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-Zheng 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, G66976, ET combined with G66976, 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. Combined treatment with ET at 6 mg/kg and G66976 alone was significantly lower than in those treated with ET at 6 mg/kg. Combined treatment with ET at 6 mg/kg and G66976 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. (Invest Ophtalmol Vis Sci. 2011;52: 8117–8122 DOI:10.1167/iovs.11-7774)

In adult mammals, optic nerve (ON) injury is always followed by permanent visual deficits because of the poor ability of retinal ganglion cells (RGCs) to regenerate after injury. So et al.1 have demonstrated that axotomized RGCs can regenerate their axons into the peripheral nerve (PN) in adult rats. However, the number of RGCs that regenerate their axons into the PN is too low to support the restoration of visual function.2 Thus, a wide variety of attempts have been made to enhance axonal regeneration of injured RGCs using the model of PN transplantation. It has been shown that intraocular elevation of cyclic adenosine monophosphate (cAMP) potentiates ciliary neurotrophic factor (CNTF)-induced regeneration of adult rat RGC axons.3 Hu et al.4 demonstrated that combining Rho inactivation with exogenous CNTF and intraretinal elevation of cAMP significantly enhanced axonal regeneration of injured RGCs more than any single treatment in adult rats. Moreover, oncomodulin, secreted by activated macrophages, has been shown to promote the regrowth of RGC axons into PN grafts.5–7 Similarly, inflammation combined with elevated cAMP and phosphatase and the deletion of the pten gene has been reported to augment axonal regeneration of injured RGCs in adult mice.8 Despite incremental improvements in axonal regeneration of RGCs, the success achieved in restoring visual function is limited in all these cases. Therefore, improving our understanding of the cellular events that inhibit axonal regeneration of injured RGCs is very important.

Etomidate (ET), a nonbarbiturate anesthetic, is a common agent of choice for the induction of rapid sequence intubation. It has been postulated that it protects neural tissues from diabetic oxidative damage9 and reduces neuronal injury and attenuate functional deficits in the hippocampi of rats subjected to kainic acid-induced neurotoxicity.10 Interestingly, Cayli et al.11 have demonstrated that ET can also promote the recovery of spinal cord function after injury. It has been well documented that ET acts at the γ-aminobutyric acid A receptor to induce anesthesia.12,13 Moreover, Yun et al.14 reported that ET can decrease the activity of excitatory amino acid transporter type 3 expressed in Xenopus oocytes by inhibition of protein kinase C (PKC). Recent studies have identified conventional PKC as a necessary component for axon growth.15,16 However, the functional role of PKC on axonal regeneration remains controversial. 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. We used G66976, a potent PKC inhibitor with high selectivity for conventional PKCs,17 and phorbol-12-myristate-13-acetate (PMA), a potent PKC activator,18 to explore the role of PKC on RGC axonal regeneration, and we measured PKC activity in animals treated with ET.

Our results showed that ET significantly potentiated the ability of RGCs to regenerate their axons into the PN in adult rats, in part by inhibiting conventional PKC.

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
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>
</tr>
<tr>
<td>ET</td>
<td>2 mg/kg, intraperitoneal injection, once a day</td>
<td>6</td>
<td>4</td>
</tr>
<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>
</tr>
<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>
<td>6</td>
<td></td>
</tr>
<tr>
<td>ET + PMA</td>
<td>ET, 6 mg/kg, intraperitoneal injection, once a day</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PMA, 5 μL, intravitreal injection, once every 5 days</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

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 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.

**PKC Activity Measurement**

Cytosolic PKC (cPKC) and membrane PKC (mPKC) were extracted from the retina as described previously, 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). 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 number 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). There was no significant difference between the animals treated with ET at 4 mg/kg and animals treated with ET at 4 mg/kg and 6 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, β, 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 CI−-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 Gö6976 significantly increased the number of regenerating RGCs. Combined treatment with ET at 6 mg/kg and Gö6976 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, myelin-associated 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 adenylate 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 adenylate cyclase isoenzyme. The inhibition of cyclic nucleotide phosphodiesterase significantly promoted RGC survival after ON transaction in adult rats. On the other hand, ET has been shown to activate PKA to reduce glutamate uptake in rat cultured glial cells. Thus, it is necessary to consider that ET may increase the intracellular cAMP level as a result of a decrease in intracellular calcium activating Ca²⁺-inhibited adenylate cyclase or Ca²⁺-activated cyclic nucleotide phosphodiesterase, which may then activate the cAMP/PKA pathway in adult rat RGCs. It has also been reported that ET can rapidly increase the phosphorylation of extracellular signal-related kinases 1/2 by activation of the α2B receptor. Activating the extracellular signal-related kinases 1/2 by increasing their phosphorylation can promote axonal regeneration of the corticospinal tract after spinal cord injury or can facilitate the proliferation of Schwann cells and enhance axonal regeneration of the injured peripheral nerve. Moreover, activation of the extracellular signal-related kinases 1/2 is required for axonal regeneration of adult RGCs induced by fibroblast growth factor-2. These findings imply that ET may also enhance axonal regeneration of RGCs by activation of extracellular signal-related kinases 1/2. Park et al. have previously demonstrated that deletion of the pten gene significantly activates the mammalian target of the rapamycin (mTOR) pathway to promote robust axon regeneration after ON injury in adult rats. mTOR is an atypical serine/threonine protein kinase and the mTORC1 pathway work independently within adult rat RGCs. Previous studies have shown that intravenous administration of ET with bolus infusion at 5 mg/kg, followed by continuous infusion at 8 to 10 mg/kg · h induces deep anesthesia in adult rats. In our studies, the rats were asleep 2 minutes after intraperitoneal injection of ET at 8 mg/kg, but they could be awakened by stabbing the hind limb with a syringe needle. This suggests that a single dose of ET at 8 mg/kg may be the anesthesia threshold in rats. It is noteworthy that the dose of ET used in our study was lower than the anesthesia threshold.

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


