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Cyclic GMP/Cyclic GMP-Dependent Protein Kinase System Prevents Excitotoxicity in an Immortalized Oligodendroglial Cell Line

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Abstract: Previously, we have demonstrated that excitotoxicity of oligodendrocyte-like cells (OLC), differentiated from immortalized rat O-2A progenitor cells (CG-4 cells), is prevented by cyclic AMP-elevating agents. We now report that some agents that elevate cyclic GMP prevent OLC excitotoxicity. Kainate-induced injury was prevented by cyclic GMP analogues (8-bromo-cyclic GMP and dibutyryl cyclic GMP), a guanylate cyclase activator [atrial natriuretic peptide (ANP)], and phosphodiesterase inhibitors [3-isobutyl-1-methylxanthine (IBMX), ibudilast, propentofylline, and rolipram]. When both forskolin and 8-bromo-cyclic GMP were added, kainate-induced injury was additively prevented. There was a strong positive correlation between suppression of kainate-induced Ca^{2+} influx and prevention of injury by these chemicals. The measurement of intracellular cyclic AMP and cyclic GMP by radioimmunoassay demonstrated the following: an increase of cyclic GMP with treatment with 8-bromo-cyclic GMP, dibutyryl cyclic GMP, and ANP; an increase of cyclic AMP with treatment with ibudilast and rolipram; and an increase of both cyclic AMP and cyclic GMP with treatment with IBMX and propentofylline. Kainate-induced Ca^{2+} influx was decreased by 8-(4-chlorophenylthiol)-guanosine-3′,5′-monophosphate, an activator of cyclic GMP-dependent protein kinase (PKG), or okadaic acid, an inhibitor of protein phosphatases 1 and 2A. RT-PCR and western blotting of OLC demonstrated transcription of PKG II gene and translation of PKG Iβ mRNA, but no translation of PKG Iα mRNA. Therefore, we concluded that the cyclic GMP/PKG system prevents OLC excitotoxicity. **Key Words:** Non-NMDA glutamate receptors—Oligodendroglia—Cyclic GMP—Ca^{2+} influx—Cyclic GMP-dependent protein kinase—Protein phosphatases.
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We have demonstrated previously that cells of the oligodendroglial lineage express most of the genes encoding non–*N*-methyl-D-aspartate (NMDA) glutamate receptor (GluR) channel proteins, but do not express functional NMDA GluR protein (Yoshioka et al., 1995, 1996*a*,*b*). In the presence of extracellular Ca^{2+}, these cells are damaged by kainate, a glutamate analogue, representing oligodendroglial excitotoxicity (Yoshioka et al., 1995). Nimodipine, a voltage-dependent Ca^{2+}-channel blocker, did not prevent kainate-induced cytotoxicity. α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) did not induce oligodendrocyte-like cell (OLC) cytotoxicity unless desensitization of AMPA-type non-NMDA GluR was blocked with cyclothiazide (Yoshioka et al., 1995). Cyclothiazide also increased death elicited by kainate, indicating that kainate excitotoxicity is mediated, at least in part, by activation of AMPA-type GluR. Kainate-elicited damage to cells of the oligodendroglial lineage has been demonstrated recently in vivo by Matute et al. (1997) and McDonald et al. (1998).

Excitotoxicity has been implicated in oligodendroglial depletion from foci of CNS ischemia or inflammation (Leifer et al., 1990; Giulian et al., 1993; Prineas et al., 1993; Raine and Wu, 1993; Wender and Szczech, 1994). In a recent study, we showed that oxygen-glucose deprivation-induced oligodendroglial damage was prevented by non-NMDA GluR antagonists or by deprivation of extracellular Ca^{2+}, but not by nimodipine, thus indicating that Ca^{2+} influx through non-NMDA GluR channels mediates oxygen-glucose deprivation-induced oligodendroglial death (Yoshioka et al., unpublished observa-

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Abbreviations used: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate; ANP, atrial natriuretic peptide; 8-Br-cGMP, 8-bromo-cyclic GMP; cAMP, cyclic AMP; cGMP, cyclic GMP; dbcGMP, dibutyryl cyclic GMP; GluR, glutamate receptor; HBSS, Hanks' balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; LDH, lactate dehydrogenase; NMDA, *N*-methyl-D-aspartate; NO, nitric oxide; OLC, oligodendrocyte-like cell(s); 8-pCPT-cGMP, 8-(4-chlorophenylthiol)-guanosine-3′,5′-monophosphate; PDE, phosphodiesterase; PKG, cyclic GMP-dependent protein kinase; RIA, radioimmunoassay; *Rp*-8-Br-cGMPS, 8-bromoguanosine-3′,5′-monophosphorothioate, *Rp*-isomer; SNAP, *S*-nitroso-*N*-acetylpenicillamine; SNP, sodium nitroprusside.

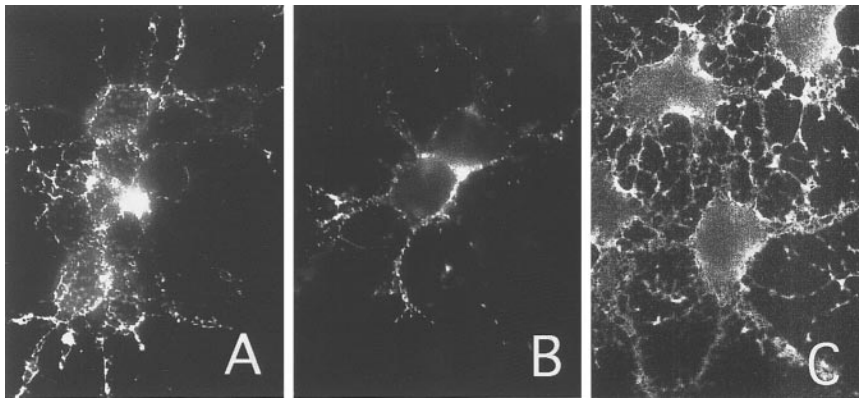

FIG. 1. Immunofluorescence staining of CG-4 OLC. The OLC 1 day after removal of B104 neuroblastoma-conditioned medium were A2B5$^+$ (**A**). The OLC 3 days after removal of B104 neuroblastoma-conditioned medium were O4$^+$ (**B**) or O1$^+$ (**C**).

tions). These results strongly suggest that decreasing Ca^{2+} influx through non-NMDA GluR would have a protective effect against oligodendroglial death in CNS ischemia.

We recently found that agents that elevate intracellular cyclic AMP (cAMP) prevented kainate-induced oligodendroglial injury by decreasing Ca^{2+} influx through non-NMDA GluR (Yoshioka et al., 1998). As agents that elevate intracellular cyclic GMP (cGMP) regulate Ca^{2+} influx and prevent neuronal injury (Weill and Greene, 1984; Garthwaite and Garthwaite, 1988; Desole et al., 1994; Meriney et al., 1994; Barger et al., 1995; Forloni et al., 1997), we wondered whether these agents prevent kainate-induced oligodendroglial death. In this study, we evaluated the effect of cGMP-elevating agents on kainate-induced oligodendroglial excitotoxicity using cultured cells of the oligodendroglial lineage.

MATERIALS AND METHODS

Cell culture

The CG-4 cells (Louis et al., 1992), an immortalized cell line established from rat O-2A progenitor cells, which demonstrate the essential characteristics of O-2A progenitor cells, including the expression of amino acid receptors, response to growth factors, and differentiation into OLC, are a useful experimental model to study the pathophysiology of oligodendroglial lineage cells.

The CG-4 cells were cultured as previously described (Louis et al., 1992). These cells were cultured with Dulbecco's modified Eagle's medium (high glucose, 25 m*M*) with 2 m*M* glutamine, N_1 components, biotin, and 30% B104 neuroblastoma-conditioned medium. Differentiation of CG-4 cells to OLC was induced by removing the neuroblastoma-conditioned medium from the culture medium (Louis et al., 1992). In the present study, OLC were used for experiments 3 days after removal of the neuroblastoma-conditioned medium.

The OLC were characterized immunocytochemically using the monoclonal antibodies A2B5, O4, O1, and glial fibrillary acidic protein. As shown in previous studies (Louis et al., 1992; Patneau et al., 1994; Pende et al., 1994), undifferentiated CG-4 cells and OLC 1 day after removal of the neuroblastoma-conditioned medium were A2B5$^+$ (Fig. 1A). The OLC 3 days after removal of the neuroblastoma-conditioned medium were O4$^+$ or O1$^+$ (Fig. 1B and C, respectively). No cells were stained with glial fibrillary acidic protein.

Evaluation of toxicity

Cultured OLC were incubated with 2 m*M* kainate for 24 h at 37°C. Cytotoxicity was quantified by measuring the activity of lactate dehydrogenase (LDH) released from the cells to the medium (Koh and Choi, 1987). LDH remaining within cells was also measured, after lysis of cells with deionized water over 1 h. The rate of LDH release into the culture medium was expressed as a percentage of the released LDH to the total LDH (i.e., LDH released into culture medium plus LDH in the cell lysate; see Younkin et al., 1993). As previously shown, indices of cell death represented by propidium iodide-positive cells and LDH release were correlated in OLC at 6 and 24 h after exposure to 2 m*M* kainate (Yoshioka et al., 1996*b*). Statistical significance was determined by Fisher's protected least significant difference following ANOVA.

$^{45}Ca^{2+}$ uptake study

OLC were incubated in Hanks' balanced salt solution (HBSS) containing 8 μCi/ml (259 kBq/ml) $^{45}CaCl_2$ at room temperature. After incubation for 10 min, cells were washed with HBSS three times and lysed with 1% Triton X-100 (vol/vol) for 10 min. Radioactivity in the lysate was measured by a liquid scintillation counter. Statistical significance was determined by Fisher's protected least significant difference following ANOVA.

Measurement of intracellular cAMP and cGMP

After exposure to cGMP-elevating agents for 24 h, cells were treated with 6% trichloroacetic acid and homogenized. After centrifugation at 3,000 rpm for 5 min, trichloroacetic acid was extracted with water-saturated ether. Intracellular cAMP and cGMP were determined by ^{125}I-cAMP and ^{125}I-cGMP radioimmunoassay (RIA) kits.

RT-PCR to detect cGMP-dependent protein kinase (PKG) II mRNA

Total RNA was extracted from CG-4 cells and normal adult Sprague–Dawley rat forebrain (Chomczynski and Sacchi, 1987). Aliquots (1 μl) were used for RT-PCR. PCR primers were designed to amplify a 617-bp PCR product for PKG II cDNA that corresponds to positions 838–1,454 of the published rat PKG II cDNA (Jarchau et al., 1994). The forward primer used was 5′-ACATTATGAGGAGAACAGCGC-3′, and the reverse primer was 5′-AAGCTCCACTCTTCCGAACC-3′. The amplification profile involved denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min, and extension at 72°C for 1 min. This cycle was repeated 30 times. Control amplifications were done either without reverse tran-

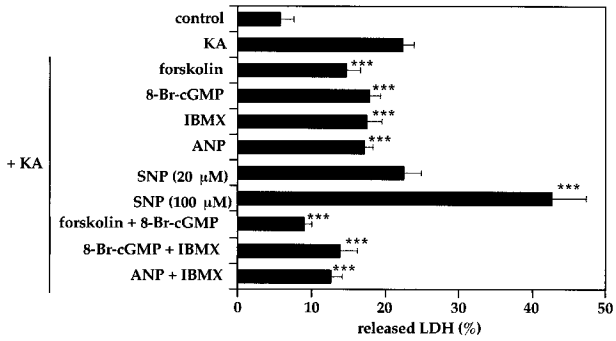

FIG. 2. LDH activity in media from OLC exposed to 2 m*M* kainate (KA) for 24 h. Forskolin at 10 μM, 500 μM 8-Br-cGMP, and 10 μM IBMX, or 100 n*M* ANP protected against kainate-induced excitotoxicity reflected by the LDH release. When both forskolin and 8-Br-cGMP were added, kainate-induced LDH release was additively decreased. Simultaneous application of 8-Br-cGMP and IBMX or ANP and IBMX additively prevented kainate-induced OLC injury. In contrast, an application of SNP, a NO donor that activates soluble guanylate cyclase, did not prevent kainate-induced injury at a 20 μM concentration. Furthermore, at 100 μM, it was toxic to OLC. Experiments were repeated at least three times with the same results. The results shown are the means ± SD (n = 6). ***$p < 0.0001$.

scriptase or without RNA. Identities of the PKG II amplification products were confirmed by Southern blotting with chemifluorescent 3′-labeled oligonucleotide. Vistra ECF 3′-oligolabeling and signal amplification modules were used (Amersham, Piscataway, NJ, U.S.A.). The oligonucleotide probe used was 5′-AGGTTGAGGAGAATCTGGCC-3′. The blot was hybridized with the probe at 42°C for 2 h and then washed with 5× saline–sodium citrate buffer plus 0.1% sodium dodecyl sulfate for 10 min at room temperature and then with 1× saline–sodium citrate buffer plus 0.1% sodium dodecyl sulfate for 15 min at 50°C. The signal was amplified with the ECF signal amplification module and detected by FluorImager SI (Molecular Dynamics).

Electrophoresis and immunoblotting

Total protein (10 μg/lane) extracted from CG-4 cells and adult rat forebrain was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Laemmli et al., 1970). After electrophoretic transfer (Towbin et al., 1979), the nitrocellulose filter was incubated in blocking buffer (Tris buffered saline/2.5% nonfat milk/10 m*M* NaN_3) for 2 h. The filter was then incubated for 4 h with rabbit polyclonal antibodies that react specifically to PKG Iα protein or react specifically to both Iα and Iβ protein, and then incubated with an alkaline phosphatase-conjugated goat anti-rabbit IgG for 2 h. Finally, the filter was developed with an alkaline phosphatase conjugate substrate kit.

Materials

Kainate, forskolin, 8-bromo-cGMP (8-Br-cGMP), dibutyryl cGMP (dbcGMP), 3-isobutyl-1-methylxanthine (IBMX), zaprinast, sodium nitroprusside (SNP), *S*-nitroso-*N*-acetylpenicillamine (SNAP), and atrial natriuretic peptide (ANP) were purchased from Sigma (St. Louis, MO, U.S.A.). LDH kit was purchased from Boehringer Mannheim (Tokyo, Japan). Rolipram was purchased from Research Biochemicals International (Natick, MA, U.S.A.). Okadaic acid was from Wako Pure Chemical Industries (Osaka, Japan). 8-Bromoguanosine-3′,5′-monophosphorothioate, *Rp*-isomer (*Rp*-8-Br-cGMPS) and 8-(4-chlorophenylthiol)-guanosine-3′,5′-monophosphate (8-pCPT-cGMP) were purchased from Biolog Life Science Institute (Bremen, Germany). Propentofylline was a gift from Hoechst Japan (Tokyo, Japan). Ibudilast was a gift from Kyorin Pharmaceutical Co. (Tokyo, Japan). $^{45}CaCl_2$, 3′-oligolabeling module, and ECF signal amplification module were purchased from Amersham (Tokyo, Japan). ^{125}I-cAMP and ^{125}I-cGMP RIA kits were purchased from Yamasa (Chiba, Japan). Rabbit polyclonal antibodies that specifically react to PKG Iα and specifically react to both Iα and Iβ were purchased from Stress Gen (Victoria, Canada). An alkaline phosphatase-conjugated goat anti-rabbit IgG was purchased from EY Laboratories (San Mateo, CA, U.S.A.). An alkaline phosphatase conjugate substrate kit was purchased from Bio-Rad (Hercules, CA, U.S.A.). RT-PCR kits were purchased from Takara (Shiga, Japan).

RESULTS

Kainate-induced excitotoxicity is prevented by cGMP-elevating agents

When OLC were exposed to 2 m*M* kainate, LDH release increased about threefold (Fig. 2). As previously shown (Yoshioka et al., 1998), 10 μM forskolin, which increased intracellular cAMP, decreased LDH released into the culture medium (n = 6). To examine the effect of various drugs that elevate intracellular cGMP on kainate-induced LDH release, these drugs were added to the kainate-containing incubation solution and incubated for 24 h. Analogues of cGMP, such as 8-Br-cGMP at 500 μM and dbcGMP at 500 μM, prevented the kainate-induced LDH release as shown in Fig. 2 and Table 1. When cells were exposed to 2 m*M* kainate, 10 μM forskolin, and 500 μM 8-Br-cGMP simultaneously, kainate-induced oligodendroglial excitotoxicity was additively prevented (Fig. 2).

We then investigated whether the drugs that inhibit cyclic nucleotide phosphodiesterase (PDE) prevent oligodendroglial excitotoxicity by increasing intracellular cGMP. We have shown previously that propentofylline, ibudilast, and IBMX prevented kainate-induced oligodendroglial excitotoxicity by elevating intracellular cAMP (Yoshioka et al., 1998). In the present study, we

TABLE 1. *LDH release assay and $^{45}Ca^{2+}$ uptake of OLC exposed to 2 m*M *kainate with various drugs*

Drugs	Released LDH (%)	$^{45}Ca^{2+}$ uptake (dpm)
Control	5.38 ± 0.51	2,109 ± 565
2 m*M* kainate (KA)	20.64 ± 2.25	12,361 ± 1,445
dbcGMP (500 μM) + KA	16.87 ± 2.07[a]	9,069 ± 890[b]
SNAP (20 μM) + KA	21.67 ± 1.53	12,645 ± 962
SNAP (100 μM) + KA	38.11 ± 5.62[b]	12,001 ± 1,214
Ibudilast (50 μM) + KA	16.89 ± 1.37[a]	8,436 ± 890[b]
Rolipram (20 μM) + KA	15.22 ± 1.96[a]	8,887 ± 1,157[b]
Propentofylline (1 m*M*) + KA	14.78 ± 2.01[a]	7,552 ± 947[b]
Zaprinast (10 μM) + KA	22.56 ± 1.98	11,967 ± 1,017

Data are presented as the means ± SD (n = 6).
[a] $p < 0.001$; [b] $p < 0.0001$.

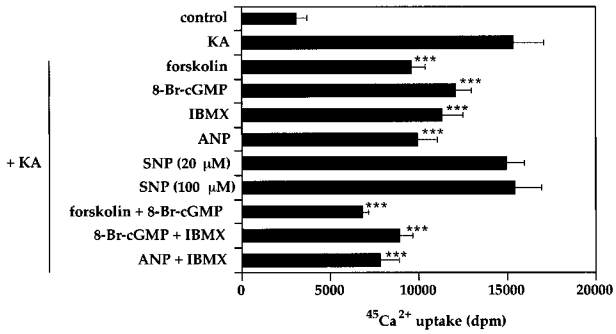

FIG. 3. $^{45}Ca^{2+}$ uptake study of OLC. Cells were incubated in HBSS containing 2 m*M* kainate (KA) and 8 μCi/ml $^{45}CaCl_2$ at room temperature for 10 min. Forskolin at 10 μM, 500 μM 8-Br-cGMP, 10 μM IBMX, or 100 n*M* ANP decreased kainate-induced Ca^{2+} influx. In the presence of both forskolin and 8-Br-cGMP, kainate-induced Ca^{2+} influx was additively decreased. The simultaneous application of 8-Br-cGMP and IBMX or ANP and IBMX additively decreased kainate-induced Ca^{2+} influx. However, SNP at 20 or 100 μM did not decrease kainate-induced Ca^{2+} influx. The experiments were performed in triplicate with the same results. The results shown are the means $\pm$ SD (n = 6). ***$p < 0.0001$.

found that kainate-induced oligodendroglial excitotoxicity was prevented by 10 μM IBMX, 50 μM ibudilast, 20 μM rolipram, or 1 m*M* propentofylline, but it was not prevented by 10 μM zaprinast (Fig. 2 and Table 1). Rolipram is a drug that specifically inhibits cAMP-specific PDE, type IV. Zaprinast is a drug that inhibits cGMP-binding cGMP-specific PDE, type V. We investigated the additive effect of a cGMP analogue and a PDE inhibitor on oligodendroglial excitotoxicity. When both 500 μM 8-Br-cGMP and 10 μM IBMX were applied simultaneously, kainate-induced OLC injury was additively prevented (Fig. 2).

We investigated the effect of drugs that activate guanylate cyclase on kainate-induced excitotoxicity. ANP (100 n*M*), which activates particulate guanylate cyclase, prevented kainate-induced injury (Fig. 2). When both 100 n*M* ANP and 10 μM IBMX were applied to cells, excitotoxicity was additively prevented. In contrast, an application of nitric oxide (NO) donors that activate soluble guanylate cyclase, such as SNP and SNAP (Table 1), did not prevent kainate-induced injury at 20 μM concentrations. Furthermore, at 100 μM, these drugs were toxic to OLC (Fig. 2 and Table 1).

Kainate-induced Ca^{2+} influx is decreased by cGMP-elevating agents

To investigate whether cGMP-elevating agents cause suppression of the kainate-induced increase in Ca^{2+} influx, we examined $^{45}Ca^{2+}$ uptake under various conditions (Fig. 3 and Table 1). The $^{45}Ca^{2+}$ uptake by OLC exposed to both 2 m*M* kainate and 10 μM forskolin for 10 min was decreased (Fig. 3). 8-Br-cGMP at 500 μM, 500 μM dbcGMP, or 10 μM IBMX also attenuated kainate-induced Ca^{2+} uptake. When both forskolin and 8-Br-cGMP were added, kainate-induced Ca^{2+} uptake was additively decreased. In the presence of both 8-Br-cGMP and IBMX, kainate-induced Ca^{2+} uptake was additively attenuated. Kainate-induced Ca^{2+} uptake with an application of 2 m*M* kainate and 100 n*M* ANP for 10 min was smaller than that by the cells exposed to 2 m*M* kainate alone (Fig. 3). Similarly, $^{45}Ca^{2+}$ uptake by cells exposed to both 2 m*M* kainate and 50 μM ibudilast, 20 μM rolipram, or 1 m*M* propentofylline was decreased, but 10 μM zaprinast did not decrease $^{45}Ca^{2+}$ uptake in OLC exposed to 2 m*M* kainate (Table 1). When 100 n*M* ANP was applied to cells with 2 m*M* kainate and 10 μM IBMX together, kainate-induced $^{45}Ca^{2+}$ uptake was additively decreased (Fig. 3). However, an application of 20 μM SNP or 20 μM SNAP did not attenuate kainate-induced Ca^{2+} uptake.

Agents used in expectation of raising cGMP level are confirmed to elevate intracellular cGMP

After treatment with a variety of agents that are expected to raise intracellular cAMP or cGMP for 24 h, intracellular cAMP and cGMP were measured using ^{125}I-cAMP and ^{125}I-cGMP RIA kits (Table 2). Forskolin (10 μM) increased intracellular cAMP, but did not increase intracellular cGMP. Treatment with 500 μM 8-Br-cGMP, 500 μM dbcGMP, or 100 n*M* ANP increased intracellular cGMP, but did not increase intracellular cAMP, supporting the view that not only intracellular cAMP, but also cGMP, is responsible for the suppression of the kainate-induced increase in Ca^{2+} influx. However, there was no correlation between intracellular cGMP and the extent to which the cells were protected. Treatment with 20 μM SNP or 20 μM SNAP increased intracellular cGMP, but did not diminish release of LDH by kainate-treated OLC, likely as a consequence of the toxicity of these NO-releasing compounds for OLC.

Then we measured intracellular cAMP and cGMP in cells exposed to PDE inhibitors. Ibudilast at 50 μM or rolipram at 20 μM increased intracellular cAMP, but did not increase intracellular cGMP, indicating that the protective effect is due to an increase of intracellular cAMP. Propentofylline at 1 m*M* or IBMX at 10 μM increased

TABLE 2. *Intracellular level of cAMP and cGMP in OLC exposed to various drugs for 24 h*

Drugs	cAMP (pmol/ml)	cGMP (fmol/ml)
Control	5.38 ± 0.51	20.34 ± 2.85
Forskolin (10 μM)	10.91 ± 1.42	23.12 ± 3.32
Ibudilast (50 μM)	10.56 ± 1.37	20.24 ± 5.89
Rolipram (20 μM)	8.27 ± 1.11	15.18 ± 3.14
Propentofylline (1 m*M*)	11.19 ± 1.17	44.10 ± 9.67
IBMX (10 μM)	7.09 ± 1.36	70.22 ± 21.56
Zaprinast (10 μM)	5.80 ± 0.38	12.64 ± 1.44
SNP (20 μM)	6.48 ± 1.62	81.00 ± 12.93
SNAP (20 μM)	5.48 ± 1.32	69.47 ± 17.56
ANP (100 n*M*)	5.59 ± 0.48	199 ± 71
ANP (100 n*M*) + IBMX (10 μM)	8.20 ± 2.60	750 ± 170
8-Br-cGMP (500 μM)	6.01 ± 0.37	2,130 ± 420
dbcGMP (500 μM)	6.11 ± 0.46	1,780 ± 310

Data are presented as the means ± SD (n = 3).

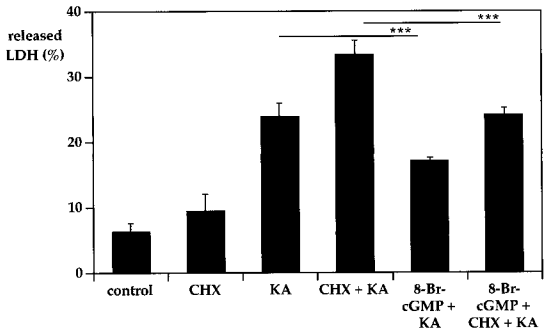

FIG. 4. LDH release assay of OLC exposed to 2 m*M* kainate (KA) for 24 h. Kainate-induced toxicity was increased by cycloheximide (CHX; 1 μM). 8-Br-cGMP (500 μM) prevented kainate-induced LDH release even when cycloheximide was present, indicating that protection by 8-Br-cGMP did not require protein synthesis. The results shown are the means ± SD (n = 6). ***$p < 0.0001$.

both intracellular cAMP and cGMP, suggesting that both intracellular cAMP and cGMP might protect against kainate toxicity. In contrast, neither cAMP nor cGMP was increased by 10 μM zaprinast, supporting the view that zaprinast does not prevent kainate toxicity.

When IBMX was applied to OLC with ANP simultaneously, both intracellular cAMP and cGMP were increased, suggesting that both intracellular cAMP and cGMP may contribute to the additive protection against OLC excitotoxicity.

Protection by cGMP-elevating agents does not require protein synthesis

To evaluate whether the protection by cGMP-elevating agents requires protein synthesis, cells were exposed to 1 μM cycloheximide during the period of incubation with 2 m*M* kainate and 500 μM 8-Br-cGMP. A treatment with 1 μM cycloheximide increased kainate-induced toxicity (Fig. 4). However, 8-Br-cGMP prevented kainate-induced LDH release even when cycloheximide was present. We concluded that protection of OLC by cGMP-elevating agents does not require protein synthesis.

PKG-dependent phosphorylation and protein phosphatases modulate kainate-induced Ca^{2+} influx and excitotoxicity

To determine whether the suppressing effect of cGMP-elevating agents on kainate-induced Ca^{2+} influx is mediated by activation of PKG, OLC were exposed to 10 μM *Rp*-8-Br-cGMPS, an inhibitor of PKG (Butt et al., 1990), during the period of incubation with 2 m*M* kainate and 500 μM 8-Br-cGMP. *Rp*-8-Br-cGMPS is an analogue of 8-Br-cGMP in which one of two exocyclic oxygen atoms in the cyclic phosphate moiety is modified by sulfur. Equatorial thio substitution leads to the *R* isomer. The suffix "*p*" indicates that *R* nomenclature refers to phosphorus. Suppression of the kainate-induced Ca^{2+} influx brought about by 8-Br-cGMP was not reversed by *Rp*-8-Br-cGMPS (Fig. 5A). In contrast, 8-pCPT-cGMP, a specific activator of PKG, at 10 μM significantly decreased kainate-induced Ca^{2+} influx. Okadaic acid (5 n*M*), an inhibitor of protein phosphatases 1 and 2A, suppressed the kainate-induced Ca^{2+} influx. The suppressing effect of okadaic acid was enhanced by 8-Br-cGMP or 8-pCPT-cGMP.

In accordance with the results of the $^{45}Ca^{2+}$ uptake experiments, *Rp*-8-Br-cGMPS did not diminish the protective effect of 8-Br-cGMP on the kainate-induced LDH release (Fig. 5B). However, 8-pCPT-cGMP prevented kainate-induced OLC injury. Okadaic acid prevented kainate-induced LDH release to some extent, as did 8-Br-cGMP or 8-pCPT-cGMP. Addition of 8-Br-cGMP to kainate-containing incubation solution caused further suppression. These results strongly suggest that the kain-

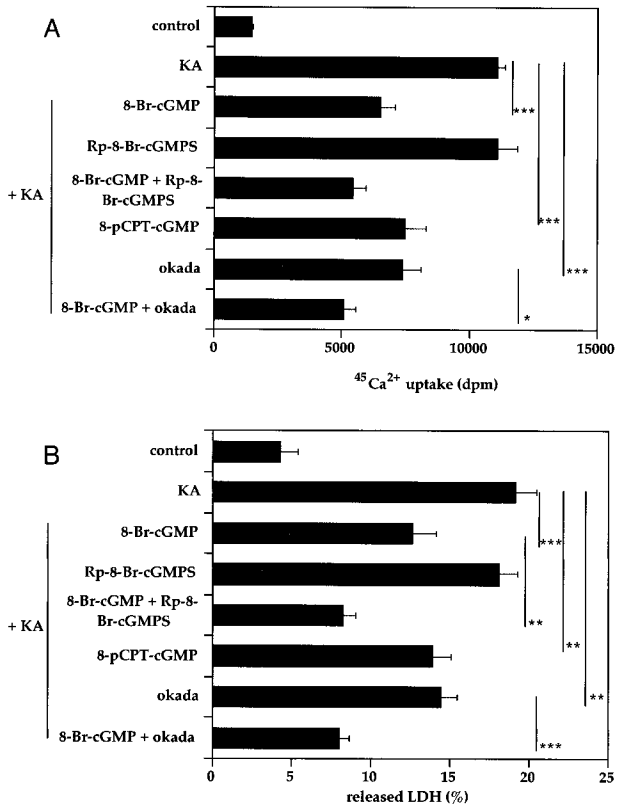

FIG. 5. A: $^{45}Ca^{2+}$ uptake study of OLC exposed to 2 m*M* kainate (KA) at room temperature for 10 min. Suppression of the kainate-induced Ca^{2+} influx brought about by 500 μM 8-Br-cGMP was not reversed by 10 μM *Rp*-8-Br-cGMPS, an inhibitor of PKG Iα. In contrast, 10 μM 8-pCPT-cGMP, a specific activator of PKG Iα, Iβ, and II, significantly decreased kainate-induced Ca^{2+} influx. Okadaic acid (okada; 5 n*M*), an inhibitor of protein phosphatases 1 and 2A, suppressed the kainate-induced Ca^{2+} influx. The suppressing effect of okadaic acid was enhanced by 8-Br-cGMP. **B:** LDH assay of OLC exposed to 2 m*M* kainate for 24 h. *Rp*-8-Br-cGMPS did not diminish the protective effect of 8-Br-cGMP on the kainate-induced LDH release. However, 8-pCPT-cGMP prevented kainate-induced OLC injury. Okadaic acid prevented kainate-induced LDH release to some extent, as did 8-Br-cGMP or 8-pCPT-cGMP. Addition of 8-Br-cGMP to kainate-containing incubation solution caused further suppression. The results shown are the means ± SD (n = 6). *$p < 0.01$; **$p < 0.001$; ***$p < 0.0001$.

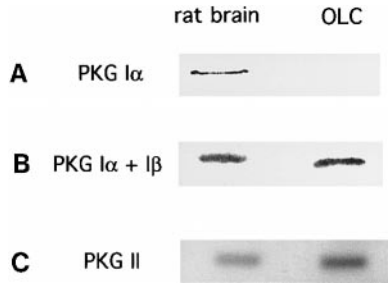

FIG. 6. Western blots for the detection of PKG Iα and Iβ proteins (**A** and **B**) and Southern blots of RT-PCR products with oligonucleotide probes for rat PKG II mRNA (**C**). The antibody, which binds to proteins encoded by the PKG Iα mRNA or both PKG Iα and PKG Iβ mRNA, labeled at 75-kDa PKG Iα or 70-kDa PKG Iα and Iβ protein on western blots from adult rat forebrain (A and B). Western blot from OLC identified protein specific to both PKG Iα and Iβ (B), but it did not identify protein specific to PKG Iα (A). Southern blots of RT-PCR products of total RNA extracted from rat forebrain and OLC showed 617-bp bands corresponding to the amplification product derived from PKG II mRNA (C).

ate-induced increase in Ca^{2+} influx is suppressed by intracellular cGMP, which may exert its effect through protein phosphorylation.

CG-4 cells transcribe PKG II gene and translate PKG Iβ mRNA, but do not translate PKG Iα mRNA

As shown by a previous study (El-Husseini et al., 1995), RT-PCR and Southern blotting of total RNA from rat forebrain demonstrated the amplification product derived from PKG II mRNA (Fig. 6). RT-PCR and Southern blotting of RNA from OLC demonstrated the amplification product derived from PKG II mRNA. Control PCRs without reverse transcriptase, or RT-PCR without added RNA, failed to yield amplification products detectable by ethidium bromide staining (data not shown).

The antibody, which binds to proteins encoded by the PKG Iα mRNA or both PKG Iα and Iβ mRNA, labeled a 75-kDa PKG Iα or 70-kDa PKG Iα and Iβ protein on western blots from adult rat forebrain (Fig. 6). Western blots from OLC labeled a 70-kDa band corresponding to PKG Iα and Iβ protein, but they did not label a 75-kDa band corresponding to PKG Iα protein (Fig. 6).

Thus, it was concluded that OLC transcribe PKG II gene and translate PKG Iβ mRNA, but do not translate PKG Iα mRNA.

DISCUSSION

The present study showed that cGMP-elevating agents, such as 8-Br-cGMP, dbcGMP, and ANP, prevent kainate-induced OLC excitotoxicity by decreasing kainate-induced Ca^{2+} influx. An increase of intracellular cGMP, but not cAMP, by these cGMP-elevating agents was confirmed by an RIA study. We found that there is a strong positive correlation between suppression of Ca^{2+} influx and prevention of cell death by these cGMP-elevating agents against kainate-induced excitotoxicity.

Our results indicate that elevations of both cAMP and cGMP prevent kainate-induced OLC excitotoxicity. When both cAMP- and cGMP-elevating agents were added simultaneously, OLC excitotoxicity was additively prevented by decreasing kainate-induced Ca^{2+} influx. In a previous study, we found that cAMP-elevating agents prevented kainate-induced OLC injury by decreasing Ca^{2+} influx through non-NMDA GluR (Yoshioka et al., 1998). Therefore, the protection by IBMX, a nonspecific PDE inhibitor, or propentofylline, which inhibits cGMP-stimulated PDE, type II, as well as cAMP-specific PDE, type IV (Meskini et al., 1994), seems to be due to an increase of both cAMP and cGMP. In contrast, protection by ibudilast or rolipram, which inhibits type IV PDE, seems to be due to an increase of cAMP (Souness et al., 1994). Zaprinast, which inhibits cGMP-binding cGMP-specific PDE, type V, did not increase cAMP or cGMP, supporting the view that zaprinast does not prevent kainate toxicity.

An increase of intracellular cGMP decreases intracellular Ca^{2+} by the binding of three cGMP receptor proteins: PKG, cGMP-regulated ion channels, and cGMP-binding cyclic nucleotide PDE (Lincoln and Cornwell, 1993). We found that 8-pCPT-cGMP, a specific activator of PKG, decreased kainate-induced Ca^{2+} influx and prevented oligodendroglial excitotoxicity. RT-PCR and western blotting of OLC demonstrated the transcription of PKG II gene and translation of PKG Iβ mRNA, but no translation of PKG Iα mRNA. Therefore, we concluded that the cGMP/PKG system protects OLC from excitotoxicity.

Although the suppression of kainate-induced Ca^{2+} influx brought about by 8-Br-cGMP was not reversed by *Rp*-8-Br-cGMPS, 8-pCPT-cGMP significantly decreased kainate-induced Ca^{2+} influx. It has been shown that *Rp*-8-Br-cGMPS specifically inhibits PKG Iα, but 8-pCPT-cGMP activates PKG Iα, Iβ, and II (Butt et al., 1990). The regulatory and catalytic properties of PKG II are distinct from those of PKG I isoforms (Gamm et al., 1995). Therefore, the different isoforms of PKG seem to determine the different response to specific activators or inhibitors. An in situ hybridization study has demonstrated that the PKG II gene is widely expressed in the rat brain (El-Husseini et al., 1995). PKG I is distributed mainly in the cerebellum (Schlichter, 1982). However, the expression of the PKG in oligodendroglial lineage cells has not been demonstrated.

The findings that the protective effect of 8-Br-cGMP against oligodendroglial excitotoxicity was not influenced by the application of cycloheximide, and that kainate-induced Ca^{2+} influx was decreased by 8-Br-cGMP during incubation for 10 min, even without preincubation, indicated that protein synthesis is not involved in the process. It has been shown that increased intracellular cGMP with or without the involvement of PKG modulates NMDA and non-NMDA GluR channels. NO decreased the responsiveness of both NMDA and non-NMDA GluR (Crepel and Jaillard, 1990; Shibuki and Okada, 1991; Manzoni et al., 1992). Somatostatin

prevented neuronal kainate toxicity by increasing intracellular cGMP (Forloni et al., 1997). Amyloid precursor protein mediated intracellular Ca^{2+} lowering and excitoprotection on target neurons by increased cGMP and activation of PKG (Barger et al., 1995; Furukawa and Mattson, 1998). NO and cGMP decreased kainate-induced current via AMPA-type non-NMDA GluR in hybrid bass retinal neurons (McMahon and Ponomareva, 1996). NO has been shown to play a role in long-term depression of cerebellar Purkinje cells by PKG-dependent phosphorylation of AMPA-type non-NMDA GluR (Ito and Karachot, 1990). The glutamate-induced current via GluR1/GluR3-injected oocytes was decreased by cGMP analogues (Keller et al., 1992). As the mechanism of PKG to modulate non-NMDA GluR, the phosphorylation of a key protein that regulates intracellular Ca^{2+} is suspected (Wang and Robinson, 1997). AMPA-type non-NMDA GluR in cerebellar Purkinje cells are phosphorylated by PKG (Nakazawa et al., 1995). Furthermore, okadaic acid, an inhibitor of phosphatases 1 and 2A, prevented excitotoxicity by decreasing kainate-induced Ca^{2+} influx. Okadaic acid additively prevented kainate-induced excitotoxicity when it was applied with 8-Br-cGMP. Thus, PKG-dependent protein phosphorylation might decrease kainate-induced Ca^{2+} influx through AMPA-type non-NMDA GluR channels, resulting in the prevention of OLC excitotoxicity.

In the present study, there was no correlation between the absolute intracellular cGMP content and the extent to which the cells were protected. The cGMP level in IBMX-applied OLC was 70.22 ± 21.56 fmol/ml and that of 8-Br-cGMP was $2{,}130 \pm 420$ fmol/ml. In spite of this large difference in the cGMP level, similar effects on both Ca^{2+} influx and LDH release were observed. However, if both IBMX and 8-Br-cGMP were applied to OLC, kainate-induced Ca^{2+} influx was additively attenuated. A similar additive effect was observed in the experiments using ANP and IBMX. Although the reason is not clear, kainate-induced OLC injury seems to be prevented more effectively by PDE inhibitors than by cGMP analogues or guanylate cyclase activators. In the case of cAMP-elevating agents preventing oligodendroglial excitotoxicity, the absolute cAMP level is not correlated with the extent to which the cells were protected (Yoshioka et al., 1998).

NO has both protective and toxic effects on neuronal cells (Jaffrey and Snyder, 1995). We found that NO generators, such as SNP and SNAP, did not prevent OLC excitotoxicity at 20 μM concentration, although the RIA study demonstrated an increase of intracellular cGMP. Furthermore, at 100 μM, these drugs were toxic to cells. The inability of SNP or SNAP to protect OLC against excitotoxicity in spite of an elevation of intracellular cGMP seems to be due to cellular toxicity. Previous studies have demonstrated a direct toxicity of NO on oligodendroglia (Mitrovic et al., 1995). NO toxicity to oligodendroglia may involve the combination of NO with superoxide to form peroxynitrite (Beckman et al., 1990). Poor detoxification of free radicals due to low glutathione and high levels of iron has been suggested to contribute to the susceptibility of oligodendroglia to hypoxia (Husain and Juurlink, 1995; Thorburne and Juurlink, 1996). Alternatively, NO may damage cells by decreasing intracellular ATP by inhibiting glyceraldehyde-3-phosphate dehydrogenase activity or by activating poly(ADP) ribosyltransferase. In the developing rat brain, NO-mediated cGMP synthesis was found in oligodendroglial precursor cells, confirming the expression of soluble guanylate cyclase in these lineage cells (Tanaka et al., 1997).

We found that ANP prevented OLC excitotoxicity by increasing intracellular cGMP, indicating that particulate guanylate cyclase is expressed in OLC. Although ANP-stimulated cGMP synthesis and expression of ANP receptors were found in astrocytes (De Vente et al., 1990; Tang et al., 1993), Tanaka et al. (1997) did not find ANP-induced cGMP synthesis in oligodendroglial lineage cells. The reason for the discrepancy between their results and ours is not clear, but may be due to the differences in the experimental systems and lineage cell development.

Finally, it may be said that some of the agents elevating cGMP reduce the cytotoxic effect of kainate by suppressing Ca^{2+} influx, and that the suppression of the kainate-induced Ca^{2+} influx is caused by the PKG-dependent substrate protein phosphorylation.

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