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MEMBRANE PROPERTIES OF SOLITARY HORIZONTAL CELLS ISOLATED FROM GOLDFISH RETINA

BY M. TACHIBANA

From the Department of Neurobiology, Harvard Medical School, Boston, MA 02115, U.S.A.

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SUMMARY

1. Solitary horizontal cells were obtained by dissociating the adult goldfish retina using the enzyme papain. The cells were identified on morphological grounds and could be kept in culture for over a week.

2. Solitary horizontal cells, penetrated with micro-electrodes, had resting potentials of about −75 mV in normal solution. When external K^+ concentration was changed, the membrane potential varied with E_K calculated from the Nernst equation.

3. All solitary horizontal cells tested showed an action potential in response to superthreshold depolarizing current pulses. The action potential had an overshoot of about +20 mV and a plateau potential lasting for several seconds.

4. The action potential appeared to be Ca-dependent for the following reasons: (*a*) TTX or low $[Na^+]$ did not affect the action potential, (*b*) Sr^{2+}, Ba^{2+} or high $[Ca^{2+}]$ enhanced the action potential, while (*c*) Co^{2+} or high $[Mg^{2+}]$ blocked it. No regenerative activity has been observed in horizontal cells in the retina but it is possible that the regenerative mechanism is suppressed normally.

5. A role for K^+ was indicated by an increase in the duration and amplitude of the action potential on the application of tetraethylammonium.

6. The steady-state current–voltage (*I–V*) curve, measured by applying constant current pulses, was S-shaped (current on the abscissa) and composed of inward- and outward-going rectifying regions and a transitional region between them. A similar non-linear *I–V* relationship has been reported *in vivo*.

7. The transitional region was characterized by a sudden potential jump and hysteresis, suggesting the presence of a 'negative resistance'. This potential jump appeared not to be produced by the Ca-conductance mechanism mentioned above, since similar jumps were observed in the presence of Co^{2+}.

INTRODUCTION

Horizontal cells, second-order neurones in the vertebrate retina, show graded responses to light illumination (MacNichol & Svaetichin, 1958; Tomita, 1963; Werblin & Dowling, 1969; Kaneko, 1970). The precise role of horizontal cells remains to be established but it has been demonstrated that one of their functions is to provide an antagonistic input to photoreceptors and/or bipolar cells: for example,

hyperpolarization of horizontal cell membrane by intracellularly applied current produces a depolarization in photoreceptors (Baylor, Fuortes & O'Bryan, 1971) and a hyperpolarization in on-centre bipolar cells (Toyoda & Tonosaki, 1978).

In order to understand the information processing in the outer plexiform layer, it is important to identify the ion-conductance mechanisms of horizontal cells. The transmitter from photoreceptors depolarizes the horizontal cell membrane by increasing the permeability to Na^+ (Kaneko & Shimazaki, 1975; Waloga & Pak, 1978) and block of its release leads to hyperpolarization of the cell membrane (Dowling & Ripps, 1973; Cervetto & Piccolino, 1974). After removal of synaptic input, the horizontal cell at rest behaves as a K electrode (Kaneko & Shimazaki, 1975). However, horizontal cells in the intact retina exhibit additional complexities: horizontal cells are also under the influence of chemical transmitter from interplexiform cells (Ehinger, Falck & Laties, 1969; Dowling, Ehinger & Hedden, 1976; Dowling & Ehinger, 1978; Hedden & Dowling, 1978), non-linearity of current–voltage relationship of the cell membrane has been reported (Trifonov, Byzov & Chailahian, 1974; Werblin, 1975; Byzov, Trifonov, Chailahian & Golubtzov, 1977) and horizontal cells are electrically coupled to neighbouring horizontal cells (Naka & Rushton, 1967; Norton, Spekreijse, Wolbarsht & Wagner, 1968; Kaneko, 1971), probably through gap junctions that have been visualized (Yamada & Ishikawa, 1965; Witkovsky & Dowling, 1969; Stell, 1972; Raviola, 1976).

A way to examine the membrane properties without such complexities in the intact retina is to study the solitary horizontal cells obtained by enzymatic dissociation of the retina. Several studies have demonstrated that the major classes of cells from vertebrate retina could be isolated and identified (Drujan & Svaetichin, 1972; Lam, 1972; Anctil, Ali & Couillard, 1973; Kaneko, Lam & Wiesel, 1976) and in some cases their physiological and biochemical properties were examined (Lam, 1975, 1976; Sarthy & Lam, 1978, 1979; Bader, MacLeish & Schwartz, 1978, 1979; Sarthy, Bridges, Lam & Kretzer, 1979).

The solitary horizontal cells studied in this paper were obtained from the goldfish retina by an improved method of tissue dissociation, and were cultured for over one week. During this time the electrical properties of the cell membrane were studied. It was found that at rest, the membrane was permeable mainly to K^+ and that the cells showed Ca-dependent action potentials and marked non-linearity in the I–V characteristics of the membrane, including inward- and outward-going rectifications and hysteresis.

METHODS

Animals

Experiments were performed on common goldfish, *Carassius auratus*, (12–20 cm total length). The goldfish were kept in aerated, dechlorinated water at room temperature and were adapted to room light by day.

Dissociation procedure

The dissociation procedure, modified from Lam (1972) and Bader *et al.* (1978), was performed in room light. A goldfish adapted to room light was pithed and the eyes were excised. The eyes were soaked in 70% ethanol for 20 sec and then were rinsed three times with solution A (Table 1). The cornea and lens were removed and the eye cups were incubated for 20 min at 28 °C in 14 ml. of an oxygenated solution A containing papain (Worthington Biochemical Corp., no. 3126), 7 u./ml.; DL-cysteine HCl (Sigma, no. C-9768), 2·7 mM; $CaCl_2$, 0·5 mM; BSA (Sigma, no. A-4378), 0·02 mg/ml.

The pH was adjusted to 7·4 at 20 °C with NaOH. The incubation took place in a 50 ml. erlenmeyer flask and the solution was gently stirred (1–2 rev/sec) with a stirring magnet. The retina was then detached from the pigment epithelium and cut into small pieces. The pieces were incubated for another 20 min in fresh enzyme solution. At the end of the second incubation, the retina was transferred to a plastic test tube and rinsed three times with 10 ml. normal solution (solution B). The retina was gently triturated in 1·5 ml. of this solution with a 5 ml. glass pipette (Corning Glass Co., type 7707-5N). An aliquot of the resulting cell suspension was mixed with an equal volume of an agarose solution at 37 °C to help immobilize the cells and facilitate micro-electrode penetration. The composition of the agarose solution was that of solution B plus agarose 3·5 mg/ml. (Miles Lab., no. 95-201-4). Each well (diameter, 1·25 cm) of modified Petri dishes (Bray, 1970) received a 35 μl. aliquot of the final cell suspension. The dishes were kept on ice for 5 min to allow the agarose to gel and then 2 ml. of culture medium was added. Two types of culture media were used: normal solution (2·6 mM-K^+, solution B) and high $[K^+]_o$ solution (10 or 26 mM-K^+, solution C or D). Since the solitary horizontal cells survived better in high $[K^+]_o$ solution and since the physiological properties were found to be almost identical to those in normal solution, most of the experiments were performed in high $[K^+]_o$ solution. The dishes were kept at 10 °C in a low temperature incubator, where filtered moist air was circulated.

Solutions and recording

A dish containing dissociated cells was placed on an inverted microscope, viewed under phase-contrast optics (Zeiss, IM-35), and perfused continuously with 0·8 ml./min of oxygenated solution (Table 1). As the solitary cells were embedded in the agarose gel, it was difficult to know the diffusion time from the medium to the cell membrane. However, from the high $[K^+]_o$ experiment, diffusion time was estimated to be 4 min (see Fig. 3).

Solitary horizontal cells were identified from their characteristic morphology (see Results) and were impaled with micro-electrodes filled with 4 M-K acetate. The electrode resistance was 150–200 MΩ. The reference electrode was an Ag–AgCl coil, immersed in a 2 M-KCl chamber which was connected to the dish with an agarose bridge. The micro-electrode was connected to a high input impedance preamplifier with an active bridge circuit and with a negative capacitance compensator (Colburn & Schwartz, 1972; Thomas, 1977). The electrical signals were observed on a oscilloscope (Tektronix, 565) and recorded on a pen recorded (Brush, 220) and on film (Grass, C4N).

Current was sometimes injected through the micro-electrode to evoke an action potential and to determine the current–voltage (I–V) relationship. Since the electrode resistance was fairly high, only currents of less than ± 300 pA were injected. The potential drops caused by the electrode resistance were not always completely compensated by the bridge circuit because of the non-linear properties of the electrode. Corrections were made for incomplete compensation by subtracting for a given current the voltage drop observed in pulling out of the cell from voltage drop measured in the cell. The data were discarded when the potential drop due to the incomplete compensation was more than 20 mV at the maximal current or when the electrode resistance changed markedly during the time of the recording. All experiments were done at room temperature (20–24 °C).

RESULTS

Morphology of solitary horizontal cells in culture

After the dissociation, solitary photoreceptor-, bipolar-, horizontal-, and Müller cells could be identified unequivocally from their characteristic morphology. Amacrine and ganglion cells could be differentiated from other classes of cells, but it was difficult to distinguish amacrine cells from ganglion cells because the axon of ganglion cell was usually lost during the dissociation. The classes of cells obtained depended on the length of incubation time in the enzyme solution and on the concentration of the enzyme.

Pl. 1 (*A*1, *A*2 and *A*3) shows a variety of solitary horizontal cells just after dissociation. They retained the characteristic morphology of horizontal cells such as

TABLE 1

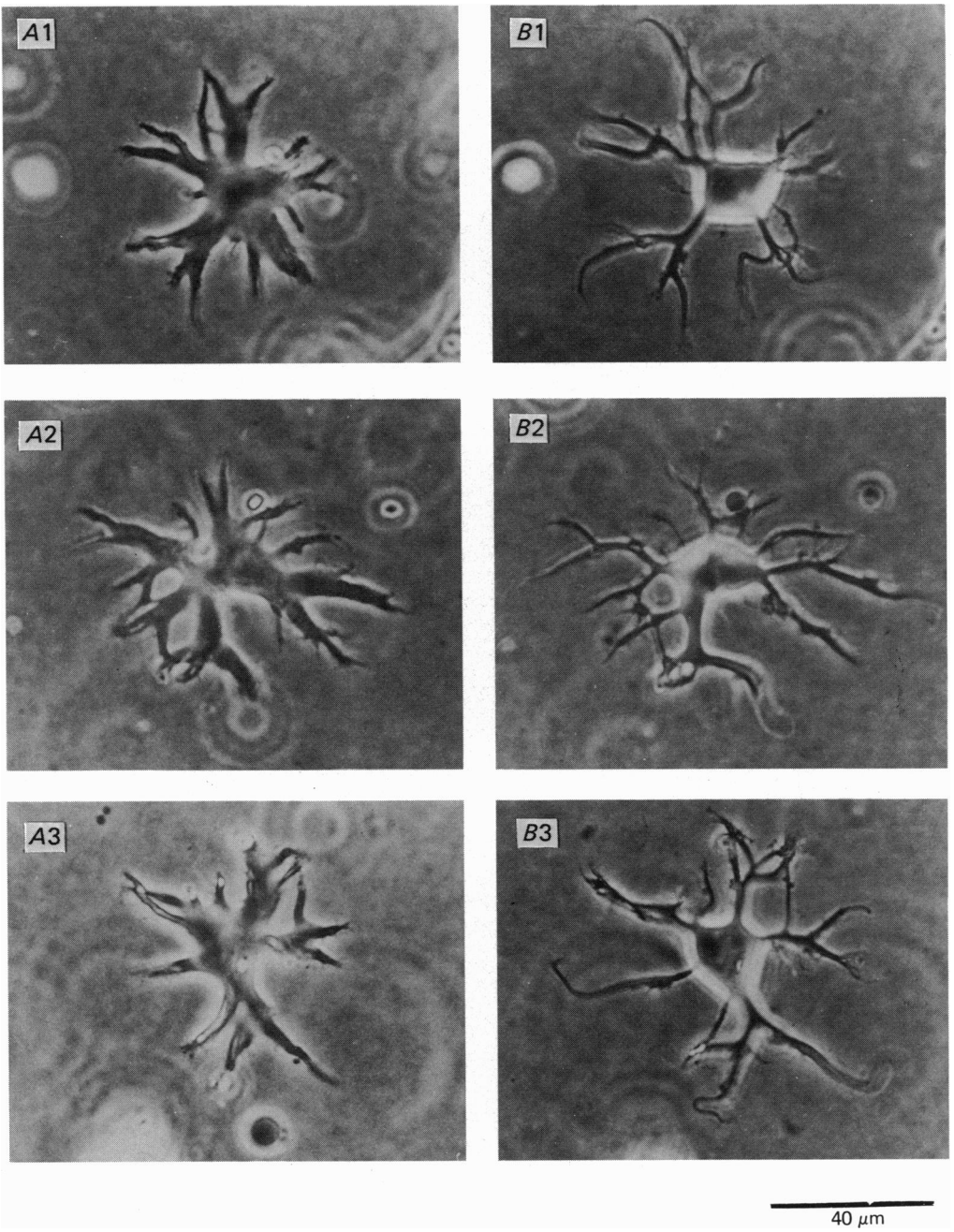
Solutions (mM)	NaCl	KCl	$NaHCO_3$	NaH_2PO_4	Na pyruvate	$MgCl_2$	$MgSO_4$	$CaCl_2$	Choline Cl	$SrCl_2$	TEA-Cl
A normal, 0 Ca^{2+}	125·0	2·6	0·5	0·5	1·0	0·5	0·5	—	—	—	—
B normal	125·0	2·6	0·5	0·5	1·0	0·5	0·5	2·5	—	—	—*
C high $[K^+]_o$ (10 mM-K^+)	117·6	10·0	0·5	0·5	1·0	0·5	0·5	2·5	—	—	—*
D high $[K^+]_o$ (26 mM-K^+)	101·6	26·0	0·5	0·5	1·0	0·5	0·5	2·5	—	—	—*
E high $[K^+]_o$ (52 mM-K^+)	75·6	52·0	0·5	0·5	1·0	0·5	0·5	2·5	—	—	—*
F high $[K^+]_o$ (78 mM-K^+)	49·6	78·0	0·5	0·5	1·0	0·5	0·5	2·5	—	—	—*
G low $[Na^+]_o$ (2·6 mM-K^+)	—	2·6	0·5	—	—	0·5	0·5	2·5	126·5	—	—*
H low $[Na^+]_o$ (5·2 mM-K^+)	—	5·2	0·5	—	—	0·5	0·5	2·5	123·9	—	—*
I low $[Na^+]_o$ (10 mM-K^+)	—	10·0	0·5	—	—	0·5	0·5	2·5	119·1	—	—*
J high $[Ca^{2+}]_o$ (10 mM-K^+)	109·6	10·0	—	—	—	—	—	10·0	—	—	—*
K high $[Ca^{2+}]_o$ (26 mM-K^+)	71·1	26·0	—	—	—	—	—	25·0	—	—	—*
L low $[Ca^{2+}]$, high $[Mg^{2+}]$	94·9	10·0	0·5	0·5	1·0	13·8	6·2	0·5	—	—	—*
M Sr^{2+}	109·6	10·0	—	—	—	—	—	—	—	10·0	—*
N TEA	105·6	10·0	0·5	0·5	1·0	0·5	0·5	2·5	—	—	12·0*

All solutions contained 16 mM-glucose, 2 mM-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid), BSA*** 0·1 mg/ml. and phenol red (final concentration 0·2%, GIBCO no. 630-5100).

* The pH was adjusted to 7·8 with NaOH.

** Ba^{2+} or Co^{2+} solution: 0·1–1·0 mM-$BaCl_2$ or 2·0–4·0 mM-$CoCl_2$ was added to solution C or D.

*** Solution A did not contain BSA.


A1
B1
A2
B2
A3
B3
40 μm

large flat cell body and thick dendrites, though the cells lacked their thick axon terminal and fine processes. When compared with horizontal cells in the intact retina, the solitary horizontal cells seemed to be somewhat swollen, probably due to the injury during the dissociation.

Pl. 1 (*B*1, *B*2 and *B*3) shows the same cells cultured for 8 days in high $[K^+]_o$ solution (10 mM-K^+, solution C). The dendrites became longer and thinner, and fine processes extended from many of these dendrites. Horizontal cells could easily be distinguished from other classes of cells in culture.

In normal solution (2·6 mM-K^+, solution B), some horizontal cells also survived over one week and began to grow processes. However, horizontal cells in normal solution were more susceptible to deterioration than those in high $[K^+]_o$ solution: the membrane became rough, vacuoles were observed, and finally the cell disappeared during the course of several days. Thus, most of the experiments were done using the cells cultured in high $[K^+]_o$ solution (10 or 26 mM-K^+, solution C or D).

After 10 days in culture, horizontal cells began to deteriorate even in high $[K^+]_o$ solution, probably because of a lack of some necessary nutrients.

Properties of morphological subclasses. According to their responses to monochromatic light (ref. Tomita, 1963) and characteristic morphologies, horizontal cells in the retina of cyprinid fish are divided into four subclasses; cone horizontal cells (H1, H2 and H3) and rod horizontal cells (rH) (Mitarai, Asano & Miyake, 1974; Hashimoto, Kato, Inokuchi & Watanabe, 1976; Stell & Lightfoot, 1975). In the present study some solitary horizontal cells were identified as H1, H2 or H3 from the characteristic morphologies, i.e. the size of cell body and the dendritic pattern. It was, however, often difficult on morphological grounds to unequivocally classify a cell into one of the four subclasses. Since there were no significant differences in resting potentials, action potentials or other membrane properties, the solitary horizontal cells will be treated without reference to specific subclass.

General properties of solitary horizontal cells in culture

A total of 108 horizontal cells whose morphology indicated no signs of deterioration under the light microscope were impaled with micro-electrodes and their membrane properties were studied. The age of the culture ranged from 1 to 9 days; 80% of the cells examined were 1–4 days old.

High $[K^+]_o$ solution. The cells cultured in high $[K^+]_o$ solution showed stable resting potentials: in 10 mM-K^+ (solution C), $-55{\cdot}5 \pm 3{\cdot}8$ mV, $n = 65$ (mean ±S.D., number of cells examined); in 26 mM-K^+ (solution D), $-39{\cdot}6 \pm 4{\cdot}8$ mV, $n = 26$. The resting potentials showed no obvious changes related to the age of the culture.

All of the cells examined in high $[K^+]_o$ solution (10 or 26 mM-K^+) produced all-or-none action potentials when they were depolarized to a critical membrane potential level. Fig. 1 shows an example of action potentials, on slow (*A*) and fast

EXPLANATION OF PLATE

Living solitary horizontal cells maintained in tissue culture in high $[K^+]_o$ solution (10 mM-K^+; solution C). Each pair (*A*1/*B*1, *A*2/*B*2 and *A*3/*B*3) shows the same cell photographed imediately after dissociation (*A*) and 8 days later (*B*). The cells are embedded in an agarose gel. The photographs were taken using phase-contrast optics.

(*B*) time bases, recorded from a cell in high $[K^+]_o$ solution (10 mM-K^+). No obvious notch was observed during the rising phase of the action potential. An overshoot was always observed ($+22{\cdot}1 \pm 9{\cdot}1$ mV, $n = 91$). After the current pulse was terminated, the membrane potential stayed at a plateau for several seconds ($3{\cdot}9 \pm 2{\cdot}5$ sec, $n = 91$) and finally returned to the original resting level. The overshoot became slightly smaller after 7 days in culture ($+16{\cdot}3 \pm 2{\cdot}3$ mV, $n = 9$).

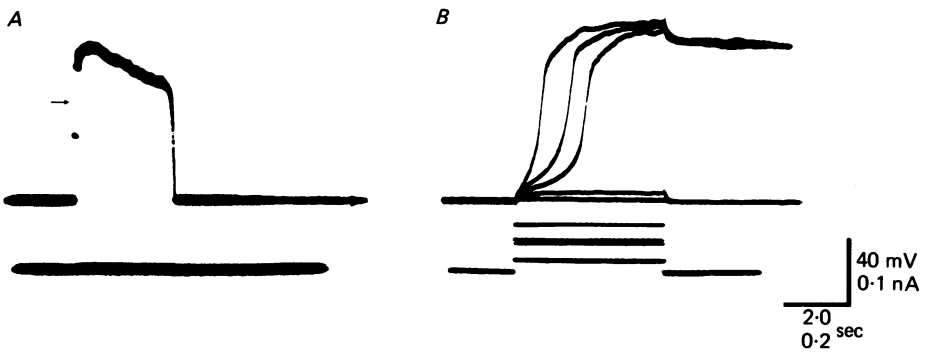


Fig. 1. Action potentials recorded from a solitary horizontal cell on slow (*A*) and fast (*B*) time bases. The cell showed a resting potential of −62 mV in high $[K^+]_o$ solution (10 mM-K^+, solution C). The duration of the applied current pulses was 45 msec (*A*) or 450 msec (*B*). The arrow indicates 0 mV. Top trace shows voltage and bottom trace shows current.

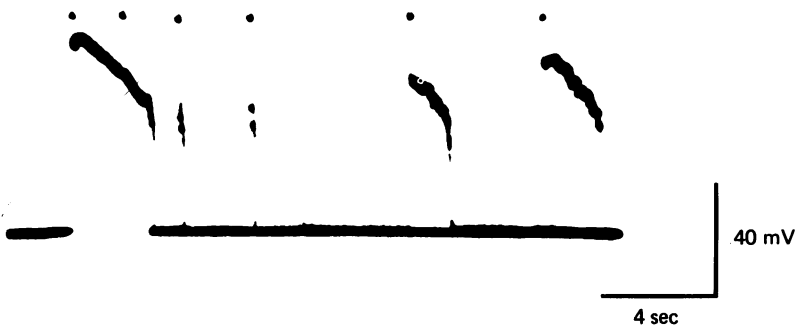


Fig. 2. Refractory period of the action potential. Double pulses of 40 msec duration were applied every min and the interval between pulses was changed (1·7, 3·6, 6·1, 11·6 and 16·1 sec). Dark dots indicate the timing of applied pulses. This Figure was made by superposition of five trials. When the second pulse was applied at 1·7, 3·6 or 6·1 sec after the onset of the first pulse, an action potential failed to occur. After a 11·6 or 16·1 sec rest, an action potential was evoked, but the amplitude was smaller and the duration was shorter than the previous action potential. The external K concentration was 26 mM (solution D) and the resting potential was −41 mV.

The input resistance was estimated to be 48 ± 19 MΩ ($n = 15$) from the voltage drop induced by a constant current of −100 pA. However, absolute values of the input resistance were difficult to measure accurately because of the prominent inward-going rectification of the cell membrane near the resting potential (see below) and the non-linearity and instability of the micro-electrodes. Thus, the input resistances were not determined routinely.

The cells exhibited absolute and relative refractoriness to repetitive firing. As shown in Fig. 2, when the second pulse fell on the plateau or just after the action potential, a second action potential failed to occur. A 20 sec interval was necessary to fully restore the action potential.

Normal solution. In cells cultured in normal solution (2·6 mM-K^+, solution B), action potentials with an overshoot ($+21{\cdot}1 \pm 7{\cdot}0$ mV, $n = 9$) were observed in response to

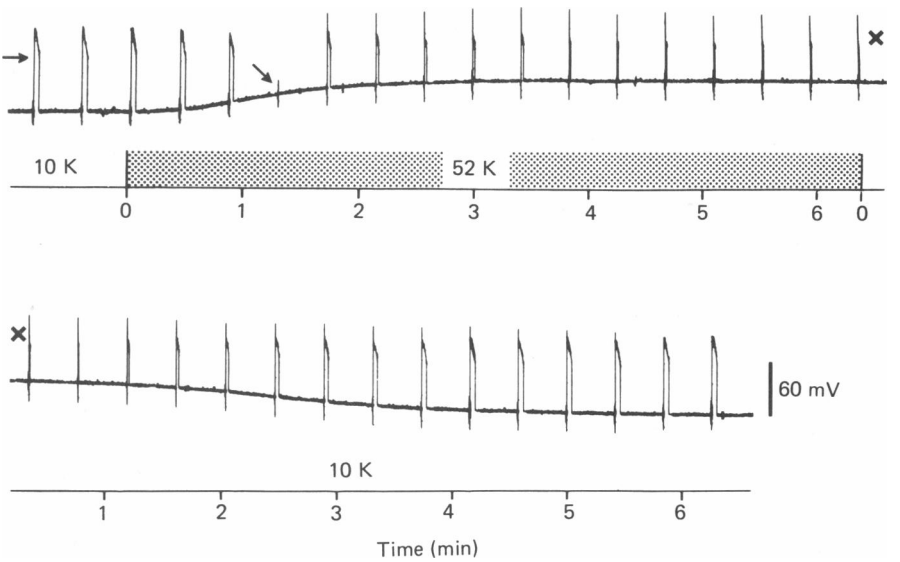


Fig. 3. Effect of external K^+ concentration on the resting potential. The upper trace shows a record of membrane potential and the lower line indicates the time and $[K^+]_o$. The record is continuous between × 's. At time labelled 0, $[K^+]_o$ was increased from 10 to 52 mM, and in 4 min the membrane potential reached a steady level. Action potentials were induced by 30 msec pulses every 25 sec. Since the action potential failed at 1·5 min (oblique arrow), the intensity of the current pulse was increased from 150 to 180 pA. Action potentials were preceded by the artifacts due to the current pulses. After the cell was perfused in 52 mM-K^+ solution for 6·4 min, the solution was changed to one containing 10 mM-K^+. A horizontal arrow shows 0 mV.

electrical stimulation. These action potentials were similar to those recorded in high $[K^+]_o$ solution.

There were, however, differences in the resting potential and its peak to peak fluctuations: for cells in normal solution, resting potentials were between −60 and −85 mV ($-73{\cdot}7 \pm 10{\cdot}7$ mV, $n = 9$) and peak to peak fluctuations were about 4 mV. Amplitude of the fluctuations could be influenced by varying the resting potential: depolarization increased the amplitude, while hyperpolarization decreased it. In eight of seventeen cells fluctuations were large enough to evoke action potentials. The large membrane fluctuations and spontaneous action potentials neither were related to the age of the culture, since they were observed in different cells irrespective of the period *in vitro* (1–9 days), nor could be explained simply by the injury current secondary to electrode penetration, since the membrane potential became stable as soon as the external K^+ concentration was increased from 2·6 to 10 mM and more.

Resting potential and its ionic mechanisms

In various excitable cells, K^+ is the main ion responsible for the resting potential. Therefore, solitary horizontal cells were perfused with test solutions in which NaCl was replaced by KCl at various concentrations. Fig. 3 shows the results of such an experiment. In the control solution (10 mM-K^+) the resting potential was −58 mV. When the perfusate was switched to the test solution (52 mM-K^+), the membrane

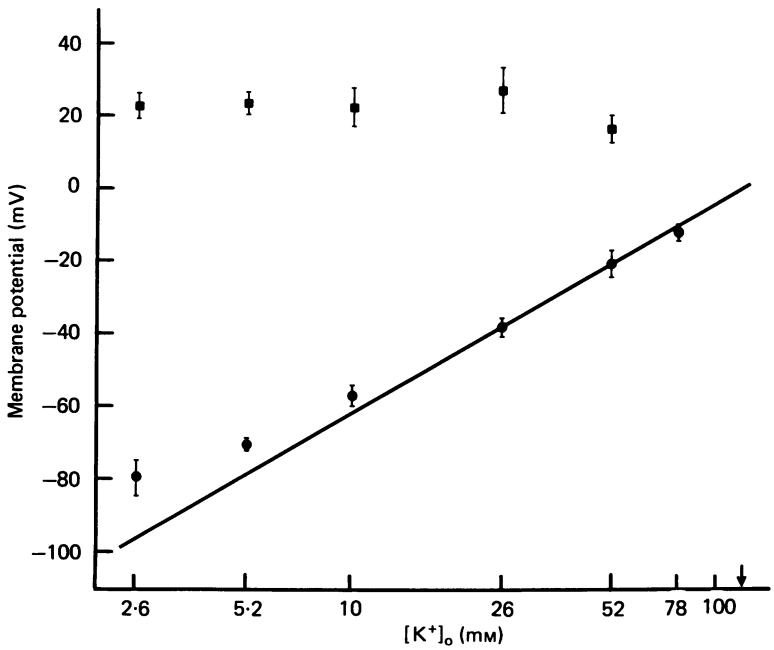


Fig. 4. Relation between the external K^+ concentration and the membrane potential. The circles represent mean resting potentials, and the vertical lines indicate standard deviation. The data at 2·6 and 5·2 mM-$[K^+]_o$ were obtained in low $[Na^+]_o$ solution to reduce the leak currents of the membrane. The line has a slope of 58 mV per decade as predicted from the Nernst equation. The internal concentration of K^+ in solitary horizontal cells were estimated to be 118 mM, and is indicated by the arrow. The mean amplitude of the action potentials (squares) was not greatly affected by $[K^+]_o$. At 78 mM-$[K^+]_o$ it was difficult to produce action potentials by short pulses, probably because of an inactivation of Ca channels. These data were obtained from fifteen cells.

potential began to depolarize within 20 sec and reached a final value of −20 mV at 4 min. When the cell was returned to the control solution, the membrane potential reverted to its original level with a slightly slower time course.

The relation between the resting potential and the external K^+ concentration is shown in Fig. 4. The data were obtained from fifteen cells. The continuous line is calculated from the Nernst equation, and has a slope of 58 mV per decade. At higher $[K^+]_o$ (26 mM and more), the potentials were in good agreement with the predicted line. At lower $[K^+]_o$, the potentials were smaller than the predicted values. Such a difference can be caused either by a leakage of the membrane produced by electrode

penetration or by additional permeability to other ions. The result shows that K^+ is the main ion responsible for the resting potential of solitary horizontal cells.

The internal concentration of K^+ was estimated from Fig. 4 to be 118 mM. A value of 125 mM was obtained from horizontal cells in the intact retina (Kaneko & Shimazaki, 1975).

Action potential and its ionic mechanisms

Effects of TTX and low $[Na^+]_o$ *on action potentials.* Na-dependent action potentials are usually completely eliminated by the application of 100–150 nM-tetrodotoxin (TTX), while Ca-dependent action potentials survive even in two orders of magnitude higher concentrations of TTX (Hagiwara & Nakajima, 1966). When solitary horizontal cells were perfused with a solution containing 38 μM-TTX for 8 min, action potentials were not eliminated.

Na-dependent action potentials are not always sensitive to TTX (ref. Narahashi, 1974). To test whether action potentials in solitary horizontal cells are TTX-resistant, Na-dependent action potentials, the cells were perfused with a low $[Na^+]_o$ solution (solution I). After 20 min of perfusion, action potentials could still be evoked by depolarizing currents. In some preparations, action potentials became slightly smaller in amplitude and shorter in duration, but it was difficult to conclude whether such changes were caused by deterioration of the cells or by the lack of Na^+, since in these cases complete recovery was not seen after the perfusate was switched to the control solution. These results suggest that in solitary horizontal cells Na^+ carried at most only a small fraction of the inward currents that produced the action potential.

Effects of divalent cations on action potentials. If the upstroke of action potentials recorded from solitary horizontal cells is produced by an influx of Ca^{2+}, the amplitude of the action potential should depend on the extracellular Ca^{2+} concentration. Fig. 5 shows the effect of a 10-fold increase in $[Ca^{2+}]_o$; in control solution (2·5 mM-Ca^{2+}) the overshoot was +42 mV, and in high $[Ca^{2+}]_o$ (25 mM-Ca^{2+}) the overshoot increased to +58 mV at 8·5 min and finally reached +62 mV at 12 min. The duration of action potential was prolonged slightly in high $[Ca^{2+}]_o$ solution. The resting potential was not significantly changed during the experiment. Table 2 shows the average increase of the overshoot and the predicted values calculated from the Nernst equation. The result suggests that near the peak of action potentials solitary horizontal cells roughly approximate a Ca electrode, although the observed values were slightly smaller than those predicted.

In all known cases, Ca-dependent action potentials are enhanced if the external Ca^{2+} is replaced by Sr^{2+} or Ba^{2+} and are suppressed by the addition of Co^{2+}, Mg^{2+} or Mn^{2+} (Hagiwara, Fukuda & Eaton, 1974). Fig. 6 shows the effect of Sr^{2+} on action potentials recorded from a solitary horizontal cell. When 2·5 mM-$[Ca^{2+}]_o$ in control solution was replaced by 10 mM-$[Sr^{2+}]_o$, the action potential became larger in amplitude and longer in duration. After a 10 min perfusion, the amplitude increased by 12 mV and duration increased 5-fold. The effect of Sr^{2+} was reversible. Increase of $[Ca^{2+}]_o$ to the same amount as $[Sr^{2+}]_o$ (10 mM-Ca^{2+}) also produced an increase in amplitude by 9 mV. The larger increase in amplitude by Sr^{2+} may be explained by the fact that in some systems the Ca-channel is more permeable to Sr^{2+} than Ca^{2+}

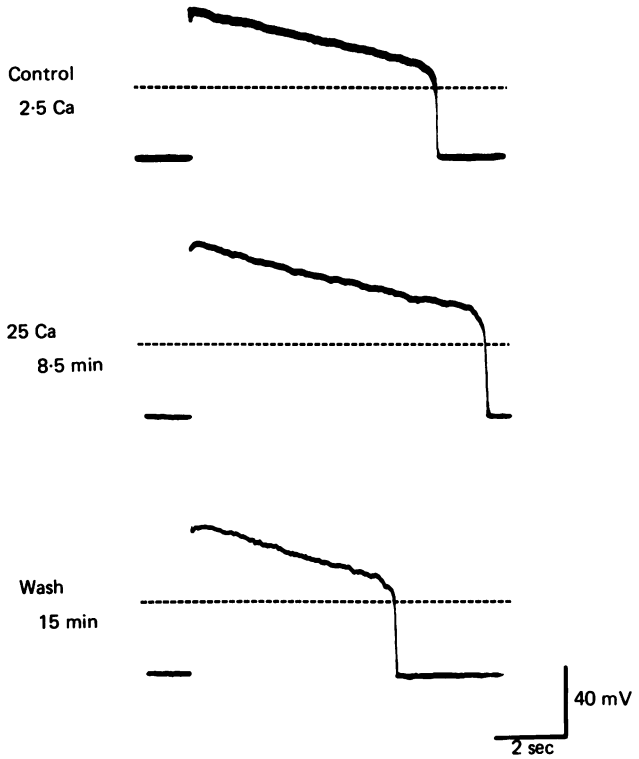


Fig. 5. Effect of high $[Ca^{2+}]_o$ on the action potential. External Ca^{2+} concentration was increased from 2·5 mM (control; solution D) to 25 mM (solution K). External K^+ concentration was always kept at 26 mM. A constant current pulse of 10 msec duration was applied to evoke action potentials. The resting potential was −41 mV during the experiment. The dashed line indicates 0 mV.

TABLE 2. Effect of high $[Ca^{2+}]_o$ on the amplitude of action potentials

Increase of $[Ca^{2+}]_o$*	Augmentation of amplitude (mean ±S.D.)	Predicted values from the Nernst equation
4×	11·0±1·2 mV	17·5 mV
8×	22·5±6·5 mV	26·2 mV
10×	25·3±3·8 mV	29·0 mV

* The control solution contained 2·5 mMCa^{2+}.

(Hagiwara *et al.* 1974). The prolongation of the duration in Sr^{2+} might be caused by the weaker effect of Sr^{2+} than Ca^{2+} on Ca^{2+} activated outward K^+ current (Gorman & Hermann, 1979), though this possibility was not examined substantially in solitary horizontal cells.

The effect of Ba^{2+} was dramatic as shown in Fig. 7. When the cell was perfused with solution C (10 mM-K^+, 2·5 mM-Ca^{2+}) containing 1 mM-Ba^{2+}, the resting potential began to depolarize, spontaneous action potentials were then induced, and finally the membrane potential became stable at a more depolarized level ($-6·6 \pm 5·4$ mV, $n = 5$). Such spontaneous action potentials (or oscillations) were observed in all seven cells

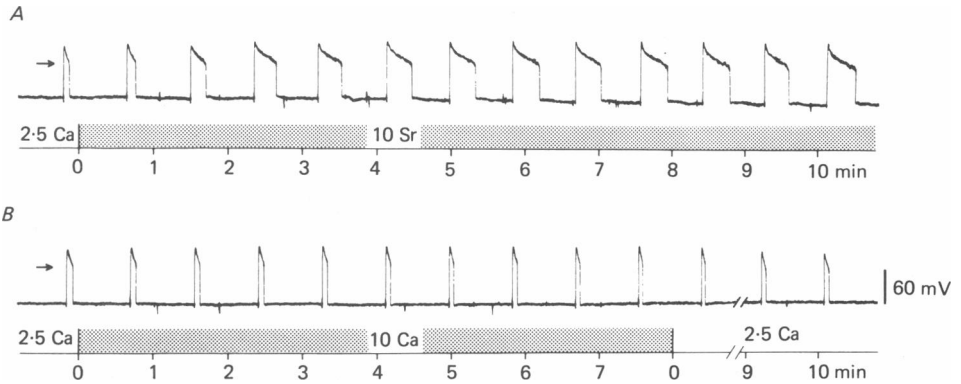


Fig. 6. Effect of Sr^{2+} on the action potential. *A*, the cell was bathed with control solution (2·5 mM-Ca^{2+}; solution C), followed by Sr^{2+} solution (10 mM-Sr^{2+}; solution M). The action potentials were induced by 10 msec pulses every 50 sec. In Sr^{2+} solution, the action potentials became larger in amplitude by 12 mV and longer in duration. *B*, the same cell was washed with control solution for 11 min, then bathed with high Ca^{2+} solution (10 mM-Ca^{2+}, solution J). The amplitude became larger by 9 mV. Finally after an 8 min perfusion in 10 mM-Ca^{2+}, the cell was washed with control solution, and the amplitude of the action potential returned to base line. In this experiment $[K^+]_o$ was kept at 10 mM. The arrows indicate 0 mV. The resting potential was −60 mV.

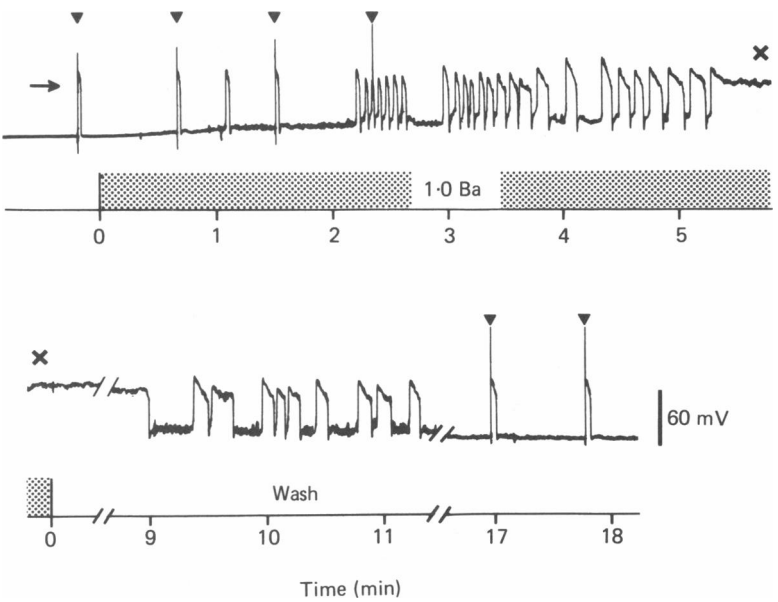


Fig. 7. Effect of Ba^{2+} on the action potential. The cell was bathed with control solution C (2·5 mM-Ca^{2+}, 10 mM-K^+), followed by Ba^{2+} solution (solution C + 1 mM-Ba^{2+}) for 6 min and then washed. Action potentials preceded by stimulus artifact were produced by 10 msec current pulses (▼); the other potential changes occurred spontaneously. The record is continuous between × 's. The arrow shows 0 mV.

examined. Similar phenomena were observed in an opposite sequence during washing. Effects of Ba^{2+} on excitable membrane have been reported to be 2-fold; first, Ba^{2+} carries more current through Ca-channel than Ca^{2+}, and secondly, Ba^{2+} suppresses the current through the K-channel (Hagiwara, Miyazaki, Moody & Patlak, 1978). Thus, Ba^{2+} may have increased the net inward current to the extent that a small depolarization was capable of inducing the spontaneous activity.

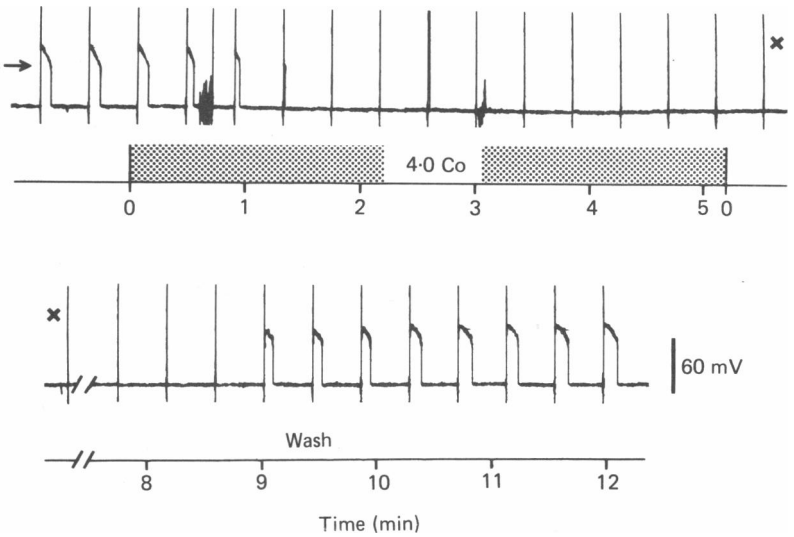


Fig. 8. Effect of Co^{2+} on the action potential. The cell was bathed with control solution D (2·5 mM-Ca^{2+}, 26 mM-K^+), followed by Co^{2+} solution (solution D+4 mM-Co^{2+}). Within 2 min after the application of Co^{2+}, the action potential induced by a 100 msec pulse disappeared; only the artifact due to the current pulse remained. At 2·6 min in Co^{2+} solution, a 1 sec pulse was applied, but no action potential was evoked. After 5·2 min of perfusion with Co^{2+} solution, the cell was washed with control medium. The action potential began to reappear at 8·2 min, and finally recovered in amplitude and duration to values comparable to those observed in the control solution. The artifacts due to current pulses were seen every 25 sec as vertical lines. At 0·7 and 3·1 min artifacts caused by electrostatic noise were recorded. The resting potential was −44 mV and remained constant throughout the record. The record is continuous betwen × 's.

In the solution containing 0·1 mM-$[Ba^{2+}]_o$, no obvious changes in resting potentials or action potentials were seen. However, under this condition one could evoke anode-break responses which were never observed in normal or high $[K^+]_o$ solutions even with a strong stimulation. This effect may be due to the suppression of K current by Ba^{2+}.

Co^{2+} has a blocking effect on Ca-dependent action potentials (ref. Hagiwara, 1974). Perfusion with 2–4 mM-Co^{2+} suppressed the action potential recorded from solitary horizontal cells (Fig. 8). Within several min after the application of Co^{2+}, without a change in the resting potential, the duration of the action potentials became shorter, the overshoot became smaller, and finally the action potentials disappeared. The blocking effect of Co^{2+} was reversible, but it was often necessary to wait for over 10 min for the recovery of action potentials. A similar blocking effect of Co^{2+} was observed in the cells cultured in normal solution.

The action potentials from solitary horizontal cells were also blocked in high [Mg^{2+}], low [Ca^{2+}] solution (20 mM-Mg^{2+}, 0·5 mM-Ca^{2+}; solution L).

The experiments on divalent cations all provide strong evidence that Ca^{2+} is the ion which carries inward current for the upstroke of action potentials in solitary horizontal cells.

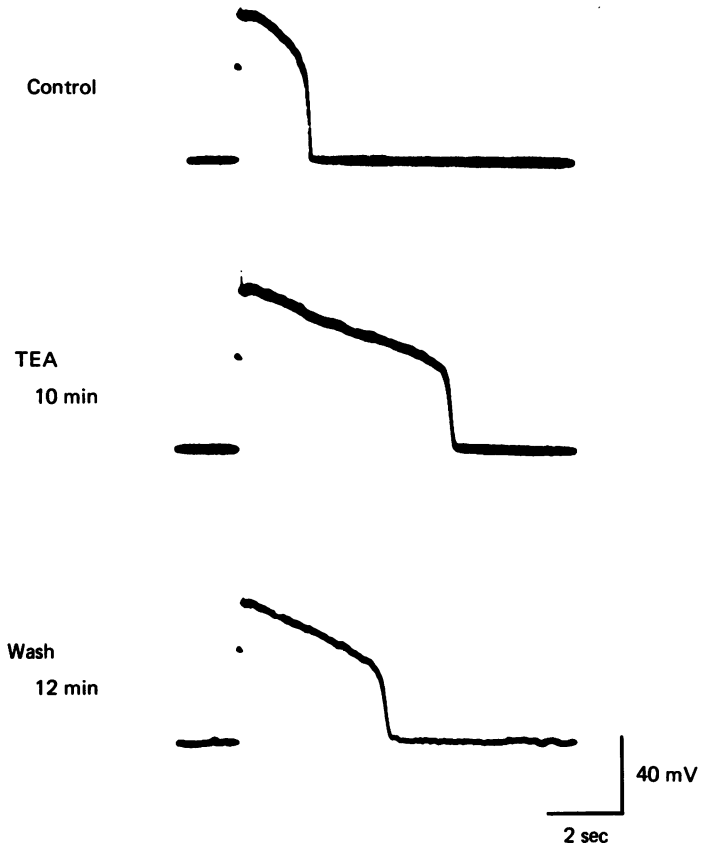


Fig. 9. Effect of tetraethylammonium (TEA) ions on the action potential. Control solution (10 mM-K^+, solution C) was switched to TEA solution (12 mM-TEA, solution N). The action potential was produced by a 50 msec pulse. The overshoot increased from +15 to +25 mV, and the duration was prolonged from 2·0 to 5·7 sec in TEA. External K^+ concentration was maintained at 10 mM. The resting potential was −60 mV during the experiment. The effect of TEA was in large part reversible. Black dots indicate current stimuli.

Effect of TEA on action potentials. Tetraethylammonium (TEA) ions are thought to block a voltage-sensitive K conductance (ref. Narahashi, 1974). In the presence of 12 mM-TEA (solution N), the duration of the action potential of solitary horizontal cells was prolonged by three-fold and the peak amplitude increased by about 10 mV (Fig. 9). This result suggests that, in addition to a Ca conductance mechanism demonstrated earlier in this paper, a K conductance mechanism is present. It is possible that an increase in a K conductance may account for the deviation of action potential amplitudes from values predicted from the Nernst equation. The effect of TEA was in large part reversible.

Current–voltage relationship of the horizontal cell membrane

Fig. 10*A* shows the voltage responses to current pulses of 5·2 sec in high $[K^+]_o$ solution (10 mM-K^+, solution C). For a depolarizing current pulse, an action potential was produced when the membrane potential reached threshold. The membrane potential returned to the resting potential at the termination of the current pulse. A larger current pulse (+85 pA and more) was followed by a plateau potential which

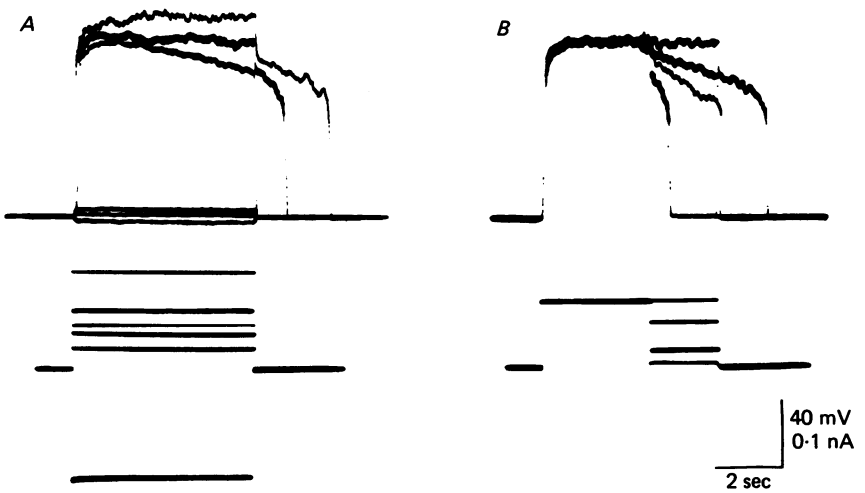


Fig. 10. Changes in membrane potential in response to current pulses of 5·2 sec (*A*) and to double pulses (*B*). *A*, the intensities of applied current pulses were −160, +32, +52, +63, +85 and +140 pA. After the termination of current pulses of +85 and +140 pA, the membrane potential stayed at a plateau level. *B*, the intensity of the conditioning pulse was +95 pA and those of test pulses were +95, +65, +25 and +5 pA. A plateau potential was evoked after the application of a +95 pA test pulse. Both were recorded from the same cell in solution C (10 mM-K^+). The resting potential was −62 mV.

outlasted the depolarizing current pulse. In contrast, when a hyperpolarizing current was applied, only a small voltage response was obtained (6 mV at −160 pA). The amplitude of these voltage responses, however, did not increase proportionally to the hyperpolarizing current intensity; for example, the amplitude was 5 mV at −100 pA (not illustrated). This suggests an active conductance mechanism. Anode-break responses were never observed at the termination of the hyperpolarizing current pulses.

The steady-state current–voltage (*I–V*) relationship was obtained by measuring the membrane potentials at the end of current pulses (Fig. 11). The *I–V* relationship showed a marked non-linearity. The slope resistance was larger at the resting potential than at more negative potentials: thus indicating inward-going (anomalous) rectification. The membrane potential jumped to a more positive level at the threshold. After the potential jump, the slope resistance gradually decreased at more positive potentials: thus indicating outward-going rectification.

The *I–V* relationship was examined in a total of twenty-two cells in normal or high $[K^+]_o$ solution, and all cells showed a similar *I–V* relationship. The slope resistance

was about 160 MΩ at the prominent outward-going rectifying portion and about 20 MΩ at the prominent inward-going rectifying portion.

The potential jump in the steady-state *I–V* curve suggests a 'negative resistance' region. To examine this possibility, a conditioning pulse technique was applied; the membrane was first depolarized to +42 mV by a conditioning pulse (intensity

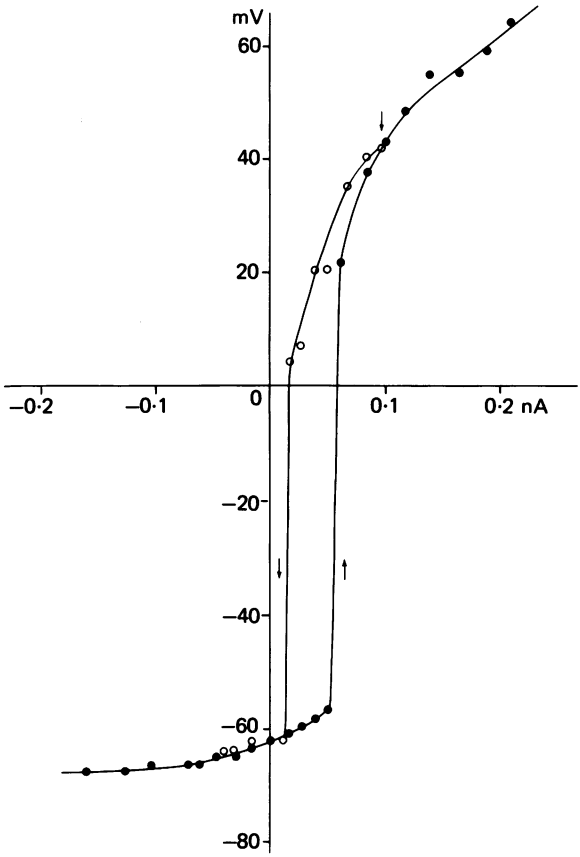


Fig. 11. Steady-state *I–V* relationship in control solution (10 mM-K^+, solution C). The data were obtained from the responses shown in Fig. 10. The membrane potentials were measured at the termination of the current pulse (5·2 sec) and corrections were made for the incomplete compensation of electrode resistance (see Methods). Filled circles and open circles were obtained from the procedures shown in Fig. 10*A* and *B*, respectively. The arrow at +95 pA indicates the intensity of the applied conditioning pulse (see Fig. 10*B*). The slope resistances of the *I–V* curve shown with filled circles were 13 MΩ at −67 mV, 97 MΩ, at −62 mV, 667 MΩ at +30 mV and 153 MΩ at +55 mV. Hysteresis was clearly observed; a depolarizing jump (upward arrow) and a hyperpolarizing jump (downward arrow) were evoked at +50 pA and at +20 pA, respectively.

+95 pA), and then hyperpolarized from that level by test pulses (Fig. 10*B*). The potential at the end of the test pulse was plotted against the current intensity (Fig. 11, open circles). The slope resistance became larger as the cell was hyperpolarized from +42 mV and became nearly infinite at about +5 mV. When a slightly smaller test pulse was applied, a potential jump to −61 mV occurred. Without a conditioning

pulse, a +50 pA test pulse evoked a depolarizing jump, but following a +95 pA conditioning pulse, a +20 pA test pulse evoked a hyperpolarizing jump. Therefore, hysteresis of the *I–V* relationship exists between these two portions, supporting the possibility of 'negative resistance'. A similar hysteresis was obtained in normal solution (solution B).

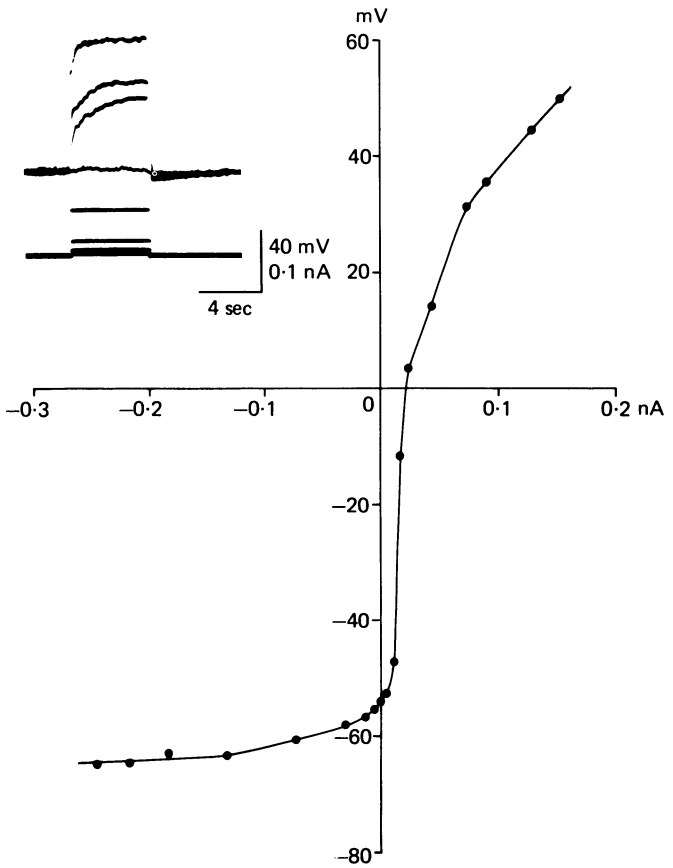


Fig. 12. Steady-state *I–V* relationship in Co^{2+} solution. Action potentials were completely blocked by addition of 4 mM-Co^{2+} to solution C (2·5 mM-Ca^{2+}, 10 mM-K^+). Then, 5 sec pulses were applied, and the *I–V* relationship was examined. The inset shows an example of the records; upper and lower traces indicate voltage and current records respectively. The rising phase of the membrane potential was very slow, indicating the lack of a Ca-dependent action potential. The potentials were measured at the termination of the pulses. Corrections were made for incomplete compensation of electrode resistance (see Methods). The *I–V* relationship was non-linear similar to Fig. 11. This indicates that non-linearity of membrane is caused not only by a Ca-dependent mechanism but also by another mechanism(s).

The potential jump in the steady state is apparently evoked by the Ca-dependent action potential demonstrated earlier in this paper. However, a similar steady-state *I–V* relationship was obtained in the presence of Co^{2+}, where no action potentials were induced (Fig. 12). Thus, it is likely that not only the Ca-conductance mechanism but also another conductance mechanism, for example, a depolarizing K inactivation,

could induce the potential jump on application of a depolarizing current (similar to starfish and tunicate oocytes in Miyazaki, Takahashi & Tsuda, 1974*a*; Miyazaki, Takahashi, Tsuda & Yoshii, 1974*b*; Miyazaki, Ohmori & Sasaki, 1975*a*, *b*).

The non-linear steady-state $I-V$ relationship may explain the long duration of the plateau potential found in solitary horizontal cells. Once the cell produces an action potential, only a small amount of residual inward current or leak current can maintain the membrane potential at the plateau level because the $I-V$ curve during the repolarization is very close to the potential axis at zero injected current. In some cases, the $I-V$ curve during the repolarization crossed the potential axis at zero current and the membrane kept the plateau level until a hyperpolarizing current was applied.

DISCUSSION

Solitary horizontal cells, which had no synaptic connexions with other cells, had resting potentials of about -75 mV in normal solution (2·6 mM-K^+). This value is comparable to the membrane potential of horizontal cells in the intact retina under bright illumination or when synaptic inputs to the horizontal cells were blocked by Co^{2+} or low [Ca^{2+}], high [Mg^{2+}] (Kaneko & Shimazaki, 1975). At rest, solitary horizontal cells showed a selective permeability to K^+, and when $[K^+]_o$ was changed, the membrane potential varied over a wide range with E_K calculated from the Nernst equation. These results strongly indicate that when the endogenous transmitter is absent, the membrane potential of the horizontal cell is determined by the distribution of K^+ across the membrane.

Solitary horizontal cells showed no obvious permeability to Na^+ at rest or during the action potential, while in the intact retina, it is reported that the depolarization of horizontal cells in the dark is caused by an increase of Na-conductance (Kaneko & Shimazaki, 1975; Waloga & Pak, 1978). Such a difference does not necessarily imply that certain properties of the solitary horizontal cell membrane have been changed by the dissociation; it may be explained if the postsynaptic membrane of the horizontal cells is selectively permeable to Na^+ under the influence of the transmitter from photoreceptors. This possibility can be examined when the transmitter from photoreceptors has been identified.

For a long time it was generally accepted that unlike other neurones, photoreceptor-, horizontal- and bipolar cells of vertebrate retina were unable to produce action potentials. However, recently, it has been shown that photoreceptors, both rods and cones, can produce Ca-dependent action potentials (Fain, Quandt & Gerschenfeld, 1977; Fain, Gerschenfeld & Quandt, 1980; Piccolino & Gerschenfeld, 1980; Gerschenfeld & Piccolino, 1980). The action potentials of photoreceptors were very labile, and it was often necessary for the cell to be perfused with a solution containing TEA, Sr^{2+} or Ba^{2+} in order to produce the action potentials. This paper demonstrates that solitary horizontal cells always produce action potentials in normal or high $[K^+]_o$ solution when the membrane potential is depolarized to a critical level by a current pulse. The results of the superfusion experiments strongly indicate that the action potential is Ca-dependent; Sr^{2+}, Ba^{2+} or high [Ca^{2+}] enhanced the action potential, while Co^{2+} or low [Ca^{2+}], high [Mg^{2+}] blocked it; TTX or low [Na^+] did not affect the action potential significantly.

The presence of action potentials in solitary horizontal cells could be related to changes in membrane properties after dissociation, but another possibility remains, namely that all normal horizontal cells have this potentiality but that the action potential is suppressed in the intact retina. It is interesting that some horizontal cells in the turtle retina show action potential-like activities when the cells were perfused with solution containing Sr^{2+} (Piccolino, Neyton & Gerschenfeld, 1980), although it is not clear whether the origin of action potential-like activities is in cones or horizontal cells.

It has been demonstrated that the receptive field of photoreceptors has an antagonistic centre-surround organization (Baylor & Fuortes, 1970; Baylor *et al.* 1971; O'Bryan, 1973; Pinto & Pak, 1974*a*, *b*; Burkhardt, 1977). The antagonistic effect is thought to be mediated by horizontal cells through reciprocal synapses; a hyperpolarizing current injected into the horizontal cell produced a depolarization of the cone membrane (Baylor *et al.* 1971). If the synaptic transmission from horizontal cells to photoreceptors is chemical and is regulated by a voltage-dependent Ca-influx, like that demonstrated for the giant synapse of squid (Katz & Miledi, 1969), the presynaptic terminal of horizontal cells would have the potentiality to produce Ca-dependent action potentials. In this case, the release of chemical transmitter from horizontal cells may occur in the dark: the horizontal cell membrane is depolarized by transmitter released from photoreceptors in the dark (Trifonov, 1968; Byzov & Trifonov, 1968), and thus Ca^{2+} enters horizontal cells resulting in the transmitter release from horizontal cells. Since a depolarization of the photoreceptor cell membrane is caused by a hyperpolarization of the horizonal cell membrane (Baylor *et al.* 1971), the transmitter of horizontal cells may be inhibitory; the depolarization of photoreceptor cell membrane may be explained as a disinhibition. This idea seems to be consistent with the suggestion that γ-aminobutyric acid may be the transmitter of some horizontal cells (Lam & Steinman, 1971; Lam, Lasater & Naka, 1978; Marc, Stell, Bok & Lam, 1978; Lam, Su, Swain, Marc, Brandon & Wu, 1979).

The solitary cells provide a favourable system for measurements of the I–V relationships, because they lack chemical and electrical synaptic inputs. In this paper non-linearity of the solitary horizontal cell membrane was clearly demonstrated. Hysteresis was found in the transitional portion of the I–V curve, and the shape of the curve was not seriously affected by the application of Co^{2+}. These results suggest that at least two active components produce the long-lasting action potential; Ca-conductance mechanism, and another conductance mechanism which seems to be related to K conductance and causes the potential jump between two rectifying portions. The precise ionic mechanisms remain to be studied with the voltage clamp technique. A similar behaviour of the cell membrane has been reported in starfish and tunicate oocytes (Miyazaki *et al.* 1974*a*, *b*, 1975*a*, *b*). It is interesting that a non-linearity and 'negative resistance' of the horizontal cell membrane has been described in the intact retina using a special technique which allows the electrically coupled horizontal cells to be polarized equally (Trifonov *et al.* 1974; Byzov *et al.* 1977).

At present, the physiological signficance of the non-linearity of the horizontal cell membrane is not clear. However, several possibilities are suggested: (1) amplification of the graded potentials generated by the subsynaptic membrane (Byzov *et al.* 1977),

(2) maintenance of the length constant by compensating the conductance decrease of the subsynaptic membrane during the light response (Werblin, 1975), (3) economy of transmitter release from photoreceptors since only a small amount of current is sufficient to keep the horizontal cell membrane depolarized, (4) delay of initiating the feed-back effect on photoreceptors due to hysteresis, (5) improved discrimination under light adaptation, that is, the conductance of the postsynaptic membrane is small due to the decrease of transmitter release from photoreceptors under light adaptation; thus the non-linearity of the horizontal cell membrane becomes explicit, resulting in the sharper slope of light intensity-response curve (ref. Normann & Perlman, 1979).

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