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LIGHT DEPENDENT CHANNELS AND EXCHANGER IN THE INTERNAL LIMITING MEMBRANE OF VERTEBRATE EYE

HYUK JUNG* AND YOU YOUNG KIM*
Department of Biochemistry, Kyungpook National University, Taegu, 702-701, Korea.

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Abstract-Calcium has a variety of functions in neuron and muscle cells and blood clotting, especially in the visual system where dark adapted rods cotransport with Na^+ into the cell. An influx of Ca^{++} flows out of the cell through the Na^+-Ca^{++} exchanger. By using a modified Ussing chamber in order to bring *in vivo* environment close, we have known that Ca^{++} blocks the activity of guanylate cyclase, in consequence, having an effect on the amplitude of electroretinogram (ERG). We have measured the Ca^{++}, K^+, and Na^+ concentration in dark and light adapted bullfrog's (Rana catesbeiana) vitreous humor. The calcium concentration of the light adapted bullfrog's vitreous humor was higher than that of the dark adapted bullfrog' s vitreous humor. This means that ion activity between the photoreceptor and vitreous humor side is light dependent and we have found that a Ca^{++} channel and Na^+-Ca^{++} exchanger exist in the vitreous humor side. Taken together permeability of Na^+, Ca^{++} and K^+ ion internal limiting membrane faced in the vitreous humor side has light-dependent activity during the illumination.

INTRODUCTION

There has been considerable speculation that decreased cytoplasmic Ca^{++} concentration of the rod outer segment in the vertebrate retina may play a role in visual adaptation.[1-6] Photoexcited rhodopsin triggers an enzymatic cascade that leads to the activation of a cGMP phosphodiesterase and rapid hydrolysis of cGMP.[4-10] Recovery of the dark state requires the resynthesis of cGMP, which is catalysed by guanylate cyclase.[2,4,11-15] The lowering of the cytosolic Ca^{++} concentration following illumination is thought to be important in stimulating cyclase activities, which increases several fold of cGMP concentration. It is evident that cGMP and Ca^{++} levels are reciprocally controlled by negative feedback.[9,10,16-22] These Ca^{++} balances are controlled by the cGMP-activated cation-specific channel and Na^+-Ca^{++} exchanger.[10,13,14,23] It is generally accepted in the present time that the visual cascade only occurs in the parts of the photoreceptor; and Ca^{++}, which is present in the photoreceptor side, plays a key role in the process of visual adaptation.[24,25] However, the above mentioned Ca^{++} transport experiments were done with ERG recording patch clamp, intracellular recording, in which the photoreceptor and scelera side of the retina were exposed to the ringer solution.[6,26,27] Therefore, those experiments were only limited to the study of the mechanism of Ca^{++} transport in the photoreceptor side. The purpose of our experiment was to study the ERG of the a-, b-wave effects in the normal ringer solution (NRS) and Ca^{++} free ringer solution (Ca^{++} FRS) treated with EDTA and EGTA and to study how Ca^{++} affects the threshold, amplitude, and regeneration time in the presence and absence of divalent cation during the light response. In addition to identify what kinds of light dependent transport systems exist between the vitreous humor side and photoreceptor during light adaptation. And the above experimental results together, we can suggest that the light-dependent ion transport system, which affect ionic movements might be exist in internal limiting membrane. Therefore we have concluded that light induced the blocking of Na^+ and Ca^{++} influx into retina and efflux of K^+ to vitreous humor. These experiments were performed using a modified Ussing chamber in order to bring *in vivo* environment close.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Dissection A bullfrog was dark-adapted for at least 2 hours before decapitation. Dissection was performed in dim red light. The eyes were enucleated, the anterior portion cut away, and the posterior eyecup portion was mounted onto the modified Ussing chamber containing Bullfrog ringer solution: 105mM NaCl, 2.5mM KCl, 2mM $MgCl_2$, 1mM $CaCl_2$, 5mM glucose, 5mM $NaHCO_3$, and 10mM HEPES buffered to a pH of 7.5.

Instrumentation The optical system contained two light paths with interposed 505nm interference filters, neutral density (ND) filters, and an electronic shutter. The stimulus beam was projected straight to deliver 200 msec flashes, which served as the stimulus, and the background beam to project a steady background light was reflected by a mirror into a parallel path. Two beams were combined through a beam splitter and evenly illuminated onto the preparation. The ERG was monitored with Ag-AgCl agar bridge electrodes, which were placed into the modified Ussing chamber, amplified with a DC pre-amplifier and

main amplifier, and displayed on a dual beam storage oscilloscope or recorded on video tape through AD/DA converter and digital data recorder. There was little deterioration of ERG sensitivity, amplitude, or waveform in most preparations during the 5-6 hours of the experiments. All data were analyzed with Axotape and plotted with Sigma plot.

Ca^{++} concentration measurement The dark-adapted eyes were exposed to a desired level of background light for a predetermined time and then, quickly dropped into liquid nitrogen. The frozen eye balls were peeled of the scelera with a razor blade to extract the vitreous humor, then separately, the volume was measured, and was digested for two hours at 100°C in predetermined amounts of nitric acid.

A Thermo Jarrell Ash atomic absorption spectrophotometer was used for Ca^{++} concentration measurement.

RESULTS

ERG changes in NRS treated with EDTA Figure 1 shows the relative amplitude(V/Vmax) of a- and b-wave and a-wave after being treated with EDTA during light response. The x-axis represents NRS, EDTA-treated NRS, and various time periods after treatment; the left y-axis represents the relative amplitude of a- and b-wave; and the right y-axis represents the relative amplitude of a-wave. We used ND 1 as a light source and the larger the ND intensity the lower the log unit was. Relative amplitude occurred in EDTA-treated NRS after 5 and 10 minutes; however, in all following time periods, that amplitude decreased. This decrease occurred because EDTA damaged or destroyed the retinal membrane and because of the chelation of divalent cation.

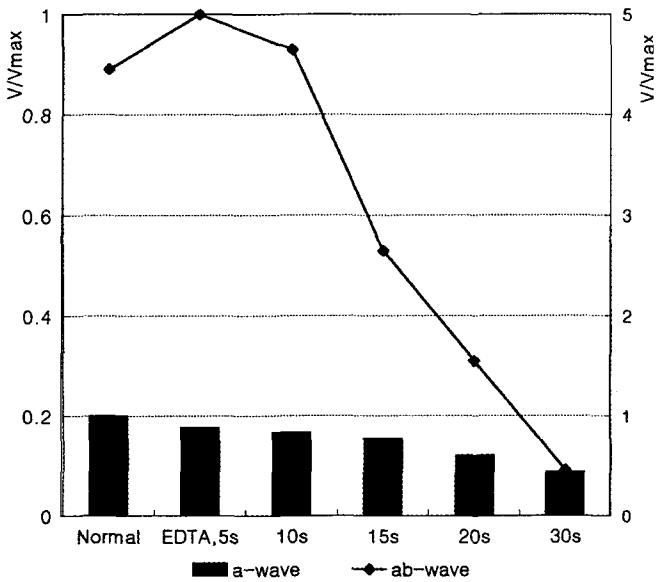

Figure 1. Relative amplitude (V/Vmax) of a- and b-wave and a-wave after adding EDTA-treated NRS(Is=ND 1).
Is : Stimulus light intensity S : Second(Time)
Left y-axis : a- and b-wave Right y-axis : a-wave

EDTA-treated Mg^{++} FRS and Ca^{++} FRS To find out the reasons behind the amplitude when NRS was treated with EDTA, we eliminated divalent cation in NRS one by one. In order to chelate Ca^{++} and other metal ions in the Mg^{++} FRS, 2mM EDTA was added and those results are plotted in Fig. 2, which shows the relative amplitude following various time periods. We observed that there was no significant change when Mg^{++} FRS replaced NRS, but when EDTA was added to this new solution, the amplitude elevation had a similar tendency of when EDTA was added to NRS. Thus, Mg^{++} had no effect of amplitude elevation after EDTA treatment. And to chelate other divalent metal ions, we used 3mM EDTA to Ca^{++} FRS, which contains the same concentration (2mM) as $MgCl_2$. Fig. 2 shows the relative amplitude after treatment of EDTA in NRS. We observed an increase in the relative amplitude when Ca^{++} FRS replaced NRS. The increase in a- and b-wave of Ca^{++} FRS decreased again because of the addition of EDTA, but the amplitude elevation due to EDTA that was shown in previous experiments did not appear. The reason for this amplitude decrease after EDTA treatment was the chelation of divalent ions and cell damage by the EDTA.

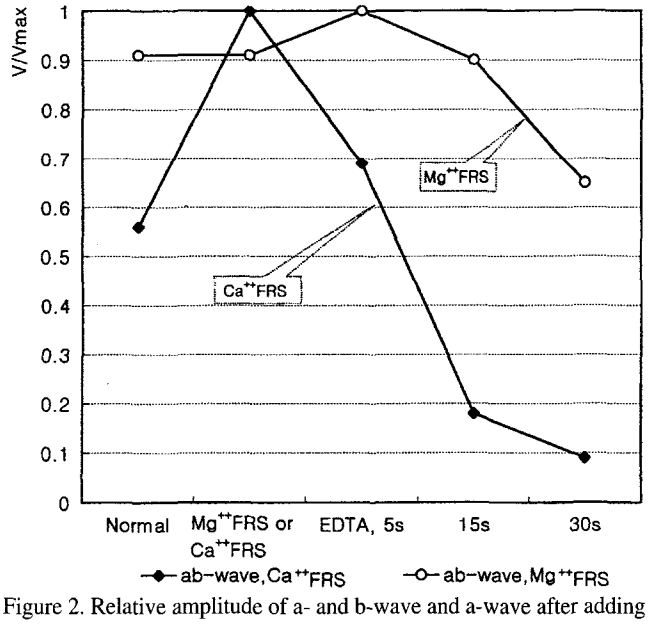

Figure 2. Relative amplitude of a- and b-wave and a-wave after adding EDTA-treated Mg^{++} FRS and Ca^{++} FRS (Is=ND 1).
s : Second (Time)

EGTA treatment in NRS In the previous experiments, we came to know that Ca^{++} affects the ERG amplitude. So, we used EGTA, which chelates only Ca^{++}, to find out the exact function of Ca^{++}. Fig. 3 shows the relative amplitude of EGTA-treated NRS. The x-axis represents NRS, EGTA-treated NRS, and various time periods after treatment. Amplitude elevation appeared because of the chelation of Ca^{++} when

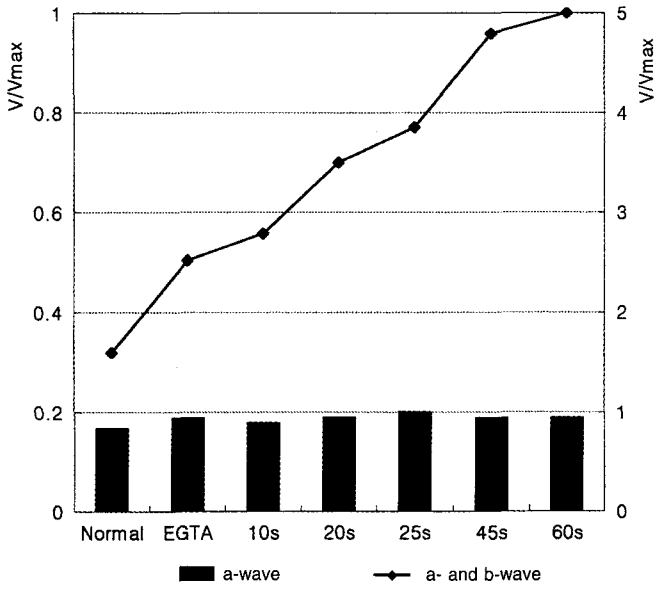

Figure 3. Relative amplitude of a- and b-wave and a-wave after adding EGTA-treated NRS.
Left y-axis : a- and b-wave Right y-axis : a-wave
s : Second (Time)

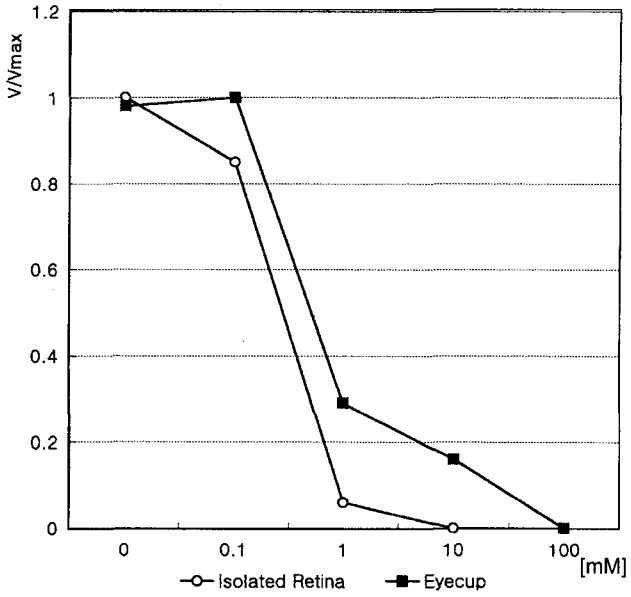

Figure 4. Relative amplitude of a-wave depending on Ca++ concentration.

EGTA was added to the NRS; also, there was no significant change in a-wave, but an increase in b-wave appeared relatively. There was no significant change in a-wave.

Comparison of ERG among vitreous humor side, scelera side, and both sides We conducted experiments to find out where Ca^{++} has the most influence among the vitreous humor, scelera, and both sides. In the case where Ca^{++} FRS was added to the vitreous humor side, that part of the retina had the most influence. However, in the case of adding Ca^{++} FRS to the scelera side and both sides, there was not a significant effect as there was in the vitreous humor side. Therefore, all following experiments involved comparison between Ca^{++} FRS and NRS in only the vitreous humor side (Data not shown).

Ca^{++} concentration that has the greatest effect to the retina : Fig. 4 shows the relative a- and b-wave amplitude change which was aroused by the exchanges of different Ca^{++} concentration in the NRS. The x-axis represents the difference of Ca^{++} ($CaCl_2$) concentration by unit of mM. The control NRS contained 1mM Ca^{++} and, as shown in Fig. 4, had a bigger difference in relative amplitude than in Ca^{++} FRS. In case of the isolated retina, the same tendency occurred. It seemed that the cell showed Ca^{++} toxicity when we added 100mM Ca^{++}.

ERG comparison between Ca^{++} FRS and NRS To experiment what effect Ca^{++} has in light adaptation between NRS and Ca^{++} FRS, first, we illuminated background and stimulus light in which each light had a different intensity. Then, we recorded ERG changes between NRS and Ca^{++} FRS. For example, Fig. 5 shows the comparison between NRS and Ca^{++} FRS at ND 3 background light. The two upper lines are NRS and Ca^{++} FRS without background light and the bottom two lines are NRS and Ca^{++} FRS with ND 3 background light. In the absence of background light, the relative amplitude wave of Ca^{++} FRS increased more than that of NRS. In the presence of background light, the same increasing tendency also appeared. However, the threshold change reduced in both cases.

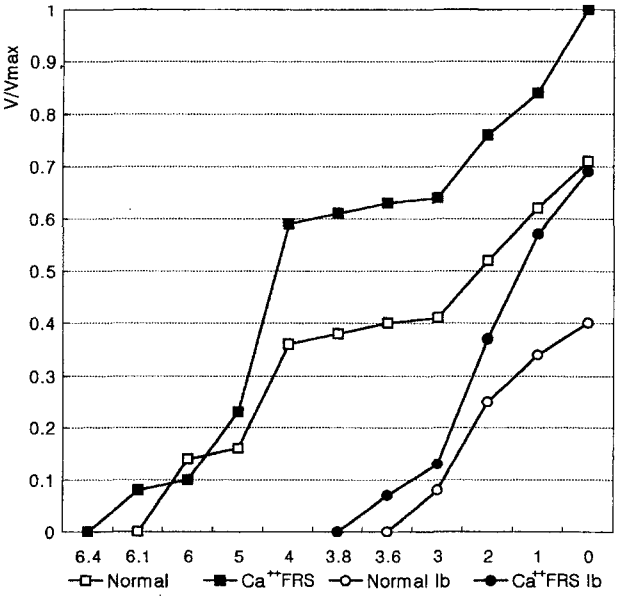

Figure 5. Relative amplitude of a- and b-wave and ND 3 background light.
Symbol on x-axis : Threshold Ib : ND 3 background light
x-axis : Light intensity

Regeneration time comparison between NRS and Ca^{++} FRS At first, after obtaining the threshold from each of NRS and Ca^{++} FRS, we exposed them to the background light and

recorded the threshold appearing time after turning off the background light as shown in Fig. 6. The stronger the background light was, the slower the regeneration time became,

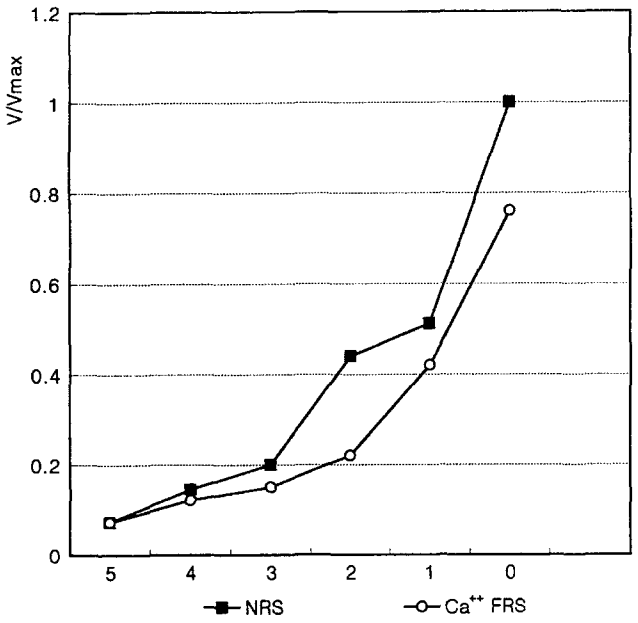

Figure 6. Regeneration time in NRS and Ca++ FRS.
x-axis : Light intensity y-axis : Threshold appoearing time

and also, Ca^{++} FRS had a shorter regeneration time than that of NRS. These results suggest that Ca^{++} has an effect on regeneration time evoked by light stimulus.

Cation concentration in vitreous humor during dark and light adaptation : Fig. 7 shows Ca^{++} , Na^{+} and K^{+} concentration differences between dark and light adaptation in the vitreous humor from a light adapted retina. Ca^{++} , Na^{+} and K^{+} concentration in the vitreous humor during light adaptation was obviously higher than during dark adaptation. These results suggest that Ca^{++} moves through a certain light dependent transport system, which might exist in the vitreous humor side during the courses of light adaptation. Based on the above results, we can accept that the Ca^{++} channel and Na^{+}-Ca^{++} exchanger is present in the side of the vitreous humor side of the retinal membrane.

Ca^{++} channel blocking We treated the Ca^{++} channel blocker (Ni^{++}, Co^{++}, Cd^{++}, Mn^{++}, Mg^{++}) as a method of preventing Ca^{++} entry to the vitreous humor side in order to confirm the existence of Ca^{++} channels. Mg^{++} FRS was used for the following experiments because the Bullfrog ringer solution contains the Ca^{++} channel blocking Mg^{++}. Comparison of the relative ERG a- and b-wave amplitude peak after Mg^{++} FRS was treated with Ca^{++} channel blocking Co^{++} (2mM $CoCl_2$) is shown in Fig. 8. The illuminated light intensity was ND 1. The x-axis represents the sorts of solution and the y-axis is the relative peak of a- and b-wave. The (+) and (-) denotes removal and addition of divalent cation in the ringer solution respectably. The a- and b-wave amplitude decreased

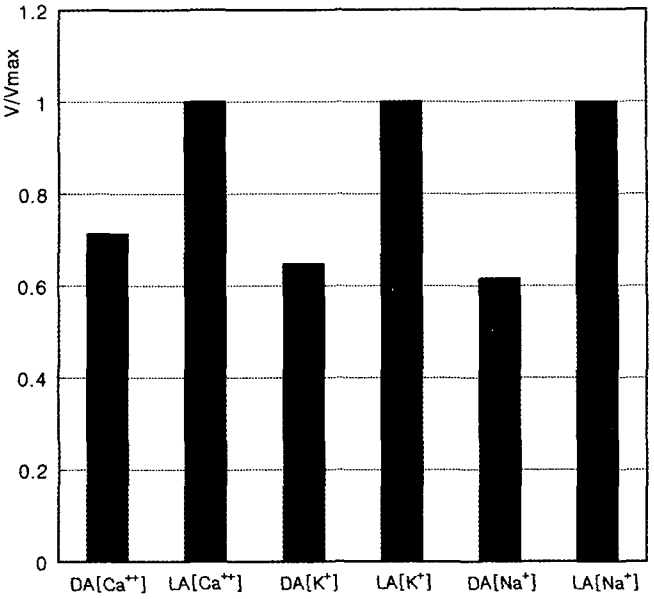

Figure 7. Ca^{++}, K^{+} and Na^{+} concentration in the vitreous humor during light and dark adaptation.
y-axis : Relative concentration of each ion
LA : light adaptation DA : dark adaptation

remarkably after Co^{++} treatment compared to the ERG response in Mg^{++} free or Mg^{++} and Ca^{++} FRS, because Ca^{++} channel was docked by Co^{++} Fig. 9 shows the examples of typical ERG wave form exposed to ND 1 stimulus light intensity after Mg^{++} free ringer solution was each treated with Cd^{++} (2mM $CdCl_2$) and Ni^{++} (2mM $NiCl_2$). There was no comparable change in the a-wave, but the b-wave was sup-

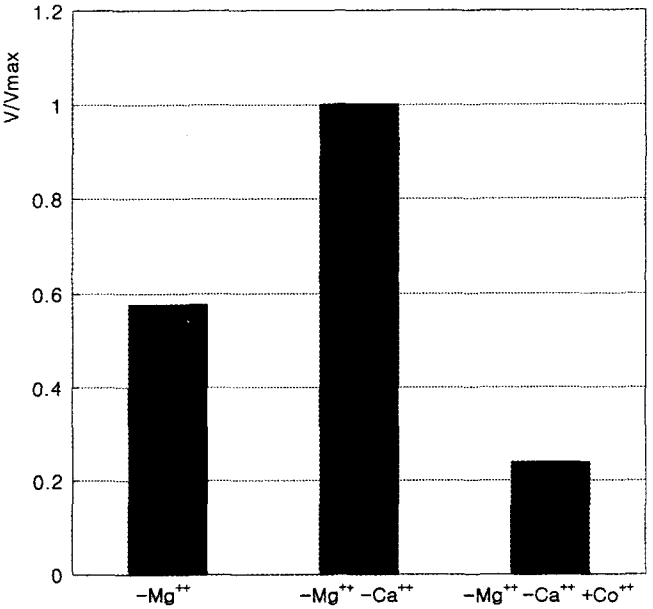

Figure 8. The relative ERG ab-wave peak after Mg^{++} FRS was treated with Ca^{++} channel blocking $CoCl_2$.
-Mg^{++}: Mg^{++} FRS. -Mg^{++}-Ca^{++}: Mg^{++} and Ca^{++} FRS
-Mg^{++}-Ca^{++}+Co^{++}: Mg^{++} and Ca^{++} FRS was treated with $CoCl_2$

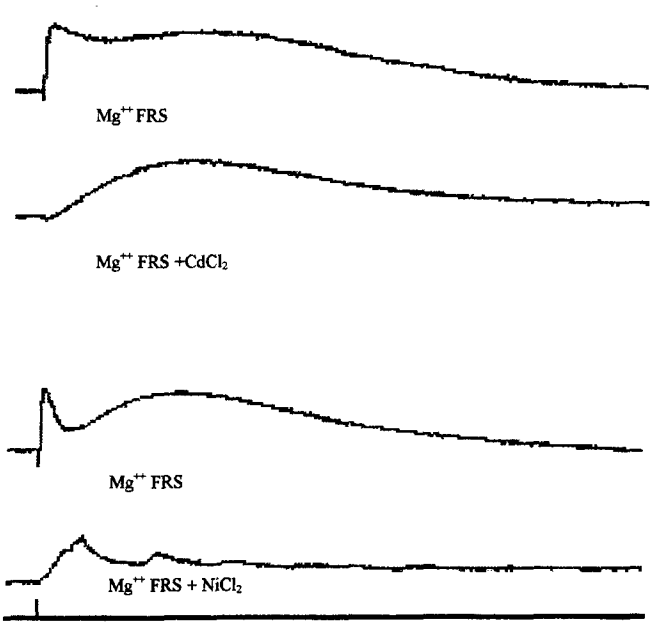

Figure 9. The ERG waveform after Mg^{++} FRS was each treated with $CdCl_2$ and $NiCl_2$.

pressed. The a-wave originated from the photoreceptor. Accordingly, these results suggest that even though the photoreceptor performed its function ie., during illumination, when the light sensitive channels are closed and inward leak of Ca^{++} is thereby suppressed, the Na^+-Ca^{++} exchanger continues to operate and the free Ca^{++} concentration falls to a lower level, the Ca^{++} channel in the vitreous membrane was blocked after blocker ($CdCl_2$, $Nicl_2$) treatment. This means that the Ca^{++} channel exists in the vitreous humor side membrane.

From previous data, K^+ and Na^+ concentration in the vitreous humor is higher during light adaptation than during dark adaptation, which implies those ions move through the Na^+-Ca^{++} exchanger. We treated Na^+-Ca^{++} exchanger blocker and activator to the vitreous humor side membrane in order to prove the existence of the exchanger. The NaCl concentration was reduced by half (52.5mM NaCl) and replaced it with choline (52.5mM), which has no effect on ERG waveform and a- and b-wave amplitude.

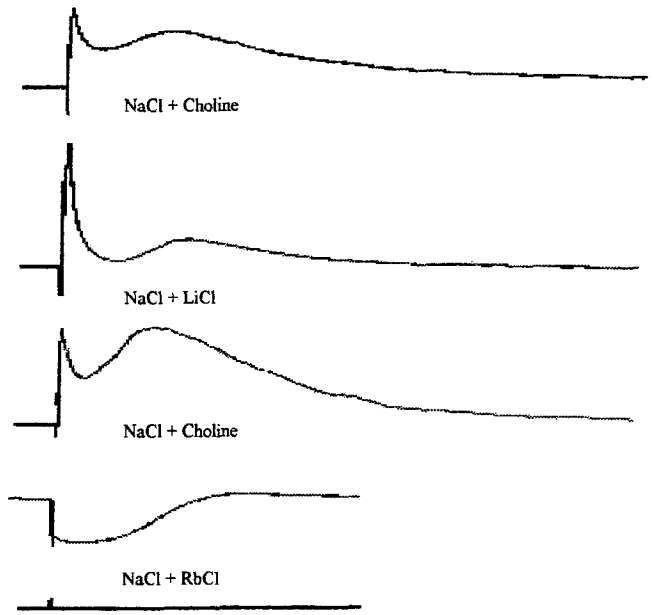

Figure 10. The ERG waveform after each LiCl and RbCl was treated in place of choline.

Fig. 10 shows the example of ERG waveform after each LiCl and RbCl was treated in replace of choline. After treating LiCl, being an activator of Na^+-Ca^{++} exchanger, the b-wave amplitude increased, but there was no comparable change of a-wave. After treating RbCl, being a blocker of Na^+-Ca^{++} exchanger, the b-wave amplitude decreased. In this case, a-wave emerged to hyperpolarize b-wave.

Blocking of Na^+-Ca^{++}exchanger The a- and b-wave amplitudes due to the replacement of the NaCl concentration by half with the monovalent cation (Li^+, Na^+, K^+, Rb^+, Cs^+) are plotted in Fig. 11. The above results are similar to those experiment to prove of the existence of Na^+-Ca^{++} exchanger in the rod outer segment. So, this means that a Na^+-Ca^{++} exchanger exists in the vitreous humor side.

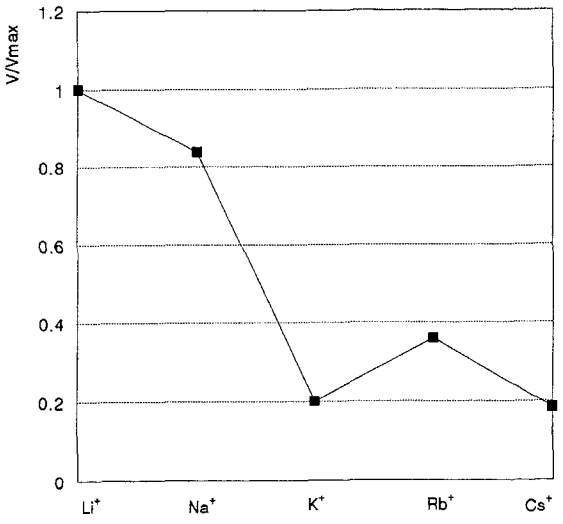

Figure 11. a- and b-wave amplitudes after monovalent cation treatment.

Treatment of Na^+ and K^+ channel blocker Treatment of Na^+ channel blockers (TTX, STX) and K^+ channel blockers (Ba^{++}, Cd^{++}, Cs^+, 4-AP and TEA) in the vitreous humor side induced the increment and decrement of ERG response. As shown fig.12, 13, 14.

DISCUSSION

The results of this study lead to the following conclusions: 1) When NRS was treated with EDTA, evidence of amplitude elevation appeared. This phenomenon was due to the

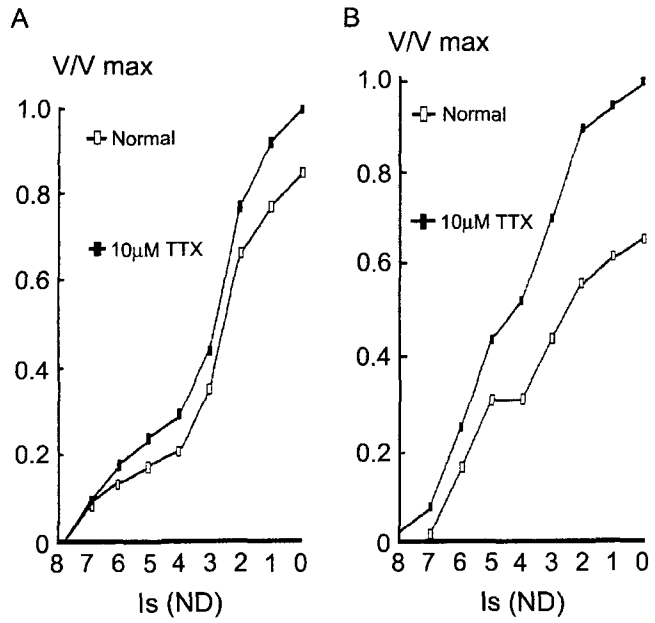

Figure 12. a- and b-wave amplitudes after 10(M TTX treatment. A : TTX treatment in the vitreous humor. B : TTX treatment in Photoreceptor.

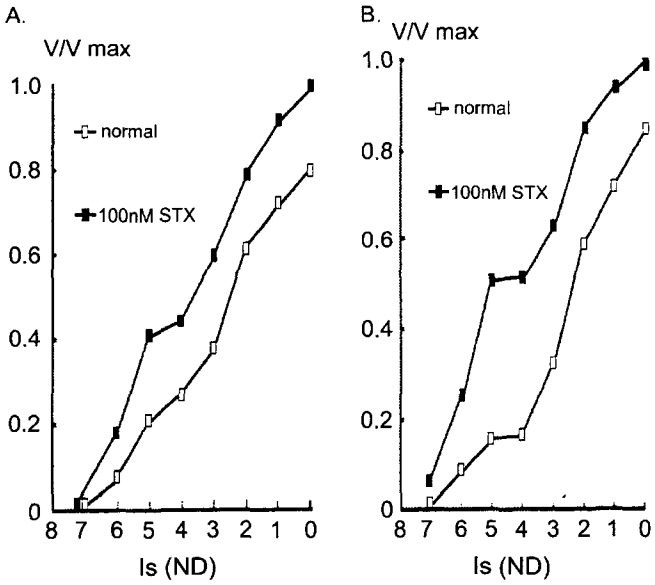

Figure 13. a- and b-wave amplitudes after 100nM STX treatment. A : STX treatment in the vitreous humor. B : STX treatment in Photoreceptor.

chelation of Ca^{++}. This result confirmed that only chelated Ca^{++} using EGTA caused an elevation in a- and b-wave amplitude. 2) The location where Ca^{++} had the highest effect on amplitude was the vitreous humor side of an eyecup. 3) After measuring the Ca^{++} concentration in the vitreous humor, during light adaptation, the concentration of Ca^{++} in the vitreous humor was higher than that in dark adaptation. So, we suggest that certain Ca^{++} transport mechanisms exist between the photoreceptor and the vitreous humor side.

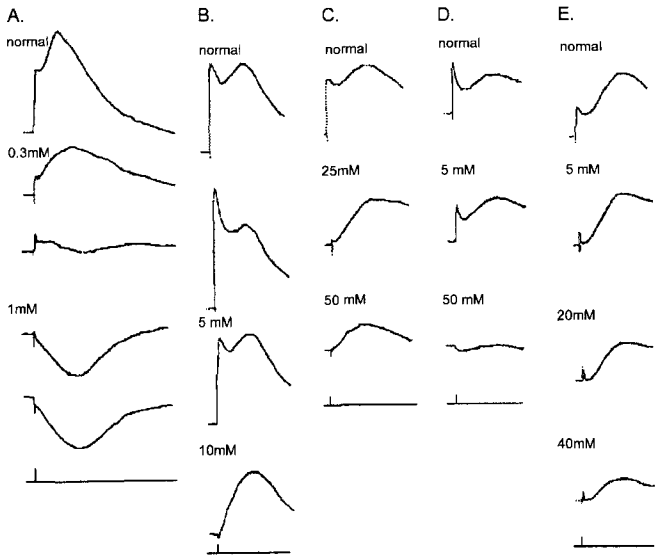

Figure 14. The ERG waveform after treatment of K^+ channel blocker. A : Ba^{++} Treatment. B : Cd^{++} Treatment. C : TEA Treatment. D : Cs^+ Treatment. E : 4-AP Treatment

4) We verified that Ca^{++} FRS has a greater effect on amplitude elevation than that of any concentration of Ca^{++} in the eyecup and the isolated retina. 5) In a variety of intensities in each the background light and stimulus light, an elevation of amplitude in ERG appeared. This elevation occurred when using Ca^{++} FRS, which was higher than when NRS was treated. Also, the threshold was reduced. 6) The regeneration time accelerated when Ca^{++} FRS was used in dark adaptation. 7) Ca^{++} concentration in the vitreous humor increased as the stimulus light intensity became higher. K^+ and Na^+ concentration in the vitreous humor is higher during light adaptation than during dark adaptation. 8) When we treated the vitreous humor with Ca^{++} channel blocker (Ni^{++}, CO^{++}, Cd^{++}, Mn^{++}, and Mg^{++}), during light adaptation, there was no comparable change in the a-wave, but the b-wave was suppressed. Even though the photoreceptor performed its function, because of the change in ionic concentration between the photoreceptor and vitreous humor, the b-wave originating from the neuron cell (bipolar cell, ganglion cell, horizontal cell, amacrine cell) and non-neuron cell (Mullar cell) was changed. This means that a Ca^{++} channel exists between the vitreous humor side and photoreceptor. 9) We reduced the NaCl concentration by half and replaced it with Li^+, Na^+, K^+, Rb^+, and Cs^+. Then, when we treated it to the vitreous humor, the b-wave was suppressed or hyperpolarized. There was no change in the a-wave, so this means that an exchanger exists in the vitreous humor side.

From these results, we have concluded that a light dependent Ca^{++} channel and Na^+-Ca^{++} exchanger exist in the vitreous humor side of vertebrate eye as shown Fig. 15.

And furthermore, these results suggest that light dependent

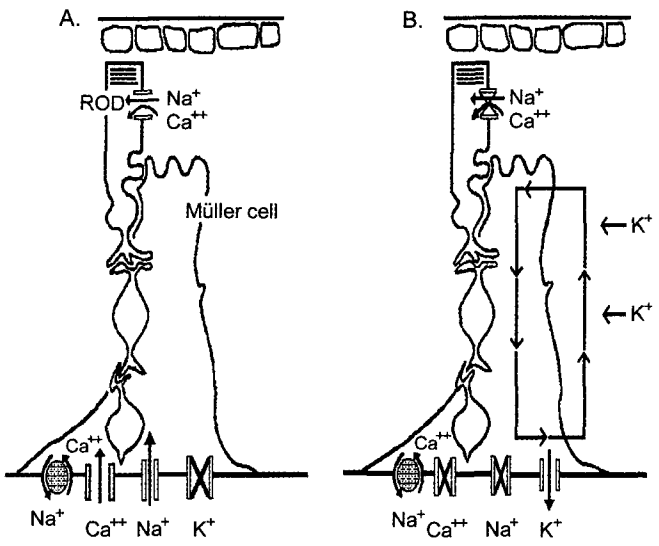

Figure 15. Change in ion transportation through the light dependent channel.
A : In dark adaptation. B : During illumination.

Na^+ and K^+ channel exist in internal limiting membrane.

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