The role of the plasmalemmal ATP-sensitive potassium channels (K_{ATP}) in induction of endothelial ischemia-reperfusion injury

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by
Dragan Gligorievski

from Kumanovo, Republic of Macedonia

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From the Institute of Physiology
Chairman: Prof. Dr. Dr. Hans-Michael Piper
of The Faculty of Medicine of the Justus Liebig University Giessen

First Supervisor and Committee Member: Prof. Dr. Dr. Hans-Michael Piper
Second Supervisor: Prof. Dr. Ulrich Decking
Committee Member: Prof. Dr. Heinz-Jürgen Thiel
Committee Member: Prof. Dr. Martin Diener

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Contents

1 INTRODUCTION

1.1 Endothelium and its barrier function

The endothelium is located at the interface between blood and the vessel wall. The cells are in close contact and form a monolayer that prevents blood cell interaction with the vessel wall as blood moves through the vessel lumen. Dependent on its localization in the vessel tree it forms a multifunctional signal-transducing surface and also serves as a barrier to the transvascular exchange of liquids and solutes. Endothelial cells (EC) modulate the tone of vascular smooth muscle cells (VSM), which in turn controls blood pressure and blood flow by adjusting the calibre of arteries and arterioles. In the microvascular bed, EC regulate the permeation of various metabolites, macromolecules and gases, as well as autocrine and paracrine factors and are involved in the regulation of cell nutrition. In all vessel types, EC are involved in blood coagulation, control of the transport between blood and tissue, movement of cells adhering to EC, wound healing, and angiogenesis. Other functions require an active response of EC to various signals of mechanical, chemical, or neuronal nature and origin. Some of these signals originate from cells of other origin, which are in contact to endothelial cells (vascular smooth muscle cells), or may originate from adhering cells (leukocytes or thrombocytes) or neighbouring endothelial cells. This signal transduction is impaired during certain pathophysiological conditions like ischemia and the subsequent reperfusion.

An essential requirement for adequate organ performance is the formation of permeability barriers that separate and maintain compartments of distinctive structure. The barrier function of endothelium regulates the transport of fluids and molecules between blood and interstitial space, largely through small intercellular pores. Actin-myosin filaments stabilise the form of the endothelial cells, and thereby also regulate the size of intercellular pores. In pathological conditions, such as ischemia and reperfusion, endothelial cell retract markedly and lose attachment to each other. This facilitates fluid and protein diffusion into the interstitium causing tissue swelling known as edema.

1.2 The role of Ca^{2+} in vascular endothelial cells during ischemia and reperfusion

Many EC functions depend on cytosolic Ca^{2+}. When metabolically impaired, endothelial cells react with a change in cytosolic Ca^{2+} concentration. Even a moderate reduction of cytosolic ATP, i.e., for 30 % leads to a pronounced increase in cytosolic Ca^{2+} in endothelial cells exposed to simulated ischemic conditions. (Noll et al. 1995, Schäfer et al. 2001). When functions of ATP-dependent ionic pumps are altered, calcium and sodium accumulate in the cell, due to their impaired extrusion.

During simulated ischemia, the endothelial cells react with a biphasic increase in cytosolic Ca^{2+}. A first rise of cytosolic Ca^{2+} is due to the release of Ca^{2+} through IP_3 sensitive Ca^{2+} channels of the endoplasmic reticulum (Ladilov et al. 2000, Schäfer et al. 2001). After the initial increase, the Ca^{2+} concentration rises further due to a secondary, "capacitative" Ca^{2+} entry from the extracellular space (Adams et al. 1989, Putney 1990, Dolor et al. 1992, Berridge 1995, Schäfer et al. 2001). Elevated cytosolic Ca^{2+} leads to formation of intercellular gaps. Gap formation causes a change in endothelial barrier permeability and leads to edema *in vivo*. This is especially noticeable in a reperfusion phase following ischemia.

Gap formation is triggered by the following mechanism: Ca^{2+} overload during ischemia-reperfusion stimulates an activation of the contractile apparatus of endothelial cells. Cell-cell contacts (tight junctions, adherence junctions) are also destabilised (Muhs et al. 1997, Schäfer et al. 2003). As a consequence, endothelial cells detach from each other. On the molecular level, contractile activation is due to the activation of the Ca^{2+}-calmodulin dependent myosin light chain kinase leading to an increase in myosin light chain-phosphorylation (Garcia et al. 1997).

1.3 The role of K_{ATP} channels in modulation of Ca^{2+} influx during ischemia and reperfusion

Ca^{2+} entry in EC occurs via different pathways, but the activation mechanisms of these entry pathways are still elusive. EC not only activate Ca^{2+} entry upon ischemia and reperfusion, but also provide a sufficiently large inwardly driving force for Ca^{2+}. There is evidence that a Na^{+}/Ca^{2+} exchanger (NCX) may shape Ca^{2+} transients activated by vasoactive agonists (Teubl et al. 1999) and it has been also shown that a

reduction of the Na^+ gradient increases Ca^{2+} via NCX in reverse mode operation (Sedova et al. 1999). Nilius et al. (2001) indicated that the driving force for Ca^{2+} entry is mainly tuned by channels that modulate electrogenesis in EC and used the following formula from Adams et al. (1993) for calculating Ca^{2+} influx (J_{Ca}):

$$J_{Ca}=2 \times N \times F \times p \times \gamma_{Ca} \times (V_M-E_{Ca})$$

where F is the Faraday constant, N is the number of channels, p is the open probability of the channel and γCa is its conductance. Ca^{2+} entry is regulated by the driving force for Ca^{2+}, i.e., the difference between membrane potential (V_M) and equilibrium potential for Ca^{2+} (E_{Ca}). Based on this formula, Ca^{2+} influx increases with plasmalemma hyperpolarisation. In an intact cell this may be antagonised by an increase in plasmalemma Ca^{2+} pump activity and Ca^{2+} extrusion via the NCX in forward mode operation favoured by a larger membrane potential.

Electrogenesis in EC is regulated by K^+ channels, but other channels, for example Cl^- channels may be involved (Nilius et al. 2001). In endothelial cells Kamouchi et al. (1999), have demonstrated that increase in K^+ channel activity and the resulting plasmalemma hyperpolarisation causes an increase in Ca^{2+} influx, as it increases the driving force for Ca^{2+} influx across the plasmalemma. Langheinrich et al. (1998) showed in endothelial capillaries that low concentrations of the K_{ATP} channels opener diazoxide induce a rapid, transient rise of cytosolic Ca^{2+} followed by a further sustained elevation. In an *in vitro* analysis of the role of K_{ATP} channels in hypoxia-anoxia in three distinct neuronal systems of rodents, Ballanyi et al. (2004) demonstrated that in dorsal vagal neurons, inhibition of K_{ATP} channels with sulfonylureas abolishes the hypoxic-anoxic hyperpolarisation which is accompanied by a moderate and sustained increase of intracellular Ca^2.

Under physiologic conditions K_{ATP} channels are inhibited, since their open-state is dependent on the cytosolic ATP level. Therefore K_{ATP} channels do not seem to play a role in regulation of the K^+ homeostasis under normal physiological circumstances. However, during ischemia the cytosolic level of ATP decreases more than 30 %, which leads to activation of the K_{ATP} channels. The role of K_{ATP} channels in Ca^{2+} overload of vascular endothelial cells under conditions of ischemia-reperfusion was in the focus of this study.

1.4 Molecular structure of K_{ATP} channels and their role in ischemia and reperfusion

K_{ATP} channels consist of an octameric complex containing two distinct types of protein subunits, four of which are inwardly rectifying potassium channels subunits ($K_{ir}6.1$ or $K_{ir}6.2$). Each K_{ir} subunit is associated with a larger regulatory sulphonylurea receptor (SUR). The molecular diversity of the K_{ATP} channels among species and tissue types is further expanded by the presence of multiple isoforms of SUR (SUR1, SUR2A, SUR2B). The SUR1 isoform is present in pancreatic K_{ATP} channels while the SUR2A and SUR2B isoforms are present in cardiac and vascular K_{ATP} channels. The structure of the K_{ATP} channels in vascular endothelium is to date not clarified. Immunohistological data from cardiac cryosections suggest $K_{ir}6.1$ protein is expressed in ventricular myocytes, as well as in smooth muscle and endothelial cells of coronary vessels and endothelial capillaries. $K_{ir}6.2$ protein expression is found predominantly in ventricular myocytes and also in endothelial cells. SUR1 subunits are not expressed in the coronary vasculature, whereas SUR2 is predominantly localised in cardiac myocytes and coronary vessels, mostly in smaller vessels (Morrissey et al. 2005).

K_{ATP} channels are characterised by dependence of their activation on the concentrations of intracellular ATP, ADP and other nucleotides (Yamada et al. 1997, Gribble et al. 1998). K_{ATP} channels can be activated pharmacologically by a chemically heterogeneous class of compounds, or K_{ATP} channel openers, and can be blocked by sulfonylurea derivatives. Reduction in cytosolic level of ATP during ischemia leads to activation of the K_{ATP} channels which enables their role in electrogenesis and thus taking a part in modulating the driving force for Ca^{2+} entry during ischemia. Unless the above named compensatory mechanisms prevail, K_{ATP} channels opening would therefore contribute to the extent of ischemic endothelial Ca^{2+} overload. It may then be expected that inhibition of K_{ATP} channels during ischemia could also decrease the development of cytosolic Ca^{2+} overload. Its reduction could decrease the level of contractile apparatus activation and thus the formation of intercellular gaps, which serve as parameters for cellular injury during ischemia and reperfusion. Inhibition of K_{ATP} channels during ischemia and

reperfusion could be used as a clinical target for prevention of endothelial barrier function failure.

1.5 Aim of the study

The main focus of this study on endothelial cells exposed to conditions of simulated ischemia and reperfusion was based on the following questions:

-Does activation of K_{ATP} channels during ischemia and reperfusion take place?

-Are K_{ATP} channels involved in the increase of cytosolic Ca^{2+} during ischemia and reperfusion?

-What influence do K_{ATP} channels have on formation of intercellular gaps?

2 MATERIALS:

2.1 Chemicals:

BAPTA (1,2-bis(o-aminophenoxy)ethane- N,N,N',N'-tetraacetic acid)	Sigma-Aldrich, Taufkirchen
BSA (Bovine Serum Albumin)	Sigma-Aldrich, Taufkirchen
Carbogen O_2/CO_2=95 %/5 % (vol/vol)	Messer Griesheim, Krefeld
Collagenase Type CLS II (322 U/mg)	Biochrom KG, Berlin
Cystein	Sigma-Aldrich, Taufkirchen
Diazoxide (7-Chloro-3-methyl-2H-1,2,4-benzothiadiazine 1,1-dioxide)	Sigma-Aldrich, Taufkirchen
$DiBAC_4(3)$ bis-(1,3-dibutylbarbituric acid) trimethine oxonol	Invitrogen, Karlsruhe
DMSO (Dimethyl Sulfoxide)	Merck, Darmstadt
EDTA (ethylenediaminetetraacetic acid)	Roth, Karlsruhe

EGTA ethylene glycol bis(2-aminoethyl ether)- N,N,N'N'-tetraacetic acid	Sigma-Aldrich, Taufkirchen
Fura-2 AM	Invitrogen, Karlsruhe
FCS (Fetal Calve Serum)	FAA Laboratories, Cölbe
Glybenclamide	Sigma-Aldrich, Taufkirchen
HEPES (4-(2-hydroxyethyl)-1- -piperazineethanesulfonic acid)	Roche Diagnostics, Mannheim
HMR1098	Sanofi-Aventis, Frankfurt
Medium199®	Biochrom, Berlin
N_2	Messer Griesheim, Krefeld
NaCN	Merck, Darmstadt
Ionomycin	Calbiochem, Bad Soden
KCN	Merck, Darmstadt
Penicillin/Streptomycin	Biochrom, Berlin
Resazurin	Sigma-Aldrich, Taufkirchen
Trypsin-EDTA	Invitrogen, Karlsruhe

2.2 Buffers

2.2.1 Buffers for cell cultivation

Standard Mixture	
Medium199®	9.8 g/l
HEPES	36 g/l
pH adjusted to **7.4** at 30 °C	

Medium for cell cultivation	
Medium199®/CO_2	
FCS	20 % (vol/vol)
Penicillin/Streptomycin	5 % (vol/vol)
pH adjusted to **7.4** at 5 % (vol/vol)CO_2	

Loading Buffer	
Medium199®/ HEPES	
FCS	2 %

All Buffers were sterile and stored at 4 °C.

2.2.2 Perfusion buffers

For the investigations several Perfusion Buffers were used. Their composition (in mM) is described bellow:

Normoxic HEPES-buffered Tyrode Buffer - pH adjusted to **7.4** at 30 °C	
NaCl	135
KCl	2.6
KH_2PO_4	1.2
$MgSO_4$	1.2
$CaCl_2$	1.3
HEPES	25

Anoxic HEPES buffered Tyrode Buffer – pH adjusted to **6.4** at 30 °C	
NaCl	135
KCl	2.6
KH_2PO_4	1.2
$MgSO_4$	1.2
$CaCl_2$	1.3
HEPES	25

Media were autoclaved in accordance with the method of Allshire et al. (1987). Before autoclaving, resazurin (1 % vol/vol) and cystein (5 mM) were added to the medium, which was then equilibrated with N_2, until a change in the colour of the medium appeared from violet to pink indicating a reduction of pO_2. Subsequently, the medium was autoclaved at 2 bars for 40 minutes after which the medium turned colourless indicating a pO_2<16 µPa.

3 METHODS

3.1 Isolation of coronary microvascular endothelial cells

Coronary microvascular endothelial cells were isolated from male Wistar rats which weighed approximately 200-300 g. The animals were bred in the experimental animal facility of the Institute of Physiology and had free access to the standard feed (Altromin) and had an unlimited access to water.

The coronary microvascular endothelial cells were isolated according to the method of Piper at al. (1982).

The following solutions were used:

Powell Medium (in mM) – pH adjusted to **7.4** by treating with carbogen	
NaCl	110
$NaHCO_3$	25
KCl	2.6
KH_2PO_4	1.2
$MgSO_4$	1.2
Glucose	11

Plating Medium	
Medium199®/CO_2	
FCS	20 %
Penicillin/Streptomycin	4 %

The animals were anaesthetised with ether and were euthanized by cervical dislocation. Subsequently, the thorax was opened, the pericardium, heart and lungs were surgically removed from the thoracic cavity simultaneously, and were immediately placed into an ice-cold isotonic NaCl solution. The tissue surrounding the heart was removed and discarded. The heart and its aorta were then attached to a Langendorff apparatus. To remove the remaining blood from the coronary blood vessels the heart was perfused with Powell medium (20 ml) for approximately two minutes. The tissue was perfused with the collagenase buffer (2-3 ml/min), for 30 minutes. During the perfusion, the medium was constantly warmed to a temperature of 37 °C and gassed with carbogen. At the end of the recirculating perfusion, the heart was removed from the Langendorff apparatus below the atria and was then mechanically minced.

The tissue was incubated for five minutes in recirculation buffer (37 ^{o}C) and gassed with carbogen. The dissociation of the endothelial cells was performed in this solution by pipetting up and down of the cell solution with a 5 ml pipette. The next step was filtration of the cell suspension through a nylon mesh (pore size 0,2 mm), in order to separate the remaining cell aggregates. The filtrate was then separated into two 50 ml Falcon tubes and then centrifuged at 25 x g for 3 minutes. The supernatant covering the cardiomyocite pellet was removed and then covered with trypsin and calcium. After two additional centrifugation steps the heart cell suspension was plated onto 10 cm cell culture plates and incubated with CO_2 for 4 hours. After incubation, the endothelial cells were washed and then covered with 30 ml medium for cell cultivation; medium was changed every 48 hours. After 7 days the cells became confluent and could be trypsinized. Prior to trypsinization, the cells were washed several times with Ca^{2+}-free HEPES-buffered Tyrode-buffer in order to remove the dead cells and other cellular debris. An additional advantage of this step is that Ca^{2+} was removed from the cell-cell communications sites and the sites between the cells, which simplifies the trypsinization. Subsequently, 5 ml of trypsin at 37 °C was added to the cells for 10 minutes. The cells were then pipetted up and down with a 1 ml pipette to break up any large clumps of cells. The solution was subsequently mixed with 125 ml plating medium (prewarmed to 37 °C) and the cell suspension was split into a series of 30 mm diameter culture dishes each containing a 25 mm diameter coverslip. This procedure was performed under sterile conditions and all the

instruments used in this process were previously sterilised. Three days later, a confluent monolayer of endothelial cells that had grown on the coverslip could be used for experiments; the number of cells on a coverslip was on average $2x10^5$ cells/cm^2.

3.2 Fluorescent microscopy measurements for determination of cytosolic Ca^{2+} concentration.

Experiments were performed with an inverse microscope, which was connected to the Till-Vision System. The excitation light sent from the UV-lamp can be modulated to the excitation spectrum of the Fura-2 AM fluorescent dye with the use of a monochromator. The selected wavelengths are 340, 360 and 380 nm for Fura-2 AM. The light is directed to a dichroic filter through a fiber optic cable. The dichroic filter then directs the light to the chamber with the loaded cell. Through this, the fura-2 AM is excited and the excitation light is directed through the dichroic filter, which then only lets light with a certain wavelength to pass through to the CCD-Camera. From there the image was detected and analysed on a computer.

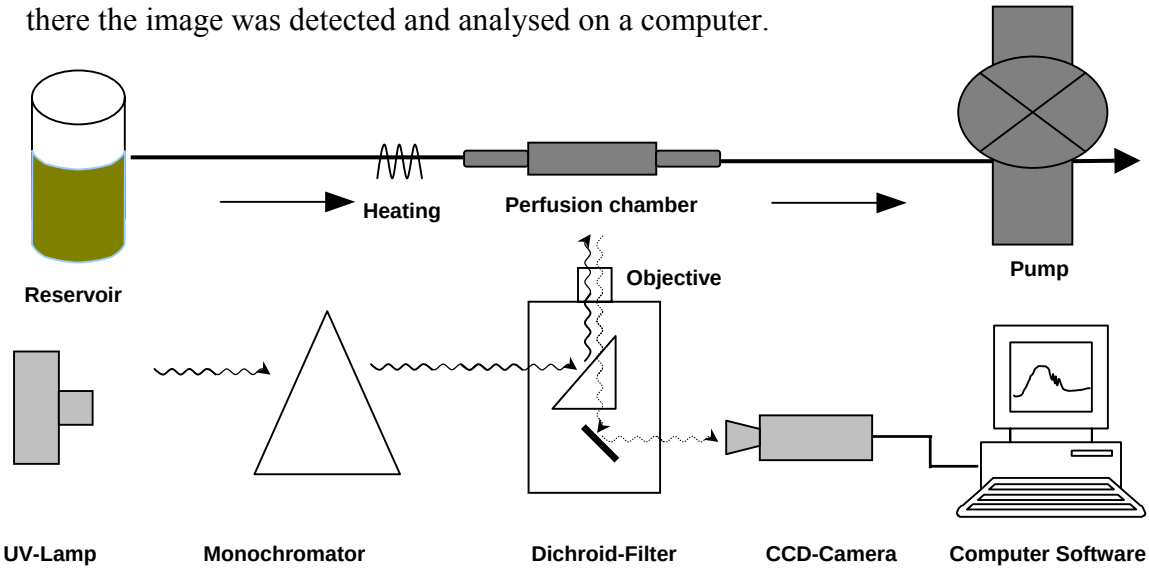


Fig.1 Graphic presentation of the experimental setup for measurement of cytosolic Ca^{2+}

3.2.1 Cell loading with Fura-2 AM and calibration of the signal

In order to determine the intracellular Ca^{2+} concentration, the cells were loaded for 90 minutes at room temperature with the fluorescent dye Fura-2 AM (2,5 µM) in HEPES buffered Medium 199. Fura-2 AM enters the cell as an acetoxymethylester where it is split by cellular esterases, thereby preventing it from permeating the cell membrane again and leaving the cell. The excitation of Fura-2 AM is done every 6 seconds with a wavelength of 340, 360 and 380 nm. The maximum emission was at 510 nm. The wavelength at 360 nm is the wavelenth where the fluorescence signal is independent of the Ca^{2+} concentration. For determination of the Ca^{2+} load, the ratio (R) between the wavelengths of 340 nm and 380 nm was used. (340 nm/380 nm). The free cytosolic Ca^{2+} concentration can be determined according to the following equation (Grynkiewicz at al. 1985):

$$[Ca^{2+}]_I = K_d \times b \times (R-R_{min})/(R_{max}-R)$$

Factor b is the ratio of the emission intensity at the excitation wavelength of 380 nm, corresponding to the R_{min} value and the emission intensity at the excitation wavelength of 380 nm, corresponding to the R_{max} value. The K_d value is a value for the affinity of the Fura-2 AM for free cytosolic Ca^{2+} ions. This value varies with the experimental conditions and is dependent on pH. Under *in vitro* conditions the K_d value of Fura-2 AM is 224 nM (Grynkiewicz et al. 1985). *In vivo*, Ladilov et al. (2000) determined a K_d value of 309 ± 8 nM (n=6) at intracellular pH 7.2, and a K_d value of 347 ± 7 nM (n=5) at intracellular pH 6.5.
For determination of the cytosolic Ca^{2+} concentration, the Fura-2 Ratio was calibrated with the K_d values determined by Ladilov et al. (2000).

Calibration solution	
NaCl	10 mM
KCl	125 mM
$MgSO_4$	1 mM
HEPES	25 mM
Ionomycin	5 µM
$CaCl_2$ or EGTA	3 mM or 5 mM
BAPTA	10 µM
pH adjusted to **7.4** with 1N KOH	

For determination of the R_{max} value (maximal ratio), 3M $CaCl_2$ was added to the perfusate, and for the determination of the R_{min} value (minimal ratio) 5 mM EGTA and 10 µM BAPTA were added to the perfusate.

3.2.2 Determination of the size of intercellular gaps.

In order to determine the formation of intercellular gaps it was necessary to determine a wavelength by which the fluorescent signal is independent of the intracellular Ca^{2+} concentration. Therefore the spectrum of wavelengths was measured at high and low Ca^{2+} concentrations and the appropriate wavelength was determined when the intensity of the signal did not change. The isosbestic value, or the value where the emission intensity of Fura-2 AM is independent of the Ca^{2+} concentration, is 360 nm.

The original sequence was binarized, i.e. pixels with Fura-2 AM emission intensity were set to “1”, whereas pixels without emission intensity were set to “0”. Pixels marked with “0” represent cell-free area (no emission intensity), whereas pixels marked with “1” represent cell-covered areas (emission intensity). These conditions were introduced to the software programme (Till Imago), which then calculates “1” pixels and “0” pixels. The region for intercellular gap determination was chosen in such a manner that at the very beginning of the experiment the size of the intercellular gaps was 6000-8000 pixels. (0). This value increased typically after 40 minutes of ischemia (pH 6.4) to 45000-55000 pixels. During reperfusion intercellular gaps increase further and with it the number of the pixels whose value was determined to be 0. With this procedure it is possible to determine the increase of intercellular gaps

compared to the size of the cells. For the analysis the number of 0 pixels during 10 minutes of normoxia at the beginning of the experiment was set to 100 %. The change in the size of the intercellular gaps during ischemia and reperfusion was then expressed as a percent compared to the normoxic value.

3.3 Determination of membrane potential with the fluorescent dye $DiBAC_4(3)$

The fluorescent dye $DiBAC_4(3)$, used for the measurement of membrane potential, binds to cellular and membrane proteins, where during hyperpolarisation of the cell membrane the dye leaves the cell and the fluorescence decreases. The dye enters depolarised cells, where it binds to the hydrophobic sites of intracellular proteins or membranes, and exhibits enhanced fluorescence and red spectral shifts. Increased depolarisation results in more influx of the anionic dye that binds to proteins resulting in increase in fluorescence. Conversely, hyperpolarisation is indicated by a decrease in fluorescence. The cells were loaded with $DiBAC_4(3)$ (1 μM) in a normoxic perfusion medium for 15 minutes and then used for ischemia and reperfusion experiments. The excitation wavelength of $DiBAC_4(3)$ is 484 nm, and the emission wavelength is 520 nm. Potential-dependent fluorescence changes generated by $DiBAC_4(3)$ are typically ~1 % per 1 mV (Scott et al. 2004).

3.4 Equipment

The cells were introduced into a gas tight perfusion chamber. The perfusion chamber has the same diameter as the coverslip and has two additional rubber rings. The rings limit the possibility that air can get into the chamber and prevent the coverslip from breaking while the perfusion chamber is assembled. The chamber is assembled by tightly screwing the upper part to the lower part. In this manner the monolayer in the chamber can be anoxically perfused using stainless steel capillaries connecting the perfusion chamber to bottles filled with normoxic or anoxic medium. A peristaltic pump attached to the chamber limits the flow of the medium through the perfusion system to a velocity of 0.5 ml/min.

The normoxic medium was aerated before and during the experiments with air, and the anoxic medium with N_2 (100 %). The temperature in the perfusion chamber was

adjusted to 30 °C. The partial O_2 pressure in the anoxic medium was determined with a polarographic Oxygen sensor directly behind the perfusion chamber and its value was less than 1 mmHg.

3.5 Experimental Protocol

The experimental protocol was set in a manner to simulate ischemia and reperfusion. All experiments started after a 10 minutes long equilibration phase in normoxic medium at pH of 7.4 without glucose. In order to simulate ischemia and reperfusion, the cells were then perfused for 40 minutes with an anoxic HEPES buffered tyrode buffer ($p0_2$<16 μPa), glucose free and with a pH of 6.4. Subsequently, the cells were reoxygenated for 30 minutes with a medium at pH of 7.4 and with 2.5 mM glucose. Pharmacological opening of K_{ATP} channels was performed with diazoxide (1 μM). During ischemia and reperfusion K_{ATP} channels were inhibited with two chemically distinct sulphonylurea derivatives: glybenclamide (10 μM; Fig.2) or HMR1098 (100 μM; Fig.3). While glybenclamide also shows inhibitory effects in ß -cell plasma-membrane K_{ATP} channels, recent advances in sulphonylurea chemistry have lead to development of tissue specific plasmalemmal K_{ATP} channel inhibitors like HMR1098 which displays selectivity for the cardiac *versus* ß -cell plasma-membrane K_{ATP} channels (Gögelein et al. 1999, Hu et al. 1999).

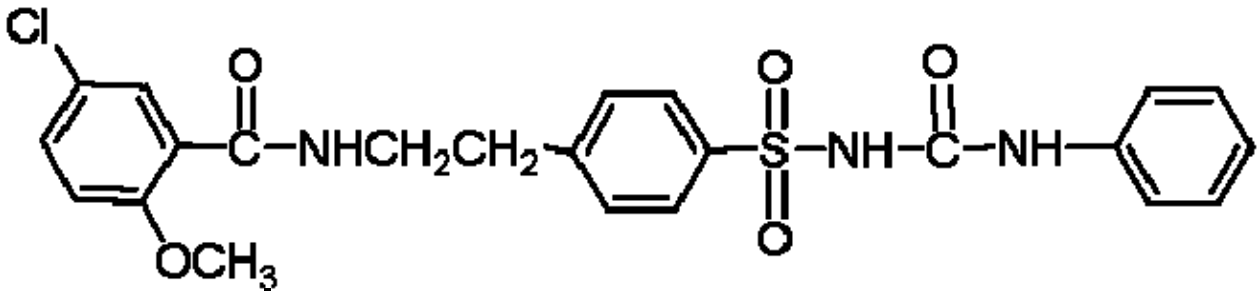


Fig.2 Chemical structure of Glybenclamide

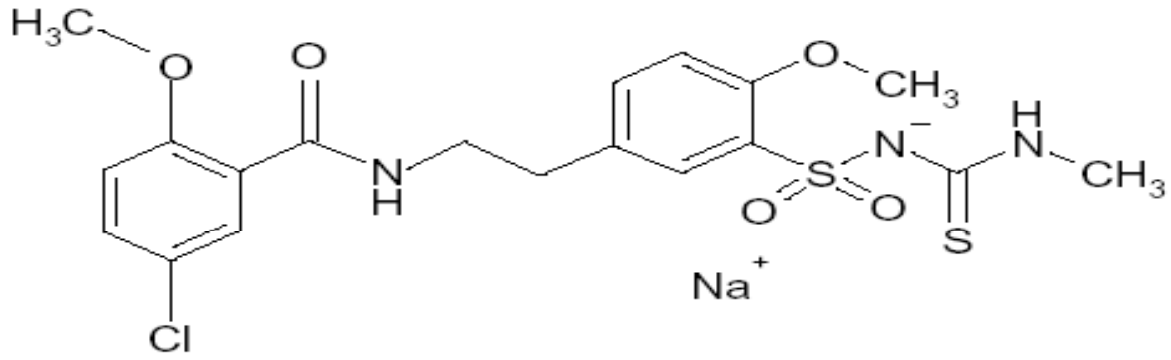
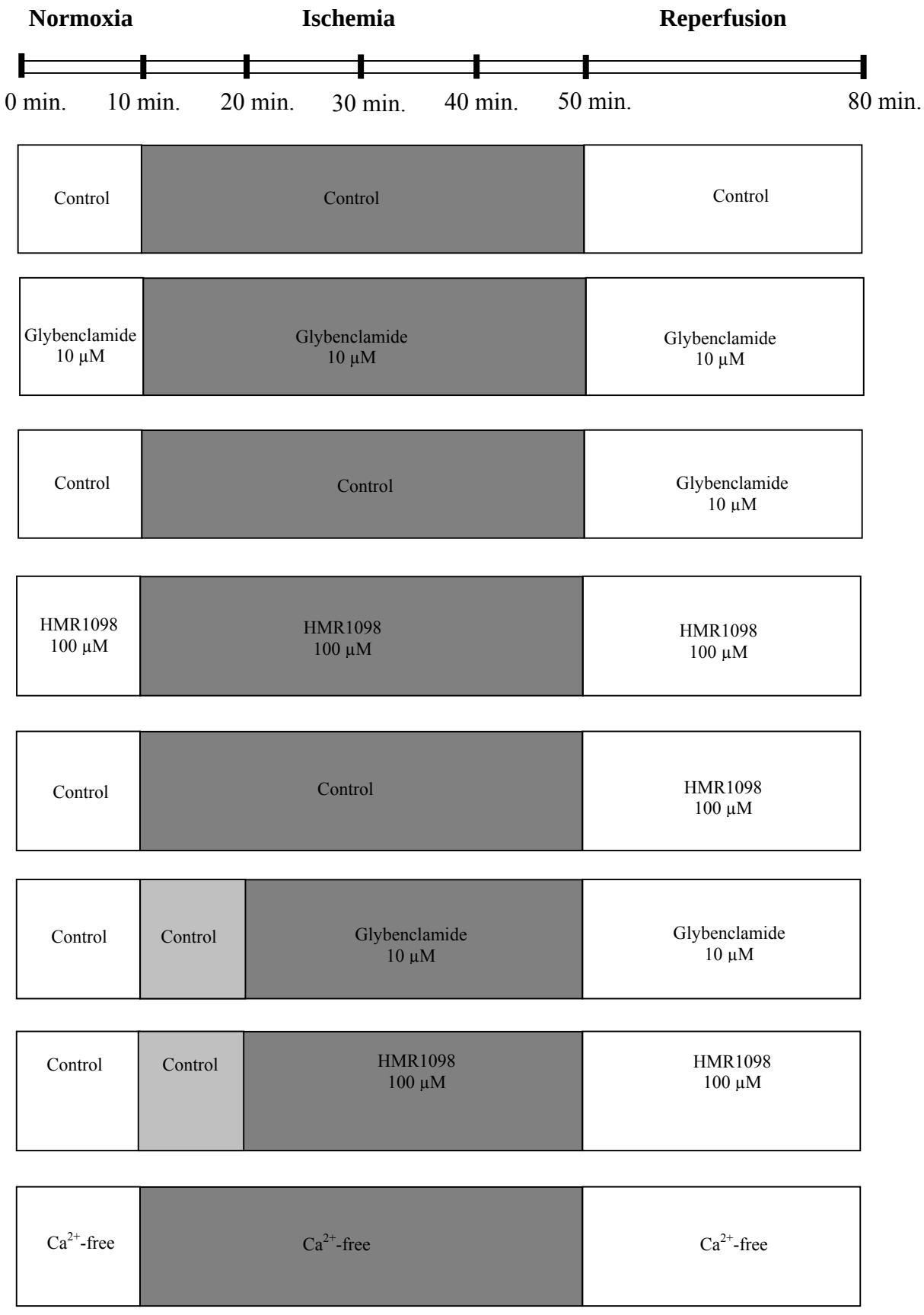


Fig.3 Chemical structure of HMR1098

3.6 Interventions in the protocol:



3.7 Statistical analysis

The data are presented as mean values ± SEM. The statistical analysis was performed by one-way ANOVA and use of the Student-Newman-Keuls-Test for post-hoc analysis (Ludbrook, 1994). The p-values $p \leq 0.05$ were considered as statistically significant.

4 RESULTS

4.1 Effects of pharmacological opening and subsequent inhibition of K_{ATP} channels on membrane potential and cytosolic Ca^{2+} load

The aim was to determine the concentration that would induce membrane hyperpolarisation through the opening of K_{ATP} channels. Experiments were performed using the K_{ATP} channel opener diazoxide in a concentration dependent manner. Fig.4 shows a representative single-cell measurement of membrane potential with $DiBAC_4(3)$. DiBAC fluorescence is shown in relative terms, where a downward direction indicates hyperpolarisation, and an upward direction indicates depolarisation. The K_{ATP} channel opener diazoxide was added after 10 minutes of normoxic perfusion in concentrations of 0.1 μM or 1 μM. While perfusion with 0.1 μM of diazoxide had no effect, perfusion with 1 μM induced a sustained hyperpolarisation of the plasmalemma.

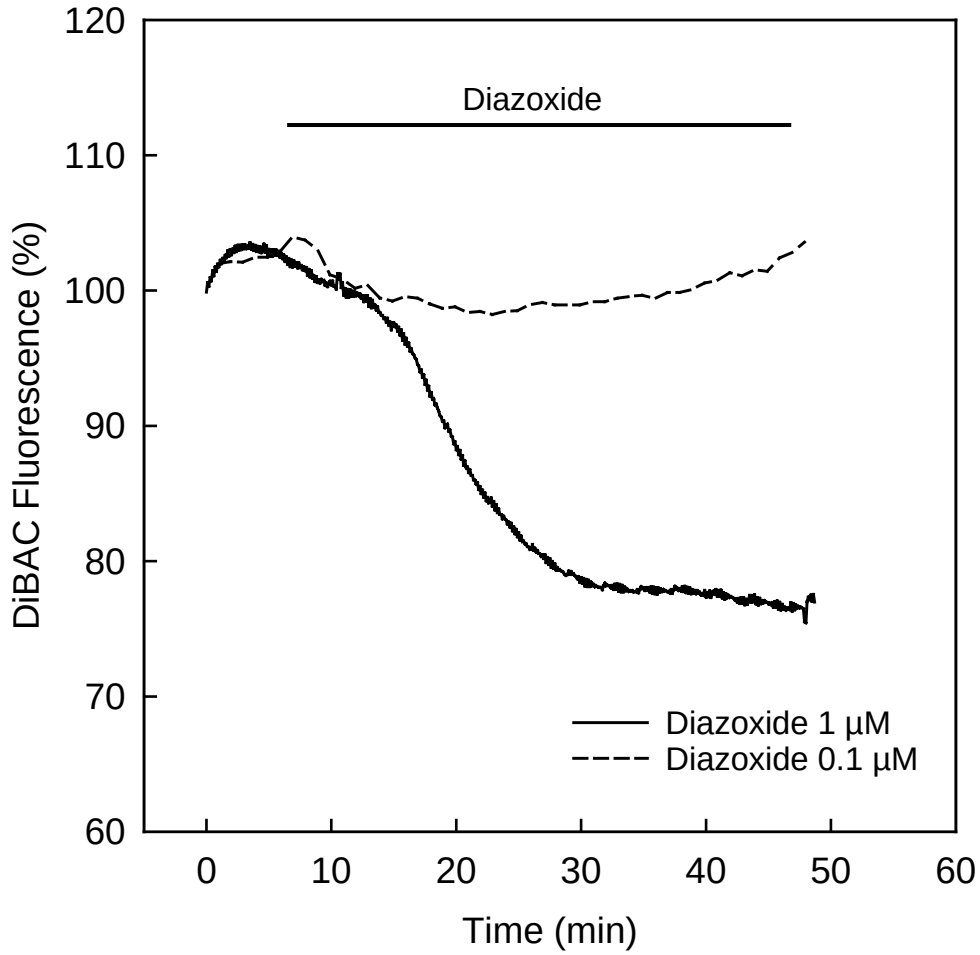


Fig.4 Representative single-cell measurement of DiBAC Fluorescence (%) during 50 minutes of normoxic perfusion with diazoxide (0.1 µM and 1 µM). Diazoxide was added after 10 minutes of perfusion.

The next step was to investigate the optimal concentration of the K_{ATP} channel inhibitors, which would reduce the diazoxide induced hyperpolarisation of the plasmalemma. Two K_{ATP} channel inhibitors, glybenclamide and HMR1098 were tested. Concentrations of 0.1 µM for glybenclamide (76.9 %; SEM 2.4 %), and 10 µM for HMR1098 (85.3 %; SEM 2.3 %) did not inhibit the diazoxide induced hyperpolarisation of the plasmalemma. 10 µM of Glybenclamide and 100 µM of HMR1098 were required to reverse the diazoxide induced hyperpolarisation of the plasmalemma. The results of a series of experiments are shown in Fig.5:

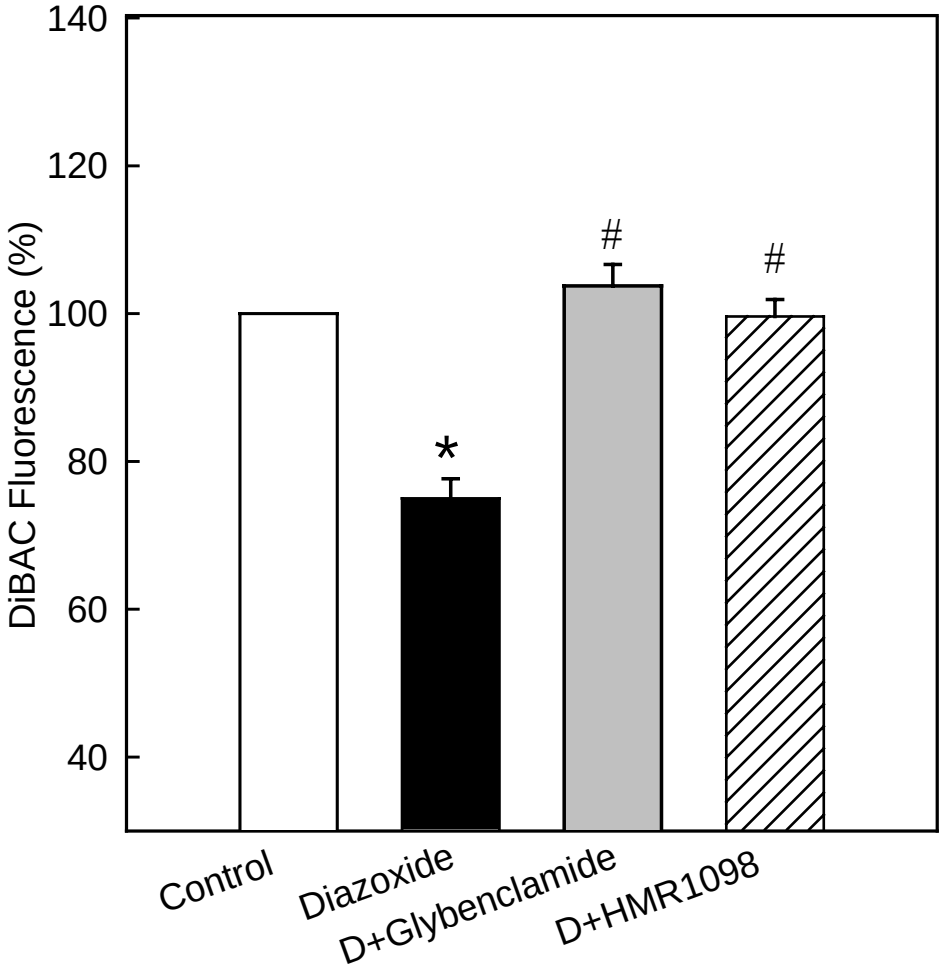


Fig.5 DiBAC fluorescence (%) at the end of 50 minutes normoxic perfusion compared to control. Cells were perfused with 1 μM diazoxide, and with glybenclamide (10 μM) or HMR1098 (100 μM). *P<0.05 vs. control; #P<0.05 vs. diazoxide 1 μM. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments).

The next experiment was to determine if the diazoxide induced hyperpolarisation of the plasmalemma influences the cytosolic Ca^{2+} load of the endothelial monolayers. After initial 10 minutes of normoxic perfusion, the cells were perfused with the K_{ATP} channel opener diazoxide (1 μM) and simultaneously perfused with different concentrations of the K_{ATP} channel inhibitors glybenclamide or HMR1098. Perfusion of the coronary endothelial monolayers with glybenclamide at a concentration of 0.1 μM or HMR1098 at a concentration of 10 μM did not inhibit the diazoxide induced Ca^{2+} overload. Concentrations of 10 μM for glybenclamide and 100 μM for HMR1098 were required to reduce the diazoxide-induced Ca^{2+} overload. The statistical analysis of two subsequent series of experiments is presented in Figures 6 and 7:

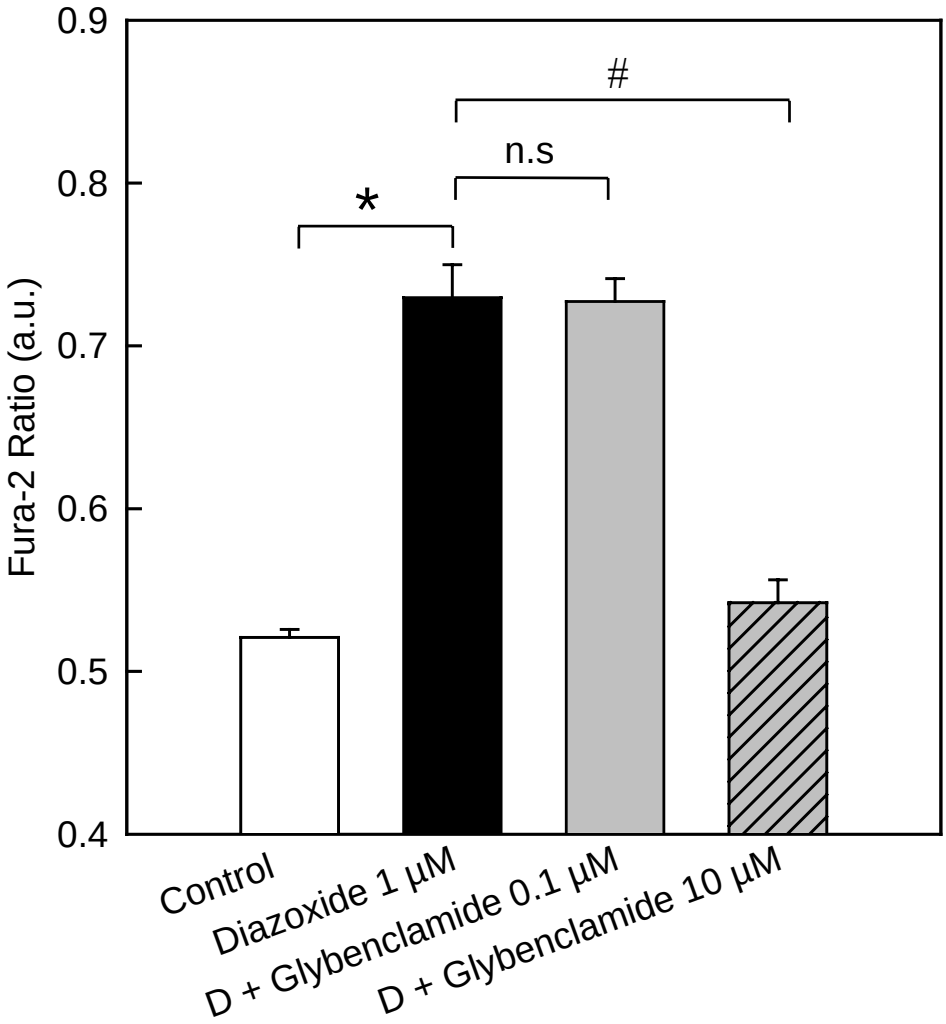


Fig.6 Fura-2 Ratio (a.u.) after 50 minutes of normoxic perfusion with 1 μM diazoxide, and simultaneous perfusion with diazoxide and different concentrations of glybenclamide (0.1 μM or 10 μM). Diazoxide and glybenclamide were added after 10 minutes from beginning of experiments. *P<0.05 vs. control; $^{\#}$P<0.05 vs. diazoxide 1 μM; n.s. (not significant) vs. diazoxide 1 μM. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments).

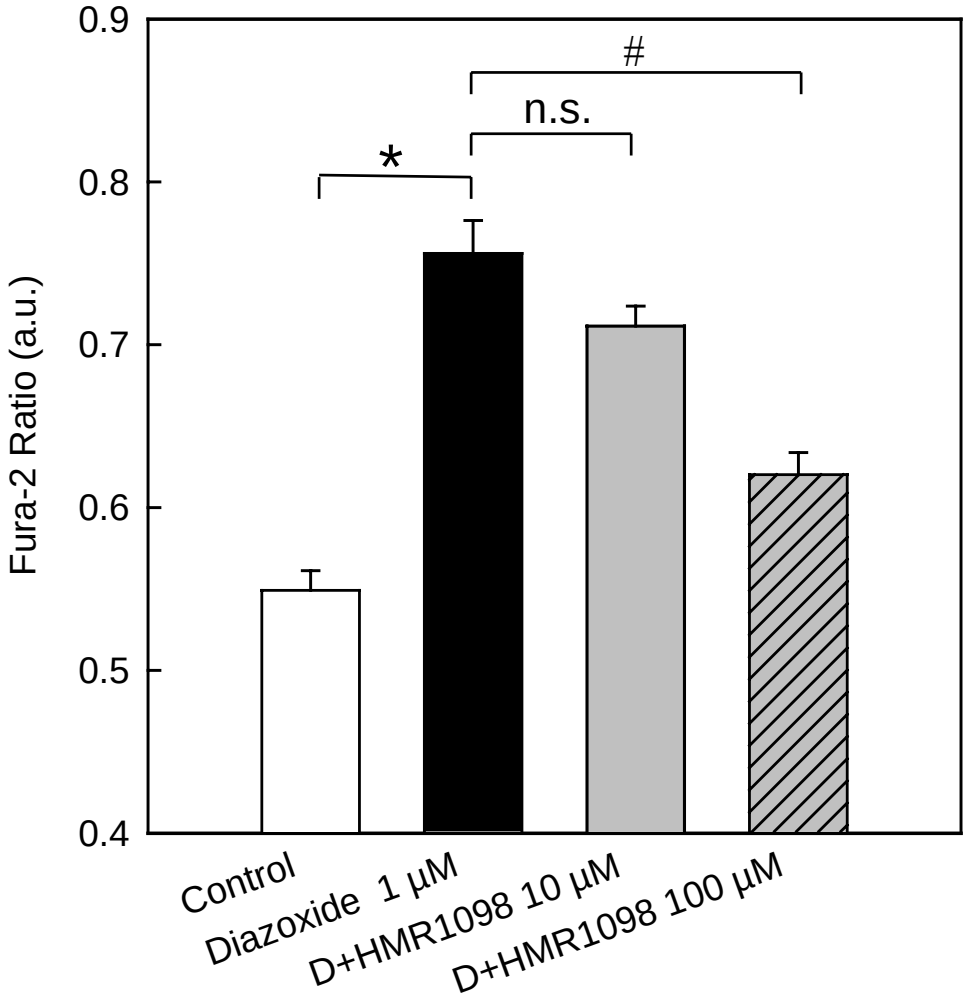


Fig.7 Fura-2 Ratio (a.u.) after 50 minutes of normoxic perfusion with 1 µM diazoxide, and simultaneous perfusion with diazoxide (1 µM) and different concentrations of HMR1098 (10 µM or 100 µM). Diazoxide and HMR1098 were added after 10 minutes of perfusion. $^{\#}P<0.05$ vs. diazoxide 1 µM; *P<0.05 vs. control; n.s. (not significant) vs. diazoxide 1 µM. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments).

4.2 Influence of ischemia and reperfusion on membrane potential

Figure 8 shows a representative single-cell measurement of membrane potential with $DiBAC_4(3)$ during ischemia and reperfusion in absence or presence of glybenclamide (10 μM). During ischemia, there is a decrease of $DiBAC_4(3)$ fluorescence indicating a hyperpolarisation of the plasmalemma. The $DiBAC_4(3)$ fluorescence returns to a normoxic level during reperfusion conditions, indicating a repolarisation. When the cell was perfused with glybenclamide, the ischemia-induced hyperpolarisation was reduced. In reperfusion, the membrane potential returns to normoxic level and is not different from control conditions.

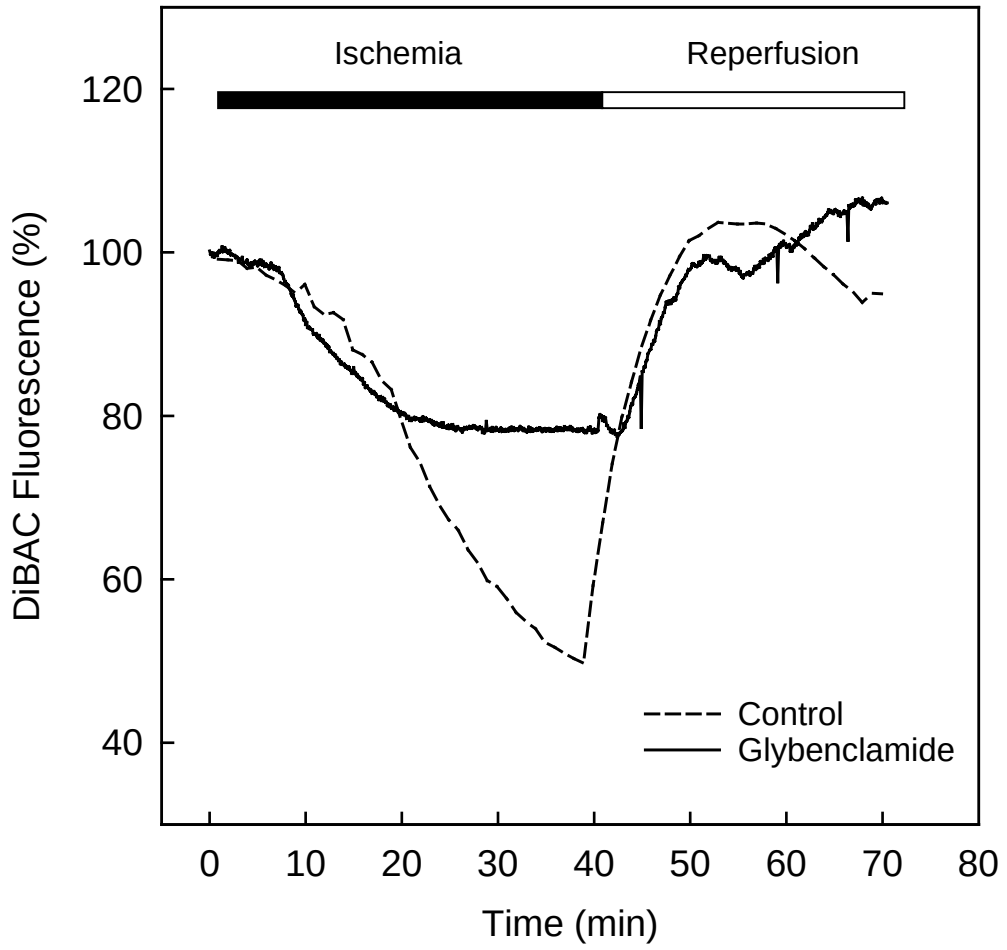


Fig.8 Single-cell measurement of DiBAC Fluorescence during ischemia and reperfusion under control conditions (dashed line) and under perfusion with glybenclamide (10 μM).

4.3 Influence of ischemia and reperfusion on cytosolic Ca^{2+} load and intercellular gap formation

Figure 9 shows a representative measurement of the Fura-2 Ratio. In the first 10 minutes of equilibration there is no increase in the cytosolic Ca^{2+} concentration. During the following 40 minutes of ischemic perfusion of the cells, a biphasic increase in cytosolic Ca^{2+} takes place. The first peak, which lasts approximately 10 minutes after onset of ischemia, is caused by the release of Ca^{2+} from the endoplasmatic reticulum. The next, more gradual increase is caused by entry of Ca^{2+} from the extracellular space (Adams et al. 1989, Putney 1990, Dolor et al. 1992, Ladilov et al. 2000, Schäfer et al. 2001). Ischemia was followed by reperfusion. During this phase, the cytosolic Ca^{2+} concentration increases further.

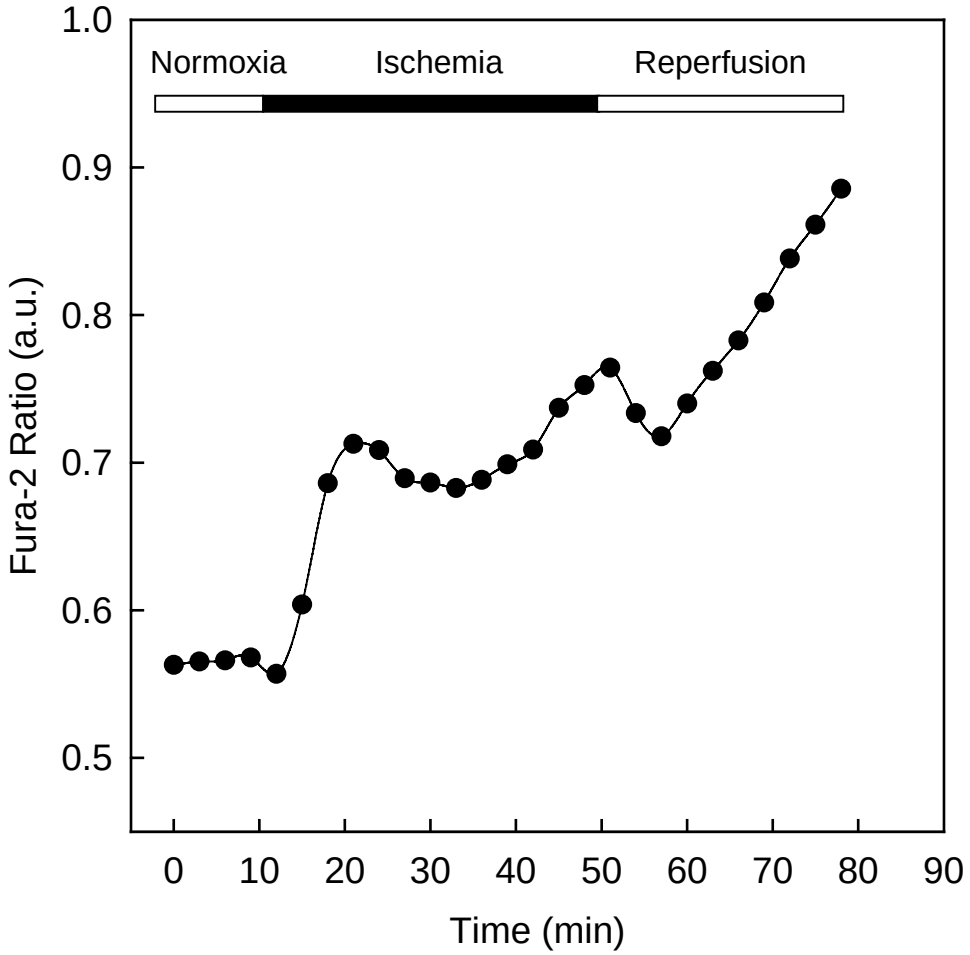


Fig.9 Representative single-cell recording of Fura-2 Ratio (a.u.). First 10 minutes are normoxic perfusion, the subsequent 40 minutes are ischemic perfusion, followed by 30 minutes reperfusion.

The other parameter measured was the formation and increase of intercellular gaps. Figure 10 shows a representative intercellular gap measurement during ischemia and reperfusion. The gaps begin forming with the onset of ischemia, and they further increase in reperfusion. It is important to notice that the increase of intercellular gaps is larger during reperfusion compared to ischemia. Intercellular gap formation was analysed as a percent of the normoxic level. The increase in intercellular gap formation reaches approximately 100 % at end of ischemia, and increases by additional 100 % in the next 30 minutes of reperfusion.

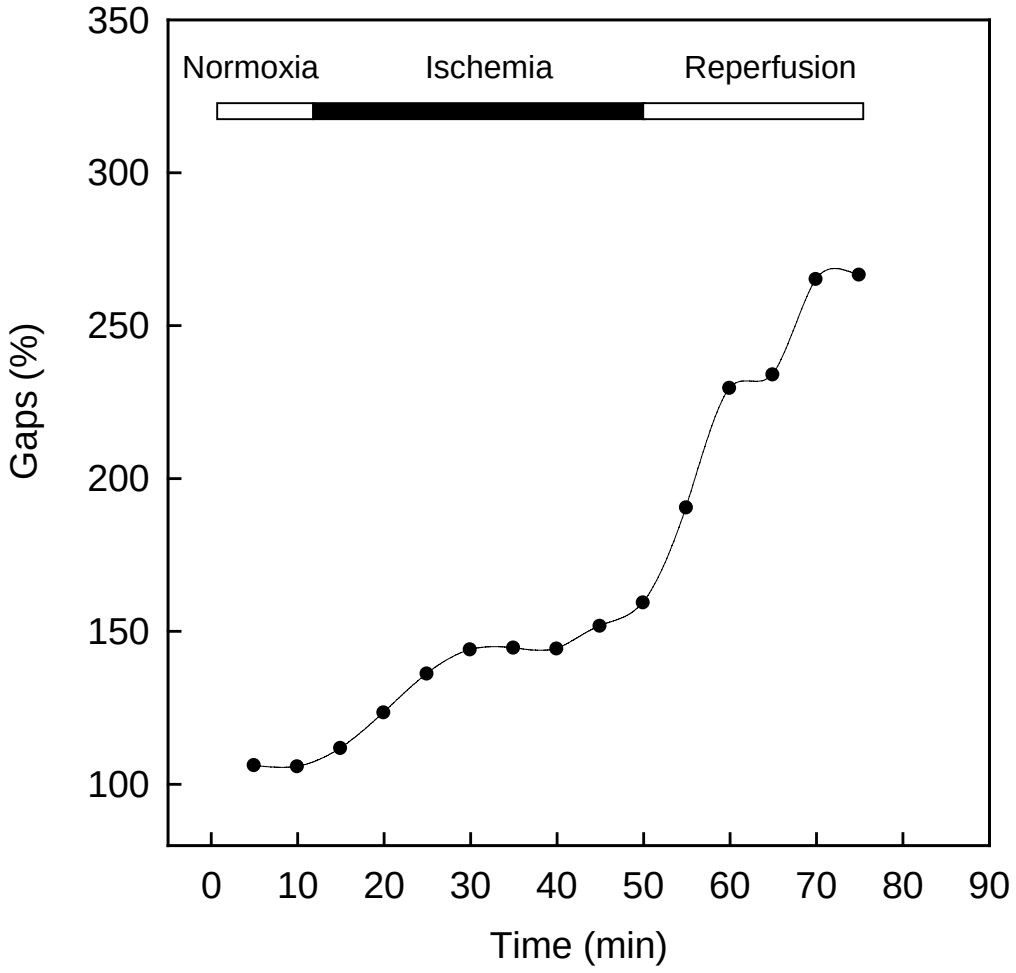


Fig.10 Analysis of intercellular gap formation during ischemia and reperfusion from a single experiment. During the first 10 minutes of normoxia there is no increase of intercellular gaps. The following 40 minutes of ischemia induce an intercellular gap increase of approximately 100 % and further 100 % increase during the subsequent reperfusion.

4.4 Influence of extracellular Ca^{2+} influx on the initial Ca^{2+} release during ischemia and reperfusion.

Cytosolic Ca^{2+} release during ischemia and reperfusion is characterised by a biphasic rise. The first, steep release is due to Ca^{2+} release from the endoplasmatic reticulum. After the initial rise, cytosolic Ca^{2+} rises further, due to Ca^{2+} influx from the extracellular space. However, when coronary microvascular endothelial cells are perfused under Ca^{2+}-free conditions, besides the expected reduction of the second Ca^{2+} increase during ischemia there is also reduction of the initial Ca^{2+} release. The results of a series of experiments where endothelial monolayers were perfused under Ca^{2+}-free conditions are showed in Fig.11.

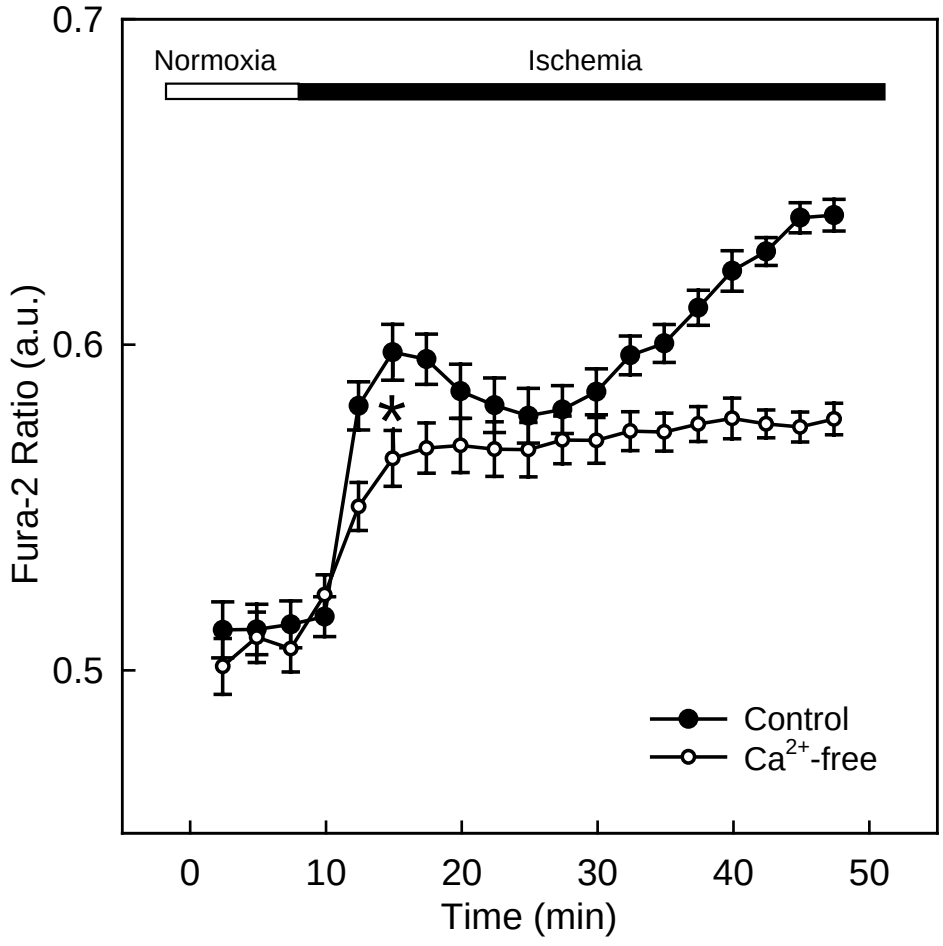


Fig.11 Time course of Fura-2 Ratio (a.u.) during ischemia under control conditions and under Ca^{2+}-free perfusion. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments). *P<0.05 vs. ischemia.

4.5 Membrane potential of coronary endothelial cells during ischemia and reperfusion and the role of K_{ATP} channels

The membrane potential of the coronary microvascular endothelial cells was measured using the fluorescent dye $DiBAC_4(3)$ (Fig.12). The statistical analysis of experiments during control conditions shows a reduction in hyperpolarisation during ischemia when cells were perfused with glybenclamide and HMR1098 throughout the entire experiment. During reperfusion, the $DiBAC_4(3)$ fluorescence returns to normoxic value.

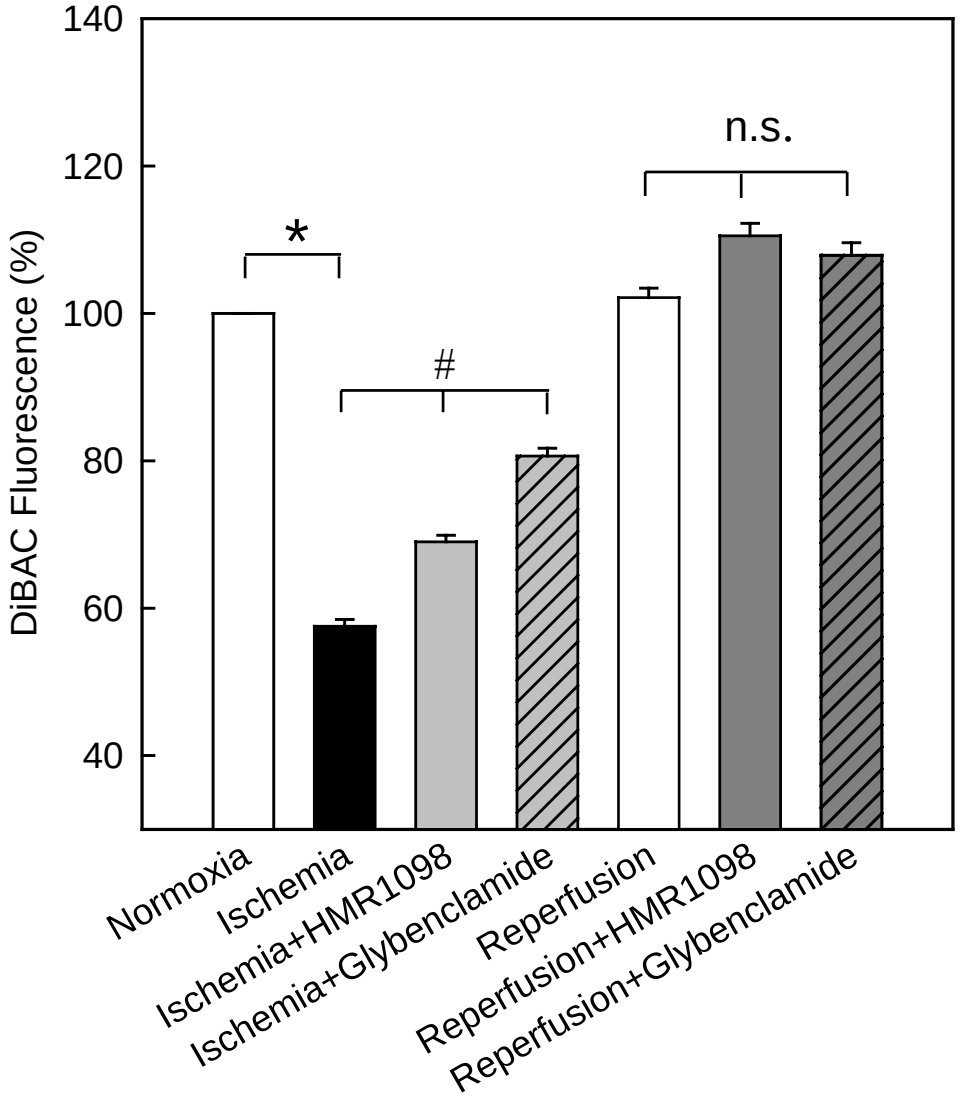


Fig.12 $DiBAC_4(3)$ Fluorescence (in % compared to normoxic control), after 40 min. ischemia with or without HMR 1098 or glybenclamide and after 30 min. reperfusion with or without HMR1098 or glybenclamide. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments). *P<0.05 vs. normoxia; #P<0.05 vs. ischemia; n.s. (not significant) vs. reperfusion.

4.6 Influence of K_{ATP} channels on cytosolic Ca^{2+} load during ischemia and reperfusion

To investigate the influence of K_{ATP} channels on the Ca^{2+} homeostasis of endothelial monolayers, the K_{ATP} channel inhibitor glybenclamide was used at concentration of 10 μM. The microvascular coronary endothelial monolayers were perfused with glybenclamide during the whole experiment. In the presence of glybenclamide the fura-2 Ratio was reduced, indicating a reduction of ischemia-induced cytosolic Ca^{2+} overload (Fig.13a).

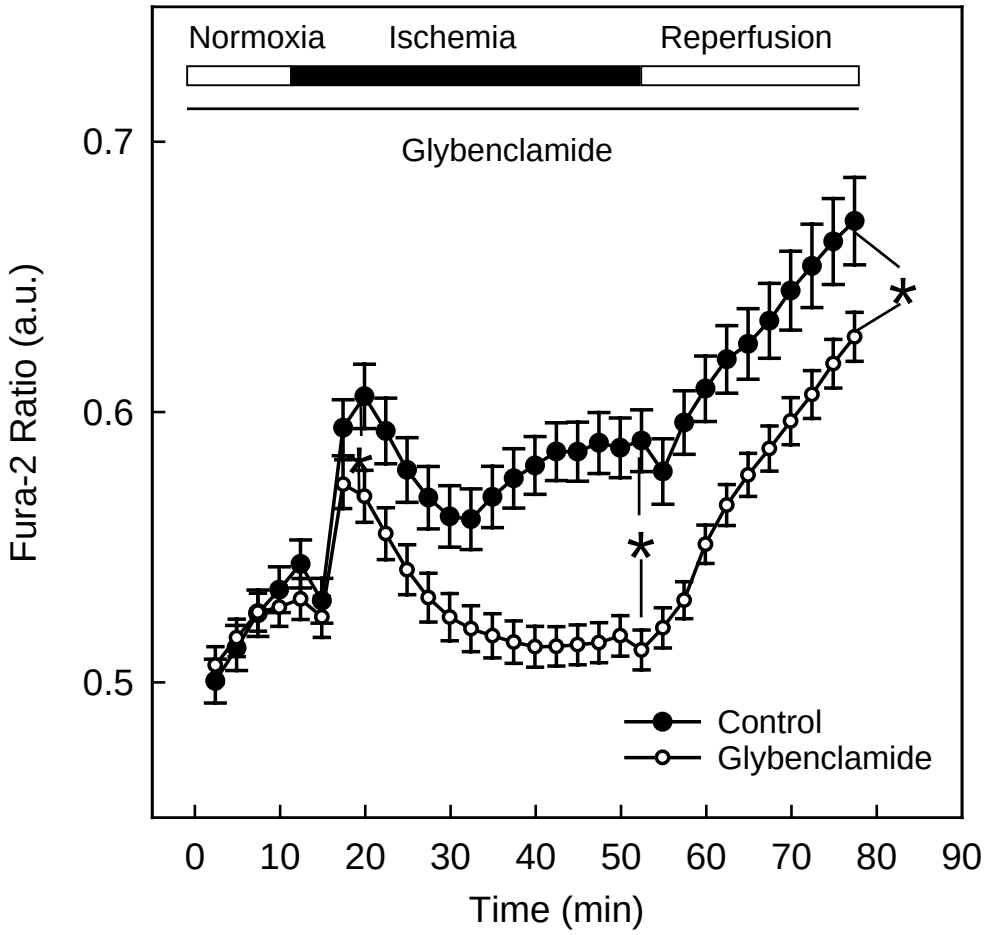


Fig.13a Time course of Fura-2 Ratio (a.u.) during ischemia and reperfusion under control conditions and under perfusion with glybenclamide (10 μM) throughout the entire experiment. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); *P<0.05 vs. control.

There is also a clear reduction formation of intercellular gaps in experiments in which the cells were perfused with glybenclamide compared to control experiments (Fig.13b).

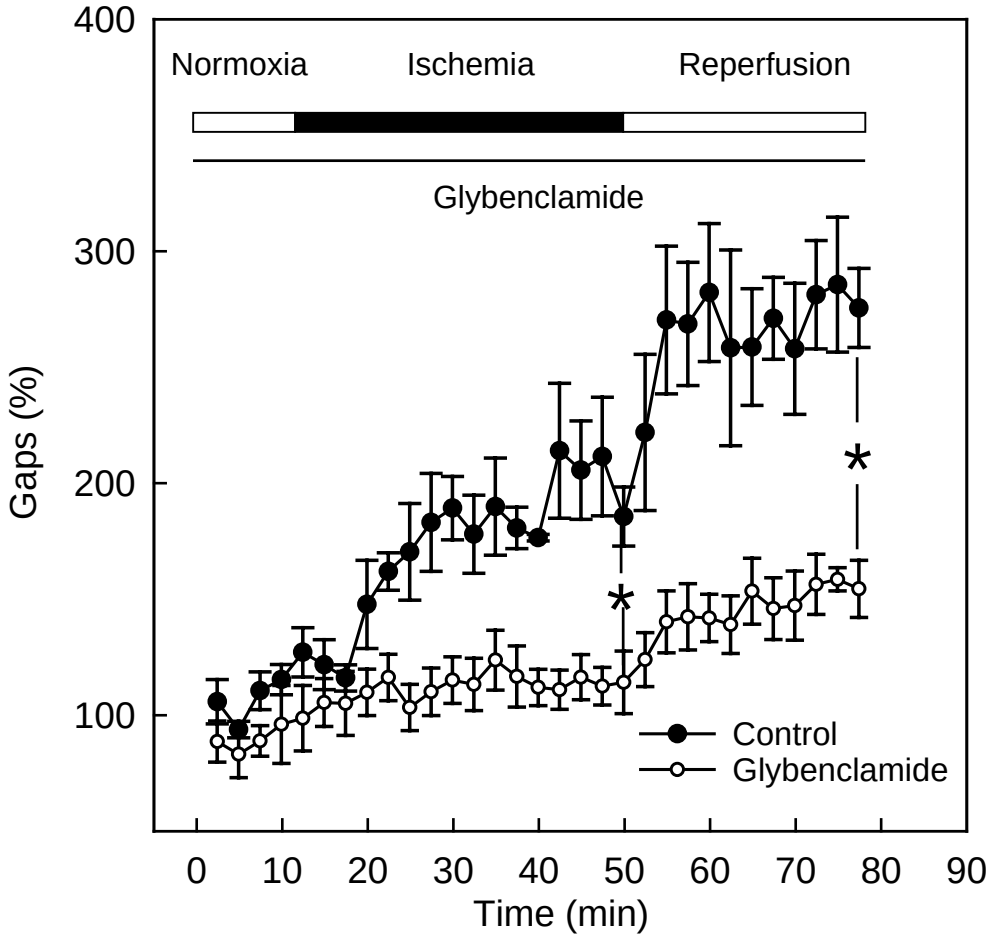


Fig.13b Time course of intercellular gap formation during ischemia and reperfusion under control conditions and under perfusion with glybenclamide (10 μM) throughout the entire experiment. Data are presented as mean values ± SEM; (n=160 cells from 6 experiments); *P<0.05 vs. control.

A chemically distinct K_{ATP} channel inhibitor, HMR1098, was used to further confirm that the plasmalemmal K_{ATP} channels influence the Ca^{2+} load during ischemia and reperfusion: HMR1098 acts specifically on the plasmalemmal K_{ATP} channels.

HMR1098 was used in concentration of 100 μM and the cells were also perfused with HMR1098 during the whole experiment. Figure 14a shows the influence of HMR1098 on the cytosolic Ca^{2+} load during ischemia and reperfusion compared to control experiments.

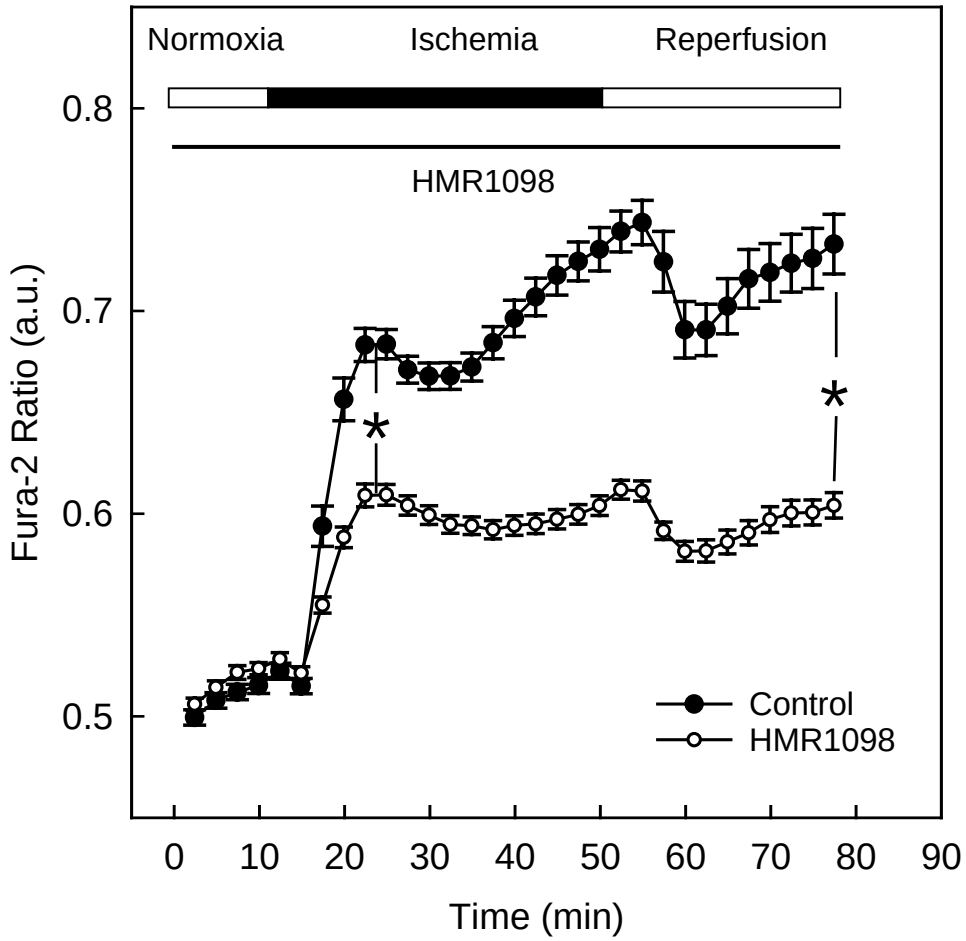


Fig.14a Time course of Fura-2 Ratio (a.u.) during ischemia and reperfusion under control conditions and under perfusion with HMR1098 (100 μM). Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); *P<0.05 vs. control.

When the formation and increase of intercellular gaps was analysed, similar results were observed. Namely, there is a clear reduction in the increase of intercellular gaps in the experiments in which the endothelial monolayers were perfused with HMR1098, compared to control experiments (Fig.14b).

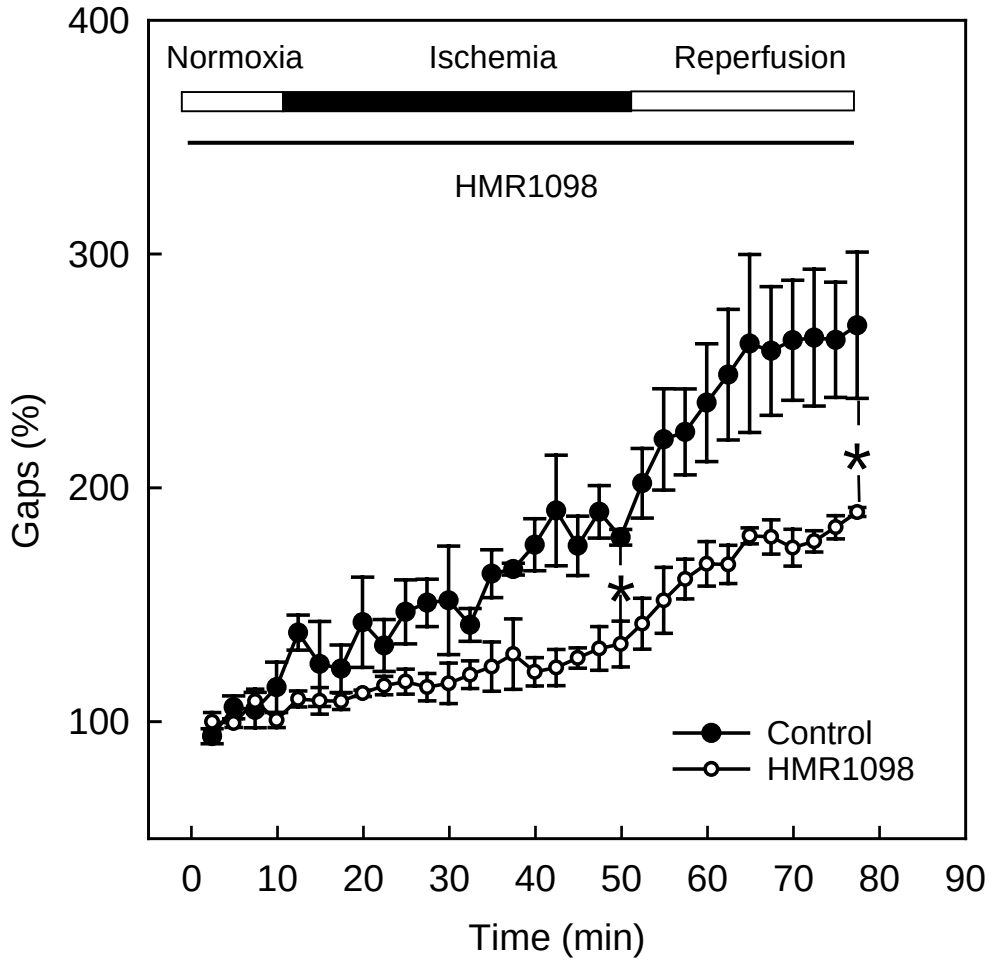


Fig.14b Time course of intercellular gap formation during ischemia and reperfusion under control conditions and under perfusion with HMR1098 (100 μM). Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); *P<0.05 vs. control.

4.7 Influence of K_{ATP} channel inhibitors on the initial Ca^{2+} release during simulated ischemia

The perfusion of the endothelial monolayers with the K_{ATP} channel inhibitors glybenclamide or HMR1098 was performed from the beginning of ischemia and reperfusion. As the results of the previous experiments show, both glybenclamide and HMR1098 reduce the initial Ca^{2+} rise during ischemia. In order to investigate if K_{ATP} channel inhibitors also influence the initial Ca^{2+} rise during ischemia, experiments were performed in which coronary microvascular endothelial monolayers were treated with glybenclamide or HMR1098 from the time point the initial Ca^{2+} release can no longer be pharmacologically influenced. In ischemia and reperfusion experiments, this was minute twelve of ischemia. In these experiments the initial Ca^{2+} rise was unaltered. The second Ca^{2+} increase, however is reduced in presence of K_{ATP} channel inhibitors. Parallel to the Ca^{2+} measurements, analysis of intercellular gap formation was also performed. The results of the planimetric analysis of the performed experiments revealed a clear reduction of intercellular gap formation during ischemia and reperfusion, when glybenclamide or HMR1098 were added from the twelfth minute of ischemia. Figures 15a and 15b show the statistical analysis, where the influence of glybenclamide on cytosolic Ca^{2+} (Fig. 15a), and on formation of intercellular gaps (Fig. 15b) was analysed, where the endothelial monolayers were perfused with glybenclamide from the peak of the initial Ca^{2+} release during ischemia until end of experiments.

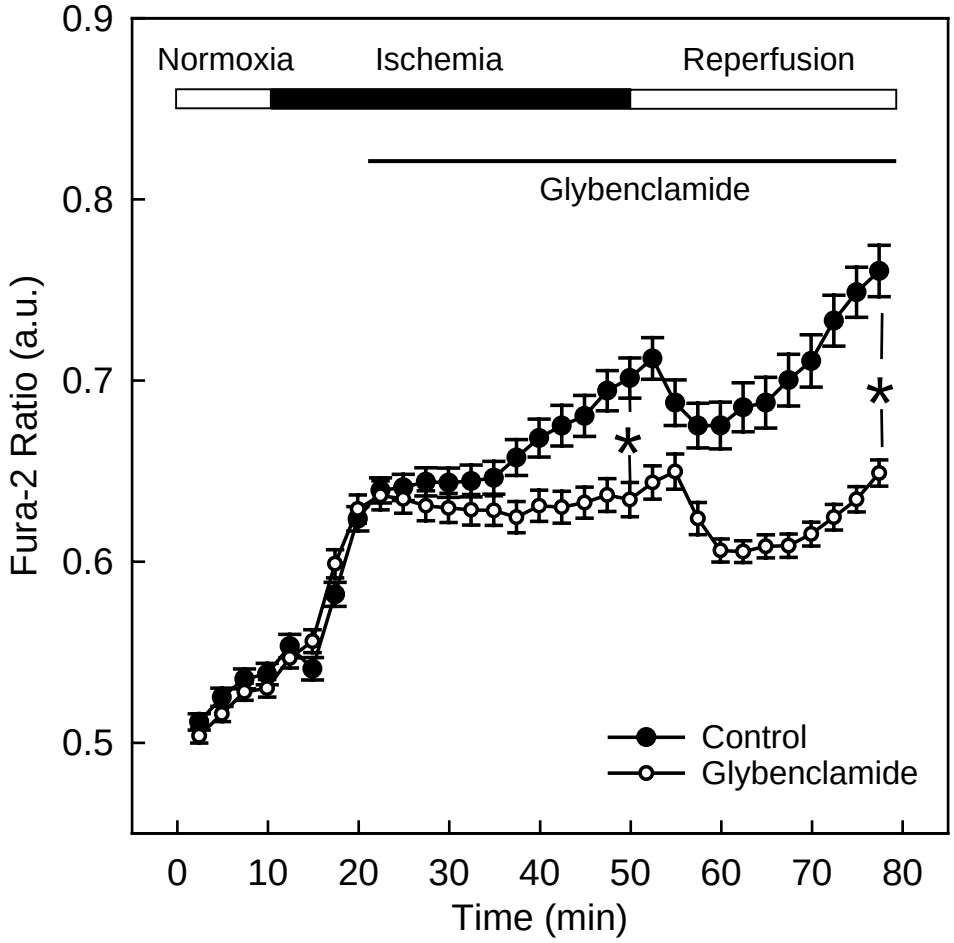


Fig.15a Time course of Fura-2 Ratio (a.u.) during ischemia and reperfusion under control conditions, and under perfusion with glybenclamide added from the twelfth minute of ischemia. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); *P<0.05 vs. control.

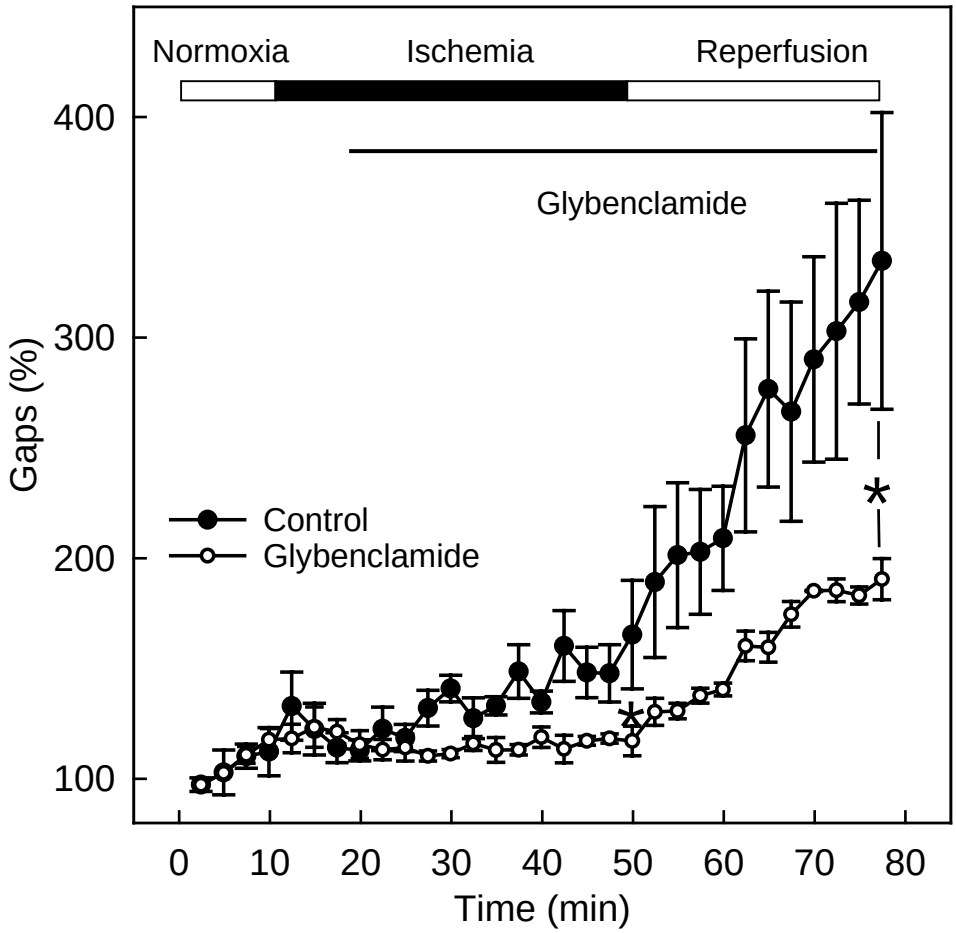


Fig.15b Time course of gap intercellular formation during ischemia and reperfusion under control conditions, and under perfusion with glybenclamide added at the twelfth minute of ischemia. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); *P<0.05 vs. control.

Experiments were also performed where the coronary microvascular endothelial monolayers treated with the chemically distinct K_{ATP} channel inhibitor, HMR1098. In this series of experiments HMR1098 was also added from the twelfth minute of ischemia. The results further confirm that K_{ATP} channel inhibitors influence the initial Ca^{2+} release during ischemia. Fig.16a shows the statistical analysis where the influence of HMR1098 on the cytosolic Ca^{2+} (Fura-2 Ratio) was investigated when HMR1098 was added from the twelfth minute of ischemia.

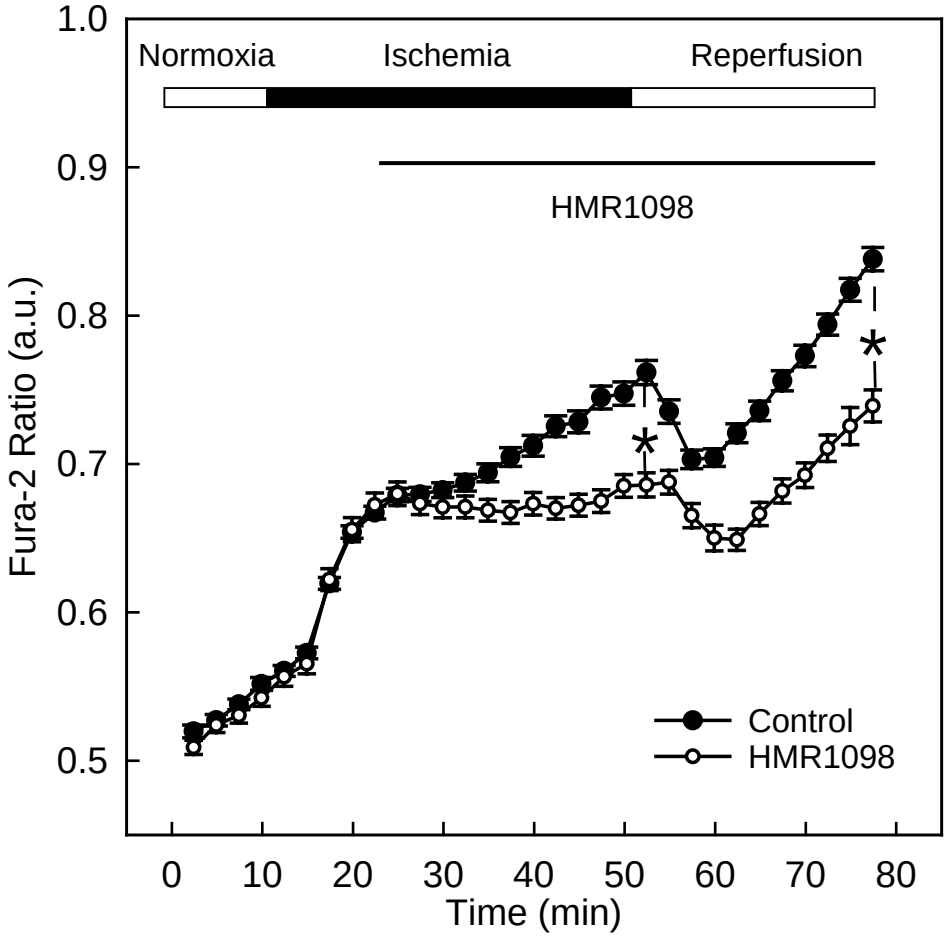


Fig.16a Time course of Fura-2 Ratio (a.u.) during ischemia and reperfusion under control conditions, and under perfusion with HMR1098 added at the twelfth minute of ischemia. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); *P<0.05 vs. control.

The analysis of intercellular gap formation also showed that perfusion of coronary microvascular endothelial monolayers with HMR1098 from the twelfth minute of ischemia until end of experiments reduce formation of intercellular gaps during ischemia and reperfusion (Fig.16b).

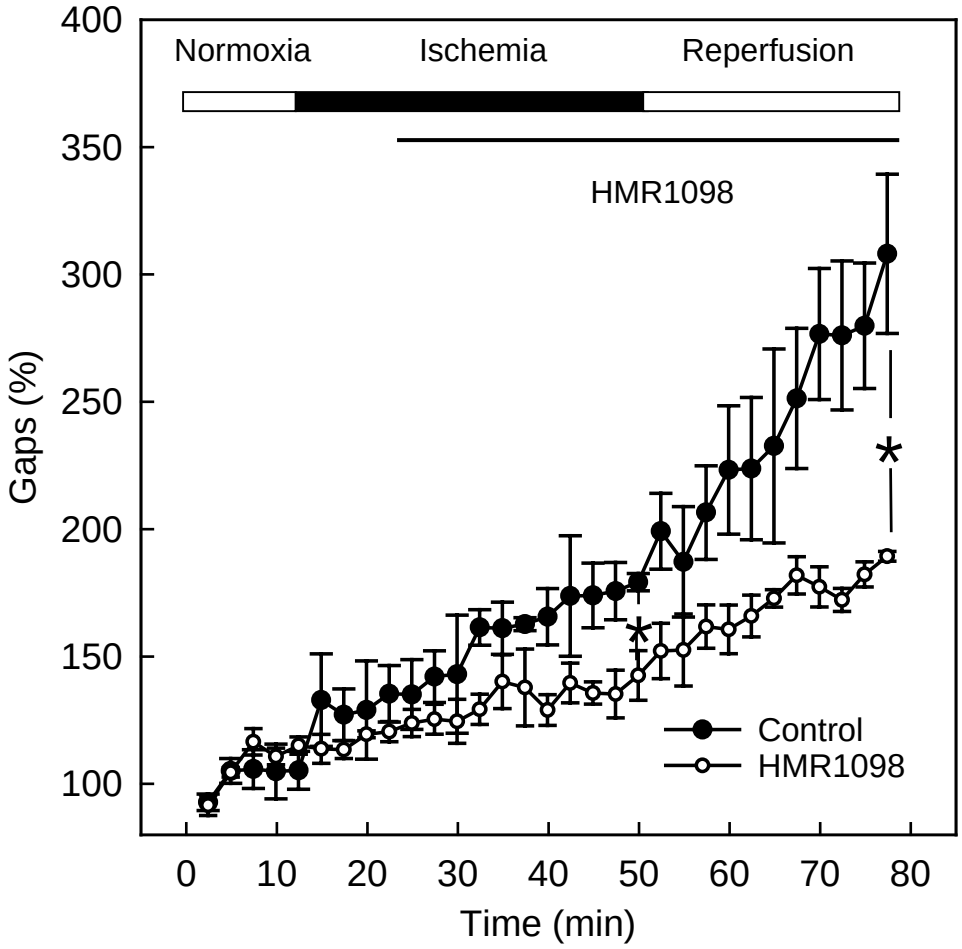


Fig.16b Time course of intercellular gap formation during ischemia and reperfusion under control conditions, and under perfusion with HMR1098 added at the twelfth minute of ischemia. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); *P<0.05 vs. control.

4.8 Influence of K_{ATP} channels on Ca^{2+} load and intercellular gap formation during reperfusion

The influence of the K_{ATP} channels on cytosolic Ca^{2+} load and intercellular gap formation in coronary microvascular endothelial cells was investigated with onset of reperfusion. The results do not show a statistically significant difference between the Fura-2 Ratio of control experiments and experiments in which endothelial monolayers were perfused with glybenclamide (Fig.17a).

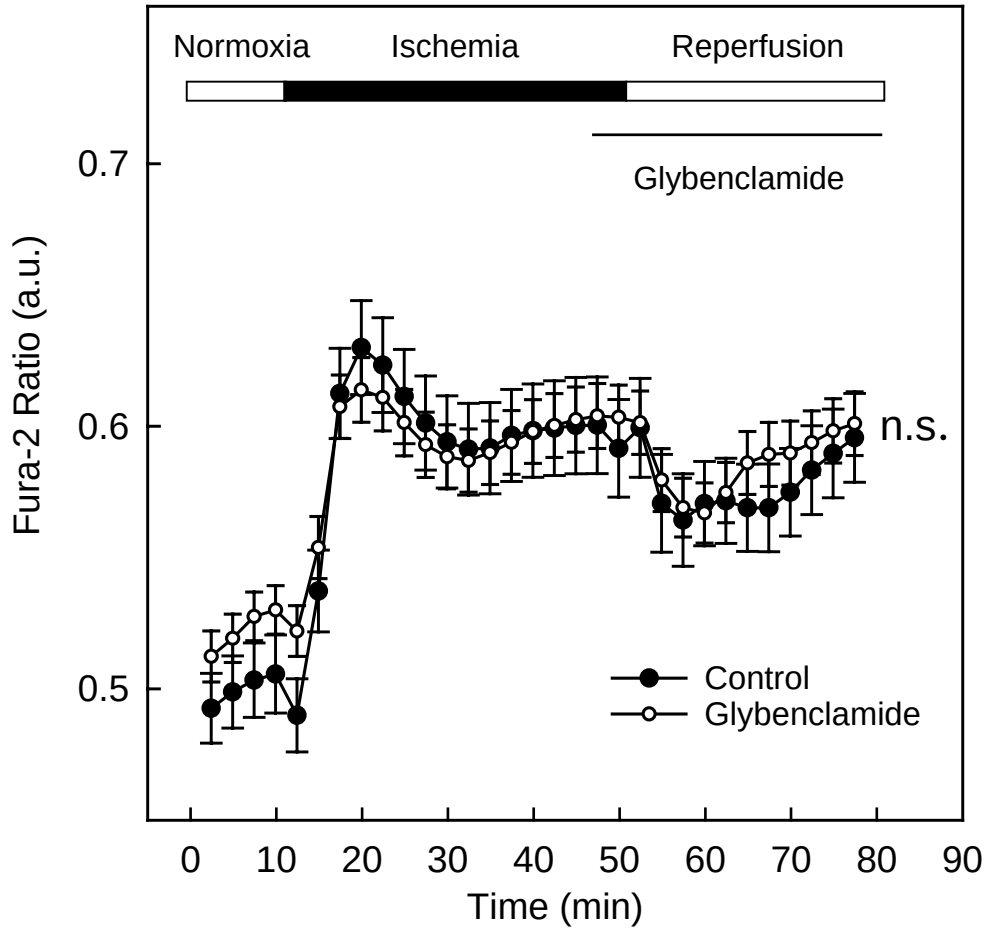


Fig.17a Time course of Fura-2 Ratio (a.u.) during ischemia and reperfusion, under control conditions, and under perfusion with glybenclamide (10 μM) only during reperfusion. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); n.s. (not significant) vs. control.

When the influence of the K_{ATP} channels on formation of intercellular gaps was investigated during reperfusion, again no significant difference was observed between control experiments and experiments where the endothelial monolayers were perfused with glybenclamide in reperfusion (Fig.17b).

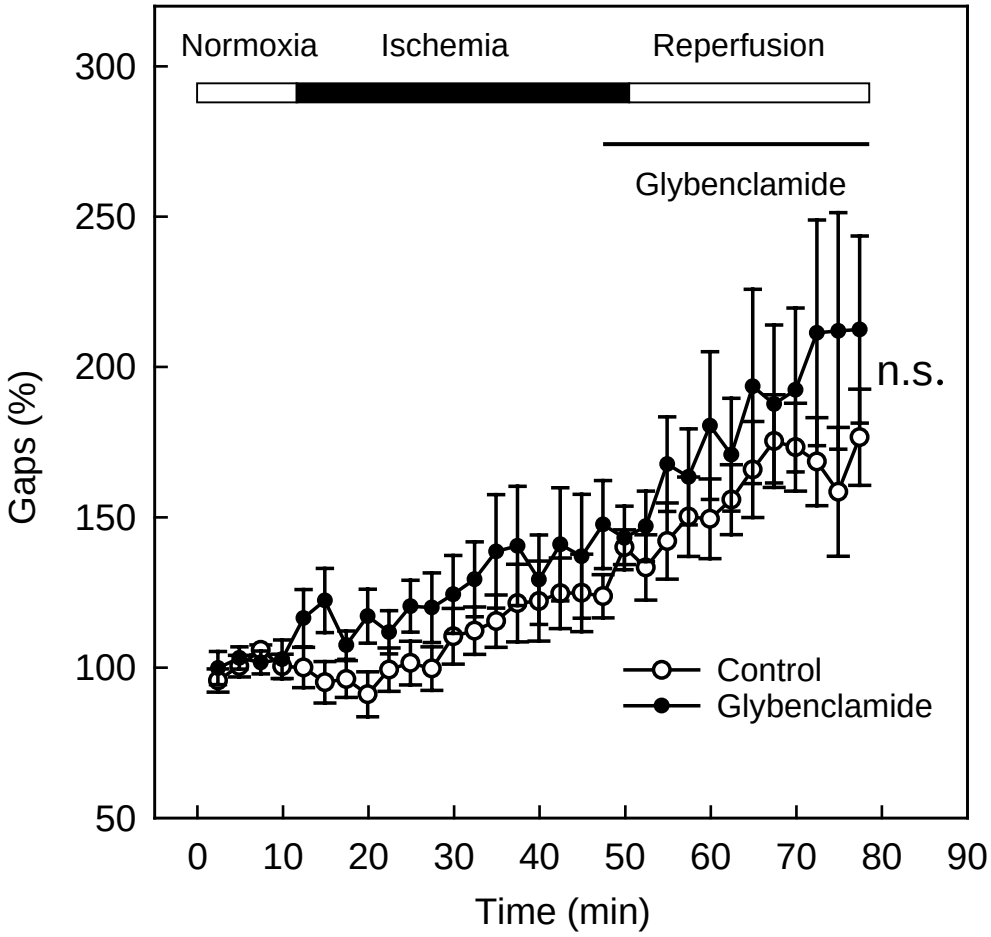


Fig.17b Time course of intercellular gap formation during ischemia and reperfusion under control conditions, and under perfusion with glybenclamide (10 µM) only during reperfusion. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); n.s. (not significant) vs. control.

Figure 18a shows the results from a series of experiments where the influence of the plasmalemmal K_{ATP} channels on cytosolic Ca^{2+} was studied when HMR 1098 was applied only during reperfusion. The results did not show a reduction in the Ca^{2+} load in reperfusion when the cells were treated with HMR1098 compared to control conditions.

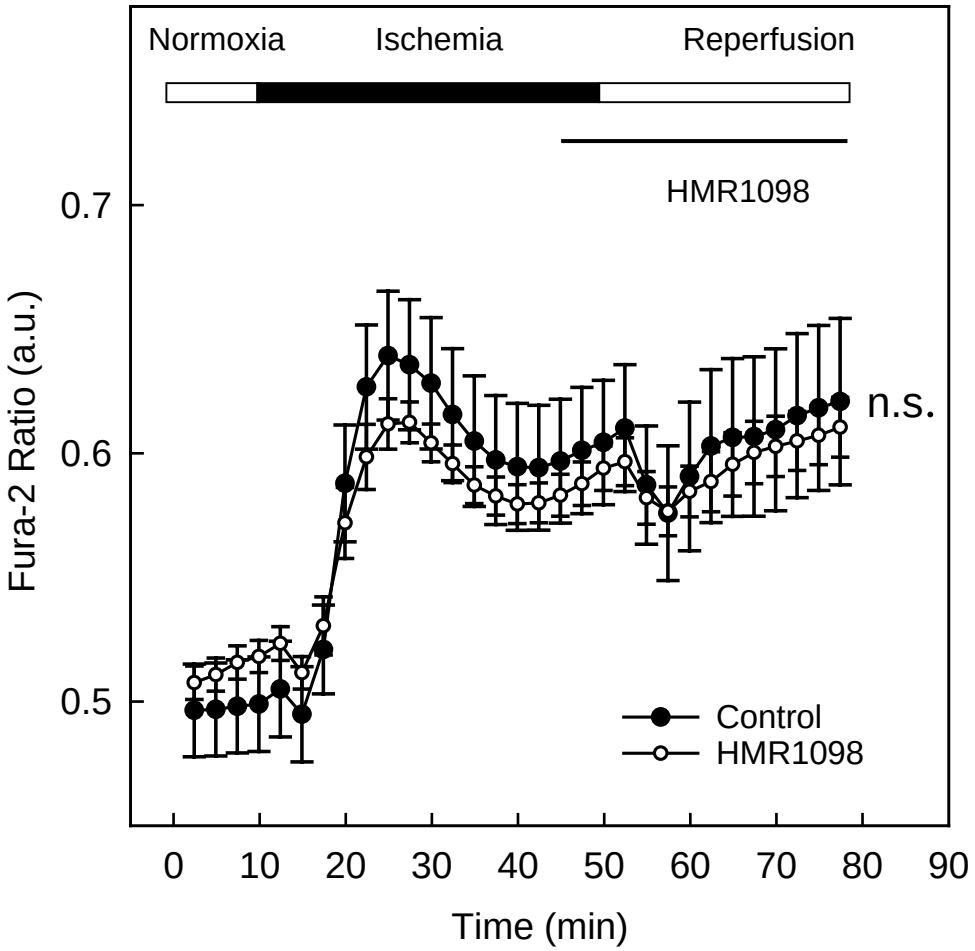


Fig.18a Time course of Fura-2 Ratio (a.u.) during ischemia and reperfusion under control conditions, and under perfusion with HMR1098 only during reperfusion. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); n.s. (not significant) vs. control.

When the formation of intercellular gaps was analysed, there was no significant reduction in intercellular gap formation under perfusion with HMR 1098 compared to control conditions (Fig. 18b).

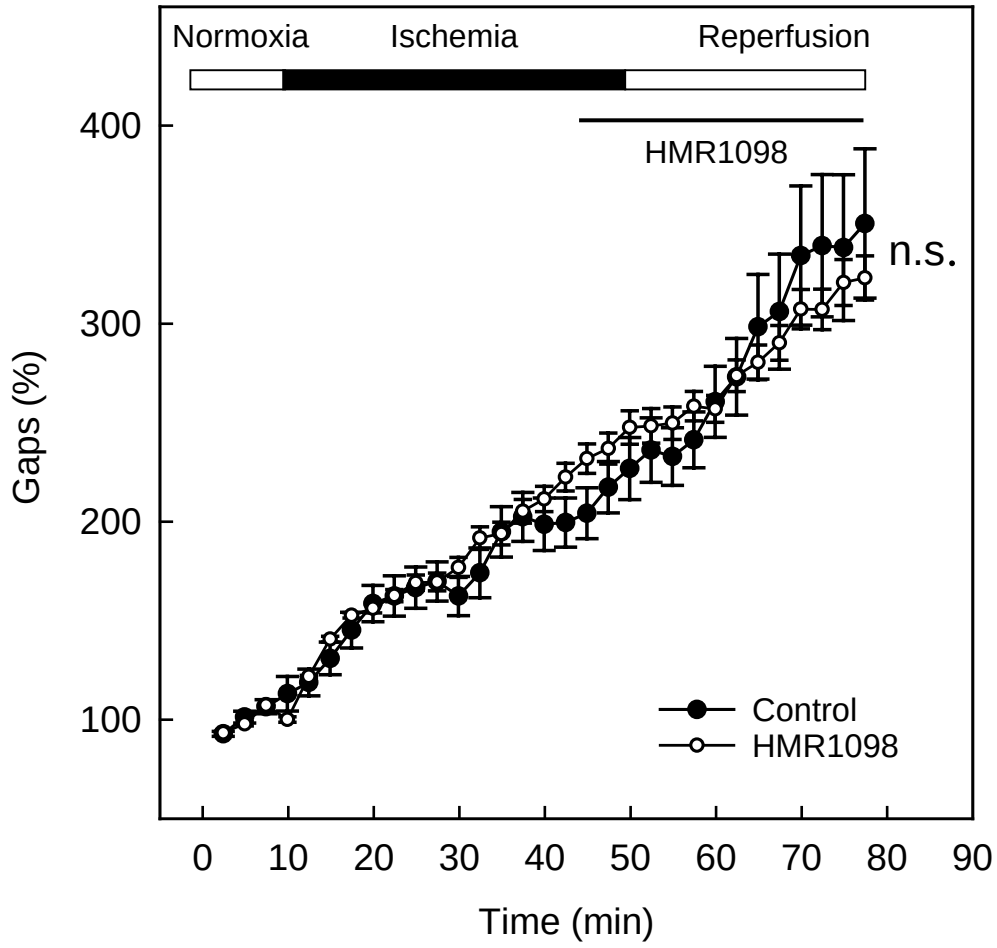


Fig.18b Time course of intercellular gap formation during ischemia and reperfusion under control conditions and under perfusion of cells with HMR1098 (100 µM) only during reperfusion. Data are presented as mean value ± SEM; (n=160 cells from 6 experiments); n.s. (not significant) vs. control.

5 DISCUSSION

The present study investigated the role of K_{ATP} channels on Ca^{2+} overload and barrier function on microvascular coronary endothelial cells under ischemia and reperfusion. The findings of this study show that ischemic conditions cause opening of plasmalemmal K_{ATP} channels and membrane hyperpolarisation. This K_{ATP} channel opening contributes to the progressive influx of Ca^{2+} into endothelial cells, the formation of intercellular gaps and hence, to failure of barrier function of endothelial monolayers under ischemic conditions. In reperfusion, K_{ATP} channels do not influence Ca^{2+} overload or barrier function.

Under normoxic conditions K_{ATP} channels are normally in a closed state and do not take part in the regulation of cellular ion homeostasis, since cytosolic levels of ATP inhibit their activation. Under normoxic conditions, application of a pharmacological K_{ATP} channel opener may therefore exert a pronounced effect. E.g., Langheinrich et al. (1997) found a glybenclamide-sensitive hyperpolarisation induced by K_{ATP} channels openers and glucose deprivation in capillaries isolated from guinea pig hearts. Some results of the present study are in agreement with those findings. Pharmacological activation of the K_{ATP} channels with use of diazoxide induced a sustained hyperpolarisation of the cell membrane in a dose-dependent manner. Application of the K_{ATP} channels inhibitors glybenclamide or the chemically distinct inhibitor HMR1098 abolished the diazoxide-induced hyperpolarisation of normoxic cells.

Exposure of endothelial cells to ischemia caused a change in their membrane potential, in form of a hyperpolarisation. When endothelial monolayers were treated with the K_{ATP} channel inhibitor glybenclamide, the hyperpolarisation of the plasmalemma was significantly reduced. Treatment of the endothelial monolayers with the chemically distinct K_{ATP} channel inhibitor HMR1098 confirmed this observation. This indicates that ischemic hyperpolarisation in endothelial cells is caused by opening of plasmalemmal K_{ATP} channels. The most likely cause is that ischemic conditions cause ATP depletion, and this then leads to activation of K_{ATP} channels. During 30 minutes of reperfusion following ischemic conditions the membrane potential returned to normoxic levels. Neither treatment with glybenclamide nor with HMR1098 influenced the membrane potential during

reperfusion. This might be expected, since a rise in cytosolic ATP concentration during reperfusion, caused by metabolic recovery, inhibits K_{ATP} channels.

Normoxic experiments on endothelial cells demonstrated that hyperpolarisation of the plasmalemma induced by pharmacological K_{ATP} channel opening is accompanied by a Ca^{2+} influx into the cell. This finding is in agreement with previous studies (Lückhoff et al. 1990, Nilius et al.1991, Kamouchi et al. 1997, Chen et al. 2001, Adams et al. 2004). Langheinrich et al. (1998) showed in coronary capillary EC that low concentrations of the K_{ATP} channel openers diazoxide and rilmakalim cause a pronounced glybenclamide-sensitive hyperpolarisation and induce a rapid Ca^{2+} transient followed by a sustained elevation of cytosolic Ca^{2+}.

The results of the present study revealed that K_{ATP} channels also contribute to the regulation of the Ca^{2+} homeostasis in EC during ischemic conditions. Treatment with K_{ATP} channel inhibitors glybenclamide or HMR1098 from onset of ischemic conditions to the end of reperfusion reduced the Ca^{2+} overload during ischemia and reperfusion. The basic features of changes in Ca^{2+} homeostasis under ischemia and reperfusion were previously described (Noll et al. 1995, Ladilov et al. 2000, Schäfer et al. 2001): Coronary EC develop a biphasic Ca^{2+} overload during ischemia, an initial rise mainly due to Ca^{2+} release from the ER and a secondary rise due to Ca^{2+} influx from the extracellular space. When applied from onset of ischemia, K_{ATP} channel inhibitors reduced the initial rise of cytosolic Ca^{2+}. Our Ca^{2+}-free experiments also showed a reduction of the first rise. This observation indicates that the first Ca^{2+} rise contains a part of Ca^{2+} influx from the extracellular space. This extracellular influx component is dependent on plasmalemmal K_{ATP} channels, probably by their effect on the rapidly developing hyperpolarisation.

To test the effect of K_{ATP} opening on the delayed Ca^{2+}-influx, glybenclamide or HMR1098 was applied at the peak of the initial Ca^{2+} rise. It was found that this postponed application of the K_{ATP} channel inhibitors still reduced the secondary Ca^{2+} increase during ischemia. It can therefore be concluded that K_{ATP} opening and plasmalemma hyperpolarisation also promotes the secondary Ca^{2+} influx during ischemia. It is likely that store-operated Ca^{2+} entry (SOC) is the mechanism that propagates the delayed Ca^{2+} influx into the cell (Adams et al. 1989, Putney 1990, Dolor et al. 1992, Berridge et al. 1995, Nilius et al. 2001). One may also discuss if activation of the NCX in reverse mode (influx of Ca^{2+} and efflux of Na^{+}) contributes

to ischemic endothelial Ca^{2+} overload. Reverse mode of NCX can be triggered by accumulation of cytosolic Na^+ and plasmalemma depolarisation. Na^+ accumulation may occur when the Na, K-ATPase fails due to a lack of energy. Berna et al. (2001) hypothesised a mechanism in endothelial cells, by which glycolysis during ischemia leads to a pronounced influx of Na^+ and consequently to activation of NCX in its reverse mode, resulting in net influx of Ca^{2+}. The finding, that ischemic conditions in endothelial cells lead to a prominent hyperpolarisation, makes it unlikely, however, that NCX is activated under these conditions since a reverse mode of activation of NCX is favoured by membrane depolarisation.

The pharmacological reduction of the Ca^{2+} overload during ischemia and reperfusion with the K_{ATP} channel inhibitors leads to reduction of endothelial barrier failure developing under these conditions. This was expected since it was previously shown that cytosolic Ca^{2+} overload during ischemia and reperfusion can trigger the failure of the endothelial barrier (Schäfer et al. 2003). The underlying cause exists in activation of the Ca^{2+}-calmodulin dependent myosin light chain kinase (MLCK). Phosphorylation of MLCK results in stress fiber formation, cell contraction and subsequent formation of intercellular gaps (Sheldon et al. 1993, Garcia et al. 1995).

The failure of the K_{ATP} channel inhibitors glybenclamide or HMR1098 to influence reperfusion-induced Ca^{2+} overload, as shown in this study, suggests that K_{ATP} channels are not involved in the mechanism causing the Ca^{2+} rise in the reperfusion phase following ischemia. Peters et al. (2007) demonstrated that reoxygenation-induced Ca^{2+} overload in endothelial cells is due to a Ca^{2+} influx. It is probably secondary to another activation of the $InsP_3$ receptor of the ER and activation of store-operated Ca^{2+} channels of the plasmalemma.

In summary, the following scheme can be proposed for the mechanism of reduction of endothelial barrier failure through inhibition of plasmalemmal K_{ATP} channels under ischemic conditions:

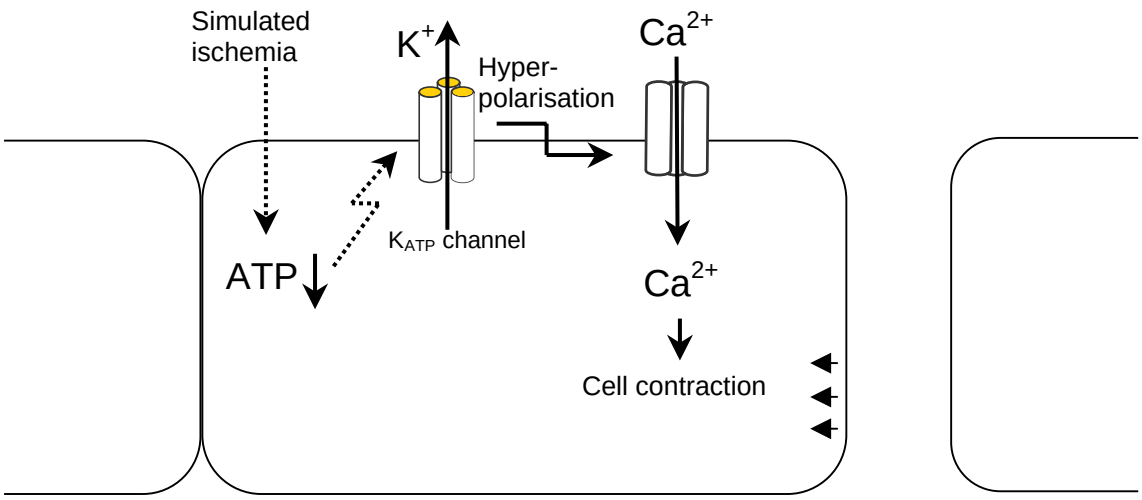


Fig.19 Proposed mechanism of action of plasmalemmal K_{ATP} channels during ischemia

ATP depletion during ischemia causes K_{ATP} channels to switch to an open state. This activation leads to a K^+ efflux, which then induces hyperpolarisation of the plasmalemma. The hyperpolarisation in turn increases the driving force for influx of Ca^{2+} into the cytosol, having as consequence a Ca^{2+} overload. The Ca^{2+} overload then activates the cellular contractile apparatus resulting in formation of intercellular gaps and barrier dysfunction (Schäfer et al. 2003). Reduction of the Ca^{2+} overload during ischemia through inhibition of plasmalemmal K_{ATP} channels reduces the Ca^{2+} overload. Concomitantly, less intercellular gaps are formed, and the endothelial barrier remains relatively stable.

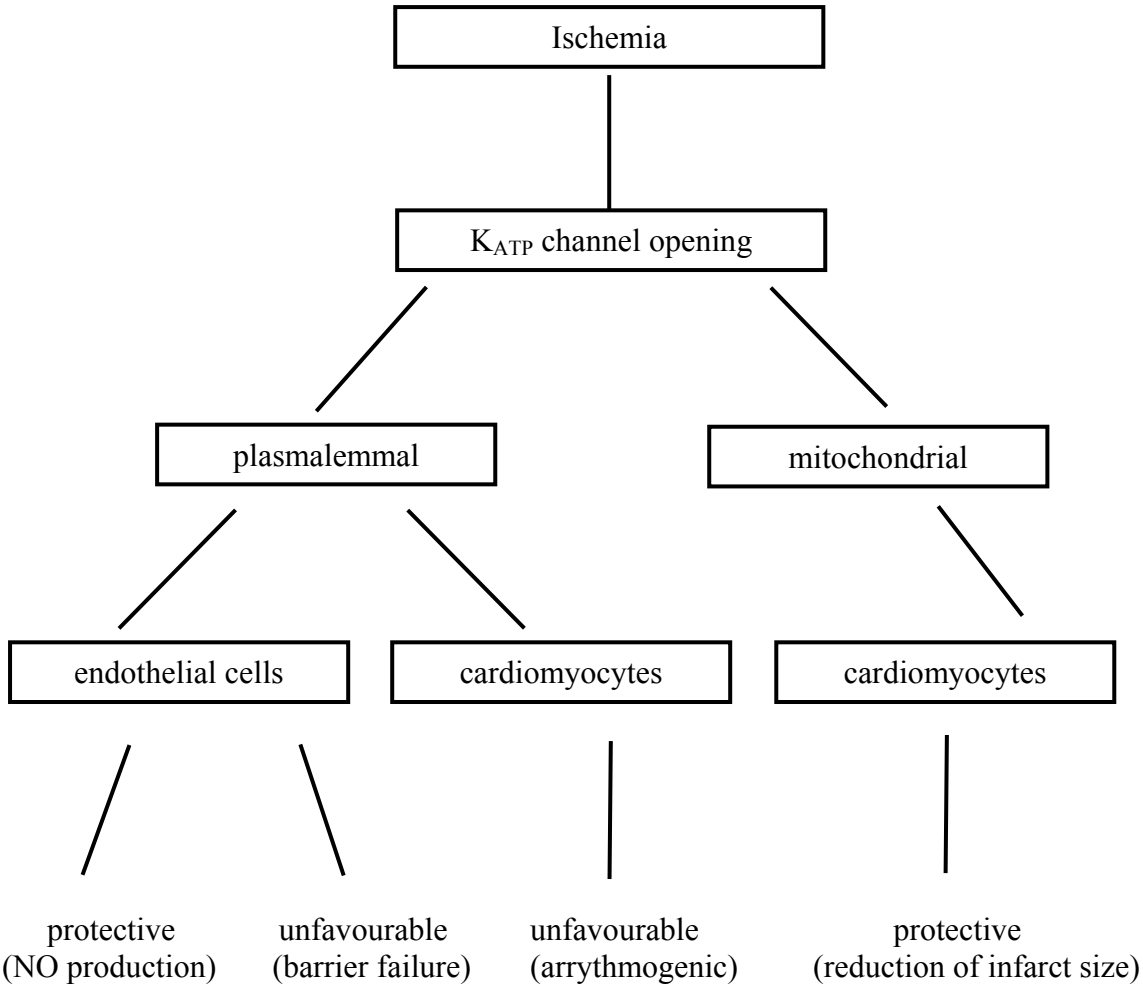
When the heart is considered as a whole the evidence for inhibition of K_{ATP} channels as protective principle is mixed, dependent on cell type and subcellular location of the K_{ATP} channel under investigation. Many studies have shown that opening rather than inhibition of K_{ATP} channels during ischemia is cardioprotective on the heart as a whole. It has also been shown that the effect of K_{ATP} channels opening mimics the protective effect of preconditioning, a phenomenon in which brief periods of ischaemia produce a phenotype in the heart and other organs in which the tissue is protected against the deleterious consequences of a subsequent more prolonged period of ischemia (Murry et al. 1986). Preconditioning produces a marked reduction in myocardial infarct size, and one of the central triggers and mediators is the opening of the mitochondrial K_{ATP} channels (Garlid et al. 1997, O'Rourke, 2000), whereas opening of plasmalemmal K_{ATP} channels during ischemia does not exert this

protective effect (Saavedra et al. 2002). In endothelial cells, Wang et al. (2007) have hypothesized that activation of endothelial K_{ATP} channels could reduce endothelial dysfunction. Their findings suggested that the activation of endothelial K_{ATP} channels activates endothelial NO synthase and inhibits the release and synthesis of endothelin-1 However, even in the area of global cardioprotection not all data indicate activation of K_{ATP} channels is protective. In an in vivo study by Picard et al. (1998) using perfused guinea pig hearts it was shown that application of K_{ATP} channel openers increases the amount of membrane damage in the cell, where application of gliburide, a K_{ATP} channel inhibitor, reduced it. A further important finding in this study was the proarrythmic effect of K_{ATP} channel opening.

Activation of the myocardial sarcolemmal K_{ATP} channels by ischemia, or by application of pharmacological agents causes shortening of the action potential duration thereby predisposing the heart to reentrant arrhythmias (Janse et al. 1989, Wilde et al. 1990). Kääb et al. (2003) demonstrated that HMR1098 is useful to prevent K_{ATP} channel-induced shortening of the action potential in human ventricular myocardium. This is explained by the fact that opening of K_{ATP} channels during ischemia or with pharmacological agents leads to efflux of K^+ which in turn shortens the duration of action potential. As a consequence, the refractory period is reduced, and this renders the myocardium more vulnerable to re-entry mechanisms and, as a consequence, ventricular fibrillation. More acidic conditions as observed in ischemia increase the sensitivity to HMR1098, indicating a more potent effect in ischemic myocardium. Thus, HMR1098 may be a useful agent to prevent action potential shortening and dispersion of repolarisation during ischemia, which may protect against ischemia induced ventricular arrhythmias.

Another area of interest in terms of cardioprotection is cardiac transplantation when hearts are deprived of blood supply for few hours. Progress in development of cardioplegic solutions has not completely eliminated the underlying challenges of ischemia and reperfusion. Methods for preservation and storage, developed initially to protect cardiomyocyte function, may be deleterious for vascular endothelium. The impairment of endothelial barrier function results in perivascular and tissue edema and this promotes graft dysfunction. The use of K_{ATP} channel inhibitors added to cardioplegic solutions may represent a promissing adjuvant to cardioprotective solutions in surgery.

Based on the above mentioned mechanisms of action of K_{ATP} channels during ischemia, the following scheme can be proposed about the potential use and development of pharmacological agents in cardioprotection having K_{ATP} channels as pharmacological targets:



The focus of future research of ischemic cardioprotection should be development of pharmacological agents that selectively block plasmalemmal K_{ATP} channels, due to their protective action both on endothelial cells as well as on cardiomyocytes. The results of this study may contribute to identifying new strategies which by including endothelium-protective effects would improve cardiac protection.

6 SUMMARY:

The aim of the present study was to investigate the role of the K_{ATP} channels on induction of the ischemia-reperfusion injury in coronary microvascular endothelial cells.
The main findings of this study are:

- Ischemia induces activation of the K_{ATP} channels in coronary microvascular endothelial cells.

- Activation of K_{ATP} channels during ischemia induces hyperpolarisation of the plasmalemma.

- Hyperpolarisation of the plasmalemma during ischemia is accompanied by a Ca^{2+} influx.

- Inhibition of the K_{ATP} channels during ischemia with glybenclamide or HMR1098 reduces the hyperpolarisation of the plasmalemma and the Ca^{2+} influx.

- Inhibition of the K_{ATP} channels during ischemia reduces the formation of intercellular gaps.

- Inhibition of the K_{ATP} channels during reperfusion does not influence the membrane potential or the Ca^{2+} load, nor does it influence the formation of intercellular gaps.

6 ZUSAMMENFASSUNG:

Die Hauptaufgabe der vorliegenden Arbeit war zu klären, welche Bedeutung die K_{ATP} –Kanäle in koronaren Endothelzellmonolayern während simulierter Ischämie und Reperfusion haben.

Die Befunde dieser Arbeit lauten wie folgt:

- Ischämie führt zur Aktivierung der K_{ATP}-Kanäle in koronaren mikrovaskulären Endothelzellen.

- Aktivierung der K_{ATP}-Kanäle während der Ischämie führt zur Hyperpolarisation der Zellmembran.

- Die K_{ATP}-Kanal-induzierte Hyperpolarisation der Zellmembran während der Ischämie wird von mit einem Ca^{2+}-Einstrom aus dem Extrazellulärraum begleitet.

- Die Inhibierung der K_{ATP}-Kanäle während der Ischämie mit Glybenclamide oder HMR1098 reduziert die Hyperpolarisation der Zellmembran und den Ca^{2+}-Einstrom.

- Eine Inhibierung der K_{ATP}-Kanäle während der Ischämie reduziert die Entstehung der interzellulären Lücken.

- Eine Inhibierung der K_{ATP}-Kanäle nur während der Reperfusion hat weder einen Einfluß auf das Membranpotential noch auf die Calciumhomöostase noch auf die Entstehung interzellulärer Lücken.

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“I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.”

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Curriculum vitae:

Last Name:	Gligorievski
First Name:	Dragan
Address:	Frankfurter Str.117, 35392 Giessen
Date of birth:	26.2.1976
Place of birth:	Kumanovo, Republic of Macedonia
Marital Status:	single

Education:

1990-1994	Gymnasium Nikola Karev, Skopje
1994-2000	Veterinary Medicine University St. Cyril and Methodius, Skopje
2003-2006	Ph.D Study, Institute of Physiology, Justus Liebig University, Giessen

Internships and work experience:

July-September 1997	Internship at the Small Animal Hospital-Fredonia, New York
September-October 1997	Internship at the Dept. of Biology-State University of New York at Fredonia
August 1998	Internship at the Small Animal Hospital-Spittal an der Drau, Austria
August 1999	DAAD Scholarship, Johannes Gutenberg University, Mainz
2000-2001	Journalist at Telma TV, Skopje
September 2001-March 2002	Internship at the German Bundestag

November 2002-December 2003	Member of the graduate training Group “Cell-cell-interaction in reproduction” JLU Giessen
June 2004 - 2007	Associate Member of the graduate training group “Vascular Biology”, Justus Liebig University, Giessen
October 2004-2006	Student Teaching Assistant in Physiology, Justus Liebig University, Giessen
November-2006	Postdoctoral Fellow , Institute of Physiology, Justus Liebig University, Giessen

Languages:

Macedonian, mother tongue
German, fluent
English, fluent
French, good
Serbian, fluent
Croatian, fluent