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Endothelium-derived hyperpolarizing factor but not NO reduces smooth muscle Ca^{2+} during acetylcholine-induced dilation of microvessels

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1 We hypothesized that nitric oxide (NO) and the endothelium-dependent hyperpolarizing factor (EDHF) may dilate microvessels by different cellular mechanisms, namely Ca^{2+}-desensitization versus decrease in intracellular free calcium.

2 Effects of acetylcholine (ACh) and the NO donors sodium nitroprusside (SNP, 0.1–10 μmol l^{-1}) and S-Nitroso-N-acetyl-D,L-penicillamine (SNAP, 0.01–10 μmol l^{-1}) on intracellular calcium ($[Ca^{2+}]_i$, fura 2) and vascular diameter (videomicroscopy) were studied in isolated resistance arteries from hamster gracilis muscle (194±12 μm) pretreated with indomethacin and norepinephrine. Membrane potential changes were determined using 1,3-dibutylbarbituric acid trimethineoxonol ($DiBAC_4(3)$).

3 ACh (0.1 and 1 μmol l^{-1})-induced dilations were associated with a $[Ca^{2+}]_i$ decrease (by 13±3 and 32±4%) and hyperpolarization of vascular smooth muscle (VSM, by 12±1% at 1 μmol l^{-1} ACh). N^{ω}-nitro-L-arginine (L-NA, 30 μmol l^{-1}) partially inhibited the dilation but did not affect VSM $[Ca^{2+}]_i$ decreases or hyperpolarization. In contrast, the K_{Ca} channel inhibitors tetrabutylammonium (TBA, 1 mmol l^{-1}) and charybdotoxin (ChTX, 1 μmol l^{-1}) abolished the ACh-induced $[Ca^{2+}]_i$ decrease and the hyperpolarization in VSM while a significant dilation remained (25 and 40%). This remaining dilation was abolished by L-NA. ChTX did not affect $[Ca^{2+}]_i$ increase and hyperpolarization in endothelial cells. SNP- or SNAP-induced dilations were not associated with decreases in VSM $[Ca^{2+}]_i$ or hyperpolarization although minor transient decreases in VSM $[Ca^{2+}]_i$ were observed at high concentrations.

4 These data suggest that ACh-induced dilations in microvessels are predominantly mediated by a factor different from NO and PGI_2, presumably EDHF. EDHF exerts dilation by activation of K_{Ca} channels and a subsequent decrease in VSM $[Ca^{2+}]_i$, NO dilates the microvessels in a calcium-independent manner.

Keywords: Endothelium-derived hyperpolarizing factor (EDHF); nitric oxide (NO); resistance arteries; K^+ channels; charybdotoxin

Abbreviations: ACh, acetylcholine; $[Ca^{2+}]_i$, intracellular calcium; ChTX, charybdotoxin; COX, cyclooxygenase; EDHF, endothelium-derived hyperpolarizing factor; L-NA, N^{ω}-nitro-L-arginine; NE, norepinephrine; NO, nitric oxide; NOS, nitric oxide synthase; PGI_2, prostacyclin; SNAP, S-Nitroso-N-acetyl-D,L-penicillamine; SNP, sodium nitroprusside; TBA, tetrabutylammonium hydrogen sulphate; VOC, voltage-operated calcium channel; VSMC, vascular smooth muscle cell

Introduction

The vasodilating effect of acetylcholine (ACh) is thought to be mediated by different endothelial factors. It is well established that nitric oxide (NO) and prostacyclin (PGI_2) play a major role in mediating this ACh-induced dilation. The fact that a distinct dilation remains even after combined inhibition of NO synthase (NOS) and cyclooxygenase (COX) suggests a role for a third factor, the so-called endothelium-derived hyperpolarizing factor (EDHF). EDHF has been demonstrated to activate potassium channels in vascular smooth muscle cells and to be active predominantly in the microcirculation (Hwa *et al*., 1994; Nagao *et al*., 1992). However, its chemical nature is still unknown. Although it has been suggested that EDHF-dependent dilations might in fact reflect incomplete inhibition of NOS or depletion of storage sites (Cohen *et al*., 1997; Davisson *et al*., 1996; Ignarro, 1990), convincing evidence for the existence of an EDHF which is distinct from NO has been provided (Mombouli *et al*., 1996; Popp *et al*., 1996). Vascular smooth muscle relaxation can be induced not only by a reduction in $[Ca^{2+}]_i$ but also by a reduction in the calcium-sensitivity of the contractile apparatus at constant $[Ca^{2+}]_i$ (Gryglewski, 1993; Hirata *et al*., 1992; Kitazawa *et al*., 1991; Pfitzer *et al*., 1993; Satoh *et al*., 1993). Hyperpolarization of vascular smooth muscle cells (VSMC) presumably inhibits the intracellular release of calcium (Yamagishi *et al*., 1992) and induces the closure of voltage-operated Ca^{2+}-channels (Robertson *et al*., 1993; Williams *et al*., 1988) and, therefore, EDHF would be expected to induce a reduction in $[Ca^{2+}]_i$. Although NO has been reported to decrease $[Ca^{2+}]_i$ in VSMCs (Andriantsitohaina *et al*., 1995; Bolotina *et al*., 1994; Lincoln & Cornwell, 1991; Lincoln *et al*., 1990; 1994; Miyakawa *et al*. 1994; Schmidt *et al*., 1993; Thornhill & Haskard, 1990), recent data suggest that NO could exert a substantial part of its dilatory effect *via* a mechanism which is independent of changes in $[Ca^{2+}]_i$ (Lee *et al*., 1997; Nishimura & van Breemen, 1989; Wu *et al*., 1996). As it is not known how NO exerts dilation in microvascular smooth muscle, we hypothesized that desensitization to Ca^{2+} could be the predominant mechanism

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through which NO induces dilation in the microcirculation. Considering that EDHF should dilate *via* a strictly Ca^{2+}-dependent mechanism, we further hypothesized that NO- and EDHF-mediated dilations of microvascular smooth muscle should exert differential effects on smooth muscle $[Ca^{2+}]_i$. We therefore studied the effects of ACh, especially after NOS and COX inhibition, on $[Ca^{2+}]_i$ and diameter in norepinephrine-preconstricted microvessels and compared this response with the effects of the NO donors SNP and SNAP as well as an endothelium-independent hyperpolarization. We report here that endogenously produced and exogenously applied NO does not decrease smooth muscle $[Ca^{2+}]_i$ in these microvessels. Since under conditions of combined NOS and COX inhibition, stimulation with ACh reduced $[Ca^{2+}]_i$ in a K_{Ca} channel inhibitor-sensitive manner, we conclude that EDHF decreases vascular tone by reducing $[Ca^{2+}]_i$ in smooth muscle cells.

Methods

Surgical preparation of small skeletal arteries

The care of the animals and the experimental procedures performed in this study were in strict accordance with the standards and guidelines provided by German animal protection laws.

Female Golden Syrian hamsters (154 ± 2 g body weight) were anaesthetized by intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1}). Side branches of the femoral artery were exposed by removing overlying bundles of the quadriceps muscle. A small artery of the second or third generation was isolated and carefully dissected from connective tissue avoiding any stretching. Throughout this preparation the vessel was continuously superfused with an ice cold 3-morpholinopropanesulphonic acid (MOPS)-buffered salt solution (for composition see 'Drugs'). The vessel segment was transferred into a temperature-controlled organ bath, cannulated with micromanipulator mounted glass micropipettes and tied off using monofile sutures (Ethicon, monophilic 11.0). The transmural pressure was maintained hydrostatically at 45 mmHg. The setup was mounted on the stage of a modified inverted microscope (Olympus, IMT-2) equipped with a $\times 20$ lens (Olympus D-APO 20 UV) and a video camera system.

$[Ca^{2+}]_i$- and diameter measurements

The organ bath temperature was slowly increased to 37°C and maintained at this level throughout the experiment. After a 20 min equilibration period, loading of the vascular smooth muscle cells with the Ca^{2+}-sensitive dye fura 2 was achieved by exchanging the pure MOPS-buffered saline in the organ bath for MOPS-buffered saline containing 2 μmol l^{-1} fura 2-acetoxymethylester (AM) and 0.5% bovine serum albumin (BSA). Dye-loading was terminated after 2 h by washing the vessel carefully with MOPS saline. Selective loading of the endothelium was achieved by perfusion (1 m l h^{-1}) of the vessel with MOPS-buffered saline containing 2 μM fura 2-acetoxymethylester (AM) and 0.5% bovine serum albumin (BSA). A change of the perfusion buffer for pure MOPS-buffered saline after 1 h terminated the loading of endothelial cells with fura 2-AM. $[Ca^{2+}]_i$ was measured according to the protocol described by Grynkiewicz *et al*. (1985). Alternating excitation wavelengths of 340 and 380 nm (selected by two monochromators, Photomed GmbH, Wedel/Holstein, Germany) were used and the fluorescence at a wavelength of 510 nm (bandpass filter 510 nm, Photomed GmbH) was recorded by means of a photomultiplier tube. The fluorescence ratio $F_{340\ nm}/F_{380\ nm}$ was calculated after subtraction of the background fluorescence (obtained after fura 2-quenching with 8 mmol l^{-1} $MnCl_2$). With the low concentration of fura 2-AM used in this study no calcium-buffering effects of the dye were determined. Loaded and unloaded vessels showed the same sensitivity to norepinephrine, angiotensin and increases of transmural pressure (myogenic response). Simultaneous to the fura 2 measurements the vessel was transilluminated at wavelengths >610 nm which did not interfere with the Ca^{2+}-related fluorescence measurements. A microscopic image was taken by a video camera (Grundig electronic FA76SIT, Germany) and stored on videotape (U-matic, Sony, Japan). The external diameter of the vessel was continuously measured online or offline by means of a calibrated digital image analysis system (laboratory made). The reproducibility of repeated measurements was ± 1.5 μm.

Membrane potential measurements

Membrane potential changes were assessed using the potential-sensitive dye bis-oxonol (bis[1,3-dibutylbarbituric acid] trimethineoxonol, [$DiBAC_4(3)$]) (Epps *et al*., 1994). Vascular smooth muscle cells of the small skeletal muscle arteries were loaded with bis-oxonol by continuous superfusion with MOPS-buffered saline containing 50 nmol l^{-1} bis-oxonol at 37°C for 30 min. All substances used during the subsequent experiments were dissolved in the superfusate containing 50 nmol l^{-1} bis-oxonol to ensure stable fluorescence. For selective loading of endothelial cells vessels were continuously perfused (0.5 ml h^{-1}) with MOPS buffer containing 50 nmol l^{-1} bis-oxonol. In this case, all other drugs were added to the organ bath. The dye bis-oxonol partitions between the cell membrane and the cytosol as a function of the membrane potential. Upon hyperpolarization dye molecules (excitation wavelength 490 nm) concentrate in the cell membranes and fluorescence intensity at the emission wavelength of 516 nm decreases. Depolarization results in the sequestration of the dye into the cytosol with increased binding to cytosolic proteins and is associated with an increase in the fluorescence intensity of bis-oxonol (Bräuner *et al*., 1984).

Experimental protocol

A total of 53 vessels from 53 animals were studied. Vascular diameter and the fura 2 fluorescence signal were recorded continuously. Experiments were started 30 min after termination of fura 2 loading. Vessels that did not develop spontaneous myogenic tone in response to 45 mmHg of transmural pressure during 30 min were excluded from the study. All experiments, except for a control series, were carried out in the presence of indomethacin (30 μmol l^{-1}), which was shown in pilot experiments to sufficiently inhibit prostaglandin synthesis. Indomethacin had no effects on either the ACh-induced dilation or decrease in vascular smooth muscle $[Ca^{2+}]_i$ ($n=5$) at any of the ACh concentrations (0.01 – 1 μmol l^{-1}) used. The viability of each vessel was assessed by its constrictor response to increasing, cumulative concentrations of NE (0.1, 0.3 and 1 μmol l^{-1}), as well as by the dilation elicited by subsequently added ACh (1 μmol l^{-1}). Because the isolated vessels developed only weak spontaneous tone all vessels were preconstricted to a level comparable to the *in vivo* situation with a standard concentration of NE (0.3 μmol l^{-1}, 2 min) prior to the addition of vasodilators (ACh, SNP, SNAP, felodipine).

To identify the relative contributions of NO and EDHF to ACh-induced vascular responses, the following series of

experiments was performed. First, the effects of increasing ACh concentrations (0.01, 0.1 and 1.0 μmol l^{-1}) on NE-preconstricted resistance arteries were tested before and after pretreatment with the irreversible NO synthase (NOS) inhibitor N^{ω}-nitro-L-arginine (L-NA). In a second series, the consequences of the application of 1 μmol l^{-1} ACh to NE-preconstricted vessels was monitored in the absence and repeated in the presence of TBA (1 mmol l^{-1}) or ChTX (1 μmol l^{-1}) in the organ bath, both of which are blockers of calcium-dependent K^+-channels, or in the presence of 30 μmol l^{-1} L-NA. To determine whether the residual response to ACh in presence of the K_{Ca}-channel inhibitors was mediated by NO, TBA as well as ChTX were applied to the organ bath in combination with L-NA in some experiments.

In complex preparations like intact small arteries the site of action of ChTX had to be characterized. It is unclear whether ChTX exclusively interferes with the effects of EDHF on the level of VSMCs or whether ChTX also reduces EDHF synthesis by interacting with endothelial K_{Ca} channels. Possible interactions of ChTX with endothelial K_{Ca} channels should affect endothelial $[Ca^{2+}]_i$ and subsequently the EDHF synthesis, which is dependent on an increase in endothelial $[Ca^{2+}]_i$. Therefore, the effects of abluminally applied ChTX on the endothelial Ca^{2+} homeostasis of L-NA/indomethacin-pretreated and ACh-stimu-

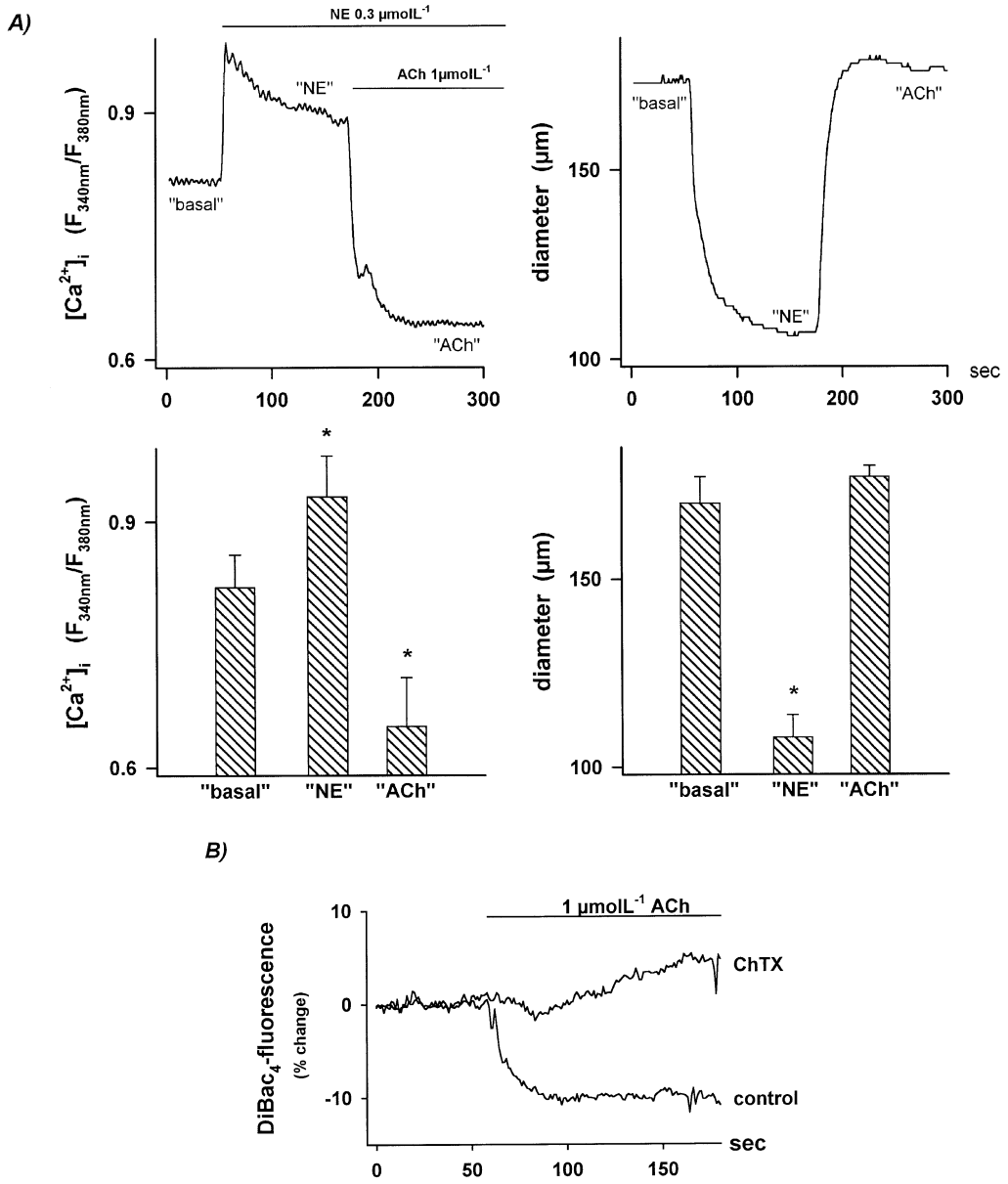

Figure 1 (A) Norepinephrine and acetylcholine-induced changes of diameter (dia) and intracellular calcium ($[Ca^{2+}]_i$) in small skeletal arteries. Small skeletal arteries were constricted with 0.3 μmol l^{-1} norepinephrine (NE) and subsequently stimulated with 1 μmol l^{-1} acetylcholine (ACh). Original recordings of changes in smooth muscle calcium ($[Ca^{2+}]_i$, ratio $F_{340\ nm}/F_{380\ nm}$) and diameter (μm) are shown in the top graphs. The box plots in the bottom panels summarize ratios and diameters under basal ('basal'), NE-stimulated ('NE') and ACh-stimulated ('ACh') conditions. (Box plots represent mean $\pm$ s.e.mean, $n=8$, * = significantly different ($P<0.05$) from basal value). (B) Original recording of acetylcholine-induced changes of membrane potential. Membrane potential changes were measured using the potential-sensitive dye $DiBAC(4)_3$. Vessels were preconstricted with 1 μmol l^{-1} NE before ACh (1 μmol l^{-1}) was added under control conditions (control) and in the presence of charybdotoxin (ChTX, 1 μmol l^{-1}). Under control conditions, the fluorescence signal decreased by $12 \pm 1\%$ after application of 1 μmol l^{-1} ACh, indicating hyperpolarization. This was completely inhibited in presence of ChTX.

lated vessels were studied. Fura fluorescence was confined to the endothelium for at least 3 h before smooth muscle cells became stained with the dye. The selective loading of endothelial and smooth muscle cells was confirmed by control experiments using double-labelling with Fura red and Calcium green. Fura red, which was detected only in endothelial cells using confocal microscopy yielded identical calcium kinetics as seen with selective endothelial Fura 2 loading, whereas simultaneous measurements of Calcium green fluorescence corresponded well to smooth muscle calcium signals obtained with Fura 2.

Effects of ACh and ChTX on membrane potential and diameter were studied in vessels which were only submaximally preconstricted with 0.1 μmol l^{-1} NE to avoid major diameter changes that could artefactually affect the bis-oxonol signal.

In a further experimental series, the effects of the dihydropyridine-type calcium antagonist felodipine (1 μmol l^{-1}), which induces immediate closure of voltage-operated calcium channels (VOCs), on $[Ca^{2+}]_i$ and vessel diameter were studied and compared to ACh effects.

Effects of a hyperpolarization other than that caused by ACh, were studied by moderately elevating the extracellular potassium concentration from 4.5 to 12.5 mmol l^{-1}. This treatment hyperpolarizes the smooth muscle cell membrane through activation of K_{IR} channels (Knot *et al*., 1996).

The endothelial dependency of the ACh-induced vascular responses was assessed by removal of the endothelium. Perfusion of the vessel with either 50 ml l^{-1} Saponin for 2 min ($n=4$) or air for 5 min ($n=5$) assured complete de-endothelialization.

The specific effects of NO on vessel diameter and $[Ca^{2+}]_i$ were studied directly by adding either sodium nitroprusside (SNP) or S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) to NE-preconstricted small arteries.

Drugs

The composition of the MOPS-buffered salt solution was as follows (mmol l^{-1}): NaCl 145, KCl 4.7, $CaCl_2$ 1.5, $MgSO_4$ 1.17, NaH_2PO_4 1.2, pyruvate 2.0, EGTA 0.02, MOPS 3.0 and glucose 5.0. Fura 2-AM and oxonol bis [1,3-dibutylbarbituric acid] trimethineoxonol ($DiBAC_4(3)$) (Molecular Probes, Oregon, U.S.A.) were dissolved in water-free dimethyl sulphoxide and stored as a 1 mmol l^{-1} stock solution (1 mg

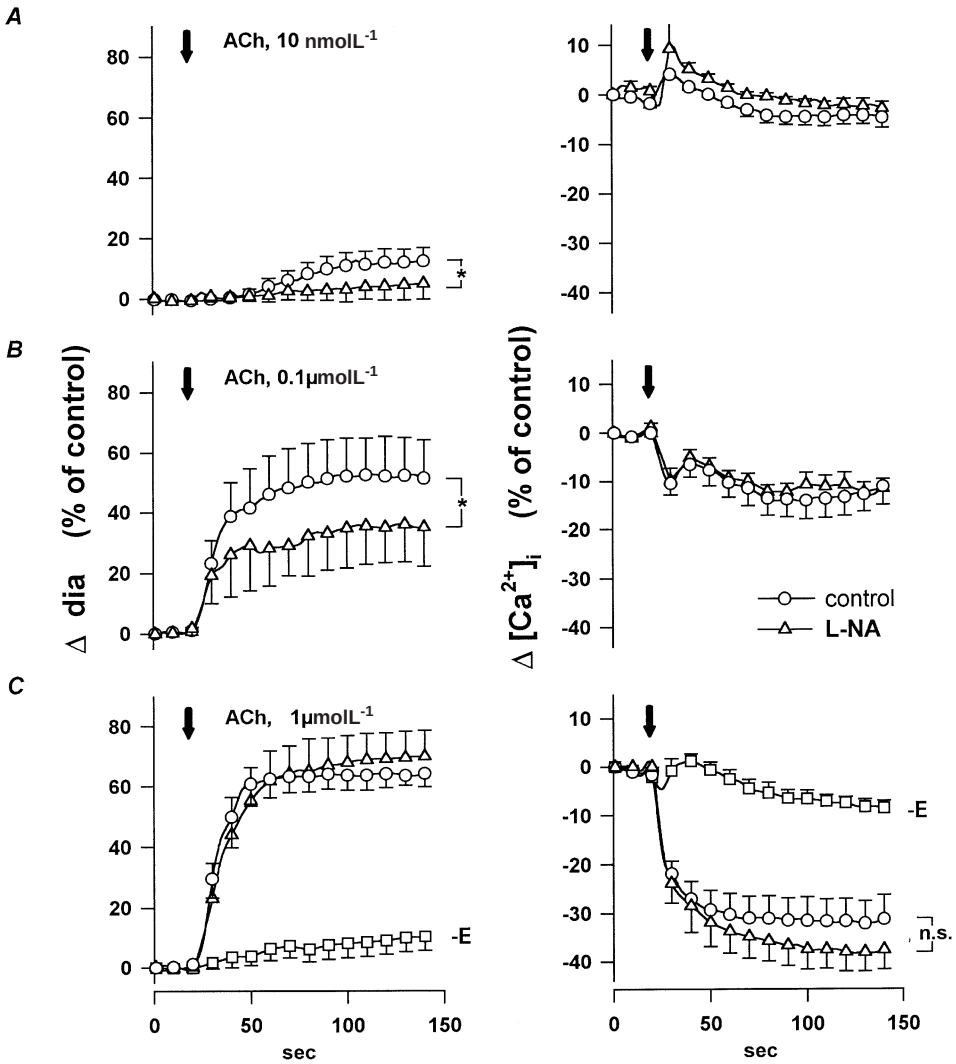

Figure 2 Effect of L-NA on ACh-induced changes of arteriolar diameter and intracellular calcium ($[Ca^{2+}]_i$) precontracted with 0.3 μM norepinephrine (NE). The arterial segments ($n=8$) were exposed to increasing concentrations of acetylcholine (ACh, from A to C) before and after treatment of the same vessels with L-NA (30 μM) and after removal of the endothelium (−E). The left-hand panel depicts diameter changes (Δ dia) while the right-hand panel shows corresponding changes of intracellular free calcium (Δ $[Ca^{2+}]_i$) in vascular smooth muscle. (Values represent mean ± s.e.mean, arrows indicate start of ACh-application; * = significantly different ($P<0.05$) from control curve; n.s. = no significant difference).

fura 2-AM in 1 ml DMSO). Fura 2-AM was diluted in MOPS-buffered salt solution containing 0.5% bovine serum albumin (BSA) to its final concentration of 2 μmol l^{-1}. $DiBAC_4(3)$ was dissolved in MOPS-buffered salt solution directly before use. The solvent DMSO had no effects neither on vascular tone nor on vascular sensitivity to vasoconstrictors or vasodilators.

Norepinephrine (NE), acetylcholine (ACh), L-NA, tetrabutylammonium hydrogen sulphate (TBA), charybdotoxin (ChTX, peptide toxin from scorpion Leiurus quinquestriatus), saponin and sodium nitroprusside (SNP) were purchased from Sigma Chemical (Deisenhofen, Germany), $MnCl_2$ from Merck (Darmstadt, Germany) and S-nitroso-N-acetyl-D,L-penicillamine (SNAP) from Alexis Chemicals (Grünberg, Germany). Felodipine was a generous gift from Astra chemicals.

Drugs were stored in stock solutions and prior to use, diluted to five times that of the final concentration. In order to assure homogenous stimulation, 1/5 of the bath volume was exchanged when substances were added to create the final bath concentration. All concentrations given in the text refer to final bath concentrations.

Statistical analysis

ACh-, SNP- or SNAP-induced dilations are expressed as per cent change (Δ dia) from control:

$$\Delta \text{ dia (per cent of control)} = ((D_{treat}/D_{NE}) \times 100) - 100$$

where D_{Treat} is the diameter under one of these treatments and D_{NE} is the steady state diameter of the NE-treated vessel. Changes of $[Ca^{2+}]_i$ were also expressed as per cent of control ratio

$$\Delta [Ca^{2+}]_i \text{ (per cent of control)} = ((R_{treat}/R_{NE}) \times 100) - 100$$

with R_{treat} being the ratio under treatment and R_{NE} the ratio under stimulation with NE alone. Due to potential errors associated with the measurements in an intact vessel (Meininger *et al.*, 1991), $[Ca^{2+}]_i$ in vascular smooth muscle

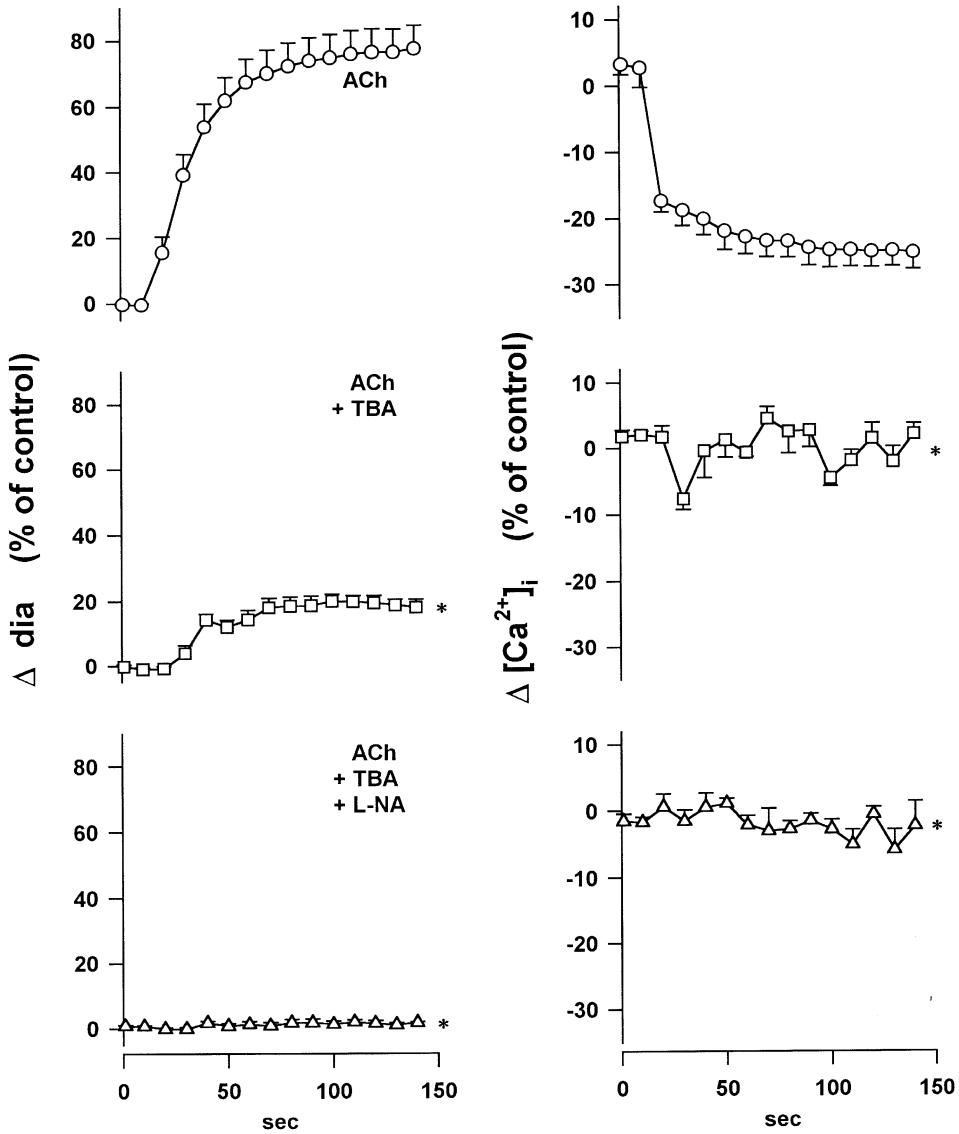

Figure 3 Effects of TBA-pretreatment (1 mmol l^{-1} and additional inhibition of NO-synthase (L-NA, 30 μmol l^{-1} on acetylcholine (ACh)-induced changes of $[Ca^{2+}]_i$ and diameter ($n=6$). 1 μmol l^{-1} ACh was applied to norepinephrine (NE, 0.3 μmol l^{-1}) - preconstricted vessels. For the sake of clarity, means $\pm$ s.e.mean are depicted only at intervals of 10 s. Connecting lines comprise all values (5 s intervals). Note that TBA induced rhythmic changes of $[Ca^{2+}]_i$ in four out of six small arteries. (* = significantly different ($P<0.05$) from ACh under control conditions, F-test; control diameters were 114 ± 5 μm (untreated), 109 ± 4 μm (TBA) and 108 ± 6 μm (TBA + L-NA). They were not significantly different).

cells can only be estimated. We therefore used ratio changes to describe per cent changes of $[Ca^{2+}]_i$. According to calibrations curves obtained in a cell free system, the range of ratios observed here (0.45–1.7) fitted well into the linear range of the calibration curve (42.2 to 1520 nmol l^{-1}) which is a prerequisite for calculating per cent changes.

ACh-induced changes in membrane potential of moderately preconstricted vessels were expressed as per cent change of the steady state bis-oxonol fluorescence under NE. In every vessel responses in the presence of pharmacological agents were studied after monitoring the response in the presence of the appropriate solvent. Thus each vessel served as its own control. All results are presented as means ± s.e.mean of *n* experiments with *n* representing the number of vessels used per experiment. Only one vessel was used per animal.

Steady state values of different experimental groups were compared using Student's *t*-test for paired data. Differences were considered to be significant at error probabilities less than 0.05 ($P<0.05$).

For comparing $[Ca^{2+}]_i$ and diameter changes over time and under different treatments, a non-linear regression analysis was employed. Briefly, the goodness of the fit to a Gompertz function was calculated first for every individual curve and then after pooling the two data sets. The individual curves were considered to be significantly different if the *F*-test indicated a significantly smaller sum of squares for the deviations in each individual fit as compared to the deviation in the fit to the pooled data (Motulsky & Ransnas, 1987).

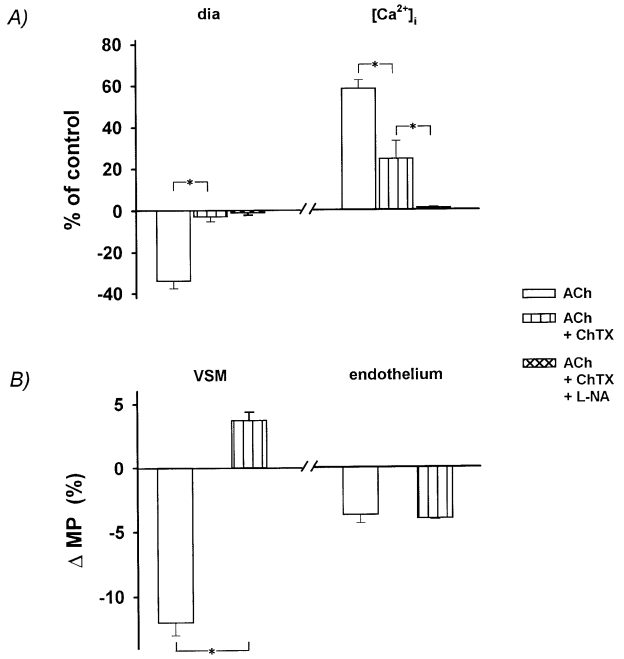

Figure 4 Effects of charybdotoxin (ChTX) on ACh-induced changes of $[Ca^{2+}]_i$, diameter (dia) and membrane potential (MP). (A) Changes in diameter and $[Ca^{2+}]_i$ after application of ACh under control conditions, in the presence of ChTX or after combined treatment with ChTX and L-NA. Columns represent the steady state values 2 min after application of 1 μmol l^{-1} ACh under control conditions, after treatment with ChTX and after combined treatment with ChTX and L-NA. ($n=5-6$; * = significant differences to ACh effects without inhibitor(s); control diameters were 85 ± 8 (ChTX) and 86 ± 8 (ChTX + L-NA)). (B) Effects of ChTX on membrane potential changes in smooth muscle and endothelial cells after application of ACh. Membrane potential measurements in vascular smooth muscle and endothelial cells were performed in separate experiments. ($n=4$; * = significant differences to ACh effects without inhibitor(s)).

Results

The small resistance arteries had a resting diameter of 194 ± 12 μm ($n=49$) and developed spontaneous tone (17 ± 7 μm) in response to 45 mmHg transmural pressure.

Effects of ACh

NE induced a $[Ca^{2+}]_i$ increase of 26 ± 3% (plateau 15 ± 2%, $n=8$) in vascular smooth muscle cells (VSMCs), which preceded a rapid constriction of the vessel from 170 ± 7 μm to 108 ± 6 μm (36.3 ± 1%, $n=8$). Application of ACh (1 μmol l^{-1}) completely reversed the constriction and elicited a rapid and sustained decrease of $[Ca^{2+}]_i$ in VSMCs to a level significantly below resting value (with $R_{basal}=0.82\pm0.04$, $R_{NE}=0.93\pm0.05$ and $R_{ACh}=0.65\pm0.06$, $n=8$). After washout of ACh and NE, $[Ca^{2+}]_i$ as well as vessel diameter returned to baseline. Virtually identical results in response to 1 μmol l^{-1} ACh were obtained in vessels preactivated by angiotensin II or by exhibiting myogenic tone after elevation of transmural pressure (data not shown). Figure 1a depicts typical original

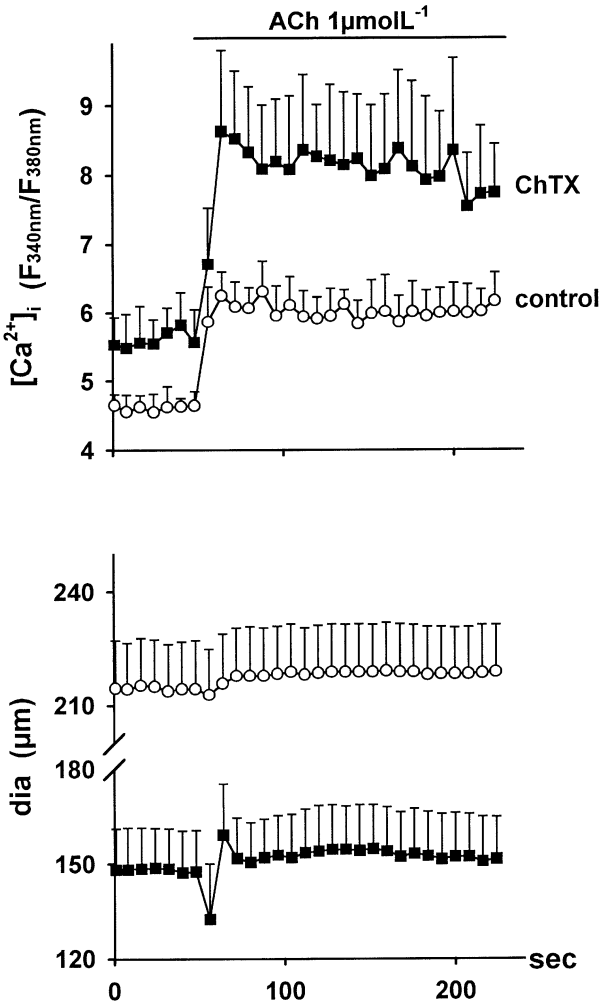

Figure 5 Effects of charybdotoxin (ChTX) on ACh-induced endothelial $[Ca^{2+}]_i$ increases. ACh (1 μmol l^{-1}) induced increases of $[Ca^{2+}]_i$ in endothelial cells of L-NA/indomethacin-pretreated vessels ($n=6$). The associated dilation completely antagonized spontaneous tone. Incubation of the vessel with ChTX (1 μmol l^{-1}, $n=6$) induced increases of resting endothelial $[Ca^{2+}]_i$ (by 19%) and constricted the vessel (by 31%). Subsequent addition of ACh induced increases of $[Ca^{2+}]_i$ as seen under control conditions but the dilation was completely inhibited.

recordings of $[Ca^{2+}]_i$ and diameter measurements in a vessel preactivated with norepinephrine. In vessels, in which the endothelium was loaded with fura 2, ACh (1 μmol l^{-1}) induced a significant increase in endothelial $[Ca^{2+}]_i$ (by $34\pm3\%$, $n=6$), which preceded the complete dilation of the vessel (Figure 5).

Assessment of changes in membrane potential using the dye bis-oxonol showed that ACh induced a significant decrease in bis oxonol fluorescence in the endothelium ($3.73\pm0.64\%$, $n=4$) and in vascular smooth muscle ($12\pm1\%$; $n=5$, Figure 1b showing a representative recording). The smooth muscle hyperpolarization coincided with a significant dilation from 75 ± 4 (preconstriction level under superfusion with 1 μmol l^{-1} NE) to $93\pm3\%$ of maximal diameter ($n=5$).

The magnitude and kinetics of the ACh-induced dilation changed with increasing concentrations of ACh. While at 10 nmol l^{-1} ACh the dilation was slow and steady (from 108 ± 6 μm to 127 ± 10 μm, $n=8$, $P<0.05$), the dilations elicited by 0.1 ($n=8$) and 1 μmol l^{-1} ($n=8$) were immediate in onset reaching 90% of the final dilation within 20 s (see Figures 1A and 2). Ten nmol l^{-1} ACh had no effect on $[Ca^{2+}]_i$, whereas 0.1 and 1 μmol l^{-1} ACh decreased $[Ca^{2+}]_i$ by 13 ± 3 and $32\pm4\%$, respectively.

The effects of ACh on diameter and $[Ca^{2+}]_i$ were abrogated after chemical or mechanical removal of the endothelium (Figure 2). The NE-induced constriction and the dilation elicited by the endothelium-independent vasodilator SNP (10 μmol l^{-1}) were unaltered by de-endothelialization (data not shown).

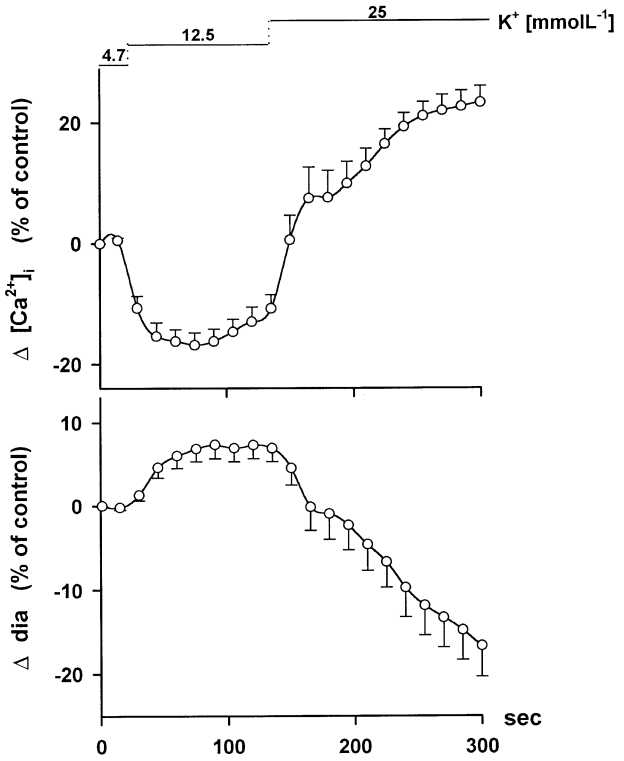

Figure 6 Effect of increasing extracellular potassium ($[K^+]_{ex}$) concentration on $[Ca^{2+}]_i$ and vascular diameter. The elevation of $[K^+]_{ex}$ concentration from 4.7 to 12.5 mmol l^{-1} induced vasodilation ($n=15$) and decreased $[Ca^{2+}]_i$ significantly ($P<0.01$), similar to observations with high concentrations of ACh. In contrast, further elevation of $[K^+]_{ex}$ to 25 mmol l^{-1} induced vasoconstriction preceded by an increase of $[Ca^{2+}]_i$ ($P<0.01$). Changes are expressed as per cent of control values (means $\pm$ s.e.mean) in NE-preconstricted vessels (absolute values see text).

Effects of NOS inhibition with L-NA

Resting and NE-stimulated $[Ca^{2+}]_i$ levels as well as the corresponding vessel diameters were not significantly altered after incubation (30 min) of the segments with L-NA (30 μmol l^{-1}). Inhibitory effects of L-NA on ACh-induced dilations were clearly dependent on the concentration of ACh applied (Figure 2). While dilations induced by low ACh (10 nmol l^{-1}) were completely abolished, dilations in response to a medium concentration of ACh (0.1 μmol l^{-1}) were only reduced by 32% ($P<0.05\%$). Dilations elicited by the highest ACh-concentration used (1 μmol l^{-1}) were not affected by L-NA. ACh-induced $[Ca^{2+}]_i$ decreases were completely unaffected by L-NA over the whole concentration range tested.

Effects of TBA and ChTX

Incubation with 1 mmol l^{-1} TBA altered neither basal diameter nor the magnitude of the NE-induced constriction (109 ± 4 μm versus 114 ± 5 μm under control conditions, $n=6$), but significantly reduced the ACh-induced dilation in these vessels by 75% ($n=6$, $P<0.05$), and abolished the concomitant decreases in $[Ca^{2+}]_i$ (Figure 3). Identical findings were obtained using the more specific K_{Ca} channel inhibitor ChTX (1 μmol l^{-1}, 30 min). Abluminally applied ChTX reduced ACh (1 μmol l^{-1})-induced dilations by 60% ($n=6$, $P<0.05$) and abolished the ACh-induced decrease in $[Ca^{2+}]_i$ ($n=6$, $P<0.05$, Figure 4). While ChTX completely blocked ACh-induced decrease in $[Ca^{2+}]_i$ and smooth muscle hyperpolarization (Figure 4) the dilation was only partially inhibited. The remaining dilation was presumably mediated by NO (see Figures 3 and 4).

In the endothelial cells of L-NA/indomethacin-pretreated vessels, ACh (1 μmol l^{-1}) increased $[Ca^{2+}]_i$ by $34\pm3\%$ ($n=6$, Figure 5). Pretreatment of the vessels with ChTX decreased resting diameter while increasing resting endothelial $[Ca^{2+}]_i$. Absolute levels of endothelial $[Ca^{2+}]_i$, and the relative $[Ca^{2+}]_i$ increase elicited by ACh ($54\pm12\%$, $n=6$) was significantly higher in the presence of ChTX than under control conditions. Under these experimental conditions the ACh-induced dilation was completely inhibited (Figure 5). The endothelial hyperpolarization as elicited by 1 μM ACh remained unaffected by ChTX treatment (Figure 4).

Effects of a combined NOS and K_{Ca}-channel blockade

As demonstrated above, neither TBA nor ChTX were able to inhibit ACh-induced dilations completely. Combined treatment of the vessels with the NOS inhibitor L-NA and either

Table 1 Dose-related effects of extracellular potassium on $[Ca^{2+}]_i$ and vascular diameter

$[K^+]_{ex}$ (mmol l^{-1})	*Vessel diameter* (μm)	Δ *diameter* (per cent of control)	*R* (per cent of control)
4.7	176 ± 5	0	0
12.5	188 ± 4	$+7.3\pm1.6$	-17.0 ± 2.0
25.0	143 ± 9	-19.0 ± 1.8	23.5 ± 5.8
37.5	117 ± 5	-23.5 ± 3.7	40.3 ± 7.4
50.0	104 ± 3	-40.9 ± 1.7	54.8 ± 8.6
75.0	95 ± 4	-45.3 ± 1.5	80.1 ± 9.7

$[K^+]_{ex}$ indicates concentration of extracellular potassium; changes of $[Ca^{2+}]_i$ are indicated as change of ratio (control $R=0.73\pm0.05$). (Values represent the mean $\pm$ s.e.mean of 15 experiments).

one of the K_{Ca}-channel blockers abolished both, ACh-induced dilations and the decrease in $[Ca^{2+}]_i$. The NOS and K^+ channel inhibitor combination (Figures 3 and 4) also resulted in a significant reduction of the basal diameter by 19% (TBA + L-NA: $n=6$, $P<0.05$) and 14% (ChTX + L-NA: $n=6$, $P<0.05$), respectively. Again, constrictor responses to NE (108 ± 6 μm for TBA + L-NA and 86 ± 8 μm for ChTX + L-NA) did not significantly differ from control. (for TBA + L-NA see Figures 3 and 4; for ChTX + L-NA, Figure 4).

Effects of an endothelium-independent hyperpolarization by low extracellular potassium

In a further series of experiments (15 vessels) the extracellular potassium concentration ($[K^+]_{ex}$) was increased in several steps from 4.7 to 75 mmol l^{-1}. Moderate increases of $[K^+]_{ex}$ (from 4.7 to 12.5 mmol l^{-1}) induced a decrease in $[Ca^{2+}]_i$ (by 17%) as well as vessel dilation (by 7.3%) (Figure 6). Both effects were abolished by barium chloride ($n=8$, data not shown), indicating the involvement of K_{IR} channels. A further stepwise increase in $[K^+]_{ex}$ concentration-dependently increased constriction and $[Ca^{2+}]_i$ (Figure 6, Table 1).

Effects of the NO donor compounds SNP and SNAP

SNP ($0.1-10$ μmol l^{-1}) dilated NE-preconstricted resistance arteries in a concentration-dependent manner (by 7.9 ± 3, 26.6 ± 6.2 and 39.2 ± 7.3%, $n=7$, Figure 7) without significantly altering $[Ca^{2+}]_i$. SNAP ($0.1-100$ μmol l^{-1}) induced dilations of $21\pm6-52\pm5$%, respectively ($n=7$, $P<0.05$). In contrast to SNP, SNAP induced initial transient decreases in $[Ca^{2+}]_i$ (by 2.9 ± 1.5, 7.0 ± 2.6, 7.6 ± 2.4 and 12.8 ± 0.9%), which returned to control levels within 10 s. For the remaining observation time (110 s), $[Ca^{2+}]_i$ levels did not differ significantly from control.

Since the NO donors and ACh induced different maximal responses, dilations of comparable magnitude were matched (10 μmol l^{-1} SNP: 39%; 1 μmol l^{-1} SNAP: 36%; 0.1 μmol l^{-1} ACh (under L-NA/indomethacin: 35%). Only the dilations in response to ACh, which are attributable to EDHF were accompanied by a significant and sustained decrease in $[Ca^{2+}]_i$ (by 11.6%, Figures 2 and 3), whereas NO-induced dilations of similar magnitude were not associated with concomitant changes in $[Ca^{2+}]_i$.

Inhibition of calcium influx through L-type calcium channels by felodipine

Application of the dihydropyridine-type calcium channel antagonist felodipine (1 μM) to NE-preconstricted small skeletal arteries ($n=9$) resulted in a complete reversal of the NE-induced constriction. The dilation was rapid in onset and maximal after 3 min. It was preceded by a fast and

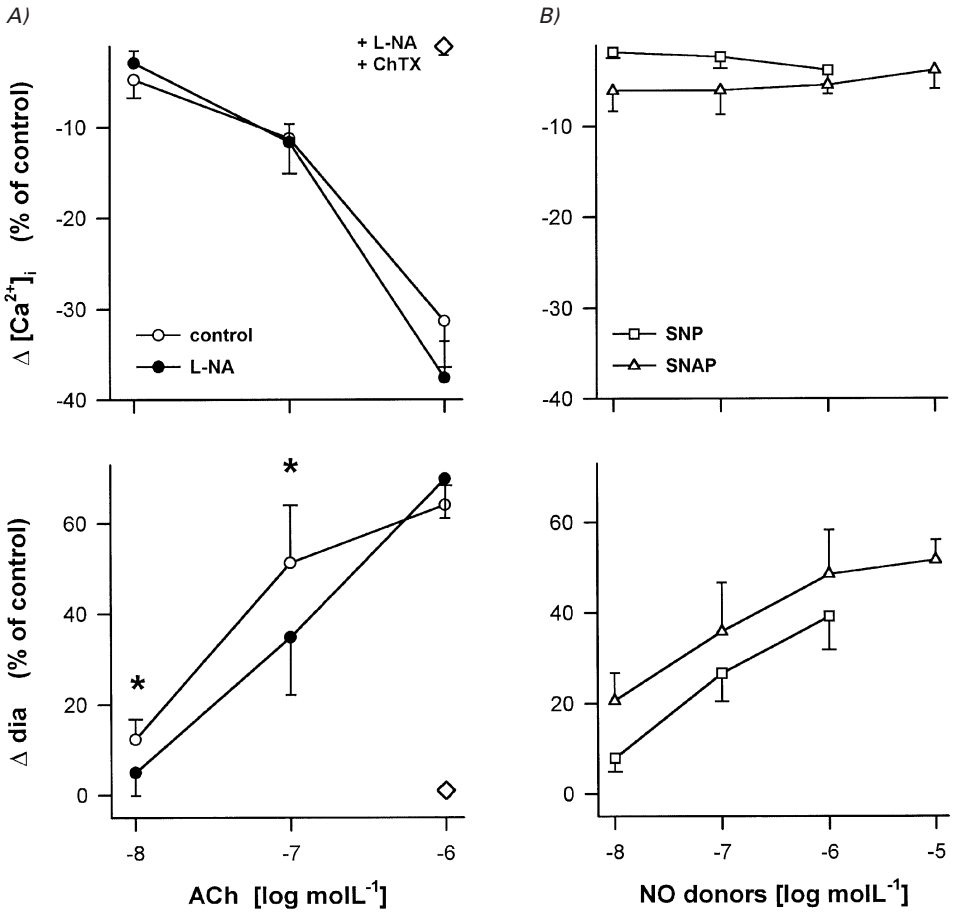

Figure 7 Dose-dependent effects of acetylcholine (ACh, (A)) and the NO donors (NOD, (B)) sodium nitroprusside (SNP) and S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) on diameter and $[Ca^{2+}]_i$. Diameter (Δ dia) and $[Ca^{2+}]_i$ values (Δ $[Ca^{2+}]_i$) represent steady state changes (per cent of control) 2 min after application of ACh or NOD to norepinephrine-preconstricted vessels. NOS-inhibition by L-NA (top panel) significantly ($*=P<0.05$) reduced the dilation at the lower ACh-concentration but did not affect the changes of $[Ca^{2+}]_i$. NOD had no effect on $[Ca^{2+}]_i$. (Values represent the mean$\pm$s.e.mean of seven (SNAP) or eight (ACh, SNP) experiments obtained at 2 min after application of ACh, SNAP or SNP to norepinephrine (NE, 0.3 μmol l^{-1})-preconstricted vessels).

sustained decrease of $[Ca^{2+}]_i$ to 16% ($P<0.005$) below the basal level (Figure 8).

Discussion

In the small skeletal muscle resistance arteries investigated in this study, ACh-induced dilations were completely dependent on the presence of the endothelium and mediated by two distinct autacoids, NO and EDHF. NO fully accounted for the dilation induced by the lowest ACh-concentration since it was completely inhibited by L-NA. However, the relative contribution of NO to the overall dilation, as derived from the inhibitory effect of L-NA, decreased with increasing concentrations of ACh. L-NA had no effect on the decrease in $[Ca^{2+}]_i$ which occurred at high concentrations of ACh. These findings indicate that in the resistance sized arteries under investigation, NO was only partly responsible for the dilations observed and that its vasodilating effect was not associated with a decrease in $[Ca^{2+}]_i$. In accordance with these observations the NO donors SNP and SNAP induced dilations which were also not associated with changes in $[Ca^{2+}]_i$. Only high supraphysiological concentrations of SNP and SNAP induced small decreases in $[Ca^{2+}]_i$. These responses were, however, transient.

There is growing evidence demonstrating that relaxation of vascular smooth muscle cells can be initiated at constant $[Ca^{2+}]_i$ *via* activation of the myosin light chain phosphatase (SMPP-1 M). Of note, in these studies the enzyme SMPP-1 M has also been demonstrated to be subject to activation by cyclic GMP, which is increased by NO in vascular smooth muscle (Lee *et al*., 1997; Wu *et al*., 1996). Therefore, it is feasible that NO may reduce the Ca^{2+}-sensitivity of the contractile apparatus rather than $[Ca^{2+}]_i$ in these small vessels. This may explain why, even at presumably maximal concentrations, NO was unable to induce complete dilation of the preparation. The same vessels were, however, completely relaxed in a NO-independent manner either by ACh, papaverine (Bolz *et al*., unpublished data) or by felodipine.

The dilator response to ACh which persisted after treatment with L-NA and indomethacin and which was especially pronounced at the highest concentration of ACh used, is most probably attributable to EDHF. In fact, our studies demonstrated that the exposure of small arteries to ACh is associated with a distinct hyperpolarization. Moreover, an endothelium-independent hyperpolarization, induced by a moderate elevation in $[K^+]_{ex}$ (Knot *et al*., 1996), elicited qualitatively similar changes in $[Ca^{2+}]_i$ and diameter as those seen with ACh. Endothelium-dependent but L-NA- and

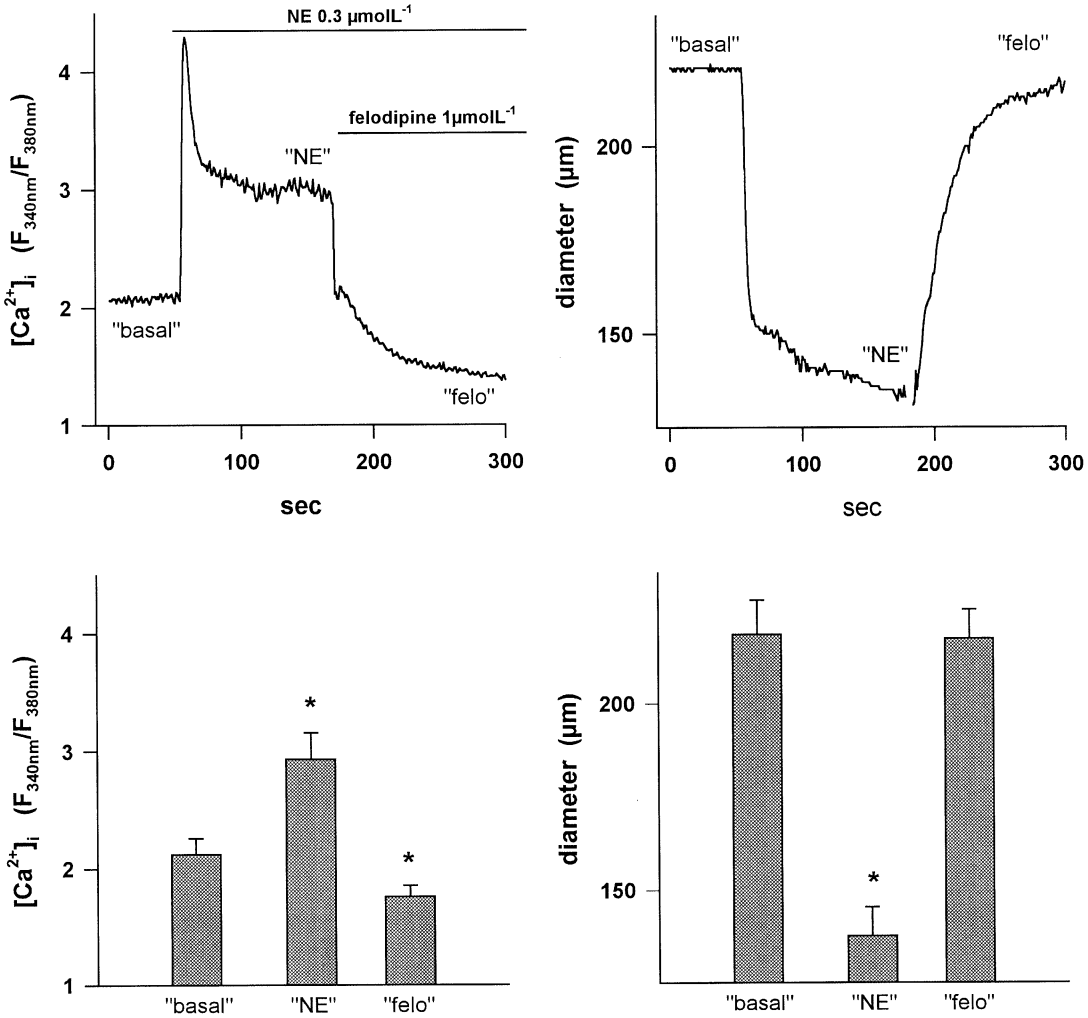

Figure 8 Effects of the calcium channel antagonist felodipine on $[Ca^{2+}]_i$ and vascular diameter in NE-preconstricted arteries. Top panels: Original recordings of the effect of felodipine on smooth muscle calcium ($[Ca^{2+}]_i$, ratio $F_{340\ nm}/F_{380\ nm}$) and corresponding diameter changes (μm). Bottom panels: Columns are summarizing calcium levels and diameters as obtained in nine experiments. Depicted are basal values and steady state conditions after NE as well as after application of felodipine. (* = significantly different ($P<0.05$) from basal value (1); for technical reasons the ratio $F_{340\ nm}/F_{380\ nm}$ in this experimental series is higher than in the other).

indomethacin-resistant dilations which were associated with hyperpolarization have been reported in a variety of vessels from different species and are thought to be mediated by EDHF. Although more than one EDHF appears to exist, recent bioassay studies revealed that the bovine coronary endothelium *in situ* releases a transferable hyperpolarizing substance that appears to be a cytochrome P450-dependent product (Hecker *et al*., 1994; Nagao *et al*., 1992).

A recent study using rabbit carotid arteries demonstrated that effects which may be ascribed to an EDHF were in fact mediated by NO, of which the synthesis was incompletely blocked (Davisson *et al*., 1996). However, the results obtained in the present study clearly demonstrate that in the small arteries investigated, the hyperpolarization and decreases in $[Ca^{2+}]_i$ induced by ACh were not mediated by NO but rather by a clearly distinct EDHF. Indeed, NO donors up to concentrations of 10 μmol l^{-1} did not affect steady state $[Ca^{2+}]_i$ and L-NA had no effect on the ACh-induced changes in $[Ca^{2+}]_i$. ACh-induced alterations in $[Ca^{2+}]_i$ were, however, inhibited in the presence of L-NA and indomethacin together with TBA or ChTX. This is consistent with our hypothesis that two independent endothelial mechanisms are active in these vessels, since the K_{Ca} channel blockers alone attenuated the ACh-induced dilation leaving a residual $[Ca^{2+}]_i$-independent component, which was abrogated by further application of L-NA.

Although the presence of K_{Ca} channels has been described both on smooth muscle and endothelial cells (Rusko *et al*., 1992), our experiments provide evidence that ChTX acted on smooth muscle only. Abluminally applied ChTX did not affect endothelial hyperpolarization which may be due to a diffusion barrier in the vascular wall and/or the absence of functional ChTX-sensitive channels in the endothelium of these vessels. In favour of the first hypothesis, we could not detect ChTX in the vascular lumen by means of HPLC (data not shown) after its abluminal application. An exclusive smooth muscle effect of ChTX is further suggested by its failure to inhibit the ACh-induced increase in endothelial $[Ca^{2+}]_i$. A direct effect of ChTX on endothelial potassium channels abolishing hyperpolarization would have reduced the driving force for calcium entry (Lückhoff & Busse, 1990; Sharma & Davis, 1994) resulting in an attenuation of the plateau phase of the calcium signal. In fact, high extracellular potassium or the K^+ channel blocker TBA abolished the Bradykinin-induced hyperpolarization and the plateau phase of the calcium increase in PAEC (Groschner *et al*., 1992). In our experiments, however, the kinetics of the endothelial calcium signal were not affected by ChTX. The absolute increase in endothelial $[Ca^{2+}]_i$ was even higher in the presence of ChTX which may be explained by myoendothelial heterocellular communication (Dora *et al*., 1997). It is therefore reasonable to conclude that ChTX did not interfere with the calcium dependent synthesis (Adeagbo & Henzel, 1998) of EDHF. The fact that ChTX did not affect endothelial hyperpolarization while completely abrogating it at the smooth muscle level also excludes endothelium-derived potassium acting as an EDHF in this particular vessel (Edwards *et al*., 1998). The hyperpolarizing effects of potassium are thought to be mediated by smooth muscle inward rectifier potassium channels (Knot *et al*., 1996) which are not affected by ChTX.

The decrease of smooth muscle $[Ca^{2+}]_i$ induced by EDHF is presumably due to a hyperpolarization dependent closure of L-type calcium channels. This is suggested by the rapid dilation as well as the decrease in $[Ca^{2+}]_i$ observed after treatment of the vessels with the calcium entry blocker felodipine. Felodipine elicited virtually identical effects with similar kinetics as ACh. This suggests that in these small arteries the maintained phase of the NE-induced contraction, classically thought to be the result of pharmacomechanical coupling, is strongly dependent on a continuous transmembranous influx of Ca^{2+} through voltage-dependent channels. Therefore any change in membrane potential, such as that induced by EDHF, should have immediate consequences on both, $[Ca^{2+}]_i$ and the level of vascular smooth muscle contraction.

We found no evidence in our particular model that prostaglandins played a significant role as mediators of the ACh-induced dilation since, consistent with previous findings in isolated rat mesenteric and canine pulmonary arteries (Gambone *et al*., 1997; McCulloch *et al*., 1997), treatment with indomethacin did not affect vascular responses to ACh. These findings are, however, at variance with *in vivo* results obtained in rat microvessels and our own intravitalmicroscopic observations in the hamster cremaster where prostaglandins contribute to the dilation. It has been reported that anaesthetics reduce the synthesis of EDHF from arachidonic acid by a cytochrome P450 enzyme (Lischke *et al*., 1995; Wit *et al*., 1999). One may speculate that the PGI_2-induced dilation is especially pronounced in anaesthetized animals, since under these conditions arachidonic acid may accumulate and the role of the arachidonic acid derived prostaglandins might be overestimated at the expense of EDHF.

In summary, ACh elicited the endothelium-dependent dilation of microvessels by two independent mechanisms which differentially affect smooth muscle $[Ca^{2+}]_i$. While the effects of NO appear to be associated with a reduction of the calcium-sensitivity of the contractile apparatus, EDHF reduced $[Ca^{2+}]_i$, probably by inhibiting voltage-dependent transmembrane Ca^{2+} influx. These results strongly suggest, that physiological concentrations of NO do not mimic the effects of EDHF in microvessels and that at least in response to endothelial stimulation with ACh, EDHF is by far the more effective dilator of microvessels. It remains to be determined whether the different mechanisms of dilation can provide a basis for synergistic effects of both dilators in the microcirculation.

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