Properties of acetylcholine-induced hyperpolarization in smooth muscle cells of the mouse mesenteric artery

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Received November 5, 2007; Accepted November 15, 2007

Abstract

The properties of smooth muscle cell hyperpolarization produced by acetylcholine (ACh) were investigated in mesenteric arteries isolated from mice. The resting membrane potential of the smooth muscle cells was about -60 mV. When ACh ($10 \mu\text{M}$) was applied for 1 min, the membrane hyperpolarized with a peak amplitude of about 5 mV which was reached in about 1 min, following which the potential slowly reverted to the resting level over about 7 min following withdrawal of ACh from the superfusate (recovery component). Exposure of the artery to 0.5 mM Ba^{2+} , an inhibitor of inward rectifier K-channels, depolarized the membrane by about 13 mV, increased the amplitude of the ACh-induced hyperpolarization to about 10 mV, and facilitated the visualization of the recovery component. Indomethacine ($10 \mu\text{M}$), an inhibitor of cyclooxygenase, inhibited the recovery component and as a consequence reduced the duration of the hyperpolarization. The ACh-induced response was not markedly altered by either N^{ω} -nitro-L-arginine ($10 \mu\text{M}$), an inhibitor of nitric oxide (NO) production, or catalase (130 U/ml), a super oxide scavenger. Exogenously applied hydrogen peroxide (H_2O_2 , $300 \mu\text{M}$) hyperpolarized the membrane by about 5 mV, which was abolished by catalase. These results suggest that in the mouse mesenteric artery, the ACh-induced hyperpolarization has two components, both an indomethacin-sensitive and an indomethacin-insensitive component. The former component may be produced by prostanoids, while the latter may be produced by factors other than NO or H_2O_2 . The results also suggested that the inward rectifier K-channels may be important for producing the resting membrane potential, but they may not be the main contributor to the ACh-induced hyperpolarization of smooth muscle cell membranes in the mouse mesenteric artery.

Key words: EDHF, hyperpolarization, prostanoids, H_2O_2 , inward rectifier K-channel

Introduction

ACh hyperpolarizes smooth muscle cell membranes of the mesenteric artery in the rabbit (Kuriyama and Suzuki, 1978; Bolton *et al.*, 1984), guinea-pig (Takata, 1980), rat (Fujii *et al.*, 1992; Fukao *et al.*, 1995) and mouse (Matoba *et al.*, 2000). The hyperpolarization is produced in an endothelium-dependent manner, and the putative mediator has been considered to be endothelium-derived hyperpolarizing factor (EDHF; Chen *et al.*, 1988; Suzuki and Chen, 1990). However, the factor contributing to the generation of the hyperpolarization is equivocal, with possible candidates being EDRF (Bolton *et al.*, 1984; Feletou and Vahoutte, 1988), arachidonic acid metabolites such as the epoxyeicosatrienoic acids (EETs) produced through activation of the enzyme P-450 epoxygenase (Hecker *et al.*, 1994), potassium ions derived from endothelial cells (Edwards *et al.*, 1998) or hydrogen peroxide (H_2O_2) produced from super oxide anions (Matoba *et al.*, 2000). In the mesenteric artery of the guinea-pig, simultaneous recording of membrane potentials from both endothelial and smooth muscle cells revealed a synchronized hyperpolarization in response to ACh. The response in smooth muscle cells, but not in endothelial cells, was inhibited by the blockade of myo-endothelial gap junctional connections (Yamamoto *et al.*, 1999), thereby suggested that the hyperpolarization of smooth muscle cells may be produced by a passive propagation of the endothelial hyperpolarization to smooth muscle cells through gap junctions (Yamamoto *et al.*, 1998; Yamamoto *et al.*, 1999).

EDHF hyperpolarizes the membrane by increasing the K-conductance, since the amplitude of hyperpolarization is reversely related to the concentration of K^+ in the superfusate (Chen *et al.*, 1988; Chen and Suzuki, 1989), and ACh increases the efflux of incorporated K^+ in an endothelium-dependent manner (Chen *et al.*, 1988). Attempts have been made to specify the species of K-channels involved in the EDHF response, however the results are controversial. A possible involvement of Ca-sensitive K-channels in the EDHF-induced hyperpolarization is suggested in the rat aorta (Popp *et al.*, 1996). The ACh-induced hyperpolarization is selectively inhibited by apamin in the rabbit mesenteric artery (Murphy and Brayden, 1995), while it is inhibited by charybdotoxin in the rat aorta (Marchenko and Sage, 1996). In some arteries such as the submucosal arterioles in the guinea-pig small intestine (Hashitani and Suzuki, 1997), and both the guinea-pig coronary artery (Nishiyama *et al.*, 1997) and carotid artery (Corriu *et al.*, 1996), the ACh-induced hyperpolarization of the smooth muscle cell membrane is inhibited by co-application of charybdotoxin and apamin, thereby suggesting a contribution of Ca-sensitive K-channels with intermediate and small conductance. A contribution by inward rectifier K-channels in the ACh-induced hyperpolarization is also suggested in the rat mesenteric artery (Edwards *et al.*, 1998). The EDHF-response is not inhibited by glibenclamide in the guinea-pig (Chen *et al.*, 1992), rat (Fujii *et al.*, 1992) and rabbit (Murphy and Brayden, 1995; Marchenko and Sage, 1996; Nishiyama *et al.*, 1998; Hattori *et al.*, 2005), thereby suggesting that ATP-sensitive K-channels are not involved in the hyperpolarization.

Experiments were carried out to investigate the properties of ACh-induced hyperpolarization in the mouse mesenteric artery. The results would indicate that the ACh-induced hyperpolarization has two components, both an indomethacin-sensitive and an indomethacin-insensitive component, with the latter component not inhibited by catalase or L-

NA. The results do not support the idea that the ACh-induced hyperpolarization is mediated by H_2O_2 in the mouse mesenteric artery.

Materials and methods

Male and female mice (C57BL/6J, 6–8 weeks old) were used. All mice were treated ethically according to the Guidelines for the Use of Experimental Animals, approved by the Animal Treatment Committee of Nagoya City University Medical School.

After anesthesia with fluoromethyl 2,2,2-trifluoro-1-(trifluoromethyl) ethyl ether (sevoflurane, Maruishi Pharm., Osaka, Japan), animals were exsanguinated by decapitation. The proximal parts of the mesenteric artery supplying the ileum were dissected, and cleaned of fatty tissue surrounding the artery. The isolated segment of the artery was immobilized by using tiny pins on the rubber plate fixed at the bottom of the recording chamber (8 mm wide $\times$ 30 mm long $\times$ 2 mm deep, with a volume of about 0.5 ml), and superfused with warmed (36°C) Krebs solution, at a constant flow rate of 2 ml min^{-1} using a peristaltic pump (SMP-23, EYELA, Tokyo Rikakikai Co. Ltd., Tokyo, Japan).

Membrane potentials were recorded from smooth muscle cells using glass capillary microelectrodes (capillary outer diameter, 1.5 mm, inner diameter 0.8 mm, with a fine filament inside; Hilgenberg, Germany) filled with 0.5 M KCl (the tip resistance ranged between 80 and 150 M Ω). Electrical responses recorded with a high input impedance amplifier (Axoclamp-2B, Axon Instruments, Inc., Foster City, California, U.S.A.) were displayed on a cathode-ray oscilloscope (SS-7602, Iwatsu, Osaka, Japan) and were also stored on a personal computer for later analysis.

The ionic composition of the Krebs solution was as follows (mM): Na^+ 137.4, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, HCO_3^- 15.5, H_2PO_4^- 1.2, Cl^- 134, and glucose 11.5. The high $[\text{K}^+]_o$ solution (13.8 mM) was prepared by changing the ratio of KCl with NaCl. These solutions were aerated with O_2 containing 5% CO_2 , and the pH of the solutions was maintained at 7.2–7.3.

Chemicals used were acetylcholine hydrochloride (ACh), atropine sulphate, catalase, indomethacin, hydrogen peroxide (H_2O_2 , 31% solution) and N^ω -nitro-L-arginine (L-NA), all supplied from Sigma Chem., MO (USA). Indomethacin was dissolved first in a small amount (about 2 ml) of 0.1 M Na_2CO_3 solution, and then made up to the final volume with distilled water as a 100 mM solution. All other chemicals were dissolved with distilled water and kept as a stock solution. These solutions were further diluted with Krebs solution, with the ratio more than 1 : 1000. The dilution of chemicals did not alter the pH of the Krebs solution.

Experimental values were expressed as the mean value $\pm$ standard deviation (S.D.). Statistic significance was tested using the Student's *t*-test, and probabilities of less than 5% ($P < 0.05$) were considered to be significant.

Results

Acetylcholine-induced hyperpolarization

At rest, the cell membranes of the smooth muscle cells of the mesenteric artery of mice

Fig. 1. ACh-induced hyperpolarizations were recorded from smooth muscle cells of the mouse mesenteric artery, in the absence (A) and presence of 0.5 mM Ba^{2+} (B), and with co-application of both Ba^{2+} and 10 μM indomethacin (C). All responses were recorded from the same tissue preparation. B and C are continuous recordings with an approximately 20 min interruption. Dotted lines in the trace indicate the level of the membrane potential before the application of ACh.

were quiescent, with a stable resting membrane potential ranging between -58 and -70 mV (mean, -59.9 ± 3.0 mV, $n=13$). Application of acetylcholine (ACh, 10 μM) for 1 min hyperpolarized the membrane, and the potential reverted to the resting level slowly after withdrawal of ACh (recovery component) (Fig. 1A). The hyperpolarization reached the peak amplitude of 3–10 mV (mean, 5 ± 1 mV, $n=7$), at 0.6–1.4 min (mean, 0.97 ± 0.3 min, $n=7$) after starting the ACh-stimulation. The recovery component ranged between 5 and 10 min (mean, 6.7 ± 2.5 min, $n=7$).

Experiments were carried out to observe the ACh-response in the presence of Ba^{2+} , a known inhibitor of inward rectifier K-channels (Hirst and van Helden, 1982). In the presence of 0.5 mM Ba^{2+} , the membrane was depolarized to -40 – -51 mV (mean, -46.4 ± 3.9 mV, $n=6$; $P<0.05$), and the amplitude of the ACh-induced hyperpolarization was doubled (Fig. 1B). These results indicate that the inward rectifier K-channels may be one of the important factors in the establishment of the resting membrane potential, but they may not be involved in the ACh-induced hyperpolarization observed in the mouse mesenteric artery, as was the case in both the guinea-pig mesenteric artery (Hashitani and Suzuki, 1997) and coronary artery (Nishiyama *et al.*, 1998).

Factors involved in the ACh-induced hyperpolarization

The effects of indomethacin on the ACh-response were examined in the presence of 0.5 mM

Fig. 2. Effects of catalase on the ACh-induced hyperpolarization of smooth muscle cell membranes in the mouse mesenteric artery. In the presence of 0.5 mM Ba^{2+} , ACh (10 μM) was applied for 1 min, in the absence (A) and presence of 130 U/ml catalase (B). C, Mean value (+ S.D.) of the peak amplitude of ACh-induced hyperpolarization ($n=4$ for both). No significant difference was observed between the two ($P>0.1$).

Ba^{2+} , with the result that two components of the ACh-induced hyperpolarization became evident (Fig. 1B). With co-application of indomethacin (10 μM) and Ba^{2+} , the average recovery component of the ACh-induced hyperpolarizations was markedly reduced (control, 2.3 ± 0.4 mV; with indomethacin, 0.9 ± 0.4 mV, $n=4$; $P<0.05$), with no significant change in either the average membrane potential (in Ba^{2+} , -41.5 ± 2.5 mV; with Ba^{2+} and indomethacin, -41.2 ± 2.7 mV; $n=4$, $P>0.05$) or the average peak amplitude of the ACh-induced hyperpolarizations (control, 12.6 ± 3.2 mV; with indomethacin, 11.7 ± 3.2 mV; $n=4$; $P>0.05$) (Fig. 1C). As a consequence, the average duration of the ACh-induced hyperpolarizations (12.6 ± 3.6 min, $n=4$) was significantly reduced to 3.6 ± 1.0 min ($n=4$). These results appear to indicate that the recovery component of the ACh-response is produced by prostanoids.

The effects of catalase on ACh-induced hyperpolarization were tested to examine the possible involvement of H_2O_2 in the response. High concentrations of catalase (>300 U/ml) did not allow continuous recording of the membrane potential from the same cell, due to the high viscosity of the solution, and therefore experiments were carried out in solutions containing 130 U/ml catalase. This concentration of catalase did not alter either the resting membrane potential (control, -58.3 ± 2.0 mV; with catalase, -58.6 ± 1.5 mV; $n=10$; $P>0.1$) or the amplitude of the hyperpolarization produced by ACh (control, 5.3 ± 1.2 mV; with catalase, 5.0 ± 1.9 mV; $n=5$; $P>0.1$). The effects of catalase were also observed in the presence of Ba^{2+} , so as to facilitate the visualization of the altered hyperpolarizing response, if any, of catalase. As shown in Fig. 2, catalase caused no significant change in the ACh-induced response.

Attempts were made to evaluate the effects of 130 U/ml catalase on membrane responses produced by H_2O_2 in the mouse mesenteric artery. Application of H_2O_2 (300 μM) hyperpolarized the membrane; the response was very slow in time course, requiring 1.7–3.3 min (2.7 ± 0.6 min, $n=7$) to reach the peak amplitude of 4.9 ± 1.9 mV ($n=7$) and the entire duration of

Fig. 3. Effects of catalase on H_2O_2 -induced hyperpolarization of smooth muscle membranes in the mouse mesenteric artery. H_2O_2 (300 μ M) was applied for 1 min, in the absence (A) and presence of 130 U/ml catalase (B). A and B were recorded from the same cell. B was recorded 10 min after application of catalase. C, Peak amplitude of H_2O_2 -induced hyperpolarization (mean $\pm$ S.D., $n=7$).

hyperpolarization ranged from 6.6 min to 14.7 min (10.8 ± 3.6 min, $n=7$), in response to 1 min stimulation with H_2O_2 (Fig. 3A). The membrane response was reversible, and required >15 min to generate a reproducible amplitude of hyperpolarization with a second H_2O_2 application (data not shown). In the presence of catalase, no response was elicited by H_2O_2 in the smooth muscle cells of the mouse mesenteric artery (Fig. 3B). The inhibitory effects of catalase on H_2O_2 -induced hyperpolarization were reversible, and the recovery required over 15 min following the removal of catalase from the superfusate (data not shown). These results suggest that H_2O_2 is not involved in ACh-induced hyperpolarization of smooth muscle cells in the mouse mesenteric artery.

The effects of L-NA on ACh-induced hyperpolarization were examined in smooth muscle cells of the mouse mesenteric artery. L-NA (10 μ M) did not alter either the resting membrane potential (-58.6 ± 1.9 mV; in L-NA, -59.3 ± 1.5 mV; $n=12$; $P>0.05$) or the peak amplitude of ACh-induced hyperpolarization (control, 4.4 ± 1.1 mV; in L-NA, 4.2 ± 1.1 mV; $n=12$; $P>0.05$). These results would appear to indicate that NO is not involved in the ACh-induced hyperpolarization in the mouse mesenteric artery.

Discussion

The present experiments revealed that in the mouse mesenteric artery, the ACh-induced hyperpolarization of the smooth muscle cell membrane is formed from two components, one which is indomethacin-sensitive and the other which is indomethacin-insensitive. The former component appeared as the recovery component following removal of ACh from the superfusate. This component may be produced by prostanoids, possibly prostacyclin, as is the case in both the guinea-pig coronary artery (Parkington *et al.*, 1993; Nishiyama *et al.*, 1998; Yajima *et al.*, 1998) and the rabbit mesenteric artery (Murphy and Brayden, 1995). A slow rate

of action may be one of the characteristics of prostanoids, as this has been observed in the electrical and mechanical responses to prostanoids in different arteries (Parkington *et al.*, 1993; Murphy and Brayden, 1995; Zhang *et al.*, 1996; Nishiyama *et al.*, 1998; Yajima *et al.*, 1998). In the mouse mesenteric artery, the recovery component was not always clear in the resting condition, but it could be clearly seen after inhibiting the inward rectifier K-channels with Ba^{2+} , possibly due to depolarization of the membrane. The indomethacin-insensitive component of the ACh-induced hyperpolarization was not inhibited by nitroarginine, suggesting that this potential was produced by factors other than prostanoids and NO.

In the mouse mesenteric artery, the ACh-induced hyperpolarization is reportedly produced by endothelial H_2O_2 , as it can be inhibited by catalase (Matoba *et al.*, 2000). However, the present experiments did not detect a contribution of H_2O_2 to the hyperpolarization response. An important reason for this failure might be related to the difference in concentration of catalase; the concentration of catalase used by Matoba *et al.* (2000) (equal to 1,250 U/ml) was not applicable to our experimental system, due to the high viscosity of the solution. The concentration of catalase tested in the present experiments (equal to 130 U/ml) was nearly maximal in allowing successful continuous recording of cell membrane activity from the same smooth muscle cell. Inhibition of the ACh-induced relaxation by catalase was also observed in the mouse aorta, but the concentrations used ranged from 800 to 2,400 U/ml (Rabelo *et al.*, 2003), which was again far beyond a concentration that could be used in our system for these electrophysiological experiments. The significant inhibition of endothelium-dependent relaxation by high concentrations of catalase in wild mice but not in LDL receptor mutant mice (Fig. 2 in Rabelo *et al.*, 2003) suggests that these concentrations of catalase inhibit endothelial functions including the production of NO. The present experiments showed that in the mouse mesenteric artery, catalase at a concentration of 130 U/ml abolished the hyperpolarization response produced by H_2O_2 , with no significant effect on the ACh-induced hyperpolarization. Thus, it seems likely that the ACh-induced hyperpolarization is not produced by H_2O_2 . This was also suggested by the difference between H_2O_2 and ACh in the time course of the membrane potential changes; the hyperpolarization produced by ACh reached peak amplitude about 1 min after its application, while with H_2O_2 -induced hyperpolarization, peak amplitude was reached in about 5 min. Similar differences were also found in the recovery process from the hyperpolarization. Thus, no supportive evidence for the contribution of H_2O_2 in the ACh-induced hyperpolarization was obtained in these experiments with the mouse mesenteric artery.

In the guinea-pig mesenteric artery, the ACh-induced hyperpolarization of the smooth muscle cell membrane is inhibited by calibdotoxin and apamin (Hashitani and Suzuki, 1997). Significant inhibition of the ACh-induced hyperpolarization by calibdotoxin is also observed in both guinea-pig coronary (Nishiyama *et al.*, 1998) and mesenteric arterioles (Yamamoto *et al.*, 1999). These observations strongly suggest that the hyperpolarization is produced by activation of intermediate conductance K-channels with some contribution of small conductance K-channels. However in the rat mesenteric artery, a possible involvement of inward rectifier K-channels in the ACh-induced hyperpolarization is suggested (Edwards *et al.*, 1998). This was also tested in the mouse mesenteric artery, by using Ba^{2+} , an inhibitor of inward-rectifier K-channels. Application of Ba^{2+} significantly depolarized the membrane, suggesting that inward

rectifier K-conductance was important in the production of the resting membrane potential, as had been previously shown in the guinea-pig mesenteric artery (Hirst and van Helden, 1982; Hashitani and Suzuki, 1997). As ACh hyperpolarized the membrane in the presence of Ba^{2+} , the contribution of inward rectifier K-channels in production of the response in the mouse mesenteric artery may be very small, if at all, unlike the case in the rat mesenteric artery (Edwards *et al.*, 1998).

In the rabbit mesenteric artery, the hyperpolarization produced by H_2O_2 is inhibited by glibenclamide, thereby suggesting an involvement of ATP-sensitive K-channels (Hattori *et al.*, 2003). Although the present experiments did not test the effects of glibenclamide on ACh-induced hyperpolarization in the mouse mesenteric artery, no contribution of ATP-sensitive K-channels in the EDHF response have been observed in many types of artery (Parkington *et al.*, 1993; Fukao *et al.*, 1995; Murphy and Brayden, 1995; Nishiyama *et al.*, 1998). These results again weaken the possibility that the ACh-induced hyperpolarization is produced by H_2O_2 in the mouse mesenteric artery.

It is concluded that in the mouse mesenteric artery, ACh hyperpolarized the smooth muscle cell membrane with two components, one which is indomethacin-sensitive and the other which is indomethacin-insensitive. The former forms the recovery component, and may be produced by prostanoids, while the latter may be produced by factors other than H_2O_2 or NO. The inward rectifier K-channels may have an important role in the maintenance of the resting membrane potential, but not in the ACh-induced hyperpolarization in smooth muscle cells of the mouse mesenteric artery.

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