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Large-conductance K^+ channel opener CGS7184 as a regulator of endothelial cell function

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ABSTRACT

Large-conductance Ca^{2+}-activated potassium (BK_{Ca}) channels are present in endothelium, but their regulatory role remains uncharacterized. The aim of the present study was to investigate the pharmacological effects of the BK_{Ca} channel opener ethyl-1-[[(4-chlorophenyl)amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxylate (CGS7184) on endothelium in the aorta and coronary circulation, particularly with regard to nitric oxide (NO)-dependent regulation of vascular tone, as well as effects of CGS7184 on NO production, calcium homeostasis, and mitochondrial function in cultured endothelial cells. The vasorelaxant action of CGS7184 was studied in coronary circulation and in the aorta using isolated perfused guinea pig heart and rat aortic rings, respectively. The effects of CGS7184 on calcium homeostasis, mitochondrial membrane potential, NO production, and mitochondrial respiration were tested in cultures of EA.hy 926 endothelial cells. The BK_{Ca} channel opener CGS7184 caused a concentration-dependent (0.03–3 μM) relaxation of the rat aorta and coronary vasodilatation in the isolated guinea pig heart. Both responses were profoundly inhibited by the nitric oxide (NO) synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) (100 μM). CGS7184 (5 μM) also increased basal NO production in EA.hy 926 cells by approximately two-fold. Moreover, CGS7184 induced a concentration-dependent (0.1–10 μM) elevation in intracellular calcium concentration. Interestingly, CGS7184 affected mitochondrial function by causing mitochondrial potential depolarization and an increase in oxygen consumption in EA.hy 926 endothelial cells. The BK_{Ca} channel opener CGS7184 activates NOS pathways and modulates mitochondrial function in the endothelium. Both effects may be triggered by the CGS7184-induced modulation of intracellular Ca^{2+} homeostasis in EA.hy 926 endothelial cells.

1. Introduction

Blood pressure and vascular flow in physiological conditions are determined by the contractile state of vascular smooth muscle cells (VSMC) located in the blood vessel wall. Contraction of VSMC is regulated by intracellular calcium concentration (Morano, 2003). In contrast, an increase in Ca^{2+} concentration in the endothelium, causes vascular relaxation through the action of endothelium-derived relaxation factors such as nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizating factor (EDHF) (Feletou and Vanhoutte, 2006; Pries et al., 2000). Calcium-activated potassium channels, specifically large-conductance Ca^{2+}-activated potassium (BK_{Ca}) channels, participate in the regulation of vascular tone (Ledoux et al., 2006).

BK_{Ca} channels belong to the group of six/seven transmembrane potassium-selective channels. These groups contain the large-conductance Ca^{2+}-activated potassium channel (BK_{Ca}, 100–300 pS) (Marty, 1981), the intermediate conductance Ca^{2+}-activated potassium channel (IK_{Ca}, 25–100 pS) (Logsdon et al., 1997), and the small conductance Ca^{2+}-activated potassium channel (SK_{Ca}, 2–25 pS) (Park, 1994). BK_{Ca} channels are tetramers of α subunits with auxiliary β subunit, present in 1:1 stoichiometry (Ghatta et al., 2006; Wei et al., 2005). These channels are activated by elevated cytosolic Ca^{2+} concentration and by membrane depolarization. These two activation processes are independent of each other (Pallotta, 1985). Their large single channel conductance results in a strong plasma membrane hyperpolarization. BK_{Ca} channel activation can also occur during excitation-contraction coupling event in VSMC when intracellular Ca^{2+} concentration is elevated and results in voltage dependent calcium channels inactivation (Jaggar et al., 2000).

BK_{Ca} channels are ubiquitous and are present in mammals, insects, and nematodes. BK_{Ca} channels were discovered in mammalian smooth muscle cells and then found in brain, bladder, cochlea, pancreatic islets

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(Coetzee et al., 1999), and endothelial cells (Dong et al., 2007; Kim et al., 2006; Rusko et al., 1992). Interestingly, putative BK_{Ca} channels were also found in the mitochondrial inner membrane of cardiomyocytes (mitoBK_{Ca}) (Xu et al., 2002), and in the human glioma cell line LN229 (Siemen et al., 1999).

The BK_{Ca} channels are activated by various potassium channel openers (Lawson, 2000). The benzimidazolones NS004 and 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazole-2-one (NS1619) have been recently identified as selective large-conductance potassium channel openers. In addition, these drugs were shown to interact with mitochondria in cardiac cells (Kicinska and Szewczyk, 2004) and glioma cells (Debska et al., 2003), where they inhibit the respiration (Szewczyk and Wojtczak, 2002). Indole carboxylate compounds such as ethyl-1-[[(4-chlorophenyl) amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxylate (CGS7184) have also been identified as BK_{Ca} channel openers. The mechanism of BK_{Ca} channel opening by CGS7184 was previously studied by investigating whole-cell BK_{Ca} current and single channel activity using the patch-clamp method in vascular smooth muscle cells (Hu et al., 1997).

In the present work, we analyzed the effects of the potassium channel opener CGS7184 on coronary flow in Langendorff-isolated perfused guinea pig heart, on vascular tone of the aortic ring in rats, on NO production and mitochondrial respiration in the EA.hy 926 endothelial cell line. We also examined the influence of this compound on calcium homeostasis in the EA.hy 926 endothelial cell line. The present work identifies new mechanisms of the BK_{Ca} channel opener CGS7184: activation of NOS pathways and modulation of mitochondrial function in endothelium that could be linked to the alteration of calcium homeostasis by CGS7184.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle's Medium (DMEM) and phosphate buffered saline (PBS) were purchased from the Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Fetal bovine serum (FBS), HAT, L-Glutamine, Penicillin-Streptomycin were obtained from GIBCO (Paisley, Scotland). DAF-FM diacetate (DAF-FM), fura-2 acetoxymethyl-ester (fura-2 AM) were purchased from Molecular Probes (Eugene, Oregon-USA) and before use were dissolved in dimethyl sulfoxide (DMSO). Since some compounds were dissolved in DMSO we carried out control experiments with this solvent under the same conditions of the experiments. Ethyl-1-[[(4-chlorophenyl)amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxylate (CGS7184) was a kind gift of Novartis (Basel, Switzerland). Phenylephrine was dissolved in deionized water and control experiments were also carried out in this solvent. N^G-nitro-L-arginine methyl ester HCl (L-NAME) were purchased from Sigma (St. Louis, MO, USA). All other chemicals used were of high grade, obtained from Sigma unless otherwise stated.

2.2. Langendorff preparation of the guinea pig heart

This investigation conforms with the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and the experimental procedures used in the present study were approved by the local Animal Research Committee of Jagiellonian University Medical College.

The techniques were performed as described previously (Chlopicki et al., 1999). Briefly, guinea pigs of both sexes with a body weight of 300–400 g were anaesthetised with pentobarbital (30–40 mg/kg body weight). Hearts were isolated, washed in ice-cold saline, and mounted in a Langendorff apparatus (Hugo Sachs Electronics, HSE). Guinea pig hearts were perfused retrogradely through the aorta under a constant perfusion pressure of 60 mmHg with Krebs–Henseleit buffer of the following composition (in mM): 118 NaCl, 4.7 KCl, 2.52 $CaCl_2$, 1.64 $MgSO_4$, 24.88 $NaHCO_3$, 1.18 KH_2P0_4, 5.55 glucose, 2.0 sodium pyruvate. The buffer was equilibrated with 95% O_2 and 5% CO_2 at 37 °C in an oxygenator with rotating disc (HSE). The hearts were paced with 273 impulses per min through two platinum electrodes placed in the right atrium. Left ventricular pressure was measured using a fluid-filled balloon inserted into the left ventricle and connected to a pressure transducer (Isotec HSE). The end diastolic pressure was adjusted to be less than 10 mmHg. The dP/dt_{max} and dP/dt_{min} values were calculated from the left ventricular pressure signal by an analogue differentiation amplifier (DIF module HSE). The values of left ventricular pressure, dP/dt_{max}, and dP/dt_{min} were used to control the quality of the preparation and only those hearts with acceptable left ventricular pressure and dP/dt parameters were used for the experiments. Coronary flow was monitored by an Ultrasonic flowmeter (HSE). Left ventricular pressure, dP/dt_{max}, dP/dt_{min}, and coronary flow were calibrated once a day prior to the experiment, continuously displayed throughout the experiment, and analyzed afterwards using specially-designed software (PSCF.EXE-IGEL, Poland).

2.3. Experimental methods

The isolated heart of the guinea pig was equilibrated at a perfusion pressure of 50 mmHg for approximately 10 min, after which the pressure was adjusted to 60 mmHg and the heart was equilibrated at the higher pressure for 10–15 min before the beginning of the experiment. Hearts were used in experiments only if the following criteria were met: (i) basal coronary flow was 7–20 ml/min, (ii) the increase in coronary flow to bolus injection of 300 pmol of ACh was >2 ml/min.

CGS7184 (0.1–3 μM), ACh (100 nM), bradykinin (3 nM), and adenosine (0.1–1 μM) were administered as intracoronary infusions lasting 1 min into the coronary circulation of the isolated guinea pig heart.

To study the contribution of NO in CGS7184-induced coronary vasodilatation, the response was induced in the absence or presence of NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME; 100 μM). For comparison, the effect of L-NAME on the endothelium-dependent vasodilation induced by ACh (100 nM), bradykinin (3 nM), adenosine (0.1–1 μM), and reactive hyperaemia (induced by coronary occlusion of 10 or 30 s) was measured.

CGS7184 was dissolved in dimethyl sulfoxide (DMSO) and all other reagents were dissolved in water. The rate of infusion took into account the value of basal coronary flow, and it never exceeded 1% of coronary flow. Infusion of DMSO alone slightly increased coronary flow by 1.03 ± 0.11 ml/min. The length of the experiment never exceeded 3 h, during which the quality of the isolated guinea pig heart remained unchanged. Results are presented as the mean ± S.E.M. Student's *t* test for paired values was used to evaluate differences before and after addition of the NOS inhibitor. A value of $p<0.05$ was accepted as being statistically significant.

2.4. Isolated aortic rings

The thoracic aorta from Wistar rats anaesthetised by thiopental (i.p. 120–150 mg/kg body weight) were removed and carefully dissected from the surrounding tissue. Each isolated aorta was placed in Krebs–Hanseleit solution, cleaned of the connective and fat tissue, and cut into rings. Next, rings were washed with Krebs–Hanseleit solution mounted between 2 hooks attached to an isometric force transducer (Biegastab K30 type 351; Hugo Sachs March-Fr, Germany) that continuously recorded the tension (Graphtec WR3320, UK). After the rings were mounted, the resting tension was increased in a stepwise fashion until 4 g was reached, after which the rings were equilibrated for 30 min. Six circular segments of the artery with lengths of 3–5 mm were used in parallel for each experiment. Aortic rings were kept in 5-ml organ baths containing pre-warmed (37 °C) Krebs–Hanseleit that was continuously

bubbled with 5% CO_2 in O_2 to maintain pH 7.4. Krebs–Hanseleit solution contained the following (in mM): 118.0 NaCl, 4.7 KCl, 2.25 $CaCl_2$, 1.64 $MgSO_4$, 1.18 KH_2PO_4, 24.88 $NaHCO_3$, 10.0 glucose, 2.2 $C_3H_3O_3Na$, and 0.05 EDTA.

After equilibration and precontraction with KCl (60 mM), aortic rings were precontracted with phenylephrine (60–80% of the maximum KCl-induced contraction) and tested for endothelium-dependent vasodilatation by treating with ACh (10 μM), and for endothelium-independent vasodilatation by treating with *S*-nitroso-*N*-acetylpenicillamine (SNAP, 10^{-5} M). Only rings that showed full vasodilatation in endothelium-dependent and -independent responses were included in experiments. Next, cumulative concentration-dependent vasodilator responses to CGS7184 were obtained in the absence or presence of L-NAME (300 μM). The aortic rings were incubated with L-NAME for at least 15 min prior to eliciting the response.

2.5. Culture of EA.hy 926 cells

EA.hy 926 is a permanent cell line derived by fusing human umbilical vein endothelial cells with the A549 cell line (Edgell et al., 1983). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 IU penicillin, 100 μg streptomycin, and 2% HAT Supplement. Cultures were maintained at 37 °C in a fully humidified atmosphere of 5% CO_2 in air. Cells were passaged twice a week (Malli et al., 2005).

2.6. Fluorescent measurements of nitric oxide

EA.hy 926 cells were cultured in 24-well plates. When the cells became 90% confluent, they were incubated with 4 μM 4-amino-5-methylamino-2′,7′-difluorescein diacetate (DAF-FM) in Krebs buffer containing (in mM): 117.3 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 1.23 $CaCl_2$, 1 lactate and 11.1 glucose, at 37 °C and 5% CO_2 in air. After 1 h, the buffer was changed to a buffer containing in addition CGS7184 or vehicle. The cells were incubated for an additional 3 h at 37 °C and 5% CO_2 in air, after which the fluorescence was measured on a C&L Dye Fluorometer (excitation/emission, 495 nm/515 nm).

2.7. Fluorescence measurements of Ca^{2+} concentration changes

Ca^{2+} concentration changes were monitored by fluorescence of the hydrolyzed form of fura-2-AM. Cells grown on coverslips were incubated with 2 μM fura-2-AM and 0.02% Pluronic F-127 (Sigma) for 45 min at 37 °C and 5% CO_2 in air in serum-free DMEM. The cells were then washed with MOPS buffer containing (in mM): 120 NaCl, 1 $MgCl_2$, 5.4 KCl, 0.33 Na_2HPO_4, 11 glucose, 30 MOPS, 5 taurine, 2 pyruvate, 1.5 glutamine, pH 7.4. Next, the coverslips were placed in cuvettes in the same buffer and analyzed on a Shimadzu RF-5301PC spectrofluorophotometer (Tokyo, Japan). The samples were excited at 340 nm and 380 nm, and the emission fluorescence was monitored at 510 nm. All fura-2 measurements were performed at room temperature.

In order to confirm intracellular Ca^{2+} changes, similar experiments were carried out to measure the fluorescence of single cells. After loading, the EA.hy 926 cells were washed twice and allowed to equilibrate for 15 min before coverslips were mounted into a chamber attached to the stage of an inverted microscope (IX 70, Olympus, Japan), which was equipped with a 20× objective. The fura-2 dye was excited at 340 nm/380 nm using a monochromator (Polychrome IV, Till Photonics, Germany). Fluorescence emission light was collected with a CCD camera (Till Photonics Imago, Germany). Results were analyzed by Till Vision software (Till Photonics, Germany).

2.8. Respiration measurements

Cell oxygen consumption was measured at 37 °C using a Clark-type electrode in a serum-free culture medium and an Oroboros high resolution respirometer. The number of EA.hy 926 cells in suspension was 1×10^6 per 1 ml of medium. The test compound, CGS7184, was added to the chamber after the respiratory flux stabilized. To uncouple oxidative phosphorylation, 1 μM CCCP was added.

2.9. Measurements of mitochondrial membrane potential

EA.hy 926 cells were cultured on rectangular glass coverslips until the cells reached 90% confluency. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′ tetraethylbenzimidazolylcarbocyanine iodide) dye. The cells were loaded with 10 μM JC-1 in Krebs buffer containing (in mM): 117.3 NaCl, 4.7 KCl, 25 $NaHCO_3$, 1.3 $MgSO_4$, 1.2 KH_2PO_4, 1.23 $CaCl_2$, 1 lactate and 11.1 glucose, at 37 °C and 5% CO_2. After 30 min of loading with JC-1, the glass coverslips were placed in a 3-ml cuvette in Krebs buffer and were allowed to equilibrate for another 30 min, after which CGS7184 or the mitochondrial uncoupler CCCP (carbonyl cyanide 3-chlorophenylhydrazone) (1 μM) was added. After an additional incubation of 30 min, changes in JC-1 fluorescence were measured. The dye undergoes a reversible change in fluorescence emission from green (527 nm) to red (590 nm) at an excitation wavelength of 490 nm as mitochondrial membrane potential increases. JC-1 aggregates in the mitochondria, resulting in red fluorescence. The relative mitochondrial potential was expressed as the ratio of fluorescence at 590 nm (red) to fluorescence at 527 nm (green), which corresponds to $\Delta\Psi_m$. Uncoupling by CCCP led to depolarization, indicating that the mitochondrial membrane was functioning normally. Measurements were performed with a Shimadzu RF-500 spectrofluorometer (Tokyo, Japan).

3. Results

3.1. Vasodilatation induced by the potassium channel opener CGS7184

The effect of potassium channel opener CGS7184 on vascular tone was tested on aortic rings (see Materials and methods). As shown in Fig. 1, the potassium channel opener CGS7184 induced a concentration-dependent vasodilatation in aortic rings. Significant vasodilatation was observed at a CGS7184 concentration of 0.1 μM reached maximum at 3 μM and could be inhibited by L-NAME. In order to

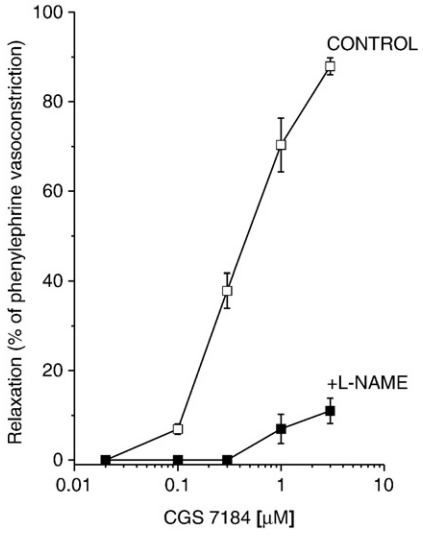

Fig. 1. Involvement of NO in the vasodilatation induced by the potassium channel opener CGS7184 in isolated rat aortic rings. Response of phenylephrine-vasoconstricted rat aortic rings to CGS7184 treatment was tested in the presence (+L-NAME, filled squares) and absence (control, empty squares) of 300 μM L-NAME. The vasodilatation caused by 10 μM ACh was defined as 100%. The bars represent the S.E.M. values (n=4–5). At all concentrations of CGS7184 used in the experiments starting from 0.1 μM data were significantly different to the control value with $p<0.05$.

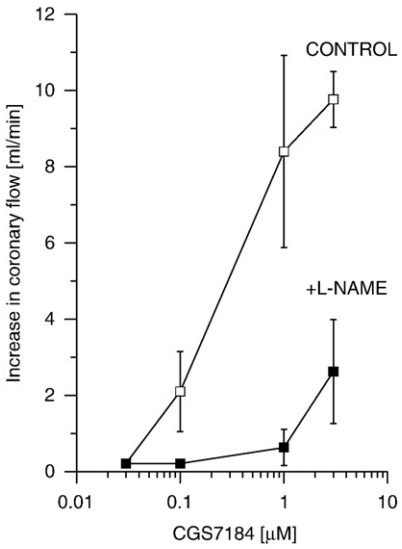

Fig. 2. Involvement of NO in the coronary flow in the isolated guinea pig heart induced by the potassium channel opener CGS7184. Changes in coronary flow caused by CGS7184 treatment in the presence (+L-NAME, filled squares) and absence (control, empty squares) of 100 μM L-NAME. The bars represent the S.E.M. values (n=4–7). At all concentrations of CGS7184 used in the experiments starting from 0.1 μM data were significantly different to the control value with $p<0.05$.

examine the role of NO in the vasodilatation induced by CGS7184, the NOS inhibitor L-NAME was added at 300 μM. Addition of L-NAME strongly inhibited CGS7184-induced aortic ring vasodilation; in fact, the L-NAME inhibited the response induced by 3 μM CGS7184 by 90% (Fig. 1).

The effect of the BK_{Ca} channel opener on coronary vascular tone was studied in the isolated guinea pig heart. Similarly to its effects on aortic ring preparations, CGS7184 caused vasodilatation in the isolated guinea pig heart and induced a concentration-dependent increase in the coronary flow (Fig. 2). Basal coronary flow was 13.6±1.4 ml/min, while the maximum of the response induced by 3 μM CGS7184 reached 9.68±0.74 ml/min. In the presence of L-NAME (100 μM), the response induced by CGS 7184 was substantially inhibited.

3.2. Measurements of cytosolic calcium concentration

The effect of CGS7184 on the intracellular concentration of calcium ions in EA.hy 926 endothelial cells is shown in Fig. 3. Addition of CGS7184 (10 μM) to the EA.hy 926 cells growing on the glass coverslip caused an elevation in Ca^{2+} levels (Fig. 3A), as detected in changes of the fluorescence ratio of cells loaded with fura-2 probe. Titration with an increasing concentration of CGS7184 caused a concentration-dependent elevation in intracellular Ca^{2+} concentration (Fig. 3B), with an IC_{50} of approximately 0.61 μM and a maximal response at 5 μM. The fura-2 fluorescence ratio at the control condition was 1.44 and after addition of 10 μM CGS7184 increase to 3.62 which corresponds to 0.13 μM and 0.64 μM free Ca^{2+} concentration, respectively (Fig. 3B). Intracellular free calcium concentration was calculated according to the equation published by Grynkiewicz et al. (1985). It is well-known that NO synthesis in endothelial cells can be directly regulated by elevation of cytosolic free calcium concentration (Fleming and Busse, 1999).

3.3. Regulation of NO synthesis in endothelial cell line EA.hy 926

Experimental results with vascular preparations demonstrated that endothelial NO was responsible for the relaxation of the aortic ring and the increase in coronary flow of isolated guinea pig heart in response to CGS7184. In turn, we measured NO synthesis in the presence of 5 μM CGS7184 in the endothelial cell line EA.hy 926. This concentration of CGS7184 caused a maximum of calcium increase in EA.hy 926 cells as measured in fura-2 experiments. As measured with fluorescent probe

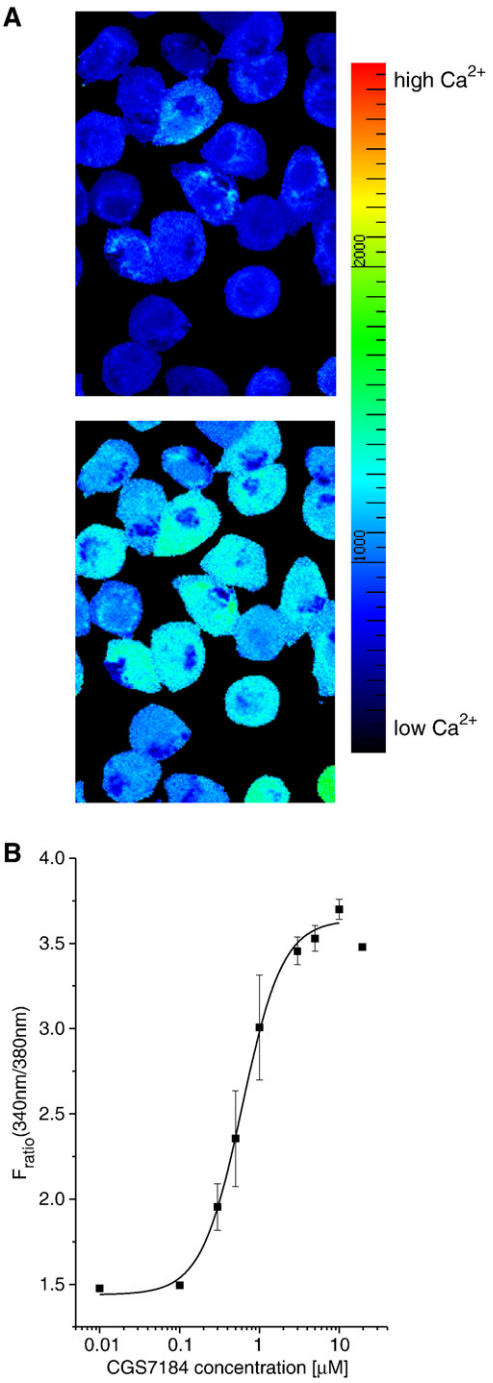

Fig. 3. Changes in cytosolic Ca^{2+} concentration in EA.hy 926 endothelial cells following addition of CGS7184. (A) Changes in the fura-2 fluorescence ratio upon addition of CGS7184. The upper image represents control conditions and the lower image shows changes in fura-2 fluorescence ratio following treatment with 5 μM CGS7184. The colored bar on the left represents the range of changes in the relative fluorescence ratio of fura-2. (B) The concentration–response curve of changes in fluorescence ratio upon titration with CGS7184. The fura-2 fluorescence was measured at 510 nm at two excitation wavelengths: 340 nm and 380 nm (see Materials and methods). Data reflect changes in Ca^{2+} cytosolic concentration in EA.hy 926 cells. The data were fit with the Hill equation, yielding a IC_{50} of 0.61 μM and Hill coefficient of 1.70. Error bars are±S.E.M. (n=3–7) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

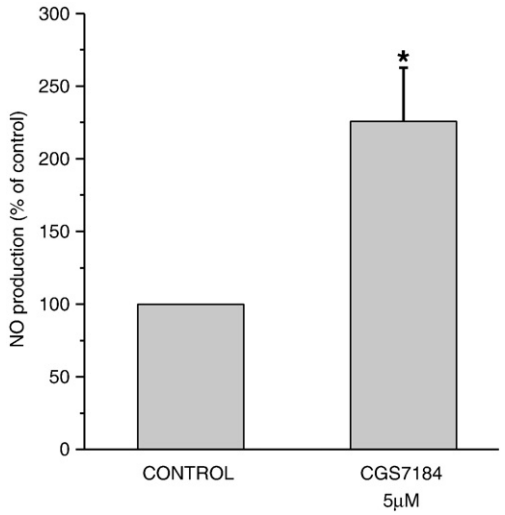

Fig. 4. Changes in NO biosynthesis upon treatment of EA.hy 926 cells with CGS7184. Treatment of the EA.hy 926 cell line with 5 μM CGS7184 caused an increase in NO biosynthesis of 125.8±36.8%. The biosynthesis was measured using the fluorescent probe DAF-2 (see Materials and methods), and the results are expressed as mean±S.E.M. (n=3). Basal NO biosynthesis under control conditions in the absence of CGS7184 was defined as 100%. *Significantly different to control value with $p<0.05$.

DAF-FM, CGS7184 (5 μM) increased NO production by 125.8±36.8% compared to control conditions (Fig. 4).

3.4. Modulation of mitochondrial function

Both NO and calcium ions may influence mitochondrial function of endothelial cells (Davidson and Duchen, 2007). Hence, we focused subsequent experiments on measuring the effects of the potassium channel opener CGS7184 on mitochondrial membrane potential in intact cells. Measurements of mitochondrial membrane potential were performed with the dye JC-1, whose fluorescence changes in response to changes in the mitochondrial membrane potential (see Materials and methods). CGS7184 (5 μM) induced a 47±13.8% mitochondrial membrane depolarization compared to the control cells (Fig. 5). Addition of the uncoupler CCCP (1 μM) to the cells loaded with JC-1 caused a larger mitochondrial membrane depolarization (73.6±2.8%).

Because mitochondrial membrane depolarization can be induced by inhibition of the mitochondrial respiratory chain or by an increase in the ion flux through the inner mitochondrial membrane, we measured oxygen consumption during CGS7184-induced membrane depolarization. For these experiments, we used EA.hy 926 cells and high-resolution respirometry.

Fig. 6 shows the respiration rate of the EA.hy 926 cell line in the presence of CGS7184. The basal respiration rate was 23±2.5 pmols/(sxml), which increased following CGS7184 exposure (Fig. 6A), suggesting that its effect on mitochondrial potential was due to an influx of ions into the mitochondrial matrix but not to inhibition of the respiratory chain. Oxygen consumption caused by 5 μM CGS7184 (Fig. 6B) could be increased further by subsequent addition of the mitochondrial uncoupler CCCP (1 μM). However, increased mitochondrial respiration was not observed using endothelial cells permeabilized with digitonin (data not shown). This suggests that CGS7184-induced changes in mitochondrial respiration are due to an influx of Ca^{2+} into the mitochondrial matrix, and not to a direct effect of the potassium channel opener on the inner mitochondrial membrane.

4. Discussion

The present study demonstrates that the potassium channel opener CGS7184 (Nardi et al., 2006) increases the biosynthesis of NO in the EA.hy 926 endothelial cell line, causes the vasodilatation of the rat aortic ring and increases coronary flow in the isolated guinea pig heart. These effects could be related to the action of CGS7184 on BK_{Ca} channels located in endothelial cells (Begg et al., 2003; Brakemeier et al., 2003; Kuhlmann et al., 2004) or BK_{Ca} channels-independent effects of CGS7184 on Ca^{2+} homeostasis. Indeed, we demonstrated that CGS7184 raised the intracellular cytosolic Ca^{2+} concentration and CGS7184-induced effects in endothelium such as the activation of NOS pathways and modulation of mitochondrial function in endothelium could be triggered by the CGS7184-induced modulation of intracellular Ca^{2+} homeostasis in EA.hy 926 endothelial cells.

CGS7184 at micromolar concentrations induced a pronounced relaxation of aortic rings that had been precontracted with phenylephrine. Addition of L-NAME, a non-specific inhibitor of nitric oxide synthases, substantially reduced the relaxation induced by CGS7184 in phenylephrine-preconstricted aortic rings. We have observed similar effects in isolated perfused guinea pig hearts, where addition of CGS7184 caused an increase in coronary flow, and this effect was blocked by the NO synthase inhibitor L-NAME. These results stay in line with the work of others that described the role of NO in the response to other widely used BK_{Ca} channel openers, NS1619 and resveratrol (Calderone et al., 2007). The less pronounced effect of L-NAME on the activity of CGS7184 at higher concentrations was most probably related to the action of CGS7184 on BK_{Ca} channels located in the VSMC, which leads to membrane hyperpolarization and to reduced entry of extracellular calcium through voltage-dependent calcium channels (Xia et al., 2002; Young et al., 2001; Zhou et al., 2001).

In order to confirm the involvement of NO in CGS7184 -induced response we measured NO production in the EA.hy 926 endothelial cell line subjected to CGS7184. The maximal used concentration of CGS7184 doubled the amount of NO produced relative to control conditions. One of the triggers of NO biosynthesis is intracellular Ca^{2+}, which activates the constitutive forms of NO synthase (Fleming and Busse, 1999; Busse and Mulsch, 1990). In fact, treatment of EA.hy 926 cells with micromolar concentrations of CGS7184 caused a rapid and significant elevation of intracellular Ca^{2+} concentration. The CGS7184 concentration capable of elevating the intracellular Ca^{2+} concentration was found to be similar to that used to relax the aortic ring and

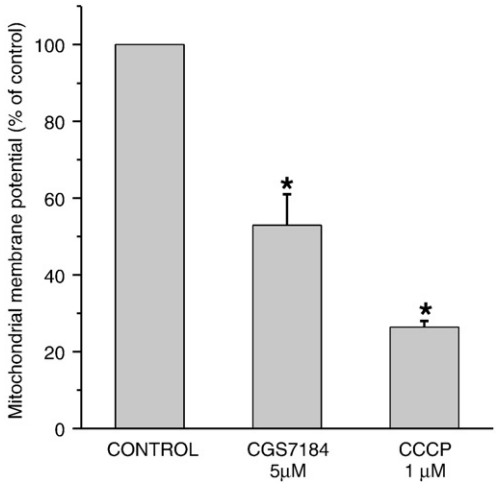

Fig. 5. Effect of CGS7184 on the mitochondrial membrane potential in the EA.hy 926 cell line. The mitochondrial membrane potential was measured as described in Materials and methods using the JC-1 fluorescent probe. Changes in mitochondrial membrane potential after addition of 5 μM CGS7184 are expressed as the percent of the control fluorescence ratio 590 nm/527 nm of the JC-1 excited at a wavelength of 490 nm. Addition of the mitochondrial membrane uncoupler CCCP (1 μM) caused the strongest depolarization of the inner mitochondrial membrane. Data are expressed as mean±S.E.M. (n=3). *Significantly different to control value with $p<0.05$.

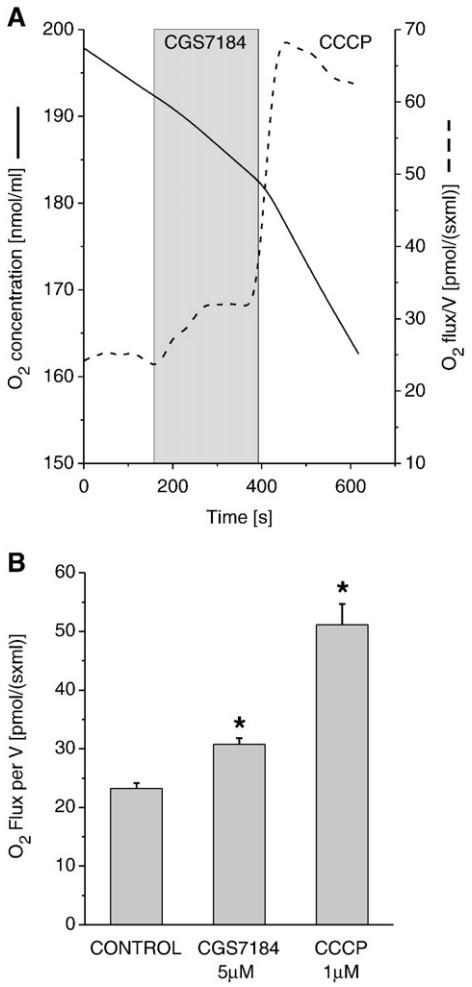

Fig. 6. Effect of GCS7184 on oxygen consumption and mitochondrial respiration in EA.hy 926 cells.(A) Changes in oxygen concentration (solid line) and respiration (dashed line) in EA.hy 926 cells upon addition of 5 μM CGS7184 and 1 μM CCCP. Measurements were performed as described in the Materials and methods section. (B) Quantitative representation of EA.hy 926 cell respiration following addition of 5 μM CGS7184 and 1 μM CCCP. Data are expressed as mean ± S.E.M. ($n = 3$). *Significantly different to control value with $p < 0.05$.

increase coronary flow. The sources of the increase in Ca^{2+} concentration induced by CGS7184 could be related to the intracellular stores and influx from the extracellular space. We tested the ability of CGS7184 to release Ca^{2+} from intracellular stores in the EA.hy 926 cell line. We detected calcium transient in the absence of extracellular Ca^{2+} after treatment with CGS7184 (data not shown) which suggest the involvement of intracellular stores in the increase of Ca^{2+} caused by CGS7184. On the other hand, in non-excitable cells including endothelial cells, the entry of Ca^{2+} is strongly influenced by the endothelial cell membrane potential and the calcium ion gradient across the plasma membrane (Luckhoff and Busse, 1990) suggesting that Ca^{2+} effect of CGS7184 could be related to the opening the of BK_{Ca} in plasma membrane of endothelial cells. Still it was recently reported that SKca but not BKca plays a major role in endothelial NO release (Stankevicius et al., 2006). Thus, it is apparent that to understand the mechanism(s) by which CGS7184 induces the release of Ca^{2+} further studies are needed.

Interestingly, recently, the BK_{Ca} channels have been detected in the inner mitochondrial membrane of various cells (Siemen et al., 1999; Xu et al., 2002). This raises the question of what is the relationship between the mitochondrial response and production of NO in endothelial cells treated with the potassium channel opener CGS7184. Potassium channel openers acting on channels located in the inner mitochondrial membrane exert a cardioprotective effect (O'Rourke, 2007). The cytoprotective role of ATP-regulated potassium channel openers are well-documented (Garlid et al., 1997; Liu et al., 1998). In addition, the present study shows an increase in NO synthesis, intracellular elevation of Ca^{2+}, and depolarization of the mitochondria of EA.hy 926 cells after addition of the BK_{Ca} channel opener CGS7184. This may suggests that the BK_{Ca} channel is present in mitochondria and its activation contributes to the endothelial response to CGS7184. In fact, mitoBK_{Ca} was detected in the LN229 glioma cell line (Siemen et al., 1999). In addition to the changes in mitochondrial potential, EA.hy 926 cells treated with CGS7184 showed an increased rate of respiration. This effect may be related to the elevation in cytosolic Ca^{2+} concentration. On the other hand it could be also linked to the direct action of the potassium channel opener CGS7184 on BK_{Ca} channels located in the inner mitochondrial membrane (Debska et al., 2003; Kicinska and Szewczyk, 2004). Further studies are needed in order to determine the molecular mechanisms of the action of potassium channel opener CGS7184 on endothelial cells.

In summary, we demonstrated that BK_{Ca} potassium channel opener CGS7184 activated NOS pathways and modulated mitochondrial function in the endothelium. Both effects may be triggered by the CGS7184-induced modulation of intracellular Ca^{2+} homeostasis in EA.hy 926 endothelial cells. The exact mechanism of CGS7184 induced endothelial response and the contribution of mitochondrial BK_{Ca} channels to this response need to be elucidated.

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