Journal of Photoscience (2000), Vol. 7(3), pp. 115 - 121

Synergism Between Zinc and Taurine in the Visual Sensitivity of the Bullfrog's Eye

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Although there are high concentrations of zinc and taurine in ocular tissue, their exact role and correlation in the visual process are not clear. The purpose of present study was to clarify this point using electroretinogram (ERG) recording and spectrophotometer measurements before and after zinc and taurine treatment in bullfrog's eye. The optimal zinc concentration used in this study was 10^{-2} M $ZnCl_2$ 120 μl/12 ml ringer solution while the optimal taurine concentration was 10^{-2} M taurine 12 μl/12 ml ringer solution. For the effects of zinc and taurine on the retinal function, the changes of ERG parameters (especially threshold and b-wave) and absorption spectra were observed before and after treatment. It is noteworthy that high concentrations of zinc and taurine present in the retinal pigment epithelium and the retina. Our results indicate that dark-adapted ERG threshold became elevated and the peak amplitude of b-wave was increased with zinc and taurine treatment. Furthermore there are some synergism effects between zinc and taurine as a result of co-treatment. In spectral scan, absorbance increment due to zinc and taurine treatment was shown over the whole range of spectral range (300-750 nm) with some differences in absorbance increment depending on the case of treatment. As the results of above we believe that zinc and taurine, which are abundant in the retinal pigment epithelium and the retina particularly, may be essential factors for visual process, have some synergism with each other and be required to improve the visual sensitivity during visual adaptation.

key words: bullfrog, electroretinogram (ERG), retina, retinal pigment epithelium (RPE), taurine, zinc

INTRODUCTION

Until now diverse observations on human zinc deficiency syndrome have been observed, such as dermatitis, depressed immunity, stunting, hypogonadism and abnormal dark adaptation and others [1-6]. Zinc has been reported to occur in more than 300 vital metalloenzymes and proteins and play a key role in genetic expression, cell division and growth. Some metalloenzymes (catalytically active metalloproteins containing stoichiometric amounts of zinc firmly bound to active sites) such as alkaline phosphatase, carbonic anhydrase and alcohol dehydrogenase [7], are present in mammalian eye tissue, especially in the retinal pigment epithelium, ciliary body, lens and Muller fibers. Furthermore zinc normally participates in vitamin A metabolism in several ways [8], including the biosynthesis of retinol-binding protein, and the enzymatic conversion of retinol to retinal [9]. Although its exact role in the visual process is not clear and its functional significance remains an enigma, the high level of zinc in ocular tissues may be related to the activity of zinc-dependent enzyme. According to the results of zinc deficiency studies in animal experimentation, zinc may be required for visual sensitivity.

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Received 15 August 2000; accepted 15 September 2000

Taurine (2-aminoethanesulfonic acid) is a β-amino sulfonic acid that is present in high concentrations in all animal tissues but it is in mammalian systems that the study of taurine generates perhaps its greatest interest due to its wide spectrum of biological activities in various tissues [10-11]. The many possible functions of tarine include the following gamut: (a) neurotransmitter (or neuromodulator) in the central nervous system (CNS); (b) stabilizer of biological membranes in many tissues which affects cardiovascular functions and protects lymphoblastoid cells and spermatozoa against loss of activity; (c) protector of rod outer segments (ROSs) from exposure to toxic levels of light and chemicals; (d) modulator of calcium binding and fluxes; and (e) inhibitor of protein phosphorylation. The retina contains an extremely high amount of taurine and in some animal species the taurine levels in the retina are the highest of any tissues [12]. Greater than half the retinal store of the free amino acid taurine is located in the photoreceptor cell layer [13]. The presence of physiological levels of taurine in the retina seems essential for the normal function of retinal cells as well. Retinal taurine depletion results in a marked decrease in the amplitude of the a- and b-waves of the electroretinogram (ERG), which are eventually abolished [14-15]. Severe damage of photoreceptor structure is also observed, characterized by disruption and twisting of the otherwise highly ordered structure of the discs membranes [16]. Vesiculation and swelling of both outer and inner segments of photoreceptors also characterizes the pathology of

taurine deficiency. At the advanced stages, the photoreceptor layer is shortened and cell loss occurs [17-18]. These finding demonstrate an important role of retinal taurine in maintaining photoreceptor cell structure and function.

An association of zinc and taurine has been suggested by observations showing that zinc deficiency results in taurine mobilization and loss through elevated concentrations in blood and urine [19]. Conversely, in taurine deficient animals, a loss of zinc in ocular tissues has been detected [20]. Therefore, it may be speculated that zinc and taurine are related to each other. Although some studies have reported, still the relationship between zinc and taurine is not clear [14, 21]. In order to clarify this point, in the present work we investigated whether or not there is a synergism between zinc and taurine, which may be required to improve visual sensitivity during visual adaptation, by ERG recording and spectrophotometer measurements.

MATERIALS AND METHODS

Sample preparation

The experiments were conducted on bullfrogs (*Rana catesbeiana*), which were dark adapted for at least one hour before decapitation. For the ERG recording, the eye was enucleated and dissected. The anterior portion of the hemi-sect eyeball was removed and the posterior portion of was quickly mounted on the modified Ussing chamber, which is located in the Faraday cage. With a continuous oxygen supply, the chamber was perfused with bullfrog ringer solution through both sides of the sclera and vitreous humor which contained 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. At room temperature the ringer solution was adjusted to a pH 7.4-7.6 and the temperature of the ringer solution was maintained at 25 ℃ during experimentation. All procedures were carried out under dimmed red light paying close attention to avoid cell damage caused by environmental light exposure.

ERG recording

Although the optical system contained two light pathways of stimulus light (Is) and background light (Ib), only stimulus light was used for this study. The stimulus beam was projected directly to deliver 200 msec flashes from the light source (12 V/100 V, halogen lamp) through a beam splitter, a 505 nm interference filter, shutter and neutral density (ND) filters. When not attenuated by neutral density filters, the stimulus light delivered was 1.95×10^{14} photons/cm^2/sec. The ERG was recorded using a fine glass micropipette agar bridge electrode. The Ag-AgCl electrode 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 recorded as the potential difference between Ag-AgCl electrodes, was amplified with a D.C. pre-amplifier (CyberAmp380) and recorded on computer through an AD/DA converter and digital data recorder.

Zinc concentration measurement

To determine the zinc concentrations in bullfrogs, various organs were separately wet weighed. To extract vitreous humor, the dark-adapted eye was dropped into liquid nitrogen quickly. The frozen eyeball was then peeled off with a razor blade to collect the vitreous humor, and the volume of the isolated vitreous humor measured. The following procedures were the same as those used with other tissues. After digestion in predetermined amounts of hot nitric acid, digests were appropriately diluted with distilled and deionized water (DDW) and analyzed by flame atomic absorption spectrophotometer (Thermo Jarrell Ash, AA-Scan1).

Taurine concentration measurement

Amino acid analyses to measurement of taurine concentration in bullfrogs followed a conventional procedure. Retinas and retinal pigment epitheliums were removed from enucleated eyes within 2 to 4 minutes, microdissected, snap-frozen in liquid nitrogen and lyophilized overnight. The freeze-dried samples were weighed, homogenized in DDW and deproteinized by the addition of sulfosalicylic acid. Other organs were prepared as the same manners. And then the clear supernatant was analyzed for amino acids on a Biochrom 20 (Pharmacia) amino acid analyzer with the use of lithium citrate buffer.

Absorption spectra scanning

To investigate how zinc and taurine affect rhodopsin regeneration absorption spectra were obtained. The eyecup sample was incubated for 1hr in normal ringer solution, zinc containing ringer solution (10^{-2} M $ZnCl_2$ 120 μl/12 ml ringer solution), taurine containing ringer solution (10-2 M taurine 12 μl/12 ml ringer solution) or zinc & taurine containing ringer solution (10^{-2} M $ZnCl_2$ 120 μl + 10^{-2} M taurine 12 μl/12 ml ringer solution). During incubation, the temperature of the incubation medium was maintained at 25 ℃ using a water bath. And pure oxygen was applied continuously to avoid cell damage from anoxia. After incubation each retina was isolated from the dissected eyecup, mounted on the sample support and set that in the photo cuvette of a spectrophotometer (Shimazu UV 160A). All spectral scans were performed in the range 300-750 nm.

RESULTS

It is remarkable that the concentrations of zinc in the retina and the retinal pigment epithelium are higher than any other organ in the bullfrog (Table 1), which suggests that the high level of zinc in these tissues may be related to the activation of zinc-dependent enzyme and play an important role in visual process.

Table 1. Zinc concentrations (in parts per million; μg/g, wet weight) in various bullfrog tissues.

Tissue	In light adaptation	In dark adaptation
Liver	20.6 ± 4.6	
Lung	11.7 ± 2.4	
Muscle	4.2 ± 0.6	
Heart	16.4 ± 1.4	
Eye	12.3 ± 0.4	13.7 ± 0.04
Vitreous humor	0.9 ± 0.02	0.9 ± 0.001
Lens	4.6 ± 0.02	4.7 ± 0.03
Retina	104.22 ± 0.28	84.1 ± 0.23
Pigment epithelium	441.73 ± 3.61	373.4 ± 6.24

The results are expressed as the mean ± standard deviation of 3-9 animals.

Table 2 shows the concentrations of taurine in various tissues of bullfrog and extremely high levels of taurine in retina, with a different aspect as compared with the distribution of zinc that is the most in the retinal pigment epithelium especially.

Table 2. Taurine concentrations (in nanomoles per milligram, dry weight) in various bullfrog tissues.

Tissue	Frog 1	Frog 2	Frog 3	Average*
Liver	-	8.12	4.60	6.36 ± 2.49
Muscle	0.68	0.65	0.78	0.70 ± 0.07
Retina	38.13	45.56	26.82	36.84 ± 9.44
Pigment epithelium	5.33	16.00	7.74	9.69 ± 5.60

*The results are expressed as the mean ± standard deviation of 3 animals.

To investigate the effects of zinc and taurine on the visual sensitivity, it was necessary to decide upon optimal concentrations of them, which are added to the normal ringer solution for further experimentation. We recorded the ERG b-wave during dark adaptation while perfusing ringer solution with different zinc or taurine concentrations into the vitreous humor side of sample. As zinc and taurine concentration increased, the relative peak amplitude of the b-wave increased (Fig. 1). The abscissa represents series of zinc or taurine concentration in the ringer solution after treatment while the ordinate represents the relative peak amplitude of the b-wave expressed as voltage. The optimal zinc concentration for the highest response was 10^{-2} M $ZnCl_2$ 120 μl/12 ml ringer solution with the result that the total zinc concentration in ringer solution became 10^{-4} M (Fig. 1-A). In the case of taurine treatment, the highest response appeared at 10^{-4} M, which was resulted from 10^{-1} M taurine 12 μl/12 ml ringer solution (Fig. 1-B). Although the highest b-wave response was obtained at this concentration mostly, in some case the response decrement was appeared at the same concentration. Therefore we determined 10^{-2} M taurine 12 μl/12 ml ringer solution as optimal taurine concentration with the result that the total taurine concentration in ringer solution became 10^{-5} M, which was shown the second-best b-wave response without the b-wave response decease at any case.

To compare specific zinc effect on ERG parameters with those of other divalent metal ions, we treated normal ringer solution with various divalent metal ions, which have similar ionized atomic radii and the same concentration as zinc.

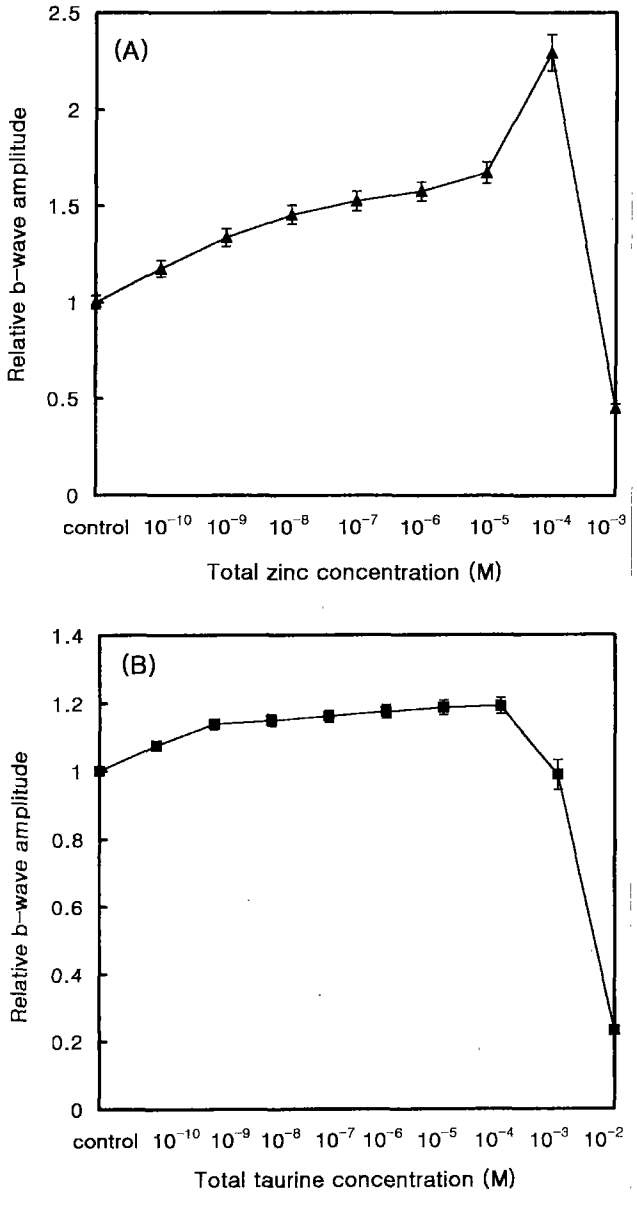

Figure 1. Optimal concentration curves of zinc and taurine. The numeric difference of the abscissa denotes total concentrations in ringer solution after treatment. The ordinate represents the relative peak amplitude of the b-wave, which standardizes that value of control used normal ringer solution. Each response was obtained under ND2 stimulus light (Is) intensity. (A) Optimal zinc concentration curve. The optimal zinc concentration for the highest response is 10^{-4} M. (B) Optimal taurine concentraton curve. The optimal taurine concentration is 10^{-5} M.

Figure 2 shows b-wave response for several divalent metal ions treatment, the abscissa represents kinds of divalent metal ions treated in ringer solution while the ordinate represents the relative peak amplitude of the b-wave expressed as voltage. There is prominent b-wave increment in case of zinc treatment and it suggests that in spite of similar ionized atomic radius zinc have more specific effects on visual sensitivity than any other divalent metal ions.

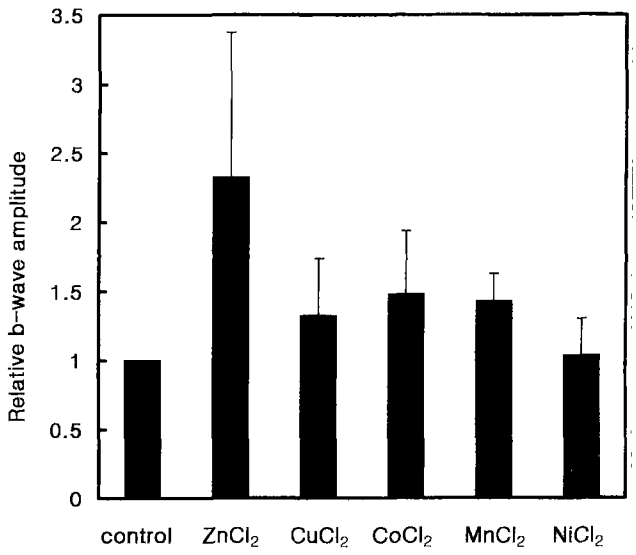

Figure 2. Comparison of various divalent metal ions' effect on the ERG b-wave increment. The abscissa represents kinds of divalent metal ions treated. The ordinate is the relative peak amplitude of the b-wave. Zinc is the most effective of all the divalent metal ions in the increment of the b-wave peak amplitude.

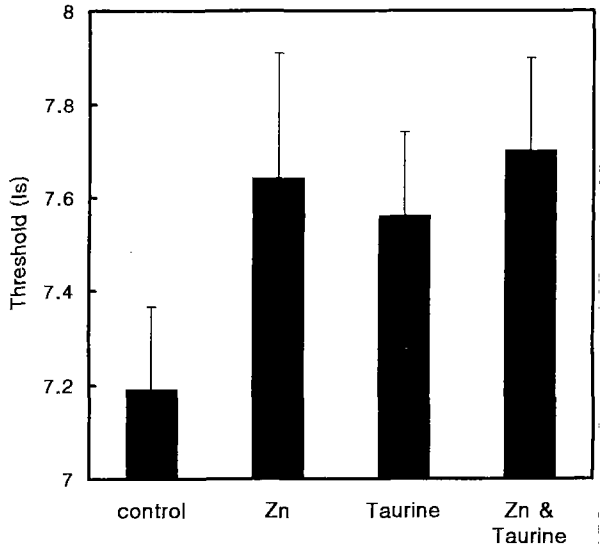

Figure 3. ERG threshold increment by zinc, taurine and zinc & taurine treatment. The abscissa represents kinds of treatments. The ordinate denotes log unit of the stimulus light (Is) intensity as threshold. Here is 10 μV of b-wave selected as a threshold criterion.

Usually threshold increment means the increase of visual sensitivity during dark adaptation. Figure 3 appears the threshold (10 μV of b-wave selected as a threshold criterion) response elevation by zinc, taurine or zinc & taurine treatment. The abscissa represents kinds of treatments and the ordinate represents the threshold flash intensity of the stimulus light in log units. All the cases of treatment show threshold elevation and there is remarkable threshold elevation with zinc & taurine treatment compared with control used normal ringer solution. This result indicates that there is some synergism between zinc and taurine in threshold increment.

Figure 4 shows comparison of b-wave response curve with kinds of treatments. The numeric difference of the abscissa represents a series of stimulus light intensity in 1 log unit while the ordinate represents the relative peak amplitude of b-wave expressed as voltage by V/Vmax which means the ratio of the maximum b-wave amplitude to the b-wave response at different conditions. Ordinarily as the stimulus light intensity increased, the b-wave amplitude increased. In the case of this result, the b-wave amplitude increase by zinc & taurine treatment is the most of all of the treatments especially.

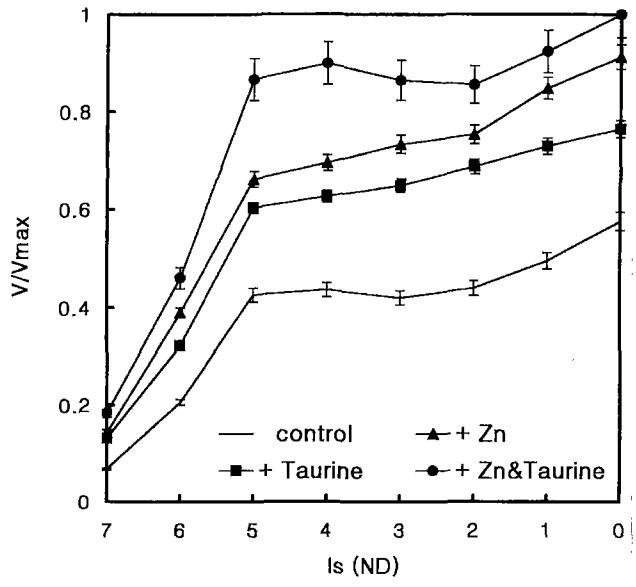

Figure 4. V-log Is curve for the relative peak amplitude of the ERG b-wave. The numeric difference of the abscissa denotes 1 log unit of stimulus light (Is) intensity attenuation. The ordinate is V/Vmax which means the ratio of the maximum b-wave amplitude after treatment to the b-wave response at different conditions.

The data illustrated in Fig. 5 represents increments in relative absorbance after treatment in spectral scan in the range of 300-750 nm. The main objective of the spectrophotometer measurement is to compare the absorbance difference caused by zinc, taurine or zinc & taurine treatment. All cases of treatment, absorbance across the spectral range is higher than that of each control. In case of zinc treatment, there is

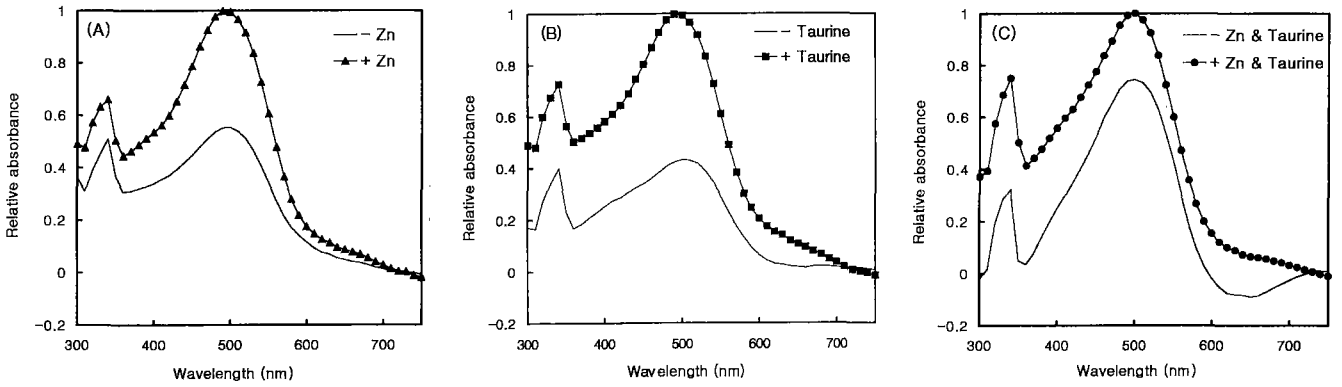

Figure 5. Absorption spectra of the bullfrog retina before and after treatment. The abscissa represents the relative absorbance which is the ratio of the maximum absorbance of the β-peak after treatment to the absorbance at different condition. The ordinate is the spectrum scanning range. (A) Absorbance difference before and after zinc treatment. (B) Absorbance difference before and after taurine treatment. (C) Absorbance difference before and after zinc & taurine treatment. Results are the mean value and the standard deviations are omitted for clarity. Typically, an isolated retina is mounted in the recording chamber of a spectrophotometer and spectral scans are taken across the range 300-750 nm at room temperature. With some differences in the absorbance increment depending on the case of treatment, there is a constant absorbance increment by treatment through the whole scanning ranges.

a maximum absorbance difference of rhodopsin about 490 nm (α-peak) while zinc & taurine treatment shows that about 340 nm (β-peak) strikingly. On the whole, absorbance difference resulted from taurine treatment is higher than any other treatment through all spectrum scanning range. This absorbance increment caused by zinc, taurine or zinc & taurine treatment suggests that these may increase the activity of alcohol dehydrogenase. Therefore, activated alcohol dehydrogenase could lead to rhodopsin regeneration and result in an increase of absorbance at 490 nm.

DISCUSSION

There are some experimental limitations to show zinc and taurine effects, for the bullfrogs used in this study were not zinc and taurine deficient as a result of dietary deficiency. However, we chose them as experimental animals because the size of the bullfrog eyeball is convenient for this type of extra cellular study. As abnormal ERG waves are reported under anoxic conditions [22], oxygen gas was supplied continuously at appropriate levels to the sample in order to avoid cell damage during the experiments.

To test the specificity of zinc, we examined the effects of other divalent metal ions with Zn^{++}, which have similar ionized atomic radii, for example Cu^{++}, Co^{++}, Mn^{++} and Ni^{++}. And we chose the peak amplitude of the b-wave out of ERG parameters as criterion to show striking difference from each divalent metal ions. As our expectation, zinc had an outstanding effect to b-wave increment than any other divalent metal ions. And zinc effect to b-wave increment is more than two times as large as normal ringer solution, which used as control, especially.

It has been reported that there are high concentrations of zinc and taurine in ocular tissue [7, 13, 23]. We had similar results by flame atomic absorption spectrophotometer and amino acid analyzer; the concentrations of zinc in the retina and the retinal pigment epithelium are higher than any other organ and that the concentration of taurine in retina is remarkable. As for a difference between things, zinc is present plentifully in the retinal pigment epithelium (retina-choroid) while taurine is in the retina.

By a preliminary experiment we chose the optimal concentrations that show outstanding peak amplitude in ERG parameters and have not any response decrement after treatment. Each of their optimal concentrations is 10^{-2} M $ZnCl_2$ 120 μl/12 ml ringer solution and 10^{-2} M taurine 12 μl/12 ml ringer solution. The ERG is a compound potential produced by layers of the retina in response to visual stimulation. The a-, b- and c-waves appear in a typical response of the dark-adapted eye to a flash and are fundamental to any discussion of ERG [24]. There is a developing consensus to the effect that the a-wave of the ERG represents the activity of the photoreceptor, while the b-wave appears to reflect the activity of Muller cells throughout the retina in response to potassium ions. The peak amplitude of the a-wave is measured from the baseline while the peak amplitude of the b-wave is distance from the peak of the a-wave to the b-wave maximum. And we chose a cut-off for the b-wave amplitude of 10 μV and determined a threshold value for the amount of stimulus light needed to produce this response. A decrease in retinal sensitivity is equivalence to an increase in the amount of stimulus light required to achieve response. The b-wave is of clinical importance in the assessment of retinal function, is easy to compare in terms of peak amplitude magnitude and is highly affected by zinc and taurine. Compared to the ERG response in normal ringer solution, the peak amplitude of b-wave remarkably increased during the visual adaptation after zinc & taurine treatment

of optimal concentration. The threshold elevation displayed the same manner as well. Although each treatment of zinc or taurine appeared outstanding peak amplitude increment than that of control, there were the most increment in case of zinc & taurine treatment. These results support that zinc and taurine play important roles for rhodopsin synthesis during visual adaptation and there is some synergism between zinc and taurine in visual process.

The absorption spectrum of rhodopsin is a reflection of the retinal's stereochemistry and its covalent linkage to opsin. The three characteristic peaks of rhodopsin's absorption spectrum are usually designated by the initial letters of the Greek alphabet, as the α-, β- and γ-peaks [25]. Of these, only the γ-peak, which is beyond 300 nm, is due to protein absorption. The electronic transition moments represent absorption in the α- and β-peaks. The α-peak results from a transition moment associated with electronic oscillations along the entire length of the conjugated bonds, while the β-peak represents transition moments which involve only partial oscillations along the bent polyene chain. Generally it is reported that bleaching by light exposure brings about a reduction of absorption [26]. To minimize variation, we tried as much as possible to use the isolated retina from the same eyeball of the same animal as control. A dramatic absorbance increase occurred after zinc and taurine treatment, throughout the wavelength range used in the present study. There were some differences in absorbance increment depending on the case of treatment. Zinc treatment showed maximal absorption difference at 490 nm, the α-peak, while zinc & taurine treatment shows maximal absorption difference at 340 nm, the β-peak. Taurine treatment obtained the most absorbance increment of all cases of treatments throughout the whole wavelength range. From these results it is suggested that zinc and taurine protect rhodopsin against bleaching by light exposure.

As described above, the present results suggest that zinc and taurine should be necessary for retinal function by improving the visual response and protecting visual pigments against light adaptation and that zinc and taurine are related to each other and there are some synergism between zinc and taurine at visual process.

Acknowledgement—We thank Kiju Hwang of KBSI (Korea Basic Science Institute)/Taegu branch for his help in amino acid analysis of bullfrog tissues.

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