Intracellular Calcium Is Regulated by Different Pathways in Horizontal Cells of the Mouse Retina

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Schubert, Timm, Reto Weiler, and Andreas Feigenspan. Intracellular calcium is regulated by different pathways in horizontal cells of the mouse retina. J Neurophysiol 96: 1278–1292, 2006. First published May 31, 2006; doi:10.1152/jn.00191.2006. Horizontal cells modulate the output of the photoreceptor to bipolar cell synapse, thereby providing the first level of lateral information processing in the vertebrate retina. Because horizontal cells do not generate sodium-based action potentials, calcium is likely to play an important role for graded potential changes as well as for intracellular events involved in the modulatory role of horizontal cells within the retinal network. Therefore we wanted to determine how the activation of glutamate receptors, voltage-gated calcium channels, and release of calcium from internal stores shape the calcium signal in horizontal cells. All horizontal cells responded to depolarizing voltage steps with sustained inward currents, which activated at around −20 mV, reached a peak amplitude of −79.1 pA at 5 mV, and reversed sign at around 66 mV. The current was insensitive to tetrodotoxin, and it was partially blocked by the L-type channel antagonists verapamil and nifedipine. The N-type channel blockers ω-conotoxin GVIA induced an additional reduction of current amplitudes. Calcium influx through ionotropic glutamate receptors was mediated by both AMPA and kainate but not by N-methyl-d-aspartate receptors. Two agonists at group I metabotropic glutamate receptor, trans-1-amino-1,3-cyclopentanedicarboxylic acid and quisqualate, had no effect. However, intracellular calcium was increased by caffeine, indicating release of calcium from internal stores via ryanodine receptors. These data show that intracellular calcium in horizontal cells is regulated by voltage-dependent L- and N-type calcium channels, ionotropic AMPA and kainate receptors, and release of calcium from internal stores after activation of ryanodine receptors.

INTRODUCTION

Calcium plays a key role in intracellular signaling, and thus its spatiotemporal distribution is tightly regulated. The concentration of intracellular free calcium reflects a complex and dynamic equilibrium of calcium entry across the cell membrane, buffering within the cytoplasm, removal by active transport systems, and storage and release from intracellular compartments (Augustine et al. 2003; Berridge et al. 2000). Extracellular calcium can enter the cell along its steep concentration gradient through voltage-gated calcium channels or after activation of calcium-permeable ionotropic glutamate receptors. Voltage-gated calcium channels are classified into two major types according to their activation threshold and their inactivation kinetics. Low-voltage-activated calcium channels (LVA or T-type calcium channels) are characterized by a relatively hyperpolarized activation threshold and a transient, fast inactivating current. High-voltage-activated (HVA) calcium channels are gated by significantly more positive potentials, and their current trajectory shows little or no inactivation. All HVA calcium channels are composed of a large α1-subunit in combination with auxiliary subunits α2δβy. Sequence variations of α1 determine the known HVA subtypes (L-, N-, P/Q-, and R-type) (Chin 1998; Jones 1998). Ionotropic glutamate receptors are generally designated into AMPA, kainate, and N-methyl-d-aspartate NMDA receptors according to their agonist specificity (Bigge 1999).

Horizontal cells are second-order neurons in the vertebrate retina that provide a lateral pathway responsible for the antagonistic surround properties of inner retinal neurons. Horizontal cells of the teleost retina express sodium and calcium channels, and activation of both channel types contributes to the voltage trajectory of their action potentials (Shingai and Christensen 1983, 1986). Conventional T-type like calcium channels are expressed in all teleost species studied so far. However, a careful electrophysiological analysis of the sustained current revealed properties of L- and P-type calcium channels (Pfeiffer-Linn and Lasater 1996). In the mammalian retina, L-type HVA calcium channels have been described only in horizontal cells of the cat (Ueda et al. 1992) and in monolayer cultures of the rabbit retina (Löhrke and Hofmann 1994), but a thorough biophysical and pharmacological characterization of voltage-gated calcium channels is still lacking.

Horizontal cells are postsynaptic to photoreceptors, and in the dark, they are depolarized by a continuous release of glutamate acting on ionotropic glutamate receptors. During the last two decades, a significant body of electrophysiological evidence on the effects of excitatory amino acids on ionotropic glutamate receptors of fish horizontal cells has accumulated. Receptors sensitive to agonists at AMPA and kainate receptors have been described in horizontal cells of all teleost species studied (Linn and Christensen 1992; O’Dell and Christensen 1989; Schmidt 1997), whereas the expression of NMDA receptors appears to be more restricted (Okada et al. 1999). The only physiological study carried out in the mammalian retina suggests the presence of AMPA and kainate receptors but not NMDA receptors (Blanco and de la Villa 1999).

In rodents, immunocytochemical data indicate expression of several subunits of ionotropic AMPA and kainate glutamate receptors on dendrites of horizontal cells. In addition to the calcium-permeable subunit GluR1 and GluR4, GluR2/3 was also found to be present in horizontal cells (Hack et al. 2001). AMPA receptors containing the GluR2 subunit are considered to have little or no calcium permeability (Hollmann et al.)
Localization of GluR6/7 on rat horizontal cell dendrites reflects expression of kainate receptors (Brandstättter et al. 1997). Immunoreactivity for the NMDA receptor subunits NR1, NR2A, and NR2B was detectable in the outer plexiform layer of the mouse retina, raising the possibility that horizontal cells express NMDA receptors (Haverkamp and Wässle 2000).

Activation of metabotropic glutamate receptors (mGluRs) can also induce changes in the intracellular calcium concentration. The metabotropic glutamate receptors belong to the family of G-protein-coupled receptors, which exert their effects by triggering intracellular signal transduction cascades. Group I mGluRs (mGluR1 and mGluR5) are coupled to phospholipase C, leading to generation of inositol 1,4,5-trisphosphate (IP₃) and subsequent release of calcium from internal stores (Berridge 1998). Calcium-induced calcium release requires activation of ryanodine receptors, and it locally amplifies the cytoplasmic calcium signal, thus acting as a positive feedback loop (Berridge 1998; Rose and Konnerth 2001; Verkhratsky and Shmigol 1996). A tight functional link coupling entry of calcium through either voltage-gated channels or ionotropic/metabotropic glutamate receptors and calcium-induced calcium release has been demonstrated in the teleost retina (Linn and Gafka 1999, 2001; Solessio and Lasater 2002). However, to our knowledge the release of calcium from intracellular stores has not been investigated at all in mammalian horizontal cells.

Here we report the contribution of voltage-gated calcium channels, ionotropic glutamate receptors, and calcium release from internal stores to the overall calcium signal in horizontal cells of the mouse retina.

**METHODS**

**Dissociation of the retina and identification of horizontal cells**

All procedures were carried out in accordance with the guidelines for animal experiments issued by the Federal Republic of Germany. As described previously (Feigenspan and Weiler 2004), 2- to 4-month-old C57Bl/6j mice were deeply anesthetized by intraperitoneal injection of a 0.1 ml solution containing equal parts of 5% ketamine (Ceva, Düsseldorf, Germany) and 1% xylazine (Ceva) and subsequently killed by cervical dislocation. After removal of the cornea, lens, vitreous body, and sclera, the retina was transferred to 1 ml of digestion buffer containing 20 U/ml papain (Worthington Biochemical, Freehold, NJ) and 200 U/ml DNase I (Sigma, Deisenhofen, Germany) in Earle’s Balanced Salt Solution (EBSS; Sigma). After 40–45 min digestion at 37°C, the retina was transferred to trituration buffer to stop papain activity (5 min, 37°C). This solution contained 1 mg/ml ovomucoid inhibitor (Worthington), 1 mg/ml bovine serum albumin (Sigma), and 100 U/ml DNase I (Sigma) in EBSS. The tissue was centrifuged at 1,000 rpm (5 min, 22°C), and the pellet was resuspended in minimum essential medium (MEM; Sigma). Subsequently, the retina was triturated with fire-polished Pasteur pipettes of decreasing open diameter, and after each trituration step, the cell suspension was carefully checked for the presence of horizontal cells. Those fractions containing horizontal cells were finally pooled, and the cell suspension was plated on glass coverslips, which had been coated with 1 mg/ml concanavalin A (Sigma). The cells were kept in an incubator in 5% CO₂-5% O₂ at 37°C. After 15–20 min, 1% fetal calf serum ( Sigma) was added to improve viability of the cells. Horizontal cell bodies and axon terminals were visually identified in the recording chamber according to their morphology (Fig. 1). Images were taken with a DFC320 digital camera (Leica, Bensheim, Germany) at a resolution of 2,088 × 1,550 pixels.

**Electrophysiological recordings**

Cells were allowed to settle ≥30 min before commencement of recordings. Coverslips with retinal neurons were placed in a recording chamber (Luigs and Neumann, Ratingen, Germany) on the stage of an upright microscope (Leica). Horizontal cell bodies and axon terminals were identified as described below using ×40 and ×63 water-immersion objectives equipped with Nomarski optics (Leica). Whole cell voltage- and current-clamp recordings were performed with an EPC9 double patch-clamp amplifier (Heka, Lambrecht, Germany). Current traces were monitored with a digital oscilloscope (Tektronix, Beaverton, OR) and directly stored to the hard disk of a personal computer. Data were acquired with a sample frequency of 10 kHz in the whole cell mode and with a frequency of 10–100 kHz in current-clamp experiments. Leak currents were subtracted prior to calcium current measurements from all current traces using a P/n protocol. Patch pipettes were pulled from borosilicate glass (1.5 mm OD, 1.275 mm ID; Hilgenberg, Malsfeld, Germany) using a horizontal electrode puller (Sutter, Novato, CA). Electrodes with a resistance ranging from 4 to 7 MΩ were connected to the amplifier with an Ag/AgCl wire. The electrode holder combined with the headstage were mounted on a mechanical, remote-controlled device attached to a three-dimensional micromanipulator (Luigs and Neumann). In whole cell experiments, the series resistance of the electrodes usually ranged between 8 and 15 MΩ and was not compensated for. However, the series resistance was carefully monitored in the time course of an experiment, and only those recordings with a stable series resistance value were considered for analysis. Drugs were applied to horizontal cells in the extracellular bath solution by a pressure-driven application system DAD-12 Superfusion System (ALA Scientific Instruments, Westbury, NY).

Isolated horizontal cells were continuously superfused (0.5 ml/min) at room temperature with an extracellular solution containing (in mM) 132 NaCl, 5.4 KCl, 5 CaCl₂, 1 MgCl₂, 5 HEPES, and 10 glucose (pH 7.4). The intracellular solution for recordings of whole cell currents contained (in mM) 120 CsCl, 20 TEA-Cl, 1 CaCl₂, 2 MgCl₂, 11

**FIG. 1.** Identification of isolated horizontal cells. A: differential interference contrast (DIC) image of an isolated horizontal cell body. B: DIC image of an axon terminal with a patch-clamp electrode attached. Both cell compartments were visually identified in the recording chamber according to their typical morphology. Scale bars: 20 μm.
EGTA, and 10 HEPES (pH 7.2). The extracellular solution for recordings in current-clamp mode contained (in mM) 137 mM NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES, and 10 glucose (pH 7.4). The intracellular solution for current-clamp recordings (in mM) 125 K-glucuronate, 10 KCl, 0.5 EGTA, and 10 HEPES (pH 7.2). All biochemicals were routinely carried out in Sigma unless otherwise noted. Stock solutions of tetrodotoxin (TTX), ω-conotoxin GVIA (both Alomone Labs, Jerusalem, Israel) and verapamil hydrochloride (Biotrend, Köln, Germany) were prepared as stock solutions in distilled water and stored at -20°C.

Fluorometric calcium imaging

For calcium imaging experiments, horizontal cells were enzymatically isolated from the retina of C57Bl/6J mice as described in the preceding text. Cells were incubated in an extracellular solution equilibrated with a mixture of 95% O₂-5% CO₂ and containing (in mM) 137 NaCl; 5.4 KCl, 1 MgCl₂, 1.8 CaCl₂, 21 NaHPO₄, 1 NaHCO₃, and 10 glucose (pH 7.4). To load the cells with calcium indicator, Fura 2/AM (25 µM) together with Pluronic 124 (0.001%; both Molecular Probes, Eugene, OR) were added for 50–60 min at 37°C, and the cells were subsequently washed for 30 min in a HEPES-buffered extracellular solution containing (in mM) 137 NaCl; 5.4 KCl, 1 MgCl₂, 1.8 CaCl₂, 5 HEPES, 10 glucose, and 0.25 CdCl₂ (pH 7.4). All calcium-imaging experiments were performed in HEPES-buffered extracellular solution unless otherwise noted. The free intracellular calcium level was monitored with a Photon Technology International calcium imaging system (PTI; Lawrenceville, NJ) using alternately the 340 and 380 nm wavelengths for excitation. Emission fluorescence images (515 nm) were recorded using an intensified CCD camera (PTI IC-100) with 768 × 494 pixel resolution and 0.5- to 1-Hz frequency and analyzed off-line in the 340/380 nm ratio mode. The ratio analysis eliminated false positive fluorescence signals based on changes of the calcium indicator concentration. The fluorescence intensity within a cell was normalized to its resting level and expressed as ΔF(t) = [F(t) − F(0)]/F(0), where F(t) is the intensity of the fluorescence at a given time t, and F(0) is the averaged fluorescence intensity of a 60-s baseline period before drug application. Where appropriate, the ΔF(t) ± SE value is given, as determined by the maximum value followed by a decay of the signal or (in a few cases with a longer calcium clearance phase extending the data acquisition period) by the steady state of the signal trace.

The glutamate receptor agonists l-glutamate (200 µM), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA; 100 µM), kainate (100 µM), N-methyl-D-aspartate (NMDA; 100 µM), 1,3-cyclopentanedicarboxylic acid (trans-ACPD; 100–200 µM), quisqualic acid (200 µM), and the glutamate receptor antagonists 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 5 µM), 2-amino-5-phosphonopentanoic acid (AP-5; 100 µM), and CdCl₂ (250 µM), as well as the ryanodine receptor agonist caffeine (10 mM) were purchased from Sigma. All glutamate receptor agonists were directly dissolved in HEPES-buffered extracellular solution and applied to the recording chamber via a gravity-fed microperfusion system. Glutamate receptor antagonists were dissolved in HEPES-buffered extracellular solution, which continuously superfused the horizontal cell preparation (2 ml/min). Experiments using the ionotropic glutamate receptor agonists glutamate, AMPA, kainate, NMDA, and Sym2081 were routinely carried out in an extracellular solution containing CdCl₂, whereas stimulation of metabotropic glutamate receptors with t-ACPD and quisqualic acid was performed in a cadmium-free solution.

Data analysis

Time constants were determined by fitting an nth-order exponential function to the charging curve

\[ y(t) = y_0 + \sum_{i=1}^{n} A_i \exp(-t/\tau_i) \]  

where \( y_0 \) denotes an offset, \( A_i \) a weighted coefficient, \( t \) the time, and \( \tau_i \) the time constant. Best fits were obtained with \( n = 2 \) for horizontal cell somata and axon terminal systems. Current-voltage relations were fitted using the Goldman-Hodgkin-Katz formalism

\[ I = \frac{I_0 - E}{1 + \exp\left(-\frac{(E - E_N)/50mV}{k}\right)} - \exp\left(-\frac{E}{50mV}\right) \]  

where \( I_0 \) denotes an offset, \( E \) the voltage, \( E_N \) the half-maximal activation voltage, \( k \) the steepness of the curve, and \( E_{rev} \), the reversal potential. The activation curve was fitted with a Boltzmann distribution

\[ y(E) = \frac{A_1 - A_2}{1 + \exp\left(\frac{E - E_0}{k}\right)} + A_2 \]  

where \( A_1 \) and \( A_2 \) denote the conductance range, and \( E_0 \) and \( k \) have the same meaning as described in the preceding text. All calculations were performed with the Pulsefit (Heka), MatLab (MathWorks, Natick, MA) and Origin software packages (Microcal, Natick, MA).

RESULTS

Identification of horizontal cells

Patch-clamp recordings and imaging experiments were performed on isolated horizontal cells obtained after enzymatical and mechanical dissociation of the mouse retina. Only one type of horizontal cell has been described in the retina of mice and rats that is characterized by a multipolar cell body and a long, thin axon extending into an elaborate axon terminal system (Jeon et al. 1998; Peichl and Gonzalez-Soriano 1994). Because the fragile axon was always ruptured during the dissociation procedure, horizontal cell bodies and axon terminal systems appeared as isolated entities in our preparation. The biophysical membrane properties were determined for both cell bodies and axon terminals. All experiments describing the regulation and pharmacology of intracellular current signaling were carried out on horizontal cell somata.

Isolated horizontal cells were identified according to their typical morphology: a medium-sized, polygonal-shaped perikaryon that measured 14 µm on average and gave rise to five to eight primary dendrites (Fig. 1A). Axon terminals were also visually recognized due to their characteristic shape (Fig. 1B). As described in detail elsewhere, visual identification of horizontal cells was confirmed by demonstrating the presence of the calcium-binding protein calbindin D-28K, using both single-cell reverse transcriptase PCR and immunocytochemistry (Feigenspan and Weiler 2004). Expression of calbindin D-28K has previously been shown in horizontal cells of the rabbit (Röhrenbeck and Weiler 1987, 1989) and the mouse retina (Haverkamp and Wässle 2000).

Input resistance and membrane time constant

Successful patch-clamp recordings could be obtained from 181 horizontal cells. Stable seals with resistances between 2 and 10 GΩ were established in ~90% of the recordings, indicating that the dissociation procedure did not impair the overall viability of the cells.
The input resistance of horizontal cell bodies and axon terminals was determined by injecting hyperpolarizing current steps in the current-clamp mode of the patch-clamp technique. Cell bodies displayed a nearly linear relation between the amplitude of the injected current and the corresponding voltage change, whereas the membrane response of axon terminals became nonlinear with increasing hyperpolarization (Fig. 2A). The input resistance of both cellular compartments was determined from the slope of a linear regression line fitted to the linear portion of the voltage-current plot (Fig. 2B). Cell bodies had an input resistance of 244 ± 1 MΩ (n = 14), and a very similar value of 236 ± 2 MΩ (n = 4) was calculated for axon terminals. We never observed a decrease in apparent input resistance with prolonged current injections (“sag”), indicating that the hyperpolarization-activated cation current I_{hi} is not expressed in horizontal cell bodies and axon terminals.

The membrane time constants of horizontal cell somata and axon terminals were measured by injecting a brief hyperpolarizing current step (1 ms, –500 pA) and fitting the resulting trajectory of voltage relaxation with an exponential function (Fig. 2C). The voltage response of horizontal cell bodies could be well fitted with a second-order exponential, which was characterized by a fast and a slow time constant with mean values of 0.609 and 6.357 ms, respectively (Table 1). The fast time constant \( \tau_f \) contributed about one half to the overall charging curve. Similarly, the voltage trajectory of axon terminals displayed a fast and a slow component (Fig. 2C). The fast time constant of axon terminals was 0.274 ms, slightly faster than the corresponding \( \tau_f \) of the cell body. It contributed 29% to the charging curve, whereas the slow time constant was 8.297 ms (71%). Comparing both compartments, the difference between either time constant was not statistically significant. However, the contribution of each time constant to the overall charging curve as well as the absolute voltage amplitudes differed significantly (\( P < 0.05 \), Student’s \( t \)-test). A bar graph of the time constants of cell bodies and axon terminals is shown in Fig. 2D. The biophysical membrane properties of horizontal cell bodies and axon terminals are summarized in Table 1.

Characterization of calcium-mediated currents in horizontal cell bodies

To block voltage-activated potassium currents, horizontal cells were dialyzed with an intracellular solution containing CsCl and TEACl. Under these experimental conditions, we never observed significant outward currents even at very depolarized holding potentials. However, as described for the calcium currents of AII amacrine cells (Habermann et al. 1992), a very small inward current component was evoked by depolarizing current steps in the current-clamp mode by negative current injections (6 steps of –20-pA increments, 400-ms duration). The axon terminal displays a nonlinear response to increasing current amplitudes. The injection of hyperpolarizing current step (1 ms, –500 pA, 1-ms duration). The voltage trajectory of axon terminals was measured by injecting a brief hyperpolarizing current step (1 ms, –500 pA) and fitting the resulting trajectory with an exponential function for both cell bodies and axon terminals. The fit is superimposed on the current trajectory. Note the different scaling of the voltage axis.

![Image](https://via.placeholder.com/150)

**TABLE 1. Membrane properties of horizontal cell bodies and axon terminals**

<table>
<thead>
<tr>
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<th>Cell Body</th>
<th>Axon Terminal</th>
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<tr>
<td>Input resistance, ( \text{MΩ} )</td>
<td>244 ± 1 (14)</td>
<td>238 ± 7 (4)</td>
</tr>
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<td>( \tau_f ), ms</td>
<td>0.609 ± 0.118 (8)</td>
<td>0.274 ± 0.131 (5)</td>
</tr>
<tr>
<td>( \tau_s ), ms</td>
<td>6.357 ± 0.961 (8)</td>
<td>8.297 ± 1.165 (5)</td>
</tr>
<tr>
<td>( A_1 ), mV</td>
<td>–19.96 ± 3.56 (8)</td>
<td>–3.96 ± 0.54 (5)*</td>
</tr>
<tr>
<td>( A_2 ), mV</td>
<td>–23.35 ± 2.45 (8)</td>
<td>–9.51 ± 0.25 (5)*</td>
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Values are means ± SE for \( n \) cells (number of cells is indicated in parentheses). \( A_i \) represent the coefficients derived from the ith-order exponential fit to the charging curves, reflecting the contribution of each time constant \( \tau_i \) to the overall charging curve. *\( P < 0.05 \) (Student’s \( t \)-test).
2003), a prolonged perfusion (>1 min) with intracellular solution was required to fully block voltage-activated potassium channels. Because of their rather depolarized activation thresholds (Fig. 3, C and D), calcium currents appeared partially masked by outwardly directed potassium currents immediately after establishing a whole cell recording. We occasionally observed voltage-dependent sodium currents, which were characterized by small, transient amplitudes (<50 pA). Sodium currents were routinely masked by calcium inward currents after complete block of potassium channels. Therefore we did not commence measurements before transient putative sodium currents immediately after the voltage step had entirely disappeared due to spontaneous run-down, potassium currents were completely blocked, and calcium currents showed a constant amplitude on repeated depolarization.

A voltage protocol for measuring the current-voltage relation of calcium channels is shown in Fig. 3A. Horizontal cell bodies were clamped at −60-mV holding potential and subse-

FIG. 3. Biophysical properties of HVA calcium channels of horizontal cell bodies. A: calcium-mediated inward currents were evoked by depolarizing voltage steps (10 steps of 10-mV increment, 100-ms duration) from a holding potential of −60 mV. Only every other step is shown for clarity. B: 3 individual current responses from the traces shown in A. Test potentials are indicated above each current trace. C: current-voltage relation as determined by the step protocol shown in A. Values represent the mean amplitudes ± SE of 7 cells. The data points were fitted with the Goldman-Hodgkin-Katz formalism (—). D: activation curve of voltage-dependent calcium channels. The data points represent the means ± SE of 7 cells. —, obtained by fitting the data with a Boltzmann function. E: histogram of steady-state current amplitudes induced by a voltage step from a holding potential of −60 mV to 10 mV for 100 ms (n = 125). The histogram was fitted with a Gaussian distribution centered around an amplitude value of −72.4 pA (R² = 0.92998; —). F: plotting the charge against the duration of the respective current steps reveals a linear relationship, indicating little inactivation of voltage-gated calcium channels. Data points representing the means ± SE of 14 cells were fitted with a linear regression line (R = 0.99948). Inset: protocol for obtaining the charge transfer function. Superimposed inward currents were recorded after stepping the membrane from a holding potential of −60 mV to 10 mV for increasing durations (100, 250, and 625 ms). Horizontal and vertical scale bars indicate 75 ms and 20 pA, respectively. G: strong depolarizations did not facilitate the overall calcium currents. Inward current amplitudes before (a) and after (b) a depolarization to 100 mV (75 ms) were not significantly different. The waveform of the voltage protocol is shown in the top trace.
frequently depolarized with increasing voltage steps of 10-mV amplitude to a maximum value of 40 mV. Significant inward currents were observed at membrane potentials greater or equal than –30 mV, reaching peak amplitudes around 5 nV (Fig. 3C). For clarity, individual current traces obtained by depolarizing the membrane from –60 to –20, 0, and 20 mV are shown in Fig. 3B. All inward currents displayed very little inactivation during the voltage step. Even when the cells were depolarized from –80-mV holding potential, we never observed a transient component, indicating that low-voltage-activated (LVA) or T-type calcium channels are not expressed in horizontal cell bodies. A current-voltage (I-V) relation was calculated by measuring the current response of seven horizontal cells according to the protocol described in the preceding text (Fig. 3C). Large depolarizations (40–70 mV) never induced outward currents, suggesting complete block of voltage-gated potassium channels. However, the cell membrane became quite unstable under these experimental conditions, and therefore voltage steps beyond 40 mV were not included in the analysis. The data points of the I-V curve were fitted with the Goldman-Hodgkin-Katz model (see METHODS) with an estimated reversal potential of 79 ± 23 mV (n = 7). We further characterized the activation properties of voltage-gated calcium channels by calculating the conductance from the current amplitudes and plotting the respective values against the applied voltage (Fig. 3D). As determined by fitting a Boltzmann function to the data points, half of the channels were open at a membrane potential $E_0$ of –11.7 ± 2.5 mV (n = 7). In addition, horizontal cell calcium channels revealed a moderately steep voltage dependence ($k = 9.98 ± 1.57$). It should be noted that gating parameters are affected by external calcium concentrations. Thus it is possible that calcium channels would activate at lower potential values when exposed to more physiological extracellular concentrations (Piccolino et al. 1996).

Peak current amplitudes measured with a voltage step from –60 to 10 mV ranged between –198 and –17 pA with an average amplitude of –79.1 ± 2.9 pA (n = 125). This value is close to the center of a Gaussian function (–72.4 pA), which was fitted to the amplitude distribution shown in the histogram of Fig. 3E. We investigated the inactivation properties of horizontal cell calcium currents by applying voltage steps from a holding potential of –60 to 10 mV of increasing duration. Calcium-mediated inward currents did not show significant inactivation even with prolonged depolarizations (Fig. 3F, inset). The charge transfer was determined by calculating the integral of the current trajectory during the depolarization. Throughout the measured range (100–650 ms), the amount of charge transferred exhibited a linear relation to the duration of the pulse (Fig. 3F). It has been shown in sympathetic neurons (Ikeda 1991; Zhu and Yakel 1997) and thalamic neurons (Kammermeier and Jones 1998) that calcium currents can be tonically inhibited, but the inhibition can be relieved with strong depolarizations. Therefore we tested whether or not depolarizations to 100 mV facilitate the total calcium current. The membrane potential of horizontal cell bodies was stepped from a holding potential of –60 to 0 mV 20 ms before and after a depolarization to 100 mV (75 ms). In 21 of 24 cells, the 100-mV prepulse failed to produce a potentiation of the overall calcium current. In the remaining three cells, the current induced by the second voltage step was increased (1.268 ± 0.111). As shown in Fig. 3G, the current amplitude induced by the second voltage step is slightly smaller, which is most likely due to inactivation of the current caused by the depolarization. The activation properties of the overall calcium current are summarized in Table 2.

We never observed a transient inward current mediated by low voltage-activated or T-type calcium channels when horizontal cells were stepped from a holding potential of –80 to –20 mV (Fig. 4A). Rather, a nonactivating inward current with an average amplitude of –49.8 ± 4.6 pA (n = 11) was elicited (Fig. 4, A and D). Depolarizing the membrane from –80 to 10 mV induced a larger current (94.9 ± 8.9 pA, n = 11), again without a significant transient component at the beginning of the voltage step (Fig. 4B). When the holding potential was increased to –30 mV to inactivate putative T-type calcium channels, a depolarizing step to 10 mV evoked a steady current of slightly smaller amplitude (Fig. 4B). Subtraction of these two current traces has been described as a standard procedure to isolate T-type channels (Bean 1985). The subtracted current trace did not show the transient kinetics typical for LVA calcium channels (Fig. 4B), suggesting that this calcium channel type is not expressed in dissociated horizontal cells of the mouse retina.

In another set of experiments, horizontal cells were stepped from –80 mV holding potential to –20 mV (100 ms), and they were immediately depolarized further to 10 mV (100 ms). As expected, the first depolarization to –20 mV elicited a nonactivating inward current without an apparent transient component (Fig. 4C). The second depolarization to 10 mV increased the inward current to an overall average amplitude of –95.8 ± 9.6 pA (n = 11; Fig. 4, C and D). This value is not significantly different from the amplitude noted in the preceding text, which was obtained after prolonged hyperpolarization of the membrane at a holding potential of –80 mV. These results confirm that horizontal cell HVA calcium channels show very little if any inactivation. In addition, they indicate that a significant amount of calcium current can still be elicited from a rather depolarized membrane potential.

Pharmacology of horizontal cell calcium currents

Unlike the calcium currents described in rod bipolar cells (Protti and Llano 1998) and AII amacrine cells (Habermann et al. 2003), activation properties of horizontal cell calcium channels resemble those reported for the majority of HVA calcium currents. Multiple types of HVA calcium channels have been identified in excitable tissue based on a combination of bio-

<table>
<thead>
<tr>
<th>Cell Body</th>
<th>Peak current, pA</th>
<th>Reversal potential, mV</th>
<th>Half-maximal activation, mV</th>
<th>$V_{max}$, mV</th>
<th>Voltage-dependence</th>
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<tr>
<td>Horizontal cell</td>
<td>–79.1 ± 2.9 (7)</td>
<td>66.4 ± 6.3 (7)</td>
<td>–11.7 ± 2.5 (7)</td>
<td>4.7 ± 2.8 (7)</td>
<td>$k = 9.98 ± 1.57$ (7)</td>
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<th>$E_{20}$, mV</th>
<th>$E_{80}$, mV</th>
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<tbody>
<tr>
<td>Horizontal cell</td>
<td>–20.0 ± 3.4 (7)</td>
<td>0.7 ± 3.9 (7)</td>
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Values are means ± SE for n cells (number of cells is indicated in parentheses). $E_{20}$ and $E_{80}$ indicate 20 and 80% activation, respectively, as obtained from fitting a Boltzmann function to the activation curves.
physical and pharmacological criteria. Depending on the identity of the α1 subunit, the family of HVA calcium channels currently contains L-, N-, P-, Q-, and R-types (Catterall 2000; Ertel et al. 2000; Fox et al. 1987). Slow, persistent L-type channels are highly sensitive to 1,4-dihydropyridines and to the phenalkylamine verapamil (Bean 1985; Bean and Mintz 1994; Hockerman et al. 1997). Slow, inactivating N-type channels are defined by their unique sensitivity to ω-conotoxin GVIA, whereas P- and Q-type channels are blocked by the funnel web spider toxin ω-agatoxin IVA (Adams et al. 1993). R-type channels are resistant to the blockers listed in the preceding text, but recently selective peptide antagonists have been identified (Bourinet et al. 2001).

In horizontal cells, currents through HVA calcium channels are enhanced by the divalent metal ion barium, but they are blocked by the transition metal ions cadmium and cobalt.

Horizontal cells were voltage-clamped at a holding potential of −60 mV and subsequently depolarized to 10 mV to evoke maximum inward currents (see Fig. 3C). To estimate possible rundown of calcium currents, this step protocol was carried out repeatedly for 5–10 min. Peak current amplitudes and tail currents of the last and first voltage step were measured, and the ratio of both values was calculated. Horizontal cell calcium channels showed no up- or downregulation of current amplitudes within this time frame (1.03 ± 0.02 and 1.04 ± 0.07, n = 7, for peak and tail currents, respectively), confirming that the pharmacological block of channel function is a specific effect (Fig. 5, E and F; Table 3). In all experiments described in the following text, inward currents and tail currents returned to predrug amplitude levels after a brief washout (1–2 min), indicating that the blocking effects are fully reversible (data not shown).

The divalent metal ions cadmium and cobalt greatly reduced calcium-mediated inward currents. The remaining peak current in the presence of cobalt (500 μM) was 0.31 ± 0.03 (n = 5), whereas cadmium (250 μM) blocked the currents to 0.46 ± 0.03 (n = 4) of control values (Fig. 5, A and E). Cadmium exerted an even stronger blocking effect on tail currents (0.30 ± 0.02, n = 4), very similar to the values obtained on peak currents with cobalt. However, cobalt did not block tail currents any further (Fig. 5F).

Assuming that the applied concentrations are saturating, these results indicate that ~70% of the depolarization-induced inward currents are mediated by HVA calcium channels. In calcium-free extracellular solution, a step depolarization also induced a measurable inward current. Its amplitude and time course was very similar to the unblocked current in the presence of either cadmium or cobalt (data not shown). Because horizontal cells did not tolerate calcium-free extracellular conditions, this was observed only in a few cases.

In the next set of experiments, we distinguished between L- and N-type calcium channels. The selective L-type channel blocker verapamil (100 μM) significantly inhibited calcium currents. Similar to the effect of cadmium noted in the preceding text, the inhibitory effect of verapamil was greater on tail currents (0.38 ± 0.06, n = 6) when compared with peak currents induced during the depolarization (0.52 ± 0.08, n = 6). Figure 5B shows that verapamil blocked all phases of the inward current with equal potency, and it had no effect on the kinetics of the current. Bay K 8644, a dihydropyridine calcium channel agonist, has been described to increase the open probability of L-type channels (Hess et al. 1984). In our hands, Bay K 8644 had a minor potentiating effect on calcium-mediated inward currents that was not statistically significant (Fig. 5, E and F). However, the dihydropyridine antagonist nifedipine (5 μM) significantly blocked calcium-mediated inward currents (Fig. 5, E and F).

Peak current amplitudes were reduced to 0.61 ± 0.03 (n = 8). The selective N-type channel blocker ω-conotoxin GVIA (1 μM) also had a pronounced effect on horizontal cell calcium currents (Fig. 5C). The antagonist blocked the calcium-mediated inward current to 0.57 ± 0.09 (n = 5). Although N-type channels have been described as transient, we never observed a differential effect of the blocker on the kinetics of the current trajectory. However, this could be due to a partial unmasking in the presence of verapamil.
of the blocker of a sodium current expressed by horizontal cells. Repeated application of either verapamil or ω-conotoxin GVIA did not reduce current amplitudes any further (data not shown). It has been reported that high concentrations of ω-conotoxin GVIA also affect L-type calcium channels.

TABLE 3. Pharmacology of horizontal cell calcium channels

<table>
<thead>
<tr>
<th>Drug</th>
<th>Peak</th>
<th>Tail</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.034 ± 0.023 (7)</td>
<td>1.040 ± 0.071 (7)</td>
</tr>
<tr>
<td>Bay K 8644 (10 μM)</td>
<td>1.075 ± 0.095 (3)</td>
<td>1.041 ± 0.183 (3)</td>
</tr>
<tr>
<td>ω-Agatoxin IVA (200 nM)</td>
<td>0.879 ± 0.054 (6)*</td>
<td>0.932 ± 0.106 (6)</td>
</tr>
<tr>
<td>ω-Conotoxin GVIA (1 μM)</td>
<td>0.573 ± 0.089 (5)**</td>
<td>0.518 ± 0.076 (5)**</td>
</tr>
<tr>
<td>Cadmium (250 μM)</td>
<td>0.463 ± 0.032 (4)**</td>
<td>0.302 ± 0.023 (4)**</td>
</tr>
<tr>
<td>Cobalt (500 μM)</td>
<td>0.314 ± 0.035 (5)**</td>
<td>0.326 ± 0.031 (5)**</td>
</tr>
<tr>
<td>Verapamil (100 μM)</td>
<td>0.515 ± 0.076 (6)**</td>
<td>0.383 ± 0.065 (6)**</td>
</tr>
<tr>
<td>Nifedipine (5 μM)</td>
<td>0.611 ± 0.026 (8)**</td>
<td>0.614 ± 0.024 (8)**</td>
</tr>
<tr>
<td>Nifedipine (5 μM) + ω-Conotoxin GVIA (1 μM)</td>
<td>0.431 ± 0.024 (8)**</td>
<td>0.465 ± 0.040 (8)**</td>
</tr>
</tbody>
</table>

Values are means ± SE for n cells (number of cells is given in parentheses). I_{Drug} and I_{Control} indicate current amplitudes in the presence and absence of a modulating agent, respectively. Peak currents were measured during the depolarization step, whereas tail currents reflect the inward currents immediately following repolarization to −60 mV. *P < 0.05; **P < 0.01 with respect to control values (Student’s t-test).

Calcium entry through ionotropic glutamate receptors

Fast postsynaptic glutamate receptors are commonly classified into three broad categories based on dicarboxylic amino (Wilkinson and Barnes 1996). Therefore we tested whether or not the blocking effects of the dihydropyridine antagonist nifedipine and ω-conotoxin GVIA are additive. In fact, ω-conotoxin GVIA produced an additional block of the current that had been already reduced by nifedipine, indicating the presence of N-type calcium channels (Fig. 5, E and F). Although the additional block was statistically significant (P < 0.01, Student’s t-test), it was apparently less than the effect of ω-conotoxin GVIA given separately, suggesting partial block of L-type calcium channels at the present concentration of this toxin.

The pronounced effects of L- and N-type channel blockers indicate that both calcium channel types play an important functional role regulating influx of calcium into horizontal cells. Finally, we tested the effect of the P/Q-type channel blocker ω-agatoxin IVA (200 nM) on horizontal cell calcium channels. Both peak and tail currents were slightly blocked by this endogenous toxin (Fig. 5D), suggesting low-level expression of P- and/or Q-type calcium channels in mouse horizontal cells. The results of the pharmacological characterization of horizontal cell calcium currents are summarized in Table 3.
acids as selective agonists: NMDA, kainate, and AMPA (Dingledine et al. 1999). Whereas NMDA receptors are highly permeable to calcium (Mayer and Westbrook 1987), the presence of the AMPA receptor subunit GluR2 renders non-NMDA receptors impermeable for calcium ions (Hollmann et al. 1991).

Horizontal cells receive glutamatergic input from photoreceptors, and in the rabbit retina, they have been described to express AMPA-type ionotropic glutamate receptors (Rivera et al. 2001). Glutamate is released from photoreceptor axon terminals in the dark, and its binding to postsynaptic ionotropic glutamate receptors leads to a depolarization of the horizontal cell membrane. This depolarization is likely to cause influx of calcium through voltage-gated channels as described above. However, glutamate receptors themselves are a potential source of calcium entry, and therefore we monitored the intracellular calcium concentration ([Ca^{2+}]) by imaging the fluorescence emitted by the calcium-sensitive dye Fura 2/AM after activation of AMPA/kainate glutamate receptor channels.

NMDA was applied to horizontal cells in a magnesium-free extracellular solution containing 1 mM glycine as an allosteric activator of NMDA receptor channels. Lowering the extracellular concentration of the divalent ion magnesium did not affect the intracellular calcium concentration as indicated by the unaltered fluorescence baseline (data not shown). We never observed a calcium signal in the presence of 100 μM NMDA [ΔF/F(t) = −0.019 ± 0.013; n = 3]. It might be conceivable that the measured cells were negatively affected by the isolation procedure in a way that glutamate did not bind because of proteolytic cleavage or internalization of the receptors. Therefore we subsequently applied the AMPA/kainate receptor agonist kainate (100 μM) to the same cells. All horizontal cells that failed to respond to NMDA showed distinct calcium signals in the presence of kainate (data not shown). These experiments indicate that isolated horizontal cells express functional ionotropic glutamate receptors of the non-NMDA type, but they do not express NMDA receptors. In addition, horizontal cell bodies were voltage-clamped at −70 mV in magnesium-free solution, and NMDA (500 μM) was applied in the presence of glycine (2 μM). We never observed an NMDA-induced membrane current in all cells tested (n = 5; Fig. 6F). To exclude the possibility that the calcium signal simply reflects activation of voltage-gated calcium channels due to kainate-induced depolarization, all experiments were carried out in the presence of cadmium (250 μM). Because LVA calcium channels are not expressed by horizontal cells, no action was taken to block T-type calcium channels.

We also applied AMPA to verify expression of AMPA/kainate receptors in mouse horizontal cells. In the presence of extracellular cadmium, application of AMPA (100 μM) resulted in a greatly increased [Ca^{2+}]; [ΔF/F(t) = 0.241 ± 0.082] in all horizontal cells tested (n = 5; Fig. 6, A and F). These experiments indicate expression by horizontal cells of non-NMDA glutamate receptor-mediated calcium influx.

**FIG. 6.** Influx of calcium through ionotropic glutamate receptors. **A:** application of AMPA (100 μM) evoked influx of calcium through AMPA/Kainate receptors. **B:** kainate-induced calcium signal was not significantly inhibited in the presence of SYM 2081 (50 nM). **C:** at higher concentrations (200 μM), SYM 2081 still induced a measurable increase in intracellular calcium. **D:** response to kainate was partially blocked by the selective AMPA receptor antagonist GYKI 52466 (200 μM). **E:** application of N-methyl-D-aspartate (NMDA, 100 μM) failed to evoke an intracellular calcium signal. **F:** likewise, in patch-clamp recordings NMDA (500 μM) did not induce a membrane current when horizontal cells were clamped at a holding potential of −70 mV. **G:** bar graph summarizing changes in intracellular calcium (means ± SE) induced by extracellular application of the drugs depicted on the left. Drugs were applied for 10 s as indicated by the bars below each trace. All experiments were carried out in the presence of 250 μM cadmium to block voltage-dependent calcium channels. Scale bars indicate the time and fluorescent changes ΔF/F(t). **P < 0.01 (Student’s t-test).**
functional glutamate receptors of the AMPA/kainate type, which lack the GluR2 subunit.

AMPA and kainate receptors are often co-expressed in neurons, and responses to kainate tend to be masked by larger AMPA receptor-mediated currents (Paternain et al. 1995). Therefore selective compounds affecting one or the other receptor type are necessary to distinguish between AMPA and kainate receptors. Introduction of a methyl group at position 4 of glutamic acid has generated a series of stereoisomers, of which the (2S,4R)-isomer (SYM 2081) proved to be a selective, high-affinity ligand for binding at kainate receptors (Gu et al. 1995). Concentrations of SYM 2081 in the nanomolar range reversibly block rapidly desensitizing currents induced by kainate, whereas at higher concentrations (>1 μM) SYM 2081 itself acts as an agonist at kainate receptors (Zhou et al. 1997). We therefore tested the effects of low concentrations of SYM 2081 (50 nM) when co-applied with kainate (100 μM). As shown in Fig. 6G, the kainate-induced calcium signal [ΔF/F(t) = 0.194 ± 0.042] was not significantly altered in the presence of SYM 2081(ΔF/F(t) = 0.209 ± 0.045; Fig. 6B). When applied in the absence of kainate at a concentration of 200 μM, SYM 2081 induced a measurable rise in cytosolic calcium [ΔF/F(t) = 0.023 ± 0.006, n = 3; Fig. 6C]. Although this concentration of SYM 2081 is saturating for kainate receptors (Donevan et al. 1998), the observed increase was much smaller when compared with the effect of kainate itself (Fig. 6G), suggesting at least partial activation by SYM 2081 of AMPA receptors. To further distinguish calcium signals due to the activation of kainate receptors from those resulting from the opening of AMPA receptors, we applied the compound GYKI 52466, which has been described as a noncompetitive blocker of non-NMDA receptors with higher affinity for AMPA receptors (Donevan and Rogawski 1993). GYKI 52466 (200 μM) significantly blocked the kainate-induced rise in [Ca2+]i [ΔF/F(t) = 0.071 ± 0.012, n = 7; Fig. 6D]. At the concentration used, GYKI 52466 has been shown to completely block AMPA receptor-mediated responses (Paternain et al. 1995), indicating that the residual signal is most likely caused by the influx of calcium through kainate receptor channels.

**Metabotropic glutamate receptors**

Synaptically released glutamate can increase [Ca2+]i, not only via ionotropic glutamate receptors but also by binding to group I metabotropic glutamate receptors (mGluRs) and subsequent release of calcium from intracellular stores via the phosphoinositide pathway. To determine whether or not mouse horizontal cells express group I mGluRs linked to inositol 1,4,5-trisphosphate (IP3)-dependent calcium release, selective mGluR agonists were applied to isolated horizontal cells.

The mixed group I and II mGluR agonist trans-ACPD (100 μM) did not change the intracellular calcium level [ΔF/F(t) = 0.013 ± 0.004], whereas application of the ryanodine receptor agonist caffeine (10 mM) to the same cells elicited significant calcium release from intracellular stores [ΔF/F(t) = 0.308 ± 0.105; n = 5; Fig. 7A]. Increasing the concentration of trans-ACPD to 200 μM did not induce a measurable calcium signal [ΔF/F(t) = 0.009 ± 0.006], whereas 10 mM caffeine again evoked marked fluorescence changes within the same cells [ΔF/F(t) = 0.231 ± 0.07; n = 6; Fig. 7B]. Although we cannot exclude the possibility that metabotropic glutamate receptors are coupled to other intracellular mechanisms affecting calcium increases (Linn and Gafka 1999, 2001), our experiments suggest that isolated horizontal cells do not express mGluRs linked to release of calcium from intracellular stores. Because activation of ryanodine receptors induced a significant calcium signal, we can rule out the possibility that the calcium stores were depleted during the isolation procedure or while loading the cells with the indicator dye.

To obtain further support for the hypothesis that mGluRs coupled to IP3 production and calcium release are not expressed by horizontal cells, we applied the endogenous ligand glutamate (200 μM) in the presence of CNQX (100 μM), AP-5 (5 μM), and cadmium (250 μM). With ionotropic glutamate receptors and HVA calcium channels blocked, glutamate was ineffective in elevating cytosolic calcium [ΔF/F(t) = 0.005 ± 0.01; n = 4]. However, the same horizontal cells responded to 10 mM caffeine showing a small but significant calcium signal [ΔF/F(t) = 0.098 ± 0.013; Fig. 7C].

Finally, we tested the selective group I mGluR agonist quisqualic acid (200 μM) together with CNQX (100 μM) to prevent a possible activation of non-NMDA glutamate receptors. As expected, quisqualic acid failed to elicit a calcium response [ΔF/F(t) = 0.001 ± 0.002], whereas a clear calcium signal could be measured after exposure to 10 mM caffeine [ΔF/F(t) = 0.1 ± 0.028; n = 5; Fig. 7D]. The average fluorescence changes are summarized in Fig. 7E. Again, these results indicate that activation of metabotropic glutamate receptors in horizontal cells of the mouse retina most likely does not evoke a direct elevation of intracellular calcium levels.

The endogenous ligands and the intracellular signal transduction cascade culminating in the activation of ryanodine receptors expressed on the membranes of internal calcium stores are currently not well understood. However, these receptors are sensitive for caffeine (Verkhratsky and Shmigol 1996). As shown above, isolated horizontal cells respond to extracellular application of caffeine with a significantly increased release of calcium [ΔF/F(t) = 0.191 ± 0.037; n = 20; Fig. 7E], suggesting the presence of ryanodine receptors on intracellular calcium stores.

Fluorescence signals based on caffeine-evoked calcium release from stores revealed different amplitudes and decay times. As a possible explanation, one could assume that calcium concentrations in intracellular stores vary among horizontal cells, maybe as a consequence of the isolation procedure. Therefore this could influence the amount of calcium released and the cytoplasmic calcium extrusion rate, thus leading to signal dissimilarity. In experiments using t-ACPD or glutamate with CNQX and CdCl2, caffeine-evoked calcium signals were found to be smaller using an extracellular solution containing CdCl2. Cadmium ions bind with a high affinity to P2X receptors expressed on the membranes of internal calcium stores. As a possible explanation, one could assume that calcium concentrations in intracellular stores vary among horizontal cells, maybe as a consequence of the isolation procedure. Therefore this could influence the amount of calcium released and the cytoplasmic calcium extrusion rate, thus leading to signal dissimilarity. In experiments using t-ACPD or glutamate with CNQX and CdCl2, caffeine-evoked calcium signals were found to be smaller using an extracellular solution containing CdCl2. Cadmium ions bind with a high affinity to P2X receptors expressed on the membranes of internal calcium stores.
DISCUSSION

In this study, we examined different pathways culminating in the elevation of intracellular calcium in mouse horizontal cells. Calcium ions can cross the cell membrane from the extracellular side via voltage-gated calcium channels and/or after binding of glutamate to non-NMDA receptors. An alter-
native entry route from intracellular stores requires activation of ryanodine receptors.

Voltage-gated calcium channels

Based on pharmacological evidence, we found that both L- and N-type calcium channels are expressed in mouse horizontal cells, whereas P/Q-type channels apparently are not involved in the regulation of intracellular calcium. Functional calcium channels have been identified in all major cell types of the mammalian retina: rod and cone photoreceptors (Morgans 2001; Morgans et al. 2005), bipolar cells (Berntson et al. 2003; de la Villa et al. 1998; Pan 2000), horizontal cells (Ueda et al. 1992), amacrine cells (Cohen 2001; Habermann et al. 2003), and ganglion cells (Schmid and Guenther 1999). Currently, four different members of the L-type subfamily have been described (Ertel et al. 2000), two of which (Ca1.3 and Ca1.4) are localized in the retina. The biophysical properties of mouse horizontal cell calcium channels are reminiscent of those described for Ca1.2 (Xu and Lipscombe 2001) and Ca1.4 (Baumann et al. 2004), whereas they differ with respect to BayK 8644 sensitivity. It has to be noted, however, that the likely presence of accessory subunits in horizontal cells has a profound impact on gating kinetics and pharmacological properties of native channels as compared with heterologous expression systems. Surprisingly, cadmium and cobalt did not completely block the depolarization-induced inward current. We have currently no explanation for this finding, but given the potent blocking effect of both transition metal ions in other neurons of the CNS, it seems unlikely that the residual current is mediated by calcium. This assumption is corroborated by the finding that we could measure an inward current in the absence of extracellular calcium.

Whereas L-type calcium channels subserve a plethora of different functions depending on cell type and intracellular localization, N-type channels are most commonly associated with calcium influx for exocytotic release of neurotransmitter. The question of whether mammalian horizontal cells release their endogenous transmitter GABA via a calcium-dependent exocytotic mechanism or mimic the transporter-driven release observed in nonmammalian retinas (Schwartz 2002) has not been answered yet. Early work has demonstrated the presence of clear-core vesicles in horizontal cell processes (Dowling et al. 1966; Fisher and Boycott 1974; Kolb 1977). In addition, the vesicular GABA transporter (VGAT), responsible for packaging of transmitter into vesicles, is expressed in mammalian horizontal cells, with highest levels in dendritic and axonal endings within the synaptic triad (Cueva et al. 2002; Haverkamp et al. 2000; Jellali et al. 2002). Recently, the presence of synaptic proteins participating in vesicular release has been demonstrated (Hirano et al. 2005). Electrophysiological studies so far failed to detect a transporter current in mammalian horizontal cells (Blanco et al. 1996; Feigenspan and Weiler 2004). Thus the experimental evidence summarized in the preceding text supports the notion that GABA is released in a conventional vesicular manner.

The expression of functional N-type calcium channel in mouse horizontal cells is in line with conventional, calcium-dependent transmitter release. Rapid, localized action of calcium is essential for the release of neurotransmitters (von Gersdorff et al. 1996). The delay between depolarization of the presynaptic membrane at the active zone and transmitter release is <1 ms, suggesting a close spatial relationship between plasma membrane calcium channels and the machinery of synaptic transmitter release. α-Conotoxin GVIA-sensitive N-type calcium channels bind to syntaxin, one of the proteins present on the presynaptic membranes, thereby placing the channel in close proximity to calcium-dependent trafficking, docking, and fusion proteins of the exocytosis complex (Bennett et al. 1992; Leveque et al. 1994). The presence of syntaxin-1 has been demonstrated in horizontal cell endings at cone and rod photoreceptor synapses (Hirano et al. 2005). Although the precise localization of N-type calcium channels in horizontal cells has to be determined, a possible co-localization of N-type calcium channels and proteins of the vesicle releasing machinery might point to a putative role of N-type calcium channels in vesicular transmitter release. However, further studies are needed to establish the mechanisms underlying transmitter release from mammalian horizontal cells. Especially, the subcellular localization of voltage-gated calcium channels has to be determined.

Gluatamate receptors

Because horizontal cell dendrites form the lateral elements of the triad structure postsynaptic to cone photoreceptors, glutamate receptors are likely to play a role in the regulation of intracellular calcium. Extracellular application of AMPA and kainate, but not NMDA, induced a significant calcium signal in mouse horizontal cell bodies. These experiments were carried out in the presence of extracellular cadmium to block HVA calcium channels. Because cadmium did not completely abolish the inward current evoked by depolarizing voltage steps, we cannot exclude that a small fraction of the glutamate-induced calcium signal could be mediated by influx of calcium through unblocked voltage-gated channels and possibly subsequent release from intracellular stores. As noted in the preceding text, however, cadmium is generally considered to be a potent blocker of HVA calcium channels, and therefore we deem this possibility rather unlikely. Although AMPA receptors are activated by kainate, the rise of intracellular calcium in the presence of the selective AMPA receptor antagonist GYKI 52466 suggests expression of both AMPA and kainate receptors. In the mouse retina, the AMPA receptor subunit GluR1 has been found on horizontal cell processes postsynaptic to cones, whereas GluR2/3 and GluR4 are expressed on horizontal cell processes postsynaptic to rods and cones (Hack et al. 2001). The presence of the edited form of GluR2 renders AMPA receptors impermeable for calcium (Hollmann et al. 1991), suggesting either expression of a heterogeneous population of glutamate receptors or the absence of GluR2 from mouse horizontal cell dendrites contacting cones. Calcium-permeable AMPA receptors have also been demonstrated in horizontal cells of the fish retina (Linn and Christensen 1992; Tachibana 1985).

To our knowledge, the presence of kainate receptors has not yet been reported in horizontal cells of the mouse retina. Whereas GluR5 and KA2 are apparently expressed by certain types of OFF cone bipolar cells in rat and mouse retina (Brandstätter et al. 1997; Haverkamp and Wässle 2000), GluR6/7 has been localized to horizontal cell processes postsynaptic to both rods and cones in the rat retina (Brandstätter et al. 1997). The
only electrophysiological study so far, carried out on isolated horizontal cells of the rabbit retina, failed to detect kainate receptor-mediated inward currents (Blanco and de la Villa 1999). Our results indicate that kainate receptors are expressed—possibly at low levels—in horizontal cells, but in contrast to AMPA-type receptors, they do not contribute significantly to the overall calcium signal. This is most likely due to the fast desensitization kinetics of kainate receptors as well as their slow recovery from desensitization. Although NMDA receptors have been found in horizontal cells of the fish retina (Linn and Christensen 1992; O’Dell and Christensen 1989), the lack of these receptors in horizontal cells of the mammalian retina has recently been reported and is in agreement with our findings (Blanco and de la Villa 1999; Hack et al. 2001; Rivera et al. 2001; Shen et al. 2004). Of course, NMDA receptors could be lost during the enzymatical isolation procedure, in which case they are preferentially located on the fine distal dendrites that are commonly ripped off during dissociation. However, this seems rather unlikely because a variety of other ligand- and voltage-gated channels are functionally expressed on isolated horizontal cells and other cells isolated from the retina using a similar protocol (Gustincich et al. 1997). Thus immunocytochemical evidence for expression of NMDA receptor subunits in the outer plexiform layer is likely to originate from NMDA receptor localization on photoreceptor terminals and/or bipolar cell dendrites (Fletcher et al. 2000; Wenzel et al. 1997). Because horizontal cells of the mouse retina most likely do not express NMDA receptors, activation of AMPA receptors and subsequent opening of voltage-gated calcium channels are the two major entry routes for calcium from the extracellular space. As described for on/off bipolar cells (DeVries 2000), the presence of AMPA and kainate receptors could represent an early filter system to segregate cone input into different temporal domains.

Group I metabotropic glutamate receptors (mGluR1 and mGluR5) are strongly activated by quisqualic acid, and they are coupled to stimulation of phospholipase C and subsequent generation of IP$_3$, subsequently leading to release of calcium from internal stores via activation of IP$_3$ receptors. Our results suggest that group I mGluRs do not participate in the regulation of the intracellular calcium concentration in mouse horizontal cells. Immunocytochemical evidence for the presence of group I mGluRs has been reported in the outer plexiform layer of the rat retina, with mGluR1 and mGluR5 being expressed in bipolar cells but not in horizontal cells (Koulen et al. 1997). With the exception of mGluR6 and mGluR8, which are localized to depolarizing bipolar cells and photoreceptors, respectively, the remaining members of the metabotropic glutamate receptors are either not expressed in the retina (mGluR3) or exclusively restricted to the level of the inner plexiform layer (Brandstätter et al. 1996; Koulen et al. 1996). Therefore mGluRs apparently do not contribute to intracellular calcium signaling in mouse horizontal cells.

**Calcium-induced-calcium-release in the horizontal cell network**

Stimulating horizontal cells with caffeine evoked calcium release from intracellular stores via ryanodine-sensitive receptors. Ryanodine receptors are calcium-release channels located in the membrane of the endoplasmatic reticulum, and they are regulated, among other factors, by intracellular calcium and cyclic ADP ribose. Calcium-induced calcium release has been described so far only for horizontal cells of the teleost retina (Linn and Gafka 2001; Linn and Christensen 1992; Solessio and Lasater 2002). Here influx of calcium through ionotropic glutamate receptors is linked to calcium release from ryanodine-sensitive stores and modulation of L-type channel activity. Horizontal cells in the mouse retina are extensively coupled by gap junctions containing connexin57, thus forming a low-resistance network promoting intercellular signal transmission within the layer of horizontal cells (Hombach et al. 2004). At present, we can only speculate on the function of calcium-induced calcium release within the horizontal cell synecytium. The endoplasmatic reticulum is a continuous network distributed throughout neurons and even expands in fine dendritic processes. This calcium store can produce slowly propagating regenerative calcium signals using a conduction system of ryanodine receptors along its surface (Berridge 1998). Because gap junctions are readily permeable for calcium, they would not impede diffusion of calcium ions between horizontal cells, and thus they are ideally suited to spread regenerative calcium waves across the network (Berridge et al. 2000; Pearson et al. 2004). Although rather speculative, the regulation of intracellular calcium within the transgap junctional network, generated through the precise orchestration of voltage-gated and ionotropic channels in conjunction with calcium-induced calcium release and several calcium-buffering mechanisms, could play a significant role in signal processing and adaptational mechanisms in the outer retina.

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