Impaired Isoproterenol-Induced Hyperpolarization in Isolated Mesenteric Arteries of Aged Rats

Koji Fujii, Uran Onaka, Kenichi Goto, Isao Abe, Masatoshi Fujishima

Abstract—Stimulation of vascular β-adrenoceptors leads to membrane hyperpolarization, presumably via the β-adrenoceptor/G, protein/adenylate cyclase signaling cascade; the ionic mechanisms of this phenomenon remain unclear. β-Adrenoceptor–mediated vascular relaxation is impaired with aging; however, little is known concerning whether β-adrenoceptor–mediated hyperpolarization is altered with aging. We sought to determine the ionic mechanisms of isoproterenol-induced hyperpolarization in the rat mesenteric resistance artery, as well as the age-related changes in isoproterenol-induced hyperpolarization and their underlying mechanisms. Isoproterenol-induced hyperpolarization was inhibited by high-K+ solution and glibenclamide (10−6 mol/L), an inhibitor of ATP-sensitive K+ channels (KATP), but not by apamin, iberiotoxin, or charybdotoxin, inhibitors of Ca2+-activated K+ channels. Isoproterenol-induced hyperpolarization was markedly less in aged rats (≥24 months) than in adults rats (12 to 20 weeks) (3×10−6 mol/L; −3.1 versus −9.9 mV; P<0.001; n=8 to 9). Cholera toxin (10−9 g/mL), an activator of Gs, evoked hyperpolarization only in adult rats. Hyperpolarization to forskolin, a direct activator of adenylate cyclase, was also reduced to some extent in aged rats (10−5 mol/L; −8.8 versus −13 mV; P<0.05; n=6), whereas hyperpolarization to levromakalim, a KATP opener, was comparable in both groups. These findings suggest that isoproterenol elicits hyperpolarization via an opening of KATP in the rat resistance artery and that isoproterenol-induced hyperpolarization is attenuated in aged rats mainly because of a defective coupling of β-adrenoceptors to adenylate cyclase and partly because of a defect at the level of adenylate cyclase, but not because of an alteration of KATP per se. (Hypertension. 1999;34:222-228.)

Key Words: receptors, adrenergic, beta ■ hyperpolarization ■ potassium channels ■ aging ■ muscle, smooth, vascular

Stimulation of β-adrenoceptors leads to vascular relaxation, which involves the following signaling cascade: β-adrenoceptor/stimulatory guanine nucleotide regulatory protein (G, protein)/adenylate cyclase/cAMP.1–4 In addition, β-agonists, eg, isoproterenol or norepinephrine, induce membrane hyperpolarization in various smooth muscle cells,5–8 and such hyperpolarization has been demonstrated to play an important role in the regulation of membrane potential of smooth muscle cells.8,9 Several recent studies suggest possible involvement of K+ channels in this hyperpolarization10–12; however, controversy exists regarding the type of K+ channels involved.10–12

β-Adrenoceptor–mediated vasodilatation is impaired with aging in both humans13,14 and animals.15–19 Several mechanisms have been proposed to account for this impairment, such as defective Gs protein16,20 and abnormality of cAMP-dependent protein kinase A (PKA).15,17 On the other hand, little is known about whether β-adrenoceptor–mediated hyperpolarization alters with aging.

The first goal of this study was to determine ionic mechanisms of isoproterenol-induced hyperpolarization in the rat mesenteric resistance artery, and the second goal was to evaluate age-related changes in isoproterenol-induced hyperpolarization and their underlying mechanisms.

Methods

Preparation of Arteries

Twelve- to 20-week-old and 24- to 26-month-old male Wistar-Kyoto rats were used in the present study. The rats were originally purchased from Charles River Co, Ltd (Atsugi, Japan) and maintained at the animal center of Kyushu University. They had free access to tap water and were fed a normal rat chow. The study protocol was approved by the Animal Experimentation Ethics Committee of Kyushu University. Systolic blood pressure was measured by the tail-cuff method. Rats were anesthetized with ether and exsanguinated. The mesenteric vascular bed was excised and placed on a plate containing cold Krebs’ solution. The third or fourth branches of the arteries (external diameter, 100 to 150 μm) were cut and cleaned of adherent connective tissue. In some preparations, the endothelium was removed by rubbing the intimal surface with small rugged pins. The absence of the endothelium was verified by the lack of hyperpolarization to acetylcholine.21

Recording of Membrane Potentials

The arteries were placed in an experimental chamber (capacity, 2 mL). Tissues were carefully pinned to a rubber bed fixed at the bottom of the chamber and were superfused with Krebs’ solution.
(36°C) bubbled with 95% O₂ and 5% CO₂ (pH 7.3 to 7.4) at a rate of 3 mL/min. The arteries were allowed to equilibrate for ≥60 minutes before recordings were started. Membrane potentials were recorded with glass capillary microelectrodes filled with 3 mol/L KCl and with tip resistances of 50 to 80 MΩ and tip potentials of <4 mV.²¹⁻²² Microelectrodes were impaled into the smooth muscle cell from the adventitial side. Criteria for successful impalement were an abrupt drop in voltage on entry of the microelectrode into the cell, a stable membrane potential for ≥2 minutes, and a sharp return of the membrane potential to zero on withdrawal of the electrode. Electric responses were monitored on an oscilloscope (VC-11, Nihon Kohden Co Ltd) and recorded with a pen writing recorder (RIG-4002, Nihon Kohden Co Ltd).

**Solutions and Drugs**

The ionic composition of Krebs’ solution was as follows (mmol/L): Na⁺ 137.4, K⁺ 5.9, Mg²⁺ 1.2, Ca²⁺ 2.5, HCO₃⁻ 15.5, H₂PO₄⁻ 1.2, Cl⁻ 134, and glucose 11.5. Drugs used were (−)-isoproterenol hydrochloride, forskolin, glibenclamide, acetylcholine chloride, dibutyryl cAMP, propranolol hydrochloride, phenolamine maleate, bumetanide, nitroprusside, nitroglycerin, and diltiazem hydrochloride (Biomol), apamin, iberiotoxin, and charybdotoxin (Peptide Institute). Levcromakalim was dissolved in ethanol. Forskolin, glibenclamide, at doses of 10⁻⁶ mol/L, depolarized the membrane by 3.6±1.7 mV (n=7) and 5.1±1.6 mV (n=6), respectively. Hyperpolarization to forskolin (10⁻⁵ mol/L), a direct activator of adenylyl cyclase,²⁸ was also inhibited by glibenclamide (−10.8±0.9 and −3.4±0.9 mV in the absence and presence of 10⁻⁵ mol/L glibenclamide; n=5; P<0.05). Cholera toxin (10⁻⁶ g/mL), a direct activator of Gs, also produced hyperpolarization, which was sensitive to glibenclamide (−10.5±0.3 and −3.0±0.2 mV in the absence and presence of 10⁻⁵ mol/L glibenclamide; n=10 and n=5, respectively; P<0.05). On the other hand, at a concentration of 10⁻⁵ mol/L, glibenclamide did not affect hyperpolarization to acetylcholine (10⁻⁶ mol/L) (−13.3±1.8 and −12.8±1.6 mV in the absence and presence of 10⁻⁵ mol/L glibenclamide; n=5). Apamin (10⁻⁶ mol/L),²⁹ iberiotoxin (3×10⁻⁸ mol/L),³⁰ and charybdotoxin (10⁻⁷ mol/L),³¹ inhibitors of small-, large-, and intermediate- and large-conductance Ca²⁺-activated K⁺ channels, respectively, had no effect on isoproterenol-induced hyperpolarization (Figure 2).

**Statistical Analysis**

Data are given as mean±SEM. The number of animals is indicated by n. The average value of membrane potentials obtained from multiple impalments was calculated for each animal. These values were then used for statistical comparison of the membrane potential between the groups. Statistical analysis was performed by 2-way ANOVA, followed by Scheffé’s test for multiple comparison or by unpaired Student’s t test. Probability values <0.05 were considered statistically significant.

**Results**

Body weight was significantly greater in aged (n=17) than in adult (n=37) rats (366.5±18.4 versus 282.8±4.7 g; P<0.001). Systolic blood pressure was also significantly higher in aged than in adult rats (157.9±4.3 versus 143.9±3.0 mm Hg; P<0.05).

Resting membrane potential of the mesenteric resistance arteries was −62.1±0.8 mV (n=37) for adult rats and −61.4±1.6 mV (n=17) for aged rats and did not differ between the 2 groups.

Isoproterenol, a relatively selective β-agonist, produced hyperpolarization in mesenteric arteries (Figures 1 to 3), which was subsequently abolished by propranolol (10⁻⁶ mol/L) (data not shown). Some characteristics of this hyperpolarization were investigated in arteries from adult rats. Isoproterenol-induced hyperpolarization was slightly but significantly inhibited by butoxamine, a relatively selective β₁-adrenoceptor antagonist,²³⁻²⁴ and was markedly inhibited by metoprolol, a selective β₁-adrenoceptor antagonist²⁵ (Figure 1). The combined application of these 2 agents nearly abolished isoproterenol-induced hyperpolarization (Figure 1). Isoproterenol-induced hyperpolarization was still observed in endothelium-rubbed preparations (3×10⁻⁶ mol/L; −8.3±0.3 mV; n=4), suggesting that isoproterenol acts directly on smooth muscle cells to elicit hyperpolarization. High-K⁺ solution (20 mmol/L) and TBA (1 mmol/L), a nonspecific blocker of K⁺ channels,²⁶ markedly inhibited isoproterenol-induced hyperpolarization (Figure 2). Glibenclamide, an inhibitor of ATP-sensitive K⁺ channels (KATP),²⁷ also inhibited isoproterenol-induced hyperpolarization (Figure 2). Glibenclamide, at doses of 10⁻⁶ and 10⁻⁵ mol/L, depolarized the membrane by 3.6±1.7 mV (n=7) and 5.1±1.6 mV (n=6), respectively. Hyperpolarization to forskolin (10⁻⁵ mol/L), a direct activator of adenylate cyclase,²⁸ was also inhibited by glibenclamide (−10.8±0.9 and −3.4±0.9 mV in the absence and presence of 10⁻⁵ mol/L glibenclamide; n=5; P<0.05). Cholera toxin (10⁻⁶ g/mL), a direct activator of Gs, also produced hyperpolarization, which was sensitive to glibenclamide (−10.5±0.3 and −3.0±0.2 mV in the absence and presence of 10⁻⁵ mol/L glibenclamide; n=10 and n=5, respectively; P<0.05). On the other hand, at a concentration of 10⁻⁵ mol/L, glibenclamide did not affect hyperpolarization to acetylcholine (10⁻⁶ mol/L) (−13.3±1.8 and −12.8±1.6 mV in the absence and presence of 10⁻⁵ mol/L glibenclamide; n=5). Apamin (10⁻⁶ mol/L),²⁹ iberiotoxin (3×10⁻⁸ mol/L),³⁰ and charybdotoxin (10⁻⁷ mol/L),³¹ inhibitors of small-, large-, and intermediate- and large-conductance Ca²⁺-activated K⁺ channels, respectively, had no effect on isoproterenol-induced hyperpolarization (Figure 2).

Isoproterenol (10⁻⁶ mol/L)–induced hyperpolarization was markedly inhibited by H-89 (10⁻⁵ mol/L), an inhibitor of PKA²² (−9.9±0.3 and −2.7±0.5 mV in the absence and presence of H-89; n=5; P<0.001). In addition, dibutyryl cAMP (10⁻⁵ mol/L), a cell-permeable analogue of cAMP,³³ produced hyperpolarization by −6.4±1.2 mV (n=5), which was reversed by application of glibenclamide (10⁻⁵ mol/L).

Isoproterenol-induced hyperpolarization was markedly less in aged rats than in adult rats (Figure 3). Pretreatment with
Phentolamine did not affect the hyperpolarization to isoproterenol in aged rats (isoproterenol \(3 \times 10^{-6}\) mol/L; \(2.1.5 \pm 0.3\) and \(2.1.3 \pm 0.3\) mV before and after treatment with \(10^{-6}\) mol/L phentolamine; \(n=4\); \(P=NS\)). Forskolin-induced hyperpolarization also tended to be smaller in aged rats than in adult rats at a concentration of \(10^{-7}\) mol/L, and the difference was statistically significant at concentrations of \(10^{-5}\) and \(10^{-4}\) mol/L (Figure 4). Hyperpolarization to levomakalim, a direct activator of KATP, was comparable between adult and aged rats (Figure 5).

Hyperpolarization to cholera toxin (\(10^{-6}\) g/mL), a direct activator of \(G_{s}\), was virtually absent in aged rats (0.7 ± 0.4 mV; \(n=11\)) and was significantly smaller in aged rats than in adult rats (−2.8 ± 0.7 mV; \(n=10\); \(P=0.01\), aged versus adult rats).

### Discussion

The present study demonstrated that (1) isoproterenol-induced hyperpolarization in the rat mesenteric resistance artery was inhibited by glibenclamide; (2) isoproterenol-induced hyperpolarization was markedly impaired in aged rats; (3) forskolin-induced hyperpolarization also tended to be reduced in aged rats; and (4) levomakalim-induced hyperpolarization was comparable between adult and aged rats. These findings suggest that isoproterenol-induced hyperpolarization is mediated by an opening of KATP in the rat mesenteric artery and is attenuated in aged rats mainly because of a defective coupling of \(\beta\)-adrenoceptor to adenylate cyclase and partly because of an abnormality at the level of adenylate cyclase, but not because of an alteration of KATP per se.

### Ionic Mechanisms of Isoproterenol-Induced Hyperpolarization

The ionic mechanisms underlying smooth muscle hyperpolarization after \(\beta\)-adrenoceptor stimulation have not been fully elucidated. Possible mechanisms include an increase in K⁺ conductance and stimulation of the electrogenic Na-K pump. Recently, with the use of a patch clamp technique, Miyoshi et al. demonstrated that the catalytic subunit of PKA activated KATP in cultured porcine coronary smooth muscle cells, whereas Sadoshima et al. showed that cAMP and PKA activated Ca²⁺-activated K⁺ channels in cultured rat aortic smooth muscle cells. With a conventional microelectrode technique, Nakashima and Vanhoutte showed that isoproterenol induced hyperpolarization through an opening of KATP in the canine saphenous vein.

In the present study, isoproterenol-induced hyperpolarization was abolished by high-K⁺ solution and was markedly inhibited by TBA, a nonspecific blocker of K⁺ channels, suggesting that the hyperpolarization was due mostly to an opening of K⁺ channels. Furthermore, isoproterenol-induced hyperpolarization was nearly abolished by glibenclamide, a selective inhibitor of KATP, but not by apamin, iberiotoxin, or charybdotoxin, inhibitors of small-, large-, and intermediate- and large-conductance Ca²⁺-activated K⁺ channels, respectively. Nonspecific inhibition of hyperpolarization by glibenclamide seems unlikely because this agent did not inhibit acetylcholine-induced hyperpolarization. These findings suggest that isoproterenol induces hyperpolarization through the opening of KATP in the rat mesenteric artery.
addition, isoproterenol-induced hyperpolarization was mimicked by forskolin, a direct activator of adenylate cyclase, and inhibited by glibenclamide. Cholera toxin, a direct activator of Gs protein, also elicited membrane hyperpolarization in adult rats, which was sensitive to glibenclamide. It thus appears that isoproterenol-induced hyperpolarization in the rat mesenteric resistance artery is achieved primarily through the following signaling cascade: \( \beta \)-adrenoceptor/Gs protein/adenylate cyclase/KATP. It remains to be determined whether cAMP/PKA is involved in the activation of KATP. However, our present findings that dibutyryl cAMP, a cell-permeable analogue of cAMP, induced glibenclamide-sensitive hyperpolarization and that isoproterenol-induced hyperpolarization was inhibited by PKA inhibitor H-89 favor the possibility that the cAMP/PKA cascade is involved in isoproterenol-induced hyperpolarization.

The predominant \( \beta \)-adrenoceptor subtype that mediates isoproterenol-induced hyperpolarization in the rat mesenteric artery might belong to a \( \beta_1 \) subtype rather than a \( \beta_2 \) subtype because metoprolol, a selective antagonist of \( \beta_1 \)-adrenoceptor, appeared to inhibit isoproterenol-induced hyperpolarization to a greater extent than butoxamine, a relatively selective \( \beta_2 \) antagonist. This finding is in agreement with that found in the canine large coronary artery, in which \( \beta_1 \)-adrenoceptor played a predominant role in vasodilation, but differs from that found in the canine saphenous vein, in which \( \beta_2 \)-adrenoceptor is mainly involved in isoproterenol-induced hyperpolarization. Further studies are necessary to fully characterize the receptor subtype involved in \( \beta \)-adrenergic hyperpolarization.

**Impaired Isoproterenol-Induced Hyperpolarization in Aged Rats**

Although several studies have shown that relaxation to isoproterenol is impaired in isolated blood vessels from aged animals, this study is the first to demonstrate that isoproterenol-induced hyperpolarization is decreased in arteries from aged rats. Stekiel et al demonstrated that in situ hyperpolarization produced by isoproterenol in cremaster muscle arterioles was markedly reduced in hypertensive, reduced renal mass rats compared with normotensive control rats. It appears, therefore, that isoproterenol-induced hyperpolarization diminishes with aging as well as in hypertension. Blood pressure was slightly but significantly higher in aged rats than in adult rats. However, isoproterenol-induced hyperpolarization was generally reduced in all the aged rats regardless of individual blood pressure, implying that the impairment in aged rats could not...
be ascribed to high blood pressure. Furthermore, it cannot be
generalized from this study alone whether the loss of β-adrenoceptor–mediated hyperpolarization contributed to the
elevation of blood pressure in aged rats.

β-Adrenoceptors are also present at presynaptic nerve terminals to facilitate the release of adrenergic neurotransmit-
ters.42 If the facilitatory action of isoproterenol on transmitter release is enhanced, the excess catecholamines released might
depolarize the membrane by acting on postsynaptic α-adrenoceptors, thereby counteracting isoproterenol-
induced hyperpolarization. However, such a mechanism is unlikely to explain the present findings, because
isoproterenol-induced hyperpolarization in aged rats was still impaired under the blockade of α-adrenoceptors with phentolamine. On the same grounds, it is unlikely that direct
stimulation of α-adrenoceptors by isoproterenol offset β-adrenoceptor–mediated hyperpolarization in aged rats. Thus,
the ability of isoproterenol to produce hyperpolarization per se may be impaired with aging.

Mechanisms Underlying Impaired Isoproterenol-Induced Hyperpolarization in Aged Rats

As mentioned previously, isoproterenol-induced hyperpolarization may be achieved through the β-adrenoceptor/Gs protein/adenylate cyclase/KATP signaling cascade. In the present
study, the age-related impairment of the maximal hyperpolarization to isoproterenol appeared to be more marked than
that to forskolin, implying that the major defect responsible for the impaired β-adrenergic hyperpolarization may lie at the
level of the β-adrenoceptor/Gs protein/adenylate cyclase coupling step, and the abnormality of adenylate cyclase may also
partially account for the impairment. The lack of hyperpolarization to chérea toxin in aged rats might suggest the
defective Gs protein.

The aforementioned assumption may be consistent with some of the previous studies in β-adrenoceptor–mediated relaxations in aged animals. Kazanietz and Enero18 reported
that relaxation to isoproterenol, but not to forskolin, was reduced in the aorta of aged rats. In their study, increases in
cAMP in response to both isoproterenol and cholera toxin were also reduced in aged rats. On the other hand, Tsujimoto
et al15 demonstrated that the sensitivity, but not the maximal response, to forskolin was reduced along with a marked
reduction in the maximal relaxation to isoproterenol in the mesenteric artery of aged rats. They also showed the reduced
relaxation response to dibutylryl cAMP in vessels from aged rats. Thus, previous studies appear to agree that the coupling
step of β-adrenoceptors to adenylate cyclase is defective in aged animals but differ as to whether adenylate cyclase
(and/or a more distal step) is altered.

The present study agrees with that of Tsujimoto et al15 in that the response to forskolin was also altered with aging, but
it differs in that the maximal hyperpolarization to forskolin was impaired in this study, whereas only the relaxation sensitivity to forskolin was reduced in their study. The present data alone are not sufficient to evaluate sensitivity to forskolin, and it thus remains to be further determined whether there may also be an age-related alteration in the sensitivity to forskolin regarding hyperpolarization. Differences between this study and previous studies may arise from differences in the vascular bed studied, vessel size, or the type of response examined, ie, hyperpolarization or relaxation. Characteristics of K<sub>ATP</sub> itself, likely a final step of β-adrenergic hyperpolarization, may not be altered with aging, because hyperpolarization to the direct K<sub>ATP</sub> opener levcromakalim was comparable between adult and aged rats in the present study.

In a previous study on the rat mesenteric artery, the number of β-adrenoceptors in 5- to 6-week-old rats was the same as in 10- to 12-month-old rats, despite a difference in isoproterenol-induced relaxation between the 2 groups. In humans the β-adrenoceptor density in lymphocytes was comparable between young and aged subjects. These findings might imply that an alteration in β-adrenoceptor density may not be the major cause of impaired isoproterenol-induced responses in aged rats. Nevertheless, because the present study did not measure β-adrenoceptor density, the possible alteration in β-adrenoceptor density in aged rat arteries remains to be determined.

Pathophysiologic Implications

β-Adrenoceptor–mediated hyperpolarization has been suggested to play an important role in the control of membrane potential by opposing α-adrenoceptor–mediated depolarization. In addition, several studies have demonstrated that the activation of K<sub>ATP</sub> contributes to the relaxation elicited by β-agonists. It is thus conceivable that an impairment of β-adrenoceptor–mediated hyperpolarization might be detrimental to the maintenance of peripheral circulation.

In conclusion, isoproterenol-induced hyperpolarization appears to be mediated by K<sub>ATP</sub> in rat mesenteric resistance arteries and is shown to be attenuated in aged rats mostly because of a defective coupling of β-adrenoceptor to adenylate cyclase and partly because of a defect at the level of adenylate cyclase, but not because of an alteration of K<sub>ATP</sub> per se.

References


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