

Structural and Functional Properties of Two Types of Horizontal Cell in the Skate Retina

ROBERT PAUL MALCHOW, HAOHUA QIAN, HARRIS RIPPS,
and JOHN E. DOWLING

From the Lions of Illinois Eye Research Institute, Department of Ophthalmology, the Department of Anatomy-Cell Biology, University of Illinois College of Medicine, Chicago, Illinois 60612; and the Department of Cellular and Developmental Biology, Harvard University, Cambridge, Massachusetts 02138

ABSTRACT Two morphologically distinct types of horizontal cell have been identified in the all-rod skate retina by light- and electron-microscopy as well as after isolation by enzymatic dissociation. The external horizontal cell is more distally positioned in the retina and has a much larger cell body than does the internal horizontal cell. However, both external and internal horizontal cells extend processes to the photoreceptor terminals where they end as lateral elements adjacent to the synaptic ribbons within the terminal invaginations.

Whole-cell voltage-clamp studies on isolated cells similar in appearance to those seen in situ showed that both types displayed five separate voltage-sensitive conductances: a TTX-sensitive sodium conductance, a calcium current, and three potassium-mediated conductances (an anomalous rectifier, a transient outward current resembling an A current, and a delayed rectifier). There was, however, a striking difference between external and internal horizontal cells in the magnitude of the current carried by the anomalous rectifier. Even after compensating for differences in the surface areas of the two cell types, the sustained inward current elicited by hyperpolarizing voltage steps was a significantly greater component of the current profile of external horizontal cells. A difference between external and internal horizontal cells was seen also in the magnitudes of their TEA-sensitive currents; larger currents were usually obtained in recordings from internal horizontal cells. However, the currents through these K^+ channels were quite small, the TEA block was often judged to be incomplete, and except for depolarizing potentials $\geq +20$ mV (i.e., outside the normal operating range of horizontal cells), this current did not provide a reliable indicator of cell type.

The fact that two classes of horizontal cell can be distinguished by their electrophysiological responses, as well as by their morphological appearance and spatial distribution in the retina, suggests that they may play different roles in the processing of visual information within the retina.

Address reprint requests to Dr. Harris Ripps, Department of Ophthalmology, University of Illinois College of Medicine, 1855 West Taylor Street, Chicago, IL 60612.

INTRODUCTION

Much of our present knowledge concerning the organizational features and adaptive properties of the vertebrate retina stems from studies on the S-potential, an intracellularly-recorded, light-evoked response derived from horizontal cells (MacNichol and Svaetichin, 1958). These second-order neurons help to shape the receptive field of more proximal elements and serve to modulate signal transmission from photoreceptors to bipolar cells (cf. Kaneko, 1987). In the mixed (rod-cone) retinæ of some species, various strategies have evolved to segregate at the horizontal cell level the responses arising from the various cone types, and to maintain separate pathways for rod- and cone-mediated signals (Stell, 1967; van Haesendonck and Missotten, 1979; Boycott, 1988; but cf. Witkovsky et al., 1988). In the cyprinid fish, for example, there appear to be several types of cone-driven horizontal cells, and only a single class of horizontal cell associated with the rod pathway (Mitarai et al., 1974; Stell, 1975; Hashimoto et al., 1976), whereas in cat the dendrites of its two types of horizontal cell insert only into cone pedicles while the electrically isolated terminal expansions of the axon-bearing horizontal cells send processes to the bases of rod spherules (Kolb, 1974).

The retinæ of some species of skate contain only a single class of rod photoreceptor (Dowling and Ripps, 1970; Szamier and Ripps, 1983), which might suggest the presence of only one type of horizontal cell. However, Stell and Witkovsky (1973) have stressed the likelihood that two types of horizontal cell are to be found in skate, and careful histological examination of the retina has confirmed the presence of two morphologically distinct cell types (Sakai et al., 1986). One is cuboidal in shape and located at the distal margin of the inner nuclear layer, and the other is a slender elongated element situated just proximally to the distal horizontal cell; examination after intracellular injection of HRP (horseradish peroxidase) reveals that neither cell type emits an axon (Sakai et al., 1986). The significance of this morphological arrangement is not known, nor is it evident that the differences in appearance represent functionally distinct classes of horizontal cell.

We have attempted in this study to address both of these issues; i.e., to determine whether the dendritic processes of these cells are organized differently within the outer plexiform (synaptic) layer, and whether the two cell types can be distinguished by their electrophysiological properties. Accordingly, we have traced, in serial electron microscopy sections of Golgi-impregnated cells, processes of the two forms of horizontal cell and examined their relation to bipolar cell dendrites and the synaptic complex within the receptor terminal. In addition, we have used enzymatic and mechanical dissociation of the retina to obtain isolated cells that were structurally similar to the two classes of horizontal cell seen in the intact retina. Whole-cell voltage-clamp recording enabled us to identify the voltage-sensitive currents of these cells, and to compare their electrical characteristics.

MATERIALS AND METHODS

Skate (*Raja erinacea* and *Raja ocellata*) were obtained from the Marine Biological Laboratory at Woods Hole, MA., and maintained for periods of up to 1 mo in a tank of circulating artificial seawater held at 14°C; a 12-h light-dark cycle was used. Prior to enucleation, animals

were anesthetized with 0.02% Tricane (MS 222; Sigma Chemical Co., St. Louis, MO), and pithed.

Electron Microscopy

Eyes were hemisected, and the posterior half of the globe was partially drained of vitreous with paper toweling before immersion in cold 2% OsO₄ in veronal acetate buffer (pH 7.8) containing 1% CaCl₂ and 40 mg/ml sucrose. After ~20 min in the cold, fixation was continued for 1 h at room temperature. The fixed tissue was dehydrated in graded ethanol-water mixtures, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined in a JEOL 100B electron microscope.

Golgi Impregnation

Pieces of eyecup were fixed for 1 h in 2.5% glutaraldehyde in 0.05 M sodium phosphate buffer, and then processed by the Golgi triple impregnation method as modified by Stell (1965). After silver impregnation, the tissue was embedded in collodion, sectioned at 90 μm, and mounted on glass slides. Individual well-impregnated cells were identified by light microscopy, and sections containing selected cells were reembedded in epoxy resin. Serial thin sections were cut, mounted on slot grids, and after staining with lead citrate, examined in the electron microscope.

Cell Dissociation

Pieces of neural retina were separated from the eyecup, and immersed for 60–75 min either in 10 ml Ringer's solution containing 5 mg papain (P4762; Sigma) and 8 mg *l*-cysteine (C7880; Sigma) pH 7.7, or in 20 ml Ringer containing 45 mg of papain (5125; Calbiochem-Behring Corp., La Jolla, CA), 30 mg *l*-cysteine, pH 7.7. The Ringer's solution contained (in millimolar): 250 NaCl, 6 KCl, 20 NaHCO₃, 1 MgCl₂, 4 CaCl₂, 0.2 NaH₂PO₄, 360 urea, 10 glucose, 5 *N*-2 hydroxyethyl-piperazine-*N'*-2-ethanesulfonic acid (HEPES), and was adjusted to pH 7.7 with NaOH. Retinae were rinsed a minimum of four times with Ringer and placed in a plastic culture tube containing ~1 ml of culture medium (L-15; GIBCO, Grand Island, NY), to which was added 102.7 mM NaCl, 350 mM urea, 5 mM glucose, and 20 mM HEPES, pH 7.7. The tissue was triturated through a flame-polished Pasteur pipette, and after several passes, was allowed to settle to the bottom of the tube. Aliquots of the supernatant containing isolated cells were placed in 35-mm petri dishes that contained 1 ml of the modified Leibowitz solution and one drop of penicillin-streptomycin solution (600-5070; GIBCO). Trituration of the tissue was repeated up to 120 times, and aliquots were drawn after each series of ~20 passages and examined for cellular content. Dishes containing good yields of horizontal cells were kept at 14°C for periods of 30 min to 5 d; no differences in results could be attributed to length of incubation. Before each experiment, the media was replaced with the standard Ringer's solution. An inverted microscope equipped with Hoffman contrast modulation optics and a video monitoring system was used to visualize the cells during the electrical recording procedures; the microscope, perfusion system, and associated microdrives were mounted on an air isolation table to reduce mechanical vibration.

Electrophysiology

Electrical recordings were obtained from external and internal horizontal cells using the whole-cell version of the patch-clamp recording technique (Hamill et al., 1981). Pipettes with tip diameters of ~1 μm were pulled from Kovar capillary tubing (i.d. 1.15 mm, o.d. 1.65 mm, #7052; Garner Glass, Claremont, CA) using a BBCH puller (World Precision Instruments, Inc., New Haven, CT) and were used without flame polishing. The recording pipettes were

filled with a solution containing 204 mM KCl, 1 mM CaCl₂, 11 mM EGTA, 2 mM MgCl₂, 2 mM MgATP, and 10 mM HEPES, pH 7.6, and connected via a chlorided silver wire to a List EPC7 patch-clamp amplifier modified to increase the capacitance compensation by a factor of two (Medical Systems, Great Neck, NY); the return circuit was via a scintered silver-silver chloride electrode connected to the culture dish by a Ringer-filled glass capillary. Three-dimensional remotely controlled manipulators (Narashige, Tokyo, Japan) were used to advance the pipette to the cell membrane. GΩ seals formed readily on the horizontal cells, often requiring little or no suction. After 30 s, the seal was broken via a combination of light suction and 1 V pulses of 100 ms duration, and a whole-cell recording configuration was achieved; for most of the recordings to be described, test-pulse responses were used to compensate for the series resistance of the electrode and cell membrane capacitance. With optimal compensation, the capacitative current settled within 2–4 ms. Moreover, due to the size of skate horizontal cells and the extent of their dendritic processes, the quality of the voltage clamp was less than perfect. This feature was examined in three external horizontal cells by monitoring the voltage with a second intracellular electrode; under these conditions, the fidelity of the clamp varied from 97 to 92% with the two electrodes separated by 50–70 μm. Preliminary experiments using intracellular electrode measurements of clamp voltage at two recording sites indicated that an accuracy of ~92% was achieved between extreme regions of the perikaryon; it was probably less accurate at the ends of the processes. The clamp procedure was controlled using a commercially available program (pClamp: version 4; Axon Instruments, Inc., Burlingame, CA) run on an IBM AT computer equipped with the 40-kHz version of the Labmaster analog interface (Scientific Solutions Inc., Solon, OH). Data were stored on a video cassette recorder (Panasonic PV-1564) after conversion through a PCM recording adaptor (R 20 PCM; A. R. Vetter, Co., Rebersburg, PA).

Drugs were added at various concentrations to the normal Ringer's solution and introduced into the culture dish via a gravity-fed superfusion system at a rate of ~5 ml/min; perfusate was removed via a suction-operated siphon. The agents used included tetrodotoxin (TTX), tetraethylammonium chloride (TEA), 4-aminopyridine (4-AP), cesium chloride, and cobalt chloride; 4-AP was obtained from Pfaltz & Bauer, Inc., Stamford, CT, all other chemicals were from Sigma Chemical Co.

RESULTS

Horizontal Cell Morphology

Fig. 1 shows light micrographs of cells that correspond to the two types of horizontal cell described by Sakai et al. (1986). The external horizontal cell, characterized by its large cell body and its distal location within the inner nuclear layer, is shown in the Golgi preparations of Figs. 1, A and D. Short dendritic branchlets emerge directly from the cell body of external horizontal cells, and appear to extend tufted or knob-like endings into the rod terminals (Fig. 1 D). The second cell type, termed the internal horizontal cell, is depicted in Fig. 1 B. These cells have much thinner cell bodies, are situated just proximal to the external horizontal cell, and send thick processes distally that must course around the perikarya of the external horizontal cells to reach the outer plexiform layer (Fig. 1 C). As the processes approach the photoreceptor terminals they emit fine branches that run toward the synaptic bases of the visual cells (Fig. 1 B).

Fig. 2 shows a low power electron micrograph that extends proximally from the receptor terminals (RT) to the inner plexiform layer (IPL). Two large external hor-

horizontal cells (*EH*) and a thinner internal horizontal cell (*IH*) occupy much of the thickness of the inner nuclear layer. Below the internal horizontal cell is an amacrine cell (*AC*) and axonal processes of bipolar cells (*B*). In skate, as in other cold-blooded vertebrates, the axonal processes of bipolar cells often run obliquely through the proximal part of the inner nuclear layer and, as here, are seen frequently in cross section (Dacheux, 1982; Ehinger et al., 1988).

The inset to Fig. 2 shows at higher magnification the synaptic region of a photoreceptor terminal in skate. Processes from horizontal and bipolar cells penetrate into a single large invagination, and two types of specialized junctions are observed

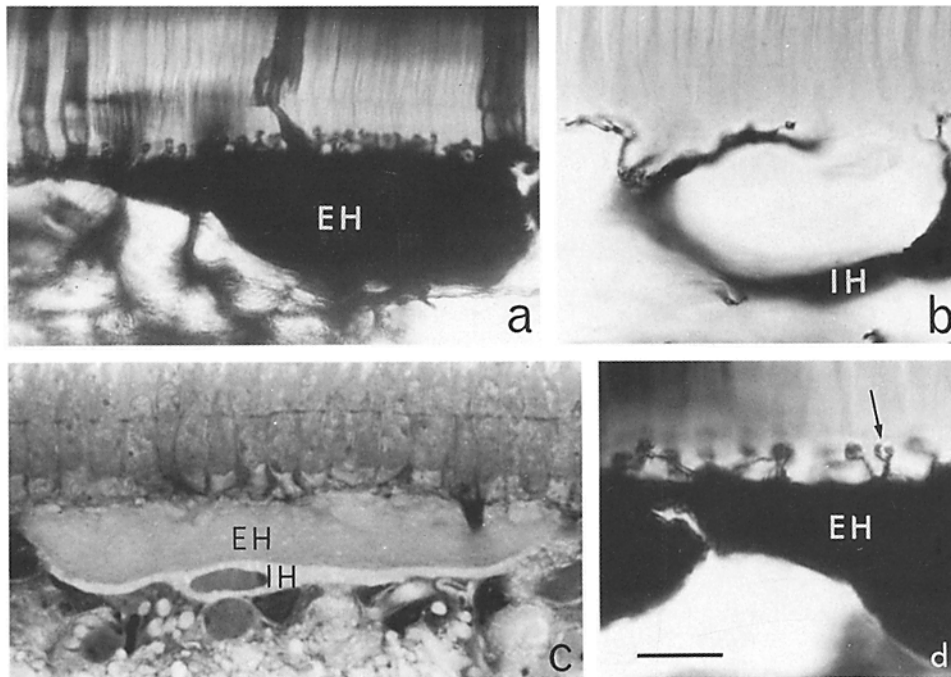


FIGURE 1. Light micrographs of transverse sections through the skate retina showing Goigi profiles (*a*, *b*, and *d*) and an unstained section (*c*) of external (*EH*) and internal (*IH*) horizontal cells. The areas include the outer nuclear layer, the synaptic endings of the photoreceptors, and the distal portion of the inner nuclear layer. Note the knob-like endings (*arrow*) emanating from a dendritic branchlet of the external horizontal cell. Bar = 10 μm in *a*, *b*, and *c*; 5.8 μm in *d*.

within the invagination: ribbon synapses (*arrowheads*) and superficial junctions (*arrow*) (Dowling, 1974). The processes positioned around the circumference of the invagination, lateral to the synaptic ribbons, have the granular cytoplasmic appearance typical of horizontal cells and their processes (*H*). The elements making the superficial type of junction usually have a clearer cytoplasm and are thought to be bipolar cell dendrites (*B*). Note the electron dense material (*arrow*) in the presynaptic cytoplasm that marks the superficial junctions in the skate.

Confirmation that the elements positioned laterally to the synaptic ribbons are

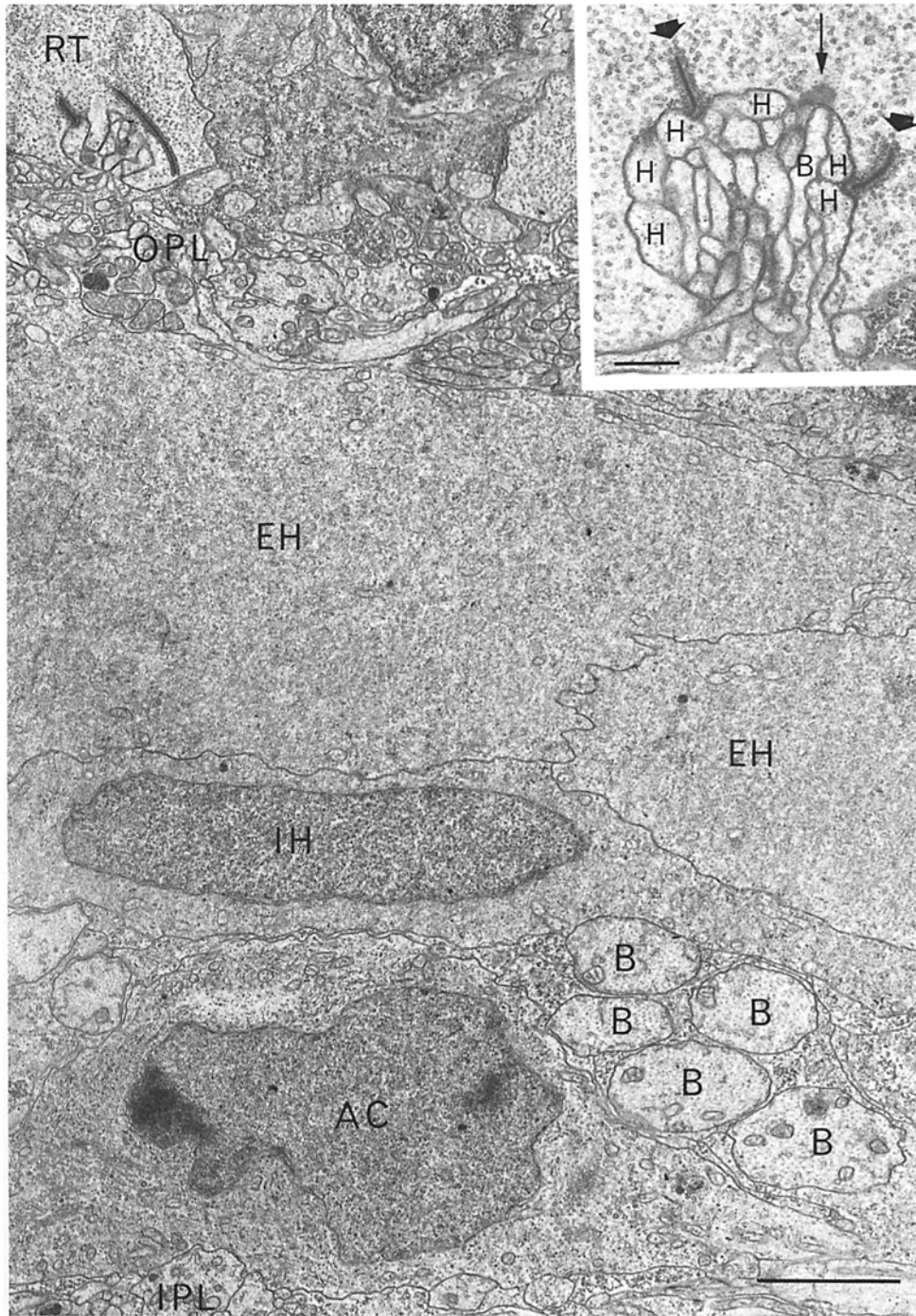


FIGURE 2

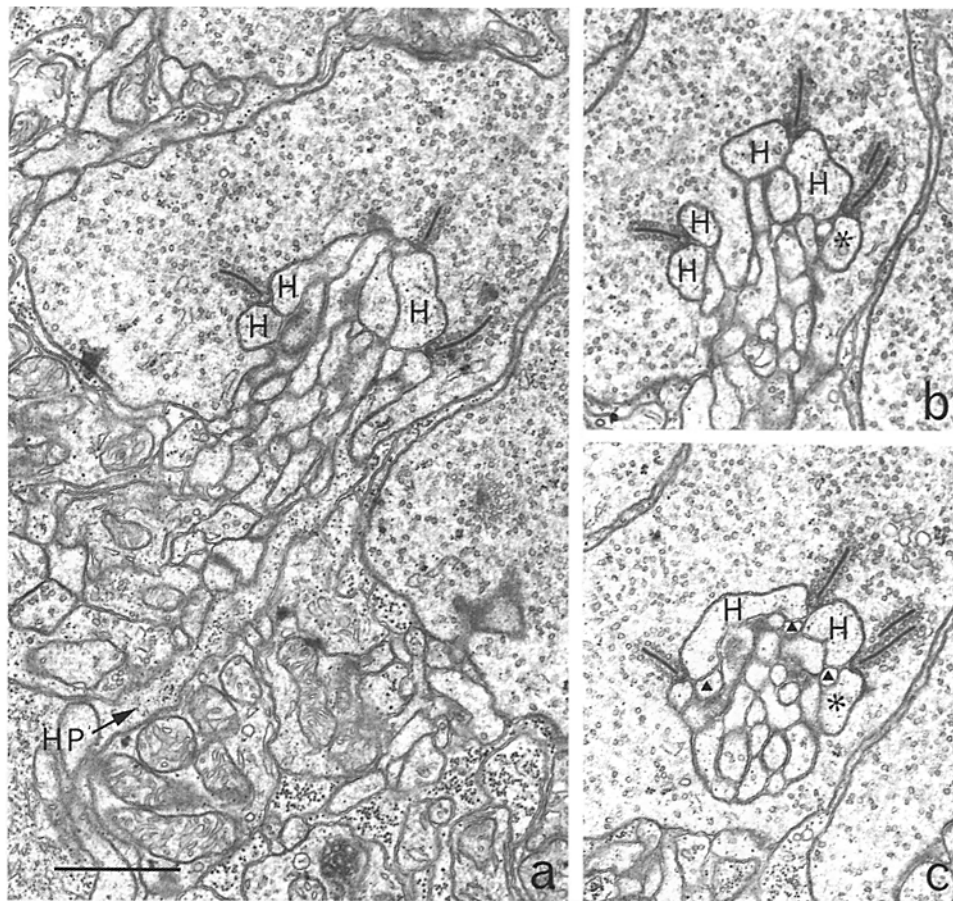


FIGURE 3. Selected serial sections through a skate photoreceptor terminal. The arrow in *a* indicates a process from a horizontal cell perikaryon (*HP*) that extends into the terminal invagination. In *b* and *c*, the identified process (*) is observed lateral to one of the synaptic ribbons. *H*, horizontal cell processes; ▲, presumed bipolar cell dendrites. Bar = 1 μ m.

derived from external horizontal cells was obtained by serial-section analysis; two serial reconstructions were performed, and processes of three horizontal cells were traced to their endings in the receptor terminal. Fig. 3 *A* shows the third section of a series in which a process from an external horizontal cell extended into the invagination of a receptor terminal and ended just short of a lateral process adjacent to

FIGURE 2. Electron micrograph of the skate retina showing a region extending from the receptor terminals (*RT*) to the inner plexiform layer (*IPL*). Included are the outer plexiform layer (*OPL*), external horizontal cell (*EH*), amacrine (*AC*) and internal horizontal (*IH*) cells, and bipolar cells axons (*B*) seen in cross section. Bar = 2 μ m. (*Inset*) The processes of second-order cells within the invagination of a photoreceptor terminal. *H*, horizontal cell processes; *B*, a bipolar cell dendrite; *arrowheads*, synaptic ribbons; *arrow*, a superficial junction. Bar = 0.5 μ m. See text for details.

one of the three synaptic ribbons observed in this terminal. By the sixth section of the series (Fig. 3 *B*), the identified horizontal cell process had pushed further into the invagination and was clearly lateral to one of the synaptic ribbons (*). Note that both in this section and in the eleventh section of the series (Fig. 3 *C*), one horizontal cell process may be lateral to two synaptic ribbons. Finally, small clear processes (*filled triangles* in Fig. 3 *C*) that are positioned more proximally in the invagination can be seen to lie between the lateral elements flanking the synaptic elements. These processes may be bipolar dendrites, completing the well-known triadic arrangement of processes postsynaptic to ribbons in the photoreceptor terminals of other species (Missotten, 1965; Stell, 1965, 1967; Dowling and Boycott, 1966).

The processes of internal horizontal cells also terminate lateral to the synaptic ribbons within the receptor terminal invaginations, as revealed by serial section analysis of Golgi-impregnated retinas. Fig. 4 *A* shows a low power electron micrograph of a portion of a Golgi-stained internal horizontal cell under a large, unstained external horizontal cell (*EH*). A process from the filled cell extends into the outer plexiform layer (*arrowhead*), and two small stained processes are seen at the level of the receptor terminals (*arrows*). Fig. 4 *B* presents a higher power micrograph of a filled process lateral to a synaptic ribbon in a receptor terminal, and Fig. 4 *C* shows a terminal containing two filled processes, both of which are lateral to synaptic ribbons. Although only one internal horizontal cell was studied in serial sections, six of the processes emanating from that cell were traced to their terminations, where they ended lateral to the synaptic ribbons.

Isolated Horizontal Cells

Horizontal cells isolated from the enzymatically dissociated skate retina are shown in Fig. 5. The cell in Fig. 5 *A* is characterized by an extremely large cell body and multiple club-like extensions that emanate from the soma. The cytoplasm appears relatively agranular, cellular organelles are difficult to discern, and even the nucleus is not usually evident. This cell closely resembles the one pictured and described by Lasater et al. (1984) in their investigation of the pharmacological properties of skate horizontal cells. A comparison with the description given by Sakai et al. (1986) and with the observations presented earlier leads us to suggest that this cell can be classified as an external horizontal cell.

A second type of cell isolated by the dissociation procedure is shown in Fig. 5 *B*. The cell bodies of these elements give a smaller, flatter appearance than the external horizontal cells, and their relatively long dendritic processes taper as they extend from the perikaryon. The nucleus, usually very prominent, seems to push out against the cell membrane, forming almost a small hillock. In overall form, these cells closely resemble the GAD-positive cells observed by Brunken et al. (1986), and tentatively identified by them as internal horizontal cells. Throughout the remainder of this paper we will refer to cells resembling those seen in Fig. 5, *A* and *B* as external and internal horizontal cells, respectively.

Voltage-Clamp Recordings

During the course of this study, 53 external horizontal cells and 46 internal horizontal cells were studied using the whole-cell voltage-clamp technique. The average

resting potential for the external horizontal cells was 69.7 ± 7 (SD) mV; the mean for internal horizontal cells was 69.5 ± 14 mV. External horizontal cells had an average capacitance of 127 ± 36 pF, while the value for internal horizontal cells was 62 ± 13 pF. Using a value of $1.77 \mu\text{F cm}^{-2}$ for the specific membrane capacitance

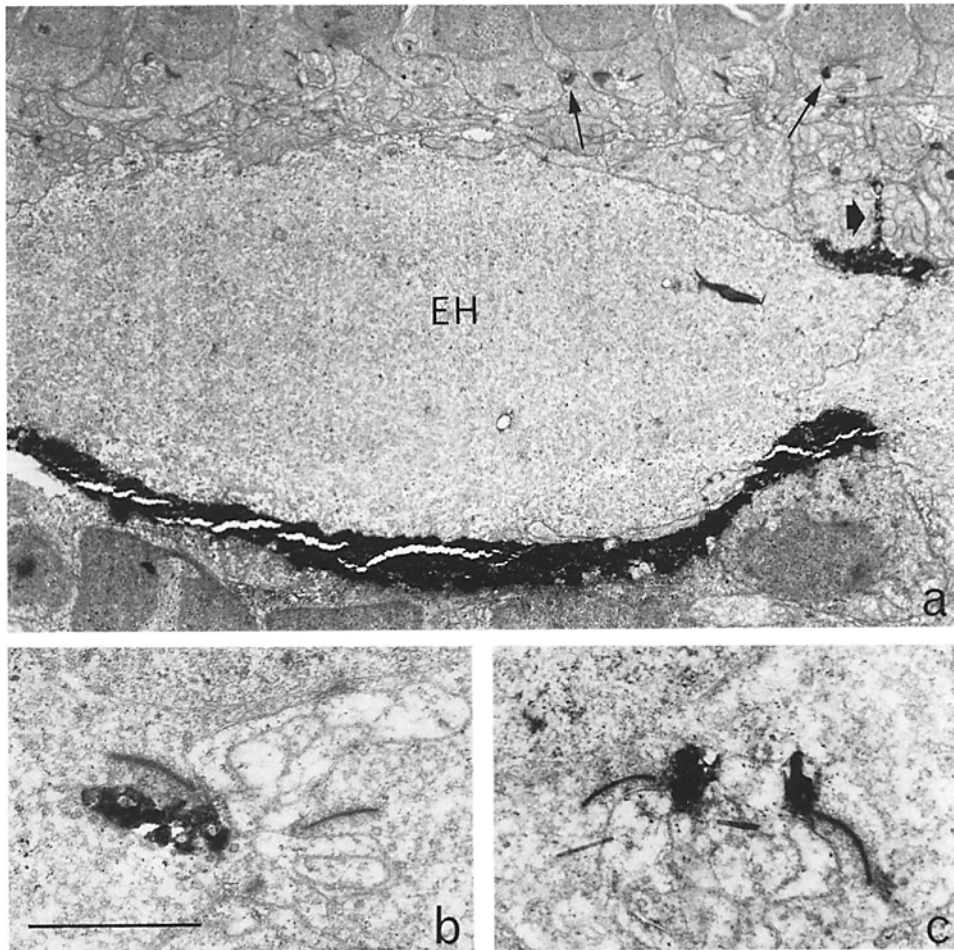


FIGURE 4. Electron micrographs showing in *a*, a Golgi-impregnated internal horizontal cell. Arrows point to stained processes in receptor terminals, the arrowhead to a process of the impregnated cell in the outer plexiform layer. *EH*, an external horizontal cell. (*b* and *c*) Processes from the impregnated cell end as elements lateral to the synaptic ribbons within the photoreceptor terminals. Bar = $6 \mu\text{m}$ in *a*, $1 \mu\text{m}$ in *b* and *c*.

(Newman, 1985), these values lead to estimated surface areas of $7,158$ and $3,491 \mu\text{m}^2$ for external and internal horizontal cells, respectively. These values compare favorably with the mean surface areas of $6,690 \pm 2,240 \mu\text{m}^2$ (external, $N = 22$) and $4,790 \pm 1,720 \mu\text{m}^2$ (internal, $N = 40$) calculated from light micrographs of isolated cells.

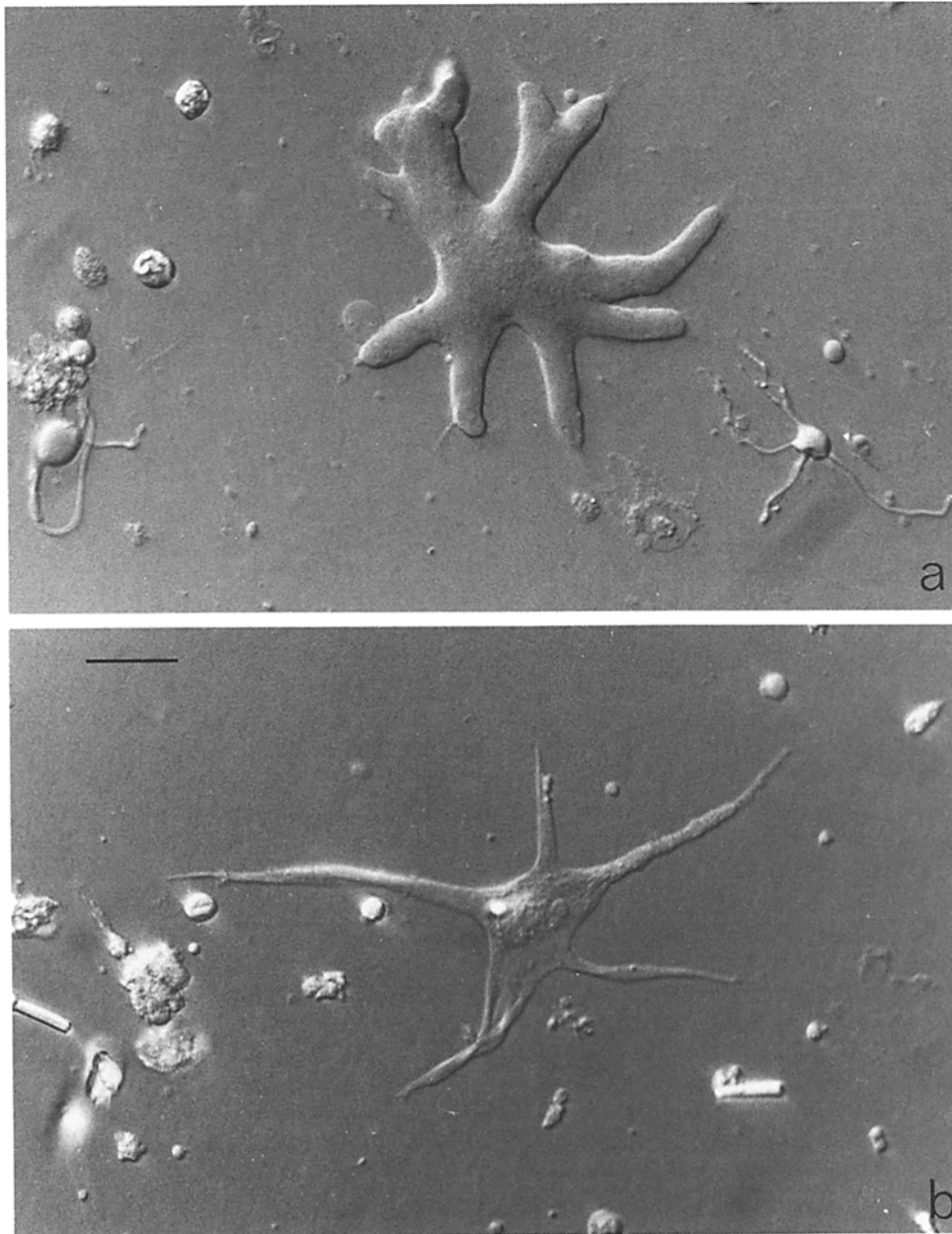


FIGURE 5. Isolated internal and external horizontal cells viewed with Hoffman modulation optics. (A) An example of an external horizontal cell. Note the large cell body, the relatively clear cytoplasm, and the stubby dendritic processes from which fine branchlets emerge. (B) An internal horizontal cell, immediately distinguishable from the external horizontal cells by the smaller perikaryon, the prominent nucleus, the graceful tapering of the dendritic processes, and the greater granularity of the cell cytoplasm. Bar = 25 μm .

Ionic Currents

By far the largest current observed in both cell types was a transient outward current that appeared when the voltage was jumped from a holding potential of -70 to -30 mV (Fig. 6 A), and increased linearly with further depolarization. This current appears to be similar to the A current described by Conner and Stevens (1971), and seen in recordings from horizontal cells of carp (Tachibana, 1983; Kaneko and Tachibana, 1985) and perch (Lasater, 1986). In agreement with their observations, we found that the current could be selectively blocked by 10 mM 4-AP (Fig. 6 B). In

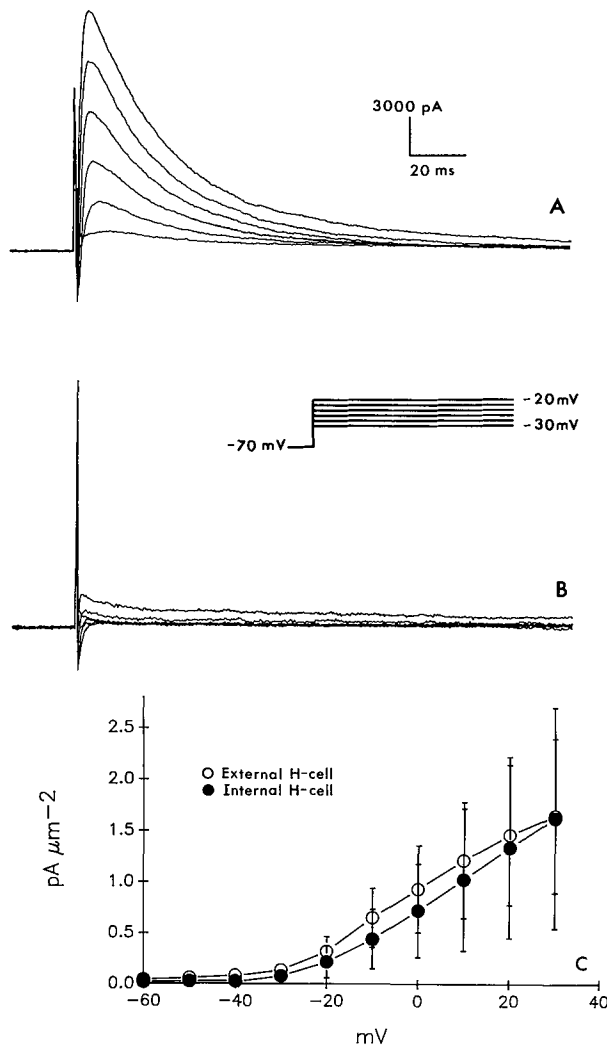


FIGURE 6. Currents blocked by 4-AP. (A) Current responses recorded under voltage clamp from an external horizontal cell superfused in Ringer; the voltage protocol is shown in the inset. Responses to commands from -30 to $+20$ mV from a holding potential of -70 mV are shown; the interval between command pulses was 2 s. After the surge in capacitive current (initial upward deflection), a fast transient inward current was observed, after which a large transient outward current was seen. (B) Current responses from the same cell using the same protocol after the cell had been superfused with 10 mM 4-AP for 2 min. The capacitive spike and the transient inward current are still present, but the transient outward current has been abolished. (C) I - V relationship of the current blocked by 4-AP. Open circles reflect values for external horizontal cells, while the values for internal horizontal cells are shown as filled circles. For each cell, currents obtained while superfusing 4-AP were subtracted from currents recorded during Ringer

superfusion; the peak currents were then determined, corrected for the estimated membrane area of the cell, and averaged with the results from other cells. The graph presents the results obtained from 9 internal and 13 external horizontal cells; error bars in this and subsequent figures represent standard deviations.

addition, when the holding potential was at -40 mV, depolarizing pulses were either ineffective or produced only small transient outward currents (data not shown). In general, the external horizontal cells generated larger peak outward currents, but the distinction disappeared when the data were corrected for differences in membrane area; i.e., the voltage-gated currents expressed in $\text{pA } \mu\text{m}^{-2}$ were not significantly different (Fig. 6 C).

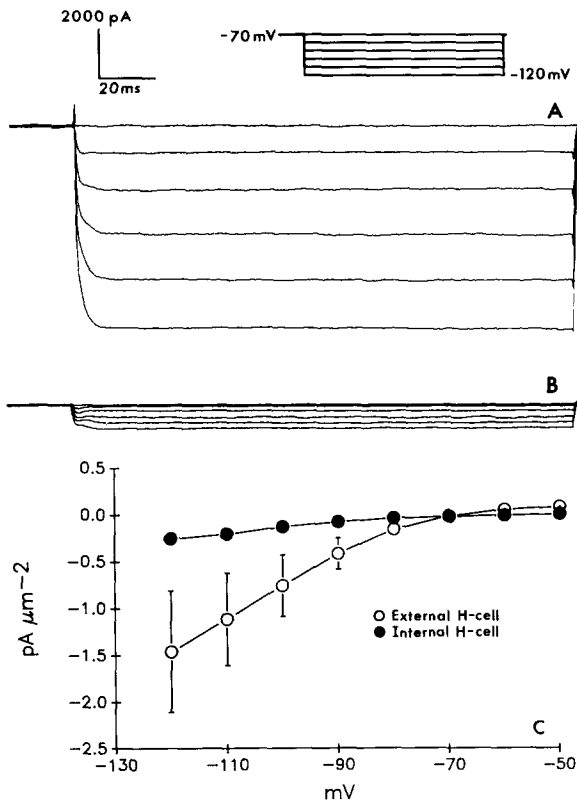


FIGURE 7. Currents produced by hyperpolarizing voltage pulses in external and internal horizontal cells. (A) Currents from one external horizontal cell. The holding potential of -70 mV was stepped from -120 to -70 mV in 10 -mV increments; 2 -s intervals separated the command pulses. After a brief capacitive spike (upward deflection), sustained inward currents were recorded; the responses turned off rapidly upon return to the holding potential. (B) Currents observed in an internal horizontal cell using the same protocol as described in A. No capacitive spike is observable in this set of traces. (C) I - V relationship for the steady inward currents produced by external horizontal cells (*open circles*) and internal horizontal cells (*closed circles*). The current induced by a specific voltage from a cell was averaged

from the middle of the trace to just before the return to the holding potential; these values were then corrected for the estimated membrane areas, and then averaged between cells. The data represent results obtained from 9 internal and 13 external horizontal cells.

Fig. 7, A and B, shows that hyperpolarizing voltage pulses elicited in both cell types sustained inward currents that mimicked the behavior of the anomalous rectifier current observed in a variety of other neuronal elements (Hodgkin and Horowitz, 1959; Hagiwara and Takahashi, 1974). The current showed no sign of inactivation during pulses lasting 6 s, and was almost completely blocked by bath application of 10 mM cesium chloride (not shown); at this concentration of cesium, there was also a slight reduction (10%) in the amplitude of the transient outward current. The current carried by the inward rectifier was activated at potentials > -70 mV, and over the range of -70 to -120 mV it increased monotonically with voltage. However, there is clearly a dramatic difference in the magnitude of the

currents induced in the two cell types. Even when differences in membrane area are taken into account, the *I-V* curves of Fig. 7 *C* show that the anomalous rectifier was much more prominent in external horizontal cells than in internal horizontal cells (Fig. 7 *C*).

Both internal and external horizontal cells exhibited fast early inward currents activated by depolarizing potentials exceeding ~ -30 mV. The current was selectively blocked by 1 μ M TTX, without significantly affecting other currents. Fig. 8 *A* shows the currents blocked by TTX; the curves were derived by algebraic subtraction of currents recorded before and after the introduction of 1 μ M TTX. Current-voltage data for two external horizontal cells are shown in Fig. 8 *B*; they indicate that the transient inward current was maximal at ~ 0 mV, and declined gradually with further depolarization. These results, coupled with the fact that replacing the sodium chloride in the Ringer solution with choline chloride markedly reduced the fast inward current (data not shown) are a good indication that the current is mediated by the opening of a sodium conductance. However, the magnitude of the current varied considerably from one horizontal cell to the next (Fig. 8 *B*), and in some cells it was not detectable at all (cf. Fig. 10 *A*). The latter finding may reflect inadequacy of the voltage clamp in this temporal domain, or perhaps masking by the very large outward currents associated with these cells. Because of these difficulties, a careful comparison between cell types was not attempted.

A current that could be blocked by 4 mM cobalt chloride or 100 μ M cadmium chloride was detected in both cell types when sodium and potassium currents were pharmacologically suppressed. Current recordings were obtained first with the cell bathed in a solution containing 4-AP, cesium, TEA, and TTX, and again after the addition of either cobalt or cadmium. Since both sets of current recordings were contaminated by leakage currents, the cobalt-sensitive current was obtained by subtracting currents at corresponding voltages recorded before and after the introduction of cobalt; the resultant data are shown in Fig. 9 *A*. The *I-V* curves of Fig. 9 *B* are typical of calcium-mediated currents seen in other preparations (Tsien, 1986); activation of the current occurred when the cell was depolarized beyond -40 mV, and maximal currents were elicited at ~ 0 – 10 mV. The voltage activation profile of this current and its sustained quality over time suggest that it may reflect the opening of L-type calcium channels (Fox et al., 1987). Further depolarization reduced the current, and in some cells there was a reversal to an outward current; this was quite variable, however, and may have been due to incomplete blockage of the sustained outward current described below. The averaged data for four internal and six external horizontal cells, shown in Fig. 9 *B*, indicate that there was no significant difference in the calcium-mediated current between the two types of horizontal cell.

In addition to the foregoing, a relatively small sustained outward current was seen in response to membrane depolarization (Fig. 10 *A*). Although 20 mM TEA could reduce the magnitude of this outward current (Fig. 10 *B*), the amount of reduction varied considerably from cell to cell. Subtracting the voltage-clamped currents obtained before and after TEA reveals the magnitude of this K^+ -mediated current (Fig. 10 *C*). In addition, there is a small transient component at the beginning of the trace that reflects perhaps a slight effect of TEA on the A-type current. The averaged *I-V* curves of the TEA-sensitive currents for external and internal horizontal

cells (Fig. 10 *D*) indicates that it appears at potentials greater than ~ -20 mV, and increases with further membrane depolarization. Although seen in both cell types, the internal horizontal cell typically exhibited larger current responses. However, the distinction is questionable owing to the small size of the current, and the fact that the TEA block appeared to be less than complete in most cells.

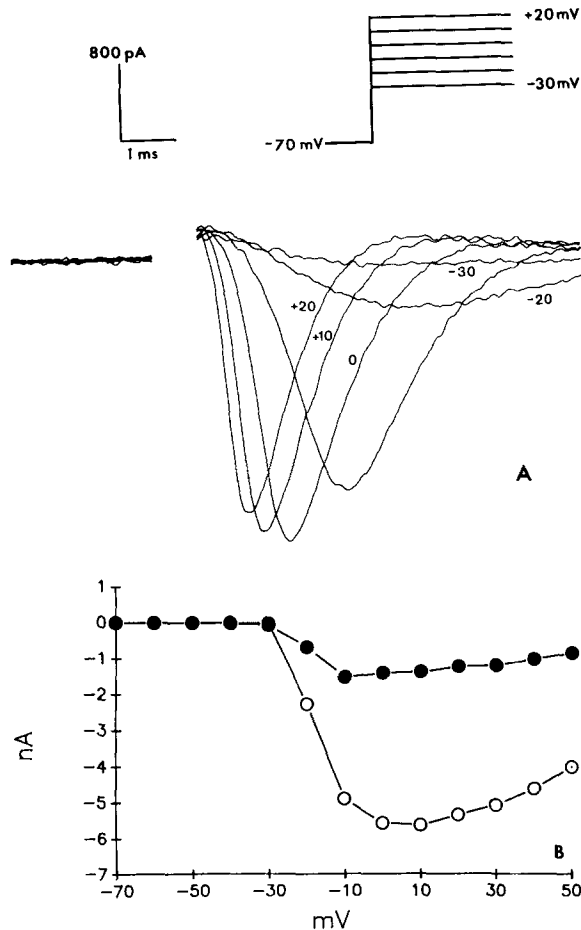


FIGURE 8. Current blocked by TTX. (A) The tracings show for one external horizontal cell the resultant currents that were obtained after responses recorded in $1 \mu\text{M}$ TTX were subtracted from those recorded in normal Ringer. The capacitive artifact (gap between the traces) has been removed for clarity. The figure illustrates results to voltage steps ranging from -20 to $+30$ mV in 10-mV increments; the holding potential was -70 mV and 2-s intervals intervened between successive commands. (B) *I-V* profile for the TTX-sensitive current from two external horizontal cells shows the variability in expression of this current. Data points represent the subtraction of currents in TTX from currents obtained while the cell was superfused in Ringer. Note that the ordinate is in nanoamperes and has not been adjusted to compensate for membrane area.

DISCUSSION

Our results show that two cell types having the morphological features of the external and internal horizontal cells described previously by Sakai et al. (1986) can be identified both *in situ* (Fig. 1) and after enzymatic dissociation of the skate retina (Fig. 5). The external (more distally placed) horizontal cells are cuboidal in shape, considerably larger than the internal horizontal cells, and send many short branchlets toward the bases of the visual cells. The internal horizontal cells appear to line the proximal border of the external elements, present a much narrower profile, and

extend long processes that intercalate between the external horizontal cells to reach the photoreceptors. Both cell types appear to make similar synaptic connections with the photoreceptors, i.e., the processes of both end as lateral elements adjacent to synaptic ribbons (Figs. 3 and 4).

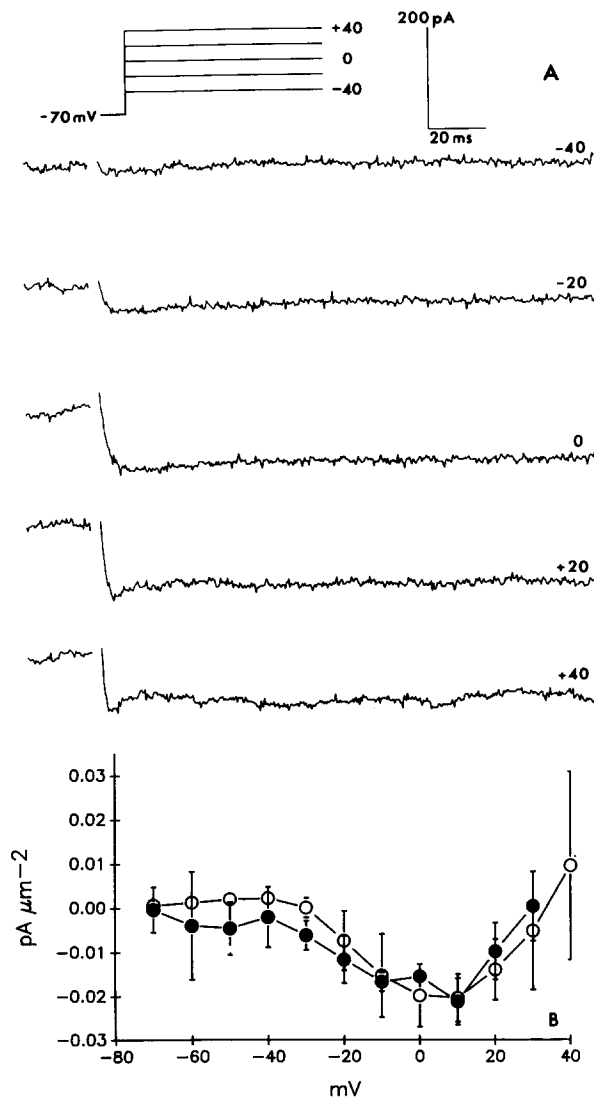


FIGURE 9. (A) Currents blocked by cobalt in an internal horizontal cell. To obtain these records, currents recorded in the presence of 10 mM 4-AP, 10 mM cesium, 20 mM TEA, 1 μM TTX, and 4 mM cobalt were subtracted from traces obtained with all of the above blockers except for cobalt. The capacitance artifact has been eliminated and the traces have been displaced for clarity. (B) *I-V* profile for the cobalt-blocked currents obtained from six external horizontal cells (*open circles*) and four internal horizontal cells (*filled circles*). Currents were recorded as described in A in the absence and presence of 4 mM cobalt. The data were obtained by averaging currents just before the return to the holding potential, subtracting the values obtained before and after cobalt, and then correcting for membrane area; error bars represent standard deviations. Inward currents are activated at potentials more depolarized than -40 mV, reach a maximum between 0 and $+10$ mV, and then become smaller, sometimes reversing in sign as the depolarization is increased.

There is only one class of receptor in the skate retina (Dowling and Ripps, 1970; Szamier and Ripps, 1983) and the receptor terminals present a rather uniform appearance; all are about the same size, and their invaginations contain roughly the same number of processes. Although it seems likely that most receptor terminals receive processes from both types of horizontal cell, the Golgi images of Fig. 1 sug-

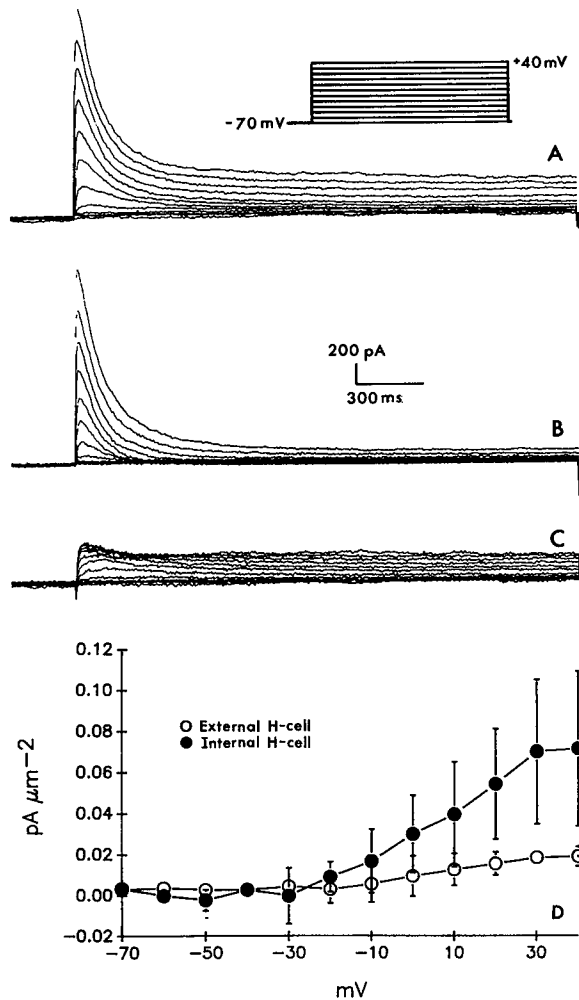


FIGURE 10. Sustained outward currents blocked by TEA. (A) Traces obtained from one internal horizontal cell while bathed in Ringer. Command voltages (*inset*) began at -70 mV and increased in steps of 10 mV to $+40$ mV. The transient outward current is seen to be followed by a sustained outward current. No fast transient inward current was observed in this cell. (B) Currents obtained from the same cell after superfusion with TEA for 2 min. The transient outward current is relatively unaffected, but the sustained outward current has been markedly reduced. (C) Subtraction of the second set of traces from the first reveals the current blocked by TEA. A small peak is evident in the initial portion of the trace, reflecting perhaps a slight reduction in the A current; the sustained outward current follows. (D) *I-V* profile for the TEA-sensitive sustained outward current averaged from four internal horizontal cells (*filled circles*) and four external horizontal cells (*open circles*). Currents from individual cells were obtained by subtraction as in C; the averaged currents produced in the final quarter of the trace were corrected for the estimated membrane area, and then averaged between cells.

gest that the external horizontal cells send many more processes to the photoreceptors than do the internal horizontal cells. Thus, not only do external horizontal cells receive a greater synaptic input, it may be that some receptor terminals provide input exclusively to the external horizontal cells. These considerations aside, there are clearly two morphologically identifiable types of horizontal cell, each with a particular location in the inner nuclear layer and a characteristic dendritic pattern. Evi-

dence that they are physiologically discrete elements stems from voltage-clamp studies of their membrane properties (see below).

Our ability to classify horizontal cells would be greatly enhanced if unique morphological and/or physiological properties were associated consistently with specific functional roles. There are, for example, other elasmobranchs in which the retinae contain both rods and cones and two or three morphologically distinct types of horizontal cell arranged in separate laminae within the distal region of the inner nuclear layer (Stell and Witkovsky, 1973; Toyoda et al., 1978). Results obtained with intracellular recordings of S-potentials indicate that each type may respond in a distinctive way to photic stimulation (Toyoda et al., 1978). Thus, color-coded horizontal cells of the stingray hyperpolarize to short-wavelength light flashes and depolarize to long-wavelength stimuli. A second type of horizontal cell receives inputs from rods and cones and there is a Purkinje shift in going from light- to dark-adapted conditions, whereas a third type subserves only rods and shows no change in response characteristics as a result of adaptive state. However, no distinguishing features have been seen thus far in S-potential recordings from skate retina. Both cell types hyperpolarize in response to light (Sakai et al., 1986), show no change in spectral properties as a result of the state of adaptation (Dowling and Ripps, 1971), and are similar in their response dynamics (Naka et al., 1988).

But now there are other, more sensitive means by which to detect functional differences between cells. With the advent of reliable cell-isolation procedures and whole-cell voltage-clamp technology, it is a relatively straightforward procedure to examine the voltage- and ligand-gated conductances of large numbers of identifiable cells under conditions that obviate problems associated with intercellular electrical coupling, and questions concerning whether drug-induced effects are direct or a result of indirect action through neighboring neurons. Thus, isolated catfish horizontal cells subserving either rod- or cone-mediated pathways are morphologically distinct, and present a unique current profile under voltage clamp (Shingai and Christensen, 1983, 1986; Schwartz, 1987). A similar situation has been seen also with individual horizontal cells of the perch retina (Dowling et al., 1985; Lasater, 1986).

These findings raise the possibility that rod- and cone-driven horizontal cells display a separate and unique set of membrane properties. Unfortunately, such parsimony does not square with the experimental evidence. Consider, for example, the results obtained on rod-driven horizontal cells. In skate, the horizontal cells receive input exclusively from rods, and both the external and internal horizontal cells exhibit five voltage-gated conductances: a TTX-sensitive sodium conductance, a calcium current, and three potassium-mediated conductances (the anomalous rectifier, a transient outward current resembling an A current, and a delayed rectifier). On the other hand, rod horizontal cells of the perch show no sign of an A current (Lasater, 1986), while the rod-driven horizontal cells of catfish lack not only the A-type current, but also the fast inward sodium current and the anomalous rectifier (Schwartz, 1987).

It could be argued that skate photoreceptors are functionally more like cones than rods, particularly when one considers their ability to respond to incremental

stimuli in the presence of background illumination that completely saturates the rods of other vertebrates (Dowling and Ripps, 1972). On this view, we might expect skate horizontal cells to display the electrical characteristics of cone-driven horizontal cells in other species. There are, in fact, instances in which the data tend to support this notion. For example, the five voltage-gated currents of skate horizontal cells have been recorded from the three classes of cone horizontal cell in perch, and as in skate, the different types of cell can be distinguished from one another both morphologically and by the magnitudes of their voltage-activated currents (Lasater, 1986). These same currents have also been described in isolated horizontal cells from goldfish retina (Tachibana, 1983), although no attempt was made in that study to distinguish between the various cell types. The parallels break down, however, when skate horizontal cells are compared with the cone-driven horizontal cells of catfish (Shingai and Christensen, 1986) and carp (Lasater, 1986); in both species, the potassium-mediated A current—the most prominent current of skate horizontal cells—is not detectable.

Thus, although there may be a clear distinction between rod- and cone-driven horizontal cells in a given species, the foregoing indicates that the interspecies variability in membrane properties is too great to categorize horizontal cells in terms of the type of photoreceptor they subserves. It would be even more difficult to apply such criteria to horizontal cells in skate, where there are two types of horizontal cell and only one class of photoreceptor. There is, of course, the possibility that the horizontal cells are arranged in two tiers merely for the purpose of increasing their population density, and that any differences in structural features are necessitated by location within the inner nuclear layer and the need to gain access to the receptor terminal. However, the electrophysiological results reveal at least one striking, perhaps important, difference in the current profiles of external and internal horizontal cells.

Although the same five voltage-dependent conductances were seen in both types of horizontal cell, differences in the anomalous rectifier currents were so great as to provide a reliable means of distinguishing physiologically between cell types. Whereas large sustained inward currents were recorded consistently from external horizontal cells in response to hyperpolarizing voltage steps, only very small, sometimes barely detectable currents were seen under comparable conditions in the internal horizontal cells (Fig. 7).

The physiological significance of the anomalous rectifier is not known, but Lasater (1986) suggests that this current tends to maintain the membrane potential of the horizontal cell in the light-stimulated (hyperpolarized) state. Thus, the anomalous rectifier may participate indirectly in excitation-response coupling of the feedback mechanism mediated by horizontal cells (Kaneko, 1987), e.g., by suppressing the depolarization-induced release of the cell's endogenous transmitter (Schwartz, 1987). Interestingly, we have found that when the anomalous rectifier current is blocked by cesium, the cell often hyperpolarizes to potentials > -100 mV (data not shown). This hyperpolarizing response may reflect the action of an electrogenic pump, e.g., a sodium/potassium exchanger. In the intact, dark-adapted retina, pump activity could serve to extrude intracellular sodium that may accumulate during the prolonged depolarization induced by the continuous release of glutamate

from photoreceptors. On this view, the current contributed by the electrogenic pump during the light response (i.e., when the cell is hyperpolarized) would be countered by activation of the anomalous rectifier. Thus, the anomalous rectifier may act to prevent the horizontal cell from becoming excessively hyperpolarized; i.e., outside the voltage range over which the cell's neurotransmitter release is modulated. However, the situation is complicated by at least two factors. The first stems from the finding that changes in the external concentration of potassium have been shown to exert a marked effect on the voltage dependence of anomalous rectifier currents (Leech and Stanfield, 1981; Perlman et al., 1988), and it is well known that light-induced changes in extracellular potassium occur throughout the retina (Oakley and Green, 1976; Kline et al., 1978). The degree to which such changes alter the horizontal cell response *in vivo* is still an unanswered question.

An additional complication arises from the observation that glutamate, a chemical suspected of being the photoreceptor's neurotransmitter (Ishida and Fain, 1981; Slaughter and Miller, 1981; Ehinger et al., 1988), acts to turn off the anomalous rectifier of horizontal cells in culture (Kaneko and Tachibana, 1985; Tachibana, 1985; Hals et al., 1986). We have also seen this phenomenon in both types of skate horizontal cell (Malchow et al., 1988). Although in some instances the glutamate effect is attributable to an artifact of pressure ejection, i.e., a reduction in the local concentration of K^+ that may have accumulated in the vicinity of nonperfused isolated cells (Perlman et al., 1988), it is not likely to have been a factor in our experiments, where glutamate was applied after the cells had been rapidly perfused (5 ml/min) with a normal Ringer solution. Nevertheless, we do not know yet whether the release of glutamate in the intact retina has any effect on the currents carried by the anomalous rectifier.

To our knowledge this is the first instance in which rod horizontal cells have been divisible into two classes based on their membrane properties, specifically, the magnitude of a voltage-gated current. Whether the striking differences in the induced current through the anomalous rectifier is reflected in the functional properties of the two cell types in the intact skate retina is not known. It is tempting to speculate that perhaps each class of horizontal cell provides input to a particular class of bipolar cell; i.e., one may form the surround of on-center cells, while the other regulates surround responses of off-center bipolar cells. However, there is no evidence to support this idea, nor is it at all clear in what ways a retina possessing a single class of photoreceptor makes use of two distinct types of horizontal cell.

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