Effect of Carbenoxolone on Arrhythmogenesis in Rat Ventricular Muscle

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Background: Connexin43 (Cx43) is a major connexin that forms gap junction (GJ) channels in the heart and is also present in the cell membrane as unopposed/non-junctional hemichannels and in the inner mitochondrial membrane. By using carbenoxolone (CBX), a blocker of Cx43, the effect of the blockade of Cx43 on Ca2+ waves and triggered arrhythmias in the myocardium with non-uniform contraction was examined.

Methods and Results: Trabeculae were obtained from rat hearts. Force, [Ca2+], and the diffusion coefficient were measured. Non-uniform contraction was produced with a 2,3-butanedione monoxime jet. Ca2+ waves were induced by electrical stimulation. Inducibility of arrhythmias was estimated based on the minimal [Ca2+]; at which arrhythmias were induced. The Ca2+ spark rate was measured in isolated single rat ventricular myocytes. CBX reduced the GJ permeability, whereas it did not change force and [Ca2+]; transients. CBX increased the Ca2+ leak from the sarcoplasmic reticulum in trabeculae and increased the Ca2+ spark rate in isolated single myocytes. CBX increased the velocity of Ca2+ waves and further increased the inducibility of arrhythmias. Modulation of mitochondrial KATP channels by diazoxide, cromakalim and 5-hydroxydecanoic acid affected the inducibility of arrhythmias increased by CBX.

Conclusions: These results suggest that in diseased hearts, Cx43 plays an important role in the occurrence of triggered arrhythmias, probably under the modulation of mitochondrial KATP channels. (Circ J 2016; 80: 76–84)

Key Words: Calcium; Carbenoxolone; Connexin43; Diazoxide

Life-threatening ventricular arrhythmias are likely to occur in diseased hearts, such as hearts with cardiac hypertrophy and failure.1 In diseased hearts, non-uniform muscle contraction commonly occurs2 as a result of muscle damage,2 heterogeneous adrenergic activation, heterogeneous protein expression or non-uniform electrical activation. In the myocardium with non-uniform contraction, regional differences in contractile strength may cause paradoxical stretching of the impaired muscle by contractions of the more viable neighboring muscle.3 During the relaxation of the more viable muscle, the paradoxical shortening of the impaired muscle dissociates Ca2+ from the myofilaments and then initiates Ca2+ waves.4 These Ca2+ waves cause delayed afterdepolarizations (DADs) through the activation of Ca2+-activated transient inward currents across the sarcolemma, and their propagation features such as velocity and amplitude are deeply involved with the formation of DADs.4 Because DADs cause arrhythmias associated with catecholamine excess,5 heart failure,6 and mutations of the ryanodine receptor or calsequestrin in the sarcoplasmic reticulum (SR),7 the velocity of Ca2+ waves is one of the determinants of arrhythmogenesis.4 In trabeculae, the propagation velocity of Ca2+ waves is determined by cellular Ca2+ loading, the open probability of the SR-Ca2+ release channels,8 and gap junction (GJ) communication.9

Connexin43 (Cx43) is a major connexin that forms GJ channels in the heart and is especially abundant in ventricular muscle.10 Not only present as GJ channels, Cx43 is also present as unopposed/non-junctional hemichannels in the cell membrane11,12 and is also found in the inner mitochondrial membrane.13 Interestingly, the presence of Cx43 is needed for cardioprotection14,16 by ischemic preconditioning and pharmacological preconditioning with diazoxide,17 a mitochondrial KATP channel opener.18,19 Although activation of mitochondrial KATP channels by diazoxide reduces ventricular arrhythmias induced by ischemia,20 it has not yet been established why Cx43 is needed when activation of mitochondrial KATP channels reduces such arrhythmias.

Carbenoxolone (CBX) has been used as a blocker of GJ...
channels in rabbit,21 guinea pig,22 dog,23 and rat hearts.24 In addition, it blocks Cx43 hemichannels25 and mitochondrial Cx43.26 Therefore, to determine what role Cx43 plays when mitochondrial \( K_{ATP} \) channels are involved in arrhythmogenesis in diseased hearts, we investigated how the blockade of Cx43 by CBX and the modulation of mitochondrial \( K_{ATP} \) channels affect the inducibility of triggered arrhythmias in the myocardium with non-uniform contraction. The results in the present study suggest that Cx43 plays an important role in arrhythmogenesis, probably under the modulation of mitochondrial \( K_{ATP} \) channels in diseased hearts.

**Methods**

**Measurements of Force, [Ca\(^{2+}\)] \(_{i}\), and Equivalent Diffusion Coefficient for Fura-2 in Rat Trabeculae**

All animal procedures were performed according to 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) and were approved by the Ethics Review Board of Tohoku University (approval reference number: 2011-33).

After the animals had been adequately anesthetized, trabeculae were dissected from the right ventricles of rat hearts. Force, [Ca\(^{2+}\)] \(_{i}\), and the equivalent diffusion coefficient for fura-2 were measured as previously reported.8,27

**Experimental Protocol With Trabeculae**

Non-uniform muscle contraction was produced with a 2,3-butanedione monoxime (BDM) jet as previously reported.28

**Measurements of Ca\(^{2+}\) Sparks in Isolated Rat Cardiac Myocytes**

Myocytes were enzymatically isolated from ventricles of rat hearts, and Ca\(^{2+}\) sparks were measured using fluo-4 and fast 2D confocal Ca\(^{2+}\) imaging.29 At different CBX concentrations (0, 5, 10, and 50 \( \mu \)mol/L), Ca\(^{2+}\) sparks were counted between stimulated transients at a steady basal Ca\(^{2+}\) level ([Ca\(^{2+}\)] \(_{o}\)=2 mmol/L, 35°C).

**Statistical Analysis**

All measurements were expressed as mean±SEM. Statistical analysis was performed with 1-way repeated-measures ANOVA, with Tukey-Kramer for multiple comparisons and a paired t-test for 2-group comparisons when the data were normally distributed. Otherwise, the Friedman test with Scheffe was used for multiple comparisons, and the Wilcoxon signed-rank test was used for 2-group comparisons unless otherwise mentioned. These analyses were performed using software for statistical analysis (Ekusen-Toukei 2012, Social Survey Research Information Co, Ltd, Tokyo, Japan). Values of P<0.05 were considered to be significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agreed to the manuscript as written.

See expanded ‘Methods’ section in Supplementary File 1.

**Results**

The effect of CBX on GJ communication was first investigated in rat ventricular trabeculae. The diffusion of fura-2 within trabeculae was recorded after a 15-min superfusion with 5 and 10 \( \mu \)mol/L CBX, and the diffusion coefficients for fura-2 were calculated. As shown in Table, the diffusion coefficients were significantly decreased after superfusion with both 5 and 10 \( \mu \)mol/L CBX, and these decreases in the diffusion coefficients lasted for at least 1 h. When the GJ permeability was calculated using the previously reported diffusion coefficient in the myoplasm (42.5×10\(^{-8}\) cm\(^2\)/s),27 it was 6.13×10\(^{-5}\) cm/s before superfusion with CBX and was reduced to 1.38×10\(^{-5}\) and 1.81×10\(^{-5}\) cm/s just after and 1 h after superfusion with 5 \( \mu \)mol/L CBX, respectively. This means that 5 \( \mu \)mol/L CBX reduces the GJ permeability to 20–30% of its initial value and that this reduction lasts for at least 1 h after superfusion. In addition, this result also suggests that 5 \( \mu \)mol/L CBX actually blocks Cx43 because GJ channels in adult rat ventricular muscle are chiefly formed by Cx43.10

CBX has been reported to affect neither the action potential in rabbit hearts21 nor the electrocardiogram in human hearts,32 and thus the effect of CBX on force and intracellular Ca\(^{2+}\) handling was next assessed in rat trabeculae. Force and [Ca\(^{2+}\)] \(_{i}\) transients were measured during electrical stimulation with 2-s intervals before and after superfusion with 5 \( \mu \)mol/L CBX ([Ca\(^{2+}\)] \(_{o}\)=2.0 mmol/L). As shown in Figure 1, 5 \( \mu \)mol/L CBX caused no significant changes in the developed force, the peak [Ca\(^{2+}\)], the diastolic [Ca\(^{2+}\)], and the time constant of the decline in [Ca\(^{2+}\)]; transients during electrical stimulation, suggesting that 5 \( \mu \)mol/L CBX has no significant effect on intracellular Ca\(^{2+}\) handling in rat cardiac muscle. To further examine the effect of CBX on intracellular Ca\(^{2+}\) handling under conditions of Ca\(^{2+}\) overload, force and [Ca\(^{2+}\)] transients were again measured at higher [Ca\(^{2+}\)]. Figure 2A shows force and changes

### Table. Effect of CBX on Diffusion Coefficients for Fura-2 in Rat Cardiac Trabeculae

<table>
<thead>
<tr>
<th>CBX Concentration</th>
<th>Control (0 h)</th>
<th>1 h</th>
<th>5 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 ( \mu )mol/L</td>
<td>25.1±4.3</td>
<td>10.4±2.1*</td>
<td>12.7±1.7*</td>
<td></td>
</tr>
<tr>
<td>10 ( \mu )mol/L</td>
<td>26.1±6.1</td>
<td>5.3±2.4*</td>
<td>1.7±0.7*</td>
<td></td>
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</tbody>
</table>

Control, 0 h, and 1 h indicate the diffusion coefficients without, just after, and 1 h after 15-min superfusion with CBX, respectively. Superfusion with CBX significantly decreased the diffusion coefficients for fura-2 in rat cardiac trabeculae. Results are expressed as mean±SEM. *P<0.05 vs. control, **P<0.01 vs. control. CBX, carbenoxolone.
To investigate which effect is more prominent in the case of CBX, the velocity of Ca\(^{2+}\) waves was measured before and after superfusion with 5 μmol/L CBX. In a trabecula with non-uniform contraction created by a regional jet of BDM, a Ca\(^{2+}\) wave occurred within the jet-exposed region after rapid electrical stimulation and was propagated through GJs within the trabecula (Figure 3A Left panel). Superfusion with 5 μmol/L CBX increased the velocity of the Ca\(^{2+}\) wave (Figure 3A Right panel). Summary data indicate that 5 μmol/L CBX did not change the developed force (P=0.23, n=12, [Ca\(^{2+}\)]\(_o\)=2.0mmol/L), but significantly increased both the velocity of Ca\(^{2+}\) waves (P=0.46), the diastolic [Ca\(^{2+}\)] (P=0.43), the diastolic Ca\(^{2+}\) leak from the SR (P=0.34, n=7, [Ca\(^{2+}\)]\(_o\)=2.0mmol/L).

Summary data indicate that 5 μmol/L CBX significantly increased the number of spontaneous increases in [Ca\(^{2+}\)] within trabeculae (Figure 2B), whereas it caused no significant change in force during electrical stimulation at 5-s intervals ([Ca\(^{2+}\)]\(_o\)=4.8±0.6 mmol/L, Figure 2C), suggesting that CBX increases diastolic Ca\(^{2+}\) leak from the SR without an increase in the Ca\(^{2+}\) loading within the SR in rat trabeculae.

It is possible that CBX causes such an increase in diastolic Ca\(^{2+}\) leak from the SR by blocking GJ channels within trabeculae. Thus, using isolated single myocytes, Ca\(^{2+}\) sparks were counted before and after superfusion with CBX (Figure 2D). Even in isolated single myocytes, 5 μmol/L CBX significantly increased the number of Ca\(^{2+}\) sparks. In addition, the number of Ca\(^{2+}\) sparks increased depending on the concentration of CBX. These results suggest that, depending on the concentration, CBX does increase diastolic Ca\(^{2+}\) leak from the SR, but not by blocking GJ channels.

It has been previously reported that the propagation velocity of Ca\(^{2+}\) waves is decreased by the reduction of GJ communication, and is increased by the increase in Ca\(^{2+}\) leak from the SR. To investigate which effect is more prominent in the case of CBX, the velocity of Ca\(^{2+}\) waves was measured before and after superfusion with 5 μmol/L CBX. In a trabecula with non-uniform contraction created by a regional jet of BDM, a Ca\(^{2+}\) wave occurred within the jet-exposed region after rapid electrical stimulation and was propagated through GJs within the trabecula (Figure 3A Left panel). Superfusion with 5 μmol/L CBX increased the velocity of the Ca\(^{2+}\) wave (Figure 3A Right panel). Summary data indicate that 5 μmol/L CBX did not change the developed force (P=0.23, n=12, [Ca\(^{2+}\)]\(_o\)=2.0mmol/L), but significantly increased both the velocity of Ca\(^{2+}\) waves (P=0.46), the diastolic [Ca\(^{2+}\)] (P=0.43), the diastolic Ca\(^{2+}\) leak from the SR (P=0.34, n=7, [Ca\(^{2+}\)]\(_o\)=2.0mmol/L).

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As an increase in the velocity of Ca\(^{2+}\) waves is deeply involved with arrhythmogenesis, the effect of 5 μmol/L CBX on the inducibility of triggered arrhythmias was then investigated. As shown in Figure 4A, before superfusion with 5 μmol/L CBX, arrhythmias were not induced even at 7 mmol/L [Ca\(^{2+}\)]\(_o\) by electrical stimulation at 300 ms intervals for 30 s, whereas they were induced at 1 mmol/L [Ca\(^{2+}\)]\(_o\) after superfusion. The minimal [Ca\(^{2+}\)]\(_o\) ([Ca\(^{2+}\)]\(_o\)\(_{\text{min}}\)) at which arrhythmias were induced by electrical stimulation was significantly decreased
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The blockade of mitochondrial K<sub>ATP</sub> channels cannot increase the inducibility of arrhythmias by itself. Interestingly, CBX decreased the [Ca<sup>2+</sup>]<sub>o</sub> at last in the presence of 5-HD (Figure 5E). These results suggest that CBX increases the inducibility of arrhythmias only when mitochondrial K<sub>ATP</sub> channels, rather than sarcolemmal K<sub>ATP</sub> channels, are closed.

Finally, the effect of CBX on ∆Ψ<sub>m</sub> was observed using TMRM fluorescence. As shown in Figure 5F, TMRM fluorescence was decreased after superfusion with 5 µmol/L CBX, and this decrease was suppressed in the presence of 100 µmol/L diazoxide, suggesting that CBX depolarizes ∆Ψ<sub>m</sub> under the modulation of mitochondrial K<sub>ATP</sub> channels.

Discussion

The present study characterized how CBX affects arrhythmogenesis in the myocardium with non-uniform contraction. To the best of our knowledge, it shows for the first time that regardless of the reduction of the GJ permeability, CBX increases the inducibility of arrhythmias and that activation of mitochondrial K<sub>ATP</sub> channels suppresses this increase. These

![Figure 2](image-url)
results suggest that Cx43 plays an important role in the occurrence of triggered arrhythmias, probably under the modulation of mitochondrial KATP channels in diseased hearts.

**CBX and GJ Communication**

It has been reported that the common logarithm of GJ permeability inversely correlates with molecular weight; the values of the permeability range from $7 \times 10^{-5}$ cm/s (mol wt 559, lissamine rhodamine) to $770 \times 10^{-5}$ cm/s (mol wt 39, K+). In the present study, the calculated GJ permeability for fura-2 was $6.13 \times 10^{-5}$ cm/s (mol wt 832), which is similar to the GJ permeability determined for molecules of a similar size. Although it is well known that high Ca2+ levels close GJs, we assume that the [Ca2+] level during the measurement of the diffusion coefficients was not high enough to affect the GJ permeability because they were measured without electrical stimulation at 0.7 mmol/L [Ca2+].

CBX has been used as a GJ blocker at concentrations ranging from 15 to 100 μmol/L in rabbit, guinea pig, and dog hearts. In guinea pig hearts, CBX at concentrations of less than 13 μmol/L did not change the conduction velocity of excitation, whereas in rat hearts, CBX even at a concentration of 10 μmol/L, affected it. The present study showed that in rat trabeculae, 5 μmol/L CBX reduced the GJ permeability to 20–30% of its initial value. It has been reported that in adult rat ventricular muscle, GJ channels are formed by Cx43, and that a reduction of Cx43 using transgenic methodology slows the conduction velocity of excitation in ventricular muscle, suggesting that CBX reduces the GJ permeability in rat trabeculae by blocking Cx43, although CBX has also been reported to block other Cx families.

In multicellular cardiac muscle, the Ca2+ movement through GJs determines the velocity of Ca2+ waves because Ca2+ waves propagate within the cardiac muscle by Ca2+-induced Ca2+ release from the SR mediated by Ca2+ diffusion through GJs. Actually, it has been previously reported that in rat trabeculae, the velocity of Ca2+ waves is decreased by the reduction of GJ communication. As for cardiac muscle excitation, ventricular
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increased the inducibility of arrhythmias, which is consistent with past reports using other experimental models. Acceleration of Ca\(^{2+}\) waves increases arrhythmogenesis through enhancement of DADs, suggesting that CBX may have increased it by the acceleration of Ca\(^{2+}\) waves, as shown in Figures 3. Therefore, these results suggest that CBX accelerates Ca\(^{2+}\) waves by an increase in Ca\(^{2+}\) leak from the SR and thereby increases arrhythmogenesis.

Diazoxide and cromakalim have been used as K\(_{ATP}\) channel openers, and diazoxide is 2000-fold more potent at the mitochondrial channel than at the surface channel. In the present study, CBX did not increase the inducibility of arrhythmias in the presence of diazoxide (Figures 5B, C) or cromakalim (Figure 5D), but increased it in the presence of 5-HD (Figure 5E), suggesting that CBX accelerates Ca\(^{2+}\) waves by an increase in Ca\(^{2+}\) leak from the SR and thereby increases arrhythmogenesis.

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In the present study, 5\(\mu\)mol/L CBX increased the number of spontaneous increases in [Ca\(^{2+}\)]\(_i\) within trabeculae and increased the number of Ca\(^{2+}\) sparks depending on its concentration in isolated single myocytes, although it did not change the developed force and [Ca\(^{2+}\)]\(_i\) transients by electrical stimulation (Figures 1, 2). These results suggest that CBX increases Ca\(^{2+}\) leak from the SR not by increasing the Ca\(^{2+}\) loading in the SR but probably by lowering the SR threshold for Ca\(^{2+}\) leak. Such an increase in Ca\(^{2+}\) leak from the SR accelerates Ca\(^{2+}\) waves because Ca\(^{2+}\) waves result from the spatial and temporal summation of Ca\(^{2+}\) sparks. As shown in Figure 4, CBX also increased the inducibility of arrhythmias, which is consistent with past reports using other experimental models. Acceleration of Ca\(^{2+}\) waves increases arrhythmogenesis through enhancement of DADs, suggesting that CBX may have increased it by the acceleration of Ca\(^{2+}\) waves, as shown in Figures 3. Therefore, these results suggest that CBX accelerates Ca\(^{2+}\) waves by an increase in Ca\(^{2+}\) leak from the SR and thereby increases arrhythmogenesis.

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As shown in Figure 5F, a decrease in TMRM fluorescence after the addition of CBX was suppressed by diazoxide, suggesting that the blockade of Cx43 depolarizes \(\Delta\phi\) and that activation of mitochondrial K\(_{ATP}\) channels suppresses this depolarization. It is still controversial as to whether diazoxide...
In diseased hearts, such as those suffering from myocardial infarction and heart failure, impaired muscle is occasionally distributed within hearts, causing regional differences in contractile strength around the muscle. Such regional differences in contractile strength may produce paradoxical stretching and shortening of the impaired muscle by contractions of the more viable neighboring muscle, thereby inducing Ca²⁺ waves and triggered arrhythmias.

The findings of the present study show that in the myocardium with non-uniform contraction, Cx43 plays some role in Ca²⁺ wave propagation and the occurrence of arrhythmias, probably under the modulation of mitochondrial KATP channels, making Cx43 an attractive therapeutic target for suppressing triggered arrhythmias in such diseased hearts.

In conclusion, the present study suggests that Cx43 plays an important role in the modulation of mitochondrial KATP channels and Ca²⁺ wave propagation, which may contribute to the occurrence of arrhythmias in diseased hearts.

**Figure 5.** Inducibility of triggered arrhythmias under the modulation of KATP channels. (A) Summary data concerning the effect of 5µmol/L carbenoxolone (CBX) on the amplitude of aftercontractions (AC force) in the presence of 100µmol/L diazoxide. CBX did not change the amplitude of the AC force (n=10, P=0.17). (B) Representative recordings of force before (Upper panel) and after superfusion with 5µmol/L CBX (Lower panel) in the presence of 100µmol/L diazoxide. Spontaneous contractions were not induced by electrical stimulation, even after superfusion with CBX. Arrows with ST indicate the moments of electrical stimulation (23.8°C, Experiment number 140303). (C) Summary data concerning the effect of CBX on the minimal Ca²⁺ concentration ([Ca²⁺]o)min at which spontaneous contractions were induced by electrical stimulation in the presence of 100µmol/L diazoxide. CBX did not change the [Ca²⁺]o)min (n=7, P=0.18). (D) Summary data concerning the effect of CBX on Ca²⁺ concentration ([Ca²⁺]o)min in the presence of 10µmol/L cromakalim. CBX did not change the [Ca²⁺]o)min (n=7, P=1.00). (E) Summary data concerning the effect of CBX on [Ca²⁺]o)min in the presence of 10µmol/L cromakalim and 200µmol/L 5-hydroxydecanoic acid (5-HD). CBX decreased the [Ca²⁺]o)min in the presence of both cromakalim and 5-HD (n=4). *P<0.05 vs. 5-HD (-) CBX (-) and 5-HD (+) CBX (-). (F) Recordings of tetramethylrhodamine methyl-ester (TMRM) fluorescence before and after superfusion of CBX in the presence (blue circles) and absence of 100µmol/L diazoxide (red circles). The Y-axis shows the changes (%) in the TMRM fluorescence ([Ca²⁺]o 2.0 mmol/L, 24.1°C, Experiment numbers 150629, 150701).
important role in the occurrence of triggered arrhythmias, probably under the modulation of the mitochondrial K<sub>ATP</sub> channels in diseased hearts.

### Study Limitations

Mouse models with chronic reduction of Cx43 GJ channels or mitochondrial Cx43 by transgenic methodology would be useful to investigate their role in arrhythmogenesis. In the present study, however, we did not use such mouse models because we could not create non-uniform muscle contraction in mouse cardiac muscle due to its smaller size. In addition, we could not find a knock-out model of Cx43 hemi-channels or mitochondrial Cx43. Furthermore, we could not find drugs that have been widely recognized as selective GJ channels or mitochondrial Cx43. Furthermore, we could not find drugs that have been widely recognized as selective GJ channels or mitochondrial Cx43. Therefore, in the present study we had to use CBX, which has the ability to block not only GJ channels but also Cx43 hemi-channels and mitochondrial Cx43.

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### Disclosures

None.

### References


Supplementary Files

Supplementary File 1

Methods

Please find supplementary file(s);