and Chavko, 1994) and rats (Yamashita et al., 1993) without showing any significant effects on the systemic blood pressure or heart rate (Gotoh et al., 1995; Nakashima and Kanamaru, 1989; Tamaki et al., 2003).

Therefore, Iganidipine, nimodipine and lomerizine are potentially useful calcium channel blockers for ophthalmic treatment. We assessed the protective effect of these drugs using RGC hypoxia damage model.

RGC cultures were obtained from retina of 5- to 7-day-old Sprague–Dawley rats according to a two-step immunopanning procedure (Barres et al., 1988; Goto et al., 2002; Inatani et al., 2001; Kashiwagi et al., 2001; Otori et al., 2003; Otori et al., 1998; Pielen et al., 2004). Briefly, retinas were dissociated into cell suspension, using Papain Dissociation System (Worthington Biochemical Corp). 50 ml flasks and 50 ml tubes were incubated with anti-rat macrophage antibody (1:50 dilution, Chemicon) and anti-rat and mouse Thy1.1 antibody (1:300 dilution, Chemicon) in phosphate buffer saline (PBS), respectively at 4 °C overnight. Antibodies were removed and cell suspension was incubated in flask for 1 h. Suspensions containing cells that did not adhere to the flask were treated for 1 h in tubes coated with Thy1.1 antibody. Cells adhering to the tube (RGCs) were resuspended in serum-free Neurobasal medium (GIBCO) supplemented with 2% B27, BDNF (40 ng/ml), CNTF (40 ng/ml) and Forskolin (10 μM) and seeded onto 13mm auto-craved cover slips, placed within 24-well plates. Cover slips were coated with 0.05 mg/ml of poly-L-lysine (GIBCO) overnight, rinsed twice with Hanks' buffered saline solution (HBSS) and then coated for 2 h with 1 μg/ml of laminin (GIBCO). RGCs were cultured for 3 days under normoxia condition (20%O_2, 5%CO_2, 37 °C) before each experiment.

On day 3, one or combination of the following drugs was added to the each culture: (1) iganidipine (Senjyu, Japan), (2) nimodipine (Sigma), (3) lomerizine (Nippon Organon, Japan), (4) ionomycin (5 μM; SIGMA), (5) EDTA (10 μM; SIGMA). Immediately after adding drugs, plates were transferred to a controlled-atmosphere incubator in which oxygen levels were reduced (hypoxic condition; i.e., a 5%CO_2, 90%N_2 and 5%O_2 mix). The concentration of O_2 was decided based on the result of preliminary experiments.

The viability of the purified RGC cell cultures was counted by LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes). For calcium imaging, the medium of RGC culture was changed with HEPES–Tyrode Buffer (composition in mM: 140.0 NaCl, 2.7 KCl, 1.0 $CaCl_2$, 12.0 $NaHCO_3$, 5.6 D-glucose, 0.37 NaH_2PO_4 and 25.0 HEPES, with pH 7.4 at 25 °C) containing 0.1% BSA and 5 μM fura-2/AM, a fluorescent calcium ion indicator. Then, these RGCs were incubated under hypoxic condition for further 60 min. The cells were subsequently washed three times with the HEPES–Tyrode Buffer to remove the dye. The resultant fluorescent images were analyzed with a fluorescence analyzer (Argus 50, Hamamatsu Photonics). The fluorescence ratio (F340/F380) was obtained by dividing, after background subtraction, the 340-nm by the 380-nm images on a pixel-by-pixel basis. The mean ratio over the area of each RGC, well separated from the edge of other RGCs, was measured. Results are indicated as mean ± SD. Data were analyzed by Mann–Whitney U test for comparison of two groups or one-way analysis of variance (ANOVA), followed by Dunnett's test for comparison of three or more groups, with $P < 0.05$ being considered to indicate significance.

The viability of RGCs was 71.9 ± 3.5% in the normal condition (n = 10). To examine the protective effect of iganidipine, nimodipine and lomerizine, RGC culture was incubated under the hypoxic condition for 12 h. The viability of control RGC (no drug in medium) was decreased to 44.0 ± 4.5% (n = 10) (Fig. 1A, $P < 0.01$). Iganidipine (n = 8), nimodipine (n = 8) and lomerizine (n = 8) ameliorated hypoxic damage (Figs. 1B–D). Application of iganidipine (0.01–1 μM), nimodipine (0.01–1 μM) and lomerizine (0.01–1 μM) reduced RGC death in a dose-dependent manner ($P < 0.01$).

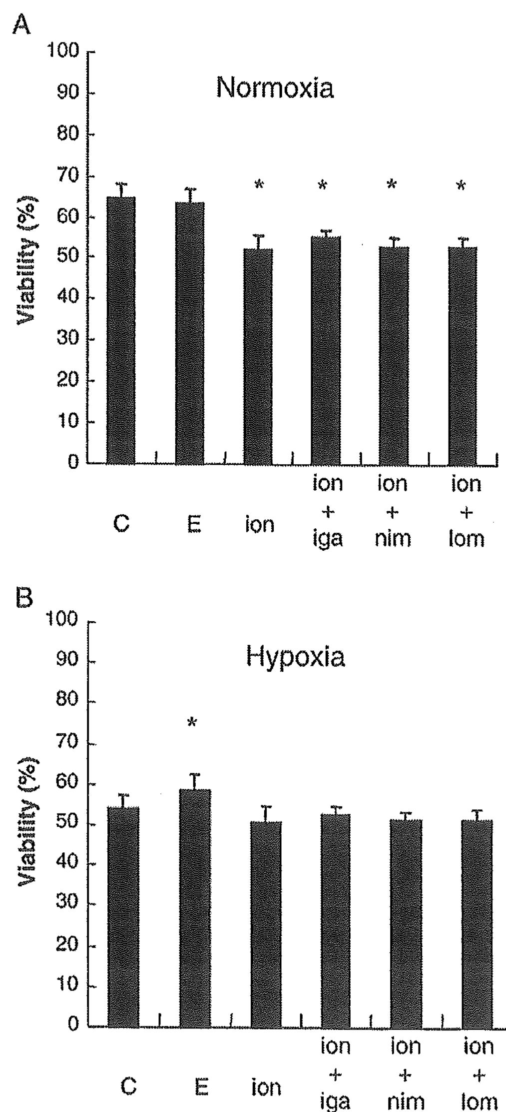

Fig. 2 – Effect of EDTA, ionomycin and calcium blockers to RGC survival rate under normoxic and hypoxic condition. (A) RGC survival rate (viability) under normal condition for 12 h. (B) Viability under hypoxic condition for 12 h. C = control, E = EDTA, ion = ionomycin, iga = iganidipine, nim = nimodipine, lom = lomerizine. ANOVA followed by Dunnett's multiple comparison test. *P < 0.01 versus control. n = 4. Viability is indicated as mean ± SD.

EDTA, which is calcium ion chelator, reduced RGC loss under hypoxic condition ($n = 4$) (Fig. 2B, $P < 0.01$). Ionomycin, which is calcium ionophore, increased RGC loss even under normoxic condition (Fig. 2A, $P < 0.01$). However, its toxicity was not blocked by calcium channel blockers (Fig. 2A).

To examine the effect of these calcium channel blockers on the intracellular calcium ion concentration ($[Ca^{2+}]_i$) in RGC under hypoxic stress, RGC was cultured under hypoxic condition for 5 h and calcium imaging was performed at the earlier timing than viability experiment because the raise of $[Ca^{2+}]_i$ was supposed to precede RGC death.

Fig. 3 shows the F340/F380 ratios obtained by calcium imaging of RGCs ($n = 6$). Calcium ion concentration in RGC increased under hypoxic condition ($P < 0.01$). Calcium channel blockers (at 0.01 μM and 1 μM) reduced increase of calcium ion concentration in RGC under hypoxic condition ($P < 0.01$).

The new aspects of this study are that hypoxia-induced RGC death is at least partly related to increase of $[Ca^{2+}]_i$ and that calcium channel blockers can protect directly RGC death from hypoxic injury.

There are several points to be discussed in the present study. First, it should be considered if the RGC viability was influenced by anaerobic respiration in our culture system. To examine this, it will be helpful to analyze the energy metabolism (e.g., lactate production) (Ben-Yoseph et al.,

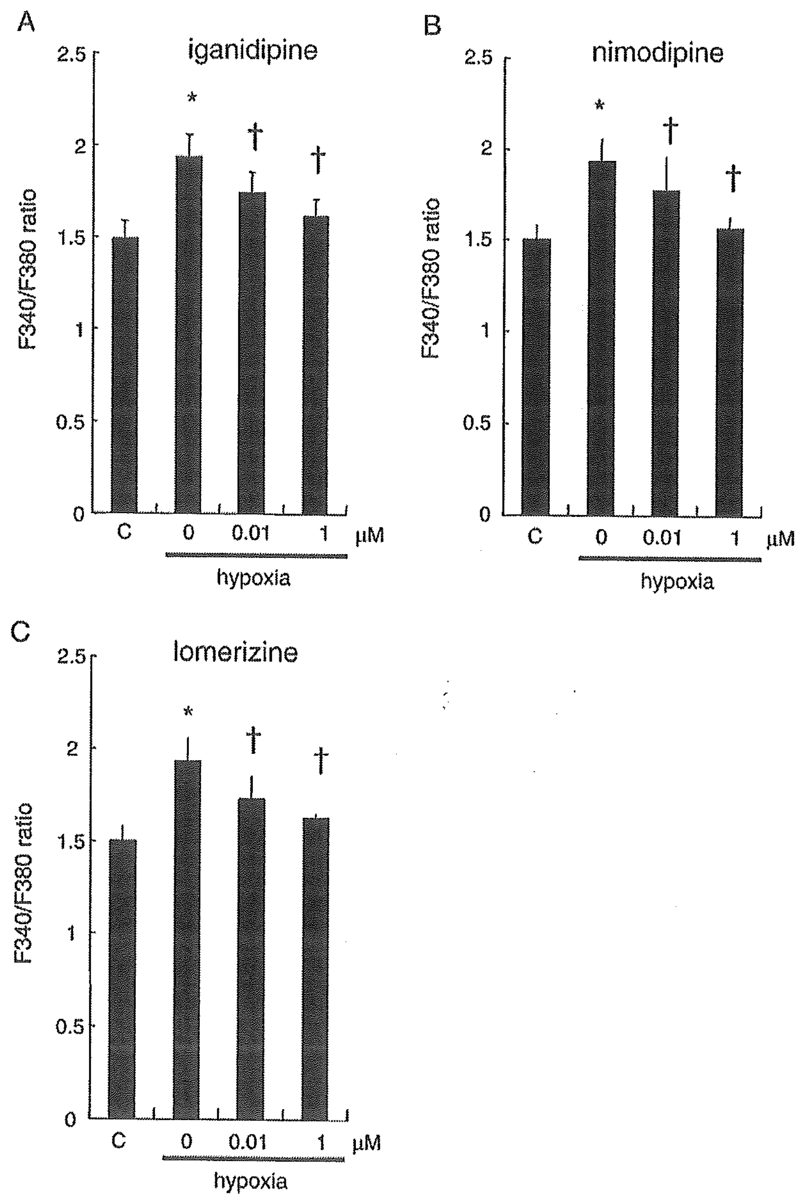

Fig. 3 – Fluorescence ratio (F340/F380) of RGC under normoxia and hypoxia. The effect of calcium channel blockers to fluorescence ratio was also indicated. (A) Iganidipine, (B) Nimodipine, (C) Lomerizine. C = control under normoxia. ANOVA followed by Dunnett's multiple comparison test. *$P < 0.01$ versus C, †$P < 0.01$ versus 0 μM. $n = 6$. Fluorescence ratio is indicated as mean ± SD.

1993; Cox et al., 1983). Unfortunately, RGC pure culture system that we adopted yielded too small amount of RGC to analyze the energy metabolism. Another culture system should be taken to examine this point. The second point is that iganidipine, nimodipine and lomerizine at the same concentration indicated almost equal neuroprotective effect at the similar extent in spite of their different properties for calcium channels. Though lomerizine exerts its neuroprotective effect partly via t-type channel expressed in the neuron (Hara et al., 1999; Mori et al., 1996), lomerizine indicated no advantage compared to other drugs. This fact suggests that t-type channel is not involved in the hypoxic RGC damage pathway, or relatively small magnitude of protective effect might indicate that the hypoxia stimulation was excessive or too small to assess the difference of effect among calcium blockers. It is also interesting whether hypoxia truly influenced the apoptosis process or function (e.g. evoked potentials) of RGC. Further extensive and careful examination should be planed to assess these points to clarify the mechanism of its neuroprotective effects.

The previous reports have shown that 10^{-8}~10^{-7} tissue concentration can be reached by administration of iganidipine, nimodipine and lomerizine (Ishii et al., 2003; Nakashima and Kanamaru, 1989; Sakai, 1994; Yan et al., 1993). Thus, these drugs may have a potential to be used clinically as ophthalmic neuroprotective drugs in consideration of their systemic side effects. The systemic side effect (for example, dizziness, headache, flushing, edema and/or systemic hypotension) is thought to be less frequent during nimodipine (Battistella et al., 1990) and lomerizine (Hara et al., 1999) treatment. Thus, these agents may be used for ophthalmic long-term oral treatment without compliance problems. Iganidipine can be given as an ophthalmic solution, which should be free of systemic effects.

In conclusion, our study indicates the possibility of direct neuroprotective effect of iganidipine, nimodipine and lomerizine on RGC death against hypoxic damage. Although further studies are required to clarify the direct mechanism of neuroprotection of calcium channel blockers, these calcium channel blockers may be candidates of ophthalmic drugs for neurodegenerative ocular diseases related to hypoxia.

REFERENCES

Barres, B.A., Silverstein, B.E., Corey, D.P., Chun, L.L., 1988. Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. Neuron 1, 791–803.

Battistella, P.A., Ruffilli, R., Moro, R., Fabiani, M., Bertoli, S., Antolini, A., Zacchello, F.A., 1990. Placebo-controlled crossover trial of nimodipine in pediatric migraine. Headache 30, 264–268.

Ben-Yoseph, O., Badar-Goffer, R.S., Morris, P.G., Bachelard, H.S., 1993. Glycerol 3-phosphate and lactate as indicators of the cerebral cytoplasmic redox state in severe and mild hypoxia respectively: a 13C- and 31P-n.m.r. study. Biochem. J. 1, 915–919.

Boehm, A.G., Breidenbach, K.A., Pillunat, L.E., Bernd, A.S., Mueller, M.F., Koeller, A.U., 2003. Visual function and perfusion of the optic nerve head after application of centrally acting calcium-channel blockers. Graefe Arch. Clin. Exp. Ophthalmol. 241, 34–38.

Bose, S., Piltz, J.R., Breton, M.E., 1995. Nimodipine, a centrally active calcium antagonist, exerts a beneficial effect on contrast sensitivity in patients with normal-tension glaucoma and in control subjects. Ophthalmology 102, 1236–1241.

Carter-Dawson, L., Crawford, M.L., Harwerth, R.S., Smith III, E.L., Feldman, R., Shen, F.F., Mitchell, C.K., Whitetree, A., 2002. Vitreal glutamate concentration in monkeys with experimental glaucoma. Invest. Ophthalmol. Visual Sci. 43, 2633–2637.

Chung, H.S., Harris, A., Evans, D.W., Kagemann, L., Garzozi, H.J., Martin, B., 1999. Vascular aspects in the pathophysiology of glaucomatous optic neuropathy. Surv. Ophthalmol. 43 (Suppl. 1), S43–S50.

Cioffi, G.A., Wang, L., 1999. Optic nerve blood flow in glaucoma. Semin. Ophthalmol. 14, 164–170.

Cox, D.W., Morris, P.G., Feeney, J., Bachelard, H.S., 1983. 31P-n.m.r. studies on cerebral energy metabolism under conditions of hypoglycaemia and hypoxia in vitro. Biochem. J. 15, 365–370.

Danielisova, V., Chavko, M., 1994. KB-2796, a calcium channel blocker, ameliorates ischemic spinal cord damage in rabbits. Neurochem. Res. 19, 1503–1507.

Flammer, J., 1994. The vascular concept of glaucoma. Surv. Ophthalmol. 38 (Suppl), S3–S6.

Flammer, J., Orgul, S., Costa, V.P., Orzalesi, N., Krieglstein, G.K., Serra, L.M., Renard, J.P., Stefansson, E., 2002. The impact of ocular blood flow in glaucoma. Prog. Retinal Eye Res. 21, 359–393.

Gaspar, A.Z., Flammer, J., Hendrickson, P., 1994. Influence of nifedipine on the visual fields of patients with optic-nerve-head diseases. Eur. J. Ophthalmol. 4, 24–28.

Goto, W., Ota, T., Morikawa, N., Otori, Y., Hara, H., Kawazu, K., Miyawaki, N., Tano, Y., 2002. Protective effects of timolol against the neuronal damage induced by glutamate and ischemia in the rat retina. Brain Res. 958, 10–19.

Gotoh, F., Fukuuchi, Y., Tashiro, K., 1995. Long-term trial of lomerizine hydrochloride for migraine. Jpn. Pharmacol. Ther. 23, 1445–1460.

Hara, H., Morita, T., Sukamoto, T., Cutrer, F.M., 1995. Lomerizine (KB2796), a new antimigraine drug. CNS Drug Rev. 1, 204–226.

Hara, H., Shimazawa, M., Sasaoka, M., Yamada, C., Iwakura, Y., Sakai, T., Maeda, Y., Yamaguchi, T., Sukamoto, T., Hashimoto, M., 1999. Selective effects of lomerizine, a novel diphenylmethylpiperazine Ca^{2+} channel blocker, on cerebral blood flow in rats and dogs. Clin. Exp. Pharmacol. Physiol. 26, 870–876.

Honkanen, R.A., Baruah, S., Zimmerman, M.B., Khanna, C.L., Weaver, Y.K., Narkiewicz, J., Waziri, R., Gehrs, K.M., Weingeist, T.A., Boldt, H.C., Folk, J.C., Russell, S.R., Kwon, Y.H., 2003. Vitreous amino acid concentrations in patients with glaucoma undergoing vitrectomy. Arch. Ophthalmol. 121, 183–188.

Inatani, M., Honjo, M., Otori, Y., Oohira, A., Kido, N., Tano, Y., Honda, Y., Tanihara, H., 2001. Inhibitory effects of neurocan and phosphacan on neurite outgrowth from retinal ganglion cells in culture. Invest. Ophthalmol. Visual Sci. 42, 1930–1938.

Ishii, K., Matsuo, H., Fukaya, Y., Tanaka, S., Sakaki, H., Waki, M., Araie, M., 2003. Iganidipine, a new water-soluble Ca^{2+} antagonist: ocular and periocular penetration after instillation. Invest. Ophthalmol. Visual Sci. 44, 1169–1177.

Ishii, K., Fukaya, Y., Araie, M., Tomita, G., 2004. Topical administration of iganidipine, a new water-soluble Ca^{2+} antagonist, increases ipsilateral optic nerve head circulation in rabbits and cynomolgus monkeys. Curr. Eye Res. 29, 67–73.

Kashiwagi, K., Iizuka, Y., Tanaka, Y., Mochizuki, S., Kajiya, F., Araie, M., Suzuki, Y., Iijima, H., Tsukahara, S., 2001. Dual action of nitric oxide on purely isolated retinal ganglion cells. Curr. Eye Res. 23, 233–239.

Kitazawa, Y., Shirai, H., Go, F.J., 1989. The effect of $Ca^{2(+)}$-antagonist

on visual field in low-tension glaucoma Graefes Arch. Clin. Exp. Ophthalmol. 227, 408–412.

Levkovitch-Verbin, H., Martin, K.R., Quigley, H.A., Baumrind, L.A., Pease, M.E., Valenta, D., 2002. Measurement of amino acid levels in the vitreous humor of rats after chronic intraocular pressure elevation or optic nerve transaction. J. Glaucoma 11, 396–405.

Luo, X., Lambrou, G.N., Sahel, J.A., Hicks, D., 2001. Hypoglycemia induces general neuronal death, whereas hypoxia and glutamate transport blockade lead to selective retinal ganglion cell death in vitro. Invest. Ophthalmol. Visual Sci. 42, 2695–2705.

Mori, Y., Mikala, G., Varadi, G., Kobayashi, T., Koch, S., Wakamori, M., Schwartz, A., 1996. Molecular pharmacology of voltage-dependent calcium channels. Jpn. J. Pharmacol. 72, 83–109.

Nakashima, M., Kanamaru, M., 1989. Clinical phase 1 study of KB-2796. Rinsho Iyaku 5, 1791–1811.

Netland, P.A., Chaturvedi, N., Dreyer, E.B., 1993. Calcium channel blockers in the management of low-tension and open-angle glaucoma. Am. J. Ophthalmol. 115, 608–613.

Osborne, N.N., Ugarte, M., Chao, M., Chidlow, G., Bae, J.H., Wood, J.P., Nash, M.S., 1999. Neuroprotection in relation to retinal ischemia and relevance to glaucoma. Surv. Ophthalmol. 43 (Suppl. 1), S102–S128.

Otori, Y., Wei, J.Y., Barnstable, C.J., 1998. Neurotoxic effects of low doses of glutamate on purified rat retinal ganglion cells. Invest. Ophthalmol. Visual Sci. 39, 972–981.

Otori, Y., Kusaka, S., Kawasaki, A., Morimura, H., Miki, A., Tano, Y., 2003. Protective effect of nilvadipine against glutamate neurotoxicity in purified retinal ganglion cells. Brain Res. 31, 213–219.

Pielen, A., Kirsch, M., Hofmann, H.D., Feuerstein, T.J., Lagreze, W.A., 2004. Retinal ganglion cell survival is enhanced by gabapentin-lactam in vitro: evidence for involvement of mitochondrial KATP channels. Graefe Arch. Clin. Exp. Ophthalmol. 242, 240–244.

Piltz, J.R., Bose, S., Lanchoney, D., 1998. The effect of nimodipine, a centrally active calcium antagonist, on visual function and muscular blood flow in patients with normal-tension glaucoma and control subjects. J. Glaucoma 7, 336–342.

Sakai, T., 1994. Yakuri to chiryo 22, 4657–4662.

Tamaki, Y., Araie, M., Fukaya, Y., Nagahara, M., Imamura, A., Honda, M., Obata, R., Tomita, G., 2003. Effects of lomerizine, a calcium channel antagonist, on retinal and optic nerve head circulation in rabbits and humans. Invest. Ophthalmol. Visual Sci. 44, 4864–4871.

Tezel, G., Wax, M.B., 2004. Hypoxia-inducible factor 1α in the glaucomatous retina and optic nerve head. Arch. Ophtalmol. 122, 1348–1356.

Tezel, G., Yang, X., 2004. Caspase-independent component of retinal ganglion cell death, in vitro. Invest. Ophthalmol. Visual Sci. 45, 4049–4059.

Tomita, G., Niwa, Y., Shinohara, H., Hayashi, N., Yamamoto, T., Kitazawa, Y., 1999. Changes in optic nerve head blood flow and retrobular hemodynamics following calcium-channel blocker treatment of normal-tension glaucoma. Int. Ophthalmol. 23, 3–10.

Toriu, N., Akaike, A., Yasuyoshi, H., Zhang, S., Kashii, S., Honda, Y., Shimazawa, M., Hara, H., 2000. Lomerizine, a Ca^{2+} channel blocker, reduces glutamate-induced neurotoxicity and ischemia/reperfusion damage in rat retina. Exp. Eye Res. 70, 475–484.

Waki, M., Sugiyama, T., Watanabe, N., Ogawa, T., Shirahase, H., Azuma, I., 2000. Effect of topically applied iganidipine dihydrochloride, a novel Ca^{2+} antagonist, on optic nerve head circulation in rabbits. Nippon Ganka Gakkai Zasshi 104, 541–546.

Yamamoto, T., Niwa, Y., Kawakami, H., Kitazawa, Y., 1998. The effect of nilvadipine, a calcium-channel blocker, on the hemodynamics of retrobulbar vessels in normal-tension glaucoma. J. Glaucoma 7, 301–305.

Yamashita, A., Ozaki, A., Ikegami, A., Hayashi, A., Hara, H., Sukamoto, T., Ito, K., 1993. Effects of a new diphenylpiperazine calcium antagonist, KB-2796, on cerebral ischemic neuronal damage in rats. Gen. Pharmacol. 24, 1473–1480.

Yan, X.F., Ding, D.Y., Liu, R.J., 1993. Studies on the bioavailability and pharmacokinetics of nimodipine tablet in human body. Yaoxue Xuebao 28, 45–49.