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Dynein Coordinates β2-Adrenoceptor-Mediated Relaxation in Normotensive and Hypertensive Rat Mesenteric Arteries

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BACKGROUND: The voltage-gated potassium channel (Kv)7.4 and Kv7.5 channels contribute to the β-adrenoceptor-mediated vasodilatation. In arteries from hypertensive rodents, the Kv7.4 channel is downregulated and function attenuated, which contributes to the reduced β-adrenoceptor-mediated vasodilatation observed in these arteries. Recently, we showed that disruption of the microtubule network, with colchicine, or inhibition of the microtubule motor protein, dynein, with ciliobrevin D, enhanced the membrane abundance and function of Kv7.4 channels in rat mesenteric arteries. This study aimed to determine whether these pharmacological compounds can improve Kv7.4 function in third-order mesenteric arteries from the spontaneously hypertensive rat, thereby restoring the β-adrenoceptor-mediated vasodilatation.

METHODS AND RESULTS: Using both wire myography and intravital microscopy, we show that ciliobrevin D enhanced the β-adrenoceptor-mediated vasodilatation by isoprenaline. This effect was inhibited partially by the Kv7 channel blocker linopirdine and was dependent on an increased functional contribution of the β2-adrenoceptor to the isoprenaline-mediated relaxation. In mesenteric arteries from the spontaneously hypertensive rat, ciliobrevin D and colchicine both improved the isoprenaline-mediated vasorelaxation and relaxation to the Kv7.2–7.5 activator, ML213. Immunostaining of isolated vascular smooth muscle cells, confirmed ciliobrevin D enhanced the membrane abundance of Kv7.4. As well as an increase in the function of Kv7.4, the functional changes were associated with an increase in the contribution of β2-adrenoceptor following isoprenaline treatment. Immunostaining experiments showed ciliobrevin D prevented isoprenaline-mediated internalization of the β2-adrenoceptor.

CONCLUSIONS: Overall, these data show that colchicine and ciliobrevin D can induce a β2-adrenoceptor-mediated vasodilatation in arteries from the spontaneously hypertensive rat as well as reinstating Kv7.4 channel function. (Hypertension. 2022;79:00–00. DOI: 10.1161/HYPERTENSIONAHA.122.19351.)

Key Words: colchicine ♦ dyneins ♦ mesenteric arteries ♦ microtubules ♦ muscle cells
The voltage-gated potassium channel, Kv7.4, in smooth muscle cells, which are known to be stimulated by secondary messengers downstream of G\(\alpha\) protein activation. Furthermore, we showed that inhibition of the microtubule motor protein, dynein, prevented retrograde trafficking of Kv7.4 channels in vascular smooth muscle cells, thereby enhancing the vasorelaxations elicited by Kv7 channel activators. Whether this dynein regulation of Kv7.4 channels impacts on \(\beta\)-adrenoceptor-mediated relaxation is still unknown.

\(\beta\)-Adrenoceptor-mediated relaxation is attenuated in arteries from hypertensive rats. Reduced expression and function of Kv7.4 channels in arteries from hypertensive rats is one of the potential mechanisms underlying the reduced \(\beta\)-adrenoceptor-mediated relaxation. However, it is unclear whether the microtubule network and dynein contribute to this pathophysiological feature of hypertension, and whether this network can be targeted to restore these relaxations.

The aim of this study was to investigate the physiological role of dynein on \(\beta\)-adrenoceptor-mediated relaxation in small mesenteric arteries from normotensive and hypertensive rats. Based on previous data, we hypothesize that dynein mediates microtubule-dependent retrograde trafficking of Kv7.4 channels, thereby blunting \(\beta\)-adrenoceptor-mediated relaxation. Herein, we show that dynein inhibition increases \(\beta\)-adrenoceptor-mediated relaxation, specifically, by preventing internalization of the receptor and also augments membrane levels and function of Kv7.4 channels in arteries from both normotensive and hypertensive rats.

What Is New?
Vascular relaxations through the \(\beta\)-adrenoceptor are physiologically important and impaired in arteries from hypertensive humans and rodents. This study reveals a novel mechanism regulating the \(\beta\)-adrenoceptor-mediated relaxations. By targeting this mechanism we can restore the relaxations in arteries from hypertensive rats.

What Is Relevant?
We highlight the importance of the microtubule network in controlling \(\beta\)-adrenoceptor-mediated vaso-relaxations. We propose that the microtubule network can be targeted to treat hypertension.

Clinical/Pathophysiological Implications?
Our findings pave the way for testing whether this mechanism can improve vasodilatations in arteries from hypertensive humans.

### METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

**Animals**

All experiments were performed in accordance with Directive 2010/63/EU on the protection of animals used for scientific purposes and approved by the national ethics committee and by local Animal Care and Use Committees.

**Statistical Analysis**

All statistical analysis was performed using GraphPad Prism 9. LogEC\(_{50}\) values for concentration-responses were determined from individual experiments by fitting data to a 4-parameter nonlinear regression analysis (bottom/hillslope/top/EC\(_{50}\)). Comparison of mean logEC\(_{50}\) values from 2 groups was performed with an unpaired \(t\) test. In myography experiments, where the control, ciliobrevin D and colchicine data were tested multiple times when comparing with additional pharmacological compounds, a 2-way ANOVA was performed on the logEC\(_{50}\) values to determine the source of variation (\(F\) ratio). The results of this analysis are reported in the figures as the \(F\) ratio (\(df\)) and \(P\) values. Following this, a 1-way ANOVA followed by a Šidák posttest was performed to test for significant differences between predetermined groups. The results from these tests are denoted in the figures (*) and provided in the results section, unless otherwise stated. For the morpholino experiments, where it was not possible to calculate logEC\(_{50}\) values, concentration-effect curves were tested with 2-way ANOVAs followed by a Bonferroni posttest with correction for multiple comparisons. Membrane expression data were compared with a 1-way ANOVA followed by a Tukey posttest or an unpaired \(t\) test, depending on the number of groups in the comparison. All data are presented as mean±SEM. Significance values are represented as follows: *\(P<0.05\), **\(P<0.01\), and ***\(P<0.001\). \(n=(x)\), number of animals used; \(n=(x)\), number of technical replicates.
RESULTS

Dynein Inhibition Enhances β-Adrenoceptor-Mediated Relaxation in Rat Mesenteric Arteries Ex Vivo and In Vivo

To investigate the functional role of dynein on β-adrenoceptor-mediated relaxation, we used the specific dynein inhibitor, ciliobrevin D, on segments of freshly isolated rat mesenteric arteries. Incubation with 10 μmol/L ciliobrevin D augmented the isoprenaline-mediated relaxation in arteries preconstricted with methoxamine (N=8–10; P<0.001; Figure 1A and 1B). The relaxation elicited by isoprenaline in the absence and presence of ciliobrevin D was independent of a functional endothelium (N=5; F[1]=0.92, P=0.39 according to a 2-way ANOVA; Figure 1B). Ciliobrevin D, at a concentration of 1 μmol/L, had no effect on the isoprenaline relaxation (data not shown). Previously, we have shown that microtubule disruption with colchicine also enhanced β-adrenoceptor-mediated vasorelaxation in rat mesenteric arteries. Using ciliobrevin D and colchicine combined, we examined whether dynein inhibition and microtubule disruption enhance β-adrenoceptor-mediated relaxation through a similar trafficking mechanism. Ciliobrevin D (10 μmol/L) combined with colchicine (500 μmol/L) enhanced the isoprenaline-mediated relaxation to a similar level as ciliobrevin D alone (N=5; P<0.001; Figure S1). Again, this effect was independent of a functional endothelium (N=5; Figure S1).

We also determined the effect of ciliobrevin D in rat mesenteric arteries under in vivo conditions using intra-vital myography. Cumulative application of isoprenaline (0.01–3.0 μmol/L) induced a small vasodilation in control (DMSO treated) arteries (N=6; Figure 1C and 1D). Incubation with ciliobrevin D (30 μmol/L) enhanced the isoprenaline-induced relaxation compared with control (Figure 1D; logEC50 of −7.3±0.1 versus −6.3±0.2; N=6–8; P=0.019 according to an unpaired t-test). These data suggest that dynein inhibition is able to enhance the isoprenaline-mediated relaxation in both ex vivo and in vivo mesenteric arteries.

Kv7 Channels Contribute to β-Adrenoceptor-Mediated Vasorelaxation in the Absence and Presence of Ciliobrevin D

We investigated if blockade of Kv7 or large-conductance Ca2+ activated K+ (BKCa) channels, 2 functional end points of β-adrenoceptor signaling11,15,24–27 could attenuate the enhanced isoprenaline-mediated relaxation in segments of mesenteric artery after incubation with ciliobrevin D. In line with previous reports,11,15 application of the Kv7 channel blocker, linopirdine (10 μmol/L), inhibited the relaxation to isoprenaline in control arteries by shifting the logEC50 from −6.9±0.1 to −5.5±0.2 (N=11; P<0.001; Figure 2A and 2C) as well as attenuating the maximal relaxation by isoprenaline (P<0.001; Figure 2D). In addition, linopirdine also attenuated the ciliobrevin D-enhanced isoprenaline-mediated relaxation by shifting the logEC50 from −7.7±0.1 to −6.5±0.2 (N=8–12; P<0.001; Figure 2A and 2C) and attenuating the maximal relaxation (P=0.02; Figure 2D). Blocking the BKCa channels with iberiotoxin (100 nmol/L) attenuated the relaxation to isoprenaline in control arteries (maximal relaxation from 92.7±2.9% to 49.0±8.8%; N=6–11; P<0.001; Figure 2B), but, in contrast to linopirdine, did not affect the ciliobrevin D-enhanced isoprenaline relaxations (logEC50 of −7.73±0.141 versus −7.74±0.246; N=7–12; P>0.99; Figure 2B and 2C).

Kv7.4 and Kv7.5 are the 2 predominant Kv7 channel isoforms expressed in vascular smooth muscle cells, where they mostly form as heteromeric Kv7.4/7.5 channels.28–30 To investigate whether the ciliobrevin D-enhanced isoprenaline relaxation was mediated through Kv7.4 channels specifically, we knocked down Kv7.4 in isolated mesenteric arteries using morpholino oligos targeting the Kv7.4 gene.30 Knockdown of Kv7.4 attenuated the isoprenaline-mediated relaxations (N=6; P=0.003 and P=0.03 at 1 and 3 μmol/L isoprenaline respectively; Figure 2E). Ciliobrevin D enhanced the isoprenaline-mediated relaxations in arteries transfected with the miss-match morpholino and was able to moderately improve relaxations in the arteries that had Kv7.4 knockdown (N=6 and 4; P=0.004 and P=0.047; Figure 2E). Since the linopirdine and morpholino experiments do not conclusively show an increased contribution of Kv7 channel function to ciliobrevin D-enhanced isoprenaline relaxation, especially at the higher concentrations of isoprenaline, we confirmed that ciliobrevin D enhances Kv7 channel function using a specific channel activator, S-1. Similar to our previous results,12 ciliobrevin D increased the relaxation to S-1 in segments of mesenteric artery (logEC50 of −5.7±0.07 to −6.2±0.1; N=10–11; P=0.004; Figure 2F). Taken together, these data suggest that Kv7 channels contribute to the isoprenaline-mediated relaxation, and in the presence of ciliobrevin D, the Kv7 channels are particularly responsible for the enhanced relaxation at lower isoprenaline concentrations than the higher concentrations, where the BKCa channel no longer has a functional role.

Increased β2-Adrenoceptor Activity Determines the Ciliobrevin D-Enhanced Isoprenaline Relaxation

To assess the role of β-adrenergic receptor subtypes in the ciliobrevin D-enhanced isoprenaline-mediated relaxation,
Figure 1. Ciliobrevin D enhances isoprenaline-mediated relaxation in rat mesenteric arteries ex vivo and in vivo.

A. Representative isometric tension recordings of rat mesenteric artery segments preconstricted with methoxamine (•) before sequentially increasing concentrations of isoprenaline were applied in control and ciliobrevin D (10 µmol/L)-treated arteries. B. Mean concentration-effect curves and logEC_{50} values to isoprenaline showing the effect of isoprenaline in endothelium intact or endothelium cells denuded (-EC) rat mesenteric artery segments in control or ciliobrevin D (10 µmol/L) preincubated artery segments. A 2-way ANOVA was performed on the logEC_{50} values to determine the source of variation – F ratio (df) and P value are denoted. Mean logEC_{50} values were compared according to a 1-way ANOVA followed by a Šídák multiple comparisons test with ***P<0.001. C. Representative traces of isoprenaline-induced vasodilation of in vivo rat mesenteric artery segments preconstricted with methoxamine (•) in control (left) and ciliobrevin D (30 µmol/L)-treated arteries.

D. Mean concentration-effect curves and logEC_{50} values to isoprenaline showing the effect of isoprenaline in in vivo rat mesenteric arteries in control and after 30 µmol/L ciliobrevin D incubation. Mean logEC_{50} values were compared according to an unpaired t test with *P<0.05.
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**Figure 2.** Ciliobrevin D-enhanced isoprenaline-mediated relaxations are partially mediated through voltage-gated potassium channel (Kv7) channels.

A, Mean concentration-effect curves showing the effects of linopirdine (10 µmol/L) and ciliobrevin D (10 µmol/L) on the isoprenaline relaxation in mesenteric artery segments. B, Mean concentration-effect curves showing the effects of iberiotoxin (IbTx; 100 nmol/L) and ciliobrevin D (10 µmol/L) on the isoprenaline relaxation in mesenteric artery segments. C, LogEC50 values showing the effects of linopirdine (10 µmol/L), iberiotoxin (IbTx; 100 nmol/L) and ciliobrevin D (10 µmol/L) on the isoprenaline relaxation in mesenteric artery segments. A 2-way ANOVA was performed on logEC50 values to determine the source of variation – F ratio (df) and P value are denoted. One-way ANOVA followed by a Šídák multiple comparisons test was performed on the mean logEC50 values with **P<0.01 and ***P<0.001. D, The maximal relaxation of mesenteric artery segments to 3 µmol/L isoprenaline showing the effects of linopirdine (10 µmol/L) and ciliobrevin D (10 µmol/L) with * and *** denoting P<0.05 and P<0.001, respectively. E, Mean isoprenaline concentration-effect curves in rat mesenteric arteries transfected with either a Kv7.4 targeted or control (miss-match) morpholino in the absence and presence of 10 µmol/L ciliobrevin D. A 2-way ANOVA followed by a Bonferroni multiple comparisons test was performed on the concentration-effect curves with *P<0.05 and ***P<0.001. F, Mean concentration-effect curves and logEC50 values to the Kv7.2–Kv7.5-specific activator S-1 showing the effect of S-1 in rat mesenteric artery segments in the absence and presence of 10 µmol/L ciliobrevin D incubation. Mean logEC50 values were compared according to an unpaired t test with **P<0.01.
we used the nonselective (propranolol), β1-selective (bisoprolol) and β2-selective (ICI 118 551) adrenoceptor antagonists. Propanolol (10 nmol/L) inhibited the control relaxation to isoprenaline (logEC50 = −5.55±0.209 versus −6.4±0.1; N=6–7; P=0.009; Figure 3A and 3D) and was able to inhibit the ciliobrevin D-enhanced isoprenaline relaxation to a similar extent (logEC50 = −5.87±0.2 versus −7.1±0.1; N=6–7; P<0.001; Figure 3A and 3D). Bisoprolol (10 nmol/L) attenuated the control isoprenaline relaxation (logEC50 = −5.6±0.3 versus −6.4±0.1; N=7; P=0.01; Figure 3B and 3D) but had no effect on the ciliobrevin D-enhanced isoprenaline-mediated relaxation (N=6–7; Figure 3B and 3D). In contrast, ICI 118 551 (100 nmol/L) had no effect on the control isoprenaline relaxations but attenuated the ciliobrevin D-enhanced isoprenaline relaxations (logEC50 = −6.3±0.1 versus −7.1±0.07; N=6–7; P=0.01; Figure 3C and 3D). These data suggest that the ciliobrevin D-improved isoprenaline relaxations are mediated through increased β2- but not β1-adrenoceptor activity.

Ciliobrevin D and Colchicine Improve the Attenuated Isoprenaline Relaxations in Hypertensive Rat Arteries

In hypertensive animals, β-adrenergic responsiveness is impaired leading to attenuated vasorelaxations.15,18,19 We, therefore, tested whether ciliobrevin D could improve the attenuated β-adrenoceptor-mediated vasorelaxations in mesentric artery segments of spontaneously hypertensive rats (SHRs). Figure 4A and 4B confirmed the isoprenaline-mediated relaxation is attenuated in mesenteric artery segments from SHRs compared with Wistar-Kyoto rat (WKY) arteries (logEC50 = −5.7±0.2 versus −7.0±0.1; N=8–9; P=0.001; Figure 4A and 4B). Ciliobrevin D enhanced the isoprenaline-mediated relaxation in SHR mesenteric artery segments (logEC50 = −7.6±0.1 versus −5.7±0.2; N=5–6; P<0.001; Figure 4A and 4B), to the same level as in the ciliobrevin D-treated WKY arteries. Previously, we have shown that microtubule disruption with colchicine also enhanced β-adrenoceptor-mediated

Figure 3. Increased β2-adrenoceptor activity contributes to the ciliobrevin D-enhanced isoprenaline relaxation.

Mean concentration-effect curves showing the effect of (A) nonspecific β-adrenoceptor antagonist, propranolol (10 nmol/L), (B) β1-selective antagonist, bisoprolol (10 nmol/L), and (C) β2-selective antagonist, ICI 118 551 (100 nmol/L) on isoprenaline-mediated relaxations in control and ciliobrevin D incubated rat mesenteric arteries. D, Mean logEC50 values showing the effect of different β-adrenoceptor antagonists in the absence and presence of ciliobrevin D. A 2-way ANOVA was performed on logEC50 values to determine the source of variation – F ratio (df) and P value are denoted. A 1-way ANOVA followed by a Šidák multiple comparisons test was performed with *P<0.05, **P<0.01, and ***P<0.001.
Figure 4. Ciliobrevin D and colchicine improve the attenuated β-adrenoceptor-mediated relaxations in hypertensive rat arteries. A, Representative isometric tension recordings of Wistar-Kyoto rat (WKY; top) and spontaneously hypertensive rat (SHR; bottom) rat mesenteric artery segments preconstricted with methoxamine (∗) before sequentially increasing concentrations of isoprenaline were applied in control, ciliobrevin D (10 µmol/L) and colchicine (500 µmol/L)-treated arteries. Mean concentration-effect curves and logEC50 values to isoprenaline showing the effect of ciliobrevin D (B) and colchicine (C) on isoprenaline-mediated relaxations in WKY and SHR mesenteric artery segments, in the absence and presence of linopirdine (10 µmol/L). A 2-way ANOVA was performed on logEC50 values to determine the source of variation – F ratio (df) and P-value are denoted. A 1-way ANOVA followed by a Šídák multiple comparisons test was performed on the mean logEC50 values with *P<0.05, **P<0.01, and ***P<0.001.
vasorelaxation in normotensive Hannover mesenteric arteries (Lindman et al.11). Figure 4A and 4C shows that the colchicine enhanced the isoprenaline-mediated relaxation in SHR arteries to a similar degree as ciliobrevin D treatment (logEC50 from −5.6±0.3 to −7.7±0.2; N=7; P<0.001).

Next, we determined whether the improved β-adrenoceptor vasorelaxations in SHR arteries following ciliobrevin D treatment was due to an increased functional contribution of Kv7 channels. Similar to our findings in normotensive Hannover mesenteric artery segments, application of linopirdine partially attenuated the control and ciliobrevin D-enhanced isoprenaline-mediated relaxations in the WKY arteries (N=5; C=$0.002 and N=6; C=0.03, respectively; Figure 4B). In addition, linopirdine partially attenuated the control and ciliobrevin D-enhanced isoprenaline-mediated relaxations in arteries from both WKY or SHR arteries (Figure 6A and 6B). In the WKY and SHR mesenteric artery segments, application of ICI 118551 (100 nmol/L) had no effect on isoprenaline relaxations under control conditions but inhibited the ciliobrevin D-enhanced isoprenaline relaxations (WKY: N=6; C<0.001 and SHR: N=7; C=0.008; Figure 6B). These findings suggest that the ciliobrevin D-enhanced isoprenaline relaxation is mediated through an increase in β2-adrenoceptor signaling.

**Kv7 Channel Function and Expression Is Enhanced in WKY and SHR After Dynein Inhibition**

To determine whether the ciliobrevin D-enhanced Kv7 channel function in WKY and SHR mesenteric arteries, we applied the Kv72 to Kv75 activator, ML213, to pre-constricted mesenteric artery segments. In segments from the SHR, relaxations to ML213 were attenuated compared with WKY arteries (logEC50 −5.3±0.2 versus −6.5±0.2; N=9; C=0.001; Figure 5A). Ciliobrevin D and colchicine treatment both enhanced the ML213 relaxation in the WKY and SHR artery segments (Figure 5A). In isolated vascular smooth muscle cells, total protein expression of Kv7.4 was decreased in the SHR compared with WKY mesenteric artery myocytes (N=3, n=8–10; C=0.003; Figure 5B and 5C). Treatment of the isolated myocytes with ciliobrevin D for 30 minutes increased the Kv7.4 membrane expression relative to total expression in both WKY and SHR mesenteric artery myocytes (N=3, n=8–10; C=0.0013; Figure 5B and 5C).

**Dynein Inhibition Increases β2-Adrenoceptor Signaling in WKY and SHR Arteries**

Application of bisoprolol (10 nmol/L) inhibited the isoprenaline relaxation in the WKY artery segments under control conditions by shifting the logEC50 from −6.9±0.2 to −6.2±0.1 (N=6–10; C=0.01) but had no effect on the control isoprenaline relaxation in the SHR artery segments (N=5–6; Figure 6B). Bisoprolol had no effect on the ciliobrevin D-enhanced isoprenaline-mediated relaxations in arteries from both WKY or SHR arteries (Figure 6A and 6B). In the WKY and SHR mesenteric artery segments, application of ICI 118551 (100 nmol/L) had no effect on isoprenaline relaxations under control conditions but inhibited the ciliobrevin D-enhanced isoprenaline relaxations (WKY: N=6; C<0.001 and SHR: N=7; C=0.008; Figure 6B). These findings suggest that the ciliobrevin D-enhanced isoprenaline relaxation is mediated through an increase in β2-adrenoceptor signaling.

**Agonist-Induced β2-Adrenoceptor Internalization Is Prevented With Ciliobrevin D**

Agonist stimulation of β-adrenoceptors can lead to desensitization and receptor internalization.31 Furthermore, it is reported that β2-adrenoceptor internalization is microtubule-dependent.32 Since dynein is an important motor protein that traffics endocytosed cargo vesicles away from the membrane to facilitate internalization,33 we examined whether dynein inhibition with ciliobrevin D can reduce agonist-induced β2-adrenoceptor internalization. Membrane abundance of β2-adrenoceptors was slightly decreased in freshly isolated mesenteric artery smooth muscle cells from SHRs compared with WKY (N=3; n=9–10; C=0.01; Figure 6C and 6D). Treatment of the isolated myocytes with isoprenaline (1 µmol/L) for 20 minutes before fixing, decreased β2-adrenoceptor membrane expression compared with nontreated artery myocytes in both WKY (N=3, n=9–10; C=0.0007) and SHR (N=3, n=9; C=0.003; Figure 6C and 6D). Strikingly, preincubation of the myocytes with ciliobrevin D (3 µmol/L) for 10 minutes before adding isoprenaline (1 µmol/L) prevented the decrease in β2-adrenoceptor membrane expression (Figure 6C and 6D). In contrast, isoprenaline had no effect on the β1-adrenoceptor membrane expression (Figure S2). These results indicate isoprenaline can cause internalization of β2-adrenoceptors in isolated mesenteric artery myocytes, which is prevented by dynein inhibition.

**DISCUSSION**

β-Adrenoceptor signaling stimulates adenylate cyclase, which elevates cAMP production in smooth muscle. Although many downstream effectors are activated via a cAMP-dependent mechanism, it is now well established that β-adrenoceptor stimulation activates voltage-gated Kv7 channels in vascular smooth muscle. Both Kv7.4 and Kv7.5 have been implicated as downstream targets of β2-adrenoceptor stimulation. However, their role in β-adrenoceptor signaling has not been fully explored in vascular smooth muscle. In this study, we confirm that Kv7 channels are stimulated following β-adrenoceptor activation and our morpholino
Figure 5. Ciliobrevin D enhances voltage-gated potassium channel (Kv)7 channel function and expression in rat mesenteric arteries. A, Mean concentration-effect curves and logEC\textsubscript{50} values to the Kv7.2–Kv7.5–specific activator ML213 showing the effect of ML213 in Wistar-Kyoto rat (WKY) and spontaneously hypertensive rat (SHR) rat mesenteric artery segments in control and after ciliobrevin D (10 µmol/L) incubation. A 2-way ANOVA was performed on logEC\textsubscript{50} values to determine the source of variation – F ratio (df) and P value are denoted. A 1-way ANOVA followed by a Šídák multiple comparisons test was performed on the mean EC\textsubscript{50} values with *P<0.05, **P<0.01, and ***P<0.001. B, Representative mid-cell section of a mesenteric artery myocyte treated with or without ciliobrevin D (3 µmol/L) and stained for Kv7.4 (green) and wheat germ agglutinin (WGA; purple). Scale bars, 5 µm. C, Mean total Kv7.4 intensity and Kv7.4 membrane intensity in cells treated with ciliobrevin D compared with nontreated cells were calculated from a single mid-cell section. Significance was determined by a 1-way ANOVA followed by a Tukey multiple comparisons test. **P<0.01.
Figure 6. Ciliobrevin D increases β2-adrenoceptor signaling in Wistar-Kyoto rat (WKY) and spontaneously hypertensive rat (SHR) arteries and prevents isoprenaline-induced β2-adrenoceptor internalization. Mean concentration-effect curves and logEC50 values to isoprenaline in the presence of β1-selective antagonist, bisoprolol (10 nmol/L) and β2-selective antagonist, ICI 118 551 (100 nmol/L), in WKY (A) and SHR (B) rat mesenteric artery segments in control and after ciliobrevin D (10 µmol/L) incubation. A 2-way ANOVA was performed on logEC50 values to determine the source of variation – F ratio (df) and P value are denoted. A 1-way ANOVA followed by a Šídák multiple comparisons test was performed on the mean logEC50 values with *P < 0.05, **P < 0.01, and ***P < 0.001. C, Representative mid-cell section of WKY and SHR mesenteric artery myocytes from nontreated (control), isoprenaline (1 µmol/L) or ciliobrevin (3 µmol/L) + isoprenaline (1 µmol/L) treated cells, stained for β2-adrenoceptors (red) and WGA (green). Scale bars, 5 µm. D, Mean membrane intensities in the different groups of treated cells were calculated from a single mid-cell section. Significance was determined by a 1-way ANOVA followed by a Tukey multiple comparisons test with *P < 0.05 and ***P < 0.001.
data suggest an important role for Kv7.4 in eliciting the relaxation in rat mesenteric arteries.

Our laboratory showed previously that microtubule disruption with colchicine and nocodazole enhanced β-adrenoceptor-mediated vasorelaxation, which was at least partially mediated by an increase in Kv7 channel function and elevated membrane levels of the Kv7.4 protein. Moreover, we showed recently that the microtubule motor protein dynein, targeted Kv7.4 channels for retrograde trafficking away from the cell membrane. In the present study, we investigated whether this dynein-dependent regulation of Kv7.4 channels influenced β-adrenoceptor-mediated vasorelaxation in vascular smooth muscle cells. Dynein inhibition with ciliobrevin D enhanced the isoprenaline relaxation in ex vivo artery segments from both Wistar Hannover and Kyoto rat strains, in a similar manner to microtubule disruption. With our in vivo myograph model, we were able to confirm the ciliobrevin D effects in a more physiological system. Interestingly, we observed a lack of control relaxation to isoprenaline with the intra vital myography. Under in vivo conditions, it is possible that β-adrenoceptor relaxations are masked but are apparent (perhaps unphysiologically) under ex vivo wire myograph conditions. However, by using intra vital myography, we show that ciliobrevin D can induce isoprenaline relaxations under in vivo conditions, thereby strengthening the translatability of our findings.

In the presence of ciliobrevin D, Kv7 channels still contributed to the isoprenaline relaxation. However, the logEC50 shift of linopirdine on the isoprenaline relaxation was comparable in the absence and presence of ciliobrevin D suggesting that there was not an enhanced contribution of Kv7 channel function in these relaxations. Conversely, at the lower concentrations of isoprenaline, the ciliobrevin D-enhanced isoprenaline relaxation is completely inhibited by linopirdine, reflecting an important contribution of Kv7 channels in mediating this ciliobrevin D effect. Moreover, although morpholino-induced Kv7.4 channel knockdown attenuated the ciliobrevin D-enhanced isoprenaline responses, it was unable to inhibit the vasorelaxation to the same extent as the Kv7.4 knockdown control artery segments. These data suggest that the enhanced isoprenaline-mediated relaxation with dynein inhibition is only partially mediated through Kv7 channels and thus cannot solely be explained by increased Kv7.4 channel function. Functional and molecular studies show that Kv7.4 and Kv7.5 are most likely to exist as a heteromeric channel in vascular smooth muscle cells with the participation of the accessory subunit KCNE4. Whether Kv7.5 channels are similarly regulated by dynein, thereby contributing to the effects observed with linopirdine, remains to be determined. Interestingly, BKCa channel inhibition had no effect on the isoprenaline-mediated relaxation in ciliobrevin D-treated arterial segments but did inhibit the isoprenaline-mediated relaxation in control segments. This is in line with our previous finding that colchicine treatment of mesenteric arteries also removed the effect of BKCa channel inhibition on isoprenaline-mediated relaxations. In the current study, we have not investigated the role of the microtubule network or dynein on BKCa channel function, however, a previous study found BKCa channel function was attenuated in mouse cerebral arteries by microtubule depolarization, which was caused by a dissociation of the channel from calcium released by the sarcoplasmic reticulum. In addition, a recent study found a functional interdependency between Kv7 and BKCa channels in the rat saphenous artery. Based on this model, colchicine or ciliobrevin D would increase the number of activated Kv7 channels following isoprenaline application and increase the hyperpolarization of the arterial myocyte, thereby reducing the functional impact of membrane depolarization by iberiotoxin. Whether BKCa channel activity is attenuated by a direct effect of dynein inhibition, or the functional impact of iberiotoxin is reduced by increased Kv7 channel activity remains to be determined.

Since the ciliobrevin D-enhanced isoprenaline responses can only partially be attributed to increased Kv7 channel function, we sought to determine upstream mechanisms underlying the increased isoprenaline response. Isoprenaline binds primarily to β1 and β2-adrenoceptors on arterial smooth muscle cells. Using selective β1- and β2-adrenoceptor antagonists, we data support a predominating role of β1-adrenoceptors underling the isoprenaline-mediated vasorelaxations in control rat mesenteric arteries. However, we found that increased β2-adrenoceptor activity was responsible for the ciliobrevin D-enhanced isoprenaline relaxation, since ICI 118551 abolished the effect of ciliobrevin D on the isoprenaline relaxation. In contrast, the β1 blocker, bisoprolol, had no effect on the isoprenaline response in the presence of ciliobrevin D, perhaps because the β2-adrenoceptor pathway now predominates. In the presence of propranolol, at a concentration known to inhibit β1/2 adrenoceptors, a relaxation at the higher concentrations of isoprenaline was still observed. It is possible this occurs through the β3-adrenoceptors found on the endothelium, whose function is normally suppressed by β1/2 adrenoceptors. We did not explore whether the β3-adrenoceptor activity was altered following treatment with ciliobrevin D, however, given that propranolol and ICI 118551 completely inhibited the improved isoprenaline relaxation, it seems unlikely that β3-adrenoceptors play a role. Thus, dynein inhibition increases the isoprenaline-induced relaxation.
Dysfunction of the β-adrenoceptor pathway is a dominant feature in blood vessels from hypertensive animals, however, little is known about the molecular mechanisms responsible. In the renal artery of the SHR, where Kv7.4 expression is reduced, isoprenaline does not cause a relaxation, in line with an attenuated relaxation to Kv7 activators. Our study confirms compromised β-adrenoceptor relaxation and Kv7 channel activity in mesenteric artery segments from hypertensive rats and reduced total Kv7.4 protein levels in SHR mesenteric myocytes, compared with normotensive controls. Based on our findings that dynein inhibition and microtubule disruption by colchicine, as previously shown, could enhance the β-adrenoceptor relaxation in normotensive rat mesenteric arteries, we tested whether cilobrevin D or colchicine could improve the attenuated β-adrenoceptor-mediated vasorelaxation in mesenteric artery segments of the hypertensive rat. Both drugs enhanced the isoprenaline response in SHR mesenteric artery segments, to the same extent as in normotensive WKY mesenteric arteries. Furthermore, dynein inhibition enhanced the diminished responses to the Kv7.2 to Kv7.5 channel activator, ML213, in SHR arteries, providing strong evidence that dynein inhibition can reinstate Kv7 channel function in arteries from hypertensive rats, which may contribute to enhanced isoprenaline relaxations, particularly at the lower concentrations. We showed previously that dynein inhibition increased the membrane levels of Kv7.4 protein in isolated smooth muscle cells from the mesenteric arteries of normotensive Wistar rats. In this study, we confirm that dynein inhibition increased the membrane levels of Kv7.4 protein in smooth muscle cells from normotensive WKY rats and also SHR. Thus, although the total expression of Kv7.4 protein is lower in vascular smooth muscle cells of the SHR, dynein inhibition can increase the functional contribution of the channel by increasing the membrane levels of the protein, thereby helping to restore the β-adrenoceptor function in arteries from hypertensive animals.

Interestingly, it has previously been shown that angiotensin II enhanced the interaction of Kv7.4 with the E3 ubiquitin ligase CHIP (C terminus of Hsp70 [heat shock protein]-interacting protein), and thereby promotes Kv7.4 ubiquitination and degradation in vascular smooth muscle cells. Dynein can associate with Hsp70 for microtubule minus-end directed transport and ultimately degradation by the aggresome. Therefore, dynein might regulate the CHIP dependent degradation of Kv7.4 channels in arteries. Whether this Kv7.4-CHIP-dynein degradation mechanism is present in mesenteric arteries and augmented in hypertension remains to be clarified.

We also investigated the effects of β1- or β2-adrenoceptor-selective antagonists on the isoprenaline responses in WKY and SHR mesenteric arteries. In the SHR, the β1-adrenoceptor response was compromised, since bisoprolol no longer had an effect on the control isoprenaline relaxation, and, as in the WKY, ICI 118 551 also had no effect on the control isoprenaline relaxation. In line with mesenteric arteries from the Wistar Hannover, we found that ICI 118 551 inhibited the cilobrevin D-enhanced isoprenaline response for both WKY and SHR artery segments. In contrast, the β1-adrenoceptor-selective antagonist, bisoprolol, did not affect the cilobrevin D-enhanced relaxation. These functional data indicate that dynein inhibition increases the functional contribution of the β2-adrenoceptors to isoprenaline-mediated relaxations. This mechanism can reinstate the isoprenaline-mediated relaxation in the SHR by functionally substituting the compromised β1-adrenoceptor with β2-adrenoceptors. Previous studies found that agonist stimulation of β-adrenoceptors can lead to internalization of the receptor, resulting in a decreased abundance of functional β-adrenoceptors in the plasma membrane. Furthermore, previous studies report that β2-adrenoceptors are more prone to agonist-induced internalization compared with β1-adrenoceptors. β-Arrestin is involved in the agonist-induced internalization of the β2-adrenoceptors. Dynein was identified in the β-arrestin interactome and other receptors are known to undergo agonist-induced internalization that is β- arrestin and microtubule dependent. In this study, we have not investigated the complex internalization process involving β-arrestin. Importantly, a previous study revealed that disruption of microtubules with nocodazole blocked the isoprenaline-induced internalization of β2-adrenoceptors. Furthermore, it has been well described that dynein is important for endosomal sorting, where portions of the plasma membrane, containing receptors and their ligands, are internalized and subsequently sorted to various locations to be recycled or degraded. That dynein plays a crucial role in this mechanism is supported by reports showing reduced and disorganized trafficking of endocytic vesicles after dynein complex disruption or inhibition. Given that internalization and endosomal trafficking of internalized membrane vesicles is microtubule and dynein dependent, we postulated that cilobrevin D can prevent β2-adrenoceptor internalization, and thereby increase the membrane abundance of the receptor, which could explain the increased β2-adrenoceptor functional contribution to isoprenaline responses observed with dynein inhibition. Our immunocytochemistry experiments revealed that β2-adrenoceptors in the membrane of both WKY and SHR mesenteric artery myocytes were decreased with isoprenaline stimulation, suggesting agonist-induced receptor internalization. Strikingly, pre-incubation of the myocytes with cilobrevin D prevented the isoprenaline-induced decrease of β2-adrenoceptors in the plasma membrane. Together with the functional myography data, these results provide compelling evidence that the motor protein dynein plays a key role in
Agriculture to the agonist-induced internalization of β2-adrenoceptors in vascular smooth muscle.

**REFERENCES**


