Characteristic Effects of β1-β1,2-Adrenergic Blocking Agent, Carvedilol, on [Ca²⁺]i in Ventricular Myocytes Compared With Those of Timolol and Atenolol

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Beta-adrenergic stimulation and the resultant Ca²⁺ load seem both to be associated with progression of heart failure as well as hypertrophy. Because the β₁, β₁,2-blocker, carvedilol, has been shown to be outstandingly beneficial in the treatment of heart failure, its direct effects on intracellular calcium ion concentration ([Ca²⁺]i), including antagonism to isoproterenol, in ventricular myocytes were investigated and then compared with a selective β₁-blocker, atenolol, and a non-selective β₁,2-blocker, timolol. At 1–300 nmol/L, carvedilol decreased the amplitude of [Ca²⁺]i by ~20% independently of its concentration, which was a similar effect to timolol. All the β-blockers at 10 nmol/L decreased the amount of cAMP, but atenolol had the least effect. Carvedilol in the 100 nmol/L order further diminished the amplitude of [Ca²⁺]i transients, and at 1000 nmol/L increased the voltage threshold for pacing myocytes. These effects were not observed with timolol or atenolol. L-type Ca²⁺ currents (I_Ca) were decreased by carvedilol in the 100 nmol/L order in a concentration dependent manner. As for the β-agonist effect, the concentrations of carvedilol, timolol, and atenolol needed to prevent the effect of isoproterenol by 50% (IC₅₀) were 1.32, 2.01, and 612 nmol/L, respectively. Furthermore, the antagonizing effect of carvedilol was dramatically sustained even after removal of the drug from the perfusate. Carvedilol exerts negative effects on [Ca²⁺]i, including inhibition of the intrinsic β-activity, reduction of I_Ca in the 1000 nmol/L order, and an increase in the threshold for pacing at ≥10 μmol/L. Data on the IC₅₀ for the isoproterenol effect suggest that carvedilol could effectively inhibit the [Ca²⁺]i load induced by catecholamines under clinical conditions. (Circ J 2003; 67: 83–90)

Key Words: β₁-blocker; Carvedilol; Calcium; Ion channels; Myocytes

Carvedilol is an β₁ and β₁,2-adrenergic blocking agent with other effects, such as a scavenger for oxidation and an inhibitor of apoptosis and has been shown to improve the prognosis of patients with congestive heart failure. Although the mechanism is not fully elucidated, some other β-blocking agents have been reported to have beneficial effects on heart failure suggesting that continuous β₁-adrenergic stimulation progresses heart failure. Carvedilol is also known to directly inhibit L-type calcium current (I_Ca) similar to Ca²⁺ antagonists, and diminishes the influx of Ca²⁺ in ventricular myocytes as well as vascular smooth muscle cells in concentrations of the 1,000 nmol/L order. However, most calcium antagonists have failed to decrease the mortality of patients with heart failure. In addition, the physiological blood concentration of carvedilol has been reported to be 100–600 nmol/L after a single oral dose or daily doses of 12.5, 25, or 50 mg. Because most (~98%) of carvedilol in the blood is thought to bind to albumin, the inhibitory effect of carvedilol on I_Ca may not be of clinical significance. The beneficial effects of carvedilol on heart failure, therefore, are most probably exerted through antagonizing β-agonists independently of blocking the L-type Ca²⁺ channel, although other effects of carvedilol such as antioxidation and anti-apoptosis might be involved.

In ventricular myocytes, β₁,2-receptors couple with the Gs protein complex, which is a positive modulator of adenyl cyclase, and directly increases I_Ca. Once β-agonists are bound to β₁,2-receptors, adenyl cyclase is activated to produce more cAMP, leading to the activation of protein kinase A (PKA). Activated PKA subsequently phosphorylates the L-type Ca²⁺ channel and phospholamban, resulting in the augmentation of I_Ca and the Ca²⁺ uptake rate of the sarcoplasmic reticulum (SR) Ca²⁺-ATPase, respectively, both of which cooperatively contribute to an increase in the SR Ca²⁺ content and enhancement of Ca²⁺ induced Ca²⁺ release from the SR (CICR). In this way, β-agonists augment the amplitude of intracellular calcium ion concentration ([Ca²⁺]i) transients, mostly through the PKA cascade as well as through direct interaction of the Gs protein with the L-type Ca²⁺ channel. Recently we have found that a β-agonist, isoproterenol, induces hypertrophic
responses, such as the activation of MAP kinase and calcineurin, through a Ca\(^{2+}\)-dependent pathway in cultured neonatal rat ventricular myocytes. Therefore, the catecholamine-induced Ca\(^{2+}\) load could be associated with hypertrophy, and in this respect, the Ca\(^{2+}\) load induced by catecholamines could be regarded as a Ca\(^{2+}\) overload-condition such as ischemia–reperfusion,20 cardiac glycoside treatment21 and so on. On the other hand, Marx et al have recently reported that hyperphosphorylation of FKBP12.6 by PKA is pathophysiologically important for aspects of heart failure such as decreased ventricular function and arrhythmia.22 In this respect, we propose the importance of the catecholamine-induced [Ca\(^{2+}\)]\(_i\) load and PKA activation for hypertrophic responses and the pathophysiology of heart failure, respectively, although it is not clear that the transition from hypertrophy to heart failure is associated with Ca\(^{2+}\) load. [Ca\(^{2+}\)]\(_i\) transients are not only direct parameters for [Ca\(^{2+}\)]\(_i\), but also can be used as a very sensitive indicator for the activity of PKA. Thus, it is suitable to utilize [Ca\(^{2+}\)]\(_i\) transients as a parameter in order to evaluate the effects of \(\beta\)-blockers.

The purposes of the present study were to elucidate whether carvedilol directly influenced [Ca\(^{2+}\)]\(_i\) cycling in beating ventricular myocytes by itself, and how much carvedilol antagonized a \(\beta\)-agonist, isoproterenol, in terms of [Ca\(^{2+}\)]\(_i\), as well as evaluating the clinical importance of these effects of carvedilol. These effects of carvedilol were also compared with those of a selective \(\beta_1\)-blocker, atenolol, and a non-selective \(\beta_1,2\)-blocker, timolol.

**Methods**

**Ventricular Myocyte Isolation**

The investigation conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). We isolated ventricular myocytes from hearts of male guinea pigs (300–500 g) as described previously.23 In brief, hearts were dissected from anesthetized animals and rapidly attached to a Langendorff perfusion system. All perfusion solutions were maintained at 37°C and pH 7.4. After perfusion with 0 mmol/L Ca\(^{2+}\) solution consisting of (mmol/L) 126 NaCl, 4.4 KCl, 1.0 MgCl\(_2\), 2.5 g/L taurine, 0.65 g/L creatine monophosphate, 0.55 g/L sodium pyruvate, 0.14 g/L NaH\(_2\)PO\(_4\), and 2 g/L glucose, the hearts were subsequently digested with 100 \(\mu\)mol/L Ca\(^{2+}\) solution containing 100 mg/dl of type II collagenase (Worthington Biochemicals, Freehold, NJ, USA) and 10 mg/dl of protease (Sigma Chemical Co, St Louis, MO, USA) for 8–12 min. Then, after the hearts were washed with 100 \(\mu\)mol/L Ca\(^{2+}\) solution containing 100 mg/dl of type II collagenase ( Worthington Biochemicals, Freehold, NJ, USA) and 10 mg/dl of protease (Sigma Chemical Co, St Louis, MO, USA), for 8–12 min. Then, after the hearts were washed with 100 \(\mu\)mol/L Ca\(^{2+}\) solution without the enzymes, both ventricles were excised, minced and shaken gently in 100 \(\mu\)mol/L Ca\(^{2+}\) solution. The cell suspension was filtered through a fine metallic tea filter. Isolated myocytes were kept in 1 mmol/L Ca\(^{2+}\) solution at room temperature, and were used within 6 h after the isolation procedure.

**Measurements of [Ca\(^{2+}\)]\(_i\) Transients**

[Ca\(^{2+}\)]\(_i\) was measured with a method described previously.23 Briefly, isolated myocytes were incubated for 30 min in 3–4 \(\mu\)mol/L fluo-3 AM containing normal HEPES, which consists of (mmol/L) 126 NaCl, 4.4 KCl, 1.0 MgCl\(_2\), 1.08

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**Fig 1.** Effects of carvedilol on [Ca\(^{2+}\)]\(_i\) in ventricular myocytes. (A) Carvedilol of 1 nmol/L decreased [Ca\(^{2+}\)]\(_i\) amplitudes and cell shortenings. (B) In a 10-\(\mu\)mol/L carvedilol solution, myocytes lost their responsiveness to field stimulation, which was rescued to some extent by increasing the pacing voltage or by wash-out of carvedilol. (C) Carvedilol (\(\bullet\)), timolol (\(\triangle\)) and atenolol (\(\Delta\)) all decreased [Ca\(^{2+}\)]\(_i\) amplitudes at tested concentrations, but carvedilol at \(\geq\)1 \(\mu\)mol/L further decreased [Ca\(^{2+}\)]\(_i\) amplitudes, which was divergent from the effects of the other two drugs. The effect of 10 \(\mu\)mol/L carvedilol was 100%, indicating the loss of responsiveness to field stimulation. Each data point is expressed as mean \(\pm\) SEM, consisting of 4–6 recordings.
CaCl₂, 13 NaOH, 11 glucose, 24 HEPES (pH 7.4 at 25°C). After washing in dye-free normal HEPES solution for more than 15 min, a single cell was isolated in the field to be excited by 485-nm light, and the emission of 530 nm was collected. The intensity of 530-nm fluorescence increases as [Ca²⁺]i increases. The flow rate of the perfusate was ~2.0 ml/min and the volume of the solution in the chamber was kept constant (~1–1.5 ml) during the experiments. Measurements were performed at room temperature (24–26°C).

Cell Shortening Measurement

Ventricular myocytes were electrically stimulated by a platinum electrode with a threshold voltage of 4 ms duration every 4 s (0.25 Hz). Both longitudinal edges of the myocytes were continuously monitored with a video motion detector, and cell shortening was measured as described previously.23 In some experiments, [Ca²⁺]i and cell shortening were simultaneously recorded.

I_Ca Measurement

I_Ca was recorded with a whole cell voltage clamp method. Ventricular myocytes were dialyzed with a microelectrode (1.5–2.5 Ω) filled with a pipette solution containing (mmol/L) 130 CsCl, 10 NaCl, 0.5 MgCl₂, 5 K₂ATP, 5.5 glucose, 10 HEPES, and 10 EGTA, which was titrated to pH 7.2 with KOH. I_Ca was activated with a voltage step to 0 mV for 400 ms from −40 mV of 100-ms duration following linear ramp step for 1 s from a holding potential of −80 mV (Fig 2A). Measurements of I_Ca were performed in normal HEPES solution at room temperature every 2 min after an initial 5-min dialysis of the cytosol with the pipette solution. To prevent the run-down phenomenon, myocytes were voltage-clamped at −80 mV between the measurements. The flow rate of the perfusate was ~0.5 ml/min, and the volume of the solution in the chamber was kept constant (~1–1.5 ml) during the experiments.

Assessment of β-Blocking Effects

Because carvedilol is lipophilic and its effect is difficult to wash out (as shown in Fig 5B), we first defined the expected amplitude of [Ca²⁺]i transients stimulated by 1 μmol/L isoproterenol with 10 records. Briefly, a fluo-3-loaded myocyte was paced for 2 min until stabilized, and then the [Ca²⁺]i transient was recorded as control data.

Consecutively, the myocyte was treated with isoproterenol for 5 min, and then the [Ca²⁺]i transient was recorded. The amplitude of the [Ca²⁺]i transient treated with isoproterenol was expressed as normalized to that of the control. The mean value of the isoproterenol-treated [Ca²⁺]i transients was 263±27% (n=10), which was defined as the expected amplitude. Assuming that without the β-blocking effect the amplitude of the [Ca²⁺]i transients should reach 263% of the control amplitudes, we then defined the formula to calculate the inhibition rate:

\[ \% \text{ inhibition} = 100 \times \frac{263-b}{263-a} \]

where a and b are, respectively, the amplitude when a myocyte is pretreated with a β-blocker and that when the myocyte is consecutively treated with the β-blocker and 1 μmol/L isoproterenol. Each [Ca²⁺]i amplitude in this formula was expressed normalized to the control [Ca²⁺]i amplitude.

Cyclic AMP (cAMP) Measurements

After the myocytes were isolated, an equal amount of cell suspension in normal HEPES solution was poured into laminin-coated dishes of 60 mm diameter. After the cells in the dishes were treated with drugs according to the protocols, the solution was vacuumed and cells were then harvested with 500 μl of the cell lysis buffer 1B included in the cAMP assay kit (BIOTRACK™, Amersham Pharmacia Biotech UK Ltd). The amount of cAMP was measured according to the manufacturer’s protocol. The amount of protein was also measured with a modified Bradford method using the dye reagent from the BIO-RAD Protein Assay (BIO-RAD Laboratories, CA, USA). The relative amount of cAMP was calculated by dividing the concentration of cAMP by that of protein, and normalizing it to the control value in each series of the experiment. Experiments were performed at room temperature.

Drug Preparation

If a drug could not be dissolved in water, it was first
dissolved in a suitable solvent at a concentration 10,000 times higher than that for use. Then the drug in the solvent was diluted with the perfusate. The solvent was dimethyl sulfoxide for carvedilol and forskolin, methanol for atenolol, and water for the others.

**Data Acquisition and Statistics**
Voltage data converted from original current signals, fluorescence signals, cell shortening and pacing signals were digitized with a 12-bit A/D converter (Digidata 1200) and then stored on personal computers using the software of P-clamp 6.0 or Axo scope 1.1 (Axon Instruments, Burlingame, CA, USA), which was also used to analyze the data. Comparison was performed with unpaired t-test, or paired t-test if data were obtained from the same cell. p<0.05 was considered statistically significant.

**Results**

**Direct Effects of Carvedilol on \([\text{Ca}^{2+}]_i\)**
We first examined the direct effects of carvedilol on \([\text{Ca}^{2+}]_i\) in ventricular myocytes and found that the amplitude of \([\text{Ca}^{2+}]_i\) was attenuated by carvedilol of 1 nmol/L, which was accompanied by decreased cell shortening (Fig 1A). Furthermore, in a 10 \(\mu\)mol/L carvedilol solution, myocytes reversibly lost their responsiveness to field stimulation (Fig 1B) and this loss of responsiveness to field stimulation was overcome by increased voltage for field stimulation. These results suggest that carvedilol has a direct negative inotropic effect on ventricular myocytes by lowering peak \([\text{Ca}^{2+}]_i\). The mean data on the \([\text{Ca}^{2+}]_i\) lowering effects of carvedilol, timolol, and atenolol are summarized in Fig 1C, showing that all of the \(\beta\)-blockers can be thought to exert negative inotropic action by diminishing the peak of \([\text{Ca}^{2+}]_i\) transients. At a concentration \(\geq 1 \mu\text{mol/L}\), carvedilol further diminished the \([\text{Ca}^{2+}]_i\) amplitude, which contrasted with the effects of timolol and atenolol. Timolol and atenolol showed neither apparent concentration-dependent attenuation of the \([\text{Ca}^{2+}]_i\) amplitude nor induced pacing failure in myocytes at tested concentrations.

**Effects on ICa**
The results for \([\text{Ca}^{2+}]_i\) just described implied that carvedilol at concentrations of the \(\mu\text{mol/L}\) order may directly decrease ICa, facilitating the decrease in \([\text{Ca}^{2+}]_i\) amplitude, so we assessed the direct effect of carvedilol on ICa. We first confirmed that ICa was stable for 5–15 min after starting the dialysis of the cytosol with the pipette solution and as shown in Fig 2A, carvedilol at 10 \(\mu\text{mol/L}\) markedly decreased ICa. Carvedilol at \(\geq 5 \mu\text{mol/L}\) directly and signifi-
cantly decreased I\(_c\) in a concentration-dependent manner (Fig 2B). From these data, it can be deduced that the [Ca\(^{2+}\)]-lowering effect of carvedilol at \(\leq 300\) nmol/L is not the result of a reduction of I\(_c\). Because 10 nmol/L carvedilol suppressed the I\(_c\) by approximately 52%, the pacing failure in a 10 \(\mu\)mol/L carvedilol solution can not be fully explained by the reduced I\(_c\).

**Alteration of Intrinsic \(\beta\)-Activity by Carvedilol**

To further investigate the mechanism of the direct negative inotropy by carvedilol, as well as that of timolol and atenolol, we performed an additional experiment. Earlier reports\(^{24,25}\) have suggested that some \(\beta\)-receptors transmit to the active form spontaneously and in a self-acting way, and that some \(\beta\)-blockers inhibit this transmission, resulting in decreased production of cAMP (inverse agonism). In this regard, we measured the amount of cAMP in myocytes treated with each \(\beta\)-blocker for 30 min. As shown in Fig 3, the amount of cAMP was significantly decreased by 10 nmol/L of carvedilol, timolol, and atenolol, although the effect of atenolol was relatively weak. Therefore, occupation of the \(\beta\)-receptors by the blockers seems to lead to attenuation of the intrinsic PKA activity, which consequently decreases the [Ca\(^{2+}\)] amplitude. These results suggest that the direct negative inotropic action by carvedilol at \(\leq 300\) nmol/L is at least in part associated with a decrease in the amount of cAMP, which is probably a result of a decrease in the intrinsic \(\beta\)-activity.

**Beta-Antagonism of Carvedilol**

We then examined the antagonism of carvedilol to isoproterenol and compared its effect with that of timolol and atenolol. First, we examined by how much 1 \(\mu\)mol/L isoproterenol increased the amplitude of [Ca\(^{2+}\)] transients and found that it markedly increased the amplitude, with after-peak humps seen in most cases (Fig 4A). On the other hand, a selective \(\beta\)-stimulator, zinterol, did not appear to affect the [Ca\(^{2+}\)] transients (Fig 4B), suggesting that the effect of isoproterenol is likely \(\beta\)-dependent. Next, after the [Ca\(^{2+}\)] transients were stabilized, myocytes were pretreated with one of the \(\beta\)-blockers for 2 min and subsequently treated with 1 \(\mu\)mol/L isoproterenol for 5 min in the presence of the \(\beta\)-blocker. We found that 10 nmol/L carvedilol effectively inhibited the [Ca\(^{2+}\)] rise, and always prevented the after-peak hump, which was observed frequently with 10 nmol/L timolol (Fig 5A). Among the tested concentrations, the minimal concentration to always eliminate the after-peak hump was 10 nmol/L, 300 nmol/L, and 10 \(\mu\)mol/L for carvedilol, timolol, and atenolol, respectively. Because the after-peak hump was observed more frequently as the \(\beta\)-stimulation increased, the inhibitory effect of carvedilol seemed to be stronger than that of timolol. At the concentration of 300 nmol/L, both carvedilol and timolol almost completely suppressed the isoproterenol-induced [Ca\(^{2+}\)] rise (Fig 5B). In contrast, atenolol appeared to have much less inhibitory effects. In addition, the time course of washing out the effects of carvedilol was much slower than timolol, implying that the binding affinity of carvedilol might be higher. The inhibitory effect of carvedilol was stronger than that of timolol as well as that of atenolol, as indicated by the IC\(_{50}\) (1.32, 2.01, and 612 nmol/L, respectively) (Fig 5C).

We also examined the inhibitory effects of these \(\beta\)-blockers on isoproterenol-induced increases in the amount of cAMP and found that 300 nmol/L carvedilol significantly prevented the isoproterenol-induced increase in the amount of cAMP, to a greater degree than timolol (Fig 5D). Atenolol did not show any apparent inhibition. In these serial experiments, atenolol seemed to be a comparatively and distinctively weak \(\beta\)-antagonist, but it was much more \(\beta\)-selective than the other two. Therefore, it seems that the \(\beta\)-activation by isoproterenol may compensate for the \(\beta\)-blocking by atenolol, resulting in apparently less inhibition of isoproterenol-induced [Ca\(^{2+}\)] enhancement. To rule out this possibility, 2 more experiments were performed. Myocytes were exposed to isoproterenol in the presence of atenolol and 1 \(\mu\)mol/L ICI-118551, a selective \(\beta\)-blocker, after pretreatment with them. There was no obvious difference in the [Ca\(^{2+}\)] amplitudes between the atenolol alone and the atenolol with ICI-118551 treatment groups (Fig 5C). Also, 100 nmol/L ICI-118551 did not significantly inhibit the effects of isoproterenol. Taken together with the data on the effect of zinterol (Fig 4B), the effects of isoproterenol on [Ca\(^{2+}\)] amplitudes were exerted almost exclusively via \(\beta\)-dependent pathway, and the \(\beta\)-activation by isoproterenol seems not to be the reason for the weak antagonizing ability of atenolol in guinea pig ventricular myocytes.

To examine the affinity of carvedilol for \(\beta\)-receptor, myocytes pretested with isoproterenol (1 \(\mu\)mol/L) were subsequently and additionally exposed to 300 nmol/L of carvedilol or timolol. Carvedilol rapidly diminished the amplitude of [Ca\(^{2+}\)] close to the control level, and the effect was sustained after carvedilol was washed out (Fig 6A). On the other hand, timolol showed only slight effects on
with a reduced ICa and/or SR Ca-ATPase function because of the L-type Ca2+ current at concentrations stronger than timolol, and much more so than atenolol, as indicated by carvedilol at 50 μmol/L. Unlike isoproterenol, carvedilol did not affect the [Ca2+]i amplitudes enhanced by forskolin (Fig 6C). Therefore, the downstream PKA pathway may not be directly modulated by carvedilol.

Among the 3 β-blockers, carvedilol possesses the strongest antagonistic actions to isoproterenol, and its binding affinity for the β-receptors is distinctive.

Discussion

In the present study, we have shown the direct [Ca2+]-lowering effects of carvedilol in guinea pig ventricular myocytes: the decrease in the intrinsic β-activity (shown as a decrease in the amount of cAMP), the significant reduction of the L-type Ca2+ current at concentrations ≥5 μmol/L, and the increase in the voltage threshold for pacing at concentrations ≥10 μmol/L.

The ability of carvedilol to prevent the isoproterenol-induced enhancement of [Ca2+] amplitudes was slightly stronger than timolol, and much more so than atenolol, as indicated by IC50 of 1.32, 2.01, and 612 nmol/L, respectively. In addition, carvedilol seems to have a relatively strong affinity for β-receptors.

Negative Modulation of [Ca2+] by Carvedilol

Our data for [Ca2+] demonstrate that carvedilol exerted a negative inotropic action even at very low concentrations (1 nmol/L) by lowering [Ca2+]. Furthermore, carvedilol significantly decreased the ICa in a concentration-dependent manner at ≥5 μmol/L. Therefore, the negative inotropic effect of carvedilol at ≤300 nmol/L is not necessarily because of the reduction of the ICa. As described24–25 the number of β-receptors is an important factor in maintaining the intrinsic β-activity of ventricular myocytes, even in the absence of catecholamines. Some β-blockers appear to prevent β-receptors from spontaneously transmitting to the activated form, resulting in decreased production of cAMP (inverse agonism). In the present study, carvedilol, timolol, and atenolol negatively modulated the [Ca2+] amplitudes and all three induced a decrease in the amount of cAMP in ventricular myocytes. A decrease in the amount of cAMP is supposed to lead to a decrease in the PKA activity, so the mechanism for the negative modulation of [Ca2+] amplitudes by the β-blockers is at least in part associated with a reduced ICa and/or SR Ca-ATPase function because of the decrease in the amount of intracellular cAMP. From this result, one might wonder why the measured ICa was not influenced by the decrease in the amount of cAMP induced by carvedilol at ≤1 μmol/L. One possible reason is that the voltage-clamped myocytes were dialyzed with a cAMP-free pipette solution, and therefore the intracellular cAMP concentration might be already very diluted. This is widely recognized as a reason for the run-down phenomenon of ICa and hence the effects of carvedilol on the amount of cAMP were no longer reflected in the size of ICa, although the activation of adenyl cyclase might locally increase cAMP and activate PKA to increase the ICa. Carvedilol at ≥5 μmol/L seems to directly reduce the ICa, but not through the cAMP-dependent pathway, to further decrease [Ca2+]i amplitudes. In a 10-μmol/L carvedilol solution, ventricular myocytes lost their responsiveness to field stimulation, which was regained when the voltage for field stimulation was increased. Because the other two β-blockers did not mimic this phenomenon, and 10-μmol/L carvedilol did not inhibit the ICa enough to eliminate [Ca2+]i transients, the pacing failure is not attributable to the alteration of the intrinsic β-activity or the inhibition of ICa. This result indicates that the L-type Ca2+ channel was not opened by pacing, suggesting that the depolarization by pacing was not enough to induce an action potential, otherwise a [Ca2+]i increase would be observed. The 0 phase of an action potential in a ventricular myocyte is composed of Na+ and Ca2+ currents. A Na+ channel blocker, tetrodotoxin (10 μmol/L), did not mimic the phenomenon, but slightly decreased the [Ca2+]i amplitudes (data not shown). From this result, the membrane depolarization by pacing probably did not reach the threshold of the Na+ channel, which is known to be approximately ~60 mV. Presumably this carvedilol-induced pacing failure was caused by decreased membrane excitability in response to electrical stimulation, which may be caused by changes in the membrane characteristics by carvedilol. Further examination is required to elucidate the mechanism underlining this intriguing finding.

Beta-Antagonism of Carvedilol

In terms of the [Ca2+], we assessed the antagonism of carvedilol to β-stimulation. Carvedilol antagonized the isoproterenol-induced β-stimulation most strongly among the 3 β-blockers we tested (Fig 5C) and the data for cAMP (Fig 5D) have clearly shown that carvedilol most effectively prevented the production of cAMP induced by isoproterenol, again suggesting that carvedilol is the strongest β-blocker. In addition, these inhibitory effects of the β-blockers on the production of cAMP by isoproterenol appear to be comparable to the data for [Ca2+]: shown in Fig 5D. In these experiments, the antagonizing effects of a β-selective blocker, atenolol, were distinctively weak, implying the functional contribution of residual β-activation by isoproterenol. However, a specific β-selective β-blocker, zintenol (1 μmol/L), did not affect the [Ca2+] amplitudes, and a specific β-blocker, ICI-118551 (100 nmol/L), exerted only a slight inhibitory effect on the isoproterenol-induced enhancement of [Ca2+]. Furthermore, the combination of atenolol and ICI-118551 had no additional antagonizing effect, suggesting that the β-activation by isoproterenol is not significantly involved in [Ca2+] regulation in guinea pig ventricular myocytes. In rat and mouse ventricular myocytes, a β-subtype has been reported in ventricular myocardium, but is almost devoid of coupling with positive inotropic action and/or increase in [Ca2+] amplitudes. Because the β-subtype has been shown to exist in guinea pig ventricular myocytes as well, our present results are very similar to those for rat and mouse. Regarding the uncoupling of β-receptor with positive inotropism, Xiao et al reviewed earlier reports and proposed 3 possible mechanisms. First, the β-receptor signaling pathway is extremely localized so that its activation leading to the production of cAMP cannot be transmitted to the phosphorylation of the L-type Ca2+ channel, phosphoholamban, and so...
on. Second, the $\alpha_2$-receptor is coupled with Gi. Third, intrinsic and/or additional PKA activation switches the coupling of the $\alpha_2$-receptor from Gs to Gi. In the present study, zinterol did not affect [Ca$^{2+}$]$i$ amplitudes, suggesting that the latter two possibilities are unlikely explanations for our results, although the first mechanism could still explain our results on the uncoupling of the $\alpha_2$-receptor with [Ca$^{2+}$]$i$ regulation. Very recently Zaccolo et al have shown that the localization of cAMP is limited by phosphodiesterases, and that the specificity of the subsequent events was tightly localized$^{30}$ which further supports the idea that the local production of cAMP via $\alpha_i$-receptor is specifically coupled with [Ca$^{2+}$]$i$ regulation.

In addition, the $\alpha_1$-blocking effect of carvedilol may have influenced our results for [Ca$^{2+}$]$i$ and cAMP. In guinea pig ventricular myocytes, $\alpha_1$-stimulation has been reported to shorten action potential duration by increasing the delayed rectifier K+ current$^{11}$ and decreasing cAMP$^{12}$ both of which contribute to a decrease in the peak of Ca$^{2+}$ transient. This hypothesis suggests that the $\alpha_1$-blocking effect of carvedilol may result in an increase in the amplitude of [Ca$^{2+}$]$i$ transients, but that seems to contradict our results of carvedilol-induced decreases in the peak of Ca$^{2+}$ transient and the amount of cAMP. Therefore, the $\alpha_1$-blockade by carvedilol may have minor additional effects in the absence of $\alpha_1$-agonists under our experimental conditions, although further experiments are necessary to prove it.

Under our experimental conditions isoproterenol increased [Ca$^{2+}$]$i$ amplitudes mainly via the $\beta$-receptor pathway, and carvedilol possessed the strongest $\alpha_1$-antagonizing effects among the 3 $\beta$-blockers, although the functional significance of $\alpha_1$-receptors and the effect of $\alpha_1$-blockade by carvedilol remain to be elucidated.

**In Vivo Kinetics of Carvedilol**

From the data on drug kinetics previously reported$^{33}$ the maximum blood concentration of atenolol could be nearly 2 $\mu$mol/L after a 50-mg oral dose, and most (97%) of atenolol is supposed to be free in human plasma$^{35}$ On the other hand, the blood concentration of carvedilol in clinical use after a single or daily oral dose of 12.5–50 mg has been reported to reach 100–600 nmol/L at maximum$^{15,16}$ and $\geq$98% is bound to albumin in blood$^{37}$ The calculated concentration of free carvedilol is approximately 2–12 nmol/L, although neither the distribution nor the local concentration in various tissues is clear. Applying these calculations to our present results, carvedilol of 2–12 nmol/L and atenolol of $\sim$2 $\mu$mol/L antagonize $\alpha_1$-stimulation in ventricular myocytes to a similar degree (Fig 5C). In addition, our data on the wash-out of (Fig 5D) and additional application of (Fig 6A) carvedilol in the presence of isoproteenol imply that the binding of carvedilol to $\alpha_1$-receptors is tight as compared with isoproteenol and timolol. The washing out of carvedilol might be related to its characteristic lipophilic structure, which when dissolved in the plasma membrane may re-surface through a diffusion theory after carvedilol is washed out from the extracellular fluid. Given that diffusion is restricted on the plasma membrane$^{35}$ carvedilol diffusing from the membrane may be retained on the surface of the membrane, which not only accumulates carvedilol, but also consequentially slows the diffusion of carvedilol from the membrane. These local kinetics of carvedilol would favor an accentuation of its effects on the surface membrane.

Carvedilol at clinical concentrations seems to effectively inhibit $\beta$-activation and the [Ca$^{2+}$]$i$-load induced by catecholamines. The characteristics of carvedilol that include a prominent $\beta$-receptor affinity and/or a lipophilic structure may contribute to its efficacy.

In conclusion, carvedilol possesses various types of direct [Ca$^{2+}$]$i$-lowering effects in ventricular myocytes, which include decreasing the intrinsic $\alpha$-activity, antagonizing $\alpha_1$-stimulation at concentrations of the nmol/L order supposedly equivalent to its clinical concentration, reducing the $I_{Ca}$ at $\geq$5 $\mu$mol/L, and increasing the threshold for pacing at $\geq$10 $\mu$mol/L. Supposing that one possible mechanism associated with the progression of hypertrophy and heart failure is catecholamine-induced [Ca$^{2+}$]$i$: load and/or PKA activation, it could be partially but effectively prevented by $\beta$-blockers. In this respect, carvedilol could be regarded as one of the $\beta$-blockers potent enough to prevent [Ca$^{2+}$]$i$: load and PKA activation in the clinical setting.

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