PKC Isozymes in the Enhanced Regrowth of Retinal Neurites after Optic Nerve Injury

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PURPOSE. To establish an in vitro model of axonal regeneration from mammalian retinal ganglion cells and to evaluate the role of PKC isozymes in promoting such retinal axon regeneration.

METHODS. Postnatal day-5 mice were subjected to optic nerve crush, and then retinal ganglion cells (RGCs) were used for culture 5 days later. RGCs were selected using anti-Thy 1.2-coated magnetic beads and plated onto a merosin substrate. Changes in axonal localization of PKC and axonal regeneration were examined in cultured RGCs by immunofluorescence. Changes in PKC isozyme mRNA levels were determined by semiquantitative reverse transcription–polymerase chain reaction (RT-PCR). The role of PKC in RGC neurite outgrowth was examined by treatment with activators or pharmacological inhibitors of PKC activity.

RESULTS. RGCs subjected to optic nerve crush injury demonstrated more rapid neurite outgrowth in vitro when compared with RGCs isolated from naïve retina. The neurites of these injury-conditioned RGCs showed both an increased rate of extension and enhanced PKC localization in culture. Injury-conditioned RGCs had elevated PKC isozyme mRNA levels, which probably contributed to the increased level of PKC protein in injury-conditioned RGC axons. Pharmacological activation of PKC enhanced neurite growth, whereas inhibition of PKC suppressed neurite growth in both the conditioned and naïve RGCs.

CONCLUSIONS. RGCs actively respond to axonal injury by regulating expression of genes that promote neurite outgrowth. PKC-α and -β isozymes are among the growth-associated proteins that are upregulated after injury. Results of pharmacological manipulation of PKC activity support the argument that increased PKC levels enhance neurite regrowth after axonal injury. (Invest Ophthalmol Vis Sci. 2003;44:2783–2790) DOI: 10.1167/iovs.02-04715

Optic nerve injuries in the adult mammalian visual system are most often detrimental to retinal axons and trigger axonal degeneration and massive retinal ganglion cell (RGC) death.1,2 Although developmental changes in the extraneuronal environment, whether in the visual system or in other parts of the central nervous system (CNS), appear to prevent the mature CNS neurons from regrowing their axons after injury,3–11 intrinsic changes in neurons also affect the capacity for axonal regrowth.12–17 Such an intrinsic change in regenerative ability of RGCs occurs when the embryonic mouse retina establishes functional CNS innervation after transplantation into the adult brain, whereas the adult retina fails to establish any connections.18,19 Nevertheless, a growing body of evidence suggests that although intrinsic developmental changes often result in reduced regenerative ability in mature RGCs, these cells can also actively respond to axonal or eye injuries and alter cellular activity or gene transcription regulation on injury, which results in heightened regenerative activity.20–25 For example, explanted retina that experienced optic nerve crush exhibited more than a 100-fold increase of neurite outgrowth when compared with explanted retina that did not experience optic nerve crush.22 A dramatic increase in mRNAs encoding transcription factors (c-Jun, KROX, and CREB2,22,23) and proteins associated with axonal outgrowth21,22,24 is seen in such regenerating RGCs. These observations suggest a renewed ability of retinal axon regeneration after injury.21,22,26,27

PKC isoforms have been implicated in promoting axonal regeneration, because their levels are increased in regenerating neurites and axons.28–32 For example, PKC isozymes α, β, and γ are all upregulated and translocated into the growth cones of regenerating sciatic nerve axons.30 This rapid increase in axonal PKC levels occurs within hours of nerve injury and has been shown to costimulate axonal regeneration with other factors.33 Conversely, inhibition of PKC has been found to decrease regenerative axonal growth.33–36 In terms of molecular interactions and growth-regulating mechanisms, PKC function has been shown to play roles in neurite outgrowth stimulated by laminin,37,38 an extracellular matrix molecule that promotes retinal axon outgrowth during development.39–41 In addition, PKC isozymes have been implicated in modulating the shape of growth cones and activating axon growth-associated proteins in hippocampal neurons in culture.42 Injury is known to condition neurons for rapid axonal regrowth in vivo and in vitro after a second injury.25,26,43 In this study, we began to address the molecular changes that occur after injury-conditioning of RGCs. Using a purified preparation of RGCs for these experiments has enabled us to focus on injury-induced changes that are intrinsic to the ganglion cells when compared with preparations that also contain non-neuronal components of the retina. We found that the increased neurite outgrowth in injury-conditioned RGCs was coupled with an increase of PKC-α and -β mRNAs in the RGCs. The intensity of PKC immunoreactivity was dramatically increased in the rapidly growing processes of the conditioned RGCs, and this was particularly apparent in the growth cones of these cultured neurons. The role of PKC isozymes in neurite outgrowth was confirmed by culturing RGCs in the presence of either a PKC activator or an inhibitor.

MATERIALS AND METHODS

Optic Nerve Crush and Retinal Cell Culture

Postnatal day 3 (P3, where the day of birth is P0) mice were anesthetized by hypothermia. The closed eyelid furrow was blade-bisected.
under a binocular dissection microscope. Optic nerves on both sides were crushed for 3 seconds 0.5 to 1 mm behind the eyeball with curved forceps. Animals were then rewarmed to 37°C in a normal air incubator before being returned to the nest. Neuretinas were dissected 5 days after surgery and dissociated by papain using a commercial system (Papain Dissociation System; Worthington Biochemicals, Lakewood, NJ), according to the manufacturer’s specifications. After digestion, retinas were pipetted into a 15-mL centrifuge tube and pelleted by centrifuging at 600 rpm for 5 minutes in ovooucmdium enzyme-inactivating buffer. Cells were dissociated by trituration with a fire-polished, long-stem glass pipette and collected by centrifuging at 800 rpm for 5 minutes. Dissociated RGCs were then selected by Thy 1.2 antibody-conjugated magnetic beads (CD90. Miltenyi Biotech, Auburn, CA) and seeded onto glass coverslips that had been sequentially coated with 0.1 mg/ml poly-lysine and 10 μg/ml merosin (Gibco-Life Technologies, Grand Island, NY). Cells were cultured in serum-free medium (Neurobasal-A) supplemented with B27 and 100 U/ml penicillin and streptomycin (all from Gibco). Brain-derived neurotrophic factor (BDNF; Gibco) was also added at 50 ng/ml to support RGC survival. All procedures were performed for the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Immunofluorescence**

For immunostaining and neurite outgrowth assay, cells were fixed in warmed (37°C) 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 0.12 M sucrose for 10 minutes, 2 days after the seeding of the purified RGCs. After three washes in PBS, fixed cells were incubated in PBS containing 0.2% Triton X-100, 10% normal goat serum, and 10% heat-inactivated horse serum for 1 hour to block nonspecific binding. The following primary antibodies were used: mouse anti-Thy 1.2 (Accurate Chemical, Westbury, NY), mouse anti-α-tubulin (Oncogene Research Products, Boston, MA), mouse anti-PKC-α (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-PKC-β (Gibco), and rabbit anti-neurofilament H, M, and 68kDa antibodies (Chemicon, Temecula, CA). Note that for Thy 1.2 immunostaining, Triton X-100 was omitted in the blocking buffer.

For tubulin immunostaining, mouse anti-α-tubulin antibody was diluted 1:500 in PBS containing 0.1% Triton X-100/1% normal goat serum/1% heat-inactivated horse serum and incubated with the cells at 4°C overnight. Cells were then rinsed in PBS three times at room temperature. For secondary antibody staining, Texas red–conjugated goat anti-mouse antibody was used at 1:200 and incubated with cells for 1 hour. For all other immunostaining, the primary and secondary antibodies were diluted 1:200 in PBS with 1% each of normal goat serum and heat-inactivated horse serum. Cells were incubated with primary antibody for 2 hours, rinsed in PBS three times, and then incubated with secondary antibody for 1 hour. Coverslips were rinsed in PBS three times and then mounted on glass slides with antifade mounting medium (Vectorshield; Vector Laboratories, Burlingame, CA). Stained cells were observed and imaged with a fluorescence photomicroscope (Nikon Instruments, Garden City, NY).

**Neurite Outgrowth Analyses**

RGCs were fixed 48 hours after plating and immunostained for α-tubulin as described earlier. Thirty to 40 randomly chosen fields were recorded as digital images (600× magnification). These stored images were used to quantify neurite growth in 150 to 300 RGCs for each experiment. For this, total cells and the number of cells with neurites were scored. Cells were considered neurite bearing when the length of the processes exceeded two cellbody diameters. Neurite length, measured from the tip of the growth cone to the edge of cell body, was measured for all neurites of each cell, to obtain the average neurite length per cell. In addition, the length of the longest neurite was recorded for each RGC scored. To analyze neurite growth, histograms from neurite measurement were generated for neurite number and length. Statistical data were drawn from these experiments to determine the significance of differences among neurite outgrowths.

**Semiquantitative RT-PCR**

Total RNA of isolated RGCs was extracted after 1-day seeding by the acid phenol-guanidium- chloroform method. 18 RNA was processed for RT-PCR, as previously described. 18,19 Briefly, a commercial cDNA synthesis kit (SMART cDNA Synthesis; Clontech, Palo Alto, CA) was used for RT reactions to generate first-strand cDNA. For standard RNA isolates from RGC cultures, 200 ng total RNA was used as a template. RNA from human placenta (Clontech) was used as a positive control. The first-strand cDNA from RT reactions was diluted 50-fold with TE buffer. PCR was performed in 100-μl reaction volumes through 45 cycles, with 10 μl of diluted first-strand cDNA used as a template. The following gene-specific primer pairs were used: PKC-α, forward, 5'-TGAATCTCTGTAAGAGATA-3' and reverse, 5'-AGAAGGCAATGCTCTGTA-3'; PKC-β, forward, 5'-CAGCTGAAGATCAGACAA-3' and reverse, 5'-ATCCCATAAGACAGAGATC-3'; PKC-δ, forward, 5'-GGCGAGATTGAGAGAGCC-3' and reverse, 5'-TGCCCTTGTGGCATTC-3'; PKC-ζ, forward, 5'-CAAGCCTGCTGGTCCATAAC-3' and reverse, 5'-TGCCAATCTACAGGGACTCT-3'; and glyceroladehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GGGAGAAAATCGCAGAATATA-3' and reverse, 5'-AGACAACCGTTGCTCAGTG-3'. For semiquantitative PCR, aliquots were removed every five cycles, starting from the end of the 30th cycle, and used for analysis. For Northern blot analysis, cDNA from PCR reaction aliquots corresponding to 400 pg of the originally extracted RNA template were fractionated on 2% agarose gel and transferred to a nylon membrane (Osmonics, Westminster, MA). After UV immobilization, blots were probed with α-[32P]dCTP-labeled cDNA overnight and washed to high stringency. 45 Binding of the probes was detected by autoradiography and quantified by densitometry. Only cDNA bands in the linear range were used for data collection. Each experiment was repeated at least three times for statistical analysis.

**RESULTS**

Enhanced axonal regeneration using mature rodent retinal explants after optic nerve crush injury has been reported. 23 To assay the regenerative capacity of injury-conditioned mammalian RGCs without extraneuronal influence, we used dissociated RGCs isolated from neonatal mouse retinas that went through a previously administered optic nerve crush.

**Enhanced Regeneration of Injury-Conditioned RGCs**

Neuretinas were isolated 5 days after optic nerve crush and used for dissociated RGC culture. Dissociated RGCs were selected by Thy 1.2 antibody-conjugated magnetic beads and seeded onto glass coverslips, which had been precoated with poly-lysine and then with merosin. Cells were cultured in serum-free medium for 2 days and were then fixed for immunostaining and neurite outgrowth assay. The mouse RGCs readily extended neurites in culture. Anti-Thy 1.2 immunostaining of these cells showed relatively uniform Thy 1.2 expression, both on the surface of cell body and in the neurites (Fig. 1a). Eighty-nine percent to 93% of the isolated cells were Thy 1.2 immunoreactive in culture. To further determine the neuronal nature of the purified cell preparation, we co labeled cultures with anti-Thy 1.2 and antineurofilament antibodies. The Thy 1.2-positive cells consistently showed positive staining with neurofilament antibodies, indicating that these isolated cells were indeed neuronal (Fig. 1b, 1c).

Neurite outgrowth of non-injury-conditioned, naive RGCs and injury-conditioned RGCs was assessed after 2 days in vitro. For this, cells were fixed and immunostained for anti-α-tubulin (Fig. 2). The tubulin staining displayed the fine structures of neuronal processes and enabled consistently reliable visualization and measurement of neurite length. The number of neurites per RGC varied, but most often multiple neurites ema-
RGCs. implying a heightened regenerative activity in the conditioned (RGCs to extend their neurites at a faster rate than naive conditioned RGCs was 75% greater than that of naive RGCs (Fig. 2).

FIGURE 2. Anti-Thy 1.2 and anti-neurofilament immunostaining of injury-conditioned RGC. (a) RGCs were selected by using the anti-Thy 1.2 antibody-conjugated magnetic beads and plated on a merosin substrate for 2 days. Thy 1.2 expression was observed along the entire neurite and on the cell body. In double immunostaining, cells positive for Thy 1.2 (b) were also positive for neurofilament (c). Scale bars, 10 μm.

Figure 1: Different morphologies of neurite outgrowth from injury-conditioned and naive RGCs. Anti-α-tubulin immunostaining of nonconditioned naive (a) and injury-conditioned (b) RGCs. The neurites of naive RGCs were generally thin and short. There was usually no observable change in growth preferences in any of the neurites. In contrast, conditioned RGCs often sent out a prominent thick neurite that showed rapid outgrowth with greater length than the rest of the neurites of the same cell. Scale bar, 10 μm.

Quantitative Analyses of Injury-Conditioned RGC Neurite Growth
To quantify neurite outgrowth, cultured RGCs (2 days in culture) were immunostained for tubulin, and randomly chosen fields were recorded by fluorescence microscopy. Batches of unmarked images of conditioned and naive RGCs were analyzed blindly for percentage of cells with neurites, average neurite length per cell, and average length of the longest neurite in each recorded field. The proportion of neurite-bearing cells was essentially identical in the injury-conditioned and naive RGCs (conditioned RGCs: 29.1% ± 2% SD; naive RGCs: 27.0% ± 3% SD). However, the length of neurites was overall greater in conditioned RGCs compared with naive RGCs (Fig. 3). The average neurite length in the conditioned RGCs was 33% greater than that of naive RGCs (P < 0.005, t-test). The average length of the longest neurite in each field of conditioned RGCs was 75% greater than that of naive RGCs (P < 0.005, t-test). These results indicate that injury conditions RGCs to extend their neurites at a faster rate than naive RGCs, implying a heightened regenerative activity in the conditioned RGCs.

Upregulation of PKC-α and -β mRNAs and Proteins in Conditioned RGCs
Studies of the peripheral nervous system have suggested that PKC isoforms may play a role in nerve regeneration. In initially profiling differences in mRNA expression of the naive and injury-conditioned RGCs by cDNA macroarrays, we detected increase in both PKC-α and -β mRNAs in conditioned RGCs (data not shown). Because PKC isoforms are ubiquitously expressed in the brain and in retina, our pure preparation of RGCs enabled us to focus on only those changes that would reflect altered signaling in the RGCs rather than changes in all retinal cells collectively. We used a semiquantitative RT-PCR method to assess directly the changes in PKC isozyme mRNAs in the injury-conditioned RGC cultures. RT-PCR for GADPH mRNA with the same template was performed for control of RNA yield between the samples. Increased levels of mRNAs encoding PKC-α, -βI, and -βII were detected in the conditioned compared with the naive RGC cultures (Fig. 4). In contrast, the levels of PKC-ζ, which has not previously been reported in retina or mouse RGCs, showed no significant difference between the two RGC conditions (Fig. 4). RT/PCR for GADPH mRNA showed essentially identical RNA levels in the injury-conditioned and naive RGC RNA templates. Although the scant starting material derived from the isolated RGCs is not amenable to further molecular analysis to assess mRNA half-life, the transcriptional change for specific PKC isoforms is clear. These data indicate that injury can generate a lasting change in the levels of mRNAs encoding proteins hypothesized to regeneration.

To further characterize the changes in PKC mRNAs, we asked whether injury conditioning changes the level and/or subcellular localization of their protein products. Similar to
other regenerating axons in vivo,28–32 we observed dramatic increases in immunoreactivity for both PKC-α and -β proteins in injury-conditioned RGCs. Both cell body and neurites showed greater immunoreactivity in the conditioned RGCs, but the difference between conditioned and naive was most apparent in the processes of the conditioned compared with the naive RGCs (Fig. 5). The growth cones of naive RGCs showed sporadic, weak PKC localization, not specific to any subcellular areas. In contrast, in the neurites of conditioned RGCs, PKC-α and -β appeared membrane associated, with intense immunoreactivity in the front, protruding edges of the growth cones, suggesting that there were both increased intensity of localization and activation of PKC isozymes.

Role of PKC Activity in RGC Neurite Outgrowth

The expression of PKC isozymes has been shown to be tightly regulated in the brain and retina.46–50 Changes in PKC expression, translocation, and activation are associated with the neuronal responses to injury and disease.28–32,51,52 Thus, although the data are suggestive that PKC-α and -β may promote RGC axonal regeneration, any PKC protein newly introduced into the conditioned RGCs would also have to be activated. To investigate, we asked what effect pharmacological activation or inhibition of PKC has on RGC neurite regeneration.

To stimulate PKC activity, purified RGCs were cultured in medium containing phorbol 12-myristate 13-acetate (PMA), a potent PKC activator.53–55 Average neurite length per cell and longest neurites in culture were determined as outlined earlier. The presence of 30 to 50 nM PMA increased neurite outgrowth in both naive RGCs and conditioned RGCs (Fig. 6). Both average neurite length and the length of longest neurites increased by approximately 30% in naive RGCs (Fig. 6). Meanwhile, PMA even further increased neurite outgrowth by conditioned RGCs. In comparing conditioned RGCs when they were treated with PMA with conditioned RGCs without PMA treatment, we observed 22% and 29% longer neurite outgrowth, in average neurite lengths for all neurites and in the longest neurites, respectively (Fig. 6). Remarkably, with 50-nM PMA treatment, both the average neurite lengths of all neurites and of the longest neurites reached about twice the length of naive RGCs without PMA treatment (Fig. 6), implying the combined neurite-promoting effect of injury conditioning and activation in these RGCs.

PKC activity was suppressed in the RGC cultures by using the PKC-specific inhibitor, chelerythrine.53,55 Conditioned and naive RGCs were treated with 0 to 2 μM chelerythrine. At this

FIGURE 3. Neurite outgrowth of naive and conditioned RGCs. The average neurite length of all neurites and the longest neurites of conditioned RGCs were 33% and 75% longer, respectively, than those of naive RGCs. Average neurite length of all neurites, average length of the longest neurite for each recorded field. Error bars, SD; P < 0.005 for both comparisons, t-test.

FIGURE 4. PKC mRNAs were increased in injury-conditioned RGCs. Quantitative PCR reactions were performed in a 100-μL volume and processed for 45 cycles by using the first-strand cDNA from reverse transcription as templates. Left: RT-PCR results from conditioned RGCs. Right: PCR results from naive RGCs. One aliquot was removed every 5 cycles starting at the end of 30th cycle for each PCR reaction. Aliquots were resolved on agarose gel, transferred to nylon membrane, and probed with designated cDNAs. To quantify original levels of mRNA, exposures in the linear range for different cDNA levels were analyzed by densitometry. After normalization for the GADPH mRNA product, PKC-α mRNA showed an approximately 50% increase, PKC-βI showed a 100% increase, and PKC-βII showed an 80% increase, whereas PKC-ζ mRNA did not change detectably (n = 3).
concentration, chelerythrine has no apparent effect on other protein kinases.31,55 After 48 hours in culture, cells were fixed, and neurite outgrowth was assessed as described earlier. More than 200 cells were randomly chosen from each inhibitor dosage, and their neurites were measured from both the naïve and conditioned RGC cultures. The PKC inhibitor reduced neurite outgrowth in both naïve RGCs and conditioned RGCs in a dose-dependent manner (Fig. 7). The decrease in neurite growth was much more pronounced in conditioned RGCs compared with naïve RGCs when PKC activity was inhibited with the increase of dosage. Eventually, neurite outgrowth was completely suppressed in both conditioned and naïve RGCs at high dose (Fig. 7). Similar to a previous report of PKC inhibition in neurons by chelerythrine,31 we did not observe cell death caused by chelerythrine treatment at the indicated doses (data not shown).

**DISCUSSION**

We found that injury-conditioned mammalian RGCs extended neurites significantly faster than non-injury-conditioned or naïve RGCs. Immunocytochemistry revealed a dramatic increase of PKC-α and -β localization in neurites of conditioned RGCs. Increase in PKC immunoreactivity was concomitant with prominent upregulation of mRNA levels of PKC-α, -βI, and -βII in injury-conditioned compared with naïve RGCs. We further show that PKC isoforms probably contribute to the rapid neurite growth in the injury-conditioned RGCs, because activation of PKC with PMA enhanced neurite outgrowth and specific inhibition of PKC suppressed it. These data suggest that the preadministered axonal injury conditions mouse RGCs for increased regenerative activity by increasing the abundance of critical mediators of intracellular signal cascades that promote neurite outgrowth.

Our observation that RGCs can be conditioned by a preadministered injury for robust new growth is consistent with the observations in previously reported retinal explant models.23,56,57 Similar findings have been reported in studies in lower vertebrate retina,58,59 in mammalian retina,23,26 and in dorsal root ganglion cells.43,45 It is especially notable that in goldfish, where optic nerve regeneration occurs naturally, there is a dramatic increase of both PKC localization and activity in the optic nerve after injury.60 Although it remains to be seen whether the increase of PKC in the optic nerve directly brings forth enhanced regeneration in vivo, these findings, together with our findings, strongly suggest that the intrinsic changes in gene expression in mammalian RGCs after injury play an essential role for neurite outgrowth and axon regeneration.

Injury-induced transcriptional changes and changes in the localization and activation of growth-associated factors

![Figure 6](http://iovs.arvojournals.org/)

**FIGURE 6.** PKC activation enhanced neurite outgrowth. PMA stimulation promoted neurite outgrowth in naïve and conditioned RGCs. Left: average length of all neurites per cell. Right: longest neurite length per field. At 50 nM of PMA treatment, both naïve and conditioned RGCs increased neurite length by approximately 30% over non-PMA treated RGCs (P < 0.005 for both). PMA-stimulated conditioned RGCs showed 22% and 29% longer neurite outgrowth, in average neurite length and longest neurite length, respectively. Comparing conditioned RGCs with 50 nM PMA treatment to untreated naïve RGCs, both average neurite length and longest neurite length increased by approximately twofold, implying the combined effect of the upregulation of PKC expression and activation in promoting neurite outgrowth. Error bars, SD.

![Figure 7](http://iovs.arvojournals.org/)

**FIGURE 7.** Inhibition of PKC activity suppressed neurite outgrowth. The presence of chelerythrine suppressed neurite outgrowth by both naïve RGCs (■) and conditioned RGCs (●), in a dose-dependent manner. However, the inhibition of PKC was more suppressive to neurite outgrowth by conditioned RGCs. Error bars, SD.
have been discovered in several different neuronal systems.\textsuperscript{20–23,25,61} In particular, various PKC isozymes have been suggested to play roles in regeneration of axons and neurites, both in vivo\textsuperscript{20–23,32} and in vitro.\textsuperscript{33–37,62,63} PKC-\(\alpha\) and -\(\beta\), specifically, are upregulated in different neuronal populations after injury.\textsuperscript{20–23} Besides axonal regeneration, they may also be involved in vivo in promoting other neuronal responses to environmental stimuli, including dendritic arbor reorganization\textsuperscript{64} and synaptic modulation.\textsuperscript{65–67} In vertebrate RGCs, PKC-\(\alpha\) and -\(\beta\) are the only two PKC isozymes previously reported.\textsuperscript{47–49} We observed significant upregulation of both PKC-\(\alpha\) and -\(\beta\) in RGCs after optic nerve injury. Another PKC isozyme, atypical PKC or PKC-\(\xi\), showed no change in mRNA level after injury. Although the mechanisms for the involvement of these PKC isozymes in RGC regeneration are not yet known, our results suggest that the upregulation of PKC levels is specific to particular isozymes and is part of the neuronal injury response of regeneration.

Our data further point toward the possibility that the upregulation of PKC isozymes in injury-conditioned RGCs is directly involved in the acceleration of neurite outgrowth. First, stimulation of PKC activation alone increases RGC neurite outgrowth. PMA treatment at 50 nM increased neurite outgrowth by approximately 30\% in the naive RGCs. Second, injury conditioning and PMA treatment synergistically increased neurite outgrowth. At 50 nM PMA, conditioned RGCs showed more than a 50\% increase in neurite outgrowth over similarly treated naive RGC, and more than a 90\% increase in neurite outgrowth over non-PMA-treated naive RGCs. This implies that activating existing PKC isozymes in naive RGCs can mimic the injury-conditioning mechanisms, but increasing the overall amount (and subcellular localization) of PKC is necessary to reach the full potential of neurite outgrowth that we observed in the conditioned RGCs. Therefore, we are intrigued in this is not surprising that in the injury-conditioned RGCs, where there was upregulation of PKC-\(\alpha\), -\(\beta\), and -\(\beta\)II mRNA (but not PKC-\(\xi\)), the PMA treatment showed almost twice as much neurite outgrowth when compared with naive RGCs without PMA treatment. This finding indicates that both upregulation of PKC levels and activation play roles in promoting regeneration in vivo. This finding may also explain why much more robust regeneration was observed in the injury-conditioned retinal explant,\textsuperscript{25,56,57} where the extraneuronal environment was more similar to that of the in vivo environment, which may activate PKC through the upregulation of growth factors.

The suppression of neurite outgrowth by chelerythrine, a PKC-specific inhibitor, was dose dependent. The DB\(_{50}\) concentration of chelerythrine was between 0.5 \(\mu\)M and 1 \(\mu\)M, with a maximum concentration at approximately 2 \(\mu\)M. These inhibitory concentrations are lower than has been reported in other neuronal systems.\textsuperscript{51} Differences in species and neuronal phenotype may account for the different effective dose in the RGCs. However, another possible explanation is that we have used purified RGCs grown in defined serum-free medium, so that the pharmacological effects of chelerythrine was not buffered by other cellular constituents. PKC inhibition attenuated neurite outgrowth by conditioned and naive RGCs. However, with the increase in chelerythrine concentration, it was apparent that the inhibition of neurite outgrowth in conditioned RGCs was more extensive than in the naive RGCs, until a near total inhibition of both of them. These data suggest a critical role for PKC isozymes in promoting rapid neurite outgrowth by conditioned RGCs, because at high chelerythrine concentration, there no longer were significant differences in neurite outgrowth between conditioned RGCs and naive RGCs.

Other intraneuronal factors had been reported to promote RGC axon/neurite outgrowth before, with the two most prominent being cAMP and Bcl-2.\textsuperscript{14,16,68–71} Detailed studies of cAMP in promoting regeneration of RGC axons and neurite outgrowth suggest that its upregulation recruits neurotrophic factor receptors to the membrane.\textsuperscript{14,68} Similarly, Bcl-2 itself does not promote neurite growth. Instead, Bcl-2 seems to augment the axon/neurite growth-promoting effect of electrical activity and neurotrophic signaling.\textsuperscript{72} Because neurotrophic factors are known to promote axon regeneration after injury,\textsuperscript{8,14,21,26} it will be interesting to see whether PKC functions downstream of neurotrophic factors during RGC neurite outgrowth.

The mode of action of PKC in RGC axon regeneration is not known. Recent evidence points toward the possibility that PKC acts upstream of focal adhesion kinase (FAK).\textsuperscript{72–74} FAK is an essential kinase for the integrin signaling pathway that is necessary for cell-adhesion–promoted neurite outgrowth on laminin.\textsuperscript{75} Both integrin-mediated cell spreading and FAK phosphorylation reportedly require PKC activity.\textsuperscript{76} Because our preparations were cultured on laminin, a subtype of laminin that promotes RGC neurite outgrowth,\textsuperscript{77} it is possible that FAK also plays a role in the enhanced neurotogenesis in the injury-conditioned RGCs. Consistent with this notion, FAK mRNA levels appear approximately two times higher in injury-conditioned RGCs by cDNA arrays and RT/PCR using gene-specific primers (Wu D-Y, Zheng JQ, Twiss JL, unpublished data, 2002). Thus, injury-induced changes in gene expression may result in increased levels of signal transduction modules to synergistically increase retinal axon regeneration. Another possibility is that PKC isozymes act directly or indirectly through phosphorylating integrin, the laminin/mammosin receptor.\textsuperscript{78–82} Future studies are needed to elucidate the signaling cascade and to determine whether increases in these protein kinases are relevant and can lead to enhanced optic nerve regeneration in vivo.

References


