Anti-apoptotic effect of cGMP in cultured astrocytes: inhibition by cGMP-dependent protein kinase of mitochondrial permeable transition pore

Kazuhiro Takuma‡, Patamawan Phuagphong†, Eibai Lee**, Koichi Mori‡, Akemichi Baba†, and Toshio Matsuda§

From the Departments of ‡Analytical Chemistry and **Pharmacology, Faculty of Pharmaceutical Sciences and High Technology Research Center, Kobe Gakuin University, Kobe 651-2180 Japan, and the Laboratories of †Molecular Neuropharmacology and §Medicinal Pharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka 565-0871, Japan

Running title: Protection by cGMP of mitochondrial dysfunction

Correspondence to: Dr. T. Matsuda, Laboratory of Medicinal Pharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, 1-6 Yamada-oka, Suita, Osaka 565-0871, JAPAN.
TEL: +81-6-6879-8161, FAX: +81-6-6879-8159, E-mail: matsuda@phs.osaka-u.ac.jp.
Reperfusion of cultured astrocytes with normal medium after exposure to hydrogen peroxide (H₂O₂)-containing medium causes apoptosis. We have recently shown that ibudilast, which has been used for bronchial asthma and cerebrovascular disorders, attenuated the H₂O₂-induced apoptosis of astrocytes via cGMP signaling pathway. This study examined the mechanism underlying the protective effect of cGMP. The membrane-permeable cGMP analog dibutryl-cGMP attenuated the H₂O₂-induced decrease in cell viability, DNA ladder formation, nuclear condensation, reduction of the mitochondrial membrane potential, cytochrome c release from mitochondria and caspase-3 activation in a dose-dependent manner in cultured astrocytes. These effects of dibutryl-cGMP were almost completely inhibited by the cGMP-dependent protein kinase (PKG) inhibitor KT5823. In isolated rat brain mitochondria, cGMP in the presence of cytosolic extract from astrocytes inhibited the mitochondrial permeability transition pore (PTP) as determined by monitoring Ca²⁺-induced mitochondrial swelling. This ability of the cytosolic extract was inactivated by heat treatment and was mimicked by exogenous PKG. The effect of cGMP on the mitochondrial swelling was blocked by KT5823. The PTP inhibitors cyclosporin A and bongkrekic acid prevented the H₂O₂-induced decrease in cell viability and caspase-3 activation. These findings demonstrate that cGMP inhibits the mitochondrial PTP via the activation of PKG, and the prevention of mitochondrial dysfunction contributes to its anti-apoptotic effect.
We previously showed that reperfusion of cultured rat astrocytes with Ca\(^{2+}\)-containing medium after exposure to Ca\(^{2+}\)-free medium caused an increase in intracellular Ca\(^{2+}\) concentration followed by delayed cell death, including apoptosis (1-3). This injury is considered to be an *in vitro* model of ischemia/reperfusion injury, because a similar paradoxical change in extracellular Ca\(^{2+}\) concentration is reported in ischemic brain tissue (4-6). Subsequently, we have found that the Ca\(^{2+}\) reperfusion injury was mimicked by reperfusion after exposure to hydrogen peroxide (H\(_2\)O\(_2\)) (3). Our previous studies using the astrocytic injury models show that heat shock protein (7), calcineurin (8), mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (9), phosphatidylinositol-3 kinase (9,10) and cGMP phosphodiesterase (11) are target molecules for anti-apoptotic drugs in astrocytic apoptosis. The role of cGMP in preventing apoptosis is also reported in B lymphocytes (12), T lymphocytes (13), eosinophils (14), motor and sympathetic neurons (15,16), hepatocytes (17,18), PC12 cells (15,19), and ovarian follicles (20). However, it is not known how cGMP prevents apoptosis signaling and supports survival.

It is considered that the mitochondrial permeability transition pore (PTP) and associated release of cytochrome c are important in the apoptotic process. Kim et al. (19) and Li et al. (18) have recently reported that cGMP inhibits caspase activation and cytochrome c release. We (11) found that ibudilast, which has been clinically used for bronchial asthma and cerebrovascular disorders, inhibited the H\(_2\)O\(_2\)-induced cytochrome c release and caspase-3 activation in a cGMP-dependent mechanism. It is not known whether cGMP acts on the mitochondrial PTP. In this study, we examined the effects of cGMP on mitochondrial dysfunction resulting in astrocytic apoptosis and on mitochondrial PTP in isolated brain mitochondria. The present study demonstrates that dibutyryl-cGMP prevents the H\(_2\)O\(_2\)-induced reduction of the mitochondrial membrane potential, cytochrome c release and caspase
activation. In addition, we demonstrate in isolated rat brain mitochondria that cGMP inhibits the mitochondrial PTP in a PKG-mediated mechanism as determined by monitoring Ca\(^{2+}\)-induced mitochondrial swelling.
MATERIALS AND METHODS

Materials—Drugs were obtained from the following sources: mouse anti-glial fibrillary acidic protein antiserum, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), cGMP, dibutyryl-cGMP, 8-(4-chlorophenylthio)-cGMP (8-pCPT-cGMP), Rp-8-{[4-chlorophenyl]thio}-guanosine-3’5’-cyclic monophosphorothioate (Rp-8-pCPT-cGMPS), isolectin B4 (Biotin labeled), cyclosporin A, Sigma Chemical Co. (St. Louis, MO); Sp-8-{[4-chlorophenyl]thio}-guanosine-3’5’-cyclic monophosphorothioate (Sp-8-pCPT-cGMPS), Biomol Research Laboratories, Inc. (Plymouth Meeting, PA); protein kinase G, KT5823, 2’-amino-3’-methoxyflavone (PD98059), bongkrekic acid, Calbiochem (La Jolla, CA); horseradish peroxidase-labeled anti-mouse Ig, Amersham Pharmacia Biotech. UK, Ltd. (Buckinghamshire, England); Hoechst 33342, 3,3’-dihexylxocarbocyanine (DiOCl(3)), Molecular Probes, Inc. (Eugene, OR); 7-amino-4-methyl-coumarin (AMC), acetyl-L-aspartyl-L-glutamiyl-L-aspartic acid α-(4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA), Peptide Institute, Inc. (Osaka, Japan); anti-cytochrome c antibody (clone 7H8.2C12), Pharmingen (San Diego, CA); wortmannin, Nacalai Tesque (Kyoto, Japan); Eagle’s minimum essential medium, Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan), tissue culture wares, Iwaki Glass Co., Ltd. (Tokyo, Japan). All other chemicals used were of the highest purity commercially available.

Astrocyte culture—Astrocytes were isolated from the cerebral cortices of 1-day-old Wistar rats as previously reported (21-23). Cells were plated in 75-ml tissue culture flasks, split once upon confluence, and plated in 24-well (for MTT assay) and 96-well (for mitochondria energization and caspase assays) plastic tissue culture plates, 60-mm (for DNA ladder) and 100-mm (for cytochrome c release analysis and cell extract preparation) plastic tissue culture dishes, and 4-well chamber slides (for fluorescence microscopy). The second cultures were grown for 14-20 days in all experiments. The cells were routinely >95% positive for glial fibrillary acidic protein, and approximately 2% of the cells showed microglia, based on positive isolectin B4 staining.

Cell viability—Reperfusion experiments were carried out as previously reported (9,10). Cells were exposed to H2O2 (100 µM)-containing Earle’s solution for 30 min, and then incubated with normal Earle’s solution for the indicated times. MTT reduction activity was measured by a colorimetric assay as reported previously (1,3). MTT reduction activity is expressed as a percentage of control.

DNA ladder and Hoechst 33342 staining—DNA was extracted and subjected to 1.8% agarose gel electrophoresis as previously reported (9,10). DNA in the gel was stained with ethidium bromide and photographed with a Polaroid (type 667) under UV light. To observe individual nuclei, the cells plated on a chamber slide were fixed with 4% formaldehyde and stained with Hoechst 3342 as previously reported (3,10). An Olympus IX70 inverted fluorescence microscope was equipped with a cooled CCD camera system (Roper Scientific, Photometrics CoolSNAP) to scan the staining nuclear images.

Measurement of Mitochondrial Energization—Mitochondria energization was determined as the retention of the mitochondria-specific dye DiOCl(3) (24). Cells were loaded with 100 nM DiOCl(3) during the last 30 min of treatment. After removing the medium, the cells were washed twice with PBS and then lysed by the addition of 100 µl of deionized water. The concentration of retained DiOCl(3) was measured with excitation at 485 nm and emission at 510 nm using a Wallac Multilabel counter.

Measurement of cytochrome c release—Cytosol and membrane fractions were prepared as previously reported (11). The protein contents of the cytosol and membrane fractions were determined by a BioRad DC protein assay (BioRad Laboratories, Hercules, CA), and 15 µg of the sample was subjected to SDS-polyacrylamide gel electrophoresis (15% polyacrylamide).
A cytochrome c antibody (1:1000) was used for immunoblotting.

Measurement of caspase activity—The activity of caspase-3-like protease in cell lysates was measured using the fluorogenic substrate Ac-DEVD-MCA (11). After reacting for 1 h at 37°C, the released 7-amino-4-methyl-coumarin levels were measured with excitation at 355 nm and emission at 460 nm using a Wallac Multilabel counter.

Cytosolic extract from astrocytes—The cytosol extracts were prepared as follows. Cells plated on 100 mm dishes were washed twice with cold phosphate-buffered saline, scraped off using a policeman, and collected by centrifugation at 300 g for 10 min at 4°C. The pellet was suspended in 100 µl of lysis buffer (320 mM sucrose, 10 mM Tris-base, pH 7.4), and disrupted with 10 strokes of a Daunce homogenizer. The homogenate was centrifuged at 105,000 g for 1 h at 4°C, and the resulting supernatant (approximately 10 mg protein/ml) was kept at –80°C.

Measurement of the MPT pore opening by swelling in isolated mitochondria—Brain mitochondria were isolated from male Wistar rats (120-140 g) by the method of Sims (25) using a discontinuous Percoll gradient centrifugation. The mitochondrial fraction was resuspended in buffer containing sucrose (320 mM) and Tris-base (10 mM, pH 7.4) and kept on ice until use. Opening of the PTP causes mitochondrial swelling that is conveniently assayed as a decrease in the light scattering (and thus absorbance) of a mitochondrial suspension. The Ca2+-induced mitochondrial swelling was assayed essentially as described by Friberg et al. (26). Rotenone, antimycin and A23187 were added 5 min before the addition of CaCl2 solution to ensure complete equilibration of calcium ions across the mitochondrial membrane under de-energized conditions. The treatment of mitochondrial suspension (0.5 mg/ml) with cGMP, the cytosolic extract or PKG was carried out for 15 min before the addition of CaCl2 solution. The calcium ion concentrations were calculated from the NTA buffering (27). Absorbance at 540 nm was measured using a Shimadzu UV1200 spectrophotometer (Kyoto, Japan). Each experiment shown is representative of at least three similar experiments performed on separate mitochondrial preparations.

Statistics—Statistical analysis of the experimental data was carried out by Student-Newman-Keuls test, Dunnett’s t test or Tukey HSD test, using a software package (Stat View 5.0) for Apple Macintosh.

RESULTS

Reperfusion after exposure of astrocytes to H2O2-containing medium causes a decrease in MTT reduction activity, DNA ladder formation, cytochrome c release from mitochondria and caspase-3-like protease activation (3). Fig. 1 shows the effects of membrane-permeable cGMP analogs on the H2O2-induced apoptosis. Dibutyryl-cGMP, 8-pCPT-cGMP and Sp-8-pCPT-cGMPS attenuated significantly the H2O2-induced decrease in MTT reduction activity in a dose-dependent manner: the effect of Sp-8-pCPT-cGMPS was more potent than those of dibutyryl-cGMP and 8-pCPT-cGMP. The protective effect of dibutyryl-cGMP was observed even in the presence of the ERK kinase inhibitor PD98059 and the phosphoinositide-3 kinase inhibitor wortmannin (data not shown). Dibutyryl-cGMP also attenuated the H2O2-induced DNA ladder formation (Fig. 2A) and nuclear condensation (Fig. 2B). Reperfusion after the exposure of astrocytes to H2O2 also caused a decrease in DiOC6(3) retention, which reflects mitochondrial membrane potential (Fig. 3A). The H2O2-induced loss of mitochondrial membrane potential was inhibited by dibutyryl-cGMP in a dose-dependent manner (Fig. 3B). Furthermore, dibutyryl-cGMP attenuated the H2O2-induced cytochrome c release from mitochondria and caspase-3-like protease activation in a dose-dependent manner (Fig. 4).

The protection by dibutyryl-cGMP against the H2O2-induced decrease in MTT reduction activity was blocked by the PKG inhibitors KT5823 and Rp-8-pCPT-cGMPS (Fig. 5A).
KT5823 also blocked the effect of dibutyryl-cGMP on mitochondrial membrane potential (Fig. 5B), cytochrome c release (Fig. 5C) and caspase-3-like protease activation (Fig. 5D).

A pilot experiment showed that exposure of purified rat brain mitochondria to 30-100 µM Ca\(^{2+}\) caused a significant decrease in absorbance, indicating mitochondrial swelling by the PTP opening (data not shown). The swelling was dependent on Ca\(^{2+}\) concentration as reported previously (26). In this study, we used 100 µM Ca\(^{2+}\) to induce mitochondrial swelling. The swelling was inhibited by the mitochondrial PTP inhibitors cyclosporin A (28) and bongkrekic acid (29) (Fig. 6A). Neither cGMP nor cytosolic extract from astrocytes affected the Ca\(^{2+}\)-induced mitochondrial swelling, but simultaneous addition of cGMP and the cytosolic extract prevented the swelling in dose-dependent manners (Fig. 6B, 6C). The effect of cGMP plus the cytosolic extract was also observed in the swelling induced by 30 µM Ca\(^{2+}\) (data not shown). The ability of the cytosolic extract was inactivated by heat-treating it (Fig. 7A) and was mimicked by exogenous PKG (Fig. 7B). The similar protection by cGMP against Ca\(^{2+}\)-induced swelling was observed even when cGMP was added together with or after Ca\(^{2+}\) (data not shown). KT5823 antagonized the effect of cGMP plus the cytosolic extract (Fig. 7C) or PKG (Fig. 7B) in inhibiting the Ca\(^{2+}\)-induced swelling. The mitochondrial PTP inhibitors cyclosporin A and bongkrekic acid inhibited the H\(_2\)O\(_2\)-induced decrease in MTT reduction activity and activation of caspase-3-like protease in cultured astrocytes (Fig. 8). In this experiment, bongkrekic acid was used at a relatively low concentration, because at 50 µM it showed cell toxicity (data not shown).

**DISCUSSION**

This study was undertaken to characterize the mechanism by which cGMP suppresses the H\(_2\)O\(_2\)-induced apoptosis in cultured astrocytes. The membrane-permeable cGMP analogs dibutyryl-cGMP, 8-pCPT-cGMP and Sp-8-pCPT-cGMPS attenuated the H\(_2\)O\(_2\)-induced decrease in cell viability. In view of the importance of mitochondria in the apoptotic process, the present study focused on the effect of cGMP on the H\(_2\)O\(_2\)-induced mitochondrial dysfunction. We showed that dibutyryl-cGMP prevented the reduction of the mitochondrial membrane potential and cytochrome c release, and consequently abolished caspase-3 activation, nuclear condensation and DNA fragmentation in cultured astrocytes. These findings suggest that cGMP inhibits the H\(_2\)O\(_2\)-induced mitochondrial dysfunction and prevents astrocyte apoptosis. The inhibitory effect of cGMP analog on caspase-3 activation and cytochrome c release was also observed in hepatocytes (17,18) and PC12 cells (19). Many of the cellular effects of cGMP are mediated by PKG. In this respect, Kim et al. (19) reported that the PKG inhibitor KT5823 reversed 8-Br-cGMP-mediated prevention of cytochrome c release and caspase-3 activation in PC12 cells. We also found that KT5823 almost completely blocked the protection provided by dibutyryl-cGMP against the H\(_2\)O\(_2\)-induced reduction of the mitochondrial membrane potential, cytochrome c release and caspase-3 activation in astrocytes. These findings suggest that cGMP analogs prevent astrocyte apoptosis via the activation of PKG, although the exact mechanism by which cGMP/PKG prevents apoptotic events is not known.

Li et al. (18) reported that cAMP as well as cGMP analogs suppressed tumor necrosis-factor α plus actinomycin D-induced apoptosis in hepatocytes. However, it is unlikely that cAMP signaling also plays a role in prevention of astrocyte apoptosis, since cAMP phosphodiesterase inhibitors do not protect astrocytes against the H\(_2\)O\(_2\)-induced cytotoxicity (11). On the other hand, we reported that the extracellular signal-regulated kinase (ERK) signal plays a role in the protective effect of T-588 and NGF against H\(_2\)O\(_2\)-induced astrocyte apoptosis (9). In view of the recent studies that cGMP activates ERK (30-32), it was considered whether the effect of cGMP reported here may be mediated by the activation of
ERK or phosphatidylinositol-3 kinase. However, the protective effect of dibutyryl-cGMP was observed even in the presence of the ERK kinase inhibitor PD98059 and the phosphoinositide-3 kinase inhibitor wortmannin.

Activation of the mitochondrial PTP has been identified as a possible common effector of the cell death of numerous cell types in response to both necrotic and apoptotic stimuli (33,34). Thus, it seems likely that the anti-apoptotic effect of cGMP may be mediated by inhibition of the mitochondrial PTP, however there are no reports on the effect of cGMP on the PTP. We demonstrate here that cGMP together with PKG, as well as cyclosporin A and bongkrekic acid, inhibits the Ca\(^{2+}\)-induced mitochondrial swelling in isolated brain mitochondria. The effect of cGMP is dose-dependent and observed even at 1 μM, suggesting that cGMP is an endogenous potent inhibitor of the PTP. The effect of cGMP on the mitochondrial PTP required the presence of the cytosolic extract and the ability of the cytosolic extract was inactivated by heat-treatment. Furthermore, the effect of cGMP plus the cytosolic extract was antagonized by KT5823, and exogenous PKG mimicked the effect of the cytosolic extract. Taken together, it is likely that cGMP inhibits the mitochondrial PTP via the activation of PKG.

The mitochondrial PTP is formed from a complex of the voltage-dependent anion channel, the adenine nucleotide translocase and cyclophilin-D at contact sites between the mitochondrial outer and inner membranes. It is regulated by numerous endogenous physiological effectors such as ions, protons, the mitochondrial transmembrane potential, the concentration of adenine nucleotides, the pyrimidine redox state, the thiol redox state, reactive oxygen species and Bcl-2 family proteins (34). Genaro et al. (12) reported that an induction of Bel-2 is involved in the anti-apoptotic effect of cGMP in splenocytes. In contrast, the effect of cGMP was observed even when it was added together with or after Ca\(^{2+}\). In view of the rapid effect of cGMP, it is unlikely that these endogenous effectors may be involved in the effect of cGMP on the mitochondrial PTP. The present study using a cell-free system suggests that cGMP interacts directly with mitochondria resulting in inhibition of the mitochondrial PTP via the activation of PKG. It is not known how cGMP/PKG signaling affects the mitochondrial PTP. Known substrates for PKG include inositol 1,4,5-trisphosphate receptor (35), DARPP-32 (36), and cGMP-binding phosphodiesterase (37) in the central nervous system. Schlossmann et al. (38) have recently identified a PKG substrate protein in smooth muscle. However, the role of these substrates in the inhibition by cGMP of the mitochondrial PTP is not clear.

There are a limited number of inhibitors of the mitochondrial PTP. The most specific inhibitor is cyclosporin A, which competitively prevents cyclophilin from interacting with specific cyclophilin-dependent binding domains of the pore. Other classes of inhibitors of the mitochondrial PTP consist of the adenine nucleotide translocase ligand bongkrekic acid (29), the phospholipase inhibitor trifluoperazine (39), the antiestrogen drug tamoxifen (40) and nitric oxide (41). Consistent with recent studies in other cells (42-44), we found that the PTP inhibitors cyclosporin A and bongkrekic acid prevented the H\(_2\)O\(_2\)-induced reduction in cell viability and caspase-3 activation in astrocytes. In view of the evidence implicating the mitochondrial PTP in ischemia-related tissue damage, it has become important to identify strategies to inhibit induction of the PTP opening. The present study demonstrates that cGMP inhibits the mitochondrial PTP opening resulting in apoptotic events via the activation of PKG. This suggests that cGMP/PKG signaling is a novel target for prevention of mitochondrial dysfunction-mediated cell death. In line with this, we have shown that cGMP phosphodiesterase inhibitors including ibudilast protect astrocytes against reperfusion injury via cGMP/PKG signaling (11).

Acknowledgments—This research was supported by grants from the Ministry of
Education, Culture, Sports, Science and Technology of Japan, The Science Research Fund of
The Japan Private School Promotional Foundation, Uehara Memorial Foundation, Hyogo
Science and Technology Association, Joint Research (B) of Kobe Gakuin University, and
Kyorin Pharmaceuticals Co., Ltd.
REFERENCES

27. Silberbach, M., Gorenc, T., Hershberger, R.E., Stork, P.J., Steyger, P.S. and Roberts,
Figure legends

FIG. 1. Effects of membrane-permeable cGMP analogs on H2O2 exposure/reperfusion-induced cell injury in cultured rat astrocytes. Cells were exposed to Earle’s solution (open circle) or 100 µM H2O2-containing Earle’s solution (closed circle) for 30 min, and then incubated with Earle's solution for 23.5 h. The indicated concentrations of dibutyryl-cGMP (A), 8-pCPT-cGMP (B) and Sp-8-pCPT-cGMPS (C) were added 30 min before H2O2 exposure and were present until assay. Results are means ± SE for 6-10 wells and were obtained from 3-5 separate experiments. *P<0.01, significantly different from control (Student-Newman-Keuls test); †P<0.01, significantly different from the values without cGMP analogs (Dunnett’s t-test).

FIG. 2. Effect of dibutyryl-cGMP on apoptosis-like cell injury induced by H2O2 exposure/reperfusion in cultured rat astrocytes. A: Effect on DNA ladder formation. Cells were exposed to normal (control) or 100 µM H2O2 for 30 min, and then incubated with Earle's solution for 5 days. Dibutyryl-cGMP was added 30 min before H2O2 exposure and was present until assay. The typical result of three independent experiments is shown (M: 100 bp marker). B: Effect on nuclear condensation. Cells were preincubated in the absence (a, b) or presence (c, d) of 100 µM H2O2 for 30 min, and then incubated with Earle's solution for 3 days. Dibutyryl-cGMP (100 µM) was added 30 min before H2O2 exposure and was present until assay (b, d).

FIG. 3. Effect of dibutyryl-cGMP on loss of mitochondrial membrane potential induced by H2O2 exposure/reperfusion in cultured rat astrocytes. A: Time course of loss of mitochondrial membrane potential. Cells were exposed to normal (open circle) or 100 µM H2O2 (closed circle) for 30 min, and then incubated with Earle's solution for the indicated time. B: Dose-response for the effect of dibutyryl-cGMP. Cells were exposed to normal (open circle) or 100 µM H2O2 (closed circle) for 30 min, and then incubated with Earle's solution for 23.5 h. Dibutyryl-cGMP was added 30 min before H2O2 exposure and was present until assay. Results are means ± SE for 8-10 wells and were obtained from two separate experiments. *P<0.01, significantly different from control (Student-Newman-Keuls test); †P<0.01, significantly different from the values without dibutyryl-cGMP (Dunnett’s t-test).

FIG. 4. Effects of dibutyryl-cGMP on cytochrome c release from mitochondria and increase in DEVDase activity induced by H2O2 exposure/reperfusion in cultured rat astrocytes. A: Effect on cytochrome c release from mitochondria. Cells were exposed to normal (control) or 100 µM H2O2 for 30 min, and then incubated with Earle's solution for 23.5 h. Dibutyryl-cGMP was added 30 min before H2O2 exposure and was present until assay. Cytochrome c (arrow) in the cytosol (upper) and mitochondrial (lower) fractions are shown. The typical results of three independent experiments are shown. B: Effect on increase in DEVDase activity. Cells were exposed to normal (open circle) or 100 µM H2O2 (closed circle) for 30 min, and then incubated with Earle's solution for 23.5 h. Dibutyryl-cGMP was added 30 min before H2O2 exposure and was present until assay. Results are means ± SE for 6 wells and were obtained from 2 separate experiments. *P<0.01, significantly different from control (Student-Newman-Keuls test); †P<0.01, significantly different from the values without dibutyryl-cGMP (Dunnett’s t-test).

FIG. 5. Effects of KT5823 and Rp-8-pCPT-cGMPS on the protection provided by dibutyryl-cGMP against H2O2 exposure/reperfusion-induced injury in cultured rat
astrocytes. Cells were exposed to normal (open columns in A, B, and D, and control in C) or 100 µM H$_2$O$_2$-containing medium (hatched columns in A, B and D, and H$_2$O$_2$-treated in C) for 30 min, and then incubated with Earle's solution for 23.5 h. Dibutyryl-cGMP (100 µM) was added 30 min before H$_2$O$_2$ exposure and present until assay. KT5823 (2 µM) and Rp-8-pCPT-cGMPS (1 µM) were added 60 min before H$_2$O$_2$ exposure and were present until assay. The columns or lanes are: 1, none; 2, dibutyryl-cGMP; 3, KT5823; 4, KT5823 plus dibutyryl-cGMP; 5, Rp-8-pCPT-cGMPS; 6, Rp-8-pCPT-cGMPS plus dibutyryl-cGMP. A: MTT assay. Results are means ± SE for 10-22 wells, and were obtained from 5-11 separate experiments. B: Loss of mitochondrial membrane potential. Results are means ± SE for 6 wells, and were obtained from two separate experiments. C: Cytochrome c (arrow) in the cytosol (upper) and mitochondrial (lower) fractions are shown. The typical results of three independent experiments are shown. D: Caspase-3-like protease activity. Results are means ± SE for 6 wells, and were obtained from two separate experiments. *P<0.05, significant from the values of dibutyryl-cGMP alone (Tukey-HSD test).

FIG. 6. Effects of cGMP and cytosolic extract from astrocytes on Ca$^{2+}$-induced swelling in purified rat brain mitochondria. Swelling was induced by addition of Ca$^{2+}$ at 0 time (arrow) (b-f). Cyclosporin A, bongkrekic acid, cGMP and cytosolic extract from astrocytes were added 15 min before Ca$^{2+}$ addition and present during the incubation. A: The effects of cyclosporin A and bongkrekic acid on the mitochondrial swelling. a, control; b, Ca$^{2+}$; c, Ca$^{2+}$ plus 1 µM cyclosporin A; d, Ca$^{2+}$ plus 5 µM bongkrekic acid. B: Dose-response for the effect of cGMP. a, none; b, Ca$^{2+}$; c, Ca$^{2+}$ and 10 µg/ml of the extract; d, Ca$^{2+}$ and the extract plus 1 µM cGMP; e, Ca$^{2+}$ and the extract plus 10 µM cGMP; f, Ca$^{2+}$ and the extract plus 100 µM cGMP. C: Dose-response for the effect of cytosolic extract from astrocytes. a, none; b, Ca$^{2+}$; c, Ca$^{2+}$ and 100 µM cGMP; d, Ca$^{2+}$ and 100 µM cGMP plus the extract at 5 µg/ml; e, Ca$^{2+}$ and 100 µM cGMP plus the extract at 10 µg/ml; f, Ca$^{2+}$ and 100 µM cGMP plus the extract at 20 µg/ml. Results are means for 3-14 measurements, and were obtained from two or three separate experiments.

FIG. 7. Involvement of protein kinase G on the inhibition by cGMP and cytosolic extract from astrocytes on Ca$^{2+}$-induced swelling in purified rat brain mitochondria. Swelling was induced by addition of Ca$^{2+}$ at 0 time (arrow) (b-e). cGMP, the extract and PKG were added 15 min before Ca$^{2+}$ addition and present during the incubation. KT5823 was added 30 min before Ca$^{2+}$ and present during the incubation. A: Effect of heat-treated cytosolic extract. The extract was heated at 90°C for 20 min before use. a, none; b, Ca$^{2+}$; c, Ca$^{2+}$ and 100 µM cGMP; d, Ca$^{2+}$ and 100 µM cGMP plus 10 µg/ml of the fresh extract; e, Ca$^{2+}$ and 100 µM cGMP plus 10 µg/ml of the heated extract. B: Effect of exogenous PKG. a, none; b, Ca$^{2+}$; c, Ca$^{2+}$ and 100 µM cGMP; d, Ca$^{2+}$ and 100 µM cGMP plus 10,000 units/ml PKG. C: Effect of the PKG inhibitor KT5823. a, none; b, Ca$^{2+}$; c, Ca$^{2+}$ and 100 µM cGMP; d, Ca$^{2+}$ and 100 µM cGMP plus 10 µg/ml of the extract; e, Ca$^{2+}$ and 100 µM cGMP plus 10,000 units/ml PKG. Results are means for 4-9 measurements, and were obtained from two or three separate experiments.

FIG. 8. Effects of cyclosporin A and bongkrekic acid on the H$_2$O$_2$-induced cell injury and increase in DEVDase activity in cultured rat astrocytes. Cells were exposed to normal (open columns) or 100 µM H$_2$O$_2$-containing medium (hatched columns) for 30 min, and then incubated with Earle's solution for 23.5 h. Cyclosporin A (1 µM) and bongkrekic acid (5 µM) were added 30 min before H$_2$O$_2$ exposure and were present until assay. A: MTT assay. Results are means ± SE for 9-19 wells, and were obtained from two or three separate
experiments. B: DEVDase activity. Results are means ± SE for 10 wells and were obtained from 2 separate experiments. *P<0.05, significant from the values without drugs (Tukey-HSD test).
<table>
<thead>
<tr>
<th>Dibutyryl-cGMP (–log M)</th>
<th>Control</th>
<th>H₂O₂-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>M 0 6 5 4</td>
<td>0 6 5 4</td>
<td></td>
</tr>
</tbody>
</table>

**FIG. 2**
FIG. 3
FIG. 4
FIG. 5
FIG. 6

Swollen mitochondria (%)
FIG. 7
FIG. 8

A

MTT reduction (%)

Cyclosporin A
Bongkrekic acid

B

DEVDase activity (μmol/mg protein)

None
Cyclosporin A
Bongkrekic acid

*
Anti-apoptotic effect of cGMP in cultured astrocytes: Inhibition by cGMP-dependent protein kinase of mitochondrial permeable transition pore
Kazuhiro Takuma, Patamawan Phuagphong, Eibai Lee, Koichi Mori, Akemichi Baba and Toshio Matsuda

J. Biol. Chem. published online October 24, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M108622200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts