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Modification of Retinal Function by Hypothermia and Hyperthermia

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Temperature-dependent electroretinogram responses were investigated in the dark adapted bullfrog eyes within the physiological temperature range 0-40°C. In hypothermic process (25→0→25°C), the amplitude of b- and c-wave decreased with lowering the temperature but increased with raising up to room temperature again. Both b-wave amplitude and threshold responses were maximal around 15°C during the temperature increment. Upon warming to room temperature again (25°C), the b-wave amplitude was approximately doubled as compared to that of control without temperature changes. During the hyperthermic process (25→40→25°C), however, the responses decreased with warming, and the wave amplitude failed to recover by cooling to 25°C again. As described above, the recoveries of ERG in both processes show the striking difference. The hypothermia induces the amplification of the b-wave, that is, enhances the retinal function with the temperature recovery toward room temperature. While the hyperthermia produces the decrease of the b-wave even though recovered to room temperature, which indicates an irreversible retina. The morphological alteration is shown both hypothermic and hyperthermic process, such as an appearance of large vacuoles and degenerating outer segments, more intense in hyperthermia, similar to light induced damage.

Key words: bullfrog, electroretinogram(ERG), retinal pigment epithelium(RPE), transmission electron microscopy (TEM), interphotoreceptor matrix (IPM)

INTRODUCTION

The electroretinogram(ERG) is a compound potential produced by the layers of the retina in response to visual stimulation. The a-, b-, and c-waves appear in a typical scotopic response of dark-adapted eye to a flash, which are fundamental to any discussion of the ERG. There has been developing consensus in this regard that the a-wave of the ERG represents activity of the photoreceptors, while the b-wave appears to represent activity of Muller cells throughout the retina in response to potassium ions [1-3]. In particular, the b-wave is of clinical importance in the assessment of retinal function and is highly affected by temperature, zinc and taurine concentration. The effects of temperature on the retinal function have been investigated in numerous species mostly in homeotherms [4-8]. In terms of experimental method, to provide temperature changes, in most cases, studies reporting the effect of retinal cooling on the ERG of mammals have made use of an *in vitro* approach where the temperature of the retina was lowered by reducing the temperature of the bathing media or injecting the infusion fluid for intraocular perfusion which is regulated various temperatures. In some cases, *in vivo* retinal cooling was performed by using whole body cooling in order to determine if some of the temperature related ERG effects previously shown could have been, in part, amplified by alterations in the physiological status of the retina due to preparation for *in vitro* study [9]. Studies reporting the effect of retinal cooling in homeotherms all showed a progressive attenuation in the amplitude with an increase in timing of all the ERG components as the retinal temperature is lowered [10-11]. The previous results indicated that temperature can affect the functional properties of photoreceptors in a variety of ways: it may directly alter the composition and characteristics of the membranes, the cytoplasm and visual molecules; it may affect certain features of the cellular metabolism, or it can exert an effect on the hormonal system of the animal, which, in turn, may influence the adaptational state of the eye and thus determine visual sensitivity [12]. However, it is not known well how the temperature induce changes in the functional properties of retina in poikilotherms due to some limitation in direct comparison of temperature effect on visual process in consideration of the species, their body temperature and the method of temperature changing.

In the work reported here, the temperature-dependent ERG responses of eyecup and isolated retina preparations of bullfrog (*Rana temporaria*), which is one of the poikilothermal animal, were investigated in the presence of various monochromatic light intensity within the physiological temperature range 0-

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40°C and recorded the ERG returning to normal temperature. The amplitudes of b- and c-wave were plotted against temperature to investigate the effect cold and heat shocks on visual responses. We compared with the previous studies it is to examine (1) the temperature effect on visual process and sensitivity in each step of temperature and during temperature changes and (2) their reversibility by restoring of the temperature by more extended temperature range. Furthermore, this modification of retinal function by cold and heat shocks was examined by (3) transmission electron microscopic (TEM) analysis to explain how the cold or heat shock as thermal stress modifies the visual function in terms of structure and determine cellular structural requirements conducive to it.

MATERIALS AND METHOD

Sample preparation

A bullfrog was dark-adapted for at least 2 hours before the eye was enucleated under dim red light. The anterior portion of eyeball cut away, and the posterior eyecup portion was mounted onto the sample holder, which was connected to modified Ussing chamber that perfused with pure oxygen saturated ringer solution which is containing: 105 mM NaCl; 2.5 mM KCl; 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM $NaHCO_3$; and 10 mM HEPES, was adjusted to a pH of 7.5. For isolated retina experiment, a retina was peeled off from posterior eyecup portions, receptor slid up, free of pigment epithelium, placed on a piece of filter paper and mounted onto the sample holder with the same manner. The temperature of the ringer solution was maintained at 25°C as control during experimentation while those of hypothermic and hyperthermic process were changed the temperature range of 0~40°C by thermo-controller (Mono-Tech Co., Refrigerating bath circulator MRC-1131D), such as the temperature was lowered from 25°C to 0°C and increased toward 25°C again (25°C→0°C→25°C) for hypothermic process while it was warmed from 25°C to 40°C and cooled toward 25°C again (25°C→40°C→25°C) for hyperthermic process. All procedures were carried out under dimmed red light paying close attention to avoid cell damage caused by environmental light exposure.

ERG recording

The stimulus beam was projected directly on the sample to deliver 200 msec flashes from the light source (12V/120W, halogen lamp) through neutral density (ND) filters, an electronic shutter and a 505 nm interference filter. When not attenuated by neutral density filters, the stimulus light delivered was 1.95×10^{14} photons/cm2/sec. The ERG was recorded using a fine glass micropipette Ag-AgCl agar bridge electrode which was filled with 3 M KCl in 3% agar and set both at the front and rear of the sample holder. The signal, which was generated as the potential difference between electrodes, was amplified with both pre-amplifier (AI 417, Axon Instruments), D.C. main-amplifier (CyberAmp380, Axon Instruments) and recorded on computer through an AD/DA converter (Digidata 1200A interface, Axon Instruments). The temperature of samples were changed from 0°C to 40°C at intervals of 5°C by alteration of the ringer solution, which is circulated through the modified Ussing chamber and contacted with eyecup preparation directly. These temperature changes are divided into two types; hypothermic and hyperthermic condition against control, which is maintained the temperature of ringer solution at 25°C (room temperature) during experiment. For the hypothermic process, the temperature of ringer solution was lowered from 25°C to 0°C and warmed to 25°C again, while for the hyperthermic process increased the temperature of ringer solution from 25°C to 40°C and cooled to 25°C again. Temperature of perfused solution was kept constant within 1°C by flowing of 50% ethylene glycol as a coolant from thermostatted bath(Mono-Tech Co., MRC-1131D). The b-wave amplitude of ERG was measured from the trough of a-wave to the peak of b-wave. All data were analyzed with Axotape and plotted with Excel program.

Transmission Electron Microscopic analysis:

The eyecups (dark and light adapted, hypothermia and hyperthermia effected) were prefixed in Karnovsky fixative for 1 hour and the rectangular tissue with size of 1 5 mm was cut from the posterior. The block was placed in Karnovsky fixative for 2 hours in a weak vacuum again. The fixed tissue block was washed twice for 20 minutes each in 0.05 M cacodylate buffer pH 7.2 and then placed in 1% osmium tetroxide in 0.05M cacodylate buffers for 2 hours. Following fixation, the tissue was washed with distilled water for 2 minutes and prestained overnight in 0.5% uranyl acetate at 4°C. The tissue was dehydrated through a graded series of ethanol (30, 50, 70, 80, 95, and 100%) for 20 minutes each and with propylene oxide for 20 minutes repeatedly twice. Infiltration was performed in the ratio 1:1 of propylene oxide: spur for 2 hours and then with 100% spur for a few hours in a mild vacuum. Finally, the tissue was embedded in flat embedding molds in spur with and the polymerization was carried out at 70°C for 8 hours. The hardened epoxy block was sectioned with the thick 0.07 μm and then stained with uranyl acetate and lead citrate and examined on a Hitachi 7100 EM.

RESULTS AND DISCUSSION

Temperature dependency of b, c-waves in Scotopic ERG during the hypothermic process

Temperature-dependent changes of the relative b- and c-wave amplitudes during hypothermic process were recorded. ERGs were elicited from the dark-adapted bullfrog eyecup at various ranges of stimulus light intensities such as ND0, ND1, ND2, and ND3. The relative b-wave response with temperature changes is shown in Figure 1(A). The abscissa represents a temperature change during hypothermic process while the ordinate represents the relative b-wave amplitude which means the ratio of the b-wave amplitude in ND0 at an early 25°C to each b-wave response. The relative b-wave amplitude decreases

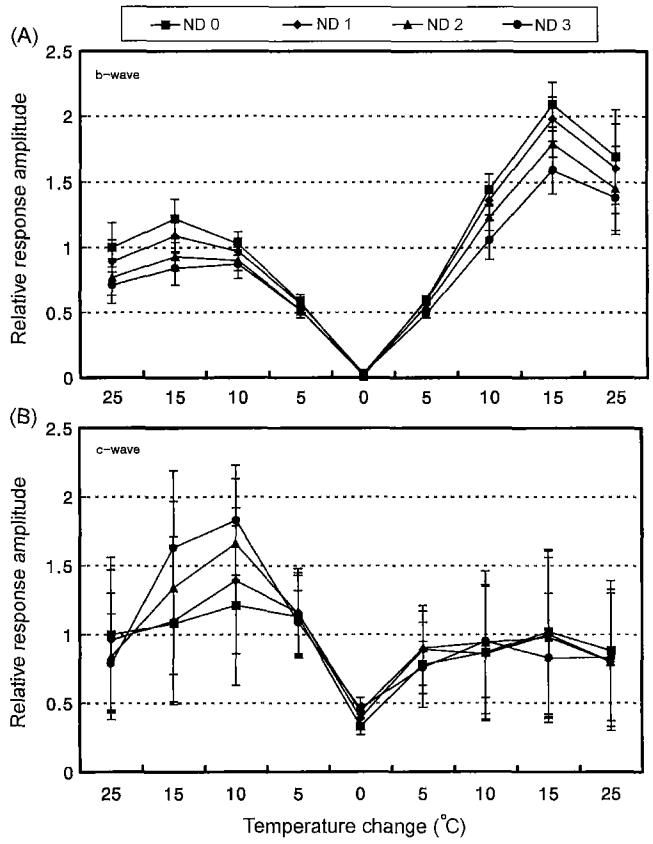

Figure 1. (A) Temperature-dependent changes of the relative amplitude responses in the b-wave of ERG with a hypothermic procedure. (B) Temperature-dependent changes of the relative c-wave amplitude responses with a hypothermic procedure. ERGs were elicited from the dark-adapted bull frog eyecup at various range of light intensities, ND3 (●), ND2 (▲), ND1 (◆), and ND0 (■).

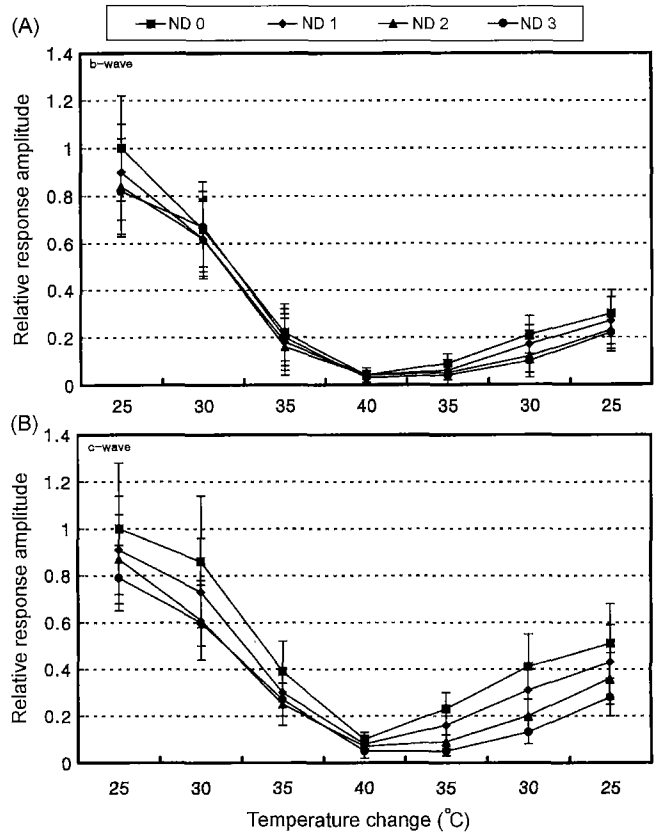

Figure 2. (A) Temperature-dependent changes of the relative b-wave amplitude responses with a hyperthermic procedure. (B) Temperature-dependent changes of the relative c-wave amplitude responses with a hyperthermic procedure. The symbols and the drawing patterns are the same as Figure 1, except that the abscissa represents a temperature change during hyperthermic process. ERGs were elicited from the dark-adapted bullfrog eyecup at various ranges of stimulus light intensities.

with temperature lowering toward 0°C and recovers with warming to 25°C. The higher stimulus light intensity shows the higher relative b-wave amplitude and it is maximized around 15°C of cooling and rising temperature, noticeably amplified at rising temperature 15°C, about x1.7 compared to control at the brightest light intensity ND 0. The relative c-wave response curves are shown in Figure 1(B). The relative amplitude of c-wave represents a tendency to decrease with temperature lowering toward 0°C after showing maximal response increment at 10°C and recover with warming to 25°C. But its temperature dependence is not as much as that of b-wave amplitude in Figure 1(A). From the above results, it is suggested that hypothermic process would improve the visual sensitivity resulted in b- and c-wave amplification and have little influence on visual recovery.

Temperature dependency of b, c-waves in Scotopic ERG during the hyperthermic process

In the temperature range 25-40°C, the b- and c-wave amplitudes were recorded at 5°C intervals upon warming toward 40°C and restoring to 25°C as shown in Figure 2(A) and 2(B) in respectively. The relative b- and c-wave amplitudes from the dark-adapted bullfrog eyecup in the hyperthermic process decreased as like that of hypothermic process. However, these changes due to hyperthermic process are much steeper than those of due to hypothermic process. And the reduced peak amplitudes due to hyperthermic process were not recovered unlike hypothermic process in spite of temperature restoration to 25°C. As the result of hyperthermic process, temperature increment toward 40°C may cause great damage to the visual sensitivity and this thermal stress could not recovered after temperature restoration.

Comparison of Hypothermia and hyperthermia effect on b-waves of eyecup and isolated retina

Figure 3 displays the recovery of b-wave amplitude in the presence of stimulus light intensity ND0 after hypothermic or hyperthermic processes in eyecup and isolated retina preparations. The abscissa represents types of treated thermal condition (control, maintained 25°C; hypo, after hypothermic process;

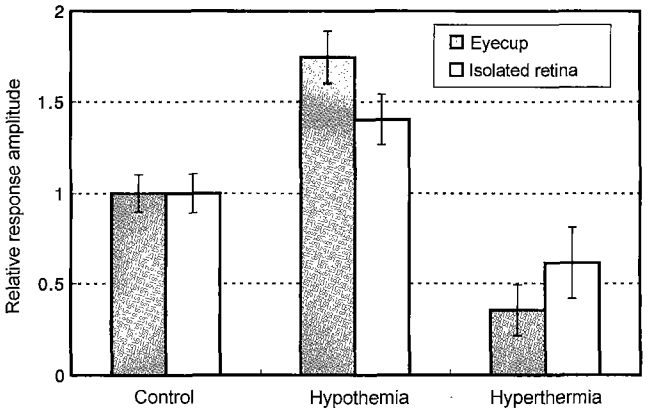

Figure 3. Comparison of temperature effect on the b-wave for the eyecup and the isolated retina preparations. Each value shows the mean of eight experiments with standard devation.

hyper, after hyperthermic process) while the ordinate represents the relative b-wave amplitude which means the ratio of the b-wave amplitude in control, without any thermal change, to each b-wave response. In the hypothermic process, b-wave amplitude of eyecup was intensified doubled. While it was reduced by half in the hyperthermic process. The increase by the hypothermia and the decrease by hyperthermia were observed respectively in both preparations but were more intense in eyecup preparation than that of isolated retina. This result indicates that temperature effect during both hypothermic and hyperthermic process is more effective in eyecup preparation with the present of the RPE than isolated retina preparation. It is suggested that the RPE may play an important role in some metabolism involved in temperature in visual process and participate in retinal function regulated by temperature.

Effects of temperature on ERG threshold

Figure 4(A) shows serial measurements of the threshold response upon hypothermic temperature change in both eyecup and isolated retina preparation. The abscissa represents a temperature change during hypothermic process while the ordinate represents threshold flash intensity of the stimulus light in log units. With temperature lowering, threshold has a tendency to decrease and shows the minimum value at 0. The decreases values exhibited a recovery with warming again and the maximum threshold recovery appeared at 15. In the case of eyecup preparation, the threshold was increased rather recovered after hypothermic process. This result suggests that hypothermic process make visual response more sensitive.

Figure 4(B) shows serial threshold changes due to hyperthermic temperature alteration, which is increased toward 40°C and then decreased to 25°C again. In both preparations threshold decrement were profoundly depend on temperature increment toward 40°C and not recovered after temperature restoration to 25°C. The temperature effect on threshold did not show a significant difference between eyecup and isolated

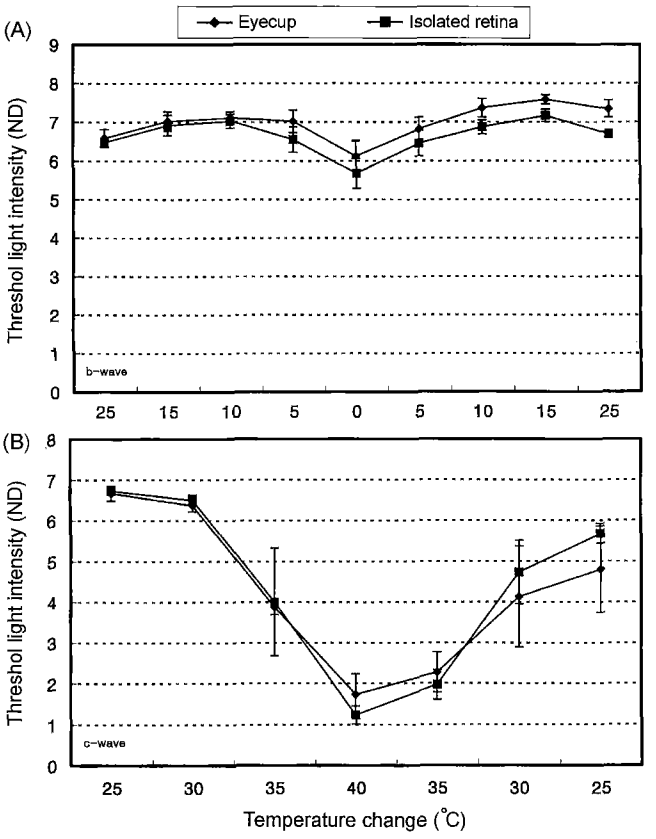

Figure 4. (A) The hypothermia effect on the ERG threshold in the eyecup (●) and the isolated retina (■). (B) The hyperthermia effect on the ERG threshold in the eyecup and the isolated retina. 10 μV of b-wave amplitude selected as a threshold criterion.

retina preparation. The threshold depends largely on the temperature changes, which induce hyperthermic process, and it was over 3 log units with the temperature change. These facts imply that hyperthermic process make visual sensitivity weakened.

Ultra structural changes associated with thermal stress, hypothermic and hyperthermic effects

In order to identify structural changes during the visual adaptation, TEM was conducted in the whole retina attached to the retinal pigment epithelium(RPE) before and after light exposure at room temperature, and the results are shown in Figure 5. After light illumination for 30 min, movement of the melanin granules is prominent, whereas most of all melanin granules are concentrated in subretinal space between the photoreceptor outer segment and the RPE in dark adaptation. In addition, the RPE displayed irregular cell shapes, vacuolization of the cytoplasm as compared with before light illumination.

We observed that the modification of retinal function by thermal stress was concomitant with a structural change in both the pigment epithelium and the outer segment of photoreceptor shown in Figure 6. The hypothermic- and hyperthermic-

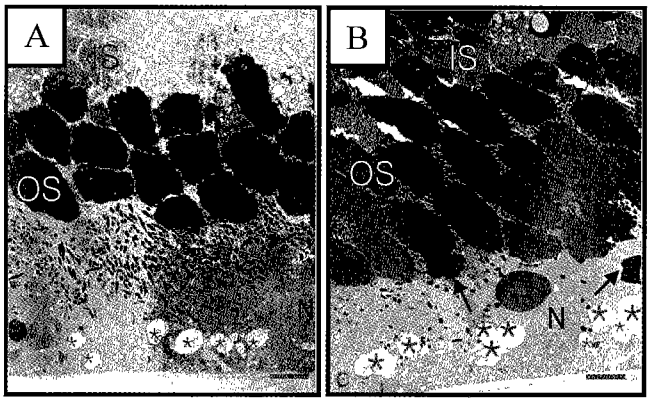

Figure 5. Transmission electron micrograph of the outer retina and the retinal pigment epithelium from bullfrog before (A) and after (B) light illumination for 30 min. It is possible to see the increase of the size and the number of vacuoles (*) and degenerating outer segments (arrows) after light exposure. Melanin granules move into the villous processes in the light and reaggregates in the base of the cell in darkness. (× 1500, scale markers = 6.6 μm) IS : rod inner segment, OS : rod outer segment, M : melanin granule, N : nucleus

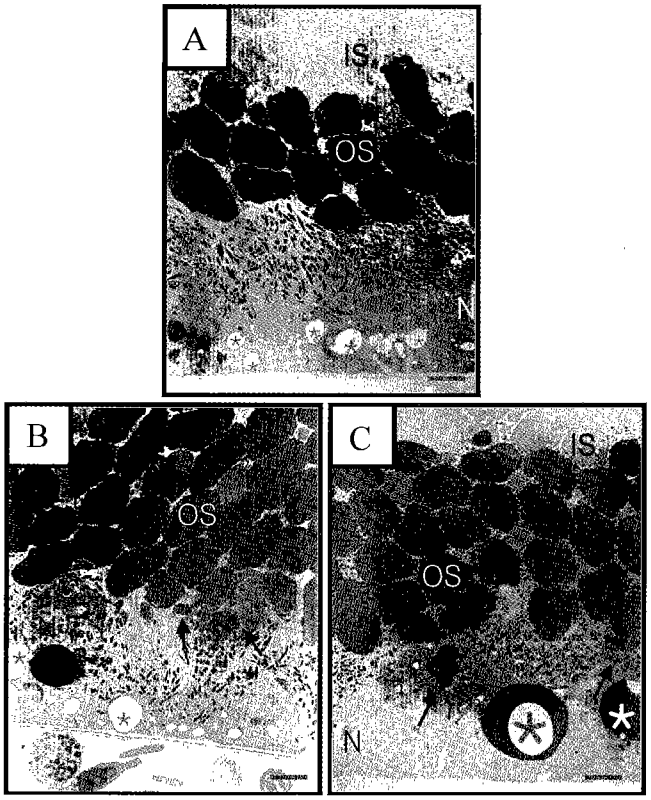

Figure 6. Transmission electron micrograph of the outer retina and the retinal pigment epithelium (RPE) in control (A), hypothermic process (B), and hyperthermic process (C). In hyperthermic process, the melanin granules move into the photoreceptor outer segment strikingly while most of them aggregate in subretinal space in control and hypothermic process. Large vacuoles (*) are present in the RPE and darken, degenerating outer segments (arrows) are shown under hypothermic process. Whereas massive disruption of the outer segment and more larger vacuoles are present under hyperthermic process. (× 1500, scale markers = 6.6 μm)

effected cells, which were restored temperature toward 25°C [Figure 6(B) and (C)], were compared to the control at 25°C without thermal change [Figure 6(A)]. The magnified views of the photoreceptor layer and the RPE showed the structural difference from the control. In the cells with hypothermic process, the RPE showed irregular and shrunken cell shapes; large vacuoles are present in the RPE and the boundary of the RPE is obscure. And degenerating outer segments are present as well. But the melanin granule is distributed between the photoreceptor outer segment and the RPE like as control. In hyperthermic process, massive disruption of the outer segment and the RPE is seen. Destruction of the RPE is characterized by numerous osmiophilic lipid droplets and disruption of the cytoplasm directly beneath the choriocapillaris. In addition, there is severe degeneration of the outer segment. Moreover, many melanin granules move into the outer segment similar to light exposure (Figure 5).

From the above, temperature change both hypothermia and hyperthermia beyond the limits of body temperature may induce morphological alteration in the outer retina and result in functional change of that. And morphological thermal stress by hyperthermia is more intense than that of hypothermic process.

So far the effect on visual function of temperature has been studied in numerous species. Especially, the researches on the temperature effect in clinical area have done with focused on reducing the retinal damage [5-7,13]. The electrophysiological experiments, related with temperature, demonstrated that with progressive retinal cooling the a- and b-waves of the ERG are gradually reduced relative to control and their peak times delayed. Whereas it was reported that light damage in visual cell is inhibited by cold temperature although the ERG wave showed the abnormalities at cold temperature. In this study, we found that hypothermia and hyperthermia, worked on the retina as thermal stress, caused the modification of retinal function after the bullfrog retina returned to a normal temperature (room temperature). In particular, both threshold and b-wave amplitude decrease with temperature lowering toward 0°C and recover with warming to 25°C during the hypothermic process. Moreover, it exhibits increment rather than recovery after hypothermic process with maximum response at late 15°C. Such a remarkable amplification of the b-wave after recovery was not observed before and the reason is still unclear. While in hyperthermia decrement of them is profound depending upon temperature increase toward 40°C and not recovered after temperature restoration to 25°C. These facts imply that hypothermic process improve visual sensitivity whereas hyperthermic process make visual sensitivity weakened by thermal stress. And next, there is some difference of temperature effect on visual sensitivity depending on sample preparation, namely temperature effect during both hypothermic

and hyperthermic process are more effective in eyecup preparation with the present of the RPE than isolated retina preparation. It is suggested that the RPE can participate in retinal function regulated by temperature.

There are some morphological alterations induced by continued light exposure for 30 min in the outer retina and the RPE, such as vacuolization of the cytoplasm of the RPE, degeneration of the outer segments, and movement of the melanin granules toward the photoreceptor outer segment. It is also suggested that there is light induced damage change in the rod outer segment and RPE morphologically.

Morphological alteration is shown both hypothermic and hyperthermic process, such as an appearance of large vacuoles, degenerating outer segments and migration of melanin granules, similar with previously reported thermal damaged charactrerictics [14]. This phenomena is more intense in hyperthermia. Unfortunately, most of melanin granule functions are not yet understood. It is reported that in homeotherms, liquid components in the vitreous humor and the cytoplasm of ocular tissues are crystallized under excessive hypothermia and finally induce severe histological damages [15,16]. Judging from the results, low temperature could increase the retinal adhesion in several ways as follows: (i) low temperature may increase the viscosity of the interphotorecepetor matrix (IPM), which has viscous properties and contributes to adhesion between the retina and the RPE, (ii) low temperature will inhibit general metabolism processes such as the RPE transport, and (iii) low temperature might reversibly affect certain properties of the cellular membrane that have been postulated to influence adhesion. This can be an important key to explain why the visual sensitivity is strikingly promoted after hypothermic process in electrophysiological study, that is, hypothermia may make retinal adhesion tight resulted in improvement of the retinol transport via IPM between the retina and the RPE for rhodopsin regeneration. With regard to hyperthermia, higher temperature may induce the structural alteration in the retina and the RPE and change the transport mechanism between them for rhodopsin regeneration resulting in reduction of threshold and the amplitude of ERG parameter. Additionally, the temperature effects in the retina could alter the protein translation machinery, which may affects on the membrane structure. Heat stress by elevation of temperature induces the hsps (heat shock protein) in the cultured retina and hsp70 mRNA species in *in vivo* retina [17-19]. It is expected that the production of such a stress protein contribute to changing membrane structure, but there is no further evidence.

Finally, adaptation to environmental stresses, such as temperature change, is essential for the survival of all living organisms. We plan further experiments to investigate the morphological, biochemical and electrophysiological effects of temperature changes in the retinal cell of the homeotherm to clarifying the temperature adaptive mechanisms of vertebrate eye.

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