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γ-Aminobutyric acid acts at axon terminals of turtle photoreceptors: Difference in sensitivity among cell types

(neurotransmitter/feedback synapse/cone/rod/retina)

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ABSTRACT **It has been proposed that horizontal cells of the vertebrate retina have a negative feedback synapse with cone photoreceptors. γ-Aminobutyric acid (GABA) has been suggested to be a neurotransmitter of monophasic horizontal cells (a subtype of horizontal cells), which have direct connections with red-sensitive and green-sensitive cones. We have examined the feedback hypothesis by measuring the GABA sensitivity of photoreceptors. To eliminate interaction with other cells, we dissociated photoreceptors from the turtle retina enzymatically. The subtype of photoreceptors was identified unequivocally on the bases of the shape of the cell and the color of the oil droplets, which are known to correlate with the spectral sensitivity. Cells were voltage-clamped using "Giga-ohm sealed" suction pipettes in the whole-cell recording configuration, and membrane currents were measured in response to GABA applied ionophoretically at various positions on the cell. It was found that red-sensitive and green-sensitive cones were highly sensitive to GABA and that the sensitivity was localized at the axon terminals. GABA-sensitivity in blue-sensitive cones and in rods was very low. GABA-induced current reversed its polarity near the equilibrium potential of chloride, suggesting that GABA increased chloride conductance. Thus, our findings are consistent with the negative feedback hypothesis.**

The center-surround antagonism of the receptive fields and color-opponency are basic properties of retinal neurons. These properties are found in very early stages of signal processing, such as photoreceptors (1–4), horizontal cells (5–10, 38), and bipolar cells (11–14, 38). To account for these properties, it has been proposed that horizontal cells make negative feedback connections to cone photoreceptors. The feedback connection was originally shown by Baylor *et al.* (1), who passed hyperpolarizing current through a horizontal cell and observed a depolarization in nearby cones. Many studies (2–4, 6–10, 15, 16, 38) support this hypothesis, but most of the evidence is circumstantial.

γ-Aminobutyric acid (GABA) has been suggested to be the neurotransmitter of a subtype of horizontal cells, the monophasic horizontal cell, which is characterized by its hyperpolarizing responses to flashes of monochromatic light of all visible wavelengths (17, 18). It has been shown that horizontal cells of this type synthesize GABA (19, 20), accumulate extrinsic GABA by a high-affinity uptake mechanism (21–23), and release GABA when exposed to high $[K^+]_0$ (23) or to L-glutamate (24). The connections between cones and horizontal cells are specific among cells of particular subtypes: in turtle, monophasic horizontal cells have a connection with red-sensitive and green-sensitive cones (25–27).

In the present study, we examined the feedback hypothesis by measuring the GABA sensitivity of various types of photoreceptors. Cells were dissociated enzymatically from the turtle retina. The use of solitary cells allowed quantitative visually-controlled application of GABA to a specific part of the cell and precluded various problems that may be encountered in experiments using whole retinal tissue, such as the indirect effects of GABA through the neural network, the diffusion barrier, or the buffering of GABA by uptake systems.

MATERIALS AND METHODS

Preparations. Solitary cells were obtained from the retina of the fresh-water turtle (*Geoclemys reevesii*, purchased from a local supplier) as described (28). Animals were dark-adapted for 1 hr and then decapitated. The eyes were removed and the opened eye cup was incubated in a solution containing 6 units of papain (Worthington) and 5 mg of collagenase (Sigma) per ml. Cells were mechanically dissociated by pipetting, seeded in a culture dish (Falcon, No. 1008), and incubated (10°C, 1–2 hr) until they attached to a concanavalin A (Sigma)-coated cover glass at the bottom of the dish. Cells were stored at 10°C and used 1 hr to 3 days after dissociation. During recording, cells were superfused at 15°C with 79 mM NaCl/10 mM KCl/2.5 mM $CaCl_2$/1 mM $MgCl_2$/16 mM glucose/2 mM Hepes/37 mM choline chloride, pH 7.4.

Recording. Membrane currents were measured in the whole-cell voltage-clamp configuration (29) by connecting suction pipettes to a low-noise current-voltage converter (EPC-5, List Electronics, Darmstadt, F.R.G.). Pipettes (≈3 μm o.d. and ≈1 μm i.d.) were filled with 120 mM KCl/5 mM EGTA/10 mM Hepes (adjusted to pH 7.4 by addition of KOH to a final concentration of ≈18 mM) and had resistances of ≈20 MΩ in the superfusing medium. After making a "Giga-ohm seal" between the pipette tip and the plasma membrane, the patch membrane at the pipette tip was ruptured by a brief strong suction. The intracellular ionic composition seemed to equilibrate rapidly with that of the pipette solution by free movement of ions through the hole produced in the cell membrane (see *Results*).

GABA Application. GABA was applied either by ionophoresis or by pressure ejection (30). For ionophoresis, the glass micropipette was filled with 1 M GABA (pH 4.0, pipette resistance ≈300 MΩ). The tip of the pipette was positioned against the cell surface under visual control [Nikon, TMD with interference optics; the microscope was also equipped with a fluorescence system to examine autofluorescence of oil droplets (31)]. GABA was applied by passing brief current pulses (duration, 1–50 msec; intensity, 1–50 nA) from a current source. A steady braking current was passed to minimize leakage of GABA. After the pipette was positioned at the axon terminal (the most sensitive spot, see *Results*), the braking current was adjusted to a critical level at which no membrane current was induced by the leaked GABA but at which the response to applied GABA remained maximal. For pressure ejection, a 20-μm-tip pi-

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Abbreviation: GABA, γ-aminobutyric acid.

Table 1. Morphological features and spectral sensitivity of turtle photoreceptors

Cell type	Oil droplet color	Spectral sensitivity
Single cones	Red	Red
	Orange*	Green
	Colorless (fluorescent†)	Red
	Colorless (nonfluorescent†)	Blue
Double cones		
Principal	Yellow*	Red
Accessory	No oil droplet	Red‡
Rods	No oil droplet	Scotopic

See refs. 31–35, 39.

*Color of the oil droplets contained in the principal member of double cones and in green-sensitive single cones appears to be different in the two genera of turtle, *Geoclemys* and *Pseudemys* (26, 27). The colors of *Geoclemys* photoreceptors are given here.

†Colorless oil droplets are further classified into two subtypes when examined under a fluorescence microscope. A recent study (31, 39) has shown tht the fluorescent colorless oil droplet is contained in red-sensitive cones whereas the nonfluorescent type is found in blue-sensitive cones.

‡The spectral sensitivity of the accessory member of double cones is controversial, but recent microspectrophotometric measurements (34, 35) and electrophysiological studies (T. Ohtsuka, personal communication) strongly suggest that it is red-sensitive.

pette containing GABA (0.1–10 μM) dissolved in the superfusate was placed $\approx$20 μm away from the cell. A 3-sec pulse of 0.4 kg/cm^2 was applied to the pipette to eject the GABA solution.

Identification of Cell Types. On the basis of cell shape and of color or fluorescence of their oil droplets, turtle photoreceptors are classified into seven types: four types of single cones, one type of double cone, and one type of rod. Each type of cone has been determined to have maximum sensitivity in either the red, or green, or blue region of the spectrum by intracellular staining (31, 32, 39) or by microspectrophotometry (33–35) (Table 1). In the present study, morphological criteria were used to identify the cell types. No light responses were obtained from solitary photoreceptors whose outer segments had been lost during dissociation.

RESULTS

All single cones that contained a red oil droplet (red-sensitive cones, n = 46) were highly sensitive to GABA. Fig. 1 illustrates an example. The lowest effective dose, determined by a pressure ejection, was $\approx$100 nM. When applied ionophoretically to the axon terminal, GABA evoked a large (>100 pA) transient inward current* in this cell, which was voltage-clamped at −60 mV (Fig. 1*B*). GABA sensitivity, as defined by the response amplitude evoked by identical doses of GABA, was highest at the cone pedicle and decreased sharply toward the distal tip; to 35% (of maximum) at the thin axon near the cell body, 13% at the cell body, $\approx$2% at the myoid and ellipsoid, and <1% at the distal tip. Similar distribution of GABA sensitivity was found in all single cones of this type. Localized sensitivity at the pedicle was further confirmed by the observation that GABA sensitivity was extremely low in single cones (each containing a red oil droplet) whose pedicles were lost during dissociation.

*As will be shown, GABA increased membrane conductance mainly to chloride ions. The polarity of the GABA-induced current may appear contradictory to what is expected for the inhibitory effect of GABA. However, it must be noted that the intracellular chloride ion concentration was nearly equal to that of the pipette solution, due to diffusion from the recording pipette, and that the driving force to chloride ions was outward at the applied holding potential of −60 mV.

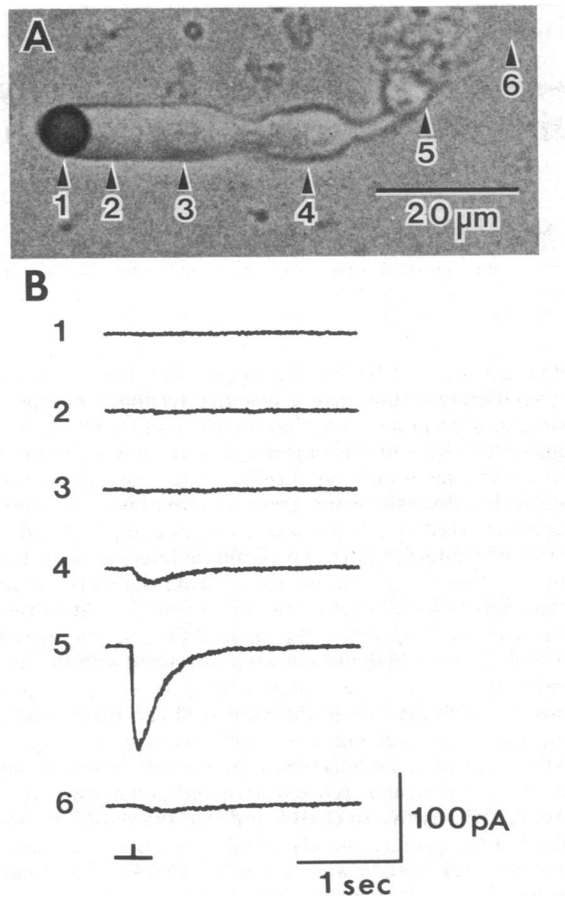

FIG. 1. (*A*) Photomicrograph of a single cone containing a red oil droplet. Numbered arrowheads indicate positions at which GABA was applied ionophoretically: oil droplet (1), ellipsoid (2), myoid (3), cell body containing the nucleus (4), cone pedicle (5), a position 15 μm away from the cone pedicle (6). (*B*) Responses of the cell shown in *A* to GABA applied ionophoretically at the numbered positions on the cell surface. The recording suction pipette was positioned at the cell body close to position 4. Holding potential was −60 mV. GABA was applied ionophoretically by passing brief current pulses (5 msec, 25 nA; time indicated by ⊥) through a fine-tip glass micropipette. At the pedicle, this dose of GABA evoked a response whose amplitude was about 1/3 of the maximum. Braking current, −5 nA. A response to GABA applied 15 μm away from the pedicle (position 6) is shown to demonstrate the limited distance of GABA diffusion.

Ionophoresis of GABA evoked responses of large amplitudes in single cones containing either an orange oil droplet (green-sensitive cones, n = 17) or a colorless oil droplet, which emitted autofluorescence when exposed to near UV light (31) (red-sensitive cones, n = 4), and in principal members of double cones, which are characterized by a yellow oil droplet (red-sensitive cones, n = 9). The maximal response amplitudes recorded in these groups of cells were within the range of variation of those of single cones with a red oil droplet (see Fig. 3*B*). GABA sensitivity was also localized to the pedicle in these types of cones.

The accessory member (red-sensitive cones, n = 5) was less sensitive to GABA than the principal member of the same double cone. Fig. 2 illustrates the peak amplitudes of GABA-induced currents recorded in the principal (*A*) and in the accessory (*B*) members of a double cone. Again, identical doses of GABA evoked the largest response amplitude in either member when GABA was applied to the pedicles. Although it has been suggested that the principal and the accessory member are electrically coupled (36), the GABA-evoked response recorded in the accessory member is prob-

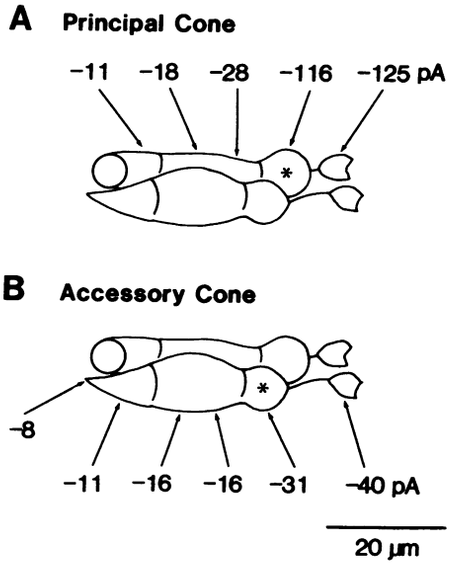

FIG. 2. Peak amplitudes of GABA-induced current (in pA) recorded in the principal (*A*) and in the accessory (*B*) member of a double cone. The principal member was identified by a yellow oil droplet, and the accessory member by the absence of an oil droplet. Asterisks indicate the position at which the recording pipette was attached. GABA was applied ionophoretically from a fine-tip glass micropipette by current pulses (10 msec, 40 nA; this dose evoked responses of ≈90% of the maximum in either member). Braking current, −10 nA. Holding potential, −60 mV. Arrows indicate the positions of the GABA pipette tip.

ably not due to the spread of the response evoked in the principal member, since similar GABA sensitivity was found in the solitary accessory member of double cones that had been detached during dissociation (n = 3).

Single cones containing nonfluorescent colorless oil droplets (blue-sensitive cones, n = 6) and rods (n = 5) showed low sensitivity to GABA. The low sensitivity was distributed uniformly over the entire cell surface. The maximum amplitude of GABA-evoked responses in these types of cells was <10% of that evoked in single cones containing red oil droplets (Fig. 3*A*). It is unlikely that the low sensitivity was due to deterioration of the cells, since in random sampling of cells within the same culture dish, cones containing red oil droplets always showed high sensitivity, whereas cones containing nonfluorescent colorless oil droplets and rods showed low sensitivity.

The amplitude of the GABA response was dose-dependent (Fig. 3*A*). The dose–response curve was sigmoidal over a range of about 1 log unit of dose (the product of the intensity and duration of the ionophoretic current). As mentioned above, the maximal response amplitudes differed from one cell type to another, but the threshold dose (50–100 pC) and the minimum dose at which the response was maximal were similar for all cell types.

GABA-induced current had a reversal potential at 6.1 ± 4.5 mV (mean ± SD, determined using the standard pipette solution and 19 single cones containing red oil droplets). The reversal potential was shifted to −17.2 ± 3.9 mV when 70% of the chloride ions in the pipette solution were replaced with nonpermeant glutamate ions (determined for 10 single cones containing red oil droplets). The magnitude of the shift in the reversal potential was close to the value estimated by the Nernst equation for chloride ions. These observations strongly suggest that the intracellular ionic composition equilibrates rapidly with that of the pipette solution and that the current induced by GABA is carried mainly by chloride

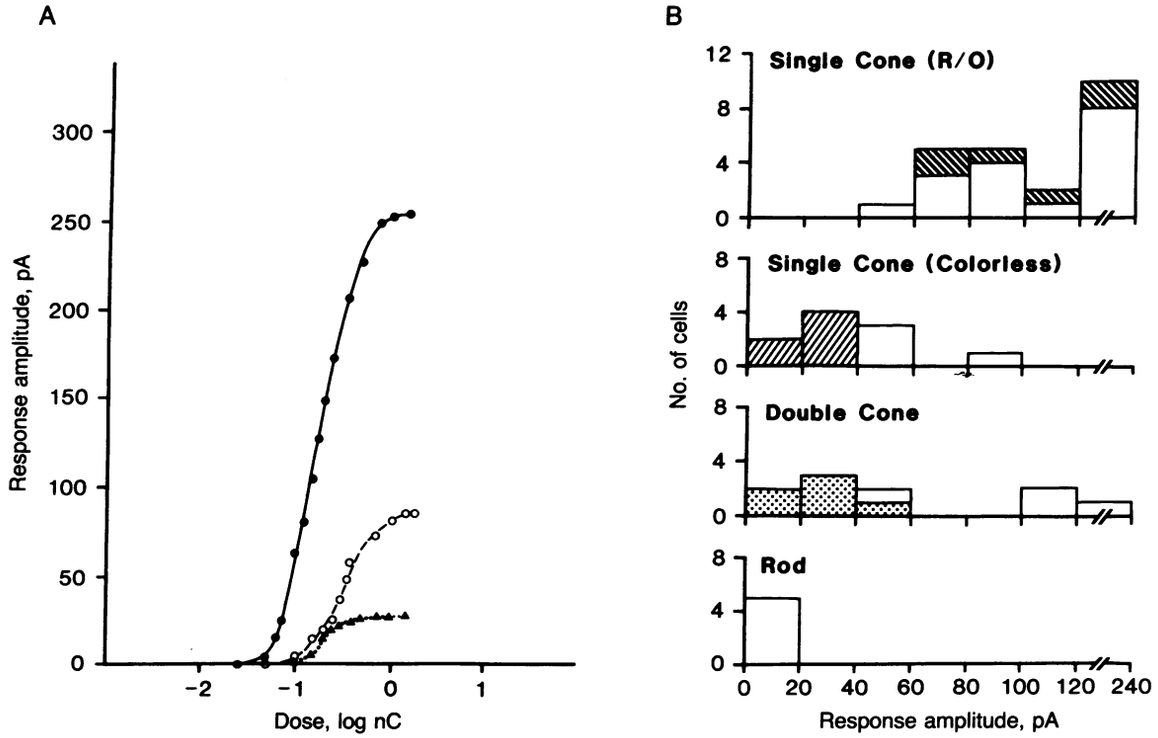

FIG. 3. (*A*) Dose–response curves of GABA examined in a single cone with a red oil droplet (●), the principal member of a double cone (○), and a single cone with a nonfluorescent colorless oil droplet (▲). GABA was applied to pedicles in all cells. The dose (nC) is the product of the intensity (varied from 5–50 nA) and duration (varied from 5–50 msec) of ionophoretic current. Braking current, −5 nA (● and ○) or −11 nA (▲). (*B*) GABA-sensitivity histogram for each morphological type of cell. Sensitivity was defined by the maximum response amplitude. The rightmost bars include cells showing response amplitudes >120 pA. Rows: top, single cones with either red (R, nonshaded) or orange (O, hatched) oil droplet; second, single cones with either fluorescent (nonshaded) or nonfluorescent (hatched) colorless oil droplet; third, principal (nonshaded) and accessory (stippled) members of double cones; bottom, rods.

ions. Reversal potentials examined in solitary photoreceptors of other types were all similar to the above value.

DISCUSSION

We have shown that turtle cones are sensitive to GABA and that the sensitivity varies among cone subtypes. Photoreceptor types were unequivocally identified on the basis of morphology, because cell shapes and colors (and fluorescence properties) of the oil droplets were well preserved in solitary cells. It is inferred that both red-sensitive (except for the accessory member of double cones) and green-sensitive cones have high sensitivity to GABA, whereas blue-sensitive cones and rods have very low sensitivity.

It is tempting to believe that GABA is the transmitter used in the interaction between the monophasic horizontal cell and red-sensitive and green-sensitive cones, since GABA was effective at a concentration as low as 100 nM and the GABA sensitivity was clearly localized to the cone pedicle. Numerous reports (10, 15, 16, 19–24, 38) indicate that GABA is the neurotransmitter of monophasic horizontal cells. Red-sensitive and green-sensitive cones are reported to have a direct connection with monophasic horizontal cells (25, 26).

Our results strongly suggest that the GABA-induced current was carried by chloride ions. The polarity of the GABA-induced current was inward when GABA was applied to solitary cells voltage-clamped at −60 mV. Since the inward current depolarizes the membrane potential, this observation appears contradictory to the expected inhibitory effect of GABA. However, under the experimental conditions, it is probable that the intracellular chloride ion concentration had been increased by rapid diffusion of ions through the ruptured hole in the cell membrane. Judging from the reversal potential, the holding potential of −60 mV generated an outward driving force to chloride ions, so that GABA induced an inward current. On the other hand, in photoreceptors *in situ*, the membrane potential is maintained at about −30 to −40 mV (1), and the equilibrium potential for chloride ions is expected to be at a more hyperpolarized level (37). GABA would, therefore, induce an outward current and, hence, a hyperpolarizing voltage response in photoreceptors *in situ*.

Morphological studies have suggested that biphasic and triphasic horizontal cells also have connections with specific subtypes of cones (25): biphasic cells, with green-sensitive and blue-sensitive cones; and triphasic cells, with blue-sensitive cones. It is also known that these types of horizontal cells neither accumulate GABA nor are able to synthesize GABA (19–22). In the present study, no blue-sensitive cones showed high sensitivity to GABA. It is unknown how biphasic and triphasic horizontal cells communicate with photoreceptors.

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1. Baylor, D. A., Fuortes, M. G. F. & O'Bryan, P. M. (1973) *J. Physiol.* **214,** 265–294.
2. O'Bryan, P. M. (1973) *J. Physiol.* **235,** 207–223.
3. Fuortes, M. G. F. & Simon, E. J. (1974) *J. Physiol.* **240,** 177–198.
4. Fuortes, M. G. F., Schwartz, E. A. & Simon, E. J. (1973) *J. Physiol.* **234,** 199–216.
5. Tomita, T. (1965) *Cold Spring Harbor Symp. Quant. Biol.* **30,** 559–566.
6. Piccolino, M. & Gerschenfeld, H. M. (1980) *Proc. R. Soc. London, Ser. B.* **206,** 439–463.
7. Gerschenfeld, H. M. & Piccolino, M. (1980) *Proc. R. Soc. London, Ser. B.* **206,** 465–480.
8. Gerschenfeld, H. M., Piccolino, M. & Neyton, J. (1980) *J. Exp. Biol.* **89,** 177–192.
9. Toyoda, J.-I., Kujiraoka, T. & Fujimoto, M. (1982) in *The S-potential*, eds. Drujan, B. & Laufer, M. (Liss, New York), pp. 151–160.
10. Murakami, M., Shimoda, Y., Nakatani, K., Miyachi, E. & Watanabe, S. (1982) *Jpn. J. Physiol.* **32,** 927–935.
11. Werblin, F. S. & Dowling, J. E. (1969) *J. Neurophysiol.* **32,** 339–355.
12. Kaneko, A. (1970) *J. Physiol.* **207,** 623–633.
13. Kaneko, A. & Tachibana, M. (1981) *Nature (London)* **293,** 220–222.
14. Yazulla, S. (1976) *Vision Res.* **16,** 737–744.
15. Murakami, M., Shimoda, Y., Nakatani, K., Miyachi, E. & Watanabe, S. (1982) *Jpn. J. Physiol.* **32,** 911–926.
16. Lam, D. M. K., Lasater, E. M. & Naka, K.-I. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 6310–6313.
17. Simon, E. J. (1973) *J. Physiol.* **230,** 199–211.
18. Saito, T., Miller, W. H. & Tomita, T. (1974) *Vision Res.* **14,** 119–123.
19. Lam, D. M. K. (1972) *J. Cell Biol.* **54,** 225–231.
20. Lam, D. M. K., Su, Y. Y. T., Swain, L., Marc, R. E., Brandon, C. & Wu, J. Y. (1979) *Nature (London)* **278,** 565–567.
21. Lam, D. M. K. & Steinman, L. (1971) *Proc. Natl. Acad. Sci. USA* **68,** 2777–2781.
22. Marc, R. E., Stell, W. K., Bok, D. & Lam, D. M. K. (1978) *J. Comp. Neurol.* **182,** 221–246.
23. Schwartz, E. A. (1982) *J. Physiol.* **323,** 211–227.
24. Miller, A. M. & Schwartz, E. A. (1983) *J. Physiol.* **334,** 325–349.
25. Leeper, H. F. (1978) *J. Comp. Neurol.* **182,** 795–810.
26. Ohtsuka, T. & Kouyama, N. (1982) *Biomed. Res.* Suppl. **3,** 1–17.
27. Kolb, H. & Jones, J. (1982) *J. Comp. Neurol.* **209,** 331–338.
28. Tachibana, M. (1983) *J. Physiol.* **345,** 329–351.
29. Hamill, O. P., Marty, A., Neher, E., Sakmann, B. & Sigworth, F. J. (1981) *Pfluegers Archiv.* **391,** 85–100.
30. Ishida, A. T., Kaneko, A. & Tachibana, M. (1984) *J. Physiol.* **348,** 255–270.
31. Ohtsuka, T. (1984) *J. Physiol. Soc. Jpn.* **46,** 435.
32. Liebman, P. A. & Granda, A. M. (1971) *Vision Res.* **11,** 105–114.
33. Ohtsuka, T. (1978) *Sens. Process.* **2,** 321–325.
34. Lipetz, L. E. & MacNichol, E. F., Jr. (1982) *Biol. Bull.* **163,** 396.
35. Lipetz, L. E. (1984) in *The Visual System: A Symposium to Honor Edward F. MacNichol*, ed. Fein, A. (Liss, New York), in press.
36. Richter, A. & Simon, E. J. (1974) *J. Physiol.* **242,** 673–683.
37. Bader, C. R., Bertrand, D. & Schwartz, E. A. (1982) *J. Physiol.* **331,** 253–284.
38. Toyoda, J.-I. & Fujimoto, M. (1983) *Vision Res.* **23,** 1143–1150.
39. Ohtsuka, T. (1984) *Neurosci. Lett.*, in press.