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Diazoxide is Protective in the Rat Retina Against Ischemic Injury Induced by Bilateral Carotid Occlusion and Glutamate-induced Degeneration

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Diazoxide (DIAZ) has been shown to be neuroprotective in animal models of different brain pathologies. However, the direct protective effect of DIAZ in different *in vivo* models of retinal degeneration has not yet been shown. Therefore, the aim of the present study was to investigate the neuroprotective role of this compound in two rodent model systems: monosodium-glutamate (MSG)- and chronic bilateral carotid artery occlusion (BCAO)-induced retinal degeneration. Rats were subjected either to s.c. MSG treatment on postnatal days 1, 5 and 9, or to BCAO at 2 months of age, followed by intravitreal DIAZ treatment. Histological examination was carried out 14 or 21 days after treatments, respectively. MSG treatment destroyed almost the entire inner retina, with the inner nuclear and ganglion cell layers being fused. DIAZ treatment significantly ameliorated the MSG-induced retinal degeneration. BCAO led to a severe degeneration of all retinal layers, and DIAZ proved to be protective also in this model. Our results may have clinical implications in reducing glutamate-induced excitotoxicity or ischemic retinal degeneration in ophthalmic diseases.

Keywords: MSG; BCAO; Retinoprotection; Ischemia

INTRODUCTION

Toxic and hypoxic injuries of the retina represent a major group of retinal pathology. Over-activation of glutamate receptors is a central contributor to neuronal cell death in the brain and in the retina in numerous pathological conditions (Vidal-Sanz *et al.*, 2000; Boldyrev *et al.*, 2004; Tsai *et al.*, 2005). Experimental elevation of glutamate concentrations can model several ophthalmic diseases. Direct increase of glutamate concentration can be reached by monosodium L-glutamate (MSG) administration, which finally leads to the destruction of the entire inner retina (Babai *et al.*, 2005). Occlusion of the common carotid arteries leads to moderate reduction in the cerebral blood flow in rats and produces a characteristic pathologic appearance of the retina, paralleling the retinopathy of carotid artery occlusive disease in humans (Slakter *et al.*, 1984; Atlasz *et al.*, 2007; Farkas *et al.*, 2007).

The events finally leading to retinal cell death are very complex, which provides an opportunity for a variety of protective pharmacological approaches. These involve attenuating glutamate-mediated excitotoxicity, reducing the detrimental effects of free radicals and increased Ca^{2+} levels, counteracting mitochondrial failure, anti-inflammatory strategies and potentiating endogenous protective

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mechanisms (Vidal-Sanz *et al.*, 2000; Barkana and Belkin, 2004; Osborne *et al.*, 2004). In the last few years, we have studied the protective effects of the neurotrophic pituitary adenylate cyclase activating polypeptide (PACAP) in MSG-induced neonatal retinal degeneration and in bilateral carotid occlusion (BCAO)-induced degeneration in adult rats. We have shown that PACAP treatment attenuates both types of retinal degeneration (Tamas *et al.*, 2004; Babai *et al.*, 2005; 2006; Kiss *et al.*, 2006; Atlasz *et al.*, 2007). We have also proven that the inhibition of pro-apoptotic signaling pathways and the activation of anti-apoptotic molecules play a role in this protective effect (Racz *et al.*, 2006a,b,c).

Mitochondrial dysfunction is involved in many key events of neuronal cell death in the retina (Osborne *et al.*, 2004). 7-chloro-3-methyl-4H-1,2,4-benzothiadiazine 1,1-dioxide (Diazoxide, DIAZ) is a mitochondrial ATP-sensitive K^+ channel-opener that has been implicated in cytoprotection in cardiac and cerebral ischemia (Busija *et al.*, 2005, Farkas *et al.*, 2007), but relatively little is known about its putative protective effects in the retina. It has been shown that DIAZ enhances survival of retinal ganglionic cells, protects retinal neurons against excitotoxicity and inhibits the glutamate-induced mitochondrial depolarization *in vitro* (Yamauchi *et al.*, 2003; Pielen *et al.*, 2004). DIAZ has also been reported to block the hypoxia-induced horizontal cell depolarization and the reduction of the light-evoked hyperpolarization *in vitro* (Hankins and Ikeda, 1993). *In vivo*, ischemic preconditioning can effectively be mimicked by DIAZ (Roth *et al.*, 2006). However, the direct protective effect of DIAZ in different *in vivo* models of retinal degeneration has not yet been shown. Therefore, the aim of the present study was to investigate the effects of local administration of DIAZ in retinal degeneration induced by neonatal MSG treatment or by BCAO-induced ischemic damage of the retina.

MATERIALS AND METHODS

Animals

Experimental animals derived from a local colony of Wistar rats. Animals were housed in individual cages, fed and watered *ad libitum*, under light/dark cycles of 12/12 h. All procedures were performed in accordance with the ethical guidelines approved by the University of Pecs (BA02/2000-20/2006).

MSG Treatment Schedule

Newborn rats (n=15) were injected s.c. by 2 mg/g body weight MSG dissolved in 100 µl physiological saline on postnatal days 1, 5 and 9 according to previous descriptions (Babai *et al.*, 2005). Normal control animals (n=5) were given the same volume of physiological saline solution. Treatments were given immediately following each MSG injection on days 1, 5 and 9. DIAZ (0.172 µg) was dissolved in 2 µl 0.01 M NaOH and phosphate buffered saline (PBS) and was injected with a Hamilton syringe into the right vitreous body of animals. The left eyes received the same volumes of vehicle treatment (PBS) and served as control MSG-treated eyes. Animals were sacrificed under anesthesia at the age of 21 days. The eyes were processed for histological observations as described below.

Bilateral Carotid Artery Occlusion (BCAO)

Adult male Wistar rats (n=12) weighing 250-300 g were subjected to permanent BCAO under isoflurane anesthesia. The carotid region was exposed through a midline cervical incision. The common carotid arteries were ligated with a 3-0 mm suture. Sham operated animals underwent the same procedure except for ligation of the carotid arteries. DIAZ (0.43 µg) was dissolved in 5 µl 0.01 M NaOH and phosphate buffered saline (PBS) and was injected with a Hamilton syringe into the right vitreous body of animals. The left eyes received the same volumes of vehicle treatment (PBS) and served as control ischemic eyes. After two weeks of survival the animals were sacrificed under anesthesia and the eyes were processed as described below.

Histology

The eyes were immediately dissected in ice-cold phosphate buffered saline and fixed in 4% paraformaldehyde dissolved in 0.1 M phosphate buffer (Sigma, Hungary). Tissues were embedded in Durcupan ACM resin (Fluka, Switzerland), cut at 2 µm and stained with toluidine blue (Sigma, Hungary). The sections were then mounted in Depex medium (Fluka, Switzerland) and examined in a Nikon Eclipse 80i microscope. Measurements were taken from the digital photographs with the NIH Image 1.55 program. Six tissue blocks from at

least three animals were prepared and central retinal areas within 1 and 2 mm from the optic nerve were used for measurements (n=2-5 measurements from one tissue block). Sections where the GCL appeared thicker than a single cell row, were excluded from evaluation. The following parameters were measured: (i) cross-section of the retina from the outer limiting membrane to the inner limiting membrane; (ii) the width of the outer and inner nuclear and plexiform layers (ONL, OPL, INL, IPL), respectively; the number of cells/100 μm section length in the ganglion cell layer (GCL).

Results are presented as mean ± S.E.M. Statistical comparisons were made using the ANOVA test followed by Tukey-B`s *post hoc* analysis.

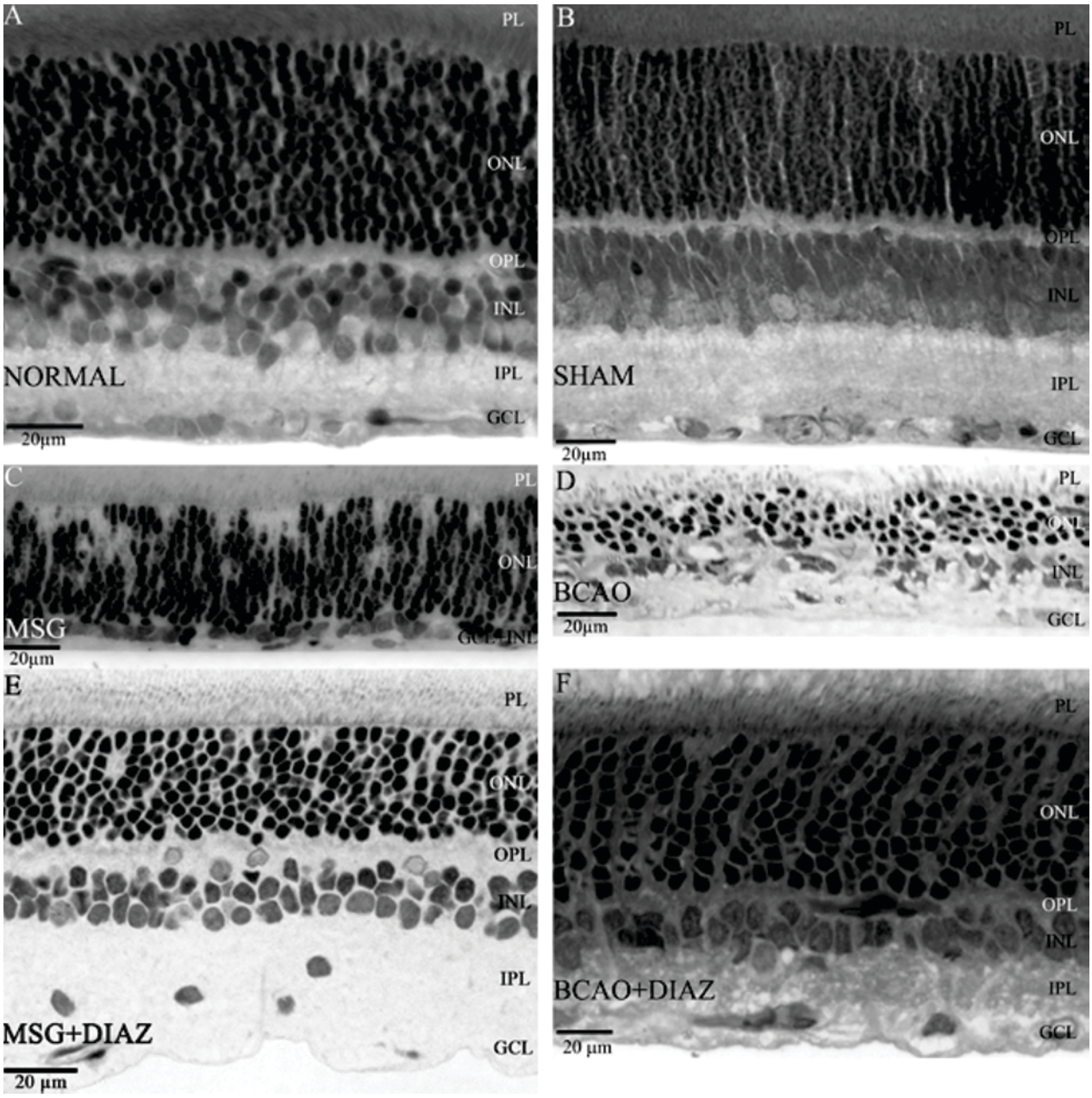

FIGURE 1 Microphotographs of representative retinas from normal animals (**A**), sham-operated control animals (**B**), MSG-treated rats (**C**) and rats with carotid occlusion (**D**). Neuroprotective effect of DIAZ in MSG-induced retinal degeneration (**E**), and in BCAO-induced ischemic insult (**F**). Abbreviations: PL: photoreceptor layer; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer; GCL: ganglion cell layer.

RESULTS

In saline-treated and sham-operated normal control preparations all the layers characteristic for the mammalian retina were well visible. Under the pigment epithelium, several rows of photoreceptors were present, followed by the thin outer synaptic layer. The INL usually consisted of 4-5 rows of cells, followed by a thick inner synaptic layer. Finally, cells in the GCL layer formed one cellular row (FIG. 1A). Retinal tissue from animals treated with MSG showed severe degeneration compared to control retinas. Much of the IPL disappeared and the INL and GCL were intermingled (FIG. 1C). As a consequence, the total thickness of the retina was significantly reduced, only the photoreceptor layer seemed unchanged (FIG. 2A). Local DIAZ treatment resulted in a retained retinal structure that was similar to that of the normal control retina (FIG. 1E, 2A). The number of cells in the GCL was not

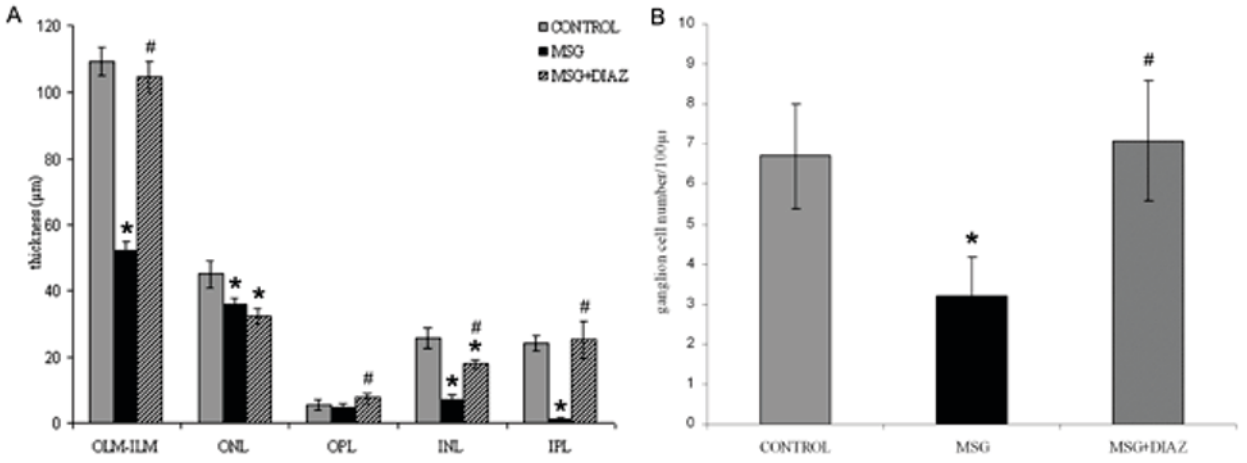

FIGURE 2 Comparison of retinal layers (**A**) and the number of cells/100 µm GCL length (**B**) in normal, MSG-treated control and MSG+DIAZ-treated retinas. Abbreviations: OLM-ILM: cross-section of the retina from the outer limiting membrane to the inner limiting membrane; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer; IPL: inner plexiform layer. **P* <0.05 compared to normal retinas; #*P* <0.05 compared to MSG-treated retinas.

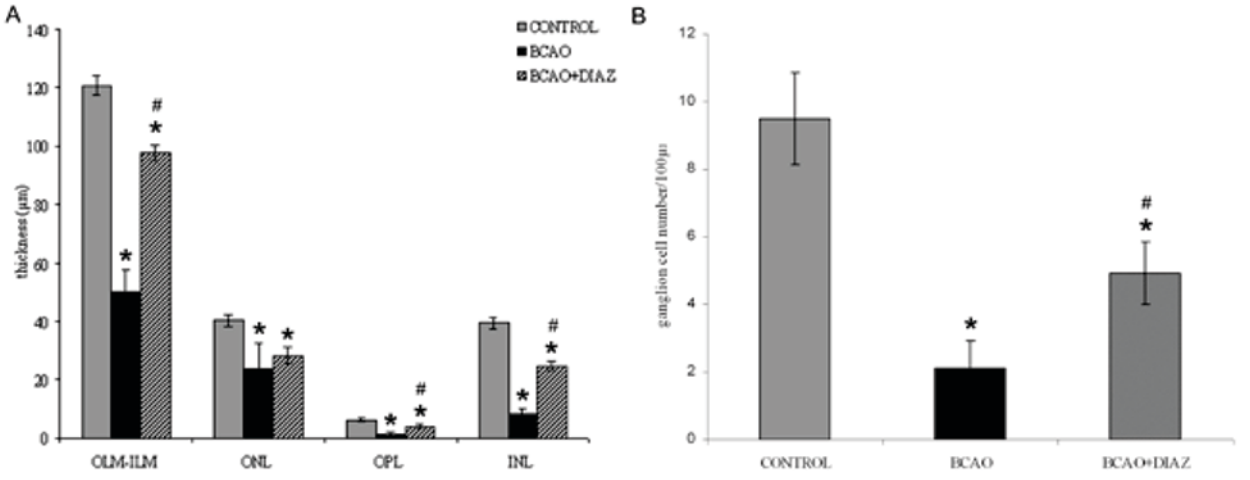

FIGURE 3 Comparison of retinal layers (**A**) and the number of cells/100 µm GCL length (**B**) in sham-operated animals, control rats with BCAO and those receiving DIAZ treatment after the carotid occlusion. Abbreviations: OLM-ILM: cross-section of the retina from the outer limiting membrane to the inner limiting membrane; ONL: outer nuclear layer; OPL: outer plexiform layer; INL: inner nuclear layer. **P* <0.05 compared to sham operated animals; #*P* <0.05 compared to BCAO-induced ischemic retinas.

significantly less than that of the untreated retina (FIG. 2B). The IPL remained visible, the INL and GCL are clearly separated at all places.

Carotid occlusion led to a severe reduction in thickness of retinal layers compared to sham-operated control rats (FIG. 1B,D). All retinal layers displayed the marks of serious degeneration. The most marked reduction in thickness was found in the plexiform layers, and as a consequence, the OLM-ILM distance was also significantly less than in control preparations (FIG. 3A). The photoreceptor layer was also reduced and many cells in the GCL also suffered degeneration as it is evidenced by the reduced number of cells in the GCL/length unit (FIG. 1D). DIAZ proved to be retinoprotective also in this model: differences between control and DIAZ-treated retinas were statistically significant in almost all retinal layers, except for the ONL (FIG. 1F, 3A). Quantitative analysis demonstrated that DIAZ administration protected the ganglionic cells in the GCL (FIG. 3B).

DISCUSSION

In the present study we showed that the severe degeneration of the inner retinal layers caused by neonatal MSG treatment or BCAO was significantly attenuated by DIAZ, a mitochondrial K^+ channel-opener. Thus, DIAZ is retinoprotective in models of retinal degeneration *in vivo*.

Glutamate is the primary excitatory neurotransmitter in the central nervous system, including the retina. Excessive glutamate receptor activation causes excitotoxic cell death. The inner retinal layers are especially vulnerable to glutamate overexcitation. Although the two models used in our present study represent two different pathomechanisms, the overexcitation of glutamate receptors plays a central role in both conditions and both models mimic the pathological increase of extracellular glutamate concentrations (Vidal-Sanz *et al.*, 2000; Yamauchi *et al.*, 2003; Osborne *et al.*, 2004). While the MSG-induced degeneration damaged mostly the inner retina (Babai *et al.*, 2005), BCAO-induced ischemic insult affected also the ONL and OPL. In the latter case many photoreceptors and possibly the second - and third-order neurons belonging to the same retinal circuitry were found to be damaged (Atlasz *et al.*, 2007).

Recent studies have indicated that glutamate leads to apoptotic cell death in the retina which can be inhibited by various anti-apoptotic compounds (Chen *et al.*, 2001). Previously, we have shown that PACAP, a neuropeptide with strong anti-apoptotic effects significantly ameliorates both types of retinal injuries (Somogyvari-Vigh and Reglodi, 2004; Babai *et al.*, 2005; Atlasz *et al.*, 2007). We have provided evidence that PACAP administration following neonatal MSG treatment is able to counteract the MSG-induced activation of caspase-3 and JNK and increases the levels of phospho-bad, ERK and CREB (Racz *et al.*, 2006a,b,c). Mitochondria are involved in many events of different forms of cell death. They play a key role in retinal apoptosis through the release of several anti- and pro-apoptotic proteins, which exert their actions through caspase dependent as well as independent way (Doonan and Cotter, 2004; Charles *et al.*, 2005). Cytochrome *c* and apoptosis inducing factor (AIF) are both pro-apoptotic signals that are released from the mitochondria and play key roles in inducing apoptosis in different types of retinal injuries (Charles *et al.*, 2005). We have shown that PACAP is able to inhibit the MSG-induced cytosolic translocation of the mitochondrial cytochrome *c* and AIF (Racz *et al.*, 2006b,c).

Another way of interfering with mitochondrial pathways is to alter ion permeability of the mitochondrial membrane. ATP-sensitive K^+ channels are located in different parts of the cell, including the inner mitochondrial membrane. The mitochondrial ATP-sensitive K^+ channels have been extensively studied in the heart and brain (Yamauchi *et al.*, 2003; Busija *et al.*, 2004). The selective activation of these channels by means of pharmacological or physiological stimuli has been shown to be cytoprotective against ischemia or chemical stress. The cardio- and neuroprotective effects of various agents are attributed to the activation of these channels (Yamauchi *et al.*, 2003; Busija *et al.*, 2004). This type of neuroprotection represents a new mechanism of protection which is not dependent on blocking glutamatergic receptors or scavanging free radicals (Busija *et al.*, 2004).

DIAZ treatment also targets the mitochondria and induces a chain of intracellular protective mechanisms (Busija *et al.*, 2004). DIAZ is a mitochondrial ATP-sensitive potassium channel opener

that is mostly used as an antihypertensive and antihypoglycemic drug in human therapy and has been applied as a neuroprotective agent (Busija *et al.*, 2004). Depending on timing of the treatment, the cytoprotective effect can be acute or delayed. It has also been demonstrated that DIAZ can induce early and late preconditioning and provides cytoprotection (Busija *et al.*, 2004). When DIAZ is used prior to the insult *in vitro*, it protects against neuronal cell death induced by oxidative stress or glutamate (Teshima *et al.*, 2003; Nagy *et al.*, 2004). *In vivo*, it has neuroprotective effects in various cerebral ischemic experimental conditions (Busija *et al.*, 2004; 2005; Domoki *et al.*, 2004; Farkas *et al.*, 2005a,b; Lenzser *et al.*, 2005). Bilateral carotid occlusion, which was also used in our present study, is mainly applied to induce cerebral hypoperfusion, where the protective effects of DIAZ have been described both using pre- and postischemic administration (Farkas *et al.*, 2004; 2005a,b; 2006).

Despite the numerous pieces of evidence showing the neuroprotective effects of DIAZ, little is known about its effects in the retina. The mitochondrial ATP-sensitive K^+ channels are also present in the retina, where the stimulatory effects of DIAZ have been reported (Sheu and Wu, 2003). *In vitro*, the opening of these channels with different agents, including DIAZ, enhances survival of retinal ganglionic cells and protects retinal neurons against glutamate-induced excitotoxicity (Yamauchi *et al.*, 2003; Pielen *et al.*, 2004). DIAZ has also been shown to inhibit the glutamate-induced mitochondrial depolarization (Yamauchi *et al.*, 2003). In an *in vitro* system, opening the mitochondrial K^+ channels has been shown to inhibit the oxygen/glucose deprivation-induced glutamate release and to be protective in a model of retinal ischemia (Jehle *et al.*, 2000). In a superfused retinal system, DIAZ has blocked the hypoxia-induced horizontal cell depolarization and the reduction of the light-evoked hyperpolarization (Hankins and Ikeda, 1993). Recently, it has been shown that these channels are required in retinal ischemic preconditioning, and preconditioning can be effectively mimicked by DIAZ (Roth *et al.*, 2006). Our present study provides *in vivo* evidence that local DIAZ administration attenuates both MSG- and ischemia-induced retinal degeneration. The mechanism could be multiple, including acute cytoprotective effects of the drug as well as early and late preconditioning. If DIAZ is available for the cells at the time of the ischemia/hypoxia or other kind of depolarization, its protective mechanism can be mediated by reduction of the mitochondrial calcium load (Domoki *et al.*, 2004). Although the morphological appearance of the two retinal degeneration models is different, our present study shows that DIAZ is able to attenuate the degeneration induced by MSG and BCAO. Comparing the present results with our earlier observations on the protective effects of PACAP, DIAZ seems to be more protective against MSG-induced retinal degeneration, while PACAP is more efficient in BCAO.

In summary, the present study showed that the severe degeneration of the inner retinal layers caused by neonatal MSG treatment or BCAO was significantly attenuated by DIAZ, which may have further clinical implication in ophthalmic diseases induced by ischemia or excitotoxicity.

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