the absence or presence of 10 µM carvedilol. (H&I) Activation time constant (τ_{act}) at −120 mV (H) and deactivation time constant (τ_{deact}) at +50 mV (I) for hHCN1 and mHCN2 as indicated. The number of independent experiments / total number of cells (n/N) are indicated in parentheses at the top of graphs. *P<0.05 vs. control
Figure 4. Biophysical characterization of carvedilol blockage on mHCN4. (A) Time course of relative mHCN4 current at −120 mV during an activation/deactivation protocol (−120 mV/+50 mV) from a holding potential of −40 mV. At the beginning of the perfusion with 10 µM carvedilol, current stimulation was stopped by holding the membrane voltage at −30 mV for 150 s. (B) The same activation/deactivation protocol was applied in the presence of 10 µM carvedilol until reaching a stable state inhibition. The cell was held at −30 mV and carvedilol was washed off for 150 s. Then, the train stimulation was resumed in the absence of carvedilol. (C) Action of carvedilol on mHCN4 channel when carvedilol was applied during steady-state activation. 10 µM carvedilol was applied for 30 s and the mHCN4 currents were recorded at −120 mV. Similar experiments were repeated at least 5 times.
Figure 5. HCN4 blockage by carvedilol depends on distinct interaction sites compared to ivabradine. (A) Amino acid sequence alignments of the S6 domain between of mHCN2 and mHCN4 channel. The reported amino acids responsible for ZD7288 or ivabradine sensitivity on mHCN2 and mHCN4 channels, respectively, are labelled red. (B) Examples of
current traces of WT, A425G and F431A mHCN2 channel in the absence or presence of 30 µM carvedilol. (C) The summarized effects of 30 µM carvedilol on WT and mutant mHCN2 channels as indicated. (D&E&F) Voltage dependent activation curves of WT (D), A425G (E) and F431A mHCN2 (F) in the absence or presence of 30 µM carvedilol as indicated. (G) ΔV_{0.5} of WT, A425G and F431A mHCN2 in the presence of 30 µM carvedilol compared to the control condition. The number of independent experiments / total number of cells (n/N) are indicated in parentheses at the top of graphs.
Table 1. $V_{0.5}$ and slope factors of HCN channels in the absence or presence of 10 µM carvedilol

<table>
<thead>
<tr>
<th>Channel</th>
<th>Control</th>
<th>10 µM carvedilol</th>
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<tr>
<td></td>
<td>$V_{0.5}$ (mV)</td>
<td>Slope factor</td>
<td>$V_{0.5}$ (mV)</td>
<td>Slope factor</td>
<td>$\triangle V_{0.5}$ (mV)</td>
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<tr>
<td>hHCN1</td>
<td>-67.7 ± 0.4</td>
<td>6.3 ± 0.4</td>
<td>-93.6 ± 1.1</td>
<td>8.3 ± 1.0</td>
<td>-24.4 ± 1.8</td>
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<tr>
<td>mHCN2</td>
<td>-97.1 ± 0.5</td>
<td>6.2 ± 0.5</td>
<td>-115.8 ± 1.4</td>
<td>7.5 ± 1.1</td>
<td>-17.7 ± 0.7</td>
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<tr>
<td>mHCN4</td>
<td>-104.0 ± 0.9</td>
<td>8.7 ± 0.8</td>
<td>-128.8 ± 6.2</td>
<td>10.9 ± 2.8</td>
<td>-26.8 ± 1.8</td>
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Table 2. EC$_{50}$ value of carvedilol on HCN channel

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<th>Subtype</th>
<th>EC$_{50}$ (µM)</th>
<th>Hill coefficient</th>
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<td>2.1 ± 1.0</td>
</tr>
<tr>
<td>mHCN2</td>
<td>7.6 ± 1.1</td>
<td>2.4 ± 0.7</td>
</tr>
<tr>
<td>mHCN4</td>
<td>4.4 ± 1.2</td>
<td>3.2 ± 1.6</td>
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