mechanisms (renin [25] and apolipoprotein B mRNA editing protein [19]), oxidative metabolism (cytochrome P450 XXVII and IIC11 [13]), Ca^{2+} metabolisms (Ca^{2+} channel β3 subunit [14], ADP-ribosyl cyclase 1 [15]), transporter (taurine transporter [17,18]), and receptors (opioid receptor δ1 [21], somatostatin receptor 4 [22], vasopressin V2 receptor [23], and mineral corticoid receptor [24]), and extracellular matrix (glypican 1 gene [26]), and up-regulated genes related to Ca^{2+} metabolism (endoplasmic reticulum Ca^{2+}ATPase [39]) and growth factor (fibroblast growth factor 2 [27–33]) were commonly recognized in both RCS and SD rat retinas (Table 1). Therefore, it seemed most likely that these changes were related to Ca^{2+} channel blocking effects by nilvadipine. In contrast, nilvadipine induced changes of apoptotic related genes (CD45 antigen [8,9], ErbA proto-oncogene [10], JAK2 [11], and tumor necrosis factor α [12]), signal transduction related genes (transducin β subunit [34], Goα subunit [44], MAP kinase 9, MAP kinase kinase 5, protein kinase C α and δ [45], and Ral B [46,47]), Ca^{2+} binding protein genes (neural visinin-like proteins 2 and 3 [38]), neurotransmitter receptor genes (GABA receptor α1 and pi subunit, GABA receptor γ3 subunit [20], NMDA receptor 2A [42], and glutamate receptor 6 [43]), transcription factor genes (V-erbA related protein [40] and NFκB transcription factor [41]), ion channel genes (voltage-gated K channel 3,4 [16] and voltage-gated K channel protein 3.1 [48]), transporter gene (kidney oligopeptide transporter [49]), immediate early gene (Arc [34–36]), and glycolysis related gene (fructose-bisphosphate aldolase A [50]) were differently regulated between RCS and SD rat retinas. These specific changes in apoptosis related genes and several genes related to signal transduction and neurotransmission were considered as results of suppression of apoptotic pathway and preservation of retinal cells caused by nilvadipine.

Among these changes, we directed our attention to the up-regulations of fibroblast growth factor 2 (FGF2) [27–33] and Arc [34–36] genes as most notable changes, because FGF2 is identified as possible therapeutic factors for RCS retinal degeneration [27–33] and Arc has recently been identified as an important factor for neuronal synaptic plasticity [34–36]. To confirm these observations, we performed real-time quantitative Taqman PCR, and Western blot analysis for FGF2 and Arc using their specific antibodies. As shown in Table 2 and Fig. 1, both FGF2 and Arc were expressed significantly higher in retinas obtained from nilvadipine treated RCS rats as compared with control RCS rat retinas.

Table 2
Taqman fold changes of FGF2 and Arc expression in nilvadipine-treated RCS retinas by Taqman quantitative PCR

	Nilvadipine treated period	
	1 week	2 weeks
FGF2	↑ 4.5 ± 0.5	↑ 5.8 ± 0.7
Arc	↑ 6.8 ± 0.3	↑ 7.4 ± 0.6

Taqman PCR was performed in 5 RNA specimens isolated from 10 in each condition. All data (threshold cycle) were standardized by GAPDH. The fold changes were relative to the retinal samples of RCS rats treated with vehicle solution.

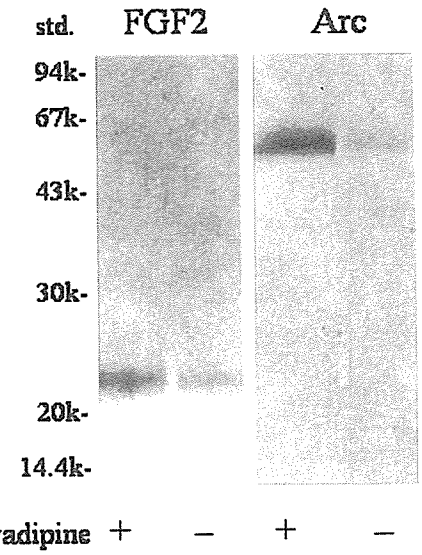

Fig. 1. Western blot analysis of FGF2 and Arc in nilvadipine-treated and untreated RCS rat retinas. Two retinas from 5-week-old RCS rats, which had been administrated nilvadipine or its vehicle solution for 2 weeks, were homogenized in 100 μl of 10 mM Hepes buffer (pH 7.5) containing 2% Tween 20. An aliquot (10 μl) was mixed with the sample buffer (10 μl) and loaded on an SDS–PAGE gel and then electrotransferred to PVDF membrane. Western blot analysis was performed using either anti-FGF2 antibody (1:2000 dilutions), or anti-Arc antibody (1:2000 dilutions), and HRP-labeled anti-rabbit IgG (1:2000 dilutions), as 1st and 2nd antibodies, respectively. The details of the Western blot are described in Materials and methods. Experiments were repeated five times using different preparations (total $n = 5$ rats, 10 retinas in each condition) and identical results were obtained.

Discussion

Recently developed microarray analysis is a very powerful tool for understanding complicated physiological, pathological, and pharmacological phenomenon [51]. By using this methodology, we found that only 30 out of 1101 genes, which are involved in a variety of cellular regulatory mechanisms, were up-regulated or down-regulated in RCS rat retina after administration of nilvadipine, which has recently been shown to have protective effects against RCS retinal degeneration. The effects upon several hormonal and neuronal receptors, ion-channels, peptide transporters, and anti-hypertensive factors regulated by these genes were considered the results of Ca^{2+} antagonist actions, an agent that was originally used as an anti-hypertensive drug. In contrast, down-regulation of apoptosis related genes most likely explains the protective effects of nilvadipine. In addition,

up-regulation of FGF2 and Arc genes seemed to be potentially important.

Fibroblast growth factors (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 [52]. Among FGFs, in vivo and in vitro studies have revealed that basic FGF (FGF 2) has been recognized as an important neuro-survival factor [53]. Several in vitro and in vivo studies have revealed that FGF2 prevented retinal degeneration [27–33]. Arc (activity-regulated cytoskeleton-associated protein) was first identified as one of the immediate-early genes in neurons [34]. 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 [35]. In addition, this specific localization of Arc mRNA was shown to be dependent on local signaling through the NMDA receptor [36].

Therefore, these observations suggest that up-regulation of FGF2 and Arc expressions upon administration of nilvadipine may play a significant role in the nilvadipine-induced protection against the RCS retinal degeneration. Microarray analysis provided us with new insight into the pharmacological mechanisms of nilvadipine-dependent protection against RCS retinal degeneration and may facilitate new therapeutic design for management of retinal degeneration.

Acknowledgments

This work was supported by grants from the Japanese Ministry of Health and Welfare, Grant-in-Aid for Scientific Research (B-11470361, B-12557145, and C-13671846) from the Japanese Ministry of Education, Sports and Culture, Naito Memorial Foundation, Ciba-Geigy Foundation for the Promotion of Science, The Mochida Memorial Foundation for Medical and Pharmaceutical Research, Uehara Memorial Foundation, Karouji Memorial Foundation for Medical Research and JRPS Research Foundation.

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Study of drug effects of calcium channel blockers on retinal degeneration of rd mouse

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Received 27 October 2003

Abstract

In the present study, we studied drug effects of Ca^{2+} antagonists on the retinal degeneration of rd mouse to evaluate their efficacy. Several kinds of Ca^{2+} antagonists, diltiazem, nicardipine, nilvadipine or nifedipine were administrated intraperitoneally and thereafter retinal morphology and functions were analyzed. In addition, we performed DNA microarray analysis both in nilvadipine treated and control retinas to understand their drug effects at molecular levels. We found that nilvadipine caused significant preservation of retinal thickness in rd mouse during the initial stage of the retinal degeneration, and nicardipine showed also significant but lesser preservation than nilvadipine. However, we recognized no preservation effects of diltiazem and nifedipine. In the total 3774 genes, the expressions of 27 genes were altered upon administration of nilvadipine, including several genes related to the apoptotic pathway, neuro-survival factor, Ca^{2+} metabolisms, and other mechanisms. It is suggested that some types of Ca^{2+} channel blockers, such as nilvadipine and nicardipine, are able to preserve photoreceptor cells in rd mouse and can potentially be used to treat some RP patients.

Retinitis pigmentosa (RP) is a disease of inherited retinal degeneration characterized by nyctalopia, ring scotoma, and bone-spicule pigmentation of the retina. So far, no effective therapy has been available for RP. Several animal models with inherited retinal degeneration have been studied in order to elucidate the molecular pathology of RP and to design an effective therapy for it [1]. The rd mouse is one of the best-studied animal models of RP in which photoreceptor degeneration starts around post-natal day 7–9 and a majority of photoreceptor cells are undetectable until after 4–6 weeks [2]. In terms of causative gene mutation of rd mouse is found in the rod photoreceptor cGMP phosphodiesterase (PDE) β-subunit and identical mutation is also recognized in patients with autosomal recessive RP [3]. This identification therefore allowed us to speculate if some therapeutic methods were beneficial for rd mouse retinal degeneration; this may also be clinically applicable to human RP patients.

Frasson et al. [4] reported rod photoreceptors were rescued by D-*cis*-diltiazem, a Ca^{2+} channel blocker in rd mouse. However, Pawlyk et al. [5] repeated the experiments but did not find any protective effects of D-*cis*-diltiazem on the rd mouse retinal degeneration. In addition, several investigators had examined drug effects of diltiazem on retinal degenerations in other animal models, such as PDE6B mutant rcd1 canine model, and rhodopsin Pro23His rat, but no retinal rescue has been found so far [6,7]. Recently, we studied drug effects of several types of Ca^{2+} channel blockers including diltiazem, nicardipine, nilvadipine, and nifedipine on the retinal degeneration of Royal College surgeons (RCS) rats, another animal model for study for RP, in which phagocytosis of retinal pigment epithelium (RPE) was affected by *Mertk* gene mutation [8]. Among these, we found that only nilvadipine caused significant protective effects [9]. In addition, we found that administration of

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0006-291X/$ - see front matter © 2003 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2003.12.034

nilvadipine to other types of photoreceptor degeneration models including rats with anti-recoverin antibody induced retinal degeneration as a cancer-associated retinopathy (CAR) model and rats with phototoxic retinal dysfunction caused significant improvement of the deteriorated retinal functions and morphology [10]. Based on this experimental experience, we assume that the regulation of intracellular Ca^{2+} levels by Ca^{2+} channel blockers may still be a potentially effective therapy to prevent progressive retinal degeneration in RP and its animal models, and think the effect of Ca^{2+} channel blockers on rd mouse should be systematically reevaluated using several types of Ca^{2+} channel blockers.

Therefore, in the present study several kinds of Ca^{2+} antagonists used in clinical practice [11–13] were administered to rd mice of 9 days of age, which is the time the degenerative changes in photoreceptor cells are known to start. Effects upon retinal function and morphology were then evaluated by electroretinography (ERG) and histological studies including light microscopy and electron microscopy. In addition, to obtain further insight into the effect of Ca^{2+} antagonist on retinal degeneration of rd mouse, DNA microarray analysis was performed.

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. Nilvadipine, diltiazem, nicardipine, and nifedipine were generous gifts from Fujisawa Pharmaceutical Japan, Tanabe Pharmaceutical Japan, Yamanouchi Pharmaceutical Japan, and Bayer Pharmaceutical Japan, respectively. Specific antibodies toward caspase 3, 9 or 14, FGF2, synaptogyrin I, and actin were purchased from Santa Cruz Biotechnology.

Drug administration to rd mice. In the present study, 9–16-day-old rd mouse reared in cyclic light conditions (12 h on/12 h off) were used. Mice were injected intramuscularly with a mixture of ketamine (80–125 mg/kg) and xylazine (9–12 mg/kg). 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 and diluted twice with physiological saline. Nicardipine and diltiazem were dissolved in phosphate-buffered saline (PBS) at 0.25 and 1 mg/ml, respectively. These Ca^{2+} antagonists and their vehicle solutions were each injected intraperitoneally (1.0 ml/kg) into rd mice every day for 7 days beginning at age 9 days. Before administration, pH of all drug solutions was adjusted at around 7.4.

Light microcopy. rd mice at age of 9 days as pretreatment control and those at age 16 days administered Ca^{2+} antagonist or its vehicle solution daily for 7 days were analyzed. Under deep ether anesthesia, animals were perfused with 100 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) (pH 7.4), and retinas were dissected out and embedded in paraffin. Retinal sections vertically through the optic disk at 4 μm thickness from nasal to temporal were prepared and stained with hematoxylin–eosin. Retinal sections were photographed, and the thickness of each retinal layer was measured at the temporal side 0.2 mm from the optic disk in 20 different sections from 20 eyeballs in each condition. Retinal layers are shown as means ± SD. Significant differences between groups were found using the Mann–Whitney test with a significance level of less than $P < 0.05$.

Electron microscopy. Nilvadipine treated and corresponding control rd mice 16 days old (3 animals for each condition) were used. Under deep ether anesthesia, each animal was perfused through the initial portion of the aorta with 100 ml of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate-buffer (pH 7.4). Tissues were dissected out, post-fixed in phosphate-buffered 1% osmium tetroxide (pH 7.4), dehydrated in an ascending series of ethanol solutions, and passed through propylene oxide. The blocks were embedded in EPON 812. Thin sections were stained in uranyl and lead salt solutions.

Electroretinography. Details of preparation, recording technique, and measurements of electroretinography (ERG) have been described previously [14]. Ten mice were studied at the age of 16 days, weighing approximately 10 g for each condition. The b-wave amplitude was determined from the bottom to the top of the ERG amplitudes are shown as means ± SD.

DNA microarray analysis. Five micrograms of total RNA from nilvadipine treated and corresponding control retinas ($n = 5$ mice, 10 eye for one analysis) isolated using ISOGEN reagent (Nippon gene, Tokyo, Japan) was reverse-transcribed into first-strand cDNA, and the double-strand cDNA was synthesized and extracted as described recently. Then the RNA amplification was performed using Ampliscribe T7 Transcription kit (Epicentre Technologies, Madison, USA) and the amplified RNA was purified with RNeasy Mini Kits (Quagen, Valencia, USA) as described recently. T7 RNA polymerase-amplified RNA (aRNA) was labeled with Cy3 and Cy5 fluorescent dyes, respectively, using Atlas Fluorescent Labeling Kit (Clontech, Palo Alto, USA). Cy3- and Cy5-labeled probes were hybridized to mouse Atlas Grass microarray 3.8 (Clontech, Palo Alto, USA) which includes 3774 genes with various functional categories, such as the gene related to oncogenes and tumor suppressors, cellular signaling, apoptosis, and transcription regulators, at 50 °C for 16 h. The microarrays were washed according to manufacturer's instructions; the slides were air-blown dried, prepared for scanning, and scanned for fluorescence with GenePrix (Axon, Union City, USA). Experiments were performed in triplicate using freshly prepared mRNA. The expression ratio was calculated by dividing fluorescence intensity of gene elements in nilvadipine treated mice by fluorescence intensity of gene elements in nilvadipine untreated mice. Genes significantly altered upon administration of nilvadipine were listed according to the following criteria: (1) Genes with a ratio of 2.0 or above or a ratio of 0.5 or below were considered up- or down-regulated. (2) Only 34 genes in which change ratio was consistently observed among the triple experiments are listed in Table 1. (3) Genes met criteria (1) but were excluded because their ratios were in different categories (normal, up-regulated or down-regulated) among the triple repeated experiments.

Taqman quantitative RT-PCR analysis. The cDNAs generated from 2 μg retinal RNA were analyzed by Taqman PCR analysis using an Applied Biosystems PRISM Sequence Detection System as described by manufacturer. The primers and probes were designed with primer-express software (Applied Biosystems) as follows:

Caspase 3 forward, 5′-AAGATACCGGTGGAGGCTGACT-3′, reverse, 5′-TAGCTTGTGCGCCTACAGCTT-3′, detection probe, 5′-TCACTTAGTCCCTTTGCATGC-3′.

Caspase 9 forward, 5′-AGTGGGCTCGCTCTGAAGAC-3′, reverse, 5′-CAGCTTTTTCCGGAGGAAGTT-3′, detection probe, TCCCTCCTTCTCAGGGTCGCCAAT-3′.

Caspase 14 forward, 5′-CTGACAGAAGAGATCACCCGACTT-3′, reverse, 5′-CCATCCCTGCCCTCTCTTTTAT-3′, detection probe, 5′-TGAAGTCCAAAGCACCCTCCGGAA-3′.

Synaptogyrin I forward, 5′-CTGCCATCGCCTTTTCGTT-3′, reverse, 5′-TCCATGTAGTCCTGGGAGAAGAG-3′, detection probe, 5′-CGTGATAGCCTTCCAGCGGTATCAGATT-3′.

FGF2 forward, 5′-GAACGCCTGGAGTCCAATAACTA-3′, reverse, 5′-CCCGTTTTGGATCCGAGTTT-3′, detection probe, 5′-ACACTTACCGGTCACGGAAATACTCCAGTT-3′.

Table 1
Significant changes in expression genes upon systemic administration with nilvadipine to rd mouse

	GenBank Accession Nos.	Ratio	Possible roles	References
Down-regulated genes[a]				
Caspase 3	NM009810	0.33 ± 0.06	Apoptosis	[15]
Caspase 9	NM015733	0.37 ± 0.08	Apoptosis	[15]
Caspase 14	NM009809	0.33 ± 0.07	Apoptosis	[15]
Programmed cell death 4	NM011050	0.24 ± 0.06	Apoptosis	[16]
Telomerase associated protein	NM009351	0.4 ± 0.07	Regulation of development and oncology	[17]
ADP-ribosyl cyclase 1	NM009763	0.33 ± 0.07	Ca^{2+} metabolism	[18]
RAB18	NM011225	0.42 ± 0.06	Endocytosis and phagocytosis	[19]
Lectin	NM008495	0.31 ± 0.08	Endocytosis and phagocytosis	[20]
Carbonyl reductase	NM007621	0.41 ± 0.05	Drug metabolism	[21]
Adenosine deaminase	NM019655	0.24 ± 0.06	Immune system	[22]
α1 Microglobulin	NM007443	0.36 ± 0.12	Immunosuppressive mechanism	[23]
Syndecan binding protein	NM016807	0.35 ± 0.06	Neuronal dendritic formation	[24]
Galanin receptor	NM010254	0.44 ± 0.06	Neuroendocrine	[25]
Up-regulated genes[b]				
TNF member 8	NM9403	2.87 ± 0.07	Apoptotic	[26]
Tropomyosin2	NM009416	2.5 ± 0.21	Ca^{2+} metabolism	[27]
GCAP2	NM00819	2.6 ± 0.17	Ca^{2+} metabolism	[28]
Crystallin α2	NM009964	3.4 ± 0.3	Heat shock protein	[29]
Crystallin γs	NM009967	3.3 ± 0.12	Heat shock protein	[29]
N-Acetylgalactosamine specific to lectin	NM010796	2.4 ± 0.04	Endocytosis and phagocytosis	[20]
GABA receptor α1 subunit	NM008067	2.7 ± 0.1	Neurotransmitter receptor	[30]
GABA receptor π subunit	NM008069	2.6 ± 0.07	Neurotransmitter receptor	[30]
Go γ subunit 4	NM010317	2.3 ± 0.14	Signal transduction	[31]
MAP kinase kinase kinase kinase4	NM008696	3.0 ± 0.06	Signal transduction	[32]
Protein kinase Cγ	NM011102	2.2 ± 0.01	Signal transduction	[32]
Protein kinase Cδ	NM011104	2.7 ± 0.04	Signal transduction	[32]
Synaptogyrin I	NM009303	3.2 ± 0.12	Neurosynaptic plasticity	[33]
Fibroblast growth factor 2	NM010198	2.8 ± 0.10	Neuro-survival factor	[34–40]

[a] Data are expressed as means ± SD. Significant changes (less than 0.5) are expressed.
[b] Data are expressed as means ± SD. Significant changes (more than 2) are expressed.

GAPDH as an internal control was amplified by using a commercially available kit (Applied Biosystems). All experiments were performed in triplicate.

Western blot. Nilvadipine- and vehicle-treated rd mice (16-day-old; 1 mouse, 2 eyes, was used for one blot) were analyzed. Analysis was performed five times with different preparations (total 5 rd mice were used for each antibody). Western blot analysis was carried out as described previously [9].

Results

After administering the four different Ca^{2+} antagonists (D-*cis*-diltiazem, nifedipine, nicardipine, and nilvadipine) and their vehicle solutions to 9-day-old rd mouse daily for 7 days, the thickness of each retinal layer was compared to study the effects of Ca^{2+} antagonists on the retinal morphology of rd mouse. As shown in Fig. 1, there were no significant differences in thickness of retinal outer layers in mice treated with D-*cis*-diltiazem, nifedipine or vehicle control solution. However, in contrast, cell layers of ONL and retinal photoreceptor outer segment layers (OS) were significantly thicker in nicardipine or nilvadipine treated mice as compared with their controls and other treatment conditions ($p < 0.01$). This suggests that nicardipine and nilvadipine have an effect for protection against degeneration of retinal layers of rd mice during its retinal degeneration. Above all, nilvadipine showed significant preservation of retinal layer. Accordingly, we analyzed nilvadipine treated and its control retina in electron microscopy. Nilvadipine treated retina showed remains of disks and synaptic structures in OPL. In contrast, control retina showed few disks remained and the fine structures in OPL were mostly destroyed (Fig. 2)

To study the effects of these Ca^{2+} antagonists on retinal function, ERG responses were measured. As shown in Fig. 3, no significant effects on the ERG responses were recognized, however, nilvadipine treated mice showed relatively higher ERG responses as compared with others ($p = 0.07$). Therefore, taken together these data suggested that administration of nilvadipine and nicaridipine had preservation effects on retinal morphology in rd mouse, but their retinal degeneration

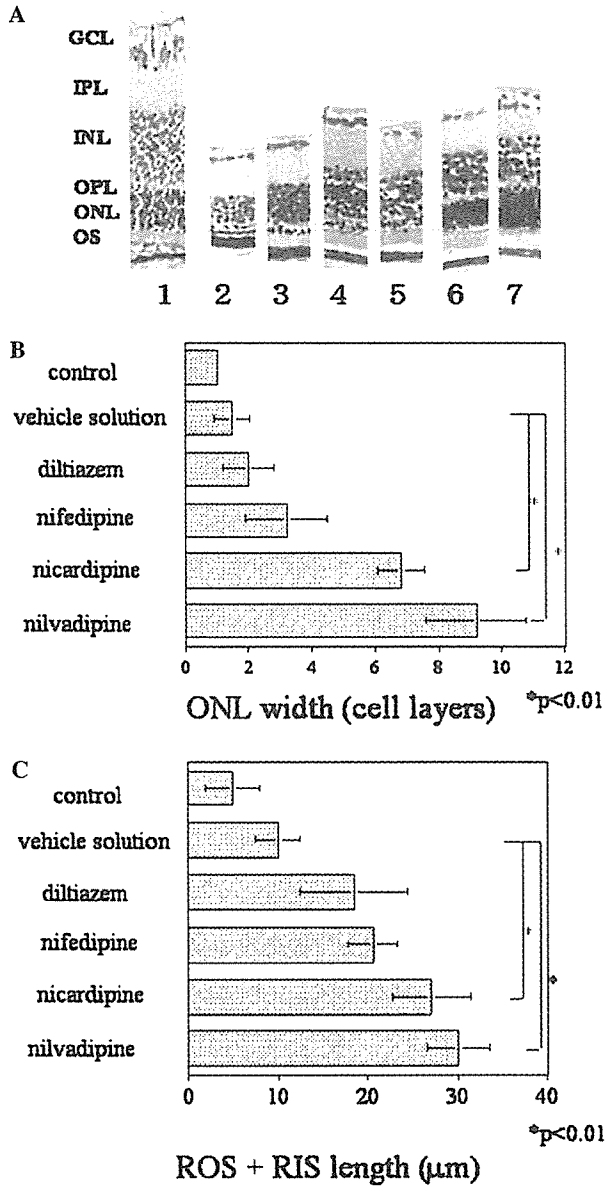

Fig. 1. Effects of several Ca^{2+} antagonists on retinal thickness and morphology in rd mouse. Hematoxylin–eosin staining of retinal sections at 0.2 mm from optic disk from rd mouse eyes at the age of 9 and 16 days treated with Ca^{2+} antagonists, diltiazem, nifedipine, nicaridipine, nilvadipine or their vehicle solutions. Photographs of the sections were taken (A), and each of the retinal layers was measured at temporal and nasal points 0.2 mm apart from optic disk from 20 different points from 20 different eyeballs, and cell layers counting of outer plexiform layer (ONL) and thickness of retinal photoreceptor outer segment layers (OS) were plotted (B,C). GCL, ganglion cell layer; IPL, inner plexiform layer; and INL, inner nuclear layer; OPL. $^*P < 0.01$ (Mann–Whitney test). 1, 9-day-old rd mouse; 2–7, 16-day-old rd mouse (2, vehicle solution 1 (for diltiazem and nicardipine); 3, vehicle solution 2 (for nifedipine and nilvadipine); 4, diltiazem; 5, nifedipine; 6, nicaridipine; and 7, nilvadipine). Scale bar = 50 μm.

was still progressive and these drug effects did not reach functional preservation levels.

In the microarray study, nilvadipine and its vehicle solution were administered to 9-day-old rd mice as above, and mRNA profiling assay was performed using atlas grass mouse 3.8 array ($n = 5$ mice; 10 retinas were used for one analysis of each conditions, and experiments were repeated three times using fresh preparations). Among 3774 of genes related to signal transduction, growth regulation, hormonal and neuronal regulations, cytoskeleton, immune response, apoptotic pathway, and other cellular regulatory mechanisms, only 16 genes were down-regulated and 18 genes were up-regulated more than twofold upon administration of nilvadipine in rd mouse retina (summarized in Table 1). Upon administration of nilvadipine, several genes related to apoptosis (caspases 3, 9, and 14 [15], programmed cell death 4 [16]), regulation of development and oncology (telomerase associated protein [17]), Ca^{2+} metabolisms (ADP-ribosyl cyclase 1 [18]), endocytosis and phagocytosis (rab18 [19], lectin [20]), drug metabolism (carbonyl reductase [21]), immune system (adenosine deaminase [22], α1 microglobulin [23]), neurodendric formation (syndecan binding protein [24]), and neuroendocrine (galanin receptor [25]) were found to be down-regulated. In contrast, apoptosis related factor genes (TNF member 8 [26]), Ca^{2+} metabolisms (tropomyosin2 [27], guanylate cyclase activating protein (GCAP)2 [28]), heat shock proteins (crystallins α2 and γs [29]), endocytosis, and phagosytosis (*N*-acetylgalactosamine [20]), neurotransmitter receptor (GABA receptor α1 and π subunits, GABA receptor γ3 subunit [30]), signal transduction related genes (Gγ subunit 4 [31], MAP 4K4, and protein kinase Cγ and δ [32]), factor for neurosynaptic plasticity (synaptogyrin I [33]), and neuro-survival factor (fibroblast growth factor (FGF) 2) [34–40] were found to be up-regulated. Among these changes, we directed our attention to the down-regulations of caspases and the up-regulations of synaptogyrin I and FGF2 as most notable changes, because caspases are recognized as important factors for apoptotic processes, FGF2 is identified as a possible therapeutic factor for retinal degeneration [34–40], and synaptogyrin I is an important factor regulating neurotransmitter release from synapses [33]. To confirm these observations, Taqman quantitative RT-PCR and Western blotting for caspases 3, 9, and 14, synaptogyrin I, and FGF2 were performed. As shown in Table 2 and Fig. 4, expressions of caspases 3, 9, and 14 were down-regulated and, in contrast, those of synaptogyrin I and FGF2 were up-regulated upon administration of nilvadipine.

Discussion

Ca^{2+} antagonists, a dihydropyridine (DHP) derivative such as nifedipine, nicaridipine, and nilvadipine, a benzothiazepine derivative such as diltiazem, and a phenylalkylamine derivative such as verapamil, have

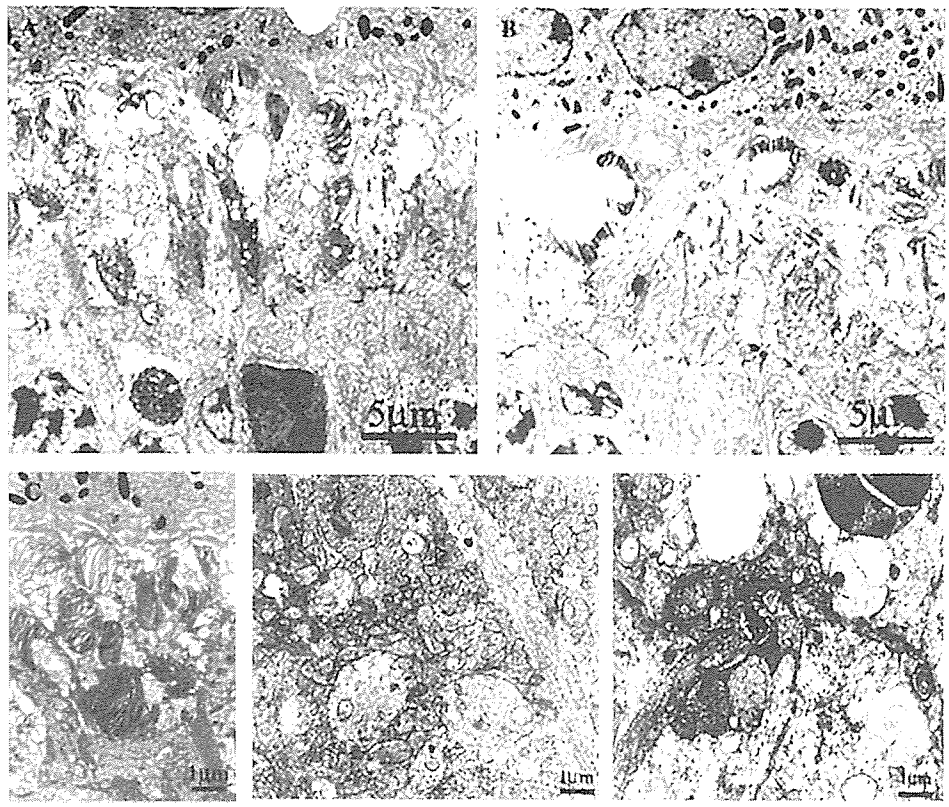

Fig. 2. Electron micrographs of nilvadipine treated (A,C,D) and vehicle-treated (B,E) retinas of 16-day-old rd mice. (A–C) Show the disk layer and (D,E) show OPL.

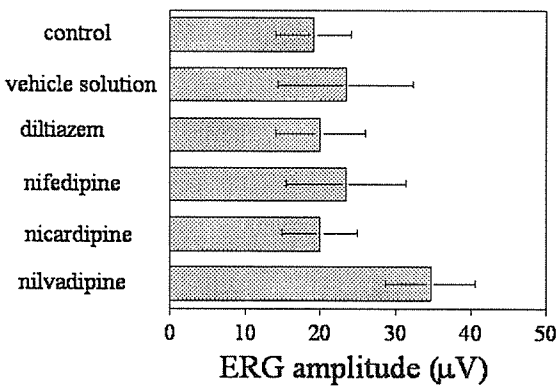

Fig. 3. Effects of Ca^{2+} antagonists on scotopic ERG in rd mice. Sixteen-day-old rd mice treated with diltiazem, nifedipine, nicardipine, nilvadipine, or their vehicle solutions for a week were subjected to scotopic ERG analysis. Twenty eyes (10 mice) were used in each condition. The amplitudes were plotted. Vehicle solution 1 (for diltiazem and nicardipine), vehicle solution 2 (for nifedipine and nilvadipine).

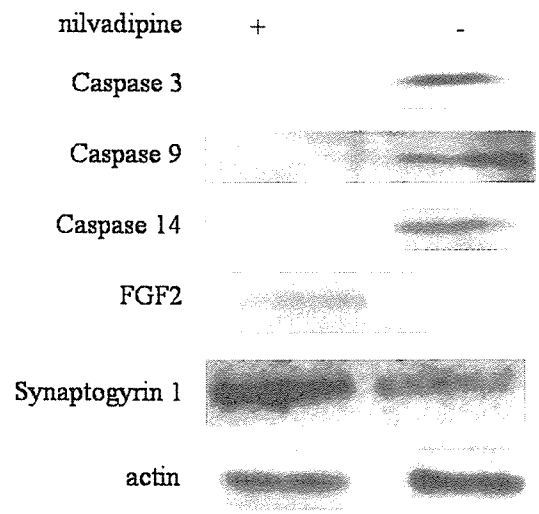

Fig. 4. Western blot analysis in nilvadipine- and vehicle-treated rd mice retinas. Two retinas from 16-day-old rd mice, which had been administered nilvadipine or its vehicle solution daily for a week, were homogenized in 50 μl of 10 mM Hepes buffer (pH 7.5) containing 2% Tween 20. An aliquot (15 μl) was mixed with the sample buffer (10 μl), loaded on an SDS–PAGE gel, and then electrotransferred to PVDF membrane. Western blot analysis was performed using either anti-caspase 3, 9, or 14 antibody (1:2000 dilutions), anti-FGF2 antibody (1:1000 dilutions), anti-synaptogyrin I antibody (1:1000) or anti-actin antibody (1:2000 dilutions), and HRP-labeled anti-rabbit IgG (1:3000 dilutions), as 1st and 2nd antibodies, respectively. Experiments were repeated five times using different preparations (total $n = 5$ rats, 10 retinas in each condition) and identical results were obtained.

been widely used as treatments for patients with systemic hypertension [41]. In the present study, among three DHPs (nilvadipine, nicaridipine, and nifedipine) and diltiazem, nilvadipine, and nicardipine had preservation effects toward rd mouse retinal degeneration. Differences observed in the effectiveness between these four Ca^{2+} antagonists may speculatively be ascribed to

Table 2
Taqman fold changes of caspases and FGF expression in nilvadipine-treated rd mouse retinas by Taqman quantitative PCR

	Taqman fold changes
Caspase 3	↓ 6.8 ± 0.5
Caspase 9	↓ 7.5 ± 0.5
Caspase 14	↓ 5.5 ± 0.5
Synaptogyrin	↑ 6.5 ± 0.5
FGF2	↑ 4.5 ± 0.5

Taqman PCR was performed in 5 RNA specimens isolated from 10 in each condition. All data (threshold cycle) were standardized by GAPDH. The fold changes were relative to the retinal samples of RCS rats treated with vehicle solution.

their differences in permeability to central nervous system (CNS) including retina and in the kinetics of Ca^{2+} channel blocking action. In fact, it was shown that nilvadipine most preferably penetrated into the CNS [42], and nilvadipine had LVA (low-voltage activated) Ca^{2+} channel blocking action in addition to the L-type HVA (high-voltage activated) Ca^{2+} channel blocking action, whereas much less effects have been reported on the LVA Ca^{2+} channel blocking action of nifedipine, nicaridipine, and diltiazem [43]. Recently, we found that nilvadipine had a preservation effect against RCS rat retinal degeneration but nicardipine and other Ca^{2+} antagonists had no effects [9]. In terms of the differences of efficacy of these Ca^{2+} antagonists, we speculated that spatial distributions of Ca^{2+} channels within retinal neuronal cells may be involved since RPE and photoreceptors are primarily affected in RCS rat and rd mouse, respectively. Therefore, taken together, our present study suggested that some types of Ca^{2+} channel blockers have a protective effect against rd mouse retinal degeneration, and nilvadipine potentially protects against retinal degeneration from several different causes.

Microarray analysis allowed us to theorize that administration of nilvadipine caused following mechanisms: (1) modulation of expressions of Ca^{2+} metabolism related proteins (ADP-ribosyl cyclase [18], tropomyosin2 [27], and GCAP2 [28]) may result from changes in cellular Ca^{2+} levels by Ca^{2+} channel blocking action; (2) modulations of expressions of apoptosis related proteins (caspases [15], programmed cell death 4 [16], telomerase associated protein [17], and TNF member 8 [26]), heat shock proteins (crystallins [29]), and endocytosis and phagocytosis related proteins (rab18 [19], lectin, and *N*-acetylgalactosamine [20]) may result from suppression of retinal cell apoptosis; and (3) modulations of expression of cellular signal transduction related proteins and neurotransmitters may be the results of preservation of neuronal cells and their networks. Similar to this, microarray analysis in our recent study identified several changes in genes involved with Ca^{2+} metabolism related proteins, apoptosis related proteins, signal transduction related proteins, and other mechanisms in RCS and control rat retinas upon administration of nilvadipine [44]. However, there were some differences in changes of gene expressions upon administration of nilvadipine between rd mouse and RCS rat retinas. In terms of the reason, we speculated that regulation of intracellular Ca^{2+} levels by Ca^{2+} channel blockers might be involved in the preservation of retinal function in different ways. For instance, upon administration of nilvadipine, expressions of several apoptosis related factors, such as TNFs, were altered in both RCS rat and rd mouse retinas. However, in rd mouse, down-regulation of expressions of caspases 3, 9, and 14 was recognized, but these changes were not observed in RCS rat retina. The caspase family of cysteine proteases is involved both in the initiation and final execution of apoptotic cell death [15]. Therefore, evidence of such down-regulation of caspases strongly suggests that nilvadipine is able to inhibit apoptotic processes in rd mouse retina.

In presynaptic nerve terminals, synaptic vesicles accumulate transmitters and release them by exocytosis. Within the synaptic vesicle, two major protein components, synaptogyrins and synaptophysins, are identified. Synaptogyrins and synaptophysins contain four transmembrane domains and a cytoplasmic C-terminal tail that is tyrosine-phosphorylated by $pp60^{c\text{-}arc}$ and fyn kinases. Functionally, several studies using knockout mice lacking synaptogyrins and/or synaptophysins suggest that both components are essential for regulation of neurotransmitter release [33]. In our current study, synaptogyrin I expression was significantly enhanced upon administration of nilvadipine, suggesting that nilvadipine may induce activation of synaptogrin-related synapse functions within retinal neurons. Interestingly in fact, Sugita et al. [45] recently reported that synaptogyrin I regulates Ca^{2+}-dependent exocytosis in PC 12 cells in terms of relationship between synaptogyrin and Ca^{2+}.

Fibroblast growth factors (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 [46]. Among FGFs, in vivo and in vitro studies have led to the recognition that basic FGF (FGF2) is an important neuro-survival factor. With regard to retinal degeneration, it was shown that FGF2 prevented the degenerative processes based upon the following observations [34–40]: (1) retinal FGF2 contents and its mRNA expression levels were significantly elevated in retinal degeneration as compared with control and (2) exogenous FGF2 caused significant neuro-survival effects on inherited retinal degeneration in RCS rat and light induced retinal damage. In fact, our recent microarray analysis revealed that expression of FGF2

was also up-regulated in RCS and control rat retinas upon administration of nilvadipine [44].

Therefore, taken together, up-regulation of synaptogrin I and FGF2, and suppressions of caspase-dependent apoptotic pathway upon administration of nilvadipine appear to be importantly involved in the nilvadipine-dependent protection against retinal degeneration in the rd mouse.

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Preservation of Retinal Morphology and Functions in Royal College Surgeons Rat by Nilvadipine, a Ca^{2+} Antagonist

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Purpose. The Royal College of Surgeons (RCS) rat is the most extensively studied animal model for understanding the molecular pathology in inherited retinal degeneration, such as retinitis pigmentosa (RP). The purpose of the present study was to evaluate the pharmacologic effects of several Ca^{2+} antagonists on the retinal degeneration of RCS rats.

Methods. Several Ca^{2+} antagonists, diltiazem, nicardipine, nilvadipine, and nifedipine, were intraperitoneally administered and retinal morphology and functions analyzed.

Results. Among the Ca^{2+} antagonists, only intraperitoneally administered nilvadipine preserved retinal morphology and electroretinogram responses in RCS rats during the initial stage of retinal degeneration. Studies using immunohistochemistry, RT-PCR, and Western blot analysis revealed significant enhancement of rhodopsin kinase and αA-crystallin expression and suppression of caspase 1 and 2 expression in the retina of nilvadipine-treated rats.

Conclusions. These data suggest that nilvadipine is beneficial for the preservation of photoreceptor cells in RCS rats and can be used to treat some patients with RP. (*Invest Ophthalmol Vis Sci.* 2002;43:919-926)

Retinitis pigmentosa (RP) is a disease of inherited retinal degeneration characterized by nyctalopia, ring scotoma, and bone-spicule pigmentation of the retina. So far, no effective therapy has been available for RP. Several animal models with inherited retinal degeneration have been studied to elucidate the pathologic molecular characteristics of RP and to design an effective therapy for it. The Royal College of Surgeons (RCS) rat, in which the retinal pigment epithelial (RPE) cell is affected by the retinal dystrophy (*rdy*) mutation and continuously expresses the *rdy* phenotype,[1] has been the most widely used animal model for the study of RP. In terms of the molecular characteristics of retinal degeneration, it has been suggested that failure of the RPE to phagocytose the shed tips of rod outer segments (ROS) debris in the RCS rat is primarily involved.[2-4]

Recently, D'Cruz et al.[5] used a positional cloning approach to study the *rdy* locus of the RCS rat, and they discovered a small deletion of RCS rat DNA that disrupts the gene encoding the receptor tyrosine kinase Mertk, which may be a molecular target for ingestion of outer segments by RPE cells. It has been shown that mutations in *Mertk* cause human RP.[6] In contrast, RCS rat photoreceptor cells are considered to be normal in their structure and functions, because they can survive after retinal RPE transplantation.[7,8] However, it has been found that several changes occur in RCS rat ROS, including protein phosphorylation levels of opsin,[9] arrestin,[10,11] and ROS,[12] which may affect quenching of the phototransduction pathway in RCS rats. In fact, we recently found significantly lower levels of mRNA expression of αA-crystallin and rhodopsin kinase (RK), which are thought to be involved in post-Golgi processing of opsin and rhodopsin phosphorylation, respectively, in RCS rats than those in the control rats at the age of 3 to 4 weeks. In contrast, expression of other photoreceptor cell-specific proteins including rhodopsin, transducin, arrestin, and recoverin were almost comparable between RCS and control rats at the age of 3 to 4 weeks.[13]

Therefore, based on these observations, we suggested that low levels of rhodopsin phosphorylation might cause misregulation of phototransduction pathways in rod photoreceptor cells, resulting in their degeneration. This idea is supported by experimental evidence that absence of rhodopsin phosphorylation in transgenic mice carrying rhodopsin mutations causes retinal degeneration.[14,15] Because recoverin, a retina-specific Ca^{2+}-binding protein, negatively regulates rhodopsin phosphorylation by rhodopsin kinase in a Ca^{2+}-dependent manner,[16] it is plausible that suppression of recoverin-dependent inhibition of rhodopsin kinase by the lowering of intracellular Ca^{2+} levels by some drugs may be effective in the preservation of photoreceptor cells in RCS rats. Frasson et al.[17] recently reported the interesting finding of rod photoreceptor rescue by D-*cis*-diltiazem, a Ca^{2+} channel blocker in a different animal model of RP, the *rd* mouse, in which the gene encoding cGMP phosphodiesterase is affected. In consideration of all these data, we assume that the regulation of intracellular Ca^{2+} levels may have potential as a therapy to prevent progressive retinal degeneration in RP and in animal models.

To test our hypothesis, several kinds of Ca^{2+} antagonists used in clinical practice[18-20] were administered to RCS rats at 3 weeks after birth—the time when degenerative changes in photoreceptor cells are known to begin. Then, the retinal function was evaluated by electroretinography (ERG), and histologic studies including light microscopy, immunohistochemistry, and electron microscopy were performed.

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Supported by Grants-in-Aid for Scientific Research B-11470361, B-12557145, and C-13671846 from the Japanese Ministry of Health and Welfare; by the Japanese Ministry of Education, Sports and Culture; Naito Memorial Foundation; the Ciba-Geigy Foundation for the Promotion of Science; the Mochida Memorial Foundation for Medical and Pharmaceutical Research; Uehara Memorial Foundation; Karouji Memorial Foundation for Medical Research, and the JRPS Research Foundation.

Submitted for publication September 10, 2001; revised December 3, 2001; accepted December 12, 2001.

Commercial relationships policy: N.

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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. Unless otherwise stated, all procedures were performed at 4°C or on ice, using ice-cold solutions. Nilvadipine, diltiazem, nicardipine, and nifedipine were generous gifts from Fujisawa Pharmaceutical Co., Tokyo, Japan; Tanabe Pharmaceutical Co., Osaka Japan; Yamanouchi Pharmaceutical Co., Tokyo, Japan; and Bayer Pharmaceutical Co., Osaka, Japan, respectively. Anti-human RK monoclonal antibody (G8),[21] in which immunoreactivities to rat rhodopsin kinase were confirmed in our previous article,[13] was generously provided by Krzysztof Palczewski (Department of Ophthalmology, University of Washington, Seattle, Washington). Anti-αA-crystallin antibody, and anti-caspase 1 and -caspase 2 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The specificity and titers of all antibodies were examined by Western blot analysis and ELISA, using rat retina soluble fractions.

Anesthesia

In the present study, 3- to 5-week-old inbred RCS ($rdy^{-/-}$) rats (Crea, Tokyo, Japan) reared in cyclic light conditions (12 hours on–12 hours off) were used. For anesthesia induction, rats inhaled diethyl ether. Once unconscious, the animals were injected intramuscularly with a mixture of ketamine (80–125 mg/kg) and xylazine (9–12 mg/kg). The adequacy of the anesthesia was tested by tail clamping, and supplemental doses of the mixture were administered intramuscularly, if needed.

Drug Administration

Nilvadipine and nifedipine were dissolved in a mixture of ethanol, polyethylene glycol 400, and distilled water (2:1:7) at a concentration of 0.1 mg/mL, diluted twice with physiological saline before use, and injected intraperitoneally (1.0 mL/kg) into anesthetized rats every day early in the morning for 2 weeks. In control rats, the same solution without nilvadipine or nifedipine (vehicle solution) was administered similarly. Nicardipine and diltiazem were dissolved in PBS at 0.25 mg/mL and 1 mg/mL, respectively, and injected intraperitoneally (1.0 mL/kg), similarly to the other agonists. As a control, the same volume of a mixture of ethanol, polyethylene glycol 400, and distilled water (2:1:7) or PBS was administered. Before administration, the pH of all drug solutions was adjusted to approximately 7.4. The concentrations of these drugs administered to RCS rats were determined by their concentrations in oral administration to human patients with hypertension for 1 day in our clinical practice (nilvadipine, 0.05–0.3 mg/kg; nifedipine, 0.1–0.5 mg/kg; nicardipine, 0.2–1.0 mg/kg; and diltiazem, 0.3–3 mg/kg).[22]

Light Microscopy

Five RCS rats each (age, 3–5 weeks; weight, 150–200 g) were studied for the control and four different Ca^{2+} antagonist administration conditions. Under deep anesthesia, animals were perfused through the initial portion of the aorta with 300 mL 4% paraformaldehyde in 0.1 M PBS (pH 7.4), and retinas were dissected and embedded in paraffin. Posterior segments (5 × 5 mm^2 containing the optic disc at center) cut from the enucleated eyes were embedded in paraffin. Retinal sections were cut vertically through the optic disc at 4 μm thickness, nasally to temporally, mounted on subbed slides, and dried. The sections were processed with hematoxylin-eosin staining after deparaffinization with graded ethanol and xylene solutions. Retinal sections were photographed, and the thickness of each retinal layer was measured at temporal and nasal points 1 mm away from the optic disc (two points per section) in five different sections from five rats in each condition. Thickness of retinal layers is shown as mean ± SD. Significant differences between groups were found using the Mann-Whitney test with a significance level of $P < 0.05$.

Electron Microscopy

Nilvadipine-treated and untreated RCS rats (three animals for each condition) were used. Under deep anesthesia, each animal was perfused through the initial portion of the aorta with 300 mL 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Tissues were dissected, postfixed in phosphate-buffered 1% osmium tetroxide (pH 7.4), dehydrated in an ascending series of ethanol solutions, and passed through propylene oxide. The blocks were embedded in Epon 812. Thin sections were stained in uranyl and lead salt solutions.

Electroretinography

Details of preparation, recording technique, and measurements of ERG responses have been described elsewhere.[23] Five RCS rats were studied (age, 3–5 weeks; weight, 150–200 g) each for control and four Ca^{2+} antagonists. Under anesthesia, each rat was laid on its side on a heating pad (at 37°C), with its head fixed in place with surgical tape in an electrically shielded room, and dark-adapted for at least 2 hours. The pupils were dilated with drops of 0.5% tropicamide. ERGs were recorded with a contact electrode equipped with a suction apparatus to fit on the cornea (Kyoto Contact Lens Co., Kyoto, Japan). A grounding electrode was placed on the ear. Response evoked by a white flash (3.5×10^2 lux, 200-ms duration) were recorded (Neuropack, model MES-3102; Nihon Kohden, Tokyo, Japan). The a-wave amplitude was determined from the baseline to the bottom of the a-wave. The b-wave amplitude was determined from the bottom of the a-wave to the top of the b-wave. ERG amplitudes are shown as mean ± SD. Significant differences between groups were found using the Mann-Whitney test with a significance level of $P < 0.05$.

Immunofluorescence Microscopy

Normal retinas of Sprague-Dawley rats (SD; 5 weeks old, three rats), nilvadipine-treated and untreated RCS rats (4 and 5 weeks old; three animals in each group) were analyzed. Animals under deep anesthesia were perfused through the initial portion of the aorta with 300 mL 4% paraformaldehyde in 0.1 PBS (pH 7.4), and retinas were dissected, embedded in optimal cutting temperature (OCT) compound (Tissue-Tek, Tokyo, Japan), and sectioned vertically through the optic disc into 14-μm-thick sections with a cryostat. Before application of the primary antibodies, sections were blocked with PBS containing 5% goat serum and 3% bovine serum albumin for 1 hour and then incubated overnight with anti-rhodopsin kinase (1:1000), anti-αA crystallin (1:500), anti-caspase 1 (1:500), or anti-caspase 2 (1:500) antibodies at 4°C. Sections were washed and incubated with fluorescein-isothiocyanate (FITC)-conjugated antibodies to mouse, goat, or rabbit IgG (Cappel, Durham, NC) for 1 hour at room temperature. Specificity control experiments were performed by omitting the primary antibodies. Sections were counterstained with iodide (TOTO-3; Molecular Probes, Eugene, OR) and observed with a confocal laser microscope (Radians 2000; Bio-Rad Laboratories, Hertfordshire, UK). Photographs were taken 1 to 2 mm from the disc.

RT-PCR Analysis and Relative Amount of α-Crystallins and RK

Total RNA from retinas was isolated using an extraction reagent (Isogen; Nippon Gene, Tokyo, Japan), according to the procedure recommended by the manufacturer. The cDNAs were generated from 2 μg retinal RNA in a 12-μL reaction, using 1 μL oligo(dT) primer (0.5 mg/mL; Gibco-BRL, Life Technologies, Inc., Rockville, MD). The reaction mixture was denatured at 70°C for 10 minutes. Four microliters first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$; Superscript; Gibco), 2 μL dithiothreitol (DTT, 0.1 M; Gibco), 1 μL deoxyribonucleoside triphosphate (dNTP, 10 mM; Gibco), 1 μL RNase inhibitor (40 U/μL; Gibco), and 1 μL reverse transcriptase (200 U/μL; Superscript II; Gibco) were added to the mix. The incubation was performed at 42°C for 50 minutes and at 70°C for 15 minutes. The PCR

amplifications were performed with 4 μL of the RT reaction, 5 μL 10× PCR buffer (200 mM Tris-HCl, 500 mM KCl), 2 μL $MgCl_2$ (50 mM), 1 μL dNTP, 5 μL sense and antisense primers (10 pM/μL), and 0.5 μL *Taq* polymerase (5 U/μL; Gibco). The PCR mix was denatured at 94°C for 4 minutes and then run for 28 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes.

The primers used for RT-PCR were as follows: 5′-ATGGACGTCACCATCCAGCA-3′, corresponding to bases 158 to 178 of the cDNA sequence and 5′-AGCTGGGCTTCTCCTCCCGT-3′ corresponding to bases 713 to 732 of the cDNA sequence for αA-crystallin,[24] with expected PCR products of 485 bp; 5′-ATGGACATAGCCATCCACCACCCCTGGAT-3′ corresponding to bases 21 to 49 of the cDNA sequence and 5′-AATCTACTTCTTAGGGGCTGCAGTGACAGC-3′ corresponding to bases 522 to 551 of the cDNA sequence for αB-crystallin,[25] with an expected PCR product of 531 bp; 5′-AAGACCAAGGGCTATGCAGGGA-3′ corresponding to bases 1226 to 1247 of the cDNA sequence and 5′-CTAGGAGATGAGACACATCCCTGA-3′ corresponding to bases 1856 to 1879 of the cDNA sequence for rhodopsin kinase,[26] with an expected PCR product of 654 bp; 5′-GTATGGAATCCTGTGGCATCC-3′ corresponding to bases 2683 to 2703 of the genomic DNA sequence and 5′-TACGCAGCTCAGTAACAGTCC-3′ corresponding to bases 3135 to 3155 of the genomic DNA sequence for β-actin,[27] with an expected PCR product of 349 bp. The amplified PCR fragments were electrophoresed on a 1.5% agarose gel containing ethidium bromide.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis

SDS-PAGE was performed by the method of Laemmli[28] using a 12.5% SDS-PAGE slab gel and a minigel apparatus (Hoeffer, San Francisco, CA). Western blot analysis was performed as described previously.[29] Briefly, after SDS-PAGE of the ROS soluble protein sample, separated proteins in the gel were electrotransferred to polyvinylidene fluoride (PVDF) membranes in 10 mM bis-tris-propane buffer (pH 8.4) containing 10% (wt/vol) methanol solution. After nonspecific binding was blocked by 5% (wt/vol) skim milk in PBS, the membrane was probed successively with antibodies and horseradish peroxidase (HRP)-labeled secondary antibodies (Funakoshi Co., Tokyo, Japan). Specific antigen-antibody binding was visualized with an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Amersham, UK), according to the method of the manufacturer.

Results

To study the effects of Ca^{2+} antagonists on retinal morphology in the RCS rat, four Ca^{2+} antagonists—diltiazem, nifedipine, nicardipine, and nilvadipine—all of which are used in clinical practice, and their vehicle solutions were systemically administered to 3-week-old RCS rats every day for 2 weeks ($n = 5$ rats, 10 eyes in each condition), and then the thickness of each retinal layer was compared among the groups. As shown in Figure 1, no significant differences were observed in thickness of retinal layers in rats treated with diltiazem, nifedipine, or nicardipine and the control rats during the 2 weeks. However, in contrast, the retinal the outer nuclear layer (ONL) and the outer segment (OS) were significantly thicker in 4- and 5-week-old RCS rats given nilvadipine than those in control rats and rats in other drug groups. This suggests that nilvadipine affords significant protection against thinning of retinal layers in the RCS rat during retinal degeneration. Electron microscopy showed that marked irregularity in the photoreceptor OS in the untreated retina (Fig. 2a). On the contrary, the structure was more preserved in nilvadipine-treated retinas (Fig. 2b). The outer plexiform layer (OPL) of untreated retinas was thinner than in the nilvadipine-treated retinas, showing the progress of the structural destruction (Figs. 3a, 3b). Although the degeneration was still observed in the OPL of nilvadipine-treated retinas, preservation of the synapse together with synaptic ribbons was noted (Fig. 3b).

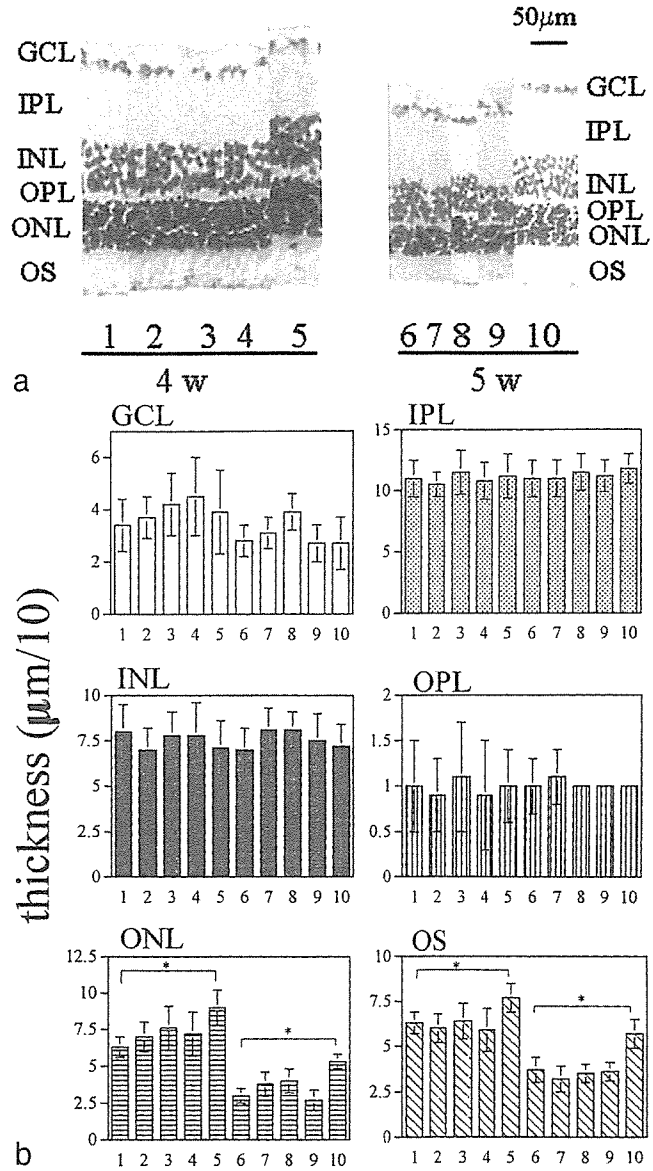

FIGURE 1. Effects of several Ca^{2+} antagonists on thickness of retinal layers of RCS rat. Hematoxylin-eosin staining of retinal sections at 1 mm from optic disc in 4- and 5-week old RCS rat eyes treated with Ca^{2+} antagonists, diltiazem, nifedipine, nicardipine, nilvadipine, or vehicle solution. Photographs of the sections were taken (**a**), and each of the retinal layers were measured at temporal and nasal points 1 mm from the optic disc in five retinas (two points per eye, total of 10 points) and the results plotted (**b**). *Lanes 1–5*: 4 weeks; *lanes 6–10*: 5 weeks; *lanes 1* and *6*: vehicle solution; *lanes 2* and *7*: diltiazem; *lanes 3* and *8*: nifedipine; *lanes 4* and *9*: nicardipine; *lanes 5* and *10*: nilvadipine. *$P < 0.01$ (Mann-Whitney test). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; OS, outer segment.

These morphologic differences between the nilvadipine-treated and untreated RCS rats were correlated with differences in retinal function by ERGs (Fig.4). Nilvadipine-treated retina showed a significant preservation of a- and b-wave amplitudes compared with the control at 4 weeks ($P < 0.001$). The preservation effect on ERG response by nilvadipine was less, but still significant ($P < 0.01$) at 5 weeks. However, other Ca^{2+} antagonists had no effect on the ERG responses. There-

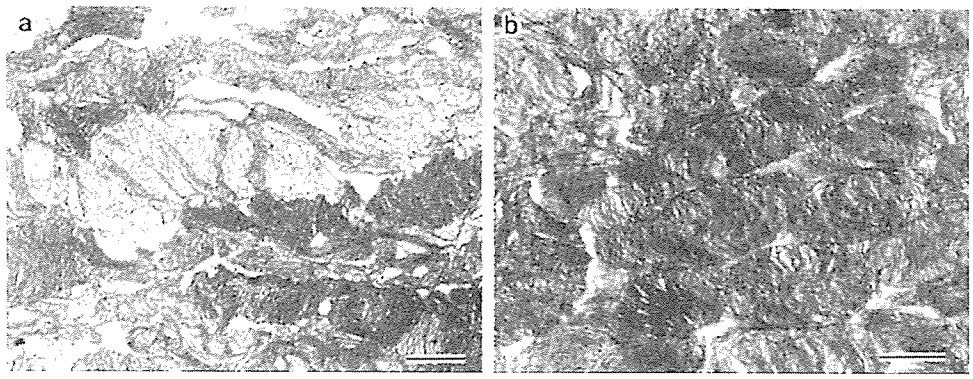

FIGURE 2. Electron micrographs of photoreceptor outer segment of untreated (a) and nilvadipine-treated (b) retina (5 weeks old). The disc arrangement is more irregular in untreated (a) than nilvadipine-treated retina (b). Scale bars, 1 μm.

fore, taken together, these data strongly suggest that administration of nilvadipine produces significant preservation of retinal morphology and function.

To further understand nilvadipine neuroprotection, RK, αA-crystallin, and caspase immunolabeling and mRNA expression were studied. In immunofluorescence microscopy, nonspecific immunoreactivity was not detected in the specificity control retinas. Although histologic disorder was observed, nilvadipine-treated retinas showed better preservation of intensities of RK and αA-crystallin expression, which has been shown to be specifically decreased in RCS rat, than untreated RCS retinas in the OS of the photoreceptor layer, both at 4 and 5 weeks (Fig. 5). These data were confirmed by RT-PCR and/or Western blot analysis. That is, as shown in Figures 6 and 7, expression of RK and αA-crystallin in retinas of 4- and 5-week-old RCS rats was enhanced by the administration of nilvadipine compared with expression in control. In contrast, expression of αB-crystallin and β-actin were almost comparable between nilvadipine-treated and untreated RCS rats (Fig. 6). Immunoreactivities to caspase 1 and 2 were detected in the cytoplasm of the ONL in the untreated RCS retina (4 weeks old), whereas the nilvadipine-treated retina showed smaller number of immunopositive cells in the ONL (Fig. 5). In 5-week-old rats, untreated RCS retina showed weaker immunopositivity than that at 4 weeks, and few positive cells were detected in nilvadipine-treated retinas. Caspase 1 and 2 immunoreactivities were not observed in the intact retina (Fig. 5). These changes between nilvadipine-treated and untreated RCS retinas were also noted in Western blot analysis (Fig. 7).

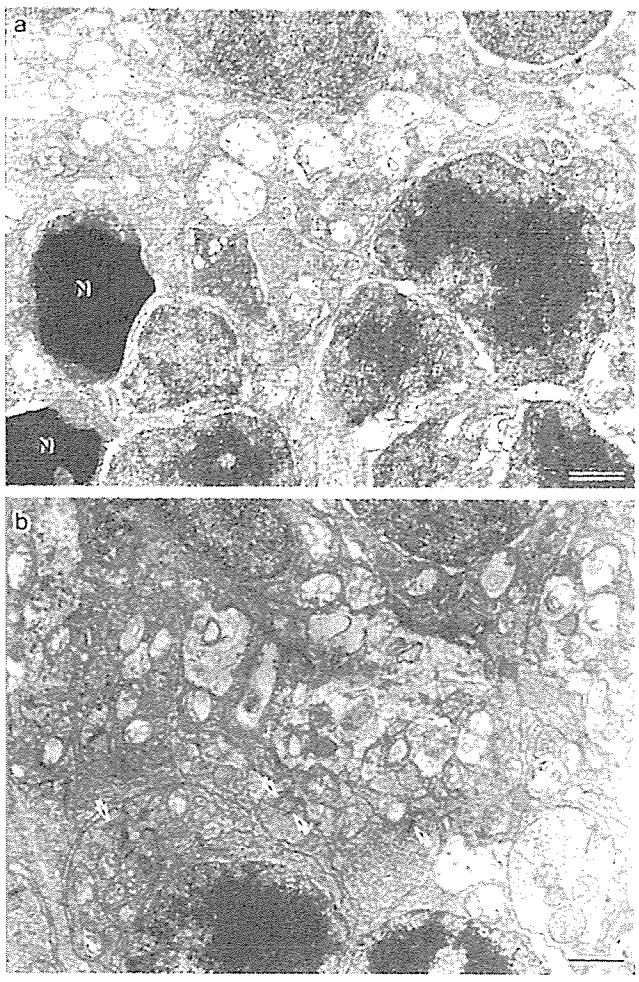

FIGURE 3. Electron micrographs of the ONL, OPL, and INL of untreated (a) and nilvadipine-treated (b) retina (4 weeks old). The INL is situated at the top and the ONL at the bottom in each micrograph. (a) In untreated retina, the decrease in thickness and the structural destruction were visible in the OPL. Apoptotic nuclei (N) are seen in the ONL. (b) The thickness of the OPL was more preserved in nilvadipine-treated retina, and synapse ribbons were visible in some photoreceptor terminals (*arrows*). INL, inner nuclear layer. Scale bars, 1 μm.

DISCUSSION

In the present study, we found that intraperitoneal administration of nilvadipine, a Ca^{2+} antagonist, had a protective effect on photoreceptor cells during retinal degeneration of RCS rat as follows: (1) In the morphology, significant preservation of the thickness of retinal outer layers was observed in light microscopic examination of photoreceptor OS structures and synapse–synaptic ribbon formation within the ONL was observed in electron microscopic examination. (2) Functionally, amplitudes of a- and b-waves of ERGs were preserved. (3) Preservation of expressions of αA-crystallin and RK were noted in immunohistochemistry, RT-PCR, and Western blot analysis. (4) Expression of caspases 1 and 2 was suppressed. However in contrast, such neuroprotective effects were not observed with other Ca^{2+} antagonists, including diltiazem, nifedipine, and nicardipine. These observations suggest that nilvadipine protects the structure and functions of photoreceptor cells by inhibition of the apoptotic process in RCS rat retinas.

Ca^{2+} antagonists, which have been widely used as treatments for systemic hypertension, inhibit the entry of calcium ion intracellularly, relax vascular smooth muscle cells, and increase regional blood flow in several organs.[18–20] The dihydropyridine (DHP) derivatives nifedipine, nicardipine, and nilvadipine; the benzothiazepine derivative diltiazem; and the phenylalkylamine derivative verapamil are the major Ca^{2+} antagonists used in clinical practice.[30] It has been shown that these drugs have different properties in the specificity and

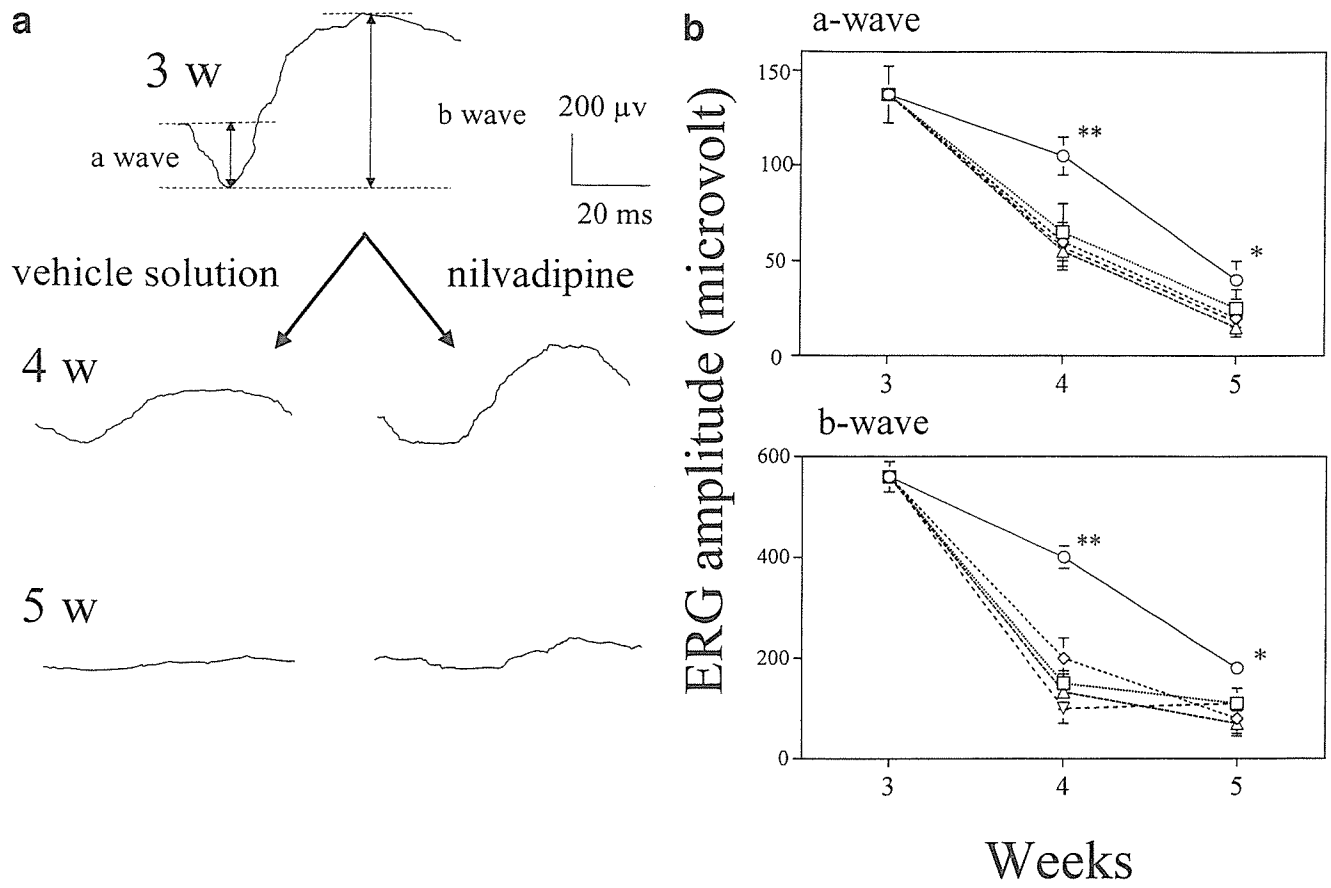

FIGURE 4. Effects of Ca^{2+} antagonists on scotopic ERG in RCS rats. RCS rats treated with diltiazem (◇), nifedipine (△), nicardipine (▽), nilvadipine (○), or their vehicle solutions (□), respectively, every day after 3 weeks of age. During the 2 weeks after the injection, scotopic ERG was recorded once a week. (**a**) Typical ERG responses of RCS rat eyes at 3 weeks of age (before operation) and at 4 and 5 weeks of age after administration of nilvadipine or vehicle solution. (**b**) ERG measurements were performed in 10 eyes (5 rats) in each condition, and the amplitudes of a-and b-waves were plotted. $^{*}P < 0.01$, $^{**}P < 0.001$ (Mann-Whitney test).

kinetics of blocking Ca^{2+} channels. On the basis of kinetics and voltage-dependent properties, the Ca^{2+} channels have been classified into two groups: One is activated by small depolarizations and is then subsequently inactivated and is called a low-voltage-activated (LVA) Ca^{2+} channel, whereas the other is activated by larger depolarizations, shows little inactivation, and is called a high-voltage-activated (HVA) Ca^{2+} channel. Based on pharmacologic properties, HVA Ca^{2+} channels can be separated further into four types (L, N, P/Q, and R).[31,32] Regarding retinal Ca^{2+} channels, it has been revealed that L-type HVA Ca^{2+} channel currents have been identified in photoreceptors of amphibians and fish and are sensitive to DHPs.[33–35] Similarly, L-type currents have been found in mammalian cone photoreceptors.[36,37] Recently, a novel Ca^{2+} channel gene, *CACN1F*, encoding α_{1F}, a retina-specific α_1 subunit of L-type HVA Ca^{2+} channels was identified,[38,39] and its immunolocalization was observed within the ONL and OPL in rat retina.[40] It has been reported that mutations in *CACN1F* cause incomplete X-linked congenital stationary night blindness (CSNB2),[38,38] in which neurotransmission between the photoreceptors and retinal bipolar cells is impaired.[41] Taken together with the fact that most DHPs and diltiazem are L-type HVA Ca^{2+} channel blockers,[30] we can reasonably speculate that these drugs react with retinal L-type HVA Ca^{2+} channels and presumably prevent photoreceptor cell death. In fact, it has recently been reported that rod photoreceptors of *rd* mice were rescued by D-*cis*-diltiazem.[17]

Among the several Ca^{2+} channel blockers, three DHPs (nilvadipine, nicardipine, and nifedipine) and diltiazem, it is not known why only nilvadipine was effective in the current study in the preservation of RCS photoreceptors, but there are several possible explanations to consider.

Preferable Transmission of Nilvadipine to the Central Nervous System, Including Retina

It has been shown that nilvadipine is a much higher hydrophobic chemical than nifedipine and nicardipine.[42,43] A pharmacokinetic study showed that [^{14}C]-nilvadipine is well distributed in various types of tissue, including brain, after intraperitoneal administration.[44] Functionally, nilvadipine increased vertebrate blood flow more effectively than nifedipine or nicardipine in dogs[45] and increased blood velocity and blood flow in the optic nerve head as well as in the choroid and retina in rabbits.[46] In the present study, we injected nilvadipine intraperitoneally at a concentration of 0.1 mg/kg. This concentration of nilvadipine is almost comparable to the concentrations (0.05–0.3 mg/kg) in oral administration to human patients with hypertension for 1 day.[22] These observations suggest that systemic administration of nilvadipine, by being able to cross the blood–brain barrier, should be able to reach cytoprotective levels within the central nervous system (CNS), including the retina. Because of this preferable transmission to the CNS, nilvadipine has in fact been used clinically in Japan for protection against neuronal cell death after the onset of CNS diseases, such as cerebral infarction.[47]

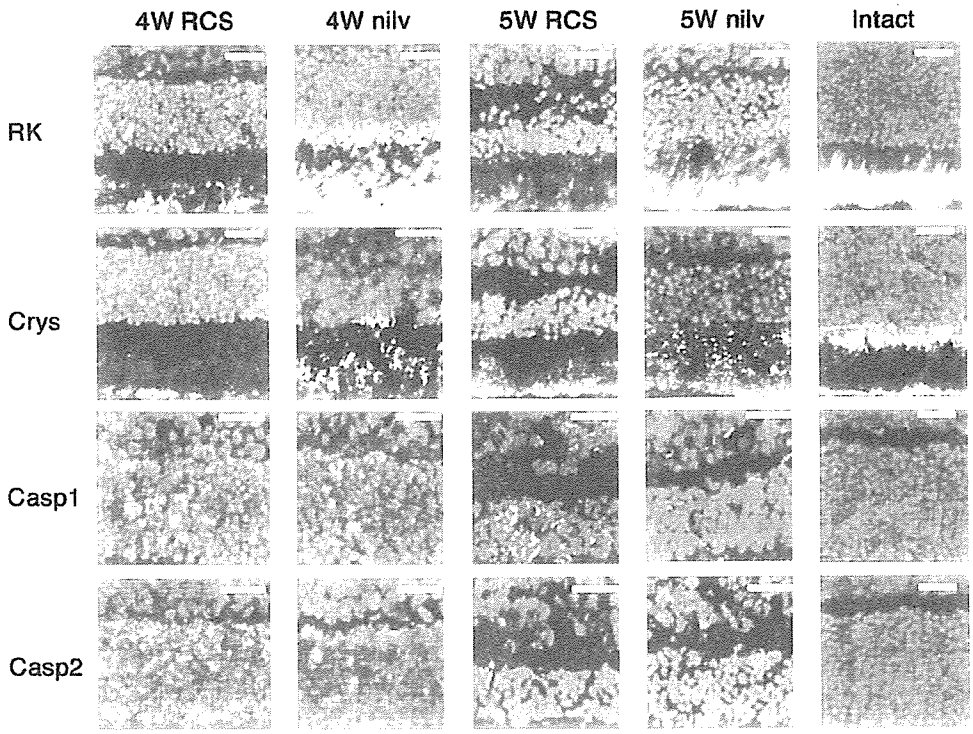

FIGURE 5. Immunohistochemical localization of RK, αA-crystallin (Crys), caspase 1 (Casp 1), and caspase 2 (Casp 2) in untreated (RCS) and nilvadipine-treated (nilv) rats 4 and 5 weeks of age. Immunohistochemical analysis of RK and αA-crystallin was also performed in the normal intact retina (intact). Scale bars, 20 μm.

LVA Ca^{2+} Channel Blocking by Nilvadipine

It has been shown that nilvadipine is an LVA and L-type HVA Ca^{2+} channel blocker.[48] However, in contrast, nifedipine, nicardipine, and diltiazem have much less effect on LVA Ca^{2+} channels.[49] This is another possible reason for nilvadipine's effect, because the presence of LVA Ca^{2+} channels has been reported in the retina.[50] In terms of the blocking of other types of HVA Ca^{2+} channels by these antagonists, Diochot et al. reported that the DHPs verapamil and diltiazem similarly block N-, P/Q- and R-type calcium currents, using sensory and motor neurons.[51] Therefore, the difference in ability to block N-, P/Q- and R-type calcium channels among these Ca^{2+} antagonists may not be involved. As another possible mechanism, it has been speculated that these Ca^{2+} antagonists may affect channels other than Ca^{2+} channels, such as Na^{+} channels, differently. In fact, it has been found that diltiazem is an Na^{+} channel blocker in ventricular myocytes.[52] However, no study has been available so far to determine the effect of nilvadipine on Na^{+} channels. Such a study is needed in the near future.

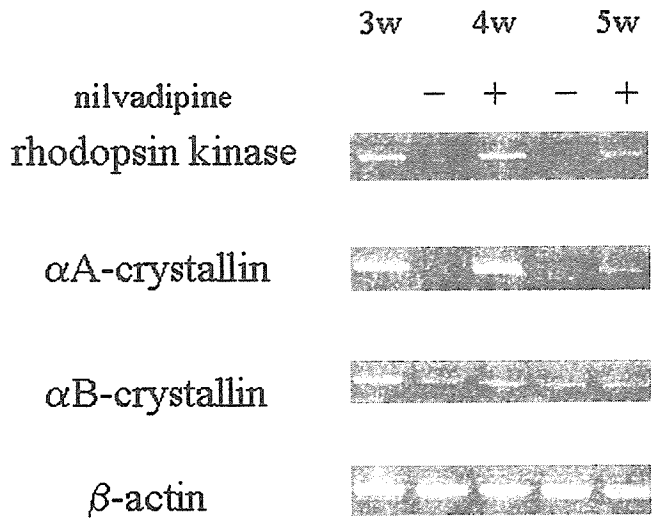

FIGURE 6. Expressions of mRNA for RK, αA-crystallin, αB-crystallin, and β-actin in retina and RPE from RCS rats administered nilvadipine (+) or vehicle solution (−). RNA (2 μg) from retinas of 3- to 5-week old RCS rats administered nilvadipine or vehicle solution was reverse-transcribed to generate a cDNA pool, and 2.2 μL from 22 μL of the cDNA pool was used for PCR, using specific primers. PCR products were evaluated by agarose gel electrophoresis and ethidium bromide staining.

An apoptotic mechanism of retinal ganglion cell death has been mainly involved in the pathologic molecular characteristics of glaucoma.[53] It has also been suggested that some of the calcium antagonists effectively retard the progression of visual field defects in some patients with glaucoma,[54–57] and especially in normal-tension glaucoma, owing to its vasodilating effects on intraocular blood flow.[54,57] In terms of the preservation effects of nilvadipine on retinal degeneration in RCS rats, we do not presently know which mechanism of action is more important, its vasodilating effect or the lowering of intracellular Ca^{2+} levels. However, the recent observation of rod photoreceptor rescue of the *rd* mouse by D-*cis*-diltiazem, another Ca^{2+} antagonist with less vasodilating action in retinal blood vessels,[17] led us to speculate that the lowering of intracellular Ca^{2+} levels by nilvadipine may be more important. If so, we can reasonably hypothesize that misregulation of Ca^{2+}-binding proteins induced by an influx of Ca^{2+} into the photoreceptor cells may be normalized by administration of a Ca^{2+} antagonist, because it is known that Ca^{2+} regulation by Ca^{2+}-binding proteins, including recoverin, guanylate cyclase-activating protein, and calmodulin, is pivotal in signal transduction mechanisms in photoreceptors.[58] We speculate that nilvadipine may also affect Ca^{2+}-dependent regulation in RPE cells in RCS rats, because signal transduction pathways regulated by tyrosine kinases, including Mertk, are known to be regulated by Ca^{2+}.[59]

As another possibility, the suppression of the Ca^{2+}-dependent apoptotic process by the Ca^{2+} antagonist was considered. In fact, it has been reported recently that caspase-dependent apoptotic pathways (caspase 1 and 2) are activated during retinal degeneration in RCS rats.[60] Similarly, a caspase 3-dependent mechanism has been shown to be involved in photo-

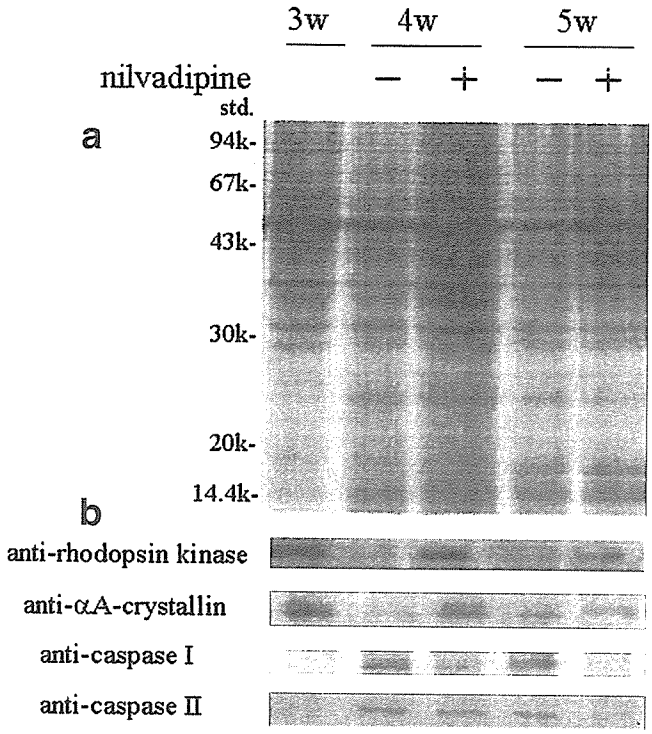

FIGURE 7. Analysis of ROS proteins in RCS and control rats by Western blot analysis using antibody against RK, αA-crystallin, caspase 1 and 2. Two retinas of 3- to 5-week-old RCS rats administered nilvadipine (+) or its vehicle solution (−) were homogenized in 100 μL of 10 mM HEPES buffer (pH 7.5) containing 2% Tween-20. An aliquot (10 μL) was mixed with the sample buffer (10 μL) and loaded on an SDS-PAGE gel (**a**) and then electrotransferred to PVDF membrane. (**b**) Western blot analysis was performed using anti-RK mAb (1:3000) or anti-α-A-crystallin (1:2000), anti-caspase I (1:2000), or anti-caspase 2 (1:2000) polyclonal antibodies.

receptor cell death in transgenic rats with the rhodopsin Ser334ter mutation.[61] This rhodopsin mutant is not phosphorylated by RK because of elimination of all the possible phosphorylation sites.[62] Therefore, it is thought that this mutant is functionally similar to RCS rats in which low levels of mRNA expression of RK has been shown.[13] However, we observed no immunoreactivities toward caspase 3 in nilvadipine-treated and untreated retinas in RCS rats (data not shown).

Recently, Bush et al.[63] reported that the Ca^{2+} antagonist D-*cis*-diltiazem had no effects on photoreceptor degeneration in the rhodopsin P23H rat. Patients with RP and mouse models of the P23H rhodopsin mutation are known to show delayed photoresponse recovery, suggesting that the quenching and adaptation processes of rhodopsin phosphorylation and its related reactions may be impaired.[64] Because these reactions are Ca^{2+}-dependent, it has been thought that Ca^{2+} antagonists may also have a protective effect on retinal degeneration in the rhodopsin P23H rat. However, protective effects by D-*cis*-diltiazem were not so significant in the rhodopsin P23H mutant rat. In our present study, nilvadipine preserved photoreceptor cell function and structure in the RCS rat, but retinal degeneration still progressed. Therefore, it seems likely that Ca^{2+} channel blockers have protective effects against retinal degeneration in some disease models, but these effects may be variable among different models, species, diseases' stages, and Ca^{2+} antagonists.

In conclusion, our findings suggest that nilvadipine may be effective in the preservation of photoreceptor cells during retinal degeneration in RCS rats. Taken together with the presence of mutations in *Mertk* in patients with RP, identical with those found in RCS rats,[6] our present observations strongly suggest that the clinical application of nilvadipine has potential benefit in the treatment of RP.

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47. 網膜色素変性モデルラットへのニルバジピン投与とカルパインの変化

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研究要旨　目的：今回我々はアポトーシスの経路としてcalpainに注目し、Royal College of Surgeons (RCS) ラットをはじめとする網膜色素変性モデルラットでの calpain 活性の差異、および RCS ラットに対してカルシウム拮抗薬ニルバジピンを投与した際の細胞質とミトコンドリア分画でのcalpain活性の変化を解析した。

方法：1）網膜色素変性モデルラットとしてRCSラット、ロドプシン遺伝子P23H, S334terトランスジェニックラットを用い、野生型である Sprague-Dawley (SD) ラットと Brawn Norway (BN) ラットとの間のcalpain活性を比較した(n=5)。

2）生後3週齢の RCS ラットにニルバジピンを連日2週間腹腔内に投与した。対照群には基剤のみを投与した。網膜ホモジェネートから細胞質とミトコンドリア画分を分離し、calpain活性を測定した。

結果：1）3種類の網膜変性モデルラットと野生型ラットの網膜 calpain 活性は、ミトコンドリア画分ではラット間での差異はなかったが、細胞質画分にて RCS ラットが有意な高活性を示した ($P < 0.05$)。

2）ニルバジピン投与後の RCS ラットでは対照群に比較してミトコンドリア画分でのcalpain活性が低下傾向を示した。

結論：RCSラットにニルバジピンを投与するとcalpain活性が低下する。この薬物が網膜でのカルシウムチャネルに影響してカルシウムの流入を調節している可能性が示唆された。

A．緒言

網膜色素変性の原因遺伝子として現在までに35種類以上の遺伝子が同定されている。このような原因遺伝子の多様性にもかかわらず、視細胞の変性はアポトーシスという共通のメカニズムにより起こるとされている。アポトーシスに関与するタンパク質として caspase 群が注目されているが、実際にはこれが唯一の経路ではなく、別に calpain や cathepsin 群などがその進行に関係していることが明らかにされつつある。今回、我々はアポトーシス関連タンパク質として calpain に注目し、網膜変性の進行過程における calpain 活性やタンパク質発現量などを比較検討した。とくに網膜変性モデルラットおよび Royal College of Surgeons (RCS)ラットにおける calpain 活性ならびにカルシウム拮抗薬投与 RCS ラット網膜における calpain 活性を比較検討した。