Acetylcholine-induced K^+ currents in smooth muscle cells of intact rat small arteries

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1. The mechanism of the sustained acetylcholine-induced endothelium-dependent hyperpolarization (EDH) in intact rat small mesenteric arteries prestimulated with noradrenaline (10^{-6} M) was investigated by means of the single microelectrode voltage-clamp method.

2. The vascular smooth muscle cells (VSMCs) in this preparation are poorly or even not coupled for the reasons that: (1) the mean input resistance R_{inp} of the clamped vascular smooth muscle increases from 120 MΩ under control conditions to 440 MΩ after application of K^+ channel blocking drugs, (2) the voltage relaxation after injection of hyperpolarizing currents has a monoexponential time course and is linearly dependent on R_{inp}, and (3) voltage steps induced by current-clamp steps are not transferred to locations in the vascular musculature 120 μm apart from the current injecting microelectrode.

3. Sustained (> 5 min) application of ACh (10^{-5} M) hyperpolarized the VSMCs by induction of a hyperpolarizing current. This effect was completely blocked by the inhibitor of the nitric oxide (NO) synthase L-NAME (10^{-3} M) but not by the inhibitor of the soluble guanylate cyclase (sGCl) Methylene Blue (MB, 10^{-4} M).

4. Application of the NO donor sodium nitroprusside (SNP, 10^{-6} M) for more than 5 min mimicked the induction of the endothelium-dependent hyperpolarizing current in vessels with destroyed endothelium. The reversal potential of this current is dependent on the extracellular K^+ concentration. The effect of SNP could also not be blocked by MB.

5. The blockers of ATP-dependent and Ca^{2+}-dependent K^+ channels, glibenclamide (Glb, 10^{-5} M) and charybdotoxin (CTX, 5×10^{-8} M), respectively, blocked a hyperpolarizing current in the VSMCs similar to the ACh- or SNP-induced current.

6. The isolated application of either Glb or CTX did not block the activation of the hyperpolarizing current by SNP. Only the combined administration of Glb and CTX blocked the SNP-induced current completely.

7. Our results suggest that in rat small mesenteric artery, ACh hyperpolarizes the VSMCs tonically by activating both ATP- and Ca^{2+}-dependent K^+ currents, only via release of NO from the endothelium without need for activation of the sGCl.

Stimulation of the endothelium by intravasal flow, hypoxia, or substances like acetylcholine (ACh), ATP, histamine, bradykinin, and thrombin can relax vascular smooth muscle cells (VSMCs) by hyperpolarization of their cell membranes. The hyperpolarization closes voltage-dependent Ca^{2+} channels leading to a reduction of the Ca^{2+} influx, an important determinant of the contractile state of VSMCs (Daut, Standen & Nelson, 1994; Kuriyama, Kitamura & Nabata, 1995; Nelson & Quayle, 1995). The endothelium-dependent hyperpolarization (EDH) of VSMCs is mediated by substances released from the endothelium. Depending on the species, the organ and the type of the stimulus, the hyperpolarizing agents are believed to be nitric oxide (NO), prostacyclin (PGI_2) and/or the so called endothelium-derived hyperpolarizing factor (EDHF). The structure of EDHF is not yet clear, but products of cytochrome P450-dependent enzymes are possible candidates. Probably several nonprostanoid, non-nitric oxide substances can act as an EDHF.

The main action of all these agents is believed to be the activation of K^+ channels in the VSMCs. Which type of K^+ channel is stimulated by the endothelium is not yet clear but it may vary with the preparation investigated (Quayle &

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Standen, 1994; Cohen & Vanhoutte, 1995; Garland, Plane, Kemp & Cocks, 1995; Harder, Campbell & Roman, 1995).

To elucidate the mechanism of endothelium-derived hyperpolarization, two types of preparations have been used predominantly. One type uses intact vessels or vessel strips which have been investigated by means of microelectrode techniques to measure the membrane potential of vascular smooth muscle (VSM). The advantage of these preparations is that the VSM cells (VSMCs) are not altered by enzymatic digestion procedures and are kept in their physiological environment within the vessel. The connective tissue, neighbouring VSMCs, the endothelium and stretch induced by transmural pressure are determinants of this environment. The disadvantage of these techniques is that conclusions concerning the ionic currents underlying the change in the membrane potential can only be made indirectly after shifting the membrane potential with different extracellular K^+ concentrations or after use of pharmacological tools in order to activate or block ionic channels in the VSMC membrane.

The other type of preparation using freshly isolated or cultured VSMCs, allows a direct measurement of ionic currents by the voltage-clamp technique. The membrane potential, as well as the composition of the intracellular and extracellular solutions, can be controlled. On the other hand, the isolation and cultivation procedures may influence the electrophysiological behaviour and the VSMCs are kept in a non-physiological environment.

We applied the single-microelectrode voltage-clamp technique to study ionic currents of VSMCs involved in the endothelium-dependent hyperpolarization. The experiments were performed in intact small resistance vessels which are important determinants of the peripheral vascular resistance and the blood supply to their corresponding organ (Mulvany & Aalkjaer, 1990). Using this method, we tried to bridge the gap between the exact electrophysiological measurements in isolated VSMCs and measurements of the membrane potential of VSMCs in intact vessel preparations. In particular we wanted to find out to what extent endothelium-derived NO contributes to, and which types of K^+ currents are involved in, the EDH in VSMCs of intact rat small mesenteric arteries.

METHODS

Drugs, solutions and abbreviations

The standard buffer solution consisted of (mM): 119 NaCl, 25 $NaHCO_3$, 4·7 KCl, 1·18 KH_2PO_4, 2·5 $CaCl_2$, 1·17 $MgSO_4$, 5·5 glucose, 0·026 EDTA. The pH was kept between 7·4 and 7·42 by equilibrating with 4% CO_2–20% O_2–76% N_2. The salts and buffers were purchased from Sigma.

Unless otherwise stated, the following drugs were obtained from Sigma. The concentrations used were (μM): 10 acetylcholine (ACh) (Serva, Heidelberg, Germany), 6000 ascorbic acid, 0·05 charybdotoxin (CTX), 1000 4,4′-diisothiocyanostilbene-2,2′-di-sulphonic acid (DIDS), 10 glibenclamide (Glb), 10 indomethacin, 125 L-arginine, 1000 L-NAME, 100 Methylene Blue (MB; Merck, Darmstadt, Germany), 1 sodium nitroprusside (SNP), 1 nisoldipine (Bayer, Leverkusen, Germany) and 1 noradrenaline (NA).

Preparation

The measurements were performed in small mesenteric arteries (inner diameter 120–200 μm, length 5–10 mm) from Wistar rats of both sexes weighing between 200 and 250 g. The rats were anaesthetized with ether and killed by exsanguination. The dissection was performed in cooled (5 °C) low Ca^{2+} (200 μM) standard buffer solution (see above) equilibrated with 4% CO_2–20% O_2–76% N_2. The isolated artery was cannulated at one end by a glass capillary (end diameter ~200 μm) and transferred into the stainless steel experimental chamber. The uncannulated end of the artery was fixed by glass needles onto the bottom of the chamber coated with Sylgard 184 (Dow Corning). The vessel was perfused via the glass cannula by means of a syringe pump (Becton-Dickinson, Alzenau, Germany) with a constant flow of 2 ml h^{-1}. The corresponding intravasal hydrostatic pressure directly behind the end of the cannula was 50 ± 10 mmHg (mean ± S.D.), as measured by a pressure transducer (RFT, Zwönitz, Germany). The extravasal solution was continuously perfused by a peristaltic pump (Ismatec, Wertheim-Mondfeld, Germany). The volume of the experimental chamber (~5 ml) was exchanged within 1 min.

The temperature of the solution in the experimental chamber was kept at 31 °C. To support the impalement of the microelectrodes, the adventitia of the vessels was exposed for 2 min to a standard buffer solution with the following modifications: 40 μM $CaCl_2$, 0 mM EDTA and 0·04 mg ml^{-1} collagenase (Worthington Biochemical Co.). This procedure did not cause any obvious change in the structure of the vessel as judged by microscopic inspection (Optiphot, Nikon, Düsseldorf, Germany).

Thereafter, the collagenase was washed out with standard buffer solution for about 30 min.

The solution was then exchanged with the control solution which consisted of the standard buffer solution supplemented with nisoldipine and DIDS to block Ca^{2+} and Cl^- currents. To prevent the formation of cyclo-oxygenase products, indomethacin was added to the intravasal solution. In preparations with intact endothelium, L-arginine was also added. The extravasal solution contained nisoldipine, DIDS and additionally noradrenaline and ascorbic acid. In the legends, currents or membrane potentials measured under these control conditions are labelled 'control'.

Electrophysiology

Microelectrodes were pulled from borosilicate glass (WPI, Berlin, Germany). The resistance of the electrodes varied between 50 and 80 MΩ after filling with 500 mM KCl. The tip of the electrode was coated with silicone oil (Merck) to reduce the capacity. The microelectrodes were impaled into the smooth muscle cell layer of the vessel via the adventitia by means of a micromanipulator with piezo translator (WPI). A single microelectrode voltage-clamp amplifier (SEC 10, NPI Electronic, Tamm, Germany) was used for electrical recordings. The remaining capacity and the resistance of the microelectrode was electronically compensated. With this amplifier, it was possible to inject very short current pulses (10–20 μs at 120 nA) through high resistance microelectrodes and to record the membrane potential accurately within the same cycle. Current–voltage switching frequencies between 25 and 35 kHz were used. Before each voltage-clamp measurement, the membrane potential (V_m) of the impaled cell was recorded in the current-clamp

mode. The membrane potential was then clamped to −40 mV. Three current ramps were subsequently elicited at a rate of 0·3 Hz by application of 500 ms voltage ramps from −90 to +20 mV. Thereafter, the membrane potential was again measured in the current-clamp mode. Afterwards, the microelectrode was withdrawn. This recording protocol usually lasted about 1 min. Criteria for accepting a record were deviations in V_m of less than ±5 mV before and after application of the three voltage ramps and a sharp drop to 0 ± 3 mV after withdrawal of the microelectrode. As the impalements were usually not stable for more than 2 min, we were not able to perform electrophysiological recordings during changes of the intra- or extravasal solution. Therefore, only sustained effects of the applied drugs were investigated. Each measurement started at least 5 min after a solution exchange. In each of the tested solutions (as indicated in the legends) current records of five to fifteen impalements were performed.

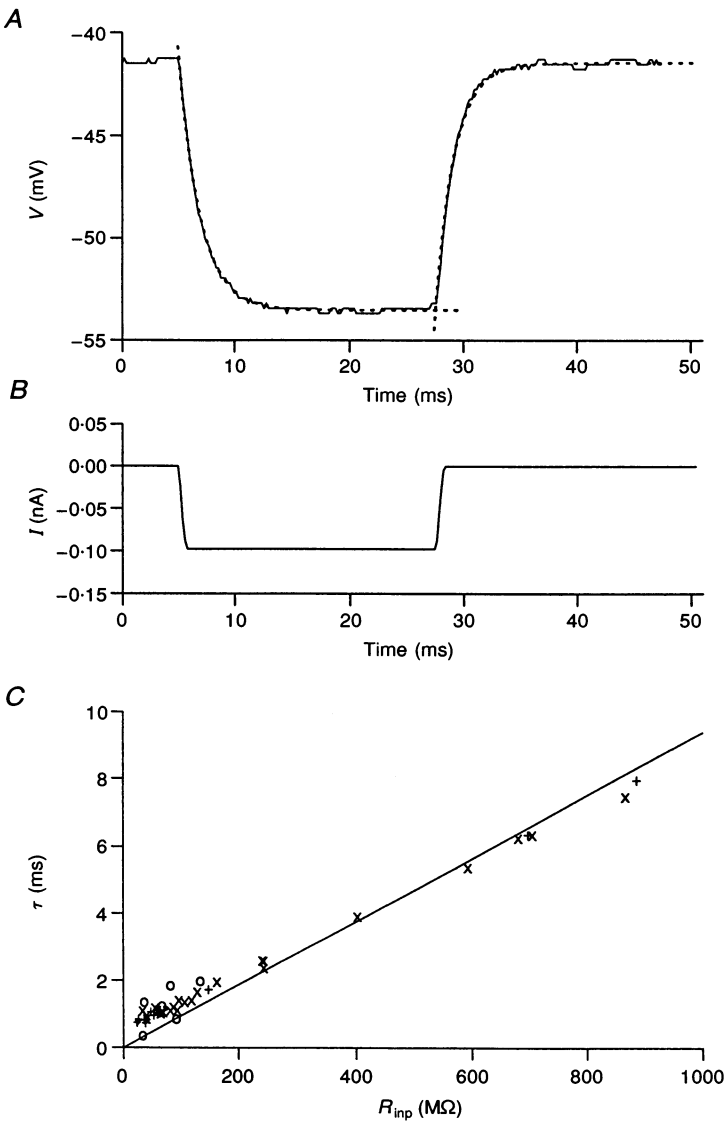

Figure 1. Passive electrical properties of smooth muscle in a mesenteric artery with endothelium

A, change of membrane potential due to injection of a hyperpolarizing current as measured in the current clamp mode. Control solution was used as intra- and extravasal solution. The time course was fitted monoexponentially with time constants of 1·9 and 1·7 ms for hyperpolarization and repolarization, respectively. From the steady-state deviation of the membrane potential of 12·9 mV, a membrane resistance of the impalement of 129 MΩ was calculated. Therefore, from $\tau = CR_{inp}$, the capacitance of the clamped cells was determined to be 14 pF. *B*, time course of the injected current. *C*, relationship between input resistance R_{inp} and time constant τ of voltage relaxation due to injected current as shown in *B*. Impalements from one vessel with control solution in the intra- and extravasal compartment with intact endothelium and intravasal application of ACh and L-arginine to the intravasal control solution (○; $n = 6$), with destroyed endothelium without ACh and L-arginine (+; $n = 10$), and with destroyed endothelium and extravasal addition of CTX and Glb (×; $n = 19$). The data were fitted by $\tau = CR_{inp}$, yielding a mean input capacitance of 9·4 pF.

For the simultaneous recording of membrane potentials of VSMCs at different locations, current-clamp recordings of one impalement were performed using the above mentioned amplifier while a second microelectrode amplifier (OC-725C Warner Instr., Hamden, CT, USA) was used to record the membrane potential of the second impalement.

Data analysis

Current signals were filtered at 1·3 kHz and sampled at 1 kHz. Data were stored and analysed on a personal computer using pCLAMP software (Axon). Mean current ramps were calculated and a linear regression of each ramp was calculated by the program Lotus (Lotus Development, München, Germany).

Unless otherwise stated, data are presented as means ± S.E.M. Comparison of means was performed by ANOVA and multiple range test using the program Statgraph (STSC Inc., Rockville, MD, USA).

RESULTS

Passive electrical properties

The input resistance R_{inp} and the input capacitance C of the smooth muscle cells were determined by injection of 0·1 nA hyperpolarizing currents in the current-clamp mode under control conditions. Figure 1 demonstrates an example of such a measurement. The time course of membrane hyperpolarization and repolarization was fitted by:

$$V(t) = V_0 + \Delta V(1-\exp((t_{on} - t)/\tau_{on})) \quad (1)$$

for the hyperpolarization ($t \geqslant t_{on}$) and

$$V(t) = V_1 + \Delta V(\exp((t_{off} - t)/\tau_{off})) \quad (2)$$

for the repolarization ($t \geqslant t_{off}$). The parameters t_{on} and t_{off} are the starting time for onset and offset of the hyper-

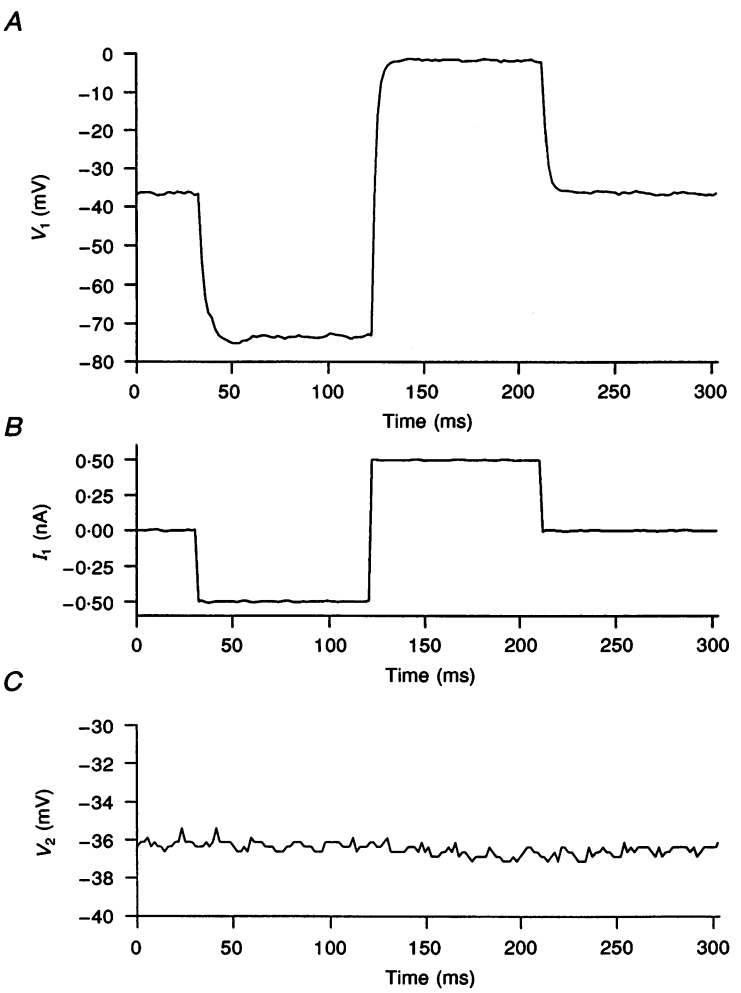

Figure 2. Measurement of electrical coupling between vascular smooth muscle cells using two microelectrodes

A, membrane potential V_1 measured by a microelectrode under current-clamp conditions. Control solution was used as intra- and extravasal solution. *B*, time course of the injected current. *C*, membrane potential V_2 measured by a second microelectrode at a distance of 120 μm from the first impalement. Note the different voltage scale compared with *A*. The line between each impalement was perpendicular to the vessel axis.

polarizing current, respectively, and V_0 and V_1 are the measured potentials V, before the onset and offset of the current step, respectively. The time constants for the change of the voltage V, τ_{on} and τ_{off}, were not significantly different under control conditions (1·66 ± 0·4 ms for τ_{on} and 1·70 ± 0·4 ms for τ_{off}, $n = 25$). From the deflections of the potential ΔV, a mean R_{inp} of 125 ± 25 MΩ ($n = 25$) was calculated under control conditions. In experiments using the standard buffer solution intra- and extravasally, R_{inp} was not significantly different (115 ± 20 MΩ, $n = 32$).

The input capacitance C of the membrane hyperpolarized by current injection was calculated by fitting the relationship between τ and R_{inp} for every impalement by:

$$\tau = CR_{inp}, \quad (3)$$

where τ is the mean of τ_{on} and τ_{off} (Fig. 1*C*). The mean capacitance was 12·3 ± 2·2 pF (96 impalements in 3 vessels).

To check the electrical coupling between VSMCs, similar experiments were performed as described above and additionally the membrane potential was measured by a second microelectrode (ME) 120–150 μm from the first impalement site. Figure 2 demonstrates a typical example of such a recording where the current injected by ME1 had no influence on the membrane potential measured by ME2. In twenty recordings, the mean voltage deviation measured at ME2 was 0·4 ± 0·3% (not significantly different from 0) of the voltage deviation at ME1.

ACh-induced endothelium-dependent increase in conductance

After prestimulation of the small mesenteric arteries with noradrenaline (NA), control current ramps were registered by depolarizing the impaled smooth muscle from −90 to +20 mV by 500 ms voltage ramps. As demonstrated in Fig. 3*A*, the membrane current showed a slight inward rectification. The mean conductance g was determined by averaging linear current–voltage regressions in the voltage range between −60 and −20 mV for the currents of every impalement. The slope conductance was significantly increased after intravasal application of acetylcholine (ACh) (for statistics, see Fig. 5). The zero current potential of the ramp current was shifted to more negative potentials indicative of a hyperpolarization of the membrane. Like the control current, the current after application of ACh displayed a slight inward rectification. Both ramp currents

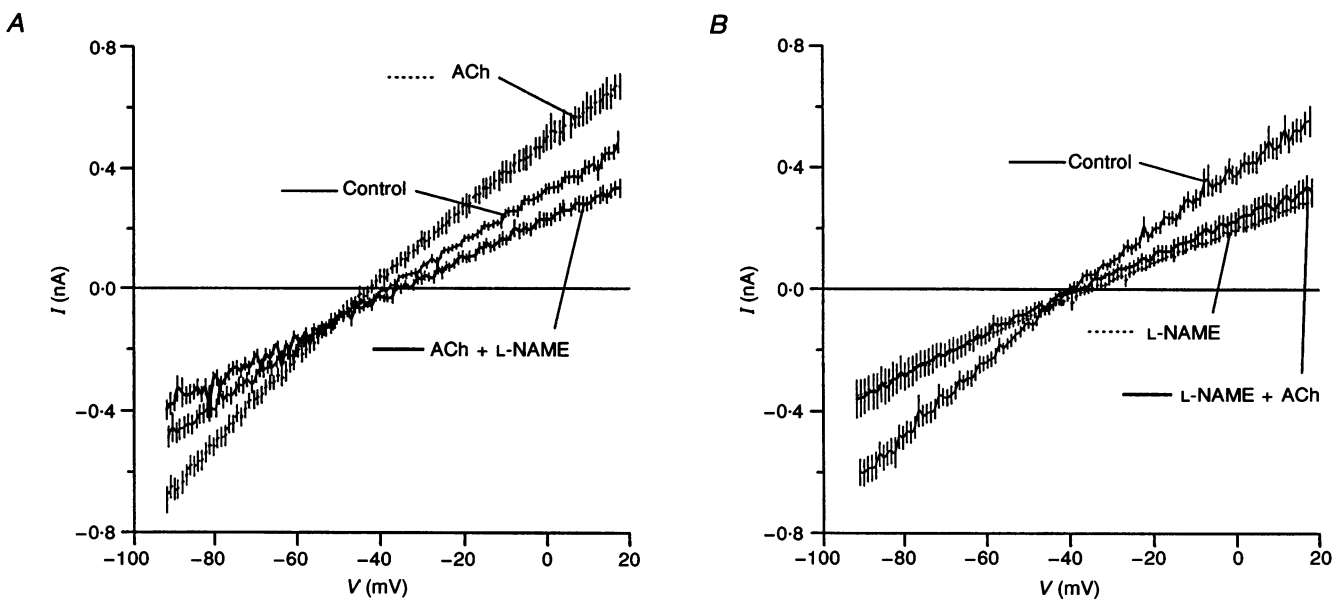

Figure 3. Effect of ACh and L-NAME on the membrane current of vascular smooth muscle

A, under control conditions the intra- and extravasal solutions were standard buffer solution plus DIDS, nisoldipine and indomethacin; the intravasal solution was supplemented with L-arginine and the extravasal solution contained noradrenaline in addition. Approximately 10 min later, acetylcholine (ACh) was added to the intravasal control solution. Again about 10 min later, the intravasal L-arginine was substituted by L-NAME (ACh + L-NAME). *B*, same protocol for the control condition as in *A*. Thereafter, L-arginine was substituted intravasally by L-NAME (L-NAME). The third mean trace was calculated from measurements after addition of ACh to the L-NAME containing intravasal solution (L-NAME + ACh). Each mean current was calculated from 12 to 19 impalements in *A* and *B*, respectively, each in 3 preparations with endothelium. The vertical bars represent ± S.E.M. Currents were evoked by clamping the smooth muscle cells by voltage ramps going from −90 to +20 mV within 500 ms.

crossed at about −55 mV. This value marks the reversal potential of the ACh-induced current. The further addition of L-NAME, a blocker of the nitric oxide synthase (NOS) (Kerwin, Lancaster & Feldman, 1995), decreased the slope conductance below the control value. The current after application of L-NAME also crossed the control current as well as the current after ACh application at about −55 mV.

In three other preparations, L-NAME was applied first to the intravasal solution. This led to a decrease in the slope conductance of the clamped smooth muscle (Fig. 3*B*) and a rightward shift of the current reversal potential. Furthermore, L-NAME prevented the ACh-inducible increase of the slope conductance. The cross-over point of the three currents was at about −45 mV.

Involvement of the soluble guanylate cyclase

It is known that many effects of endothelial nitric oxide (NO) are mediated by stimulation of the soluble guanylate cyclase (sGCl), Therefore, we tested to see if inhibition of this enzyme by Methylene Blue (MB; Ignarro *et al.* 1985) affected the ionic currents in vascular smooth muscle. As shown in Fig. 4, MB reduces the ramp current and shifts the reversal potential in a positive direction. The increase in slope conductance due to ACh application to the vascular endothelium, however, is not blocked by MB.

Figure 5 summarizes the results of current measurements in rat small mesenteric arteries with intact endothelium.

Application of ACh to the vessel lumen significantly increased the slope conductance of the vascular smooth muscle. This was accompanied by a hyperpolarization of the membrane. The block of the NOS as well as of the sGCl caused a reduction in conductance and a depolarization of the membrane. The effect of ACh on membrane potential and conductance was blocked by the NOS-antagonist L-NAME but not by inhibition of the sGCl.

Simulation of the ACh effect by sodium nitroprusside

From the experiments with intact endothelium preparations it can be concluded that the stimulation of ionic currents by intravasal ACh in vascular smooth muscle is mediated by NO. Therefore, the next experiments were performed to test whether sodium nitroprusside (SNP) could mimic the effect of ACh on the membrane currents of VSMCs. The endothelium was severely damaged by perfusion with air for 1 min to suppress the endogenous release of NO. The success of this procedure was confirmed in foregoing experiments by testing the reduction to less than 10% of the relaxing effect of 10 μM ACh on the vessel. Some relaxing effect remained, however, indicating an incomplete removal of the endothelium. More rigourous procedures like rubbing or perfusion of detergents to completely destroy the endothelium were not used because they also damaged the VSMC, resulting in a drastic increase in unsuccessful impalements.

In Fig. 6, a mean ramp current from impalements under control conditions in vascular smooth muscle is demonstrated.

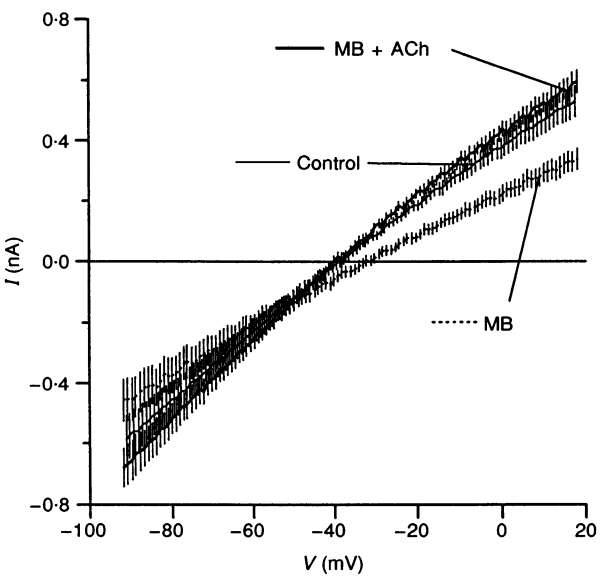

Figure 4. Effect of blocking the soluble guanylate cyclase by Methylene Blue on endothelium-dependent current induction

About 10 min after measurement of the control currents, Methylene Blue (MB) was added to the extravasal solution. A further 10 min later, ACh was applied intravasally (MB + ACh). Mean currents ± S.E.M. from 13 to 18 impalements in 3 preparations.

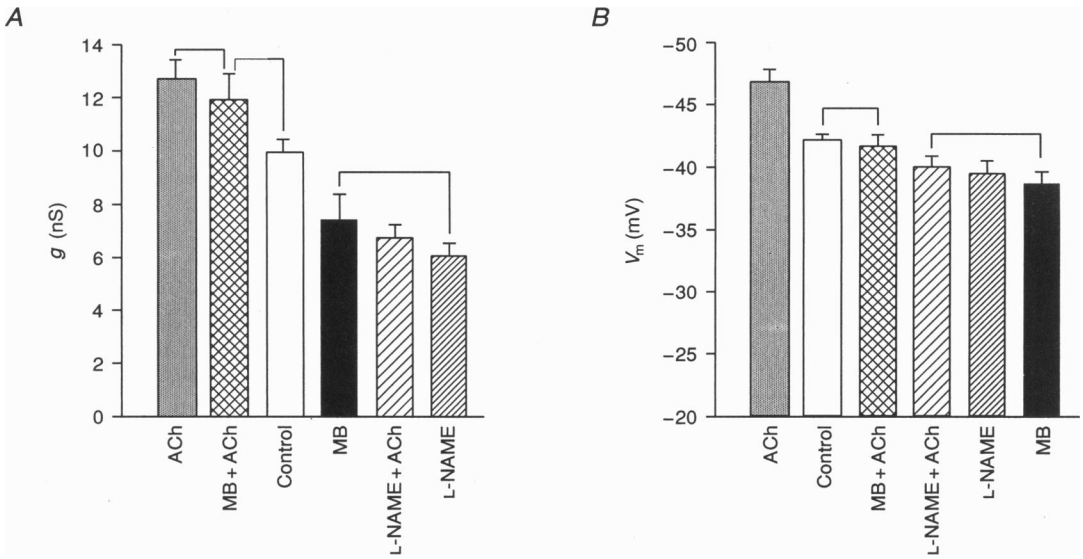

Figure 5. Block of the ACh-induced hyperpolarizing current by L-NAME but not by Methylene Blue

Statistics of the measurements demonstrated in Figs 3 and 4. *A*, slope conductance *g* of the membrane current ramps between −60 and −20 mV. *B*, membrane potential of the vascular smooth muscle measured in the current-clamp mode 1 to 3 s before application of the voltage ramps in the voltage-clamp mode. Columns of statistically not different means are sandwiched by brackets. Mean values ± S.E.M. of 10 to 58 impalements in 3 to 9 preparations with endothelium.

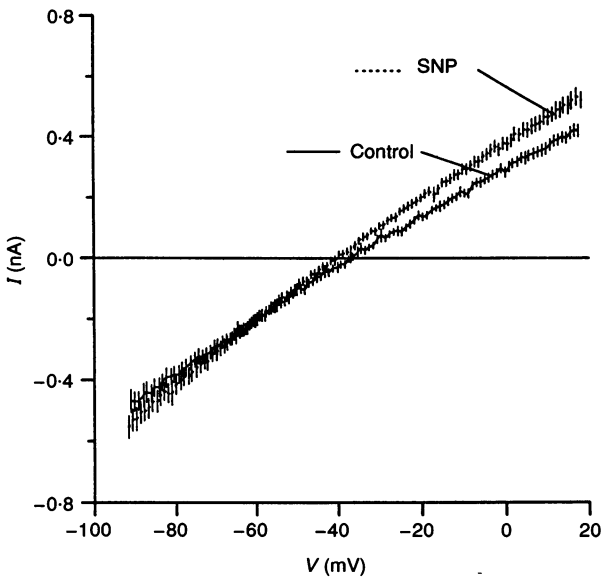

Figure 6. Effect of sodium nitroprusside on the membrane current of vascular smooth muscle without endothelium

Under control conditions, control solution was used as intra- and extravasal solution. Sodium nitroprusside (SNP) was added to the intravasal solution about 10 min after registration of the control currents. Means ± S.E.M. of 37 (control) and 29 (SNP) impalements, respectively, in 8 preparations.

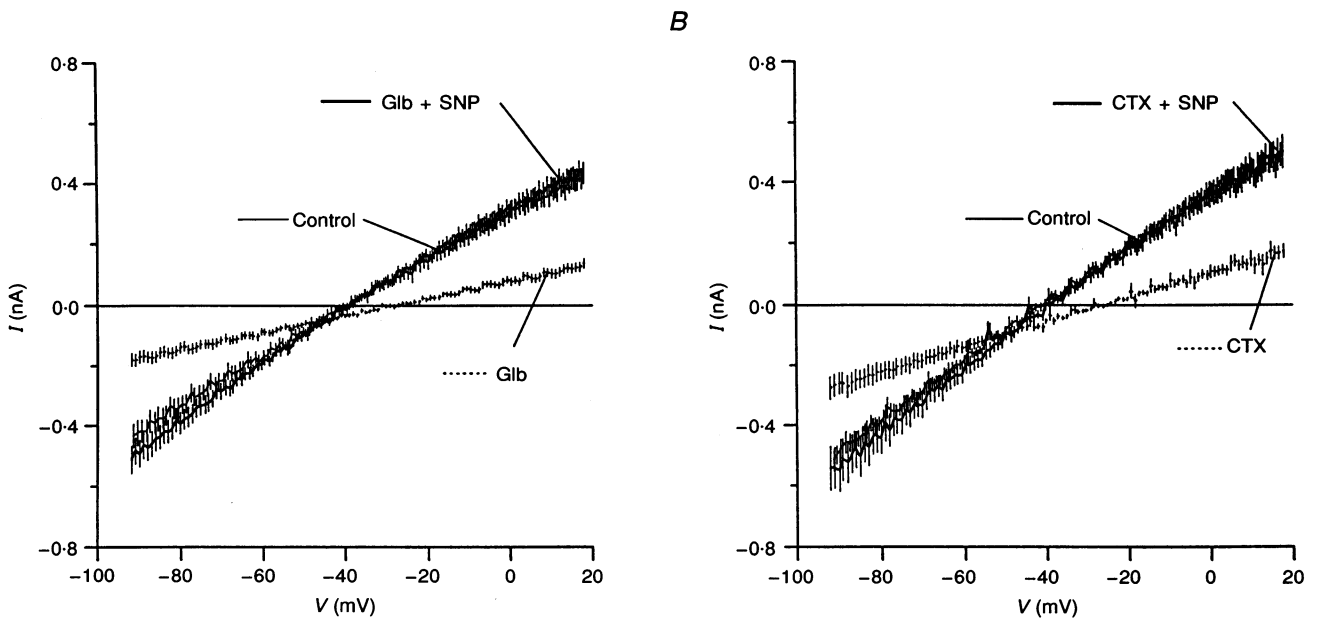

Figure 7. Effect of glibenclamide or charybdotoxin, respectively, on NA- and SNP-induced currents of vascular smooth muscle without endothelium

Same control conditions as in Fig. 6. *A*, application of glibenclamide (Glb) to the extravasal solution. Subsequent additional application of SNP to the intravasal solution (Glb + SNP). *B*, extravasal application of CTX. Membrane current after the following supplementary intravasal addition of SNP (CTX + SNP). Means ± S.E.M. of 12 to 26 impalements in 4 preparations each.

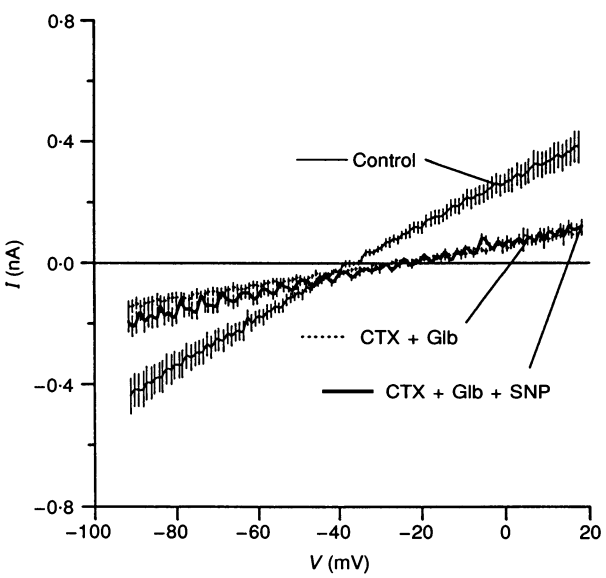

Figure 8. Effect of the combined action of charybdotoxin and glibenclamide on NA- and SNP-induced currents of vascular smooth muscle without endothelium

The same control conditions were used as in Fig. 6. Current after application of the combination of charybdotoxin and glibenclamide (CTX + Glb) to the extravasal solution. Current measured during additional intravasal administration of SNP (CTX + Glb + SNP). Means ± S.E.M. of 14 to 21 impalements in 3 preparations without endothelium.

The amplitude and the amount of inward rectification of this current is similar to the control current measured in vessels with intact endothelium (see Figs 5 and 11). The intravasal application of SNP increased the slope conductance and hyperpolarized the membrane (for statistics, see Fig. 11). The intersection of the control current and the current after addition of SNP is in the range of −55 mV, i.e. near the reversal potential of the ACh-induced and the L-NAME-inhibited current.

Pharmacology of the NO-dependent ionic current

The reversal potential of the ACh- and SNP-induced current was measured in the region of −50 mV. As chloride currents were permanently blocked under all experimental conditions it is likely that the increase in the slope conductance of VSMCs was due to stimulation of K^+ currents. To gain further insight into the type of currents involved in vascular smooth muscle cells (Kuriyama *et al.* 1995; Nelson & Quayle, 1995), the effects of glibenclamide (Glb) and charybdotoxin (CTX), specific blockers of ATP-dependent ($I_{K(ATP)}$) and Ca^{2+}-dependent ($I_{BK(Ca)}$) K^+ currents, respectively, were tested.

The effect of K^+ channel blockers on the smooth muscle current is shown in Fig. 7*A*. The intravasal application of Glb caused a drastic reduction of the slope conductance accompanied by a depolarization of the membrane. The subsequent additional application of SNP to the vessel lumen reversed this effect of Glb, i.e. the slope conductance and potential again reached nearly the control values. The three mean currents crossed at about −45 mV.

The effect of CTX was very similar to the action of Glb (Fig. 7*B*). Intravasal administration of CTX alone decreased the slope conductance and depolarized the smooth muscle. The addition of SNP enhanced the conductance and repolarized the membrane to the control values again. The reversal potentials of the CTX-blocked and SNP-evoked currents were near −50 mV.

The combined action of Glb and CTX is illustrated in Fig. 8. Application of both K^+ channel blockers caused a prominent reduction in slope conductance and depolarization. Furthermore, the enhancement of the conductance as well as the hyperpolarization due to an intravasal application of SNP was now blocked. The reversal potential of the current blocked by the combined action of Glb and CTX was at about −45 mV.

The results shown in Figs 7 and 8 point to the fact that SNP may lead to the opening of K^+ channels in the membrane of VSMCs. This was confirmed by the finding that the reversal potential of the SNP-induced current was strongly dependent on the extracellular K^+ concentration (Fig. 9).

The involvement of the sGCl in the stimulation of ionic currents was again tested by blocking the enzyme with MB. As demonstrated in Fig. 10, MB depolarized the vascular smooth muscle by reducing a hyperpolarizing current. The SNP-evoked increase in slope conductance and hyperpolarization of the membrane, however, were not blocked by MB. The MB-blocked, as well as the subsequently SNP-stimulated current reversed at about −45 mV.

The statistical evaluation of the measurements in endothelium-denuded vessels is depicted in Fig. 11. A significant increase in the slope conductance was associated in most cases with a significant depolarization of the membrane. This indicates that the blockers used inhibit

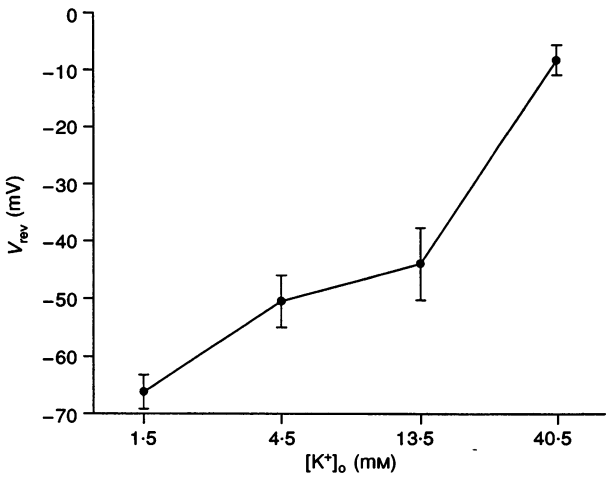

Figure 9. Dependence of the reversal potential of the SNP-induced current on the extracellular K^+ concentration

Same protocol as Fig. 6. The reversal potential of the SNP-induced current was considered as the crossing-over point of the mean ramp current from 8 to 15 impalements each, under control conditions and after intravasal application of SNP, respectively. Means ± S.E.M. of 3 to 5 preparations without endothelium.

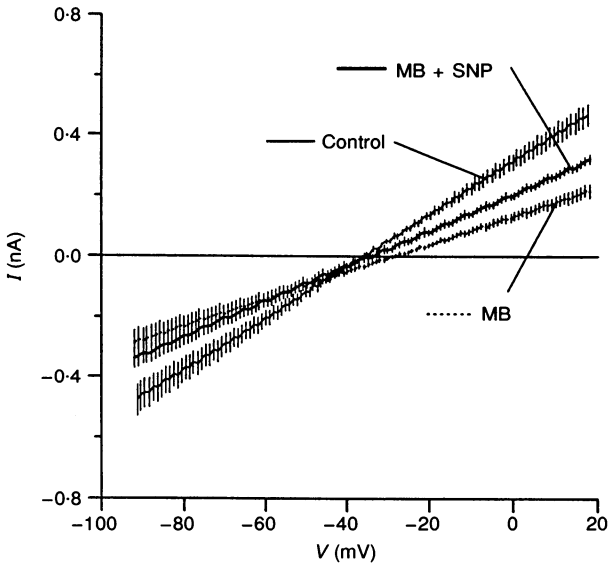

Figure 10. Effect of Methylene Blue on SNP-dependent current induction

Same control conditions (control) as in Fig. 6. Ramp current after supplementation of the bathing solution with Methylene Blue (MB). Current measured during additional intravasal administration of SNP (MB + SNP). Means ± S.E.M. of 12 to 17 impalements in 3 preparations.

hyperpolarizing currents in the vascular smooth muscle. The combined block of $I_{K_{ATP}}$ and $I_{BK(Ca)}$ reduced the slope conductance to 25% of the control value. This indicates that under our control conditions, i.e. if Ca^{2+} channels and chloride channels are blocked, the membrane conductance is dominated by these K^+ channels. This may also explain that the further enhancement of the K^+ conductance after application of SNP was not accompanied by a significant hyperpolarization.

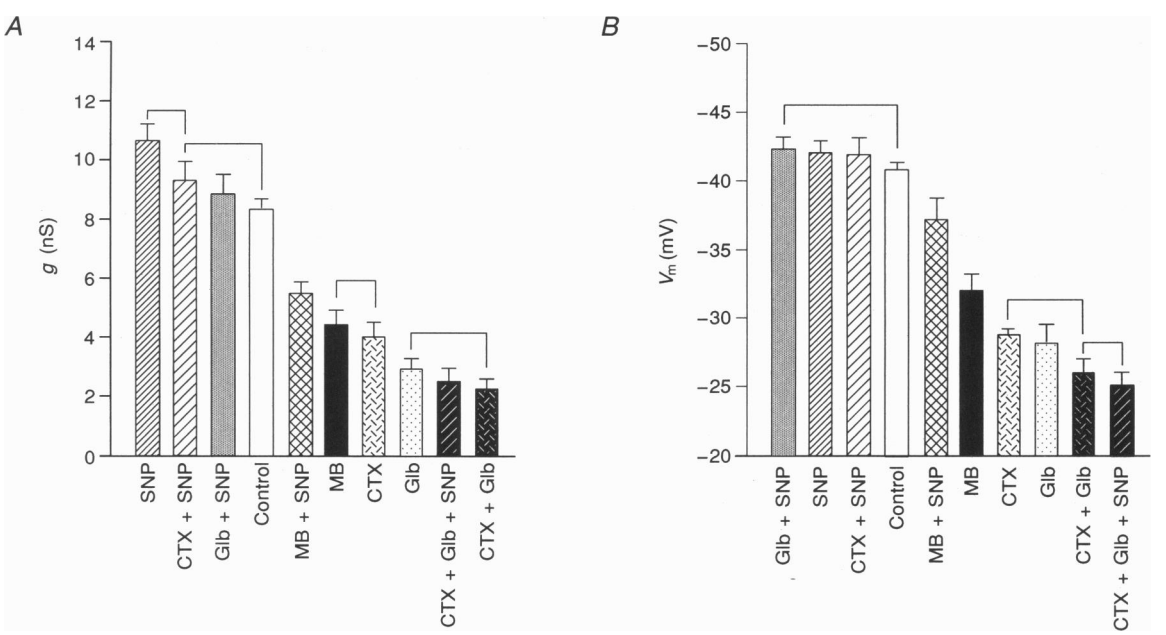

Figure 11. Characteristics of the SNP-induced membrane current in vascular smooth muscle without endothelium

Statistics of the slope of the current ramps (*g*) between −60 and −20 mV (*A*) and the membrane potential (*B*) for the experiments demonstrated in Figs 6, 7, 8 and 10. Columns of statistically not different means are sandwiched by brackets. Mean values ± S.E.M. of 14 to 96 impalements in 3 to 22 preparations.

DISCUSSION

Experimental design

The aim of the study was to investigate the ionic currents in vascular smooth muscle cells mediating the endothelium-dependent hyperpolarization within an intact vessel preparation. To focus on K^+ currents, Ca^{2+}-activated Cl^-currents which may also contribute to the ACh-induced change in the membrane potential (Cohen & Vanhoutte, 1995) were inhibited directly (by DIDS) and indirectly (by nisoldipine). The block of the Ca^{2+} influx via L-type Ca^{2+} currents by nisoldipine also reduced the contractile responses of the vessels to the tested substances. Therefore, the indirect effects of these tools on the electrophysiological behaviour of the VSM exerted by changing the intravasal hydrostatic pressure and shear stress of the endothelium, were also reduced by nisoldipine. Other experimental parameters, for example partial pressure of oxygen, flow, pressure, and nearly isotonic contractions were chosen to match physiological conditions as closely as possible.

Passive electrical behaviour

An investigation of the passive electrical properties of VSM in large vessels has been carried out in several experiments in which current was applied to an extracellular partition of the vessel. In this case, it has been shown that VSMs had cable-like properties. The electrotonic potentials decayed monoexponentially with time and distance from the partition of current stimulation. Length constants of about 0·4–2·4 mm and time constants in the range of 60–450 ms were measured (Casteels, Kitamura, Kuriyama & Suzuki, 1977; Mekata, 1980; Kajiwara, 1982; Surprenant, Neild & Holman, 1987). When current was applied intracellularly through microelectrodes, the time constants of voltage relaxation were found in most cases to be much shorter, i.e. in the range of 1·5–30 ms. This was attributed to three-dimensional current spread through electrical connections between single smooth muscle cells (Tomita, 1975; Mekata, 1980; Surprenant *et al.* 1987). It has been pointed out that if a VSM behaves like an electrical syncytium, the input resistance measured by micro-electrodes is mainly determined by the internal resistance and is extremely insensitive to changes in membrane resistance. Therefore, the microelectrode technique would be inadequate to draw conclusions about the involvement of membrane currents in the regulation of vascular tone (Jack, Noble & Tsien, 1975; Tomita, 1975). In the present preparation, a short mean time constant of about 1·7 ms was also measured. However, the following findings are contradictory to a strong electrical coupling between the vascular smooth muscle cells investigated here. (1) The input resistance ($R_{inp} = 1/g$) was changed from about 120 MΩ under control conditions (without endothelium) to 440 MΩ by application of well-known blockers of K^+ channels of the VSMC membrane (see Figs 1*C* and 11). (2) Under control conditions and also in standard saline solution without blocking Ca^{2+} and Cl^- currents, R_{inp} was obviously larger than measured in preparations with supposed three-dimensional current spread in which a value of R_{inp} of 5–40 MΩ was determined (Mekata, 1980; Surprenant *et al.* 1987). Short time constants of the decay of electrotonic potentials (2–5 ms) together with relatively high R_{inp} (about 180 MΩ) values have also been observed by Mekata (1980) in some impalements in dog circumflex artery. (3) The time course of the voltage relaxation after application of current steps was obviously monoexponential and linearly dependent on R_{inp}. This is also contradictory to the behaviour of an electrical syncytium where the voltage response to a step of current was described by a sum of two error functions (Jack *et al.* 1975) or, in a simplified model, by the sum of two exponentials (Tomita, 1975). In VSMCs of arterioles of guinea-pig submucosa which are electrically connected, Hirst & Neild (1978) found two phases of decay of the electrotonic potential with an exponential time course of the slow component. (4) Electrotonic potentials are not transferred between VSMCs 100 to 150 μm apart.

These findings mean that we can speculate that in our preparation the VSMCs were poorly coupled. Nevertheless, inhomogeneities in the voltage-clamped tissue cannot be ruled out. The method used was at least able to give qualitative information about which changes (decrease or increase) in hyperpolarizing or depolarizing currents were responsible for shifts of the membrane potential of the VSMCs.

Actions of acetylcholine

The hyperpolarizing effect of ACh on the membrane potential of rat mesenteric VSMCs was shown to be caused by activation of a hyperpolarizing current. The reversal potential of the ACh-induced current was found at about −55 mV. This was in the range of the SNP-induced, as well as the L-NAME-, MB-, CTX- and Glb-blocked currents of between −55 and −45 mV. This implies that all these currents were K^+ currents. This assumption is supported by the finding that the reversal potential of the SNP-induced current is dependent on the extracellular K^+ concentration. The large scattering in the reversal potentials may be due to the inability of the microelectrode method to isolate the currents completely. The reversal potential for K^+ currents would be expected to be more negative than −70 mV (Nelson & Quayle, 1995). The reason for this discrepancy is probably not the additional activation by ACh of other currents with reversal potentials positive to −40 mV, like chloride, non-specific cation or leak currents. In order to shift the reversal potential always in the positive direction, these currents should have the same pharmacological characteristics as the supposed K^+ currents. Large errors in the measurement of the membrane potentials are also probably not responsible for the positive reversal potential of the K^+ currents, as the membrane potential measured in standard buffer solution of −43·2 ± 9·2 mV (mean ± S.D., 254 cells, 30 vessels) is near to the value of −49·7 mV reported for pressurized rat mesenteric arteries (Schubert, Wesselman, Nilsson & Mulvany, 1996).

Spatial inhomogeneities in the voltage clamp as a fundamental source of error can be excluded for the following reason. If the voltage of the VSM cell membrane is clamped at values negative to the resting membrane potential, the clamped voltage for membranes apart from the microelectrode (where the voltage is measured) would be less negative in the case of spatial inhomogeneity. This would mean that the measured inward current which is the sum of currents from different parts of the VSM membrane is smaller than in the case of a homogeneous clamp. Therefore, the real current–voltage relationship would be steeper than the current–voltage relationship measured by the microelectrode. This error rises with a decreasing space constant of the clamped VSM, i.e. with increasing membrane conductance. As a result, the true crossing-over point of two current–voltage relationships would be shifted to more positive potentials.

One possible explanation for the positive reversal potential of the supposed K^+ currents is a reduced gradient of K^+ concentrations from the intracellular to the extracellular side of the VSMC membrane. This may be caused by a continuous K^+ outward current induced by the sustained depolarization due to the stretch of the VSM and the chronic application of NA (Schubert *et al.* 1996). This K^+ current may not have been fully compensated by the Na^+–K^+-ATPase leading to change of the intra- and extracellular K^+ concentrations. The deviation from a pure K^+ electrode of the dependence of the reversal potential (V_{rev}) of the SNP-induced current on the extracellular potassium concentration also supports this assumption.

The ACh-induced hyperpolarization of the VSMC membrane occurs with an increase in membrane conductance in rat pulmonary artery (Chen & Suzuki, 1989). Further evidence for the involvement of K^+ currents in the endothelium-dependent hyperpolarization came from the observations of an accompanying increased rubidium efflux in rat aorta (Chen, Suzuki & Weston, 1988; Taylor, Southerton, Weston & Baker, 1988). The finding that an increase in the extracellular K^+ concentration reduced the endothelium-dependent hyperpolarization (Chen & Suzuki, 1989; Fujii *et al.* 1992; Plane & Garland, 1993; Parkington, Tonta, Coleman & Tare, 1995) seems also to confirm the role of K^+ channels in the ACh-dependent hyperpolarization. However, as long as the equilibrium potential for Cl^- ions is positive to the equilibrium potential for K^+ and positive to the resting potential, a closure of Cl^- channels can also explain the observed reduction of the hyperpolarization.

Involvement of NO in the induction of the hyperpolarizing current

The application of the NOS antagonist L-NAME reduced the membrane conductance and depolarized the VSM. This implies that a tonic release of NO by the endothelium is responsible for maintaining the activity of a hyperpolarizing current. Furthermore, L-NAME also abolishes the stimulating effect of ACh on the hyperpolarizing current. This indicates that NO is the endothelium-derived hyperpolarizing factor in the present experiments. In several preparations it was shown that especially the transient component of the endothelium-dependent hyperpolarization (EDH) was not altered by antagonists of the NOS whereas the tonic component was blocked (Nagao & Vanhoutte, 1992; Vanheel, Van de Voorde & Leusen, 1994; Parkington *et al.* 1995). So the possibility exists that the transient component of the EDH is not mediated by NO and, therefore, is independent of NOS antagonists (for review, see Garland *et al.* 1995). The sustained component of the EDH, on the other hand, may be NO dependent. Due to the difficulty in maintaining stable impalements in VSM, recordings of the membrane potential too often do not last long enough to discriminate between the phasic and tonic components of the EDH. Moreover, the proportions of these components may vary in different preparations. In the present experiments, the electrophysiological measurements started at least 5 min after the intravasal application of ACh. Therefore, only the tonic component of the EDH could be measured.

It has been assumed that the EDH is caused by ACh-induced hyperpolarization of the endothelium, conducted to the VSMC due to electrical coupling between both cell types (Cohen & Vanhoutte, 1995). NOS blockers are without effect on the ACh-induced hyperpolarization in endothelium (Chen & Cheung, 1992). However, in the experiments described here L-NAME blocked the ACh-dependent increase in the hyperpolarizing current. Therefore, it is unlikely that in the present preparation the ACh-induced hyperpolarization was caused in the endothelium and transmitted to the VSMCs which might occur in the arterioles (Daut *et al.* 1994).

Blocking the resting activity of the soluble guanylate cyclase by MB reduced the slope conductance of the VSM. The reversal potential of the MB-blocked current points to the fact that a resting level of cGMP retains a part of the K^+ conductance of the VSM. This corresponds to the findings that the cGMP-dependent protein kinase activates Ca^{2+}-dependent K^+ currents in VSMCs (Robertson, Schubert, Hescheler & Nelson, 1993; Archer, Huang, Hampl, Nelson, Shultz & Weir, 1994). MB, however, does not block the stimulating effect of ACh or SNP on the membrane conductance. So it may be concluded that NO mediates the tonic EDH in the present preparation but not via a cGMP-dependent pathway. The absence of an effect of MB in rat mesenteric artery is in contrast to the findings in guinea-pig uterine artery and in cultured cells of porcine coronary artery where the NO-induced hyperpolarization was abolished by MB (Tare, Parkington, Coleman, Neild & Dusting, 1990; Miyoshi, Nakaya & Moritoki, 1994). According to the present findings, in isolated VSMCs of rat pulmonary artery the whole cell K^+ current was not

different from the control value after the simultaneous application of NO and MB (Archer *et al.* 1994). Unfortunately, the authors do not report about the K^+ current after administration of MB alone. In rabbit thoracic aorta it was found that NO activates single Ca^{2+}-dependent K^+ channels without requiring intracellular cGMP, and that MB did not block the NO-dependent relaxation of rabbit aortic rings (Bolotina, Najibi, Palacino, Pagano & Cohen, 1994).

Characterization of the current underlying EDH

The effect of ACh on the VSM could be mimicked by administration of SNP. The increase in membrane conductance is significant but smaller than after application of ACh in preparations with intact endothelium. One explanation for this is the incomplete removal of the endothelium. This assumption is supported by the finding that the membrane conductance of the VSM is smaller after administration of L-NAME than after destruction of the endothelium. Furthermore, after intravasal application of air, MB was still able to reduce the conductance of the VSM membrane probably by blocking the activity of the sGCl induced by the release of NO from the remaining endothelium. This residual production of NO may become suppressed by the application of SNP, thereby reducing the overall stimulating effect of SNP on the membrane conductance of the VSM (Rand & Garland, 1992). Nevertheless, qualitative conclusions about the currents induced by SNP in the VSMC membrane can be drawn irrespective of the presence of remaining endothelium.

The effect of SNP can be blocked completely only by the combination of Glb and CTX. This demonstrates for the first time, to our knowledge, that NO activates ATP-, as well as Ca^{2+}-dependent, K^+ channels in one vascular preparation. It is known that NO can activate either ATP- (Miyoshi *et al.* 1994) or Ca^{2+}-dependent K^+ channels (Tang & Zheng, 1993; Archer *et al.* 1994, 1996; Bolotina *et al.* 1994; Miyoshi & Nakaya, 1994) in isolated VSMCs. Moreover, it is well established that endothelium-dependent or NO-induced hyperpolarizations in vascular smooth muscle can be blocked by either Glb (Brayden, 1990; Plane & Garland, 1993; Murphy & Brayden, 1995; Parkington *et al.* 1995; Plane *et al.* 1995) or CTX (Bolotina *et al.* 1994). On the other hand, in several preparations no effect of Glb (Chen, Yamamoto, Miwa & Suzuki, 1991; Fujii *et al.* 1992; Van de Voorde, Vanheel & Leusen, 1992; Murphy & Brayden, 1995; Parkington *et al.* 1995) or the specific blocker of Ca^{2+}-dependent K^+ channels iberotoxin (Murphy & Brayden, 1995), on the EDH was found. This illustrates again the diversity of the mechanisms of the EDH depending on the preparation and the experimental conditions.

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Acknowledgements

The authors would like to thank G. Isenberg for reading the manuscript and helpful discussions. This work was supported by the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (01 ZZ 9105, TV 1·6).

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Received 5 July 1996; accepted 27 January 1997.