Materials and Methods

Preparation

Adult goldfish, *Carassius auratus* (8-9 cm in body length), were purchased from a local supplier, and transferred to a clear-sided holding tank. These fish were maintained under natural (outdoor) lighting for 1-4 weeks prior to use, and fed 3 times per week. Each animal was then pithed, and eyes enucleated under room light. Retinal slices were prepared as illustrated in Figure 3. First of all, a hole (3-4 mm in diameter) was cut in the cornea, and the lens and the cornea removed. The remaining portion of eye was cut radially, into six sectors, extending roughly two-thirds of the distance from the iris and *ora serrata* to the optic disc. The vitreous was removed after exposure to 1 mg/ml hyaluronidase (Sigma, USA) for 1 minute. A piece of one of the temporal sectors was then cut off, and placed vitreous-side-down on a piece of filter paper (0.45 μm pore size; Millipore, USA). The temporal parts of the eyecup sectors were always used to minimize variability. After removal of the sclera and pigmented epithelium, the remaining sector of isolated retina was sliced transretinally together with the filter paper, at 200 μm thickness, on a McIlwain tissue chopper (Mickle Lab. Engineering, England). These slices were transferred to a perfusion chamber having a volume of about 1 ml and attached to a cover glass at the bottom of the chamber by a small amount of Vaseline at both ends of the filter paper (Fig. 4). The chamber was put on the stage of an upright microscope (Axioscope; Carl Zeiss, Germany). They were superfused continuously with the experimental solution (see below), and viewed through a 63X water-immersion objective with differential interference contrast (Nomarski) optics.

Electrophysiological Recordings

Whole-cell patch-clamp recordings were performed in the ruptured-patch mode

(Hamill et al., 1981) with an Axopatch 1D amplifier (Axon Instruments, USA). After the electrode tip was gently positioned on the cell surface, a gigaohm-seal was achieved by a gentle suction. To obtain the whole-cell patch-recording configuration, further suction was applied, resulting in rupture of the patch of membrane beneath the pipette. This made possible to measure membrane currents through voltage-gated ion channels (Fig. 5). The current monitor output was analog-filtered at a corner frequency of 5 kHz (Fig. 12-13, 16-19, 24, 29) or 1 kHz (Fig. 21-22, 25-28) (using the Bessel filter built into the amplifier), and sampled at a frequency of 100 kHz (Fig. 12-13, 16-19, 24, 29) or 2 kHz (Fig. 21-22, 25-28). In some cases, capacitive currents were reduced as much as possible by adjustment of the amplifier cancellation circuitry. Leak subtraction was not performed if the whole-cell currents showed no nonlinearities. Otherwise, capacitive and leak currents were reduced by linear, P/5 leak subtraction (Ishida, 1991). Currents less than 10 pA were regarded as negligibly small in consideration of the ability of the recording system.

Patch pipettes were pulled with a vertical electrode puller (PC-10; Narishige, Japan) from borosilicate glass capillaries (Hilgenberg, Germany). Compositions of pipette solutions used are listed in Table 1. Solution A was used for recording the voltage-gated Na^+ currents and solution B was used for recording the gap junctional currents. To eliminate outward K^+ currents, all pipette solutions contained 110-115 mM CsCl and 30 mM tetraethylammonium-cloride (TEA-Cl). cAMP (5 mM) was sometimes added in the pipette solution.

Pipette tip resistances ranged from 4 to 8 MΩ. Current recordings were used only if the series resistance was less than 10 MΩ. Series resistance compensation (less than 80 %) was applied when recording large currents. Membrane potentials are not corrected for voltage errors due to uncompensated series resistance, but they are corrected for the liquid junction potential between the pipette and bath solutions. The recording bath was grounded via an agar bridge. Current recordings were obtained at room temperature (RT). Current amplitudes and current-voltage relations were

measured using pCLAMP software (ver. 5.5.1; Axon Instruments), and data are presented as mean ± standard error (SE).

Experimental solutions and drugs

During whole-cell current recordings, retinal slices were continuously superfused by gravity at a rate of about 2 ml/min with the experimental solution (Table 2). Solution A was used for making slice preparation, solution B for recording the voltage-gated Na^+ currents, and solution B and C for studying the gap junctional currents. In each experimental solution, TEA-Cl (Fluka, Switzerland) and 4-aminopyridine (4-AP) (Wako, Japan) were used to minimize voltage-gated K^+ currents, and $CoCl_2$ was used to reduce voltage-gated Ca^{2+} currents (Bindokas and Ishida, 1996). Voltage-gated Na^+ current was identified either by blockade with extracellular 1 μM tetrodotoxin (TTX) or by replacement of Na^+ by the large organic cation, tris+ (cf. Ishida, 1991,1995).

In the experiment of gap junction between cells, the extracellular pH was either reduced by the addition of HCl or raised by NaOH. In some experiments, either a mixture of dopamine (100 μM) and L-ascorbic acid (1 mg/ml), 8-bromo-cAMP (200 μM), halothane (5 mM) or retinoic acid (300 μM) was mixed with the above experimental solution just before use and applied within 5 min.

Intracellular staining

To visualize the morphology of cells from which currents were recorded, all pipette solutions contained 0.2 mg/ml Lucifer Yellow CH (LY) (dipotassium salt; Sigma). LY diffused gradually into the cell during whole-cell recordings. In some experiments, both LY and 0.5 mg/ml biocytin (Sigma) were iontophoretically injected into single current-recorded cells by repetitive negative current pulses (-1 nA, 40 Hz, for up to 10 min). This allowed us not only to identify the morphology of the injected cells, but also to examine tracer coupling between cells.

The retinal slices containing the biocytin-injected cells were transferred to a 4% paraformaldehyde solution in a 0.1 M phosphate buffer (PB; pH 7.4), and left 6h at 4°C or 3h at RT. To visualize the localization of biocytin, they were further incubated in the avidin-biotin complex solution (Vector Laboratories, USA) for 4-5 days at 4°C and then treated with 3,3'-diaminobenzidine-tetrahydro-chloride (DAB) solution (Vector Laboratories). Such a staining protocol is diagrammatically illustrated in Figure 6. Translucent and/or epifluorescent images of cells were displayed on a video monitor through a CCD camera (C5985; Hamamatsu Photonix, Japan) and stored on computer hard disk (Macintosh; Apple Computer, USA). The morphological properties of cells were analyzed using Photoshop 3.0 (Adobe, USA) software.

Immunohistochemistry

Eye cups were hemisected and fixed overnight in Zamboni's solution (Zamboni and DeMartino, 1967) at 4°C. The tissues were rinsed in 0.02 M phosphate-buffered saline (PBS; pH 7.4), equilibrated in 30% sucrose in 0.2 M PB for several hours and then placed in embedding medium (O.C.T. Compound; Miles Scientific, USA). The tissues were cryosectioned at a thickness of about 20 μm and sections were thaw-mounted onto gelatin-coated cover glasses. Sections were rinsed in 0.02 M PBS, 1% Triton X-100 in PBS, and PBS for 15 minutes each at RT. They were incubated with the human autoantibody to proliferating cell nuclear antigen (PCNA), developed by Takasaki et al. (1981), for 9 hours at 4°C. After the sections were rinsed as described above, the secondary antibody, FITC (fluorescein isothiocyanate)-conjugated goat anti-human IgG (Sigma), was applied to the sections for 3 hours at RT. The dilution of the primary and secondary antibodies was 1:1,000 and 1:250, respectively, in 0.02 M PBS containing 0.1% Triton X-100. The immunohistochemical protocol described above is diagrammatically illustrated in Figure 7. PCNA-immunopositive cells were examined and photographed under epifluorescence illumination (Nikon Fluophoto, Japan).