Misregulation of rhodopsin phosphorylation and dephosphorylation found in P23H rat retinal degeneration

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Abstract: To examine rhodopsin (Rho) functions in P23H rat, kinetics of Rho regeneration and dephosphorylation were investigated by spectrophotometric analysis and immunofluorescence labeling method using specific antibodies toward phosphorylated 334Ser or 338Ser site. Rho dephosphorylation at both sites was extremely delayed in P23H retina as compared to normal ones. Kinetics of Rho regeneration was not altered between normal and P23H rats under dark adaptation. Next, to study the effects of several Ca^{2+} channel blockers on this model, retinal function and morphology were evaluated. Among them, nilvadipine showed a significant protective effect against P23H retinal degeneration. Neurotrophic factor, fibroblast growth factor-2 and Arc, known to suppress the apoptosis in the central nervous system, were significantly upregulated upon administration of nilvadipine. The present study indicates that misregulation of Rho phosphorylation may be involved as an important step in retinal degeneration of P23H and administration of nilvadipine may be a potential therapeutic agent for the retinal degenerations.

Keywords: rhodopsin, P23H rat, retinitis pigmentosa, mutation

Introduction

Retinitis pigmentosa (RP), a progressive hereditary retinal degeneration, is caused by the result of various genetic mutations (van Soest et al 1999). Among the most common are mutations in the rhodopsin (Rho) gene, including P23H (Dryja 1992). The P23H Rho mutation causes autosomal dominant (ad) retinal degeneration (Berson et al 1991). Retinal dysfunction due to abnormal disc morphology is involved in the molecular pathology causing the retinal degeneration in this mutation (Liu et al 1997). Impaired activation of the phototransduction cascade and recovery from activation are observed in human adRP patients with P23H mutation (Birch et al 1995), and the etiology of these mutations were studied expensively using animal models. P23H mice experiments demonstrated delayed photo-response recovery by double-flash measurements (Goto et al 1996). Thus, similarly to human adRP patients with P23H mutation, recovery from activation is also slow in the P23H mouse model. These observations suggest that the P23H model animal may be functionally impaired in the recovery state of biochemical reactions such as kinetics of Rho phosphorylation and dephosphorylation, binding of arrestin, and activities of guanylate cyclase and cGMP phosphodiesterase. Interestingly, lower levels of mRNA expressions of α-A crystalline and Rho kinase (RK), which are involved in post-Golgi processing of opsin and Rho phosphorylation, respectively, in Royal College Surgeons (RCS) rats (Maeda et al 2002) in which the retinal pigment epithelium (RPE) cell is affected (Mullen and LaVail 1976) by a mutation in the gene encoding the receptor tyrosine kinase *Mertk* (D'Cruz et al 2000; Gal et al 2000).

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However, expressions of other photoreceptor cell specific proteins including Rho, transducin, arrestin and recoverin were almost comparable between RCS and control rats (Maeda et al 2002). Recently, we demonstrated that dephosphorylation of Rho were extremely delayed in RCS rat retinas during the dark adaptation by a newly developed method to analyze *in vivo* Rho phosphorylation employing imunohistochemistry with specific antibodies toward phosphorylated Rho at specific sites (Ohguro et al 2003). Similarly, we also found significant high levels of Rho phosphorylation in light-induced stress rat retinas (Ishikawa et al 2006) and cancer-associated retinopathy (CAR) model (Ohguro et al 2001). Therefore taken together, we hypothesized that abnormal kinetics of Rho phosphorylation and dephosphorylation may contribute to persistent misregulation of phototransduction processes in retinal degeneration. If this is the case, photosensitive Rho levels may be insufficient in comparison to deactivated forms of Rho, photolyzed Rho and phosphorylated Rho, and to normalize this imbalance might be a candidate therapy for retinal degeneration. As regards this therapy, we speculated a possible mechanism; modulation of Rho phosphorylation and dephosphorylation kinetics by Ca^{2+}.

Ca^{2+} ions may play a significant role in the cell death by apoptosis, and is a critical factor regulating the recovery of the photoresponse (Nicotera and Orrenius 1992). Delayed recovery could therefore result from abnormal Ca^{2+} ion movement or abnormal levels within cytosol of the photoreceptor cells (Koch and Stryer 1988). In fact, Rho phosphorylation by RK is exclusively regulated in a Ca^{2+}-dependent manner by a photoreceptor specific Ca^{2+}-binding protein called recoverin (Kawamura 1991). Therefore, it is plausible that suppression of recoverin-dependent inhibition of RK by the lowering of intracellular Ca^{2+} levels by some drugs may be efficient for the preservation of photoreceptor cells in retinal degeneration. Indeed, some types of Ca^{2+} channel blockers have been identified to have preservation effects against retinal degeneration models (Frasson et al 1999; Yamazaki et al 2002). As described above, if the kinetics of Rho phosphorylation and dephosphorylation were impaired and photosensitive Rho levels were really insufficient in comparison to phosphorylated Rho in P23H rat model, it would be of great interest to test the effects of Ca^{2+} channel blockers for therapy.

Therefore, in the present study, to gain new insights into the mechanism of retinal degeneration in P23H rats, photoreceptor functions including Rho regeneration, and Rho phosphorylation and dephosphorylation were systematically studied and the effects of several Ca^{2+} channel blockers were also investigated.

Materials and methods

All experimental procedures were designed to conform to both the ARVO statement for Use of Animals in Ophthalmic and Vision Research and our own institution's guidelines.

Animals

Homozygous breeders of transgenic rats carrying Rho mutation of P23H (line 2, albino) were kindly provided by Prof. Matthew LaVail (University of California, San Francisco, CA). Heterozygous P23H rats were produced by mating homozygous breeders with wild-type Sprague Dawley (SD) rats. In the present study, 4- to 8-week-old SD rats (Crea, Tokyo, Japan) and heterozygous P23H rats reared in cyclic light conditions (12 h on/12 h off) were used.

Electroretinogram measurement

Details of preparation, recording technique and measurements of electroretinograms (ERG) have been described elsewhere (Machida et al 2000). Under anesthesia, the rats were laid on its side with its head fixed in place with surgical tape in an electrically shielded room for overnight dark-adaptation. The pupils were dilated with drops of 0.5 % tropicamide. ERGs were recorded with a contact electrode (Kyoto Contact Lens Co., Kyoto, Japan). A grounding electrode was placed on the ear. Responses evoked by white flashes (3.5×10^2 lx, 200 ms duration) using a Ganzfeld dome (SG-2002, Meiyo Co., Japan) were recorded and analyzed with PowerLab Scope version 3.7 (ADI instruments Ltd., Japan).

Light and immunofluorescence microscopy

Enucleated eyes were fixed with methacarn (60% methanol, 30% chloroform, 10% glacial acetic acid) overnight, dehydrated, and embedded in paraffin. Posterior segments of eyes cut from the enucleated eyes were embedded in paraffin. Retinal sections were cut vertically through the optic disc at 2-μm thickness, mounted on subbed slides and dried. The sections were processed with hematoxylin-eosin staining after deparaffinization. For evaluation of photoreceptor cell survival, the sections including the full length of the retina from optic nerve head through the ora serrata were taken photo and rows of cell nuclei in outer nuclear layer (ONL) were counted at 200-μm intervals along whole horizontal retinal axis.

For immunofluorescence labeling, deparaffinized sections were blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin for an hour and then incubated overnight with anti-P-Rho334 antibody, anti-P-Rho338 antibody (1:500), anti-Arc antibody, or anti-FGF2 (1:200) at 4 °C. Sections were washed and incubated with fluorescein-isothiocyanate (FITC) or Cy3-conjugated antibodies to rabbit IgG (Cappel, Durham, NC) for an hour at room temperature. Specificity controls were obtained by omitting the primary antibodies. Sections were observed by a fluorescence microscope (Olympus, model BH-2, Tokyo, Japan) using suitable filters.

Drug administration

Nilvadipine and nifedipine were dissolved in a mixture of ethanol: polyethylene glycol 400:distilled water (2:1:7) at a concentration of 0.1 mg/ml, diluted twice with physiological saline before use, and injected intraperitoneally (1 ml/kg) into anesthetized rats daily for 4 weeks. Nicardipine and D-*cis*-diltiazem were dissolved in PBS at 0.25 mg/ml and 1 mg/ml, respectively and injected intraperitoneally (1 ml/kg) as above. As control for them, each vehicle solution was administrated as above. The concentrations of these drugs administrated to rats were determined by their concentrations of systemic oral administrations to human patients with hypertension for one day in our clinical practice (Triggle 1981).

Regenerated Rho concentration

Rho regeneration was determined by photospectrometric analysis as described previously (Ohguro et al 1995). Briefly, rats were exposed by illumination condition (650 lux) for 6 h and then subjected to dark adaptation. At different times of the dark adaptation, rats were euthanized and eyes were enucleated and cut into two, anterior and posterior segments. The posterior segments were homogenized with 10 mM Hepes buffer, pH 7.5, containing 10 mM dodesyl β-maltoside and 20 mM hydroxylamine by a glass/glass homogenizer. The sample was centrifuged at 20,000 × g and the spectra were recorded before and after complete bleaching. Rho concentrations were determined from difference in A_{500} before and after bleaching as above by assuming ε_{500} to be 41,000 and the molecular weight to be 38,000.

Results

As shown in Figure 1, retinal degenerative P23H rat strains showed age-dependent thinning of retinal outer layers as described previously (Machida et al 2000), whereas there was no significant difference in the morphology between control rats at Day 20. In the present study, to get an insight into which steps in the phototransduction cascade were affected in this strain, selected biochemical reactions in photoreceptor cells were evaluated, including Rho phosphorylation and dephosphorylation, and Rho regeneration rates that represent the critical step of quenching the photoexcitation, and recovery phase after photoexcitation, respectively.

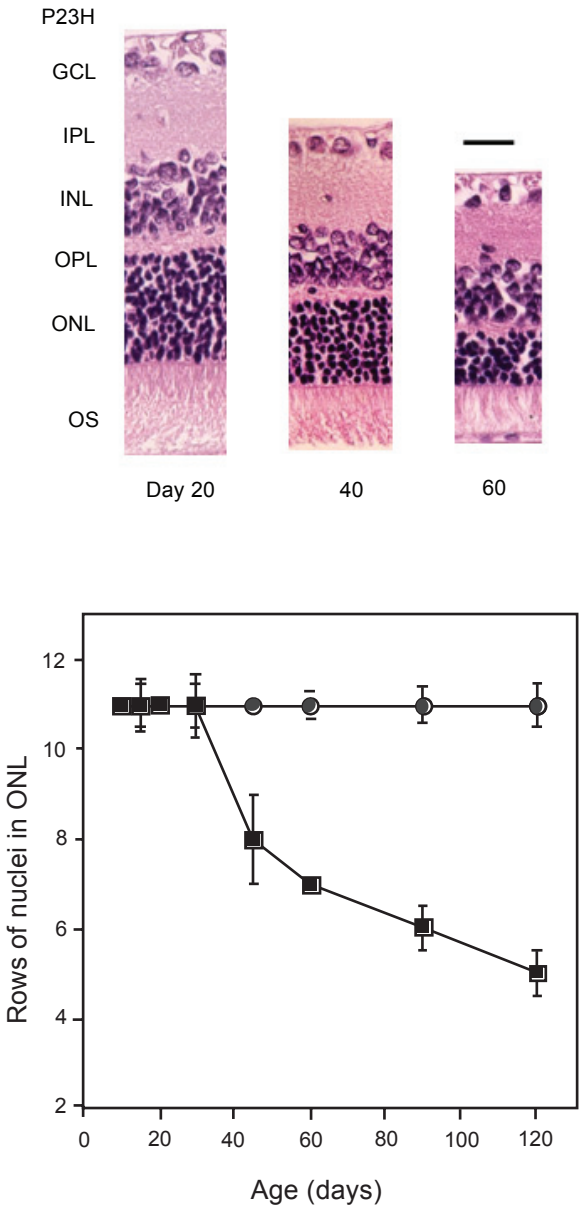

Figure 1 Retinal function and morphology in P23H rats during their development. Changes in retinal morphology and function by ERG of P23H rat are demonstrated. Hematoxyline-eosine staining of retinal sections at 1 mm from optic disc of P23H rat eyes during different ages were photographed (**A**), and rows of ONL nuclei were analyzed (**B**) as described in Materials and methods.
Notes: Scale bar, 25 μm; Closed circle (SD rat); closed square (P23H).

To assess Rho function, regeneration rate after bleach with constant light (650 lux) was firstly examined with P23H rats at the age of Day 20 without evidence of morphological retinal degeneration. Prior bleaching, the amount of Rho was compared and no significant difference was observed between different genetic back grounds. When rats were exposed to constant light for 6 h and then kept in the dark, Rho was completely regenerated in 2 h in both animals employed in the study. Rates of Rho regeneration of P23H strains during dark adaptation from light bleach conditions were almost identical with normal strain, suggesting Rho regeneration was not affected (Figure 2).

Next, to evaluate quenching function of photoreceptor, Rho phosphorylation and dephosphorylation were assessed by immunohistochemical technique using specific antibodies against phosphorylated 334Ser or 338Ser, both of which have been identified as major sites of phosphorylation in Rho *in vivo* (Ohguro et al 1995). In the control SD rat retina, 334Ser and 338Ser antibody specifically recognized rod outer segments (ROS) of light-adapted but not of dark-adapted retina, and its immunopositivities were then gradually diminished from base to tip of ROS following dark adaptation as described previously (Ohguro et al 2003) (data not shown).

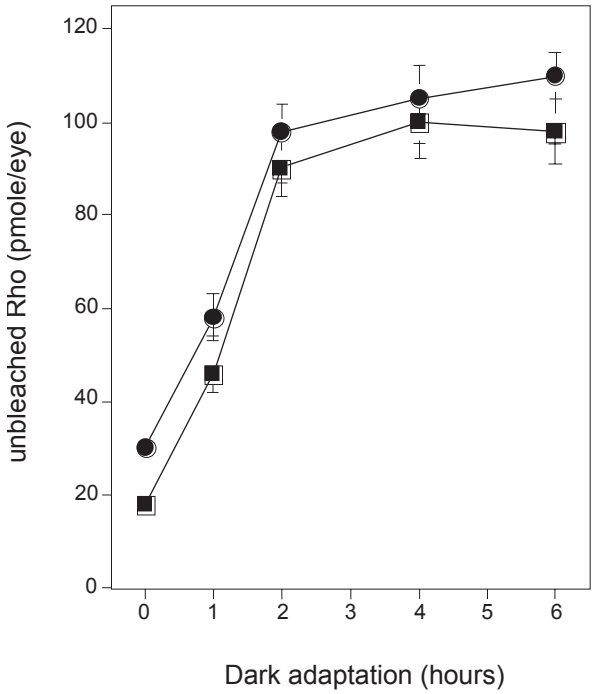

Figure 2 Time course of Rho regeneration during dark adaptation. Control or P23H rats (Day 20) were dark-adapted from regular room light (650 lux). At several time points, 0, 1, 2, 4, and 6 h, rats were euthanized and enucleated eyes were processed to direct Rho concentration analysis as described in the Materials and Methods. For one analysis two eyes from one rat were used. Experiments were performed in triplicate using fresh preparations.
Notes: Closed circle (SD rat); closed square (P23H).

The kinetics of dephosphorylation of phosphorylated 334Ser and 338Ser were determined by measuring the vertical lengths of immunofluorescence labeled ROS during the dark adaptation. Dephosphorylation of 334Ser and 338 Ser went to completion within 4 h in SD rats. In contrast, in P23H rat dephosphorylation of these sites was extremely prolonged (Figure 3).

We next studied the effects of several Ca^{2+} channel blockers, which are candidates as drugs beneficial for retinal degeneration based upon previous studies (Frasson et al 1999; Ohguro et al 2001; Yamazaki et al 2002; Ishikawa et al 2006). Four different Ca^{2+} channel blockers, D-*cis*-diltiazem, nifedipine, nicardipine, and nilvadipine, which are used in clinical practice, and their vehicle solutions were systemically administrated to 4-week-old P23H rats daily for four weeks (n = 5 rats, 10 eyes in each condition), and then the retinal function and morphology

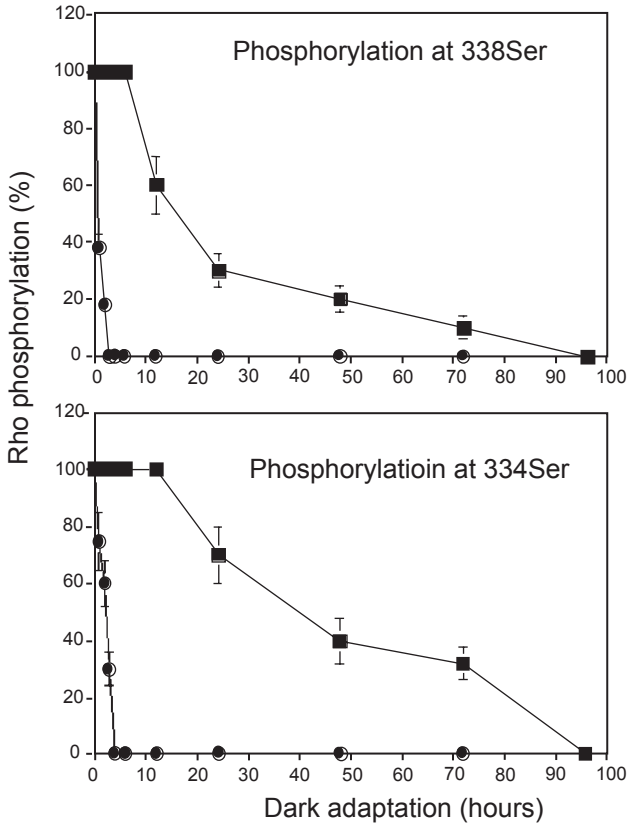

Figure 3 Kinetics of dephosphorylation in phosphorylated 334Ser and 338Ser in control and P23H rats. Four-week-old control and P23H rats were exposed to regular room light (650 lux) for 6 h and then were maintained under dark condition. Rho phosphorylation level was evaluated at three different time points (0, 1, 2, 3, 4, 6, 12, 24, 48, 72, and 96 h, n = 3 per group), with immunofluorescence labeling by anti-P-Rho antibodies. Vertical length of photoreceptor outer segment layers and that of fluorescence labeling was measured at temporal points 1.0 mm apart from optic disc from 6 different points from 3 different eyeballs and their ratios were plotted.
Notes: Data are expressed as the mean ± SD; Closed circle (SD rat); closed square (P23H rat).

were compared among these drugs. As shown in Figure 4, there was no significant difference in rats treated with D-*cis*-diltiazem, nifedipine or nicardipine and their control rats; however, significant preservation effects in retinal function by ERG and morphology (upper panel) were observed in P23H rats administrated with nilvadipine. To estimate the nilvadipine-dependent preservation effects against P23H retinal degenerations, retinal expressions of FGF2 and Arc, which were significantly enhanced in nilvadipine-treated RCS rat as compared to the control (Sato et al 2003), were immunohistochemically investigated. As shown in Figure 4, lower panel, marked enhancement

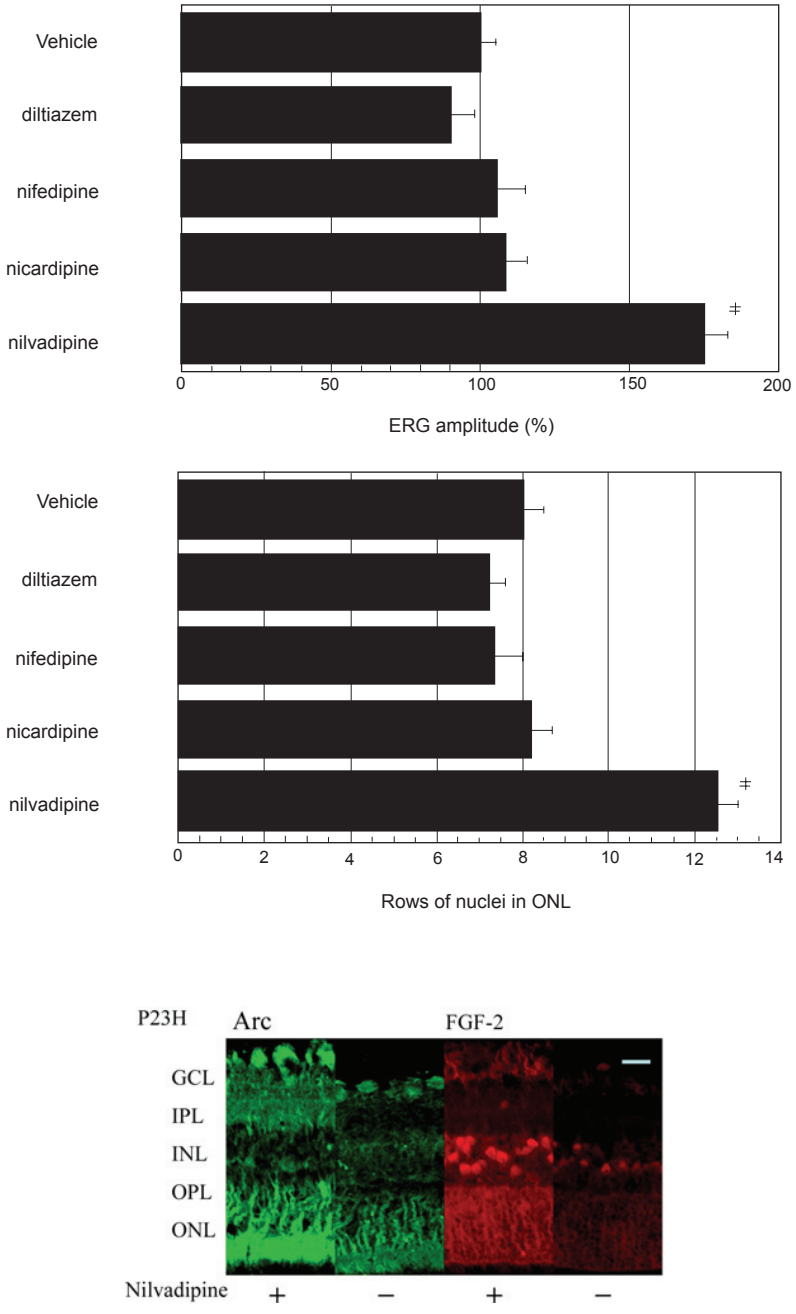

Figure 4 Effects of several Ca^{2+} channel blockers on retinal function and morphology in P23H rats. P23H rat were treated with Ca^{2+} channel blockers, D-cis-diltiazem, nifedipine, nicaridipine nilvadipine or their vehicle solutions and thereafter retinal function by ERG and morphological analysis (upper panel) were performed. ERG measurements were performed in 10 eyes (5 rats) in each condition, and b-wave amplitudes of drug treated rats were compared with those of vehicle treated rats. Hematoxyline-eosine stained retinal sections at 1mm from optic disc of P23H rat eyes treated as above were photographed, and rows of ONL nuclei were analyzed as described in Materials and methods.
Notes: *$P < 0.01$ (Mann-Whitney test). Nilvadipine- or vehicle-treated 4-week-old P23H rats were sacrificed and their retinal sections were subjected to immunofluorescence labeling by anti-FGF2 or anti-Arc antibodies (lower panel). Scale bar, 25 μm.
Abbreviations: GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer, OPL, outer plexiform layer; ONL, outer nuclear layer; OS, outer segment.

of the immunoreactivities of FGF2 and Arc was observed in nilvadipine-treated P23H rats in contrast to their controls.

Discussion

It has been hypothesized that quenching mechanisms during the phototransduction pathway are functionally affected in RCS rat, based upon the experimental evidence of several alterations in expressions of opsin (Nir et al 1987), and ROS protein phosphorylation levels (Heth and Schmidt 1992). Recently, to test this hypothesis, we developed specific antibodies toward Rho phosphorylated at 334Ser or 338Ser sites (Ohguro et al 2003), major phosphorylation sites in Rho *in vivo* (Ohguro et al 1995), and studied the kinetics of dephosphorylation of phosphorylated photolyzed Rho in living retina. During dark adaptation of normal control rats, dephosphorylated at 338Ser and 334Ser sites were completed within several hours (0–4 h). In contrast, the same immunoreactivities directed toward phosphorylated-338Ser and -334Ser sites resulted in a diminution at between 4 to 7 days in RCS rat retinas under dark adaptation (Ohguro et al 2003). Such prolonged kinetics of the Rho dephosphorylation during dark adaptation was also evident in light-stress-induced retinal degeneration (Ishikawa et al 2006), and significant enhancement of the phosphorylated form of Rho as well in a model of cancer-associated retinopathy (CAR), which is produced by intravitreous administration of anti-recoverin antibody to rats (Ohguro et al 2001). In addition, P23H rats in this study showed changes in Rho dephosphorylation kinetics during phototransduction cascade reactions. Consideration of the above suggests that deficits in regulation of phototransduction cascade by Rho phosphorylation and dephosphorylation is presumably a common mechanism responsible for most retinal photoreceptor degeneration. In fact, this hypothesis is supported by the previous observation that retinitis pigmentosa patients (Birch et al 1995) and mouse model (Goto et al 1996) with Rho mutations already known to show delayed photo-response recovery, suggesting that the quenching and adaptation process by Rho phosphorylation is impaired, in addition to a suggested disease mechanism of mislocalization of Rho within the photoreceptor cells (Green et al 2000). The status of Rho phosphorylation and dephosphorylation of the P23H rats after nilvadipine administration is very critical issue to prove our speculation that mis-regulation of Rho phosphorylation and dephosphorylation kinetics is the mechanism causing retinal degeneration and this may be normalized by administration of the suitable Ca channel blocker. Nevertheless, analysis of Rho dephosphorylation is very difficult at present because the retinal degeneration is also progressive during the drug administration period (4 weeks) and degenerative outer segments is not suitable for the proper analysis using immunohistochemical assay as above. Thus, we have to develop a more precise and reliable assay system for evaluating Rho phosphorylation and dephosphorylation at real-time *in vivo*. Therefore this is our next project.

Since the reactions responsible for quenching and adaptation of the phototransduction are Ca^{2+}-dependent (Koch and Stryer 1988), it is reasonable to speculate that Ca^{2+} channel blockers may have a potentially beneficial effect on the retinal degeneration. Frasson and colleagues (1999) first described the rescue of rod photoreceptor cells by a Ca^{2+} channel blocker, D-*cis*-diltiazem, in *rd* mouse, in which the gene encoding cGMP phosphodiesterase is affected. However Bush and colleagues (2000) claimed that D-*cis*-diltiazem had no effects on the photoreceptor degeneration in the Rho P23H rat. Therefore, the protective effect of Ca^{2+} channel blocker against retinal degeneration is controversial. Recently, to clarify this ambiguity regarding the effect of Ca^{2+} channel blocker, we systematically studied the effects of several kinds of Ca^{2+} channel blockers, including D-*cis*-diltiazem, nicardipine, nilvadipine and nifedipine against several models with retinal degeneration. From among these, only nilvadipine had a preservative effect on photoreceptor cells during retinal degeneration of RCS rat (Yamazaki et al 2002), light-stress-induced rat (Ishikawa et al 2006) and CAR model rat (Ohguro et al 2001), whereas, other Ca^{2+} channel blockers had no effects. Surprisingly, the present study consistently showed that nilvadipine also had protective effects against P23H retinal degeneration. Among four Ca^{2+} channel blockers used in the present study, three dihydropyridine (DHP) (nilvadipine, nicaridipine, and nifedipine) and diltiazem, it was revealed that nilvadipine is a much higher hydrophobic chemical and well distributed in various types of tissue, including brain, after the systemic administration than others (Tokuma et al 1987; Suzuki et al 1988). Thus, as to the reason why only nilvadipine was effective for the retinal degeneration we speculated the occurrence of preferable transmission of nilvadipine to the central nervous system, including retina as compared with other Ca^{2+} channel blockers. It was also revealed that nilvadipine had also a low-voltage-activated Ca^{2+} channel blocking action in addition to the L-type high-voltage-activated Ca^{2+} channel blocking action (Ishibashi et al 1998). However, in contrast, much less effects on a LVA Ca^{2+} channel blocking action of nifedipine, nicaridipine and diltiazem have been reported (Akaike et al 1989).

Thus this may be another possibility since presence of retinal LVA Ca^{2+} channels has been reported (Guenther et al 1994).

In our previous study, to elucidate the molecular mechanism responsible for the drug effects of Ca^{2+} channel blocker nilvadipine on the retinal degeneration, we analyzed altered gene expression by mRNA profiling assay and found that neurotrophic factor, fibroblast growth factor-2 (FGF-2) and Arc, known to suppress the apoptosis in the central nervous system were remarkably up-regulated among 1101 genes commonly expressed in rodent in nilvadipine-treated RCS rat (Sato et al 2003). Thus, we suggested that systemic administration of nilvadipine to RCS rats increases the expression of endogenous FGF-2 and Arc in retina, and potentially have a protective effect against retinal degeneration. In the present study, nilvadipine-induced enhancement of the endogenous FGF-2 and Arc expressions were also recognized in P23H model. FGFs constitute a large family of polypeptides that are important in the regulation of cell growth and differentiation and play a key role in oncogenesis and developmental processes including limb formation, mesoderm induction and neuronal development (Wagner 1991). Among FGFs, *in vivo* and *in vitro* studies have revealed that basic FGF (FGF-2) has been recognized as an important neuro-survival factor. Several *in vitro* and *in vivo* studies have revealed that FGF-2 prevented retinal degeneration (Faktorovich et al 1990; Steward and Worley 2001). Arc (activity-regulated cytoskeleton-associated protein) was first identified as one of the immediate-early genes in neurons (Steward and Worley 2001). It was shown that Arc mRNA is constitutively expressed within the cell body, but is delivered into dendrites and accumulated at synapses upon an appropriate stimulus, such as a single electroconvulsive seizure (Lyford et al 1995). In addition, this specific localization of Arc mRNA was shown to be dependent on local signaling through the NMDA receptor (Steward and Worley 2001). Thus if these factors indeed cause beneficial effects against retinal degenerations, nilvadipine-induced enhancement of endogenous these factors is a promising candidate therapeutic strategy for retinal degeneration.

Disclosure

The authors report no conflicts of interest in this work.

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