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The effect of the calcium-antagonist nitrendipine on intracellular calcium concentration in endothelial cells

[1]Aida Salameh, Gunhild Schomecker, Katja Breitkopf, Stefan Dhein & Wolfgang Klaus

Institut für Pharmakologie, Universität zu Köln,Gleuelerstr. 24, 50931 Köln, Germany

1 Nitrendipine induces NO-release from coronary vascular endothelium presumably by activating endothelial NO-synthase. We have investigated whether this effect may be mediated by an influence on the intracellular calcium in endothelial cells.

2 Bovine aortic endothelial cells (BAEC) were incubated with Fura-2/AM (1 μM) for 30 min and Fura-2 fluorescence was measured at 510 nm in response to chopped excitation with both 340 and 380 nm. The ratio 340/380 nm (known to reflect changes in intracellular calcium) was calculated from these data.

3 Nitrendipine (0.1 to 100 μM) led to a significant, concentration-dependent, monophasic increase in $[Ca^{2+}]_i$ in suspended BAEC by 11 ± 2 nM (0.1 μM), 23 ± 3 nM (1 μM), 34 ± 4 nM (10 μM) and by 47 ± 5 nM (100 μM) from a control level of 118 ± 10 nM.

4 This elevation of intracellular calcium was prevented by pretreatment of BAECs with gadolinium (100 μM) or by incubation with calcium free saline solution. In contrast, the application of 0.3 μM thapsigargin did not abolish the nitrendipine-induced calcium signal. In additional experiments it was shown that the nitrendipine-induced NO-release (as measured with the oxy-haemoglobin-method could also be inhibited by gadolinium and was absent in calcium-free solution.

5 Thus, nitrendipine elevates intracellular calcium in suspended BAECs in a concentration-dependent manner. This elevation is mainly due to a gadolinium-sensitive calcium influx from the extracellular space rather than a calcium release from intracellular stores.

Keywords: Endothelium; calcium; Fura-2; nitrendipine; dihydropyridines; nitric oxide

Introduction

1,4-Dihydropyridines are rather potent and often used vasodilator drugs, exhibiting antispastic and antihypertensive properties (Meyer *et al.*, 1984). The mechanism underlying the vasodilator properties of these drugs has been proposed to be primarily based on their inhibition of voltage-sensitive L-type calcium channels (Tsien, 1983; Janis *et al.*, 1985) located on vascular smooth muscle cells. In addition, we have recently suggested that the activation of the nitric oxide synthase (NOS) present in vascular endothelium and the consequent enhanced formation of nitric oxide (NO) may well contribute to the vasodilator properties of these agents (Günther *et al.*, 1991; 1992; Dhein *et al.*, 1994; 1995).

NO, which is identical to 'EDRF' (Ignarro *et al.*, 1987), the endothelium-derived relaxing factor discovered in 1980 by Furchgott and Zawadzki, is synthesized by endothelial cells from L-arginine and causes vasodilatation by elevating the guanosine 3':5'-cyclic monophosphate (cyclic GMP)-levels in smooth muscle cells (Förstermann *et al.*, 1986). Because of the calcium-dependency of endothelial constitutive NOS (Winquist *et al.*, 1985; Pamler & Moncada, 1989; Förstermann *et al.*, 1993) the influence of various 1,4 dihydropyridines on basal or stimulated NO-release was studied. However, the results are controversial: some authors did not observe any effect with 1,4 dihydropyridines (Spedding *et al.*, 1986; Mügge *et al.*, 1991) whereas other authors demonstrated inhibition of stimulated NO-release by 1,4-dihydropyridines (Singer & Peach 1982; Rubany *et al.*, 1985).

However, the finding of Günther *et al.* (1991) and Dhein *et al.* (1994, 1995) that nitrendipine induces NO-release in porcine isolated coronary arteries and that the nitrendipine-induced vasodilatation was sensitive to treatment with the NO-synthase inhibitor N^G-nitro-L-arginine (L-NOARG), raises the question whether there is an interaction between 1,4-dihydropyridine vasodilator drugs, endothelial calcium and NO-release. This study was undertaken to elucidate whether the 1,4-dihydropyridine nitrendipine is able to elevate intracellular calcium in endothelial cells, which may help to explain the observed increase in NO-release.

[1] Author for correspondence.

Methods

Cell culture

Bovine aortic endothelial cells (BAEC) were harvested (according to Zink, 1991) from calf thoracic aorta (10 aortae per preparation) in the following manner: the cells were disaggregated by treatment with 1 mg ml^{-1} dispase for 15 min at 37°C, rinsed off with DMEM (Dulbecco's modified Eagle's medium containing 10% foetal calf serum, 3 mM glutamine, 30 mM HEPES, 6320 u l^{-1} penicillin, 100 mg l^{-1} streptomycin buffered at pH 7.4) centrifuged (100 *g* for 10 min) and, after resuspension with DMEM, seeded in plastic Petri dishes (9.4 cm^2) coated with 0.2% gelatin. The cells were held in an incubator (Heraeus type B5060 EC/CO_2) at 37°C, saturated humidity and 5% CO_2. After they had reached confluency (5-7 days), the endothelial cells were subcultured in 25 cm^2 plastic flasks with a split ratio of 1:2 and they were fed three times a week. Purity of the culture was tested by von Willebrand factor-staining and by uptake of Dil-Ac-LDL (Jaffe *et al.*, 1973; Voyta *et al.*, 1984) demonstrating a 97 ± 2.3% positive staining for factor VIII and a 98 ± 1.5% positive staining for Dil-Ac-LDL. Passages 1-3 were used for the calcium- and passages 2 for the NO-measurements.

Ca^{2+} measurements

Confluent cells of one flask were harvested by treatment with 0.05% trypsin and 0.02% EDTA. The trypsinisation was performed under optical control by a Nikon inverse microscope, and after cell detachment immediately stopped with DMEM plus 10% foetal calf serum to prevent cell damage.

Cells were centrifuged (5 min at 100 g), resuspended in DMEM without calf serum and incubated with Fura-2/AM (1 μM for 30 min at 37°C). After a postincubation period (30 min at 37°C), to facilitate the Fura-2 ester hydrolysis, the cells were washed with a HEPES-buffered saline solution (HeBSS) containing (mM): NaCl 145, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, glucose 5 and HEPES 10, buffered at pH 7.4 and they were resuspended in the same buffer at a final concentration of 5×10^5 cells ml^{-1}. All experiments were performed at 37°C and at pH 7.4. During the experiments the endothelial cells were stirred in a quartz cuvette at 150 r.p.m. or at 100, 150, 200, 250 r.p.m. as indicated.

The experiments were started at stable ratio values (R) after an equilibration period of 5–10 min. R is the ratio of fluorescence of the Fura-2 dye excited with 340/380 nm and measured at 510 nm (emission wavelength). Fluorescence was measured for a period of 5 min after addition of the various drugs and the intracellular calcium concentration was calculated according to the following equation: $[Ca^{2+}]_i$ (nM) $= K_d \cdot S_f/S_b \cdot (R - R_{min})/(R_{max} - R)$ (Grynkiewicz et al., 1985). R_{max} (= maximum ratio of Ca^{2+} bound dye) was obtained after cell lysis with digitonin (2 mM) in a 2.5 mM Ca^{2+} solution (McCormack & Cobbold, 1991) and R_{min} (= minimum ratio of Ca^{2+}-free dye) was assessed as the ratio after treatment with 10 mM EGTA. S_f/S_b is the ratio of fluorescence values for Ca^{2+}-free and Ca^{2+}-bound dye excited at 380nm in the Ca^{2+} free and 2.5 mM Ca^{2+} solution, respectively. All ratio values were registered after subtraction of the autofluorescence of the endothelial cell and intracellular calcium was calculated on the basis of $K_d = 224$ nM. For excitation we used a xenon lamp (type: XBO 75W/2) equipped with a computer controlled chopper and 340/380 nm monochromators and for detection a photomultiplier (type SN 92-021, Amko) linked to the PC-system for data acquisition (system: Amko LTI Deltascan). The sampling rate could be varied between 0.1 and 500 Hz and was normally adjusted to 1 sample s^{-1}.

Measurement of nitric oxide

For measurement of nitric oxide the endothelial cells, seeded on glass microcarrier beads (diameter 150–210 μm, density 1.02 g per cm^3; Sigma, Deisenhofen (Germany)), were transferred to a spinner flask and stirred slowly on a magnetic stirrer (H+P Labortechnik, Oberschleißheim (Germany)) inside the incubator at 37°C and 5% CO_2 to facilitate cell attachment. After 2 h the stirring speed was adjusted so that the microbeads remained suspended in medium. Every day 1/2 of the culture medium was replaced and the cell growth was examined until confluency was reached. The endothelial cells, attached to the microcarrier beads, were then used for the NO-measurements.

For that purpose the DMEM medium was discharged and the cells were washed and incubated in HeBSS. The experiments, following the experimental protocol of Günther et al. (1992), were performed in a recirculating system consisting of a heated organ bath (37°C), a peristaltic pump and a cuvette.

NO was measured according to the method described by Feelisch and Noack (1987). This method is based on the oxidation of oxyhaemoglobin to methaemoglobin in the presence of NO, which is accompanied by a shift in the absorption spectrum of oxyhaemoglobin allowing the measurement of NO released by endothelial cells.

Oxyhaemoglobin was prepared in the following manner: about 30 mg haemoglobin were dissolved in PBS and saturated with O_2 for 10 min. Thereafter, an excess of sodium dithionite was added to reduce methaemoglobin and the solution was gassed with O_2 for another 15 min. Desalting and purification of oxyhaemoglobin was carried out by gel-filtration through a Sephadex G 25 column (Pharmacia, Freiburg (Germany)).

The difference spectrum of oxyhaemoglobin versus methaemoglobin was measured with a Beckman spectrophotometer (DU 7500). The analytical wavelength of methaemoglobin was determined at 401 nm (= absorption maximum), the background wavelength at 411 nm (= isobestic point) and the extinction difference (ΔE) between 401 and 411 nm was recorded, according to Feelisch and Noack (1987). The adaption of the method has been validated and described previously (Günther et al., 1992).

Each experiment was performed at 37°C and pH 7.4 and started after an equilibration period of 15 min with the addition of 4 μM oxyhaemoglobin and with the measurement of the basal NO-release for a minimum of 5 min. Thereafter, cumulative concentrations of nitrendipine (from 0.01 nM to 100 μM) were added and ΔE was measured for 5 min. The nitrendipine stimulated NO release was calculated as the 'area under the curve' (AUC) for each measurement period.

To validate our NO-measurements increasing concentrations of oxyhaemoglobin were quantitatively oxidized by 10% NO gas, wavelength scans were measured and the extinction rates of the differences at 401 and 411 nm were plotted versus the concentrations of oxyhaemoglobin used. According to the difference spectrum shown in the article of Feelisch and Noack (1987) a maximum extinction difference between oxyhaemoglobin and methaemoglobin was reached at 401 nm with the isobestic point at 411 nm and a minimum at 420 nm. The molar extinction coefficient was calculated as 23.27 ± 1.05 mM^{-1} cm^{-1} with a correlation coefficient of 0.99 from these data.

The spontaneous de-oxygenation of oxyhaemoglobin was determined in the organ bath (HeBSS, 37°C) in the presence of cell-free microbeads over 45 min. The resulting degradation curve was subtracted from all curves obtained in the cell experiments before the calculation of the AUC.

Materials

ATP (adenosine 5'triphosphate), digitonin, EGTA (ethylene glycol-bis(β-aminoethylether) N,N,N,N-tetraacetic acid)), Fura-2/AM, thapsigargin, gadolinium, HEPES (4-[2-hydro-

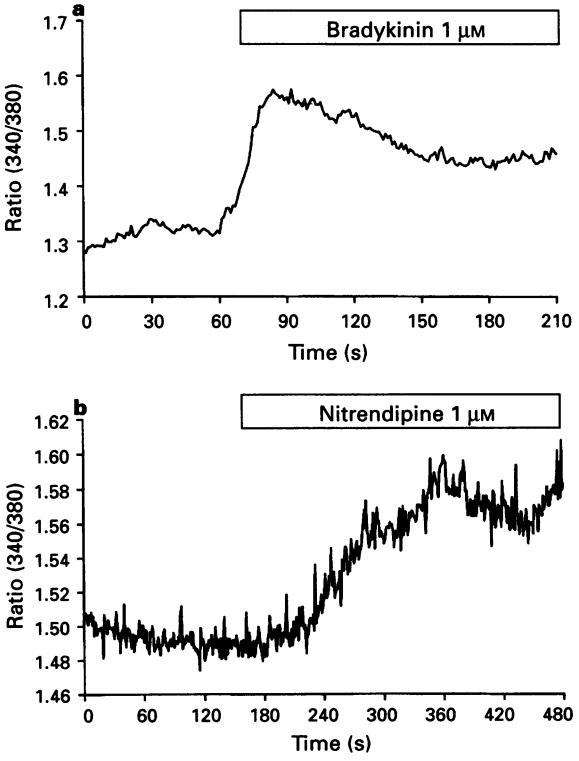

Figure 1 (a) Original trace of the 340/380 ratio of fluorescence of Fura-2 loaded bovine endothelial cells under the influence of 1 μM bradykinin. (b) Original trace of the 340/380 ratio of fluorescence of Fura-2 loaded bovine endothelial cells under the influence of 1 μM nitrendipine.

xyethyl] 1-piperazine-ethanesulphonic acid]), glutamine, gelatine, histamine, haemoglobin, penicillin G, streptomycin, PBS (Dulbecco's phosphate buffered saline, without Ca^{2+} and Mg^{2+}), trypsin, antibody to von Willebrand factor (rabbit) and anti-rabbit IgG FITC-linked antibody were obtained from Sigma, Deisenhofen, (Germany), Dil-Ac-LDL (1,1'-dioctadecyl-3,3,3',33'-tetra-methylindo-carbocyanine acetylated-low density lipoprotein) was purchased from Paesel+Lorei, Frankfurt (Germany), DMEM (Dulbecco's modified Eagle's medium without glutamine) and foetal calf serum were purchased from Gibco BRL, Eggenstein, (Germany) and dispase was obtained from Boehringer Mannheim, (Germany). The 1,4-dihyropyridine nitrendipine was supplied by Bayer Leverkusen, (Germany). All other chemicals were obtained from Merck, Darmstadt, (Germany).

Statistical analysis

All values are given as means ± s.e.mean of at least 6 experiments (i.e. 6 different cell lines). For statistical analysis we used Wilcoxon-test and U-test at a level of significance of $P<0.05$.

Results

In all cultures investigated, more than 98% of the tested cells incorporated Dil-Ac-LDL and expressed von Willebrand's factor antigen.

For comparing nitrendipine with agonists which raise the intracellular calcium in endothelial cells, cells of the same line and the same passage were treated either with 1 μM bradykinin or with 100 μM ATP resulting in a rise of $[Ca^{2+}]_i$ (peak) from 116±4 nM to 377±39 vM (bradykinin, $n=7$, $P<0.05$) or from 119±5 nM to 632±21 nM (ATP, $n=6$, $P<0.05$).

To find out how nitrendipine affects the intracellular calcium concentration, a single concentration (1 μM) of the calcium antagonist was applied, resulting in a slow monophasic increase $[Ca^{2+}]_i$, in contrast to an administration of bradykinin (same concentration) which exhibited a rapid increase in $[Ca^{2+}]_i$ (peak) with a sustained second phase (plateau) (Figure 1 shows original traces of the ratio of bovine aortic endothelial cells after the addition of nitrendipine and bradykinin (BK)).

Administration of increasing, cumulative concentrations of nitrendipine resulted in a significant concentration-dependent elevation of intracellular calcium from 118±10 nM (control)

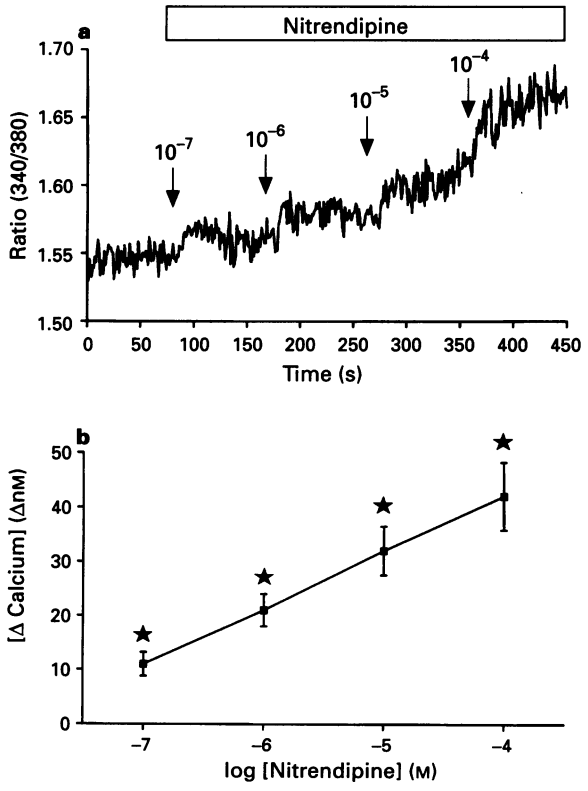

Figure 2 Cumulative concentration-response curve of the influence of nitrendipine on $[Ca^{2+}]_i$ in fura-2-loaded bovine endothelial cells. (a) The 340/380 ratio under the influence of increasing concentrations of nitrendipine; trace from an original experiment. (b) The influence of nitrendipine on $[Ca^{2+}]_i$ measured as Δ calcium (Δ nM) (control calcium 118±10 nM). Data shown are means ($n=15$) and vertical lines indicate s.e.mean. Significance ($P<0.05$) compared to control is indicated by a solid star.

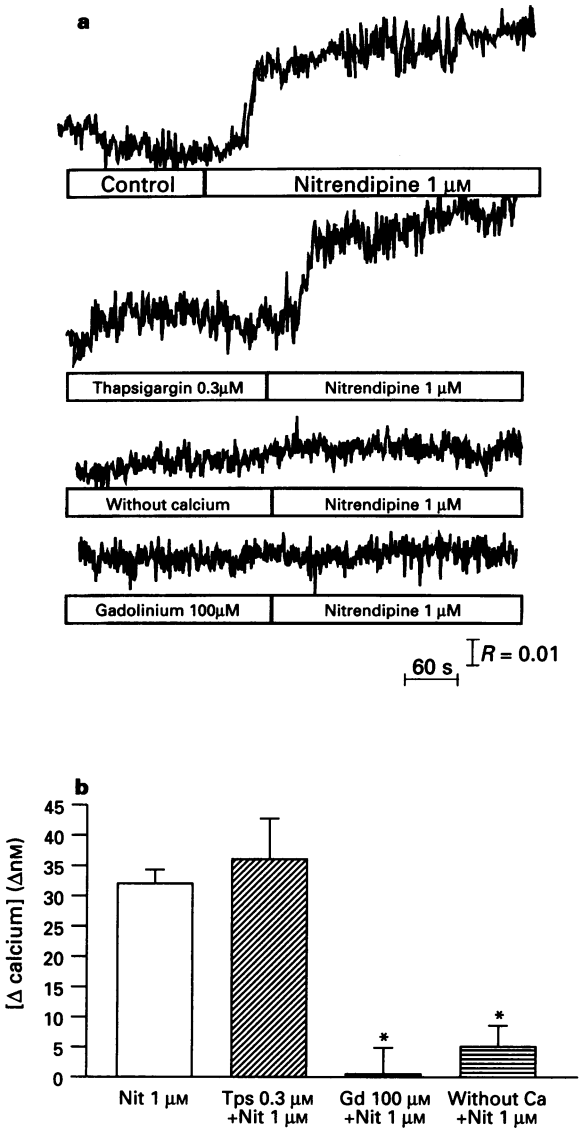

Figure 3 (a) Original recordings showing 340/380 ratio from experiments on fura-2-loaded bovine endothelial cells which were exposed to 1 μM nitrendipine with or without pretreatment as indicated. For reference a Δ 340/380 ratio of $R=0.01$ is given. For further details see below and text (Methods section). (b) Influence of gadolinium (Gd), thapsigargin (Tps) or calcium free HeBSS (without Ca) on the nitrendipine-induced calcium signal in endothelial cells. Shown are the changes in $[Ca^{2+}]_i$ measured as Δ calcium (Δ nM) after application of 1 μM nitrendipine alone and after 5 min pretreatment with Gd, 10–15 min pretreatment with Tps or 15 min incubation without calcium as indicated. All values are given as means ± s.e.mean of $n=6$. Significance ($P<0.05$; U-test) compared to the control nitrendipine-induced response is indicated by a solid star.

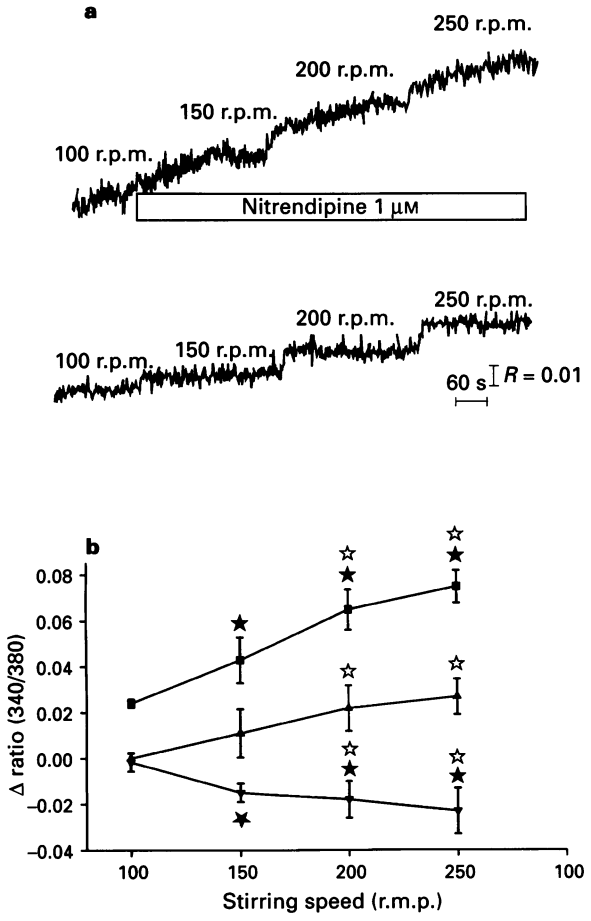

Figure 4 (a) Original recordings from experiments on the influence of increasing stirring speeds on the 340/380 ratio of fura-2-loaded bovine endothelial cells in the absence (control, lower trace) or presence of 1 μM nitrendipine (upper trace). For reference as Δ 340/380 ratio of $R=0.01$ is given. Protocol as depicted in the figure. (b) Quantitative influence of an increase in stirring speed (simulating an increase in shear stress) on endothelial $[Ca^{2+}]_i$ given as the Δ ratio (340/380) between the ratio at 100 r.p.m. and that at the actual stirring speed. In order to measure the influence of the stirring speed most accurately the experiments were carried out according to the following protocol: for a control (▲) cells were stirred at 100 r.p.m., than switched to either 150, 200 or 250 r.p.m. and finally back again to 100 r.p.m. Only if the control value at 100 r.p.m. was reached again, experiments were continued for further evaluation. In the experiments with drug treatment cells were first stirred at 100 r.p.m., then exposed to either 150, 200 or 250 r.p.m. in the presence of the drug and finally back to 100 r.p.m. (i.e. in 1 experiment only 1 change in stirring speed was investigated). Each single data point (given as mean±s.e.mean) is thus representative of $n=6$ single experiments. (■) The influence of 1 μM nitrendipine on the $[Ca^{2+}]_i$. (▼) The influence of 100 μM gadolinium on the stirring speed-dependent increase in 340/380 ratio. Significant changes are marked by an open star (significance versus 100 r.p.m.) or by a solid star indicating significance between the nitrendipine series or gadolinium series versus the control series ($P<0.05$).

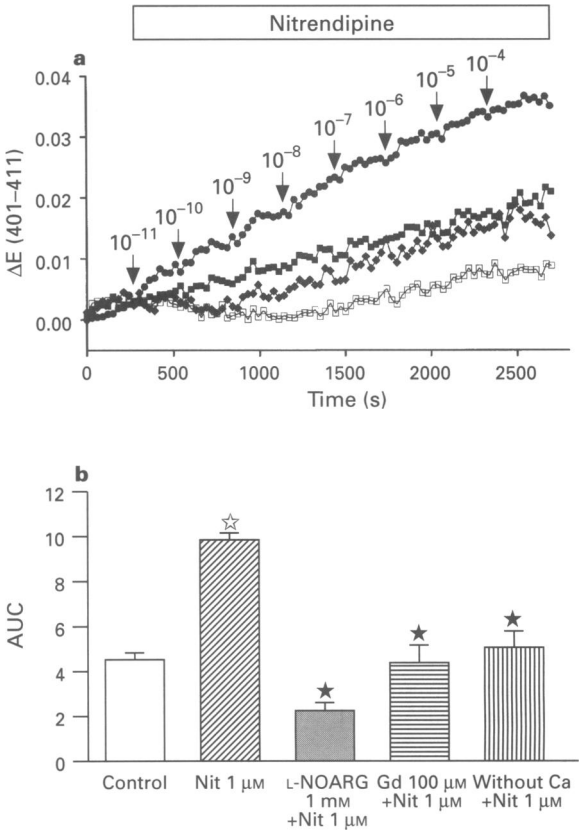

Figure 5 (a) Original recordings from experiments on the formation of methaemoglobin under the influence of cumulative concentrations of nitrendipine before (●) or after pretreatment with gadolinium (◆) or L-NOARG (□), or in a calcium free solution (■). Methaemoglobin formation was assessed as the difference in extinction between 401 and 411 nm. (b) Formation of methaemoglobin indicating NO-release given as the area under the curve (AUC) of the difference in extinction between 401 nm and the isobestic point 411 nm assessed over a period of 5 min (within which steady state conditions were reached). The first column shows the basal nitric oxide release, given as an increase in methaemoglobin formation, the subsequent columns the stimulation of nitric oxide release by 1 μM nitrendipine and the effect of additional 1 mM N^G-nitro-L-arginine (L-NOARG) or 100 μM gadolinium (Gd). The last column shows the results obtained from cells which were exposed to calcium free HeBSS (without Ca). All values are given as means±s.e.mean of $n=6$ experiments. Significance compared to 1 μM nitrendipine alone is given by a solid star ($P<0.05$) and significance compared to control, i.e. the basal methaemoglobin formation, is indicated by an open star ($P<0.05$). For further details see Methods section.

by 11±2 nM (0.1 μM), 23±3 nM (1 μM), 34±4 nM (10 μM and by 47±5 nM (100 μM) ($n=15$) (Figure 2). Steady state conditions were reached within 3 min.

In the following experiments, to elucidate the site of action of nitrendipine, the endothelial cells were pretreated for 5 min with either gadolinium (100 μM), which is supposed to block shear stress activated calcium channels (Oliver & Chase, 1992), or thapsigargin (0.3 μM), which inhibits the endoplasmatic calcium ATPase (Gericke *et al.*, 1993) or with a calcium free HeBSS to find out whether extracellular and/or intracellular calcium is needed for the nitrendipine-induced calcium signal. After this pretreatment period, a single concentration of nitrendipine (1 μM) was applied and the resulting calcium-signal was measured. In cell free control experiments the direct influence of gadolinium and thapsigargin on Fura-2 fluorescence was tested and no interaction of these two drugs with Fura-2 fluorescence could be determined; in particular no quenching of the dye occurred. In additional control experiments it was verified that gadolinium pretreatment did not interfere with the calcium increase obtained with 1 μM bradykinin (BK without Gd^{3+} 355±15 nM increase in intracellular calcium; with Gd^{3+}: 336±23 nM increase; control calcium: 122±6 nM). In further control experiments it was also verified that 10–15 min thapsigargin-pretreatment did completely abolish the calcium signal evoked by 1 μM bradykinin (BK without thapsigargin: 318±25 nM increase in intracellular calcium; BK after thapsigargin-pretreatment: 5±8 nM increase; control calcium: 114±8 nM) indicating an emptying of the intracellular calcium stores. The application of thapsigargin itself resulted in an increase in intracellular calcium (from 114±8 nM to 345±85 nM) reaching a plateau within 5–10 min. The experiment was continued if there was no more change in intracellular calcium for 5 min.

The nitrendipine-induced calcium signal was completely

abolished by Gd^{3+} ($n=6$, $P<0.05$). Similarly calcium free HeBSS inhibited the nitrendipine-induced rise in $[Ca^{2+}]_i$. Thapsigargin, which itself, in this experimental series, elevated the intracellular calcium from 111 ± 4 to 293 ± 39 nM did not abolish the nitrendipine-induced calcium signal: application of 1 μM nitrendipine further elevated significantly the intracellular calcium by about 35 nM ($n=6$, $P<0.05$) (Figure 3).

To test the hypothesis that the shear stress might have an influence on the nitrendipine-induced calcium signal we exposed the endothelial cells to various stirring speeds (from 100 to 250 r.p.m.) before the administration of 1 μM nitrendipine. We found that the stirring speed itself increased the 340/380 ratio (100 r.p.m.: 1.02 ± 0.017; 150 r.p.m.: $+0.011\pm0.01$; 200 r.p.m.: $+0.022\pm0.01$; 250 r.p.m.: $+0.027\pm0.0078$). This was further enhanced by nitrendipine. Figure 4 shows the increase in Δ ratio by the stirring speed before and after application of 1 μM nitrendipine and the reduction in Δ ratio after the application of 100 μM Gd^{3+} at the various stirring speeds.

To complete our study we also tested the influence of nitrendipine on the release of NO from BAECs: from a control level, which represents the basal release of NO, administration of cumulative concentrations of nitrendipine resulted in an increase in extinction rate ($\Delta E_{401-411}$), indicating a concentration-dependent release of NO, measured as the area under the curve ($n=8$). In control experiments, in which microbeads without endothelial cells were tested, it was found that there was no interaction between nitrendipine and oxy-haemoglobin (data not shown). Furthermore pre-incubation of endothelial cells with the NO-synthase inhibitor L-NOARG (1 mM) completely blocked the nitrendipine stimulated release of NO ($n=6$) (Figure 5).

To find out whether nitrendipine stimulates the release of NO by a gadolinium-sensitive calcium influx, BAECs were either pre-incubated with gadolinium (100 μM) or in a calcium free HeBSS and 1 μM nitrendipine was applied. Figure 5 shows the results of these experiments: both gadolinium and calcium free HeBSS prevented the nitrendipine-stimulated release of NO ($n=6$).

Discussion

Our results indicate that nitrendipine elevates the intracellular calcium in endothelial cells in a concentration-dependent manner. This elevation is not likely to be mediated via an interaction with L-type calcium-channels, as endothelial cells from macrovascular arteries do not express L-type channels in contrast to microvascular endothelium (Colden-Stanfield *et al.*, 1987; Bossu *et al.*, 1992).

Nevertheless, it was found that the effect of nitrendipine increased with increasing stirring speeds, i.e. with increasing shear stresses. In addition, the effect of nitrendipine was blocked by removing extracellular calcium and by application of the trivalent lanthanide gadolinium, which is known to block shear stress activated cation selective channels on endothelial cells (Naruse & Sokabe, 1993; Hishikawa *et al.*, 1995). This may indicate that nitrendipine possibly enhances the calcium influx into the cell via these channels.

In contrast to an administration of bradykinin, which shows a rapid calcium increase (peak) followed by a sustained elevation at a lower plateau level, nitrendipine leads to a slow monophasic increase in $[Ca^{2+}]_i$. This might indicate that the nitrendipine response does not predominantly involve calcium release from intracellular stores. This is also supported by the finding that the nitrendipine response was insensitive to thapsigargin pretreatment, which is known to deplete the intracellular calcium stores. In control experiments complete depletion of the intracellular calcium stores with 0.3 μM thapsigargin was determined by the absence of a bradykinin-induced calcium peak, a response known to reflect calcium release from intracellular stores.

In addition to its sensitivity to L-NOARG, the release of NO induced by nitrendipine was also sensitive to $[Ca^{2+}]_e$ and Gd^{3+}. Hence, we conclude that the elevation of $[Ca^{2+}]_i$ in BAECs seen with nitrendipine might at least in part be responsible for the observed release of NO.

However, the concentrations necessary for NO-release were lower than those needed for calcium increase and thus it is at present uncertain whether there is a causal connection between the two phenomena. On the other hand, this difference in the concentration-response curves may be due to the fact that different models were used: suspended, i.e. one involved non-confluent endothelial cells and the other, cells grown as a monolayer on microcarrier beads, i.e. confluent cells, which might behave differently from non-confluent cells with regard to sensitivity to shear stress because of their different physical properties.

Elevation of endothelial calcium and NO-release by nitrendipine probably participate in the vasodilator effect of this drug, as shown by Günther *et al.* (1992) and Dhein *et al.* (1995). Thus, nitrendipine and presumably some other 1,4-dihydropyridines can act as calcium agonists at endothelial cells, in addition to their well known calcium antagonistic properties at smooth muscle cells.

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