Enhancement of Axonal Regeneration of Retinal Ganglion Cells in Adult Rats by Etomidate: Involvement of Protein Kinase C

Zhao-Xi Xu,* Sbang-Zhen Qin, Guo-Zbeng Xu, Jun-Min Hu, and Lian-Ting Ma*

PURPOSE. To investigate the effect of etomidate (ET) on axonal regeneration of retinal ganglion cells (RGCs) in adult rats.

METHODS. The optic nerve was transected intravitreally at 1 mm from the optic disc, and an autologous peripheral nerve was transplanted onto the ocular ON stump in adult rats. Then the animals were treated with ET, G6976, ET combined with G6976, phorbol-12-myristate-13-acetate (PMA), or ET combined with PMA. Four weeks after grafting, the number of regenerating RGCs labeled retrogradely with neuronal retrograde tracer was counted in all animals, and the activity of membrane protein kinase C (mPKC) and cytoplasmic PKC (cPKC) was measured in ET-treated animals.

RESULTS. The number of regenerating RGCs significantly increased when the dose of ET was increased from 2 mg/kg to 6 mg/kg, whereas the ratio of mPKC activity to cPKC activity increased when the dose of ET was increased from 2 mg/kg to 6 mg/kg. Combination treatment with ET at 6 mg/kg and G6976 alone was significantly lower than in those treated with ET at 6 mg/kg. Combined treatment with ET at 6 mg/kg and G6976 alone did not increase the number of regenerating RGCs. In contrast, PMA, a potent PKC activator, partially abolished the positive effect of ET on the axonal regeneration of axotomized RGCs.

CONCLUSIONS. These results suggest that ET promotes axonal regeneration of RGCs in adult rats, in part by inhibiting conventional PKC.

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Materials and Methods

Animals and Grouping

Eighty adult female Sprague-Dawley rats (The Laboratory Animal Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China), each weighing 200 to 220 g, were used in the present study. The experiments in this study adhered to the AVRO Statement for the Use of Animals in Ophthalmic and Vision Research. All efforts were made to minimize animal suffering. Table 1 summarizes...
the experimental groups, drug administrations, and numbers of animals used.

**Surgical Procedures and Drug Administration**

The surgical procedures of ON transection and PN transplantation were performed as demonstrated by You et al.19 Briefly, while animals were under deep anesthesia induced by intraperitoneal injection of 1% pentobarbital sodium (50 mg/kg body weight; Sigma, St. Louis, MO), the left ON was exposed through the superior temporal approach (Fig. 1A) and then was completely transected at 1 mm from the optic disc (Fig. 1B), taking care to avoid the ophthalmic artery. A segment (approximately 2 cm) of PN (the autologous sciatic nerve) was obtained from the right hind limb and sutured onto the ocular stump of the transected ON with a 10–0 suture (Fig. 1C). The remaining part of the graft was laid subcutaneously over the skull.

Etomidate (ET; Etomidate-Lipuro; B. Braun, Melsungen, Germany) or 10% lipid emulsion (vehicle; Baxter, Berkshire, UK) with equal volumes of ET at 2 mg/kg was administered by intraperitoneal injection immediately after the PN was sutured onto the ON stump and then once a day until the day animals were killed.

Gö6976 (Sigma) and PMA (Sigma) were dissolved in dimethylsulfoxide (DMSO; Sigma) and then diluted with phosphate-buffered saline (PBS; 0.1 M, pH 7.4; Sigma) with a final concentration of 5 μM. The mean volume of the rat vitreous space is 56 μL.20 Thus, adding 5 μL Gö6976 or PMA at a concentration of 5 μM resulted in a final concentration of 446 nM in the rat vitreous space. The effective concentrations of Gö6976 and PMA were 7.9 nM17 and 200 nM,18 respectively. Therefore, 5 μL Gö6976, PMA, or DMSO was then injected into the rat vitreous space at the cornea-sclera junction (Fig. 1D), as demonstrated by Zhi et al.21 In all animals, the first injection was made immediately after the PN was sutured onto the ON stump. Additional injections were made thereafter every 5 days.

**Labeling and Counting of Regenerating RGCs**

All the animals were allowed to survive for 4 weeks after surgery. The procedures for labeling regenerating RGCs have been described previously.22–24 ET had been shown to increase the number of regenerating RGCs when administered at 2 mg/kg.19,25 The mice were killed by cervical dislocation, and the eyes were enucleated immediately. For counting, the superotemporal quadrant of the retina 1 mm from the optic disc was prepared for immunohistochemistry. The regenerating RGCs were labeled with neuronal retrograde tracer (Fig. 1A). More regenerating RGCs were detected in the group treated with ET at 2 mg/kg (D) than in the control (B) or vehicle (C) group. (B–D) Graphs were obtained from the superotemporal quadrant of the retina 1 mm from the optic disc.

### Table 1. Experimental Groups, Drug Administration, and Number of Animals Used

<table>
<thead>
<tr>
<th>Groups</th>
<th>Drug Administration</th>
<th>Counting of RGCs</th>
<th>Assay of PKC activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>None</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Vehicle</td>
<td>10% lipid emulsion, intraperitoneal injection, once a day</td>
<td>6</td>
<td>4</td>
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<tr>
<td>ET</td>
<td>2 mg/kg, intraperitoneal injection, once a day</td>
<td>6</td>
<td>4</td>
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<tr>
<td></td>
<td>4 mg/kg, intraperitoneal injection, once a day</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>6 mg/kg, intraperitoneal injection, once a day</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>DMSO</td>
<td>5 μL, intravitreal injection, once every 5 days</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Gö6976</td>
<td>5 μL, intravitreal injection, once every 5 days</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>ET + Gö6976</td>
<td>ET, 6 mg/kg, intraperitoneal injection, once a day</td>
<td>6</td>
<td></td>
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<tr>
<td></td>
<td>Gö6976, 5 μL, intravitreal injection, once every 5 days</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>PMA</td>
<td>5 μL, intravitreal injection, once every 5 days</td>
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<tr>
<td>ET + PMA</td>
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<td></td>
<td>PMA, 5 μL, intravitreal injection, once every 5 days</td>
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**Figure 1.** Microphotograph of regenerating RGCs labeled with neuronal retrograde tracer (A). More regenerating RGCs were detected in the group treated with ET at 2 mg/kg (D) than in the control (B) or vehicle (C) group. (B–D) Graphs were obtained from the superotemporal quadrant of the retina 1 mm from the optic disc.
Four days before an animal was killed, the PN graft was exposed and severed at approximately 1 cm from the attachment site. RGCs regenerating their axons into the PN graft were labeled by placing a small piece (approximately 3 mm³) of gel foam soaked in 5% neuronal retrograde tracer (Fluoro-Gold; Fluorochrome, Inc., Denver, CO) at the end of the graft (Fig. 1E).

The left eye was enucleated immediately after the animal was killed with an overdose of sodium pentobarbital. The retina was dissected in 4% paraformaldehyde (Merck, Darmstadt, Germany) in PBS, postfixed in the same fixative for 1 hour, and rinsed three times in PBS for 5 minutes each. It was then flatmounted in glycerol on a glass slide and covered with a coverslip. The neuronal retrograde tracer-labeled regenerating RGCs were counted in the whole retina under a fluorescence microscope using an ultraviolet filter.19,23

**PKC Activity Measurement**

Cytosolic PKC (cPKC) and membrane PKC (mPKC) were extracted from the retina as described previously,24 with slight modifications. After the animal was killed, the retina was dissected from the eyeball, weighed immediately, and homogenized for 5 minutes on ice in approximately 100 mg/mL buffer A (20 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 5 mM dithiothreitol [DTT], and protease inhibitor). The homogenate was then centrifuged at 10,000 g for 1 hour at 4°C, and the supernatant was collected to assay the activity of cPKC. The pellet was then resuspended in buffer B (20 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 5 mmol/L DTT, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride), sonicated six times for 10 seconds each, and allowed to dissolve for 1 hour at 4°C before overnight incubation at 4°C. Supernatant was collected from homogenates of the retina to assay the activity of mPKC using the same centrifugation conditions described. PKC activity assay was performed using a nonradioactive protein kinase assay kit (PepTag; Promega, Madison, WI), and the activity ratio of mPKC to cPKC (m-PKC/c-PKC) was then calculated.

**Statistical Analysis**

Data were analyzed using the predictive analysis software (SPSS 18.0; IBM, Somers, NY). Results were expressed as mean ± SD. Statistical significance was evaluated by one-way ANOVA, followed by Tukey’s test. Differences were considered significant when $P < 0.05$.

**RESULTS**

**Enhancement of ET on Axonal Regeneration of RGCs**

Neuronal retrograde tracer-labeled regenerating RGCs were observed throughout the whole retina in all animals. Regenerating RGCs usually had enlarged cell bodies with simplified dendrites, as previously reported (Fig. 1A).19,23,25

The number of regenerating RGCs in animals treated with ET at 2 mg/kg (2054 ± 349 cells/retina; Figs. 1D, 2) was significantly greater than that in control animals (1032 ± 147 cells/retina; $P < 0.01$; Figs. 1B, 2) or in animals treated with vehicle (1114 ± 179 cells/retina; $P < 0.01$; Figs. 1C, 2). However, there was no statistical difference between control and vehicle groups ($P > 0.05$; Fig. 2).

The numbers of regenerating RGCs in animals treated with ET at 4 mg/kg and 6 mg/kg were 2853 ± 498 cells/retina and 4118 ± 615 cells/retina, respectively, and the number of regenerating RGCs in animals treated with ET at 6 mg/kg was fourfold higher than in control animals. The number of regenerating RGCs increased markedly when the dose of ET was increased (Fig. 2).

**PKC Activity Assay**

The activity ratio of m-PKC/c-PKC significantly decreased in animals treated with ET at 2 mg/kg compared with control animals ($P < 0.05$; Fig. 3) or animals treated with vehicle ($P < 0.05$; Fig. 3) and was markedly higher than in animals treated with ET at 4 mg/kg ($P < 0.05$; Fig. 3). However, there was no significant difference between animals treated with ET at 4 mg/kg and at 6 mg/kg ($P > 0.05$).

**Enhancement of Gö6976 on Axonal Regeneration of RGCs**

Our results showed that the number of regenerating RGCs in animals treated with Gö6976 (3374 ± 491 cells/retina) was also significantly higher than that in control animals (1032 ± 147 cells/retina; $P < 0.01$; Fig. 4) and in animals treated with DMSO (1214 ± 194 cells/retina; $P < 0.01$; Fig. 4). There was no significant difference between the control and DMSO groups ($P > 0.05$; Fig. 4).

The number of regenerating RGCs in animals treated with Gö6976 was similar to that in animals treated with ET at 4 mg/kg and markedly less than that in animals treated with ET at 6 mg/kg ($P < 0.05$; Fig. 4). However, there was no statistical difference between the animals treated with ET at 6 mg/kg and the animals treated with Gö6976 combined with ET at 6 mg/kg ($P > 0.05$; Fig. 4).

**Inhibitory Effect of PMA on Axonal Regeneration of RGCs**

The number of regenerating RGCs in animals treated with PMA alone (470 ± 87 cells/retina) was significantly lower than that
in the control group (P < 0.05; Fig. 5) or in animals treated with DMSO (P < 0.05; Fig. 5). When animals were treated with ET at 6 mg/kg combined with PMA, the number of regenerating RGCs was significantly reduced (1529 ± 187 cells/retina) compared with that in animals treated with ET at 6 mg/kg (4118 ± 615 cells/retina) (P < 0.05; Fig. 5) but was markedly higher than that in control animals (P < 0.05; Fig. 5).

**DISCUSSION**

In the present study, we used the model of PN transplantation to investigate the effect of ET on axonal regeneration of RGCs in adult rats. Our results showed that ET led to a significant increase in the number of regenerating RGCs and a decrease in the activity ratio of m-PKC/c-PKC when the dose of ET was increased from 2 mg/kg to 6 mg/kg. These results indicate that PKC plays a crucial role in mediating the ET-induced increase in the number of regenerating RGCs.

PKC represents a family of second messenger-dependent serine/threonine kinases and consists of multiple isoforms that are subdivided into conventional (α, βI, βII, γ), novel (δ, ε, η, θ), and atypical (ζ, ι/λ) families, according to their sensitivity to diacylglycerol and Ca²⁺. The conventional isoforms are regulated by diacylglycerol and Ca²⁺. On activation of conventional PKCs, the level of intracellular Ca²⁺ increases, and c-PKC translocates to the plasma membrane and converts to m-PKC, thereby increasing the activity ratio of m-PKC/c-PKC.

In our study, the activity ratio of m-PKC/c-PKC decreased significantly in ET-treated animals, indicating that ET has a powerful inhibitory effect on PKC activity. As an intravenous general anesthetic, ET is thought to produce anesthesia by modulating or activating ionotrophic Cl⁻-permeable γ-aminobutyric acid A receptor. However, Patel et al. showed that ET substantially reduced glutamate release in the hippocampus in adult rats subjected to ischemia. Glutamate, the most abundant excitatory neurotransmitter in the vertebrate nervous system, activates glutamate receptors such as N-methyl-D-aspartate receptor, resulting in excessively high intracellular Ca²⁺. Yoles et al. reported that the level of intracellular glutamate significantly increased after ON injury in rats. Moreover, ET has been shown to decrease L-type calcium currents by altering the kinetics of the channel to favor the closed state in myocytes from guinea pig ventricles. Additionally, ET has been shown to inhibit Ca²⁺ release from the sarcoplasmic reticulum in airway myocytes, which plays a vital role on the activation of PKCs. Therefore, ET may inhibit PKC activity by decreasing Ca²⁺ influx, attenuating Ca²⁺ release from the sarcoplasmic reticulum, or both.

We also found that Go6976 significantly increased the number of regenerating RGCs. Combined treatment with ET at 6 mg/kg and Go6976 did not induce more regenerating RGCs. These results imply that ET may enhance the regeneration of RGCs possibly by the inhibition of conventional PKCs. In adult mammalian CNS, myelin inhibitory molecules, such as Nogo-A, myelinated glycoprotein, and oligodendrocyte myelin glycoprotein, bind to Nogo receptor to mediate the inhibition of axonal regeneration after injury. Because Nogo receptor is glycosylphosphatidylinositol-linked and lacks an intracellular domain to transduce the inhibitory signals, efforts have been made to identify its coreceptor. p75NTR has been implicated in the transduction of intracellular inhibitory signals, and the intramembrane proteolysis of p75NTR is dependent on conventional PKCs. Sivasankaran et al. demonstrated that, in adult the rat, pharmacologic inhibition of conventional PKCs could promote the regeneration of dorsal column axons across and beyond the lesion site after spinal cord injury. Consistent with their findings, our experiments also showed that the inhibition of conventional PKCs can promote the regeneration of RGC axons into the PN in adult rats.

In vitro, cultures of the rat anterior-horn neurons of the spinal cord from embryonic day 14 elevation of PKC activity has been found to be significantly correlated with neurite outgrowth. Ghoumari et al. reported that the inhibition of PKC prevented mouse Purkinje cell death but did not affect axonal regeneration in organotypic cultures from embryonic day 18 to postnatal day 10. Interestingly, it has been shown in cultures of explanted retina after ON crush that the activation of PKC significantly promotes axonal regeneration in goldfish. The differences in the effects of PKC on axonal regeneration reported thus far in the literature may be due to the differences in animal species, the neuronal phenotype, and the growth microenvironment of neurons. It has been shown that the inhibition of neurite outgrowth and growth cone collapse by myelin-associated glycoprotein or Nogo can be converted to neurite extension and growth cone spreading by inhibition of conventional PKCs in rat neurons from postnatal day 7, and neurite growth can be abolished by inhibiting inositol 1,4,5-triphosphate in chick neurons from embryonic day 6. Therefore, it appears that a balance between conventional PKC and inositol 1,4,5-triphosphate activity mediates the regulation of axon regeneration by myelin-derived proteins.
Interestingly, the number of regenerating RGCs in animals treated with G60976 was significantly lower than that in animals treated with ET at 6 mg/kg. Combined treatment with ET at 6 mg/kg and PMA partially abolished the positive effect of ET on RGC axon regeneration. These results suggest that ET, in addition to inhibiting conventional PKCs, affects the axonal regeneration of RGCs by other mechanisms in adult rats. Studies have demonstrated that activation of the cAMP/PKA pathway suppresses many inhibitors of neurite outgrowth, including myelin-associated glycoprotein, and thus promotes axon outgrowth in the adult mammalian CNS. Because PKA is a cAMP-dependent protein kinase, the activation of PKA requires elevation of the cAMP level in cells. To some extent, the intracellular cAMP level is dependent on the balance of adenylate cyclase and cyclic nucleotide phosphodiesterase because the activation of adenylyl cyclase and the inhibition of cyclic nucleotide phosphodiesterase results in elevation of the intracellular cAMP level. It has been shown that rodents have a calcium-inhibited adenylyl cyclase isomorph. The inhibition of cyclic nucleotide phosphodiesterase significantly promoted RGC survival after ON transection in adult rats. On regeneration of the corticospinal tract after spinal cord injury, the phosphorylation of PKC and PKA may facilitate the proliferation of Schwann cells and enhance axonal regeneration of RGCs by other mechanisms in adult rats. Studies have shown that ET at a subanesthetic dose markedly potentiated the axonal regeneration of RGCs in vivo; this effect was mediated at least in part by the inhibition of conventional PKCs. ET may, therefore, be useful in the treatment of injured or degenerated CNS.

In conclusion, our results showed that ET at a subanesthetic dose markedly potentiated the axonal regeneration of RGCs in vivo; this effect was mediated at least in part by the inhibition of conventional PKCs. ET may, therefore, be useful in the treatment of injured or degenerated CNS.

References


