Published in final edited form as:

J Neurosci Methods. 2004 August 15; 137(1): 25–35. doi:10.1016/j.jneumeth.2004.02.008.

Dissociation of Retinal Ganglion Cells Without Enzymes

Yuki Hayashida, Gloria J. Partida, and Andrew T. Ishida

Section of Neurobiology, Physiology, and Behavior, University of California, Davis, California 95616

Abstract

We describe here methods for dissociating retinal ganglion cells from adult goldfish and rat without proteolytic enzymes, and show responses of ganglion cells isolated this way to step-wise voltage changes and fluctuating current injections. Taking advantage of the laminar organization of vertebrate retinas, photoreceptors and other cells were lifted away from the distal side of freshly isolated goldfish retinas, after contact with pieces of membrane filter. Likewise, cells were sliced away from the distal side of freshly isolated rat retinas, after these adhered to a membrane filter. The remaining portions of retina were incubated in an enzyme-free, low Ca^{2+} solution, and triturated. After aliquots of the resulting cell suspension were plated, ganglion cells could be identified by dye retrogradely transported via the optic nerve. These cells showed no obvious morphological degeneration for several days of culture. Perforated-patch whole-cell recordings showed that the goldfish ganglion cells spike tonically in response to depolarizing constant current injections, that these spikes are temporally precise in response to fluctuating current injections, and that the largest voltage-gated Na^+ currents of these cells were larger than those of ganglion cells isolated with a neutral protease.

Keywords

enzyme-free dissociation; retinal ganglion cell; spikes; Na^+ current; goldfish; rat

1. Introduction

Unraveling how the electrophysiological properties of single neurons contribute to the functional output of neural circuits is complicated because ion conductances can be altered by neurotransmitters, neuromodulators, and signaling cascades. The unmodulated properties of neurons and their conductances might be studied *in situ* after pharmacologically blocking inputs from surrounding neurons. However, it is hard to know if this kind of isolation is complete and without unintended side-effects. Therefore, many laboratories use dissociated cells *in vitro*, particularly for recordings with patch electrodes (Hamill et al., 1981). The vast majority of protocols currently used to dissociate neural tissues into single cells rely on mechanical trituration after incubation in proteolytic enzymes (e.g., trypsin, papain, dispase, nagarse, pronase; see Drujan and Svaetichin, 1972; Lam, 1975; Kay and Wong, 1986; Huettner and Baughman 1986; Vaughan and Fisher, 1987; Montague and Friedlander, 1989; Mody et al., 1989). Unfortunately, several studies have shown that proteolytic enzymes can alter the amplitude, kinetics, localization, and pharmacological properties of voltage- and ligand-gated ion currents; these effects have been observed when enzymes are applied not only intracellularly but also extracellularly (e.g., Rojas and Armstrong, 1971; Lee et al., 1977; Hestrin and Korenbrot, 1987; Budde et al., 1994; Shen et al., 1995; Hermann et al.,

*corresponding author: Andrew Ishida, Ph.D., Section of Neurobiology, Physiology, and Behavior, University of California, One Shields Avenue, Davis, CA 95616 USA, tel: +1-530-752-3569, fax: +1-530-752-3569, atishida@ucdavis.edu.

1997; Armstrong and Roberts, 1998), and even as briefly as 1–3 min (Holt et al., 2001). These results have led to attempts to isolate cells solely by mechanical means.

A few studies have reported the dissociation of single neurons from young or adult vertebrate brain with the use of needles (Gündel et al., 1990), vibrodissection (Vorobjev, 1991), and tissue printing (Kotecha et al., 1997). Other studies have shown that striatal neurons, hair cells, and hippocampal neurons can be dissociated by trituration after incubation in media containing a lowered Ca^{2+} ion concentration (Surmeier et al., 1988; Shigemoto and Ohmori, 1991; Barbosa et al., 1996). Perhaps the simplest procedures of all have been the isolation of photoreceptors by slicing, shaking, or pipetting retinas (Werblin, 1978; Dearry and Burnside, 1986; Leibovic 1986), and isolations of retinal ganglion cells by passage through sieves and syringes (Hu and Ritch, 1997). Despite these successes, most published protocols for dissociating neurons incorporate exposure to proteolytic enzymes prior to the step that mechanically separates cells, e.g., by trituration, sublayer separation, or tissue printing (e.g., Shiosaka et al., 1984; Barres, 1992; Sato et al., 1994; MacDonald and Hochman, 1997; Henne et al., 2000).

Here, we describe a method to dissociate adult goldfish retinal ganglion cells without proteolytic enzymes. This method combines incubation in a low- Ca^{2+} medium (Trube, 1983), sublayer separation (Shiosaka et al., 1984), and gradual trituration. We then show responses of cells isolated this way to constant and fluctuating current injections under current clamp, and whole-cell currents elicited under voltage clamp, in perforated-patch mode. Lastly, we demonstrate that retinal ganglion cells can be dissociated from adult rat retinas by modifying part of the protocol used to dissociate goldfish retinas.

2. Materials and Methods

All animal care and experimental protocols described below were approved by the Animal Use and Care Administrative Advisory Committee of the University of California, Davis. The sources of chemicals used in this study are listed in Section 2.5, following the description of our methods.

2.1 Animals

Goldfish (*Carassius auratus*; 1–2 years old; body length, 9–16 cm) were obtained from a commercial fish farm (Dutchman Creek, Merced, CA) and maintained outdoors in a 300-gal holding tank without artificial lighting. At least two weeks before experiments, fish were transferred to indoor holding tanks and maintained in a temperature-controlled room (17–18 °C) on a 12-hr/12-hr light/dark cycle.

Sprague-Dawley rats (female; P21–P28; ~150 g) were obtained from a commercial supplier (Harlan Bioproducts; Indianapolis, IN) and housed in standard cages at room temperature (~23 °C) on a 12-hr/12-hr light/dark cycle.

2.2 Prelabeling retinal ganglion cells

Goldfish retinal ganglion cells were identified by one of two methods described in detail elsewhere (Ishida and Cohen 1988; Tabata and Ishida, 1996). Briefly stated, one method was retrograde filling of the somata by insertion of rhodamine B dextran (RB dextran) granules into an incision made with a 33-gauge hypodermic needle in the optic nerve, 2–4 days before each dissociation. The labeling of ganglion cells was confirmed in whole-mount under epifluorescence illumination (Fig. 1A–C). The alternative method was to allow the nucleoli of ganglion cells to coalesce and expand, for 2–3 weeks after optic nerve crush, before dissociations. These operations were performed monocularly, while fish were anesthetized with MS-222.

Rat retinal ganglion cells were also identified by retrograde filling of the somata with RB dextran, following a procedure similar to that described by Cook and Mobbs (1988). Rats were sacrificed by a lethal dose of sodium pentobarbital (50 mg/ml; 1.5 cc/kg, i.p.). Their eyes were enucleated with approximately 2 mm of optic nerve intact, passed through 70% ethanol, and rinsed in dH₂O. Each eye was then placed in a 1.5 ml Eppendorff tube that contained 0.4 ml of RB dextran solution (1 mg dissolved in 0.4 ml water, and filtered through an 0.2 μ m membrane filter). The eye settled in the conically shaped bottom of the tube, so that the cut end of the optic nerve was exposed to the external solution. After storage for 6–20 hours at 4 °C, the eyes were rinsed and hemisected, and the retinas isolated. Some of these retinas were examined in whole-mount on a laser scanning confocal microscope (FV-300, Olympus; Melville, NY); the others were dissociated as described below. Examination of the retinas under epifluorescence illumination revealed retrogradely transported dye in somata within the ganglion cell layer (Fig. 1D–E), and not in other cell layers.

2.3 Removing the outer retina

Goldfish were dark-adapted for at least 20 min to facilitate separation of the neural retina from the pigment epithelium. The following procedures were then carried out under dim red light. Goldfish were killed by cervical-spinal transection and pithed. The eye on the side that had been operated on to identify ganglion cells was enucleated, rinsed in 70% ethanol or chilled Hanks' solution (HEPES-buffered), and hemisected along the *ora serata* (Fig. 2A). The posterior “eyecup” portion was placed vitreous-side down on a membrane filter (0.45 μ m pore). The sclera and pigment epithelium were dissected off, leaving the neural retina and vitreous body on the filter. The retina was then covered with 2–3 ml of chilled, low-Ca²⁺ solution and incubated for 3–5 min (first incubation). This low-Ca²⁺ solution contained (in mM): 140 sucrose, 2.5 KCl, 70 CsOH, 20 NaOH, 1 NaH₂PO₄, 15 CaCl₂, 20 EDTA, 11 D-glucose, 15 HEPES, 0.1 glutathione, 1 kynurenic acid, 0.0005 or 0.001 tetrodotoxin (TTX), and 0.025 mg/ml DNaseI. The estimated free Ca²⁺ concentration was 100–200 nM. The pH was adjusted to 7.2 with HCl. The osmolality was 290–300 mOsmol/kg. TTX and kynurenic acid were included to help suppress Na⁺ spikes and glutamate receptor activation, as in other studies (e.g., Segal and Furshpan, 1990). After this incubation, the retina and adhering filter were placed onto the support screen of a disc filter holder. Negative pressure was applied to the filter holder with a 12-cc syringe, until the peripheral edges of the retina adhered to the filter. The retinal surface was covered with 1 or 2 drops of the low-Ca²⁺ solution, whenever necessary, to keep it moist. A membrane filter was then laid over the photoreceptor side of the retina and peeled off after 5–10 sec. Successful peeling was gauged by the loss of pink color from the photoreceptor side, due to removal of unbleached photoreceptors. Immediately after this peeling, the retina was covered with a few more drops of the low-Ca²⁺ solution. This process was repeated 2–3 times so that the outer retinal neurons were removed (Shiosaka et al., 1985); we made no attempt to identify the cell types removed. The remaining retinal tissue (containing the ganglion cell layer and other inner retinal neurons) was transferred to a Petri dish filled with the low-Ca²⁺ solution and detached from the filter.

Adult rat retinas were treated as described above, with the modifications described below (see Fig. 2B). The eye that had been incubated in the RB dextran solution to label ganglion cells was rinsed and hemisected. The eyecup was placed in a plastic dish filled with chilled Hanks' solution (HEPES-buffered, supplemented with 0.5 or 1 μ M TTX, and adjusted to pH 7.3 with HCl). The retina was separated from the sclera, rinsed in chilled low-Ca²⁺ solution, and incubated in approximately 5 ml of this solution for 3–5 min (first incubation). The low-Ca²⁺ solution was then removed and a membrane filter was laid over the photoreceptor side of the retina. This retina and filter were placed filter side down onto the support screen of a

disc filter holder, and negative pressure was slowly applied via the filter holder until the photoreceptors were pulled against the filter. As described above, the retinal surface was covered with low- Ca^{2+} solution whenever necessary to prevent drying. The retinal tissue was then sliced with a razor blade, manually and parallel to the filter surface, leaving the photoreceptor-side of the retina on the filter.

2.4 Cell dissociation

After removal of the outer retina as described above, the remaining retinal tissue was incubated in 15 ml of the low- Ca^{2+} solution for 20–30 min at room temperature (second incubation). After this incubation, the retina was transferred to a 5-ml plastic tube filled with L-15 medium. This medium had been adjusted to pH 7.2 or 7.3 with HCl, and supplemented with 0.5 or 1 μM TTX, 0.025 mg/ml DNase I, and (in some instances) 1 mg/ml bovine serum albumin (BSA). After rinsing in this solution, the retinal tissue was triturated with Pasteur pipettes whose tips were cut and fire-polished to openings ranging in diameter from around 0.2 to 5 mm. The retina was triturated slowly and smoothly, by gently drawing the trituration solution and retinal pieces into the trituration pipette over the course of 2–4 sec, and likewise emptying it in 2–4 sec. As this process was repeated, the retinal pieces dissociated into a suspensions of cells and smaller pieces of retina, and the trituration solution became cloudy. When this happened, the undissociated retinal pieces were allowed to sink to the bottom of the trituration tube, transferred with a pipette to a new tube containing fresh L-15 medium, and triturated with a smaller pipette. Trituration pipettes were selected to have an opening approximately half the diameter of the largest retinal pieces at hand. The retinal pieces usually dissociated completely after a total of 30–45 min of trituration. Gradual dissociations generally resulted in better cell quality, based on the brightness of the cell body under phase-contrast optics, cell viability after plating, and the stability of electrophysiological recordings. Based on our experience with this method, we generally used the cell suspensions yielded by the final one or two rounds of trituration for the types of experiments described below, and did not routinely examine the cell suspensions in the other trituration tubes.

Cells were plated in 35-mm Petri dishes that had been modified by cutting a 13-mm hole in the center of each dish bottom, and attaching a glass coverslip over these holes with Sylgard. The upper side of the coverslip was then coated with succinyl-concanavalin A (Bornens et al., 1976) or poly-L-lysine, and the dishes rinsed and air-dried. Several drops of the final cell suspension were placed onto the glass area of these culture dishes, and after allowing cells to settle down for 5–10 min each dish was filled with 3–4 ml of a 50:50 mixture of HEPES-buffered Hanks' solution and L-15 medium. This mixture was supplemented with 0.5 mg/ml BSA, 0.5 mg/ml cholesterol, and 1 % BSA-free B-27, and the pH was adjusted to 7.2 or 7.3 with HCl. Two hours later, the culture medium was replaced by a fresh aliquot (3–4 ml). The dissociated cells were left on a vibration-isolation table for 0.5–5 days at room temperature ($\sim 23^\circ\text{C}$) and photographed through phase-contrast optics (IM35, Zeiss). Prior to the electrophysiological recordings shown below, goldfish retinal cells were allowed to adhere firmly to the dish bottoms, by leaving them untouched for 12–24 hours after plating. During this time, cells were stored either at room temperature or at $\sim 15^\circ\text{C}$. The colder temperature inhibited neurite outgrowth (Ishida and Cheng, 1991), and thereby afforded better measurements of current kinetics and voltage-sensitivities during voltage-clamp recordings (e.g. Armstrong and Gilly, 1992). Current-clamp recordings could be made from cells stored at either room temperature or 15°C . The spike amplitude and firing patterns appeared similar in these cells, even though long neurites were found on some of the cells stored at room temperature.

2.4 Electrophysiology

Culture dishes containing dissociated cells were rinsed and filled with an extracellular “bath” solution. This solution contained (in mM): 140 NaCl, 3.5 KCl, 3.4 MgCl₂, 0.1 CaCl₂, 10 D-glucose, 5 HEPES. The pH was adjusted to 7.4 with NaOH. The osmolality was 280 mOsmol/kg. The lowered Ca²⁺ and elevated Mg²⁺ concentrations in this solution blocked the voltage-gated Ca²⁺ current that could normally be activated from the holding potential used in our measurements (Vaquero et al., 2001). Voltage- and current-clamp recordings were made from ganglion cells in the perforated-patch whole-cell configuration (Horn and Marty, 1988) using a single-electrode voltage-clamp amplifier (npi electronic, SEC-05LX; Tamm, Germany). Patch electrodes were pulled from thick-walled borosilicate glass capillaries (Sutter Instrument Co., Novato CA) to tip resistances of 2–5 MΩ and coated with Sigmacoat. The tip of these electrodes were filled with a “pipette solution” that contained (in mM): 110 K-D-gluconic acid, 15 KCl, 15 NaOH, 2.6 MgCl₂, 0.34 CaCl₂, 1 EGTA, 10 HEPES. The pH was adjusted to 7.4 with methanesulfonic acid, and the osmolality was adjusted with sucrose to 260 mOsmol/kg. Pipette shanks were filled with this solution after the addition at 1:200 of a solution containing 2 mg amphotericin B and 3 mg Pluronic F-127 in 60 μL dimethylsulfoxide. When measured in continuous voltage-clamp mode with patch-clamp amplifiers (Axoclamp 2B and Axopatch 1D, Axon Instruments, Union City, CA), the series resistance using these electrodes and solutions in perforated-patch mode is typically 10–30 MΩ (not shown). The current and voltage signals were both recorded in discontinuous voltage- or current-clamp mode (cf. Finkel and Redman, 1984). The patch electrode time constant was counterbalanced with the supercharging and feedback capacity compensation circuits in the amplifier (Richter et al., 1996). The switching frequency and duty cycle of the amplifier were set to 40–70 kHz and 1/4 (current injection/potential recording), respectively. The “integrator” circuit in the amplifier was utilized and voltage-clamp feedback gain was increased as much as possible (typically until an amplifier gain dial setting of 5–10; Richter et al., 1996). In voltage-clamp recordings, a brief supercharging pulse was manually superimposed on command voltage step to improve the rising and falling time constants of the voltage control (Armstrong and Chow, 1987). In some instances, the membrane potential under current-clamp was measured in bridge-balance mode. The output signals from the amplifier were analog-filtered (4–20 kHz, 2-pole Bessel) and digitally sampled (10–50 kHz). To reduce noise, the recorded signals were digitally filtered off-line (0.5–4 kHz, 8-pole Bessel). The recording bath was grounded via an agar bridge, and all experiments were performed at room temperature (~23 °C).

2.5 Sources of chemicals and other materials

The following chemicals were obtained from Sigma-Aldrich (St. Louis, MO): bovine serum albumin (#A8806), cholesterol (#C3045), DNase I (#D4527), EDTA (#E6758), K-D-gluconic acid (#G9005), glutathione (#G6529), kynurenic acid (#K3375), MS-222 (#A5040), poly-L-lysine (#P7890), rhodamine B dextran 20S (#R9006), Sigmacote (#SL2), succinyl-concanavalin A (#L3885), tetraethylammonium-Cl (#T2265). The following chemicals were obtained from other sources: B-27 (without bovine serum albumin, #99-0254DG; Life Technologies, Grand Island, NY), CaCl₂ (#190464K; BDH Laboratory Supplies, Poole, England), CsCl (#813061; ICN Biomedical, Aurora, OH), CsOH (#101328; ICN Biomedical), dimethylsulfoxide (#317275; Calbiochem, La Jolla, CA), L-15 (#41300-039; GIBCO Invitrogen, Grand Island, NY), sodium pentobarbital solution (#NDC 0074-3778-05; Abbott Laboratories, North Chicago, IL), Pluronic F-127 (#P6867; Molecular Probes, Eugene, OR), succinyl-concanavalin A (#L1102-25; EY Laboratories, San Mateo, CA), Sylgard 184 (Dow Corning, Midland, MI), tetraethylammonium-Cl (#584128; Calbiochem), tetrodotoxin (#584411, Calbiochem). All chemicals not listed here were obtained from Sigma-Aldrich.

The following items were also used in the protocols described here: membrane filter (#HAWP01300; Millipore, Billerica, MA); disc filter holder (#SX00-013-00; Millipore, Billerica, MA); teflon-coated razor blade (GEM, #121-3; Ted Pella, Redding, CA).

3. Results

3.1 Dissociation of goldfish retinal ganglion cells

Fig. 3A–E show retinal ganglion cells dissociated from adult goldfish. Under phase-contrast optics, the somata were translucent and phase-bright. When the level of focus was brought near the center of each soma, the cell membrane stood out as a phase-dark line outlining each cell. Because cells were plated at relatively low density, individual ganglion cells were typically separated by at least 10 μm from other cells, and there were usually fewer than 50 ganglion cells per dish. However, some ganglion cells were found near other ganglion cells (Fig. 3E), possibly because some aliquots contained relatively large numbers of these cells. At room temperature, neurite outgrowth was commonly seen several hours after plating (e.g., Fig. 3B, D, E). These neurites were typically phase-dark and stout near the soma, and tapered with distance from each soma (e.g. Fig. 3B). As in previous studies (Ishida and Cheng 1991), palmate growth cones decorated the neurite tips just after they emerged from the soma, and these growth cones became more compact as the neurites grew longer. These cells and neurites showed no obvious morphological degeneration for as long as 5 days at room temperature. Cells identified as ganglion cells by retrograde fills with RB dextran (Fig. 3A–B) and by nucleolar expansion (Fig. 3C–E) could both be obtained by the present dissociation protocol. The latter cells, having no fluorescent dye in their cytoplasm, were useful for immunofluorescence studies (Chock, Hayashida and Ishida, unpublished) and electrophysiological experiments (see next section).

3.2 Current and voltage recordings

We next measured voltage-gated ion currents and action potentials, in perforated-patch whole-cell mode, from ganglion cells dissociated by the methods described above. For voltage-clamp recordings, cells without long neurites (e.g., Fig. 3C) were used to minimize space clamp errors. Fig. 4A shows the currents and membrane potentials recorded from the soma of a ganglion cell under discontinuous voltage-clamp (see Materials and Methods). The voltage traces (upper traces, Fig. 4A) show that the membrane potential did not differ by more than 2.5 mV from the command potential specified in the voltage-jump protocol and that this discrepancy occurred during the rising phase of the rapid inward current (see Fig. 4A, *inset*), despite the relatively high series resistance during this recording (see Materials and Methods) and despite the relatively large amplitude of the clamp currents (lower traces, Fig. 4A). Under these recording conditions, depolarizations to voltages more positive than -45 mV activated an inward current and a more slowly activating outward current. These currents are the sum of voltage-gated Na^+ current and a mixture of K^+ and mixed cation currents, because voltage-gated Ca^{2+} currents were blocked during these recordings (see Materials and Methods). As one might expect from the timecourse of ganglion cell action potentials (see below), the inward current reached its peak amplitude within less than 1 msec after the step change in membrane potential, and the net current during the jump to 0 mV changed from inward to outward within less than a msec after the peak of the inward current. No inactivation of the outward current was seen during the 5-msec depolarizations used here. Plots of the current amplitude versus command potential show that the inward current increased smoothly in amplitude between -50 mV and its maximum amplitude at around -20 mV, and that the outward current amplitude increased almost ohmically between 0 and $+25$ mV.

In previous studies, voltage-gated Na⁺ currents were recorded from enzymatically dissociated cells, and long (≥ 100 msec) step depolarizations activated current with a “persistent” (i.e., non-inactivating) component (goldfish: Hidaka and Ishida, 1998; rat: Vaquero, Hayashida and Ishida, unpublished). Although records of small currents are relatively noisy in recordings with the type of amplifier used here (cf. Finkel and Redman, 1984), Fig. 4C shows this persistent component at -52 mV and that it was preceded by a “transient” component at this membrane potential. This implies that the voltage-gated Na⁺ current of retinal ganglion cells normally consists of transient and persistent components.

The voltage-gated Na⁺ current in some of the ganglion cells dissociated without enzymes reached substantially larger maximum amplitudes (10–30 nA, see Fig. 4D) than those of ganglion cells dissociated from the same species with enzymes (< 10 nA, Hidaka and Ishida, 1998; Hayashida and Ishida, unpublished). We have not yet identified the basis for this difference. However, it is not due merely to differences in cell size, recording stability, clamp speed and/or voltage control because Figures 3 and 5 show that the size and shape of cells used in the present study are no different than those of enzymatically dissociated cells (e.g., Ishida and Cheng, 1991), because the amplitude of low-threshold Ca²⁺ currents are similar in cells dissociated by the method described here and cells dissociated using enzymes (Lee et al., 2003), and because the stability and length (~ 20 min) of whole-cell recordings here were similar to those from cells dissociated using enzymes (Tabata and Ishida, 1999; Vaquero et al., 2001).

Recordings could be made with the patch electrode tip positioned on somata, and from ganglion cell neurites. In the experiment shown in Fig. 5, for example, the electrode tip can be seen at the lower right quadrant of the photographed field (Fig. 5A), at the end of the neurite of the soma approximately $140\ \mu\text{m}$ away, in the upper left quadrant of this panel. In bridge-balance mode, step-wise constant current or fluctuating current was injected from the patch electrode and resulting membrane potential change was recorded. During depolarizing current injections, action potentials (spikes) fired repetitively (traces *i* to *iv*, Fig. 5B). After termination of hyperpolarizing current injections, so-called ‘off-response’ spikes fired (traces *vii* and *viii*). When a fluctuating current was injected, a temporally sparse pattern of spikes was observed (Fig. 5C). When the same fluctuating current was injected multiple times, the same spike number and timing were elicited by each stimulus episode (traces *i* – *iii*, and inset at the bottom of this panel). This behavior resembles that found in a number of other spiking neurons (e.g., Mainen and Sejnowski, 1995). These results suggest that retinal ganglion cells dissociated by the present method could be useful for various types of electrophysiological measurements.

3.1 Dissociation of retinal ganglion cells from adult rat

Lastly, we tested whether the methods described here can dissociate ganglion cells from other species, and used adult rat retina for this purpose. Since the vitreous body detaches more easily from the rat retina than from the goldfish retina, a different procedure was used to remove distal cells from the retina. Negative pressure was applied to a filter attached to the photoreceptor-side of the rat retina, rather than to a filter attached to the ganglion cell-side as in the goldfish retina (Fig. 2). This allowed the distal retina to be sliced away from the proximal retina, and minimized the possibility of aspirating the ganglion cell layer into the filter. The subsequent steps (trituration and plating) were the same for both goldfish and rat. The dissociated ganglion cells were identified by retrograde labeling with RB dextran (Materials and Methods). As shown in Fig. 3, the dissociated rat ganglion cells appeared phase bright with distinct edges (*F–I*). The somata resembled those of rat retinal ganglion cells described previously, being round in profile and as large as $10\text{--}15\ \mu\text{m}$ in diameter (Fukuda 1977; Guenther et al. 1994). Although we have typically found fewer ganglion cells after dissociating rat retinas than goldfish retinas, the dissociated rat cells, like the goldfish

cells, also showed no obvious signs of morphological degeneration for as long as 5 days at room temperature.

4. Discussion

Three conclusions are supported by the results of this study. First, we have shown that ganglion cells can be dissociated from adult goldfish and adult rat retinas by a combination of peeling or slicing, incubation in low- Ca^{2+} medium, and trituration; proteolytic enzymes are not necessary to dissociate these cells. Secondly, we found that sustained depolarizations elicit tonic spike firing, that terminating sustained hyperpolarizations trigger brief spike volleys, and that fluctuating current injections elicit temporally precise spikes; these spikes resemble those recorded from ganglion cells *in situ* (e.g., Baylor and Fettiplace, 1979; Diamond and Copenhagen, 1995; O'Brien et al., 2002; Madon and Owen, 2003). Thirdly, our ability to record spikes and voltage-gated currents in perforated-patch whole-cell mode from these cells indicates that the membrane surface of these cells need not be exposed to enzymes before forming high-resistance seals with patch electrodes (cf. Hamill et al., 1981). Two previous studies showed that retinal ganglion cells can be isolated mechanically with procedures quite different from ours (Giulian 1980; Hu and Ritch 1997). While those studies agree with ours in finding that retinal ganglion cells can be dissociated without enzymes, it remains to be seen if any of these methods offers advantages over the others, because the earlier studies did not measure any electrophysiological properties or responses.

Because retinas are not more than 200–250 μm thick, it was not feasible to test how much the success of this method depends on peeling or slicing versus the other steps of our protocol. However, our experience is that all three phases of our protocol are necessary. Firstly, as reported by other investigators, we found that only photoreceptors separate from retinas either spontaneously after isolation or slicing retinas (Werblin 1978). We, and other laboratories, therefore incorporate trituration into methods to dissociate retinas, as do protocols used to dissociate other regions of CNS (Kay and Wong 1986; Huettner and Baughman 1986).

Secondly, we and others have found that agitating retinas in Ca^{2+} -containing medium dislodges only photoreceptors (Dearry and Burnside 1986; Bindokas et al. 1994). We report here that ganglion cells can be dissociated from pieces of retina after incubation in a low- Ca^{2+} solution. Although previously published protocols incubated retinas in divalent cation-free solutions to foster proteolytic enzyme activity (e.g., Vaughan and Fisher 1987; Montague and Friedlander 1989), we find that inclusion of proteolytic enzymes is not necessary to dissociate ganglion cells. This is consistent with previous findings in other tissues (e.g., Surmeier et al., 1988; Shigemoto and Ohmori, 1991; Barbosa et al., 1996).

Thirdly, we removed cells from the distal side of the retinas we dissociated for various reasons. One was to facilitate infiltration of the inner retinal layers by our low- Ca^{2+} solution, because recent studies showed that exposure of whole retinas to low- Ca^{2+} solutions does not effectively lower extracellular Ca^{2+} concentrations within the retinas (see Dmitriev et al. 1999). An empirical reason was that when whole retinas were incubated in low- Ca^{2+} solution without peeling or slicing, we found that they did not dissociate readily during subsequent trituration. Moreover, removing cells from the distal side of these retinas minimized contamination of our cell cultures by cellular debris, especially from damaged photoreceptors.

Our finding that ganglion cells dissociated with and without enzymes are similar in size and shape, and that voltage-gated Na^+ current amplitudes were larger in some of the cells dissociated without enzymes, is consistent with two possibilities. First, these protocols might

have yielded cell subtypes that had different current densities *in situ*. In this case, one might expect the spikes of these cells to differ, and we are not aware of current- or voltage-clamp recordings that reflect this. Alternatively, the Na⁺ current density and related properties may be sensitive to some difference(s) between the dissociation protocols. Slightly different solutions and culture media, for example, were used. However, we are unaware of reports that the extra ingredients used here (cholesterol, succinyl-concanavalin A, and B-27, and DNase I) increase Na⁺ currents by amounts consistent with the difference we have seen. Although it is too early to know how the Na⁺ currents of cells dissociated by the protocols presented here compare with those of cells *in situ*, our results suggest that this comparison can be made without proteolytic enzymes.

Lastly, the enzyme-free protocol we present here, like previously published dissociations using enzymes (Sarthy et al. 1983; Leifer et al. 1984; Barnstable and Dräger 1984; Barres et al. 1988; Montague and Friedlander 1989; Ishida and Cheng 1991; Kaneda and Kaneko 1991; Skaliara et al. 1993; Guenther et al. 1994; Fig. 1A of Taschenberger and Grantyn 1995), yield ganglion cell somata that, when first plated, lack axons and typically show little or no dendritic arbor. This favors detailed studies of somatic channels, but it also implies that studies of ion channels in axons and dendrites will entail other dissociation methods, or use of other preparations (e.g., Velte and Masland 1999).

Acknowledgments

This work was supported by NIH grant EY 08120 (to A.T.I.) and National Eye Institute Core grant P30 EY12576. The authors thank Sherwin C. Lee for help with confocal imaging and for comments on the manuscript.

References

- Armstrong CE, Roberts WM. Electrical properties of frog saccular hair cells: distortion by enzymatic dissociation. *J Neurosci*. 1998; 18:2962–2973. [PubMed: 9526013]
- Armstrong CM, Chow RH. Supercharging: A method for improving patch-clamp performance. *Biophys J*. 1987; 52:133–136. [PubMed: 2440491]
- Armstrong CM, Gilly WF. Access resistance and space clamp problems associated with whole-cell patch clamping. *Methods Enzymol*. 1992; 207:100–122. [PubMed: 1528114]
- Barbosa CTF, Alkondon M, Aracava Y, Maelicke A, Albuquerque EX. Ligand-gated ion channels in acutely dissociated rat hippocampal neurons with long dendrites. *Neurosci Lett*. 1996; 210:177–180. [PubMed: 8805124]
- Barnstable CJ, Dräger UC. Thy-1 antigen: a ganglion cell specific marker in rodent retina. *Neuroscience*. 1984; 11:847–855. [PubMed: 6146113]
- Barres, BA. The tissue print method. In: Kettenmann, H.; Grantyn, R., editors. *Practical Electrophysiological Methods*. Wiley-Liss; New York: 1992. p. 96–100.
- Barres BA, Silverstein BE, Corey DP, Chun LL. Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. *Neuron*. 1988; 1:791–803. [PubMed: 2908449]
- Baylor DA, Fettiplace R. Synaptic drive and impulse generation in ganglion cells of turtle retina. *J Physiol*. 1979; 288:107–127. [PubMed: 469712]
- Bindokas VP, Yoshikawa M, Ishida AT. Na⁺-Ca²⁺ exchanger-like immunoreactivity and regulation of intracellular Ca²⁺ levels in fish retinal ganglion cells. *J Neurophysiol*. 1994; 72:47–55. [PubMed: 7965029]
- Bornens M, Karsenti E, Avrameas S. Cooperative binding of concanavalin A to thymocytes at 4 degrees C and micro-redistribution of concanavalin A receptors. *Eur J Biochem*. 1976; 65:61–69. [PubMed: 1278183]
- Budde T, White JA, Kay AR. Hyperpolarization-activated Na⁺-K⁺ current (I_h) in neocortical neurons is blocked by external proteolysis and internal TEA. *J Neurophysiol*. 1994; 72:2737–2742. [PubMed: 7534826]

- Cook A, Mobbs P. Application of FITC-peroxidase to optic nerve identifies viable ganglion cells in slice and isolated cell preparations of the tiger salamander retina. *J Physiol.* 1988; 401:8P.
- Deary A, Burnside B. Dopaminergic regulation of cone retinomotor movement in isolated teleost retinas: I. Induction of cone contraction is mediated by D2 receptors. *J Neurochem.* 1986; 46:1006–1021. [PubMed: 2869104]
- Diamond JS, Copenhagen DR. The relationship between light-evoked synaptic excitation and spiking behaviour of salamander retinal ganglion cells. *J Physiol.* 1995; 487:711–725. [PubMed: 8544133]
- Dmitriev A, Pignatelli A, Piccolino M. Resistance of retinal extracellular space to Ca^{2+} level decrease: implications for the synaptic effects of divalent cations. *J Neurophysiol.* 1999; 82:283–289. [PubMed: 10400957]
- Drujan BD, Svaetichin G. Characterization of different classes of isolated retinal cells. *Vision Res.* 1972; 12:1777–1780. [PubMed: 5079247]
- Finkel AS, Redman S. Theory and operation of a single microelectrode voltage clamp. *J Neurosci Methods.* 1984; 11:101–127. [PubMed: 6482502]
- Fukuda Y. A three-group classification of rat retinal ganglion cells: Histological and physiological studies. *Brain Res.* 1977; 119:327–344. [PubMed: 830390]
- Giulian D. Isolation of ganglion cells from the retina. *Brain Res.* 1980; 189:135–155. [PubMed: 7363082]
- Gündel J, Steinhäuser, Matthies H. Proteolytic enzymes do not destroy the *N*-methyl-D-aspartate (NMDA) sensitivity of acutely isolated hippocampal CA1 and CA3 neurons from postnatal rats. *Neurosci Lett.* 1990; 119:249–252. [PubMed: 1704112]
- Guenther E, Schmid S, Grantyn R, Zrenner E. In vitro identification of retinal ganglion cells in culture without the need of dye labeling. *J Neurosci Methods.* 1994; 51:177–181. [PubMed: 7914251]
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ. Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* 1981; 391:85–100.
- Henne J, Pottering S, Jeserich G. Voltage-gated potassium channels in retinal ganglion cells of trout: a combined biophysical, pharmacological, and single-cell RT-PCR approach. *J Neurosci Res.* 2000; 62:629–637. [PubMed: 11104501]
- Hermann PM, Lukowiak K, Wildering WC, Bulloch AGM. Pronase acutely modifies high voltage-activated calcium currents and cell properties of *Lymnaea* neurons. *Eur J Neurosci.* 1997; 9:2624–2633. [PubMed: 9517468]
- Hestrin S, Korenbrot JJ. Voltage-activated potassium channels in the plasma membrane of rod outer segments: a possible effect of enzymatic cell dissociation. *J Neurosci.* 1987; 7:3072–3080. [PubMed: 2444674]
- Holt JC, Lioudyno M, Athas G, Garcia MM, Perin P, Guth PS. The effect of proteolytic enzymes on the $\alpha 9$ -nicotinic receptor-mediated response in isolated frog vestibular hair cells. *Hearing Res.* 2001; 152:25–42.
- Horn R, Marty A. Muscarinic activation of ionic currents measured by a new whole-cell recording method. *J Gen Physiol.* 1988; 92:145–159. [PubMed: 2459299]
- Hu DN, Ritch R. Tissue culture of adult human retinal ganglion cells. *J Glaucoma.* 1997; 6:37–43. [PubMed: 9075079]
- Huettner JE, Baughman RW. Primary culture of identified neurons from the visual cortex of postnatal rats. *J Neurosci.* 1986; 6:3044–3060. [PubMed: 3760948]
- Ishida AT, Cheng MH. Cold inhibits neurite outgrowth from single retinal ganglion cells isolated from adult goldfish. *Exp Eye Res.* 1991; 52:175–191. [PubMed: 2013300]
- Ishida AT, Cohen BN. GABA-activated whole-cell currents in isolated retinal ganglion cells. *J Neurophysiol.* 1988; 60:381–396. [PubMed: 3171634]
- Kaneda M, Kaneko A. Voltage-gated sodium currents in isolated retinal ganglion cells of the cat: relation between the inactivation kinetics and the cell type. *Neurosci Res.* 1991; 11:261–275. [PubMed: 1661876]
- Kay AR, Wong RKS. Isolation of neurons suitable for patch-clamping from adult mammalian central nervous systems. *J Neurosci Methods.* 1986; 16:227–238. [PubMed: 3523050]

- Kotecha SA, Eley DW, Turner RW. Tissue printed cells from teleost electrosensory and cerebellar structures. *J Comp Neurol*. 1997; 386:277–292. [PubMed: 9295152]
- Lam DMK. Synaptic chemistry of identified cells in the vertebrate retina. *Cold Spr Harb Symp Quant Biol*. 1975; 40:571–579.
- Lee KS, Akaike N, Brown AM. Trypsin inhibits the action of tetrodotoxin on neurons. *Nature*. 1977; 265:751–753. [PubMed: 859585]
- Lee SC, Hayashida Y, Ishida AT. Availability of low-threshold Ca^{2+} current in retinal ganglion cells. *J Neurophysiol*. 2003; 90:3888–3901. [PubMed: 14665686]
- Leifer D, Lipton SA, Barnstable CJ, Masland RH. Monoclonal antibody to Thy-1 enhances regeneration of processes by rat retinal ganglion cells in culture. *Science*. 1984; 224:303–306. [PubMed: 6143400]
- MacDonald SC, Hochman S. A variation of the tissue print technique for studying isolated spinal cord cells in situ. *Neurosci Lett*. 1997; 223:85–88. [PubMed: 9089679]
- Madon T, Owen WG. Precision and patterning of retinal ganglion cell spike trains. *Invest Ophthalmol Vis Sci*. 2003; 44:E-Abstract 5190.
- Mainen ZF, Sejnowski TJ. Reliability of spike timing in neocortical neurons. *Science*. 1995; 268:1503–1506. [PubMed: 7770778]
- Mody I, Salter MW, MacDonald JF. Whole-cell voltage-clamp recordings in granule cells acutely isolated from hippocampal slices of adult or aged rats. *Neurosci Lett*. 1989; 96:70–75. [PubMed: 2538782]
- Montague PR, Friedlander MJ. Expression of an intrinsic growth strategy by mammalian retinal neurons. *Proc Natl Acad Sci USA*. 1989; 86:7223–7227. [PubMed: 2780566]
- O'Brien BJ, Isayama T, Richardson R, Berson DM. Intrinsic physiological properties of cat retinal ganglion cells. *J Physiol*. 2002; 538:787–802. [PubMed: 11826165]
- Richter DW, Pierrefiche O, Lalley PM, Polder HR. Voltage-clamp analysis of neurons within deep layers of the brain. *J Neurosci Methods*. 1996; 67:121–131. [PubMed: 8872877]
- Rojas E, Armstrong C. Sodium conductance activation without inactivation in pronase-perfused axons. *Nature New Biol*. 1971; 229:177–178. [PubMed: 5280106]
- Sato T, Hirono J, Tonoike M, Takebayashi M. Tuning specificities to aliphatic odorants in mouse olfactory receptor neurons and their local distribution. *J Neurophysiol*. 1994; 72:2980–2989. [PubMed: 7897503]
- Sarthy PV, Curtis BM, Catterall WA. Retrograde labeling, enrichment, and characterization of retinal ganglion cells from the neonatal rat. *J Neurosci*. 1983; 3:2532–2544. [PubMed: 6140302]
- Segal MM, Furshpan EJ. Epileptiform activity in microcultures containing small numbers of hippocampal neurons. *J Neurophysiol*. 1990; 64:1390–1399. [PubMed: 2283535]
- Shen J, Watanabe S-I, Kaneko A. Cell dissociation with papain reduces the density of cGMP-activated channels of the retinal rod. *Jpn J Physiol*. 1995; 45:151–164. [PubMed: 7544417]
- Shigemoto T, Ohmori H. Muscarinic receptor hyperpolarizes cochlear hair cells of chick by activating Ca^{2+} -activated K^{+} channels. *J Physiol*. 1991; 442:669–690. [PubMed: 1798048]
- Shiosaka S, Kiyama H, Tohyama M. A simple method for the separation of retinal sublayers from the entire retina with special reference to application for cell culture. *J Neurosci Methods*. 1984; 10:229–235. [PubMed: 6376962]
- Skaliora I, Scobey RP, Chalupa LM. Prenatal development of excitability in cat retinal ganglion cells: action potentials and sodium currents. *J Neurosci*. 1993; 13:313–323. [PubMed: 8423477]
- Retina, Sterling P. *The Synaptic Organization of the Brain*. Shepherd, GM., editor. Oxford University Press; New York: 1998. p. 205-253.
- Surmeier DJ, Kita H, Kitai SJ. The expression of γ -aminobutyric acid and leu-enkephalin immunoreactivity in primary monolayer cultures of rat striatum. *Dev Brain Res*. 1988; 42:265–282.
- Tabata T, Ishida AT. Transient and sustained depolarization of retinal ganglion cells by I_{h} . *J Neurophysiol*. 1996; 75:1932–1943. [PubMed: 8734592]
- Tabata T, Ishida AT. A zinc-dependent Cl^{-} current in neuronal somata. *J Neurosci*. 1999; 19:5195–5204. [PubMed: 10377331]

- Taschenberger H, Grantyn R. Several types of Ca^{2+} channels mediate glutamatergic synaptic responses to activation of single Thy-1-immunolabeled rat retinal ganglion neurons. *J Neurosci*. 1995; 15:2240–2254. [PubMed: 7891163]
- Trube, G. Enzymatic dispersion of heart and other tissues. In: Sakmann, B.; Neher, E., editors. *Single-Channel Recording*. Plenum; New York: 1983. p. 69-76.
- Vaquero CF, Pignatelli A, Partida GJ, Ishida AT. A dopamine- and protein kinase A-dependent mechanism for network adaptation in retinal ganglion cells. *J Neurosci*. 2001; 21:8624–8635. [PubMed: 11606650]
- Vaughan DK, Fisher SK. The distribution of F-actin in cells isolated from vertebrate retinas. *Exp Eye Res*. 1987; 44:393–406. [PubMed: 3496232]
- Velte TJ, Masland RH. Action potentials in the dendrites of retinal ganglion cells. *J Neurophysiol*. 1999; 81:1412–1417. [PubMed: 10085366]
- Vorobjev VS. Vibrodissociation of sliced mammalian nervous tissue. *J Neurosci Methods*. 1991; 38:145–150. [PubMed: 1784118]
- Werblin FS. Transmission along and between rods in the tiger salamander retina. *J Physiol*. 1978; 280:449–470. [PubMed: 211229]

Fig. 1.

Ganglion cells retrogradely labeled with RB dextran, in whole-mount view. A goldfish retina photographed on 35 mm film at different magnifications under epifluorescence illumination (A–C). A rat retina imaged with a laser scanning confocal microscope (D–E). All panels displayed in grayscale, with rhodamine fluorescence rendered white. A and D show ganglion cell axon bundles extending radially away from the optic nerve head centered at the left-hand edge of each panel. B shows a slightly higher magnification view of the goldfish retina, with ganglion cell axons and somata within the focal plane. C oriented with the axon fascicles extending diagonally across the field of view, showing somata sprinkled in between the axons, and dendritic processes extending away from the somata. Note that a dendritic process tapers and branches as it emerges from the large soma in the middle of the field. E shows dye-filled rat retinal ganglion cell axon fascicles extending diagonally across the field of view, with round, dye-filled somata among them.

Fig. 2.

Methods to remove distal retinal cells. The steps at the far left and far right sides show that, after removing the anterior portion and lens from goldfish and rat eyes, the goldfish retinas were processed vitreous-side down (A), whereas the rat retinas were processed photoreceptor-side down (B). These approaches were based on differences in the amounts of adhering vitreous. The right side of panel A shows that the distal cells of the goldfish retina were removed with pieces of membrane filter (cf. Shiosaka et al. 1985). The left side of panel B shows that the distal cells of the rat retina adhered to a membrane filter when the proximal retina was sliced off. The proximal portions of these retinas were then incubated in low- Ca^{2+} solution (*inset*, center bottom) and triturated (not shown). PE, pigment epithelium;

MF, membrane filter; FSS, filter support screen; NP, negative pressure; RB, razor blade. See Materials and Methods for the composition of the low- Ca^{2+} solution.

Fig. 3.

Dissociated retinal ganglion cells of goldfish and rat. (A), (B) Goldfish ganglion cells identified by retrograde staining. The paired micrographs in each panel show the same field under phase-contrast optics (left) and epifluorescence illumination (right). In the latter, rhodamine fluorescence is rendered white, and it appears concentrated into small granules in each cell body. These cells were photographed 36 hours after plating. (C)–(E) Goldfish ganglion cells identified by nucleolar expansion. The nucleoli of these cells are slightly out the plane of focus, were phase-dark and had coalesced as described elsewhere (Ishida and Cohen, 1988). The cells of (C), (D), and (E), were photographed after 14, 16 and 22 hours after plating, respectively. Three ganglion cells can be seen in (E). (F)–(I) Ganglion cells dissociated from rat retina. Paired images of two different cells (F, G), showing the same field under phase-contrast optics (left) and epifluorescence illumination (right, with rhodamine fluorescence rendered white). These cells were photographed 20–22 hours after plating. Paired images of two other rat retinal ganglion cells (H, I), showing the same field under differential interference contrast optics (DIC, left) and by confocal laser scanning (right, with rhodamine fluorescence rendered white). These cells were imaged 26 hours after plating.

Fig. 4.

Perforated-patch whole-cell voltage-clamp measurements from ganglion cell somata. (A) Membrane potential (upper traces) and whole-cell current (lower traces) were both measured in discontinuous voltage-clamp mode. The holding potential was -72 mV, and the test potential was increased from -47 to $+23$ mV, in 5-mV steps. The inset superimposes the measured membrane potential (solid line) over the intended potential (-22 mV; dashed line) and shows that the largest voltage error during all of the recordings from this cell was less than 2.5 mV. Comparison of the voltage and current traces shows that this error occurred during the rising phase of the inward current. The current traces are shown with no leak subtraction. Also, the current from the beginning of each voltage step to 250 μs thereafter are not plotted because membrane capacitive currents cannot be electronically compensated with the amplifier used. (B) Current amplitude vs. membrane potential for the data in (A). Filled circles plot the maximum inward current at each test potential; open circles plot the maximum outward current at each test potential. (C) Voltage-gated Na^+ current activated at a relatively negative test potential (-52 mV). The holding potential was -72 mV, and the pulse duration was 100 msec. The current trace is the difference between currents before and after application of TTX ($1\mu\text{M}$). (D) Voltage-gated Na^+ current of relatively large amplitude. The holding potential was -72 mV, and the test potentials were -37 , -22 and -7 mV. Peak amplitude of inward Na^+ current reached approx. 28 nA (lower trace) during the

depolarization to -22 mV. Difference between the command potential and membrane potential is 8 mV at most (upper trace). Leak current subtraction was carried out off-line. In *C* and *D*, the bath solution contained (in mM): 110 NaCl, 3 CsCl, 30 tetraethylammonium-Cl, 2.4 MgCl₂, 0.1 CaCl₂, 10 D-glucose, 5 HEPES. The pH was adjusted to 7.4 with CsOH, and the osmolality was 280 mOsmol/kg. The pipette solution contained (in mM): 140 CsOH, 15 NaCl, 2.6 MgCl₂, 0.34 CaCl₂, 1 EGTA, 10 HEPES. The pH was adjusted to 7.4 with methanesulfonic acid, and the osmolality was adjusted with sucrose to 260 mOsmo/kg. The amphotericin B concentration in the patch electrode was as described in Materials and Methods.

Fig. 5.

Perforated-patch current-clamp measurement with electrode positioned on a ganglion cell neurite. (A) Micrograph of a ganglion cell with neurites extending toward the upper left and lower right corners of the field viewed. Arrow indicates the recording site (approx. 140 μm from the edge of soma). Single arrowhead indicates tip of the patch electrode (broken). Double arrowhead indicates the patch electrode shank (out of focus). (B) Membrane potential change (traces *i-viii*) in response to step-wise constant current injection. The injected currents were 49, 41, 32, 23, 15 pA in the positive direction and 35, 46, 52 pA in the negative direction, as plotted by the traces superimposed at the top of B. (C) Membrane potential changes in response to fluctuating current injections. The time constant and the standard deviation of the current fluctuation (upper trace) were approx. 3.5 ms and 29 pA, respectively (Gaussian distribution, cf. Mainen and Sejnowski, 1995). The same waveform of current was injected in three episodes and the corresponding voltage responses were shown in *i* to *iii*. The *inset* superimposes the voltage trace segments that are bracketed by the dotted lines. In both (B) and (C), the resting membrane potential of the recording site was held between -71 and -76 mV (dashed horizontal lines). All recordings were made in bridge-balance mode.