Intracellular and Extracellular Chloride Ions in the Horizontal Cells of the Stingray Retina

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Abstract Intracellular and extracellular concentrations of chloride ($[\text{Cl}^-]_i$, $[\text{Cl}^-]_o$) ions in the horizontal cells of the stingray retina were studied by means of ion-selective microelectrodes. The electrodes used were of the double-barreled type and Corning's #477315 resin was used as the exchanger. The average $[\text{Cl}^-]_o$ and $[\text{Cl}^-]_i$ in the L-type cells were 320 and 130 mM, respectively ($n=37$), and the equilibrium potential (E_{Cl}) was calculated as -23.3 mV. In some cases, dark membrane potentials were more positive than the E_{Cl} . With white light stimulus, the membrane hyperpolarized to a more negative level than E_{Cl} . Based on the experiments described above, the hypothesis that the hyperpolarizing response of horizontal cells is due to the permeability change of the membrane to chloride ion was excluded in the stingray retina.

Key words: stingray, retina, horizontal cell, chloride ion, ion-selective electrode.

In the vertebrate retina, horizontal cells respond with hyperpolarization to white light stimulus. It has been postulated that the depolarizing transmitter substance is continuously liberated from the photoreceptor terminals in the dark and the release is suppressed by light, resulting in a hyperpolarization of the horizontal cell [1–3]. From a perfusion experiment using Na^+ -free Ringer solution, Kaneko and Shimazaki [4] and Waloga and Pak [5] suggested that the transmitter depolarizes horizontal cells by increasing Na^+ permeability (sodium hypothesis). However, Miller and Dacheux [6] assumed that the transmembrane movement of chloride ions is critical for the light responsiveness of the horizontal cell activity (chloride hypothesis).

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The employment of a liquid ion-selective microelectrode enables us to measure the activity of an ion directly [7, 8]. To minimize the error caused by damaging the cell membrane with an electrode, the ratio of the size of a cell to the tip diameter of the electrode should be as large as possible. In general, fish retinae have large horizontal cells, especially those of the elasmobranch [9–12]. The horizontal cells in the retina of ray have been studied taking advantage of their large size [13–15].

In the present study, using ion-selective microelectrodes, the intracellular concentration (ionic activities) of chloride ions in the horizontal cells of the stingray retina were measured directly to examine these hypotheses concerning horizontal cell activity.

MATERIALS AND METHODS

1. *Preparation.* Stingrays, *Dasyatis akajei*, of about 30 cm body width, were dark-adapted for at least 1 h in a tank filled with aerated natural sea water. After decapitation, the eyes were excised and an eye-cup was cut into four to five pieces using a razor blade. The pieces were kept in a refrigerator in a light-tight, moist chamber until used. A recording chamber was covered by a translucent plastic plate, which had a hole for insertion of an intracellular electrode, and moisturized oxygen was continuously supplied to avoid dehydration of the preparation. In the present experiment, perfusion of the retina was not performed because it was difficult to isolate the retina.

2. *Electrode and amplifier-recording system.* The concentration of chloride ions was measured by a double-barreled-type liquid ion-exchanger microelectrode after a modification of Walker's method [7]. A double-barreled glass pipette with a single tip diameter of less than $2\ \mu\text{m}$ was made by the conventional method. Before filling the ion exchanger (Corning, #477315) into one barrel (exchanger barrel), the tip portion of the barrel was coated with 0.3% silicone oil (Corning, #1107) in acetone and baked at 200°C for 1 h [16]. The remaining part of the exchanger barrel was then filled with 0.2 M KCl. The other barrel was filled with 0.2 M CH_3COOK and used as a reference electrode. This barrel also served for recording the membrane potential and the light responses. A chlorided silver plate beneath the preparation served as the indifferent electrode.

Each double-barreled electrode was calibrated in a set of 5 ($\text{NaCl} + \text{Na}_2\text{SO}_4$) solutions. The concentration of chloride ions in each solution was 20, 50, 100, 200, and 300 mM. The cationic concentration was adjusted to 300 mM. Figure 1 is an example of the calibration curve for the electrodes. It shows a sensitivity of 54.5 mV/decade in the range between 20 to 300 mM Cl^- . The mean sensitivity of the electrodes employed in the present study was 52.4 mV/decade, and 90% of the final response levels were reached within 2 s. The sensitivity calibration was performed before and after the experiment. If the sensitivity differed by more than 5%, the data were discarded. Since in the present study the sensitivity was calibrated for ionic concentration, no correction was made for the difference between the activity

Fig. 1. An example of a calibration curve for the double-barreled Cl⁻-selective electrodes. The potentials (V_{Cl}) are plotted against the concentrations ($[Cl^-]$). The dotted line is a linear regression line, which shows a sensitivity of 54.5 mV/decade in the range between 20 to 300 mM Cl⁻.

and the concentration. All values are given as mean $\pm$ SE.

The microelectrode was connected to a high input impedance differential electrometer (WPI, Duo 773). The outputs were fed to a storage oscilloscope (Kikusui, COS5020-ST) and then to a tape recorder. Responses stored on magnetic tape were subsequently reproduced by an *X-Y-T* recorder (Graphtech, MC3735).

3. *Photic stimulation.* A flash of diffuse white light was used to stimulate the retina. The intensity was about 0.5 lm/m² and the duration was in the range of 0.1 and 30 s. A red LED powered by a battery was also used for the source of red light.

RESULTS

The stingray retina has three sublayers of horizontal cells, each with different morphological and electrical characteristics [15]. They are L-type, SL-type, and C-type cells and are located in the distal (external), middle and proximal (internal) layer, respectively. L-type cells respond with only hyperpolarization irrespective of the light stimuli wavelength. SL-type cells respond like L-type cells but with a slower and sustained time course. C-type cells are depolarized by red light but hyperpolarized by light with a short wavelength. The size of these cells is 40–50 μ m in diameter and 5–15 μ m in thickness. On the other hand, photoreceptors and

Fig. 2. A measurement of Cl^- concentration in an L-type horizontal cell of the stingray retina. A double-barreled ion-selective electrode was inserted to record the membrane potential (upper trace) and Cl^- concentration (middle trace). White light stimuli with three different durations were applied at each mark in the lower trace. The numbers show the durations in s. After withdrawal of the electrode, a negative pulse was applied through the indifferent electrode to check the balance of the differential recordings (arrow). The sensitivity of the electrode was calibrated before and after the experiment.

bipolar cells are much smaller than horizontal cells. Thus, at the distal half of the retina, stable intracellular recordings could be obtained only from horizontal cells.

Figure 2 shows an example of recordings from L-type horizontal cells. The upper trace shows membrane potential recorded by the reference barrel. The voltage difference between the exchanger barrel and the reference barrel, that reflects the concentration of chloride ion, is shown in the middle trace (ionic trace). The lower trace shows timing of the light stimuli.

Before the insertion of the electrode into the horizontal cells, the potential recorded by the reference barrel was set to 0 mV and the extracellular Cl^- concentration ($[\text{Cl}^-]_o$) was later calculated based on the calibration data. When the electrode penetrated a horizontal cell, the dark membrane potential (E_m) was recorded from the reference barrel, and a flash of light caused a hyperpolarizing response. The time course of the response was affected by the state of adaptation of the retina. In the present study, the adaptation level corresponded to a mesopic to photopic state.

Upon penetration into a horizontal cell, a large positive deflection appeared in the ionic trace. This means that the intracellular concentration of Cl^- ($[\text{Cl}^-]_i$) was lower than $[\text{Cl}^-]_o$. The data for the calculation of the mean ionic concentration

were collected when the largest and most stable membrane potential was recorded. Ionic concentrations of L- and C-type cells were collected and used for calculation of the equilibrium potential (E_{Cl}). The cell shown in Fig. 2 showed a dark membrane potential of -22 mV and $[Cl^-]_o$ and $[Cl^-]_i$ were 288 and 117 mM, respectively.

After the recording, the electrode was pulled out from the cell and a negative (hyperpolarizing) pulse of about 2 to 5 s in duration was applied via the indifferent electrode to simulate the horizontal cell response. This procedure provided calibration of the membrane potential and also checked the balance of the differential recording (Fig. 2, arrow). The large transients on the ionic trace evoked at the beginning and cessation of the pulse were capacitive artifacts. The artifact subsided within several seconds. This means that the concentration level after several seconds from the initiation or cessation of the light stimulus represented the real value. In the cell shown in Fig. 2, a change in $[Cl^-]_i$ was not detectable. Even a flash of light of 30 s in duration could not elicit any concentration change in such a cell. In some cases, however, a small decrease in $[Cl^-]_i$ associated with the light response was observed. An increase of $[Cl^-]_i$, however, was never observed.

In the same manner, E_m and $[Cl^-]$ were obtained from 37 horizontal cells in 5 retinas. E_m ranged from -3.6 to -57 mV. The small membrane potential does not mean the deterioration of the cells because stable dark membrane potentials and huge (about 90 mV max) light responses could be recorded from these cells. The mean value of E_m was -26.5 ± 2.6 mV. The estimated values of $[Cl^-]_o$ and $[Cl^-]_i$ ranged from 222 to 460 mM and from 76 to 256 mM, with mean values of 320 ± 11 and 130 ± 7 mM, respectively. E_{Cl} was estimated to be -23.3 ± 1.0 mV.

Figure 3 shows the relationship of E_m and E_{Cl} . The graph seems to indicate that E_{Cl} remains at almost a constant level irrespective of E_m . However, the E_m of about 40% of the measured cells was more positive than E_{Cl} .

Only two recordings were performed from C-type cells. Figure 4 shows one of the recordings. The cell responded with hyperpolarization to white light and with depolarization to red light. In the dark, the intracellular level of $[Cl^-]$ was lower than the extracellular one. In this case, a small increase in $[Cl^-]_i$ was observed when the retina was illuminated with red light. In both cells, the depolarizing potential by red light was more positive than the calculated E_{Cl} (-24.8 mV).

DISCUSSION

Kaneko and Shimazaki [4] and Waloga and Pak [5] have shown in carp and axolotl retinas that the light-induced responses of horizontal cells decrease in low- Na^+ media and are abolished in Na^+ -free media. Kaneko and Shimazaki [4] also reported that the membrane potentials of the carp horizontal cells, which are recorded after blocking of the synaptic transmission, depend on the concentration of potassium ion in the extracellular medium ($[K^+]_o$). Djamgoz and Laming [17] measured the intracellular K^+ ($[K^+]_i$) of horizontal cells in the roach retina using

Fig. 3. The relationship between the membrane potential (E_m) and the calculated equilibrium potential (E_{Cl}). The solid line is a linear regression line with a correlation coefficient of $+0.207$ (significant at $p < 0.05$). The dotted line shows equality, $E_{Cl} = E_m$.

Fig. 4. A recording of membrane potential and intracellular Cl^- concentration in a C-type horizontal cell of the stingray retina. "R" on the lower trace indicates that the red stimulus was applied at the time point. Other experimental conditions are the same as in Fig. 2.

a K⁺-selective ion electrode and reported that the equilibrium potential (E_K) is more negative than the membrane potential in the dark. Our preliminary results in the stingray retina also showed that the Na⁺ equilibrium potential was positive and that E_K was much more negative than the dark membrane potentials [18]. All these reports support the hypothesis that Na⁺ participates in the dark membrane potential of the horizontal cells and K⁺ takes part in the membrane potential during illumination.

Toyoda *et al.* [19] observed that low-chloride Ringer, after blocking of the synaptic transmission by Co²⁺, did not depolarize the horizontal cell membrane in the carp retina. On the contrary, Miller and Dacheux [6] found in the mudpuppy retina that responses of horizontal cells were abolished by removal of external Cl⁻ without appreciable changes in photoreceptor responses. They assumed that horizontal cells maintain internal chloride at a relatively high concentration such that E_{Cl} is more positive than E_K . Upon illumination, the release of transmitter from photoreceptors is blocked and the membrane permeability for Cl⁻ decreases, resulting in the hyperpolarization of horizontal cells.

In the present study, estimated $[Cl^-]_o$ varied over a two-fold range depending on the preparation, but in each measurement $[Cl^-]_o$ was always higher than $[Cl^-]_i$. The elasmobranchs adjust their osmotic levels using a special organic substance, urea. This may partly explain the variability of $[Cl^-]_o$ among the preparations. But, other factors such as the dehydration and the interference of some other ions should still be taken into account. The mean value was, nevertheless, very close to the concentration of Ringer solutions for *Raja* (311.8 mM) [20].

The mean E_{Cl} was about -23 mV, which was almost equal to the mean membrane potential level (-27 mV) in the dark. It seems that the E_{Cl} is kept almost constant irrespective of the membrane potential (Fig. 3). Some horizontal cells, however, actually had membrane potentials less than -20 mV as shown in the results section. That is, the membrane potential is more positive than E_{Cl} . Thus, it is not adequate to assume that the transmitter released from photoreceptors in the dark makes the horizontal cell membrane permeable only to Cl⁻ in the stingray retina. Some ions that have an equilibrium potential more positive than that of chloride might be involved.

Now, glutamate is supposed to be the best candidate for the transmitter substance released from photoreceptor terminals. An application of glutamate elicits a depolarizing response in horizontal cells even after their enzymatic dissociation from the retina [21, 22]. Tachibana [23] and Hals *et al.* [24] showed in the goldfish and the catfish, respectively, that glutamate opens the cationic channels but not anionic channels of the dissociated horizontal cells. It is, therefore, difficult at present to explain why horizontal cells are hyperpolarized and their responses are suppressed in the Cl⁻-free medium. The difference might be attributable to the species difference.

Djamgoz and Laming [25] have reported a decrease in $[Cl^-]_i$ during light-evoked hyperpolarization in luminosity type (L1) horizontal cells of the roach. The

depolarization by red light in the C-type horizontal cells was accompanied by an increase in $[Cl^-]_i$. Similar changes in $[Cl^-]_i$ were observed in some horizontal cells in the stingrays. These changes are opposite to those expected from the ionic mechanism so far suggested. The discrepancy might be accounted for by ionic flow through the non-synaptic membrane. Hyperpolarization of the membrane will either increase an outflow or decrease an inflow of chloride ions through passive membrane sites. The position of the electrode tip might also be an important factor. A further experiment is needed to resolve this question.

REFERENCES

1. Trifonov Yu A: Study of transmission between photoreceptors and horizontal cells by means of electric stimulation of the retina. *Biofizika* **13**: 809–814, 1968
2. Toyoda J, Nosaki H, and Tomita T: Light-induced resistance changes in single photoreceptors of *Necturus* and *Gekko*. *Vision Res* **9**: 453–463, 1969
3. Cervetto L and Piccolino M: Synaptic transmission between photoreceptors and horizontal cells in the turtle retina. *Science* **183**: 417–419, 1974
4. Kaneko A and Shimazaki H: Effects of external ions on the synaptic transmission from photoreceptors to horizontal cells in the carp retina. *J Physiol (Lond)* **252**: 509–522, 1975
5. Waloga G and Pak WL: Horizontal cell potentials: Dependence on external sodium ion concentration. *Science* **191**: 964–966, 1976
6. Miller RF and Dacheux RF: Synaptic organization and ionic basis of on and off channels in mudpuppy retina. I. Intracellular analysis of chloride sensitive electrogenic properties to receptors, horizontal cells, bipolar cells, and amacrine cells. *J Gen Physiol* **67**: 639–659, 1976
7. Walker JL: Ion specific liquid ion-exchanger microelectrodes. *Anal Chem* **43**: 89A–92A, 1971
8. Tsien RY: Liquid sensors for ion-selective microelectrodes. *Trends Neurosci* **3**: 219–221, 1980
9. Yamada E and Ishikawa T: The fine structure of the horizontal cells in some vertebrate retinæ. *Cold Spring Harb Symp Quant Biol* **30**: 383–392, 1965
10. Kaneko A: Electrical connexions between horizontal cells in the dogfish retina. *J. Physiol (Lond)* **213**: 95–105, 1971
11. Naka K-I and Witkovsky P: Dogfish ganglion cell discharge resulting from extrinsic polarization of the horizontal cells. *J Physiol (Lond)* **223**: 449–460, 1972
12. Stell WK and Witkovsky P: Retinal structure in the smooth dogfish *Mustelus canis*: Light microscopy of photoreceptor and horizontal cells. *J Comp Neurol* **148**: 33–46, 1973
13. Dowling JE and Ripps H: S-potentials in the skate retina: intracellular recordings during light and dark adaptation. *J Gen Physiol* **58**: 163–189, 1971
14. Dowling JE and Ripps H: Effect of magnesium on horizontal cell activity in the skate retina. *Nature* **242**: 101–103, 1973
15. Toyoda J, Saito T, and Kondo H: Three types of horizontal cells in the stingray retina: Their morphology and physiology. *J Comp Neurol* **179**: 569–579, 1978

16. Fujimoto M and Kubota T: Physicochemical properties of a liquid ion exchanger microelectrode and its application to biological fluids. *Jpn J Physiol* **26**: 631-650, 1976
17. Djamgoz MBA and Laming PJ: Intracellular potassium activities of horizontal cells and extracellular potassium activity in isolated retinæ of a cyprinid fish. *Vision Res* **27**: 711-721, 1987
18. Fujimoto M, Yanase H, and Katayama J: Ionic concentrations of H1 horizontal cells in the stingray retina measured by ion-selective microelectrode. *Dobutsu Seiri* **6**: 193, 1989
19. Toyoda, J, Fujimoto M, and Tonosaki K: S-potential and Cl⁻ activity in the retina of carp. *J Physiol Soc Jpn* **39**: 360-361, 1977 (In Japanese)
20. Burton RF: Ringer solutions and Physiological Salines, John Wright & Sons Ltd, Bristol, p 132, 1975
21. Lasater EM and Dowling JE: Carp horizontal cells in culture respond selectively to L-glutamate and its agonists. *Proc Natl Acad Sci USA* **79**: 936-940, 1982
22. Ishida AT, Kaneko A, and Tachibana M: Responses of solitary retinal horizontal cells from *Carassius auratus* to L-glutamate and related amino acids. *J Physiol (Lond)* **348**: 255-270, 1984
23. Tachibana M: Permeability changes induced by L-glutamate in solitary retinal horizontal cells isolated from *Carassius auratus*. *J Physiol (Lond)* **358**: 153-167, 1985
24. Hals G, Christensen BN, O'Dell T, Christensen M, and Shingai R: Voltage-clamp analysis of currents produced by glutamate and some glutamate analogues on horizontal cells isolated from the catfish retina. *J Neurophysiol* **56**: 19-31, 1986
25. Djamgoz MBA and Laming PJ: Micro-electrode measurements and functional aspects of chloride activity in cyprinid fish retina: Extracellular activity and intracellular activities of L- and C-type horizontal cells. *Vision Res* **27**: 1481-1489, 1987