

G. DEBSKA-VIELHABER¹, M.M. GODLEWSKI², A. KICINSKA¹, J. SKALSKA¹, B. KULAWIAK¹,
M. PIWONSKA¹, K. ZABLOCKI³, W.S. KUNZ⁴, A. SZEWCZYK¹, T. MOTYL²

LARGE-CONDUCTANCE K⁺ CHANNEL OPENERS INDUCE DEATH OF HUMAN GLIOMA CELLS

¹Laboratory of Intracellular Ion Channels, Nencki Institute of Experimental Biology, Warsaw, Poland; ²Department of Physiological Sciences, Faculty of Veterinary Medicine, SGGW, Warsaw, Poland; ³Laboratory of Bioenergetics, Biomembranes and Metabolic Regulation, Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland; ⁴Department of Epileptology, University Bonn Medical Center, Bonn, Germany

Large-conductance Ca²⁺-activated K⁺ channels (BK_{Ca} channels) are highly expressed in human glioma cells. It has been reported that BK_{Ca} channels are present in the inner mitochondrial membrane of the human glioma cell line LN229. In the present study we investigated whether BK_{Ca}-channel openers, such as CGS7181 (ethyl 2-hydroxy-1-[[[4-methylphenyl]amino]oxo]-6-trifluoromethyl-1H-indole-3-carboxylate) and CGS7184 (ethyl 1-[[[4-chlorophenyl]amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxylate), affect the functioning of LN229 glioma cell mitochondria *in situ*. In the micromolar concentration range CGS7181 and CGS7184 induced glioma cell death. Morphological and cytometric analyses confirmed that both substances trigger the glioma cell death. This effect was not inhibited by the pan-caspase inhibitor z-VAD-fmk. Lack of DNA laddering, PARP cleavage, and caspase 3 activation suggested that glioma cell death was not of the apoptotic type. We examined the effect of CGS7184 on mitochondrial membrane potential and mitochondrial respiration. Potassium channel opener CGS7184 increased cell respiration and induced mitochondrial membrane depolarization. The latter was dependent on the presence of Ca²⁺ in the external medium. It was shown that CGS7184 induced an increase of cytosolic Ca²⁺ concentration due to endoplasmic reticulum store depletion. In conclusion, our results show that CGS7181 and CGS7184 induce glioma cell death by increasing the cytosolic calcium concentration followed by activation of calpains.

Key words: glioma cells, mitochondria, respiratory chain, ion channels, potassium channel openers, calpains

INTRODUCTION

Large-conductance Ca²⁺-activated K⁺ channels (BK_{Ca} channels) are commonly expressed in cells derived from the most common human brain tumors, namely meningiomas (1) and gliomas (2). BK_{Ca} channels are activated by membrane depolarization and by an increase in cytosolic Ca²⁺. These channels belong to the superfamily of voltage-dependent K⁺ channels with six membrane spanning domains (S1-S6) and with a seventh membrane - spanning domain (called S0) (3). Large conductance K⁺ currents in glioma cells are mediated by an isoform of BK_{Ca} channel, termed gBK, that contains a 34-amino-acid insert at splice site 2 (4).

In nonexcitable cells, such as epithelial, endothelial or glial cells, BK_{Ca} channels may contribute to diverse biological

functions such as osmoregulation (5), cell proliferation (6) and cell migration (7). Examination of biopsies from patients with malignant gliomas has revealed specific overexpression of BK_{Ca} channels in gliomas when compared with nonmalignant human cortical tissues (4). These results suggest that proliferating glial cells (with glioma cells representing the most extreme case) revert to a developmental biophysical phenotype that induces BK_{Ca} channel expression.

In addition to the plasma membrane, the BK_{Ca} channel was identified by patch-clamp techniques in the mitochondrial membrane (mitoBK_{Ca} channel) of the human glioma cell line LN229 (8). This channel has a conductance of 295 pS and is activated by Ca²⁺. Like most BK-type channels, the mitochondrial channel is blocked by charybdotoxin in a voltage dependent manner (8). Recently, a mitoBK_{Ca} channel was found

Abbreviations: mitoK_{ATP} channel: mitochondrial ATP-regulated potassium channel; K_{ATP} channel: plasma membrane ATP-regulated potassium channel; KCOs: potassium channel openers; BK_{Ca}Cos: calcium activated potassium channel openers; CGS7181: ethyl 2-hydroxy-1-[[[4-methylphenyl]amino]oxo]-6-trifluoromethyl-1H-indole-3-carboxylate; CGS7184: ethyl 1-[[[4-chlorophenyl]amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxylate; TMRE: tetramethylrhodamine ethyl ester; BK_{Ca} channel: calcium activated potassium channel; mitoBK_{Ca}: mitochondrial calcium activated potassium channel; DNP: 2,4-dinitrophenol; CCCP: carbonyl cyanide m-chlorophenylhydrazone; ChTx: charybdotoxin; PBS: phosphate buffered saline; 7-AAD: 7-aminoactinomycin D; PI: propidium iodide

also in cardiac ventricular myocytes (9, 10). It was observed that the mitoBK_{Ca} channel significantly contributes to mitochondrial myocytes uptaking K⁺ and that this was induced by an opener of the mitoBK_{Ca} channel, NS1619. Xu concluded that the mitoBK_{Ca} channel protects the heart against infarction (9). Recently, differential distribution of BK_{Ca} channel β 4 subunit in rat brain was shown (11).

The intracellular targets for potassium channel openers (KCOs) have recently attracted attention mainly due to the interaction of the KCOs with mitochondria. Originally, KCOs were identified as openers of two classes of plasma membrane K⁺ channels: ATP-regulated K⁺ channels and BK_{Ca} channels (12). Some KCOs may directly interact with the potassium channels present in different intracellular membranes (13). In particular, the mitochondrial ion channels seem to be important intracellular targets for KCOs. Mitochondrial ion channels play an important role in such cellular events as apoptosis (14), exocytosis (15) and synaptic transmission (16). KCOs act on the potassium channels present in the heart (17), brain (18, 19) and skeletal muscle mitochondria (20, 21), but also in amoeba mitochondria (22). Recently, it was found that KCOs acting on the mitochondrial ATP-regulated potassium channel (mitoK_{ATP} channel) may play an important role as inducers of ischemic preconditioning in the heart (13, 23, 24). Additionally, activation of the mitoK_{ATP} protects neurons and astroglia against injury and cell death (25).

A variety of small synthetic molecules, for example the benzimidazole NS1619 (26) or indole carboxylate compounds such as CGS7181 (ethyl 2-hydroxy-1-[[[4-methylphenyl]amino]oxo]-6-trifluoromethyl-1H-indole-3-carboxylate) and CGS7184 (ethyl 1-[[[4-chlorophenyl]amino]oxo]-2-hydroxy-6-trifluoromethyl-1H-indole-3-carboxylate) have been identified as selective large-conductance Ca²⁺-activated K⁺ channel openers (BK_{Ca}COs) (12). Recently, potassium channel openers such as NS1619, have been shown to inhibit the mitochondrial respiratory chain in cardiac mitochondria (27) and in the human glioma cell line LN229 (28). Additionally, the BK_{Ca} channel opener NS1619 was shown to reduce the migration of 1321N1 human glioma cells (29) and to inhibit ROS production of isolated rat brain mitochondria (30). The mechanism of BK_{Ca} channel opening by CGS7181 and CGS7184 (in micromolar concentration range) was studied by investigating whole-cell BK_{Ca} current and single BK_{Ca} channel activity using the patch-clamp method in vascular smooth muscle cells (31).

In the present study, we evaluated the role of indole carboxylate type BK_{Ca} channel openers on the functioning of LN229 glioma cells. Both BK_{Ca}COs, CGS7181 and CGS7184, were found to induce glioma cell death in the micromolar concentration range. This effect was accompanied by morphology changes of the cell nuclei and externalization of phosphatidylserine without activation of the caspase apoptotic pathway. Furthermore, the BK_{Ca}COs caused an influx of calcium ions from the extracellular space into the cytosol, followed by activation of calpains and finally by cell death.

MATERIALS AND METHODS

Materials

All reagents for cell culture were purchased from Life Technologies, Inc. (GibcoBRL, UK) and from Sigma-Aldrich Co Ltd. (UK). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) was obtained from Molecular Probes (Eugene, OR, USA). The potassium channel openers CGS7181 and CGS7184 were provided by Dr. S. Hu (Novartis, USA). All other reagents were obtained from Sigma-Aldrich Co Ltd. (UK).

Cell culture

Glioma cell line LN229 was routinely cultured in high-glucose (4 g/l) DMEM medium supplemented with 5% fetal calf serum, 4 mM glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. The cells were incubated in an atmosphere containing 5% CO₂/95% air at 37°C.

Cell injury assay

Lactate dehydrogenase (LDH) enzyme activity was assayed using commercial kit (Roche Molecular Biochemicals, Germany). In the first step NAD⁺ is reduced to NADH/H⁺ by the LDH-catalyzed conversion of lactate to pyruvate. In a second step a catalyst (diaphorase) transfers H⁺/H from NADH/H⁺ to a yellow tetrazolium salt which is reduced to a red formazan compound. The absorbance at 492 nm was measured with a spectrophotometer (UV-160A, Shimadzu). The reference wavelength was 690 nm. Release of LDH into the medium was assayed at the indicated times of incubation and expressed as an index of cell injury, defined as the percentage of total releasable LDH, yielded by lysis of LN229 cells in 0.1% Triton X-100.

Morphological changes and DNA measurements

Measurements with a Laser Scanning Cytometer (LSC) were performed as previously described (32). In brief, cell death was analyzed by LSC (CompuCytte Corp., Boston, MA, USA) equipped with a 488 nm argon laser as an excitation source. Far-red fluorescence of 7-AAD was measured using a combination of dichroic mirrors and filters transmitting at 520 \pm 20 nm and >650 nm, respectively. Nuclear fluorescence of 7-AAD was measured within the area outlined by the "integration contour", located 2 pixels outside the "threshold contour" triggered by the far-red fluorescence. At least 5000 cells per sample were measured. The obtained results were analyzed by Microsoft Excel 2000 software (Microsoft Corporation, Redmond, WA, USA). Dying cells were relocated and morphological changes were confirmed by FV-500 confocal imaging (Olympus Poland).

Confocal images of phosphatidylserine externalization and cell death

The cells were stained with FITC-conjugated annexin V and propidium iodide (PI) according to the manufacturer's instructions (Annexin-V-Fluos Staining Kit, Roche). Confocal images were acquired with the use of an FV-500 confocal scanning system. Combination of excitation and emission was 488 nm Argon laser beam vs. 505-525 nm filter for Annexin V-FITC and 543 nm HeNe laser beam vs. 560 nm filter for PI.

Immunoblot analysis

Cells (5 x 10⁵) were washed and solubilized in 0.5 ml of RIPA buffer (50 mM Tris-HCl, pH = 7.4, 1% NP-40, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 10 μ g/ml aprotinin, 2 μ g/ml leupeptin, 5 μ g/ml PMSF, 5 μ g/ml Na₃VO₄, 5 μ g/ml NaF. Protein was determined by the method of Folin. Aliquots of protein (30 μ g) were separated by 12 %SDS-PAGE (for PARP detection) or 17% SDS-PAGE (for caspase-3 detection), and electrotransferred to nitrocellulose membrane (Amersham). The following primary antibodies were used: mouse monoclonal anti-PARP (1:2000, Biomol, SA-250), rabbit polyclonal anti-caspase-3 (1:1000, Pharmingen). As secondary antibodies anti-rabbit IgG and anti-mouse-HRP IgG (1:5000, Amersham) were used. Immunocomplexes were visualised using the enhanced

chemiluminescence detection system (ELC, Amersham Pharmacia Biotech).

Analysis of mitochondrial membrane potential

Changes in $\Delta\Psi_m$ were monitored by the uptake of fluorescent dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide). Cells were incubated with 0.5 μM JC-1 in DMEM medium for 15 min at 37°C. Afterwards the cells were washed twice with PBS and resuspended in KR-Hepes buffer. Stained cells were immediately analyzed with a FACSCalibur instrument (Becton Dickinson, San Jose, CA, USA) equipped with a single 488 argon laser. The following filter combination was used: 530 nm for FL1 channel and 617 nm for FL2 channel. The values of photomultipliers (MPT) were set logarithmically. Red fluorescence (FL2) corresponds to the J-aggregate form of JC-1 and is proportional to $\Delta\Psi_m$. The FL1-FL2 compensation was 3.7% and compensation FL2-FL1 9.5%. A minimum of 10000 cells per sample were measured and analyzed with the Cell Quest software package.

Cellular respiration measurements

Cellular respiration was measured with a Clark type oxygen electrode (Yellow Springs Instruments, OH). The incubation medium (KR-Hepes) contained 121 mM NaCl, 25 mM HEPES, 5 mM NaHCO_3 , 4.7 mM KCl, 1.2 mM KH_2PO_4 , 1.2 mM MgSO_4 , 2.0 mM CaCl_2 , 10 mM glucose, pH = 7.4, at 30°C.

Cytosolic calcium concentration measurements

Glioma cells plated on glass coverslips were loaded with 1 μM fura-2AM at 37°C for 15 min in standard medium consisting of 132 mM NaCl, 5 mM KCl, 25 mM HEPES, 1 mM MgCl_2 , 0.5 mM NaH_2PO_4 , 1 mM pyruvate, 5 mM glucose, pH = 7.2. Where indicated, 2 μM ionomycin, 1 μM CGS7184 or 2 mM Ca^{2+} were added. Before the Ca^{2+} measurement the cells on coverslips were washed twice with standard medium supplemented with 0.1 mM CaCl_2 , and coverslips were put into a cuvette in standard medium supplemented with 50 μM EGTA and cytosolic $[\text{Ca}^{2+}]$ was monitored fluorimetrically. The fluorescence was measured at 30°C in a Shimadzu RF5000 fluorimeter (Tokyo, Japan) set in the ratio mode using 340 nm/380 nm as excitation wavelengths and 510 nm as the emission wavelength. The time resolution of the measurements was 1 s. Ca^{2+} concentration was calibrated for cells in each run using 3 mM externally added Ca^{2+} and 3 μM ionomycin plus 0.0003% digitonin (final concentration).

Measurements of calpain activity

Calpain activity was measured according to modified methods of Bronk and Gores, 1993. Briefly, 2.5×10^5 cells were suspended in 1 ml KR-Hepes medium. Thereafter, the cell permeable protease substrate t-Boc-Leu-Met-CMAC was added to give a final concentration of 10 μM . The non-fluorescent t-Boc-Leu-Met-CMAC enters cells and becomes an intracellular substrate for conjugation to a thiol. Following thiol conjugation to the aminocoumarin moiety the compound remains non-fluorescent, but becomes membrane-impermeant. Subsequent intracellular proteolytic cleavage of the t-Boc-Leu-Met-CMAC-thiol conjugate between the Met and CMAC-thiol moieties liberates and unquenches the highly fluorescent, membrane impermeant CMAC-thiol conjugate. The cellular fluorescence of the CMAC-thiol conjugate was monitored at 25°C in a 1 ml quartz cuvette using a spectrofluorimeter (Shimadzu RF 5000,

Japan). The samples were excited at 380 nm and the CMAC-thiol conjugate fluorescence was registered at 460 nm.

Statistical analysis

Student's t-test was used to determine the statistical differences between various experimental and control groups. P values <0.05 were considered significant.

RESULTS

In order to determine the effects of BK_{Ca}COs on cell viability we performed measurements of lactate dehydrogenase (LDH) release upon treatment of LN229 glioma cells with various channel openers. Fig. 1 shows the result of channels openers CGS7184 and CGS7181 on cell injury. Incubation in the presence of 1 μM CGS7184 or CGS7181 induced cell injury between 6 – 18 h of incubation (Fig. 1A). Similar results were obtained with Trypan Blue measurements (data not shown).

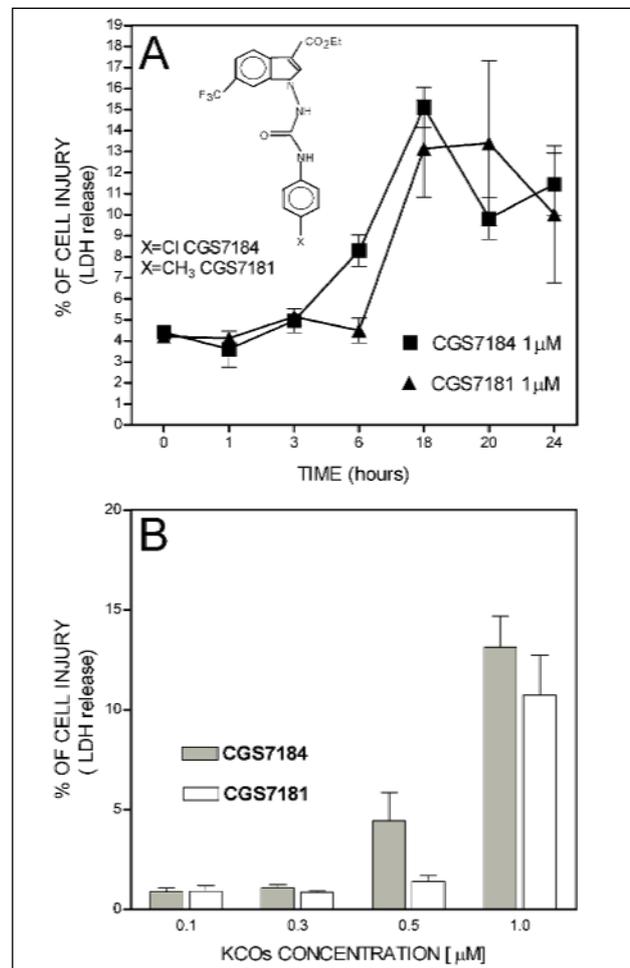


Fig 1. Effects of channel openers CGS7181 and CGS7184 on lactate dehydrogenase (LDH) release from LN229 glioma cells. (A) Time course of 1 μM CGS7184 (squares) and 1 μM CGS7181 (triangles) effect on LDH release from glioma cells. Data from triplicate time points were averaged and reported as means \pm SD. (B) Concentration dependence of CGS7184 and CGS7181 action on cell injury induction. Glioma cells were incubated for 18 h in DMEM medium supplemented with 0.1% FBS and 0.1, 0.3, 0.5 and 1 μM potassium channel openers. Cell injury in the absence of the channel openers was 2 \pm 0.6% (n=5).

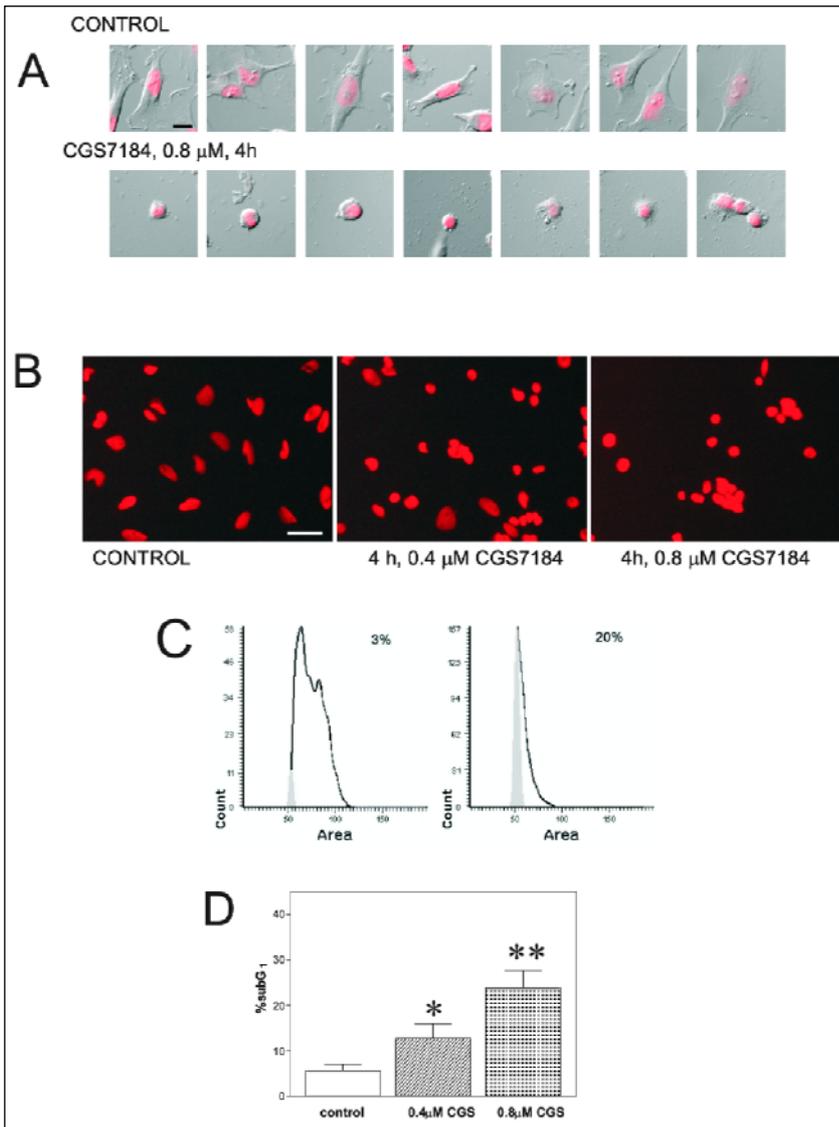


Fig 2. Morphological changes in glioma cell line LN229 treated with CGS7184. (A) Glioma cells were incubated in DMEM medium supplemented with 0.1% FBS for 4 hours before staining with 7-AAD. Confocal images with Nomarski contrast of control cells (upper panel) and cells treated with 0.8 μ M CGS7184 (lower panel) showing cell shrinkage and nucleus condensation. Scale bar = 5 μ m. (B) Changes in nucleus morphology of glioma control cells (left) and cells treated with 0.4 μ M CGS7184 (middle) and 0.8 μ M CGS7184 (right). Excitation and emission wavelengths were 546 and 647 nm, respectively. Scale bar = 5 μ m. (C) DNA histograms of glioma cells treated with CGS7184. Representative graphs show results of scanning cytometry analysis as described in Materials and Methods. Increase in subG1 area (grey) upon treatment with 0.8 μ M CGS7184 and decrease in S/G2M is clearly visible. (D) Quantitative analysis of the percentage of glioma cells in subG1 phase upon treatment with CGS7184 (CGS). Data represent the mean \pm SD from three separate experiments. *P<0.05, **P<0.005 compared with control (cells incubated with 0.1% DMSO).

Because the channel opener CGS7184 appeared to be slightly more potent (Fig. 1B) than CGS7181, CGS7184 was used for further studies.

To verify the molecular mechanism of cell death induced by potassium channel openers cell nuclei were stained with the fluorescent probe 7-aminoactinomycin D (7-AAD) and analyzed with laser scanning cytometry (LSC) and confocal microscopy (Fig. 2). Sub-micromolar concentrations of CGS7184 induced strong cell shape changes (Fig. 2A) similar to those typical for cells dying *via* apoptosis. Furthermore, chromatin condensation (Fig. 2B) was observed in images relocated from LSC. The LSC analysis of the cell nuclei size revealed that CGS7184 treatment induced an increase in the number of cells in the subG1 area (from 3% to 20%) of DNA distribution histograms with simultaneous decrease of cells in the S and G2M phases of the cell cycle (Fig. 2C). The proapoptotic action of CGS7184 was dose-dependent (Fig. 2D), reaching a significant level at 0.4 μ M concentration.

Cells that are in earlier stages of apoptosis are positive in Annexin V staining but they exclude propidium iodide (PI). In the late stages of apoptosis or in necrosis cells incorporate PI due to permeabilization of the plasma membrane. Necrotic cells are PI-positive with Annexin V staining. The cytotoxicity of CGS7184 was evaluated with Annexin V/PI staining (Fig. 3). We observed

that 0.8 μ M CGS7184 induced a fast, within 30 min, phosphatidylserine (PS) externalization measured with FITC-labeled Annexin-V (Fig. 3, panel A). This was followed by PI incorporation occurring within the next 30 min (Fig. 3, panel B).

The above observations suggest that the potassium channel opener induces LN229 glioma cell death probably *via* an apoptotic mechanism. For further evaluation of the CGS7184-induced apoptosis in glioma cells, analysis of DNA fragmentation was done by gel electrophoresis. However, no DNA laddering was found in the CGS7184-treated cells (Fig. 4A). Moreover, we were unable to observe PARP cleavage or caspase 3 activation upon treatment of LN229 glioma cells with 1 μ M CGS7184 (Fig. 4B and 4C). Also the caspase inhibitor z-VAD-fmk did not lower cell death upon 1 μ M CGS7184 treatment (Fig. 4D). All results suggested that the BK_{Ca}COs applied induced glioma cell death *via* necrosis or an atypical apoptosis pathway.

Fig. 5 shows that 3 μ M CGS7184 induced rapid mitochondrial depolarization, as measured with the fluorescent probe JC-1 (Fig. 5A). Mitochondrial depolarization is believed to be an early hallmark of apoptosis. Hence, this result would suggest that CGS7184 stimulates glioma cell death through activation of the mitochondrial apoptotic pathway, apparently by directly dissipating the mitochondrial membrane potential.

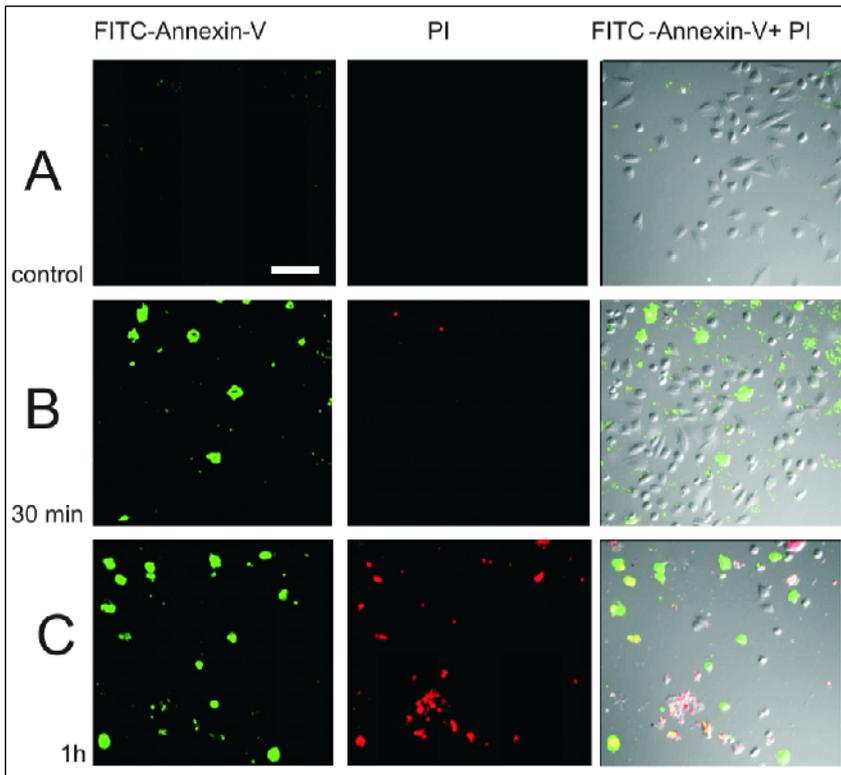


Fig 3. Effect of potassium channel opener CGS7184 on phosphatidylserine externalization in glioma cell line LN229. (A) Confocal images of control glioma cells in the absence of potassium channel opener. (B) Cells incubated in the presence of 0.8 μM CGS7184 for 30 min. (C) Cells incubated with CGS7184 for 1 h. Left to right, cells stained with FITC-conjugated Annexin V, propidium iodide, and combined confocal images with Nomarski contrast. Late apoptotic cells show positive staining (FITC-conjugated Annexin V and PI) while secondary necrotic cells show only PI-positive staining. Data are representative of three independent experiments. Scale bar = 10 μM .

Moreover, valinomycin and the mitochondrial uncoupler FCCP also induced depolarization of mitochondria in LN229 glioma cells (*Fig. 5B*).

In order to exclude the possibility that the observed mitochondrial depolarization is caused by respiratory chain inhibition by CGS7184, cell respiration was measured. We observed an increase of mitochondrial respiration (*Fig. 6A*) suggesting that the CGS7184 effect is not due to respiratory chain inhibition or apoptosis induction but rather it is caused by an influx of cations into the mitochondrial matrix. The effect of CGS7184 on cell respiration was not observed in the absence of external Ca^{2+} or in the presence of a BK_{Ca} channel inhibitor – charybdotoxin (*Fig. 6B*). This suggested that the influx of Ca^{2+} from the external medium, driven by plasma membrane hyperpolarization, contributes to the increase of cell respiration.

Fig. 7 shows that CGS7184 affects cytosolic Ca^{2+} concentration in glioma cells. Addition of CaCl_2 to the cell bath resulted in an only slight increase in $[\text{Ca}^{2+}]_{\text{c}}$, indicating the impermeability of the plasma membrane to Ca^{2+} . Subsequent addition of CGS7184 caused a significant increase of $[\text{Ca}^{2+}]_{\text{c}}$ resulting from Ca^{2+} entry into the cells (*Fig. 7A*). When CGS7184 was added to a Ca^{2+} -free cell bath a substantial $[\text{Ca}^{2+}]_{\text{c}}$ transient was observed due to Ca^{2+} release from the intracellular calcium stores. Addition of CaCl_2 following depletion of the intracellular calcium stores led to an immediate increase in $[\text{Ca}^{2+}]_{\text{c}}$ (*Fig. 7B*). This pattern of $[\text{Ca}^{2+}]_{\text{c}}$ changes was the same as that observed in glioma cells (and many other cell lines) exposed to thapsigargin instead of CGS7184 (data not shown). This suggests that the depletion of calcium stores by CGS7184 opens store-operated calcium channels and activates capacitative Ca^{2+} entry. Alternatively, calcium ions influx into the cell may be induced by transient changes in cytosolic calcium concentration following depletion of endoplasmic reticulum store. An addition of ionomycin to cells pretreated with CGS7184 in the absence of extracellular calcium did not influence $[\text{Ca}^{2+}]_{\text{c}}$. Conversely, CGS7184 did not increase $[\text{Ca}^{2+}]_{\text{c}}$ when applied after addition of ionomycin. This indicates

that the CGS7184-releasable pool of intracellular stored Ca^{2+} and that available for ionomycin completely overlap (*Fig. 7C, 7D*).

Calpains, a family of intracellular cysteine proteases, have been implicated in many cellular processes, such as regulation of signal transduction pathways. Overactivation of calpains following disturbance in calcium homeostasis is a hallmark of many pathological conditions leading to cell death. Hence, we performed measurements of calpain activity in LN229 glioma cells with the use of the fluorescent probe t-Boc-Leu-Met-CMAC. *Fig. 8* shows that 1 μM CGS7184 induces calpain activation observed as an increase of fluorescence at 460 nm.

DISCUSSION

It is believed that ion channels in glioma cells may contribute to invasive migration and proliferation, leading to the malignant behavior of these cells during brain tumor propagation (2, 33-35). Hence, the potassium channels, such as BK_{Ca} channels generally present in glioma cells (2) may represent novel therapeutic targets in the treatment of brain tumors. Additionally, the presence of the BK_{Ca} channel in mitochondria of human glioma LN229 cell line was shown with the use of the patch-clamp technique (8). This suggests a pleiotropic place of action of the BK_{Ca} channel openers in glioma cells, similar as for K_{ATP} channels in cardiac or brain tissue (13).

The BK_{Ca} channels are activated by various potassium channels openers (for review see (36)). The benzimidazolones such as NS1619 have been identified as large conductance potassium channel openers acting both on plasma membrane and mitochondrial channels (37). Indole carboxylate compounds such CGS7181 and CGS7184 have also been identify as BK_{Ca} channel opener. The mechanism of BK_{Ca} channel opening by these substances was previously studied by investing whole-cell BK_{Ca} current and single channel activity using the patch-clamp method in vascular smooth muscle cells (31). With threshold micromolar

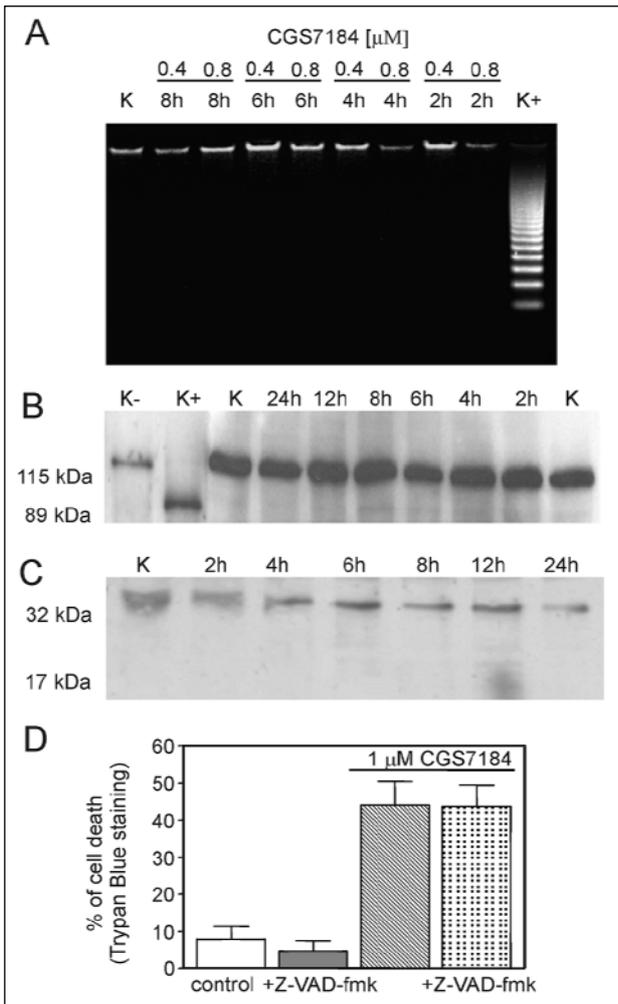


Fig. 4. Potassium channel opener CGS7184 does not induce DNA fragmentation, PARP cleavage or caspase activation. (A) No DNA laddering characteristic for late apoptosis was observed in cells treated with 1 μ M CGS7184 for the time indicated. For all assays glioma cells were incubated in DMEM medium supplemented with 0.1% FBS. (B) Western blot analysis of PARP cleavage in glioma cells treated with 1 μ M CGS7184. The experiment was performed with mouse monoclonal anti-PARP antibody (SA-250) which detects 115 kDa PARP and 89 kDa apoptosis-related cleavage fragment. As a negative control (K-) whole cell extract of human HL60 leukemia cells was used and as positive control (K+) whole cell extract of human HL60 leukemia cells induced to undergo apoptosis by etoposide (SK-003, Biomol). (C) Western blot analysis of caspase-3 cleavage in glioma cells treated with 1 μ M CGS7184. The antibodies used recognize both the 32 kDa unprocessed pro-caspase-3 and the 17 kDa subunit of the active caspase-3. (D) Quantitative analyses of the percentage of cell death after exposure to 1 μ M CGS7184 in the presence and absence of pan-caspase inhibitor z-VAD-fmk (50 μ M). Cell survival was measured using the Trypan blue exclusion assay as described in Material and Methods. Results are presented as the mean \pm SD (n=3). The cells were incubated with the channel opener for 6 hours.

concentrations these compounds caused a drastic and reversible increase the channel open probability. It was concluded that CGS7181 and its analogs directly open large conductance potassium channel from either side of the membrane (31). In

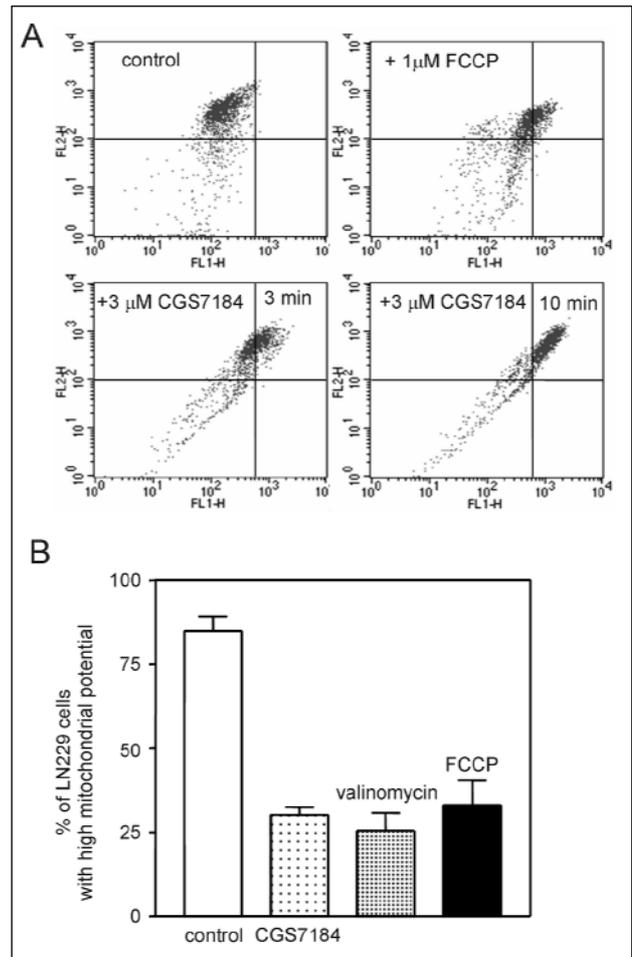


Fig. 5. Effect of BK_{Ca} channel opener CGS7184 on mitochondrial membrane potential in intact glioma cell line LN229. (A) Representative histograms of the changes of mitochondrial membrane potential after cell treatment with 1 μ M FCCP and 3 μ M CGS7184. The assays of membrane potential in cell suspension were performed using flow cytometry and fluorescent dye JC-1 at 25°C as described in Materials and Methods. (B) Depolarization of mitochondrial membrane potential in glioma cells treated with 3 μ M CGS7184, 0.1 μ M valinomycin, or 1 μ M FCCP. The results are expressed as percentage of cells with high mitochondrial potential. Data represent the mean \pm SD from five independent experiments.

addition these substances were shown to modulate cellular function independent on potassium channel modulation. For example, NS1619 were shown to interact with mitochondria in cardiac cells (27) and glioma cells (28) were they inhibit the cellular respiration. Recently, we have shown that CGS7184 regulates endothelial cell function: affecting calcium homeostasis and NO synthesis (38).

Channel openers induce LN229 glioma cell death

The potassium channel openers acting on mitochondrial K⁺ channels are considered to be cytoprotective. These concerns mainly channel openers such as diazoxide or BMS-191095 acting on the mitoK_{ATP} channel in various tissues (39). Recently, the channel opener NS1619 acting on mitochondrial BK_{Ca} channel was shown to have a cardioprotective action (9). In contrast, we observed glioma cell injury caused by BK_{Ca} channel openers such as CGS7184 and CGS7181 in the present study.

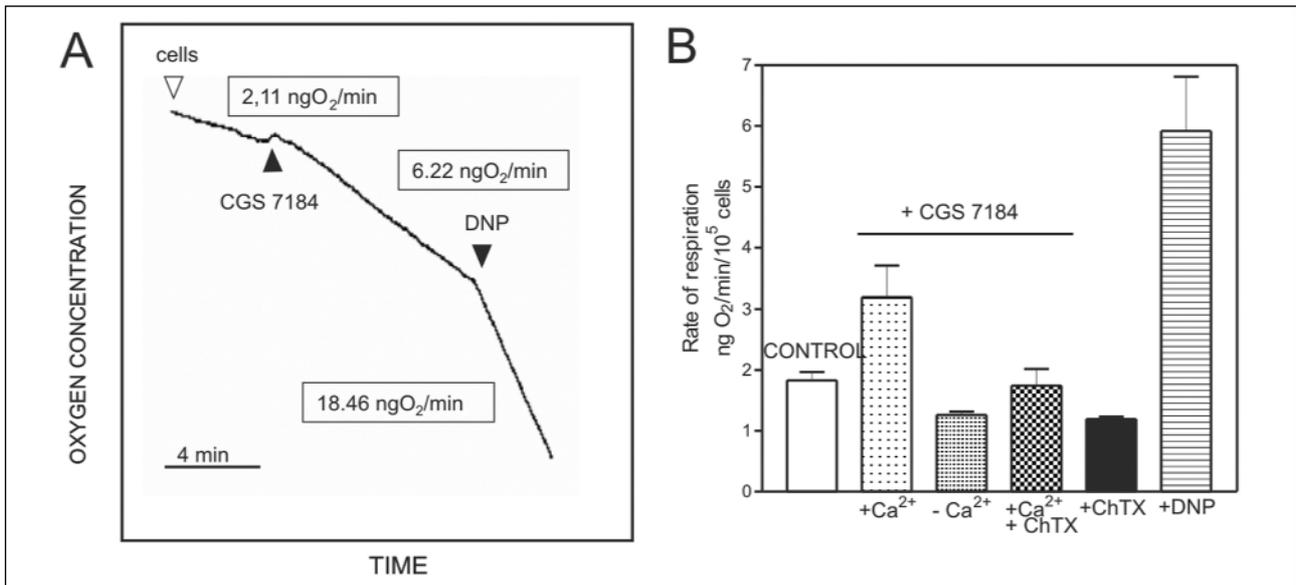


Fig. 6. Inhibitory effect of BK_{Ca} channel opener CGS7184 on respiratory rate in glioma cell line LN229. (A) Increase of rate respiration after addition of 3 μ M CGS7184. Maximal respiration rate was observed after cell treatment with 100 μ M DNP. (B) Effect of BK_{Ca} channel inhibitor charybdotoxin (ChTX) on increased cell respiration induced by 3 μ M CGS7184 in medium containing calcium (2 mM) and without calcium. Data represent the mean \pm SD from three separate experiments.

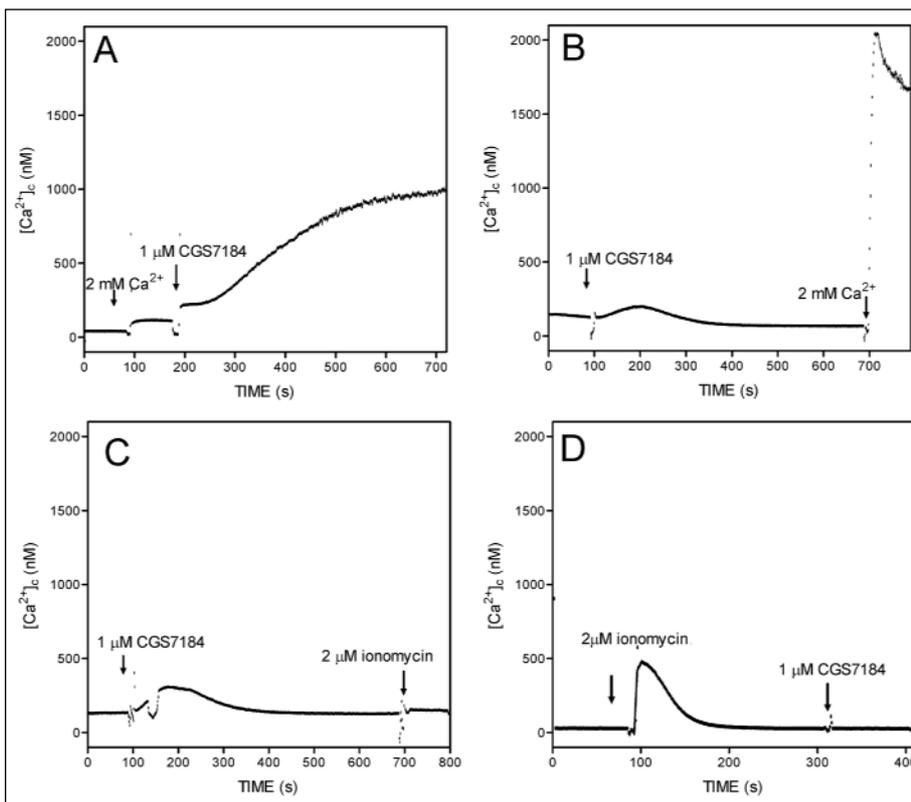


Fig. 7. Effect of BK_{Ca} channel opener CGS7184 on Ca²⁺ release from intracellular stores. (A) Ca²⁺ influx after cell treatment with 1 μ M CGS7184 in the presence of 2 mM Ca²⁺ in medium. Glioma cells were loaded with 1 μ M fura-2/AM in extracellular medium at 37°C for 15 min. Presented traces are representative for four separate experiments. (B) Ca²⁺ release from intracellular stores after cell treatment with 1 μ M CGS7184 in the absence of Ca²⁺ in extracellular medium. (C) Effect of 2 μ M ionomycin on Ca²⁺ release from intracellular stores of glioma cells pretreated with 1 μ M CGS7184. (D) Effect of 1 μ M CGS7184 on Ca²⁺ release from intracellular stores of glioma cells pretreated with 2 μ M ionomycin.

Despite the shrinkage of cell nuclei and fast PS exposure upon CGS7184 treatment no other apoptotic symptoms, such as caspase activation or DNA laddering, were observed.

Indirect action of CGS7184 channel opener on glioma cell mitochondria

In addition to cell injury, depolarization of glioma cell mitochondria was observed upon treatment with the BK_{Ca} channel

opener CGS7184. This was consistent with the previous observation of the presence of mitoBK_{Ca} channel in LN229 glioma cell mitochondria (8). In parallel an increase in glioma cell mitochondria respiration was observed. The effect of CGS7184 on respiration was dependent on the presence of Ca²⁺ outside the cells. Additionally, these effects were abolished by charybdotoxin, suggesting that plasma membrane BK_{Ca} channel plays a role in the amplification of Ca²⁺ influx into the cytosol of glioma cells. In fact, we have also shown the presence of BK_{Ca} channels in the plasma

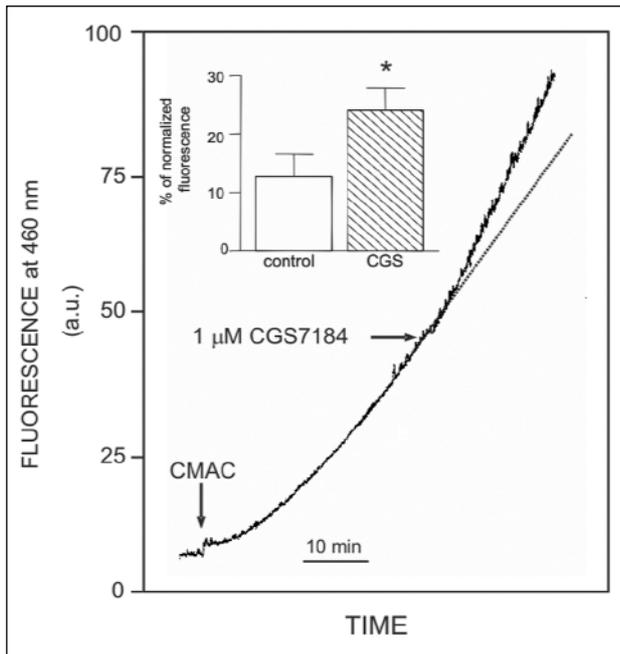


Fig. 8. Effect of BK_{Ca} channel opener CGS7184 on calpain activity in human glioma cell line LN229. Cell suspension (1.5×10^5 cells/ml) in KR-Hepes buffer was loaded with $10 \mu\text{M}$ calpain substrate Boc-Leu-Met-CMAC and fluorescence was measured over 45-50 min. Excitation and emission wavelengths were 380 nm and 460 nm, respectively. Accumulation of calpain hydrolytic product was measured before and after addition of $1 \mu\text{M}$ CGS7184. A representative trace is shown ($n=6$). Inset presents percentage of basal (before addition of $1 \mu\text{M}$ CGS7184 or 0.1% DMSO) activity of calpain in LN229 cells. Data represent the mean \pm SD from three independent experiments. * - $P < 0.001$ compared with control after cell treatment with 0.1% DMSO.

membrane of this cell line (28). Hence, further experiments were focused on the effects of CGS7184 on calcium homeostasis.

Modulation of Ca²⁺ homeostasis leads to calpain activation

CGS7184-induced activation of Ca²⁺ entry (following endoplasmic reticulum Ca²⁺ release) into glioma cells and the consequent increased [Ca²⁺]_c seems to be responsible for the decrease of the mitochondrial potential and the resulting stimulated mitochondrial oxygen consumption. It is likely that the CGS7184-evoked high [Ca²⁺]_c supports plasma membrane BK_{Ca} channel activity. This effect may be independent of and additive to the CGS7184 action as a channel opener. Therefore, this "side effect" of CGS7184 on cellular calcium homeostasis should be considered when this drug is used in the study of both mitochondrial and plasma membrane BK_{Ca} channels.

Calcium release from the endoplasmic reticulum can be caused by inhibition of calcium ATPase by CGS7184 or may be due to an increase in the ER permeability to calcium ions caused by this drug. The molecular mechanism of CGS-type potassium channel opener action on ER calcium release/uptake needs further clarification.

The increase of cytosolic calcium concentration upon CGS7184 treatment of glioma cells followed by activation of calpains is probably the main phenomenon leading to LN229 glioma cell death (*Fig. 9*). Recently, calpains have been increasingly implicated in apoptosis-like programmed cell death (40). Classical apoptosis pathways that requires caspase-3

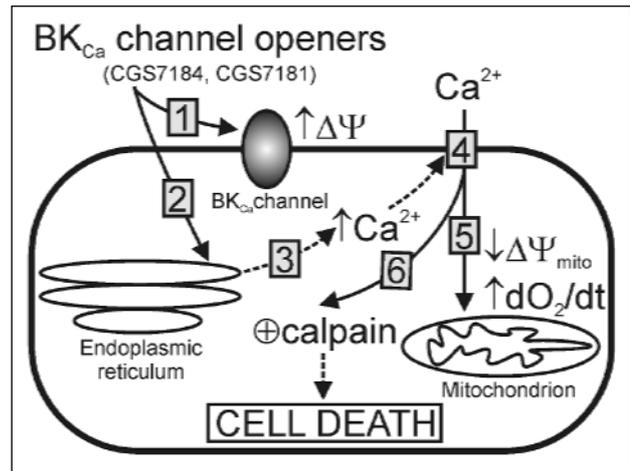


Fig. 9. Hypothetical pathway of BK_{Ca} channel opener action inducing glioma cell death. BK_{Ca} channel openers such as CGS7184 and CGS7181 act on the BK_{Ca} channel present in the plasma membrane of glioma cells (1). In parallel these drugs interact with the endoplasmic reticulum (2) leading to an increase of cytosolic Ca²⁺ concentration (3). This further stimulates a massive influx of calcium ions *via* calcium induced calcium transport system into the cytosol (4). Mitochondrial uptake of calcium leads to an increase of mitochondrial respiration and mitochondrial membrane depolarization (5). In parallel, calcium ions lead to calpain stimulation (⊕) and cell death (6).

activity is chromatin fragmentation. Like caspases, calpains are a family of cytosolic cysteine proteases, but require binding of Ca²⁺ for their activation (41). Although CGS-type channel openers induced cell death was not accompanied by chromatin fragmentation, nuclear condensation and cell shrinkage was clearly evident. Apoptosis-like cell death frequently involves alternative intracellular proteases such as calpains. Recently, ceramide induced apoptosis of medulloblastoma cells was associated with the activation of calpains but not caspases (42).

The direct contribution of mitochondrial depolarization and/or the increase of mitochondrial respiration (upon channel opener action) to glioma cell death are problematic. We were unable to prove activation by CGS-type openers BK_{Ca} channels present in glioma cell mitochondria. On the other hand our results may suggest an important role of mitochondria and endoplasmic reticulum cross-talk in cell death, as previously postulated (43). A similar problem concerns the cellular specificity of CGS7184 action. Preliminary studies on the hippocampal cell line HT22 indicate that higher concentrations of CGS7184 are needed to induce injury similar to that caused in the LN229 glioma cell line (data not shown). In contrast, C2C12 and L6 myoblast cell lines are more vulnerable to CGS7184 treatment than LN229 glioma cells (data not shown). Understanding these mechanisms can provide a starting point for therapeutic strategies to treat tumor growth.

In summary, we have shown that the potassium channel openers CGS7181 and CGS7184 induce glioma cell death. This effect is due to the modulation of calcium homeostasis by BK_{Ca} channel openers leading to activation of calpains.

Acknowledgements: We are indebted to Dr. N. de Tribolet (Lausanne, Switzerland) for the gift of the LN229 glioma cells. The authors are grateful to Beata Kuzniarska for technical support. This work was supported by the Nencki Institute of Experimental Biology and Polish Mitochondrial Network, by State Committee for Scientific Research grant No. 6P04A01219,

3P04A05025, P-N/031/2006 and PBZ-MIN-001/P05/11, and a grant from SGGW No. 50406020013.

Conflict of interests: None declared.

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Received: September 26, 2008

Accepted: November 6, 2009

Author's address: Prof. Adam Szewczyk, Laboratory of Intracellular Ion Channels, Department of Biochemistry, Nencki Institute of Experimental Biology, 3 Pasteur St., 02-096 Warsaw, Poland; Phone: (4822)6598571; Fax: (4822)8225342, E-mail: adam@nencki.gov.pl