Acta Med. Okayama, 2003
Vol. 57, No. 5, pp. 257-260

Acta Medica Okayama
http://www.lib.okayama-u.ac.jp/www/acta/

Original Article

A Simple Method for Screening Photoelectric Dyes towards Their Use for Retinal Prostheses

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Photoelectric dyes absorb light and convert photon energy to electric potentials. To test whether these dyes could be used for retinal prostheses, a simple *in vitro* screening system was developed. Retinal neurons were cultured from the eyes of chick embryos at the 10-day embryonic stage, at which time no retinal photoreceptor cells have yet developed. Intracellular calcium elevation was observed with Fluo-4 in cultured retinal neurons before and after photoelectric dye was applied at varying concentrations to the culture medium. Five of 7 photoelectric dyes tested in this *in vitro* system induced intracellular calcium elevation in cultured chick retinal neurons. The intracellular calcium elevation generated by the 5 photoelectric dyes was blocked by extracellular calcium depletion in the case of all 5 dyes, and, except for one dye, by the presence of voltage-gated calcium channel blockers. The photoelectric dyes absorbed light under an inverted microscope and stimulated retinal neurons. This simple *in vitro* system allows the screening of photoelectric dyes which can be used for retinal prostheses.

Key words: photoelectric dye (pigment), chick retinal neurons, intracellular calcium, retinal prostheses, retinal implant

The retina has photoreceptor cells which absorb light and convert photon energy to the electric potentials of the cell membrane. Photoreceptor cell loss caused by hereditary retinal dystrophy, such as retinitis pigmentosa, and also by age-related macular degeneration and diabetic retinopathy leads to blindness, and no treatment is currently available to rescue photoreceptor cell loss and restore vision. Studies have found that in these patients, the remaining retinal neurons and circuits can be electrically stimulated to evoke the sensation of light [1]. Based on these clinical findings, and due to the successful clinical application of cochlear implants, retinal implants or retinal prostheses are being considered as a future treatment for this type of photoreceptor cell loss [2-4]. The major problems with photodiodes, which have been used up to now to develop retinal prostheses, are their large size and poor biocompatibility. In this study, as a better candidate for retinal prostheses, photoelectric dyes were tested to see whether they would stimulate retinal neurons in culture, as a simple *in vitro* screening system.

Received January 16, 2003; accepted April 24, 2003.
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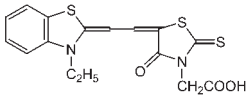
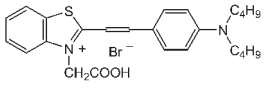
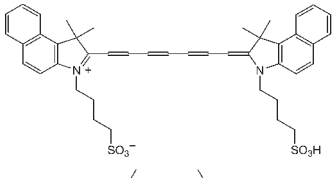
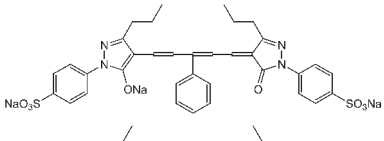
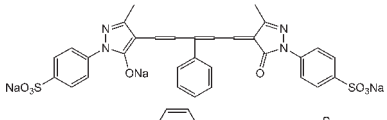
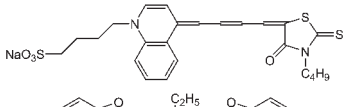
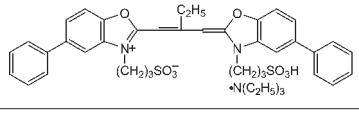
Materials and Methods

Egg shells were disinfected with 70% alcohol, and the 10-day-old chick embryos were removed from a small opening. The eyes at this embryonic stage were used, since no retinal photoreceptor cells have developed yet at this stage [5]. The eyes were enucleated and cut at the midperiphery of the globe, and the anterior halves were removed together with the vitreous. The retina at this

embryonic stage could be easily pealed off the eye cup. After peeling, the retinas were incubated in 0.25% trypsin and 1 mM ethylenediaminetetraacetic acid (EDTA) in Ca^{2+}, Mg^{2+} free Hanks' balanced salt solution (HBSS: Gibco BRL, Gaithersburg, MD, USA) to disperse the retinal cells [5, 6]. The retinal cells were then washed with Dulbecco's modified Eagle's medium (DMEM: Nissui, Tokyo, Japan) after centrifugation, and plated at a concentration of 6×10^6 cell/10 mL in a 24-well multidish (Nunc, Naperville, IL, USA) containing DMEM supplemented with 10% fetal calf serum (FCS), 100 mg/L streptomycin, and 100 mg/L ampicillin. Half of the medium was changed 3 times a week. Culture dishes were used for the following experiments between the third and fifth days, when neurons formed the predominant population [7].

The cells in the wells of a 24-well multidish were loaded with 10 μM of Fluo-4 acetoxymethyl ester (Fluo-4/AM: Molecular Probe, Eugene, OR, USA) for 30 min at 37 C in DMEM, and incubated further in DMEM without Fluo-4/AM. The multidish with 200 μL of HEPES buffered saline (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 10 mM glucose, pH 7.4) in each well [8] was placed on the stage of an inverted microscope (Olympus IX71, Tokyo, Japan) attached to a CCD camera (Olympus DP50). Fluorescence was observed with an excitation wavelength of 470–490 nm and a transmission wavelength of 510–550 nm before and after the addition of 10 μL of the photoelectric dyes (Table 1, Hayashibara Biochemical

Table 1 Structure and characteristics of photoelectric dyes and their effect on retinal neurons in culture

Code	Structure	Molecular weight	Peak absorption wavelength (nm)	Intracellular calcium elevation in retinal neurons			Autofluorescence
				Response	Minimum Concentration (μg/ml)	Calcicludine inhibition	
NK-2045		378.49	520	No	–	–	Yes
NK-5962		503.50	539	Yes	0.5	Yes	No
NK-5078		752.98	784	No	–	–	No
NK-3630		768.74	650	Yes	0.0005	Yes	No
NK-3041		712.64	649	Yes	0.005	Yes	No
NK-2761		526.67	716	Yes	0.005	Yes	No
NK-1952		802.01	504	Yes	0.05	No	No

Laboratories, Okayama, Japan) at each concentration. The images were captured with an × 4 objective lens at an exposure of 1/4 second into a computer loaded with the Viewfinder Lite software program (Viewfinder Lite, Version 1.0, Olympus).

To determine the minimal concentration of dyes needed to induce calcium elevation in retinal cells, the dye solution was tested from the concentration of 100 μg/ml (final concentration at 5 μg/ml), and then diluted in a 10-fold series until no response in the retinal cells was observed. Testing at each concentration of the dyes was repeated 3 times using different wells of retinal cells. The final concentration of the dyes at 0.5 μg/ml was used to test the inhibition of the calcium response in the retinal cells by the presence of calcicludine, a potent inhibitor of the L-, N-, and P-type high-threshold calcium channels, at the final concentration of 2.5 μM, and also by the presence of amiodarone, a non-selective ion channel blocker, at the same final concentration.

Results

The structure of the 7 photoelectric dyes tested in this study and the results are summarized in Table 1. Five dyes (NK-5962, NK-3630, NK-3041, NK-2761, and NK-1952) generated intracellular calcium elevation observed as a fluorescence increase in the retinal neurons (Fig. 1), while the other 2 dyes (NK-2045 and NK-5078) did not induce intracellular calcium elevation. The 10-fold dilution series of the dyes, which was performed to determine the minimal concentration needed to induce calcium elevation, showed all or no response between certain points in the dilution series. Methanol or dimethylsulfoxide used as a solvent for the dyes did not induce any fluorescence changes.

Fluorescence changes observed after the addition of 4 of the dyes (NK-5962, NK-3630, NK-3041, and NK-2761) were inhibited by the presence of calcicludine, a potent inhibitor of the L-, N-, and P-type high-threshold calcium channels, at the final concentration of 2.5 μM (Fig. 1), and also by the presence of amiodarone, a non-selective ion channel blocker, at the same final

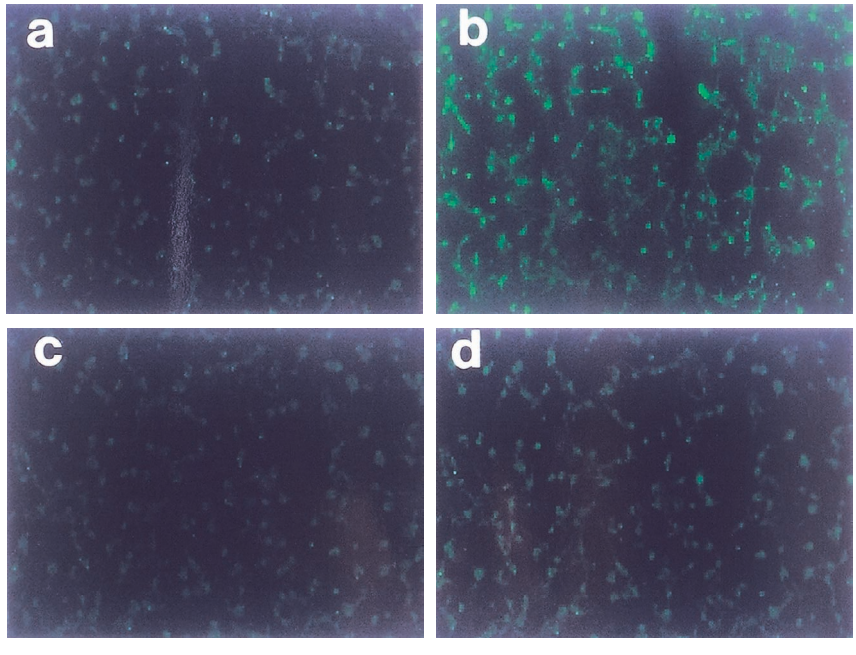


Fig. 1 The effect of a photoelectric dye, NK-3041 (see Table 1 for the structure), on intracellular calcium observed as the fluorescence of Fluo-4 in cultured chick retinal neurons. In comparison with the fluorescence before the addition of the dye (a), the fluorescence immediately increased after the addition of the dye at the final concentration of 0.5 μg/mL (b). In the presence of calcicludine, a voltage-gated calcium channel blocker, at the final concentration of 2.5 μM, no fluorescence changes were observed between before (c) and after (d) the addition of the dye.

concentration. In contrast, the fluorescence increase induced by one dye (NK-1952) was not inhibited by calcicludine or amiodarone. The depletion of calcium ion from the culture medium also led to no fluorescence changes after the addition of all 5 dyes.

Discussion

In this study, Fluo-4, a calcium-sensitive dye taken up by retinal neurons, was excited by light with wavelengths ranging from 470 to 490 nm, and fluorescence was observed through a filter passing light with wavelengths between 510 and 550 nm. As would be expected, one photoelectric dye (NK-5078), which absorbed light with wavelengths of 600 nm or longer, did not stimulate retinal neurons under light with wavelengths from 470 to 490 nm. Another dye (NK-2045) did not induce any response in the retinal neurons, although it was able to absorb the wavelengths ranging from 470 to 490 nm used in this study. The photoelectric dyes which induced a positive response in the retinal neurons tested showed no common characteristics in their molecular structures.

A major problem in this assay system is that the light used to stimulate the photoelectric dyes could not be separated from the light used to observe the intracellular calcium response in the retinal cells. Since intracellular calcium elevation was observed by Fluo-4 with the absorption maximum at 494 nm and the emission maximum at 516 nm in this study, light with a wavelength of 470–490 nm was used to stimulate both the photoelectric dyes and Fluo-4. This range of the wavelengths of Fluo-4 naturally limits the dyes which can be screened in this assay system. The development of a new assay system to use light with different ranges of wavelength to stimulate photoelectric dyes and calcium indicators separately at the same time would be a solution to testing photoelectric dyes with other spectrums.

Intracellular calcium elevation in the retinal neurons was observed immediately after the addition of the dyes to the culture medium. This fact indicates that the dyes in the medium converted the photon energy of the light to electric potentials, which then stimulated the neurons. Another possibility is that the dyes in the medium rapidly diffused, and then might have adhered to the cell membranes of the neurons. The intracellular calcium elevation induced by 4 of the dyes was not observed in the presence of a calcium channel blocker, calcicludine, which is sensitive to all types of voltage-gated calcium channels. Furthermore, the depletion of extracellular calcium also resulted in no fluorescence changes following the addition of the dyes. These facts indicate that the intracellular calcium elevation is generated by a flow of extracellular calcium ions through voltage-gated calcium channels in retinal neurons. The intracellular calcium elevation caused by one dye (NK-1952) was not inhibited by the voltage-gated calcium channel blocker, indicating that the intracellular calcium elevation was generated by different ways other than the voltage-gated calcium channels. One possibility is that this dye might serve as a kind of ionophore.

In future studies, we plan to solidify photoelectric dyes into a thin film and implant them surgically, either on the surface of the retina or beneath it. Retinal neurons would be cultured on a thin film containing photoelectric dyes for *in vitro* testing. The biological safety and stability of photoelectric dyes could also be tested in these kinds of *in vitro* and *in vivo* studies. The combination of photoelectric dyes with different spectrums of absorption might lead to color sensation by a retinal prosthesis. The simple screening method described in this study will help search for photoelectric dyes which might be suitable for a retinal prosthesis or for retinal implants.

Acknowledgments. The author would like to thank Sadaharu Suga, Ph.D. and Yasufumi Dan-oh, Ph.D. from Hayashibara Biochemical Laboratories, Okayama City, Japan, for providing the photoelectric dyes.

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