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The potassium channel opener CGS7184 activates Ca^{2+} release from the endoplasmic reticulum

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ABSTRACT

CGS7184 (ethyl 1-[[(4-chlorophenyl)amino]oxo]-2-hydroxy-6-trifluoromethyl-1*H*-indole-3-carboxylate) is a synthetic large-conductance Ca^{2+}-activated potassium (BK_{Ca}) channel opener. The existing literature suggests that potassium channels are involved in cardioprotection, particularly during ischemia-reperfusion events. However, the cellular mechanisms mediating the effects of CGS7184 remain unclear. In the present study, we investigated the effect of the BK_{Ca} channel opener CGS7184 on Ca^{2+} homeostasis in H9C2 and C2C12 cell lines, Ca^{2+} uptake by isolated sarcoplasmic reticulum (SR) vesicles, SR Ca^{2+}-ATPase (SERCA) activity, and single-channel properties of the ryanodine receptor calcium release channel (RYR2) when incorporated into a planar lipid bilayer. The effects of CGS7184 on calcium homeostasis in C2C12 and H9C2 cell lines were measured with a Fura-2 fluorescent indicator. The BK_{Ca} channel opener CGS7184, when added to the H9C2 and C2C12 cells, caused a concentration-dependent release of calcium from internal stores. Calcium accumulation by the SR vesicles isolated from cardiac and skeletal muscle was inhibited by CGS7184 with a half-maximal inhibition value of 0.45 ± 0.04 μM and 0.37 ± 0.03 μM, respectively. The results of the present study indicate that the BK_{Ca} channel opener CGS7184 modulates cytosolic Ca^{2+} concentration in H9C2 and C1C12 cells due to its interaction with the endoplasmic reticulum (ER). CGS7184 approximately doubled the opening probability of RYR2 channels; however, the compound seemed to most strongly affect channels with a higher control activity. These results strongly suggest that the BK_{Ca} channel opener CGS7184 affects intracellular calcium homeostasis by interacting with the sarcoplasmic reticulum RYR2 channels.

1. Introduction

Large-conductance Ca^{2+}-activated potassium (BK_{Ca}) channels are present in a variety of electrically excitable and non-excitable cells. BK_{Ca} channels are involved in cytoprotection during ischemia-reperfusion, neuronal secretion, hypertension, erectile responses, and cell metastasis (Cui et al., 2009; Eichhorn and Dobrev, 2007; Feletou et al., 2009). They link membrane excitability with intracellular Ca^{2+} signalling and are important in smooth muscle contraction (Sah, 1996). BK_{Ca} channels belong to a family of K^+ channels that are activated by membrane depolarisation or elevated cytosolic Ca^{2+} concentrations. These channels comprise a unique class of ion channels (Cui et al., 2009; Ghatta et al., 2006; Sah, 1996). Beneficial effects of BK_{Ca} channels on neuronal survival have been attributed primarily to plasma membrane hyperpolarisation, as BK_{Ca} channels are voltage- and calcium-dependent potassium channels whose activation tends to reduce cellular excitability (Runden-Pran et al., 2002). BK_{Ca} channels are also present as inner mitochondrial membrane ($mitoBK_{Ca}$) channels. The first observed $mitoBK_{Ca}$ channel was noted in LN229 cells using a patch-clamp technique (Siemen et al., 1999). Recently, $mitoBK_{Ca}$ channels were discovered in inner mitochondrial membranes of various cell types (Szewczyk et al., 2009).

BK_{Ca} channels are modulated by natural and synthetic compounds (Bentzen et al., 2007; Calderone et al., 2007; Candia et al., 1992; Nardi and Olesen, 2008; Sakamoto et al., 2008; Wang et al., 2008; Zhang et al., 2010). The stimulatory activities of NS004, NS1619, and NS1608 have been studied extensively and are well documented in cloned and native BK_{Ca} channels (Edwards et al., 1994; Hu and Kim, 1996; Olesen et al., 1994; Xu et al., 1994). The major limitations of this class of compounds are their weak potency and insufficient selectivity, which render them inadequate as pharmacological probes or therapeutic agents (Edwards et al., 1994; Holland et al., 1996; Hu and Kim, 1996; Xu et al., 1994). In contrast ethyl 1-[[(4-chlorophenyl)-amino]oxo]-2-hydroxy-6-trifluoromethyl-1*H*-indole-3-carboxylate

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(CGS7184), a more potent compound, has been found to produce BK_{Ca} channel-opening effect with a threshold effective concentration slightly below 0.1 μM when assessed using inside-out patches in rat and guinea pig bladder cells (Hu et al., 1997).

Recently, we demonstrated that CGS7184 reduced reactive oxygen species production by respiratory chain complex I in mitochondria isolated from rat brains (Kulawiak et al., 2008). We have shown that CGS7184 affects mitochondrial function by changes in mitochondrial potential, oxygen consumption in glioma and EA.hy 926 cells, and activation of nitric oxide synthase pathways in EA.hy 926 cells (Debska-Vielhaber et al., 2009). An additional effect of CGS7184 on glioma cells is cell death, which is caused by an increase in cytosolic Ca^{2+} concentration followed by the activation of calpains (Debska-Vielhaber et al., 2009). The effects triggered by CGS7184 in EA.hy 926 and glioma cells seem to be related to modulation of intracellular Ca^{2+} homeostasis (Wrzosek et al., 2009).

In the present study, our aim was to identify targets for the large-conductance potassium channel opener CGS7184 in the context of Ca^{2+} homeostasis. A direct effect of CGS7184 on SR, especially the ryanodine receptor calcium release (RYR2) channel was observed.

2. Methods

2.1. Chemicals

Dulbecco's modified Eagle's medium and phosphate-buffered saline were purchased from the Institute of Immunology and Experimental Therapy (Wroclaw, Poland). Foetal bovine serum, L-glutamine, and penicillin-streptomycin were obtained from GIBCO (Paisley, Scotland). Fura-2 acetoxymethyl-ester (Fura-2 AM) was purchased from molecular probes (Eugene, Oregon) and dissolved in dimethyl sulfoxide (DMSO) before use. Because some compounds of interest were dissolved in DMSO, we also used this solvent for control experiments. CGS7184 was a kind gift from Novartis (Basel, Switzerland). All other chemicals used were of high grade and obtained from Sigma unless otherwise stated. Our investigations involving animals conform to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health, and the experimental procedures used in the present study were approved by the local animal research committee of the Nencki Institute of Experimental Biology.

2.2. Isolation of sarcoplasmic reticulum (SR) vesicles from skeletal muscle

The SR vesicles from skeletal muscle were prepared as described previously (Chu et al., 1988) with some modifications (Suko and Hellmann, 1998). Briefly, Wistar rats weighing approximately 250 g were sacrificed. All remaining procedures were performed at 0–4 °C. The white back muscles and the leg muscles (fast twitch muscle) were quickly excised; rinsed in homogenising medium; trimmed of fat, connective tissue, and red muscle; and minced. A 50 g portion of ground muscle was homogenised with 500 ml of homogenizing medium (consisting of 5 mM imidazole pH 7.4, 100 mM NaCl, and 0.1 mM PMSF adjusted with HCl at room temperature) in a Waring blender for 1.5 min at maximal speed. The homogenates were centrifuged in a Sorvall RC 6 Plus centrifuge with a SLA-1500 rotor for 20 min at 4000 × *g*. The supernatant was filtered through layers of cheesecloth and centrifuged in a Sorvall RC 6 Plus centrifuge with a SLA-1500 rotor for 20 min at 5465 × *g*. The supernatant was filtered through layers of cheesecloth and centrifuged in a Sorvall centrifuge with a Type 45 Ti rotor for 60 min at 110,000 × *g*. The pellets were combined and resuspended in resuspension medium consisting of 5 mM imidazole-HCl (pH 7.4), 600 mM KCl, 250 mM sucrose, and 0.1 mM PMSF. Each microsomal fraction was loaded on a sucrose step-gradient constructed of 45%, 38%, 34%, 32%, and 27% sucrose (w/w) in 5 mM imidazole-HCl (pH 7.4) and 100 mM NaCl. The gradient was centrifuged in a Sorvall centrifuge with an AH-629 rotor for 3 h at 110,000 × *g*. The 32–34% and 38–45% fractions were collected and diluted with 10 mM HEPES–Tris pH 7.4, 100 mM NaCl, and 0.1 mM PMSF and sedimented for 1 h at 125,000 × *g*. The pellets were resuspended in storage buffer consisting of 10 mM HEPES–Tris pH 7.4, 100 mM NaCl, 250 mM sucrose, and 0.1 mM PMSF to give a protein concentration of approximately 10 mg/ml, divided into appropriate aliquots, snap-frozen in liquid nitrogen, and stored at −70 °C until use.

2.3. Isolation of sarcoplasmic reticulum vesicles from rat cardiac muscle

Cardiac muscle SR vesicles were isolated from Wistar rat ventricular tissue according to a previously described method (Buck et al., 1999) with a few modifications, such as the omission of the sucrose gradient step (Chamberlain et al., 1983). Briefly, three animals weighing between 250 and 350 g were anesthetised with a sublethal dose of Nembutal (30 mg/kg) and sacrificed by cervical dislocation. The hearts were immediately removed and immersed in washing solution (0.154 M NaCl, 0.29 M sucrose) at room temperature and then transferred to ice-cold washing solution. The ventricles were trimmed of fat, atria, connective tissue, and large vessels, blotted, and weighted. The isolated ventricles were minced and homogenised in 7 volumes of homogenisation solution composed of 300 mM sucrose, 0.5 mM DTT, 0.1 mM PMSF, and 20 mM K/HEPES (pH 7.4) at 4 °C for 60 s in a Waring blender. The homogenate was centrifuged for 20 min at 9200 × *g* in a SS-34 rotor in a Sorvall RC 6 plus centrifuge. A crude microsomal fraction was obtained from the supernatant by centrifugation for 60 min at 90,000 × *g* in Beckman Type 60 Ti rotor. The soft pellets were resuspended for salt washing in solution (290 mM sucrose, 650 mM KCl, 0.5 mM DTT, 10 mM MOPS, pH 6.8) via manual homogenisation in a Potter tissue grinder with a Teflon pestle. After 60 min incubation on ice, the solution was centrifuged for 10 min at 4400 × *g* in a SS-34 rotor to remove large aggregates. The supernatants were centrifuged for 60 min at 100,000 × *g* in a Beckman Type 60 Ti rotor. The pellet was resuspended by manual homogenisation in a Potter tissue grinder to a protein concentration of 5–10 mg/ml in solution (290 mM sucrose, 200 mM KCl, 5 mM MOPS, pH 6.8). The samples were then snap-frozen in liquid nitrogen and stored at −70 °C until use.

2.4. Calcium uptake measurements

The calcium uptake of the SR was measured at 37 °C based on Fura-2 pentapotassium salt fluorescence assaying (Kargacin et al., 2000; Wrzosek et al., 1992) with modifications. Briefly, fluorescence was measured using a SPEX Fluorolog fluorimeter (SPEX Instrument Inc., USA). Fura-2 free acid (1 μM) and SR vesicles were added to 3 ml of uptake buffer composed of 20 mM HEPES–Tris pH 7.0, 100 mM KCl, 100 mM sucrose, 2 mM $MgCl_2$, 5 mM oxalate, 2 mM NaN_3, 1.1 mM creatine phosphate, and 3 U/ml creatine phosphokinase in a 3-ml cuvette. Ca^{2+} uptake was initiated by the addition of 1 mM ATP. The free Ca^{2+} concentration, when not buffered by EGTA, was greater than 10 μM. The excitation wavelengths (340 nm and 380 nm, with emission at 510 nm) were alternated every 0.2 s, and the 340 nm/380 nm fluorescence ratio was determined at 1 s intervals.