ELSEVIER

European Journal of Pharmacology 322 (1997) 185–190

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Effect of ouabain on adenosine receptor-mediated hyperpolarization in porcine coronary artery smooth muscle

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Received 19 November 1996; accepted 20 December 1996

Abstract

We investigated the effect of inhibitors of endothelium-derived nitric oxide and sodium-potassium (Na^+-K^+) pumps on adenosine receptor-mediated hyperpolarization of porcine coronary artery smooth muscle with and without endothelium. The average resting membrane potential (RMP) in porcine coronary artery smooth muscle was -51.5 ± 0.2 and -50.7 ± 0.2 mV, in the presence and absence of endothelium, respectively. Neither ouabain, *N*-nitro-L-arginine methyl ester (L-NAME) nor ouabain and L-NAME in combination significantly affected the resting membrane potential in the absence of vasodilator agonists. Adenosine agonists, 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine at 10^{-5} M caused a significant increase in RMP with intact endothelium and caused a smaller but significant increase in RMP in the absence of endothelium. Ouabain (10^{-5} M) in the absence of L-NAME significantly reduced hyperpolarization due to 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine in the presence of endothelium. However, in the absence of endothelium, its inhibitory effect was not significant. When ouabain plus L-NAME (10^{-5} M) were given simultaneously, the hyperpolarization caused by adenosine agonists was significantly further attenuated nearly to the RMP level. Attenuation of the response to 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine by ouabain was not reversed by the nitric oxide precursor, L-arginine (10^{-4} M) both in the presence and absence of endothelium. These results suggest that hyperpolarization of vascular smooth muscle of the porcine coronary artery by adenosine agonists is at least partly endothelium dependent and possibly involves the Na^+-K^+ pump and the release of nitric oxide.

Keywords: Smooth muscle, vascular; *N*-Nitro-L-arginine methyl ester (L-NAME); Ouabain; Adenosine receptor; Coronary circulation; EDHF (endothelium-derived hyperpolarizing factor)

1. Introduction

The cardiovascular effects of adenosine have been shown to be mediated by a number of different receptor subtypes (Collis and Hourani, 1993). Adenosine causes an increase in coronary blood flow through its interaction with vascular adenosine receptors. In general, the vasodilator effects of adenosine are thought to be mediated through the A_2 receptor subtype via adenylate cyclase activation and G protein mediation (Kennedy and Burnstock, 1985). Evidence has accumulated that both adenosine A_1 and A_2 receptors are present on smooth muscle cells including vascular smooth muscle cells of the coronary artery (Mill and Gewirtz, 1990; Olanrewaju and Mustafa, 1996). It has been reported previously that adenosine-induced relaxation of human coronary artery could be inhibited by ouabain, an agent that inhibits the sodium potassium (Na^+-K^+) pump (Sabouni and Mustafa, 1989). Activation of the Na^+-K^+ pump which is present in all cells has been reported to hyperpolarize vascular smooth muscle (Haddy, 1983; Feletou and Vanhoutte, 1988). This mechanism may be induced by an unknown diffusible factor named endothelium-derived hyperpolarizing factor (EDHF) since the electrical event can be transferred from tissues with endothelium to those without endothelium (Chen and Suzuki, 1991). It has been suggested that human plasma contains endogenous digitalis-like substances which inhibit Na^+/K^+-ATPase and cause vascular contraction (De Wardener et al., 1987). Among other factors, relaxation of isolated blood vessels has been attributed to hyperpolarization of the cell membrane resulting from direct activation of the Na^+/K^+-ATPase in canine femoral artery and

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PII S0014-2999(96)00992-2

rabbit ear artery (De Mey and Vanhoutte, 1980). Nitric oxide (NO) is synthesized in various cell types through the conversion of L-arginine to citrulline by NO synthase. It has been shown that vascular smooth muscle also has the ability to produce NO or NO-related substances and that L-arginine-induced responses are in part mediated by the smooth muscle-derived NO (Schini and Vanhoutte, 1991; Wood et al., 1990). It appears that concomitant factors may mediate adenosine-induced vasodilation. The contributions of Na^+/K^+-ATPase and EDRF-induced hyperpolarizations in smooth muscle responses to endogenous and/or exogenous adenosine agonists remain unclear. This study examines the effect of inhibitors of endothelium-derived NO and Na^+-K^+ pumps on adenosine receptor-mediated hyperpolarization of porcine coronary artery smooth muscle with and without endothelium.

2. Materials and methods

Porcine hearts were obtained from a local slaughterhouse within 10 min of death and transported to the laboratory in oxygenated (95% O_2/5% CO_2) ice-cold Krebs-Henseleit buffer of the following composition (mM): NaCl, 118; KCl, 4.7; $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 2.5; $CaCl_2$, 2.5; glucose, 11 (4°C; pH 7.4). Electrophysiological experiments were performed carefully and correctly as described previously (Olanrewaju et al., 1995). Briefly, left anterior descending coronary arteries were dissected, cleaned of fat, connective tissues and prepared for the experiments. The vessels were then split open to form a sheet. The arterial sheets were placed and pinned down in an organ bath (10 ml) maintained at 37°C with the adventitial side downward to allow the penetration of microelectrodes from the intimal surface of the blood vessels. The organ bath had a constant flow system. The vascular sheets (with and without endothelium) were continually superfused throughout the experimental period with an oxygenated Krebs-Henseleit buffer solution (37°C, pH 7.4). Care was taken to preserve the endothelium intact. For observations on endothelium-denuded tissues, endothelial cells were removed by a gentle rubbing of the intimal surface of the vessel with a buffer-moistened cotton swab and lack of response to bradykinin (10^{-5} M) was used to confirm removal of endothelium (Chen and Suzuki, 1991; Olanrewaju et al., 1995).

Glass capillary microelectrodes filled with 3 M KCl (tip resistance, 40–80 MΩ) were made from a borosilicate glass tube (1.5 mm o.d.; Sutter Instrument, Movato, CA, USA). Transmembrane potentials were recorded with glass microelectrodes mounted on a micromanipulator, and monitored under a microscope to ensure that it penetrated deeper than the endothelial cell layer. Electrical responses displayed on a cathode-ray oscilloscope (VC-9A, Nihon-Kohden, Tokyo, Japan) and on a digital voltameter were tabulated. Proper impalements were accepted only when a sudden change in voltage was observed on the oscilloscope trace and the indicated potential was maintained for at least 3 min. Measurements were disregarded when membrane potential decreased spontaneously, indicating cell damage. Moreover, the electrode tip resistance was monitored before and after impalement to avoid potential changes caused by electrode artifacts.

Resting membrane potential with and without endothelium was recorded after equilibration. In the present study, experiments were conducted with 10^{-5} M ouabain, *N*-nitro-L-arginine methyl ester (L-NAME), or a combination of both by continuously superfusing the tissues with either or both of these inhibitors for 15 min and recording changes in membrane potential. From the dose-dependent preliminary experiments, ouabain at 10^{-5} M concentration was effective in inhibiting hyperpolarization induced by 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine (data not shown). L-NAME at 10^{-5} M concentration has also been shown to be effective in inhibiting hyperpolarization induced by 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine (Olanrewaju et al., 1995). L-NAME has been reported to be a more potent inhibitor of NO synthase than N^G-monomethyl-L-arginine (Gross et al., 1990; Rees et al., 1990; Olanrewaju et al., 1995). The concentration (10^{-5} M) of the adenosine receptor agonists used in the present experiments has been shown to produce hyperpolarization in isolated human and porcine coronary arteries (Olanrewaju et al., 1995). In these procedures, membrane potentials were recorded before (control) and 15 min after the initiation of continuous superfusion with oxygenated buffer containing the adenosine receptor agonists. The adenosine agonist-induced hyperpolarization was recorded after membrane potential was maintained for at least 3 min. All experiments were conducted in the presence of indomethacin (10^{-5} M) to rule out the involvement of prostanoids.

We next studied the effect of the Na^+/K^+-ATPase inhibitor, ouabain, in adenosine receptor-mediated hyperpolarization of porcine coronary artery smooth muscle with and without endothelium. The effect of the agonists was determined after equilibration and preincubating the same tissues for 15 min with 10^{-5} M ouabain and/or 10^{-5} M L-NAME. The effect of adenosine agonists on smooth muscle resting membrane potential was repeated with fresh tissue as described above while ouabain (10^{-5} M) was included in the perfusate (agonist) and the hyperpolarization effect of 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine were re-evaluated to assess direct effect of ouabain on adenosine agonist-induced hyperpolarization in porcine coronary artery smooth muscle. In subsequent experiments the tissue was continuously superfused with 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine in combination with L-NAME. After a period of 15 min, L-arginine (10^{-4} M) was included in the perfusate (agonist plus L-NAME) and the hyperpolarization effect of 2-chloroadenosine or 5′-*N*-ethylcarboxami-

doadenosine was re-evaluated to assess the reversibility effect of L-NAME.

2.1. Data analysis

Responses of smooth muscle to adenosine agonists were observed and recorded from 10 different cells from the same tissue and the pooled data from coronary artery tissue from several different hearts (4–5 separate experiments) were expressed as mean ± standard error of the mean (S.E.M.). Statistical significances were determined by Student's *t*-test for paired or unpaired observations and when more than two means were compared, one-way analysis of variance was used. Values were considered to be statistically different when P was smaller than 0.05.

2.2. Chemicals and drugs

Indomethacin, bradykinin, L-arginine, ouabain and *N*-nitro-L-arginine methyl ester (L-NAME) were purchased from Sigma (St. Louis, MO, USA). 5′-*N*-Ethylcarboxamidoadenosine and 2-chloroadenosine were purchased from Research Biochemicals (Wayland, MA, USA). Each antagonist/inhibitor solution was added to each agonist in the buffer reservoir, and the blood vessels were continually superfused with the mixture throughout the test periods. All drugs were dissolved in distilled water to make stock solutions with the exceptions of 5′-*N*-ethylcarboxamidoadenosine, L-NAME and indomethacin which were prepared in 90% ethanol at 10^{-2} M concentration. Na_2CO_3 (5 N) was used to adjust the pH of the indomethacin solution to physiological range. All stocks were further diluted by 1000 times or more in distilled water or buffer. Ethanol used to dissolve drugs at the final concentration (0.01%) did not, in itself, affect the electrical responses at the final bath concentration.

3. Results

The average resting membrane potential (RMP) for porcine coronary artery was -51.5 ± 0.2 mV with endothelium and -50.7 ± 0.2 mV without endothelium. There was no significant difference in RMP of endothelium-intact and denuded tissues (Fig. 1). Separate application of either ouabain, L-NAME or combination of ouabain and L-NAME at 10^{-5} M to porcine coronary artery tissue had no significant effect on resting membrane potential in the absence of vasodilator agonists (Fig. 1). Bradykinin increased resting membrane potential significantly only in the presence of endothelium. Lack of response to bradykinin (10^{-5} M) was used to confirm the absence of functional endothelium. Indomethacin, an inhibitor of cycloxygenase, does not inhibit the hyperpolarization mediated by 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine, confirming the previous suggestion that the endothelium-derived vasoactive substance(s) released by adenosine agonists in porcine coronary artery is not a prostanoid metabolite of arachidonic acid (Abebe et al., 1995; Olanrewaju et al., 1995).

Separate application of 2-chloroadenosine (10^{-5} M) and 5′-*N*-ethylcarboxamidoadenosine (10^{-5} M) caused significant hyperpolarization in intact and endothelium-denuded arteries (Figs. 2 and 3). In all experiments, hyperpolarization due to adenosine agonists was significantly more pronounced in the presence of endothelium. The addition of ouabain (10^{-5} M) to the perfusate containing adenosine agonists in the absence of L-NAME drastically

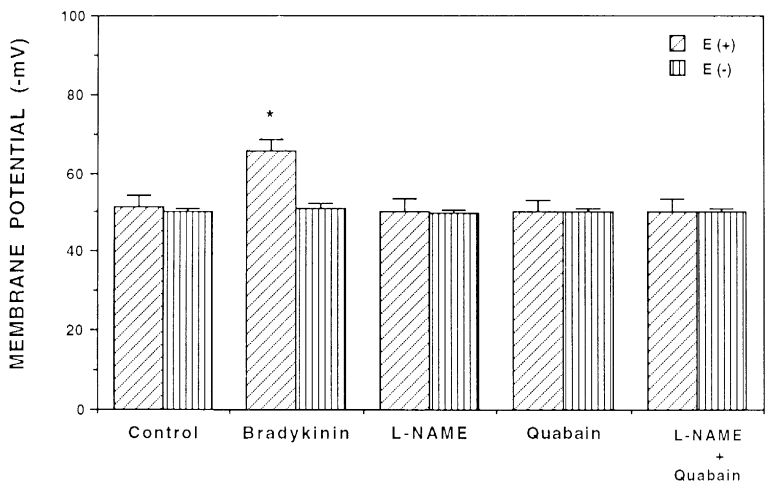

Fig. 1. Effect of 10^{-5} M ouabain, *N*-nitro-L-arginine methyl ester (L-NAME, 10^{-5} M) or combination of both on resting membrane potential in porcine coronary artery branches. Values are means ± S.E from 6 hearts in each series, each representing 10 impalements in endothelium-intact (E^+) and -denuded (E^-) arteries. * Significantly different from controls at $P < 0.05$.

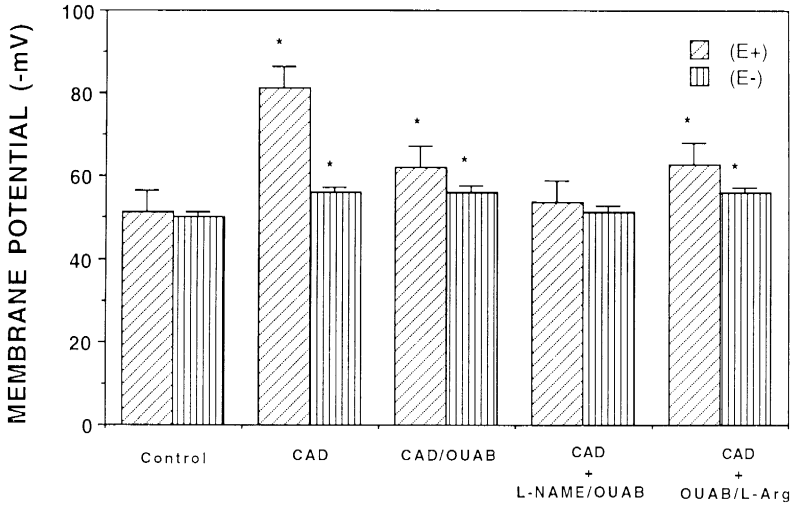

Fig. 2. Effect of 2-chloroadenosine (CAD) on resting membrane potential in porcine coronary artery branches in the presence of EDRF blocker (L-NAME, 10^{-5} M) and ouabain (10^{-5} M) and influence of 10^{-4} M L-arginine (L-Arg). Values are means ± S.E. from 6 hearts in each series, each representing 10 impalements in endothelium-intact (E^+) and -denuded (E^-) arteries. * Significantly different from controls at $P < 0.05$.

and significantly attenuated hyperpolarization due to 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine in the presence of endothelium. However, in the absence of endothelium, its inhibitory effect was not significant. Addition of L-NAME to ouabain-containing solution significantly further attenuated the hyperpolarizing effects of 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine more than ouabain alone in the presence of endothelium (Figs. 2 and 3). L-Arginine (10^{-4} M), an NO precursor, significantly reversed the attenuation of the response due to 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine by L-NAME especially in the presence of endothelium (data not shown), confirming our previous report (Olanrewaju et al., 1995). However, attenuation of the response to 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine by ouabain (10^{-5} M) was not reversed by L-arginine (10^{-4} M) both in the presence and absence of endothelium (Figs. 2 and 3).

4. Discussion

The present study demonstrates that adenosine agonists, 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine, produced hyperpolarization that was more pronounced in endothelium-intact than endothelium-denuded tissues and

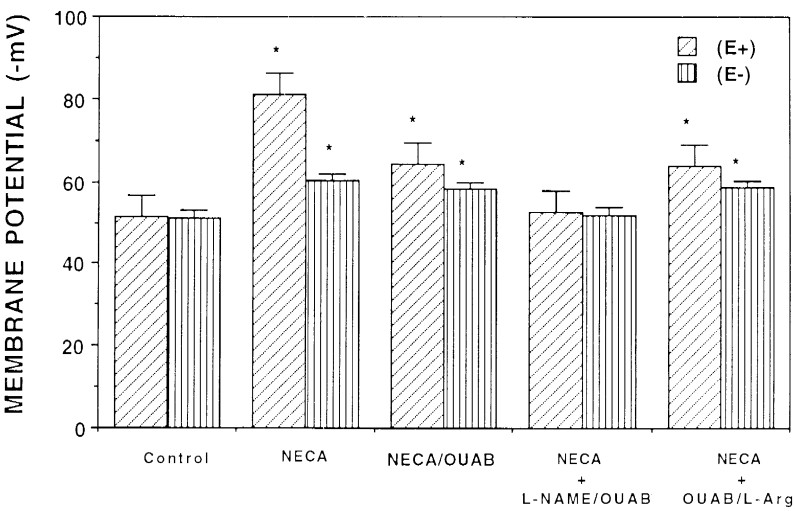

Fig. 3. Effect of 5′-*N*-ethylcarboxamidoadenosine (NECA) on resting membrane potential in porcine coronary artery branches in the presence of EDRF blocker (L-NAME, 10^{-5} M) and ouabain (10^{-5} M) and influence of 10^{-4} M L-arginine (L-Arg). Values are means ± S.E. from 6 hearts in each series, each representing 10 impalements in endothelium-intact (E^+) and -denuded (E^-) arteries. * Significantly different from controls at $P < 0.05$.

this is in agreement with our previous report (Olanrewaju et al., 1995). The endothelium-dependent effect of adenosine agonists appears to be primarily due to the release of a diffusable hyperpolarizing factor(s) following the activation of adenosine receptors present on endothelial cells (Chen and Suzuki, 1991). There was no significant difference in resting membrane potential of endothelium-intact and denuded tissues and this may indicate that there is no contribution of endothelium-derived hyperpolarizing factor to the resting membrane potential as has been reported previously (Chen and Suzuki, 1991; Olanrewaju et al., 1995). The hyperpolarization of vascular smooth muscle cells produced by adenosine agonists in the presence of indomethacin ruled out the possibility of the responses being mediated by prostanoid metabolites of arachidonic acid. A possible candidate may be endothelium-derived hyperpolarizing factor (EDHF), which has been previously shown to produce smooth muscle hyperpolarization that was not mainly mediated by NO (Chen and Suzuki, 1991; Olanrewaju et al., 1995). The hyperpolarization produced by adenosine agonists in porcine coronary artery was attenuated by ouabain and the results are in agreement with those observed in canine coronary artery (Feletou and Vanhoutte, 1988). In contrast, the present results are not in agreement with those observed in the rabbit ear artery (Suzuki, 1988) and in the dog coronary artery (Chen et al., 1989) where the attenuation effect of ouabain on hyperpolarization was not observed. Thus, the mechanisms underlying adenosine-induced hyperpolarization have yet to be clearly established. The ability of endothelial cells to release vasoactive substances varies between tissues and species (Vanhoutte et al., 1986). At present, there is no physiological explanation for the species differences in the role of coronary endothelial NO. The mechanism by which EDHF causes hyperpolarization appears to vary with the tissue, species, concentration of the drug applied, level of resting membrane potential, state of stretch of the tissue and the endothelial stimulant studied (Parkington et al., 1993). One possibility is that vasodilator agonists stimulate cAMP-dependent phosphorylation to enhance electrogenic Na^+-K^+ pump activity (activation of Na^+/K^+-ATPase) thereby hyperpolarizing smooth muscle. In the present study a 15 min incubation period with ouabain was imposed to avoid the acute transient effects on vascular smooth muscle by ouabain as we were interested in the steady-state effects of Na^+ pump inhibition. The present results suggest the possibility that attenuation by ouabain of the hyperpolarizing responses to 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine was probably a result of changes in membrane potential due to Na^+-K^+ pump inhibition in coronary smooth muscle. The sensitivity of Na^+/K^+-ATPase to ouabain for the inhibition of hyperpolarization due to adenosine agonists appears to differ between endothelial and vascular smooth muscles, the former being more sensitive to ouabain than the latter.

The hyperpolarization observed in the absence of endothelium which was inhibited by L-NAME in the present study could be due to release of NO or NO-related substances as a result of direct stimulation of the A_2 adenosine receptors on smooth muscle (Schini and Vanhoutte, 1991; Olanrewaju et al., 1995). The existence of NO in endothelium-denuded tissue may be due to several factors, such as the ability of vascular smooth muscle to produce a relaxing factor (vascular smooth muscle-derived relaxing factor, MDRF) with pharmacologic and chemical properties similar to those of NO (Wood et al., 1990). Furthermore, maybe guanylate and adenylate cyclase systems operate in a positive synergism, possibly through the inhibition of phosphodiesterase (Maurice et al., 1991). However, the physiological significance and pharmacological value of synergism between activators of guanylyl and adenylyl cyclases in vascular smooth muscle and other tissues remain unclear. Among other suggestions, hyperpolarization may be due to activation of ATP-sensitive K^+ channels by cyclic AMP-dependent phosphorylation thus mediating membrane hyperpolarization as previously reported in the porcine coronary artery (Miyoshi and Nakaya, 1993).

Microscopic examination showed an occasional fragment of endothelial cells on the luminal wall of some of the tissues, but organ-bath experiments indicated nonfunctional fragmented endothelial cells with bradykinin, which evokes vasorelaxation in endothelium-intact arteries (Abebe et al., 1995). It should be noted that in the present study, hyperpolarization induced by adenosine agonists was not completely blocked by ouabain, indicating that there is more than one mechanism of action, as with all other vasoactive substances studied to date. Addition of L-NAME plus ouabain almost completely attenuated the hyperpolarization effects of 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine, especially in the presence of endothelium indicating the involvement of the NO pathway. Attenuation of adenosine agonist-induced hyperpolarization by L-NAME (10^{-5} M) which was significantly reversed by L-arginine (10^{-4} M) mainly in the presence of endothelium has been reported previously (Olanrewaju et al., 1995). However, L-arginine (10^{-4} M) was unable to reverse the attenuation of the response to 2-chloroadenosine and 5′-*N*-ethylcarboxamidoadenosine by ouabain (10^{-5} M) both in the presence and absence of endothelium. Our results suggest the involvement of both Na^+-K^+ pump and NO in adenosine receptor-mediated hyperpolarization of porcine coronary artery vascular smooth muscle. Consideration of the relationship between the inhibition by L-NAME of the ouabain-resistant component of the adenosine-induced hyperpolarization and ion channels involved is required. It remains to be seen if the ouabain-sensitive component of the 2-chloroadenosine- or 5′-*N*-ethylcarboxamidoadenosine-induced hyperpolarization is also sensitive to L-NAME. These results suggest that the hyperpolarizing responses due to adenosine agonists are genetrated by different factors and as such are in agreement with our

previous report (Olanrewaju et al., 1995). Cardiac glycosides such as ouabain are specific inhibitors of this electrogenic pump. The concentration (10^{-5} M) of ouabain used in the present study was sufficient to produce an inhibitory effect on the isolated porcine coronary smooth muscle hyperpolarization induced by adenosine agonists.

In summary, our results indicate that activation of adenosine receptors in porcine coronary artery results in largely endothelium-dependent and to a lesser extent endothelium-independent hyperpolarization of vascular smooth muscle which involves the electrogenic Na^+-K^+ pump and the release of NO. Involvement of other factors insensitive to blockade by ouabain such as activation of K^+-ATP channels cannot be ruled out in endothelium-dependent and -independent hyperpolarization of vascular smooth muscle.

Acknowledgements

The authors thank Drs. Edward R. Seidel and James S. Wild for thoughtful review of the manuscript. This work was supported by HL 27339 and a Minority Investigator Research Supplement Award to H.A.O. from NHLBI.

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