Meclofenamic Acid Blocks Electrical Synapses of Retinal AII Amacrine and on-Cone Bipolar Cells

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INTRODUCTION

Gap junctions are found in a variety of cells and tissues and correspond to intercellular contacts where the major structural elements are formed by transmembrane proteins termed connexins (Kumar and Gilula 1996). Each gap junction contains a number of intercellular ion channels, or connexons, with each connexon corresponding to two hemi connexons, one in each of the neighboring cell membranes. A hemi connexon consists of six connexins arranged in a hexagonal pattern. Gap junctions serve a variety of functions related to intercellular diffusion of small molecules and passage of electric current (electrical synapses). In the CNS, most electrical synapses are found between neurons of the same type (homologous gap junctions), whereas electrical synapses between different types of neurons (heterologous gap junctions) occur less frequently (Galarreta and Hestrin 2001).

In the retina, gap junctions are found between a variety of neurons, both in the inner and outer plexiform layers. Considerable attention has been directed to the functional role and properties of electrical synapses in the rod pathway of the mammalian retina (reviewed by Bloomfield and Dacheux 2001). AII amacrine cells are an important part of this circuit and integrate and transmit scotopic visual signals. They receive glutamatergic synaptic input from rod bipolar cells (Singer and Diamond 2003; Veruki et al. 2003) and are interconnected by gap junctions (Kolb and Famiglietti 1974), thereby forming a network of electrically coupled neurons (Veruki and Hartveit 2002a). In addition to these homologous gap junctions, AII amacrine cells make extensive heterologous gap junctions with on-cone bipolar cells (Chun et al. 1993; Kolb and Famiglietti 1974; McGuire et al. 1984; Strettoi et al. 1992, 1994). The gap junctions between AII amacrine cells and on-cone bipolar cells also correspond functionally to electrical synapses (Trexler et al. 2005; Veruki and Hartveit 2002b) and constitute the pathway whereby rod signals are conveyed to the on-system.

There is strong evidence that the neuron-specific connexin36 (Cx36) is expressed by AII amacrine cells and is the subunit of the homologous gap junctions (Feigenspan et al. 2001; Mills et al. 2001). With respect to the heterologous gap junctions between AII amacrine cells and on-cone bipolar cells, there is evidence that they contain Cx36 on the AII side and either Cx36 or Cx45 on the bipolar side (Dedek et al. 2006; Han and Massey 2005; Lin et al. 2005; Maxeiner et al. 2005).

To investigate the functional role of electrical synapses composed of specific connexins, the development of connexin-deficient (knockout) mice has been very valuable. For example, Cx36-deficient mice display strongly reduced tracer coupling between AII amacrine cells and between AII amacrine cells and on-cone bipolar cells, as well as elimination of rod-mediated, on-center responses at the ganglion cell level (Deans et al. 2002). However, knockout animals can display secondary, compensatory changes in addition to the lack of a specific gene. Indeed, there is evidence for compensatory morphological and electrophysiological changes at the single-neuron level in Cx36-deficient animals (De Zeeuw et al. 2003). Furthermore, genetic elimination typically means that the functional block of a specific connexin is irreversible. Thus it is highly desirable to have access to pharmacological agents that would selectively and reversibly block gap junction channels. Unfortunately, there are very few agents that block gap junction channels and most of these have nonspecific effects on other channel types. Recently, a series of compounds, with known effects on gap junction channel coupling, was examined with respect to the ability to block tracer coupling through gap junctions between specific neurons in the mammalian retina (Pan et al. 2007). Of the compounds tested, meclofenamic acid (MFA) displayed many desirable properties as a gap junction...
antagonist: high potency, water solubility, and relatively fast reversibility. These properties suggested that MFA could also be a useful drug for investigating functional properties and roles of gap junction coupling and electrical synapses in the retina. The suitability of MFA in physiological studies is not yet established, however, and particular attention has to be paid to the possible dissociation between tracer coupling and electrical coupling, with only electrical coupling being directly relevant for rapid transmission of visual signals.

Here, we have applied dual whole cell voltage-clamp recording to pairs of AII amacrine cells and pairs of AII amacrine cells and ON-cone bipolar cells to directly measure the junctional conductance ($G_j$) between coupled cells. We found that MFA completely and reversibly blocked the electrical synapse currents in a concentration-dependent manner. Surprisingly, however, whereas a small reduction of the junctional conductance could be observed within minutes of application, the time to complete block was typically 20–40 min and the time to maximum recovery was typically 1 h. This suggests that great care should be exercised when interpreting the results of experiments where MFA has been used to block electrical synapses when the junctional conductance has not been directly monitored.

METHODS

General aspects of the methods have previously been described in detail (Hartveit 1996; Veruki and Hartveit 2002a). Albino rats (4–7 wk postnatal) were deeply anesthetized with isoflurane in oxygen and killed by cervical dislocation (procedure approved under the surveillance of the Norwegian Animal Research Authority). Retinal slices were visualized with a $\times$40 water-immersion objective (Olympus BX51WI) and infrared gradient contrast videomicroscopy (Luigs & Neumann Feinmechanik und Elektrotechnik, Ratingen, Germany; Dondt et al. 1998). Recordings were carried out at room temperature (22–25°C).

Solutions and drugs

The extracellular perfusing solution was continuously bubbled with 95% O$_2$-5% CO$_2$ and had the following composition (in mM): 125 NaCl, 25 NaHCO$_3$, 2.5 KCl, 2.5 CaCl$_2$, 1 MgCl$_2$, and 10 glucose (pH 7.4). Whole cell recordings were performed with high-resistance pipettes (25–35 MΩ) pulled from borosilicate glass (GC150-11; Harvard Apparatus, Edenbridge, UK) and filled with the following solution (in mM): 125 K-gluc, 5 KCl, 8 NaCl, 0.2 EGTA, 10 Heps, and 4 MgATP (pH was adjusted to 7.3 with KOH). Lucifer yellow was added at a concentration of 1 mg/ml. The theoretical liquid junction potential was calculated with the computer program JCaldW (Molecular Devices, Sunnyvale, CA) and membrane holding potentials were corrected for the liquid junction potential off-line.

During recordings, $\alpha$-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), $\gamma$-aminobutyric acid type A (GABA$\alpha$), and glycine receptors were blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), bicuculline, and strychnine, respectively. Voltage-gated Na$^+$ channels were blocked by tetrodotoxin (TTX). Drugs were added directly to the extracellular solution used to perfuse the slices. The concentrations of drugs were as follows (in μM; supplier Tocris Bioscience, Avonmouth, UK, unless otherwise noted): 10 bicuculline methchloride; 10 CNQX disodium salt; 1, 10, or 100 2-[(2,6-dichloro-3-methylphenyl)aminomethyl]benzoic acid sodium salt [meclofenamic acid [MFA] sodium salt; Sigma], 1 strychnine, 0.3 TTX. MFA sodium salt was stored as a stock solution in water (100 mM) at −20°C and diluted to the final concentration on the day of the experiment.

Electrophysiological recording and data acquisition

For paired recordings between AII amacrine cells or between AII amacrine cells and ON-cone bipolar cells, we used high-resistance pipettes (25–35 MΩ) with long, thin tips. This allowed us to obtain stable and long-lasting whole cell recordings of electrically coupled cells under conditions of reduced intracellular washout (Veruki et al. 2008). Voltage clamp was achieved by using two discontinuous (switched) single-electrode voltage-clamp (DSEVC) amplifiers (SEC-05LX-BF; npi elektronik, Tamm, Germany), each controlled by one of two instances of PatchMaster software (HEKA Elektronik, Lambrecht/Pfalz, Germany) in a "master–slave" configuration, with both instances running on the same computer (Mac OS X version 10.4). Because the potential is measured at a time when no current flows across the recording electrode, problems caused by voltage drops across nonzero series resistance ($R_s$) are reduced and potentially totally avoided. The switching frequency was set to 35–40 kHz, synchronized between the two amplifiers (Müller et al. 1999), and the duty cycle was set to 1/4. Before sampling, current and voltage signals were low-pass filtered (analog four-pole Bessel filter) with a corner frequency (~3 dB) 15 of the inverse of the sampling interval (100 μs). The voltage-clamp gain and the proportional-integral controller were adjusted to give the fastest possible voltage response with minimal overshoot and ringing.

The headstage output voltage signal from both amplifiers was monitored on an oscilloscope throughout each recording. For each amplifier, application of voltage protocols and digital sampling of the analog signals were performed by an LIH8+8 laboratory interface (HEKA Elektronik). The start of sampling by the “slave” interface was hardware triggered from the “master” interface via a digital line.

Imaging

For a few cell pairs, a series of fluorescence images at closely spaced focal planes was acquired with a digital CCD camera (CoolSnap ES; Photometrics/Roper Scientific, Tucson, AZ) controlled by μManager software (www.micro-manager.org) running under Windows XP. During image acquisition, exposure to UV-light was controlled by an electronic shutter (Uniblitz VCM-D1, Vincent Associates, Rochester, NY). Subsequently, the images were assembled in a montage to illustrate the cells’ morphology (Adobe Photoshop 7.0, Adobe Systems).

Data analysis

To estimate the steady-state $G_j$ between the two cells of a physiologically coupled pair, we used current responses obtained with dual voltage-clamp recordings. For the calculations, we assumed an equivalent-circuit model (Veruki et al. 2008) and that $R_s$ was effectively zero. This means that the junction current corresponds to the current evoked in the postsynaptic cell and $G_j$ can be calculated directly from Ohm’s law (Müller et al. 1999). Each measurement point was obtained by plotting the junction current ($I_j$) versus the junction voltage ($V_j$) and by calculating $G_j$ as the slope of a straight line fitted to the $I_j$–$V_j$ relationship (averaged for both directions of coupling). Approximately three such measurements of $G_j$ were obtained per minute. In all experiments, the strength of electrical coupling between a pair of cells was estimated on-line throughout the recording period (PatchMaster).

Off-line data analysis was performed with FitMaster (HEKA Elektronik) and IGOR Pro (WaveMetrics, Lake Oswego, OR). Data are presented as mean ± SE (n = number of cell pairs) and percentages are presented as percentage of control. Statistical analysis was performed using Student’s two-tailed t-test (paired). For illustration purposes, most raw data records were low-pass filtered (digital non-lagging Gaussian filter; ~3 dB at 0.5–1 kHz).
RESULTS

Identification of AII amacrine cells and on-cone bipolar cells in retinal slices

All amacrine cells constitute a homogeneous population of cells (Wässle et al. 1993) and were targeted for recording according to the size and location of the cell body in the inner nuclear layer and the thick primary dendrite descending into the inner plexiform layer (Fig. 1A). On-cone bipolar cells are a heterogeneous group encompassing four to five cell types (Euler et al. 1996; Hartveit 1997) and their cell bodies tend to be located distally in the inner nuclear layer, proximally to the row of cell bodies of rod bipolar cells (Euler and Wässle 1995). For recording AII amacrine cell pairs with a high probability of physiological coupling, we targeted neighboring cells that were judged to have spatially overlapping, potentially contacting, arboreal dendrites in the proximal part of the inner plexiform layer (n = 11 cell pairs). For recording cell pairs of AII amacrine cells and on-cone bipolar cells with a high probability of physiological coupling, we targeted cells with somata that were as close as possible along a vertical line across the retina (Fig. 1C; n = 4 cell pairs). All cells were filled with Lucifer yellow and, at the end of each recording, fluorescence microscopy allowed visualization of both cells’ complete morphology (Fig. 1, B and D).

Electrical coupling between AII amacrine cells

With both cells of a pair of AII amacrine cells in the whole cell voltage-clamp configuration, we tested for electrical coupling by applying voltage pulses to one cell (“presynaptic”; Fig. 2A) and recording the current responses in both the pulsed and the nonpulsed (“postsynaptic”) cell (Fig. 2, B and C; Veruki and Hartveit 2002a). In the presynaptic cell, hyperpolarizing and depolarizing voltage pulses evoked inward and outward currents, respectively (Fig. 2, B and C). When cells were physiologically coupled, the hyperpolarizing and depolarizing voltage pulses applied to the presynaptic cell evoked outward and inward currents, respectively, in the postsynaptic cell (Fig. 2, B and C).

For each direction of coupling, we plotted the $I_j$ versus the $V_j$ and calculated $G_j$ as the slope of a straight line fitted to the $I_j$–$V_j$ relationship (Fig. 2, D and E). The conductance was very similar for both directions of measurement (Fig. 2, D and E; Veruki and Hartveit 2002a) and $G_j$ for a cell pair was calculated as the average of the conductance values measured in each direction (350 ± 44 pS; n = 11 cell pairs; range 170–600 pS). In recordings with high-resistance pipettes, $G_j$ is stable over time and recordings can last for several hours, most likely because the high-resistance pipettes reduce intracellular washout (Veruki et al. 2008). An example of long-term stability of $G_j$ in paired recordings of AII amacrine cells with this technique can be seen in Fig. 4C of Veruki et al. (2008).

MFA reversibly blocks electrical coupling between AII amacrine cells

We examined the effect of MFA on the electrical coupling between AII amacrine cells by repeated measurements of $I_j$–$V_j$ relationships (approximately every 20 s), first in the control condition to establish a baseline, then during bath application of MFA (100 μM), and finally after washout of the drug. $G_j$ was calculated as the slope of a straight line fitted to the $I_j$–$V_j$ relationship. For the cell pair illustrated in Fig. 3, the baseline $G_j$ was ∼400 pS (Fig. 3A). When MFA was applied in the bath, $G_j$ was slowly reduced (Fig. 3A). The latency to onset of the reduction was ∼9 min. After ∼40 min of drug application, no electrical coupling could be

![FIG. 1. All amacrine cells and on-cone bipolar cells in in vitro retinal slice preparation. A: a pair of electrically coupled AII amacrine cells (cell bodies marked by arrows) in an in vitro slice from rat retina, visualized with infrared gradient contrast videomicroscopy. The retinal layers are indicated by abbreviations (ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer). Scale bar: 10 μm. B: composite fluorescence photomicrograph of the pair of AII amacrine cells in A after filling with Lucifer yellow. Photomicrographs were taken after about 80 min of recording, including periods with exposure to 10 and 100 μM meclofenamic acid. Due to a slanted orientation of the slice, the flattened projection of the images makes the cells appear shorter than they are. Scale bar: 10 μm. C1 and C2: an AII amacrine cell electrically coupled to an on-cone bipolar cell in an in vitro slice from rat retina, visualized with infrared gradient contrast videomicroscopy. In C1, focus is on cell body of the AII amacrine cell (marked by arrow) and in C2, focus is on cell body of the on-cone bipolar cell (marked by arrow). Scale bar: 10 μm (C1 and C2). D: composite fluorescence photomicrograph of the AII amacrine and on-cone bipolar cells in C (1, 2) after filling with Lucifer yellow. Arrows point to axon and axon terminal of bipolar cell. Recording pipette of AII amacrine cell is out of focus. Photomicrographs were taken after about 10 min of recording. Scale bar: 10 μm.](http://jn.physiology.org/)}
observed (Fig. 3, A and B). When MFA was washed out by switching to control solution, the electrical coupling slowly recovered and reached 86% of the control level after a washout period of 90 min (Fig. 3, A and B).

With 100 μM MFA, the electrical coupling was completely blocked for all AII amacrine cell pairs tested (7/7). The latency to onset, measured as the time from application of MFA to 10% of total block, was 5.3 ± 0.7 min (n = 7; range 3.1–8.7 min). The latency to full block, measured as the time from application of MFA to 90% of total block, was 20.2 ± 2.3 min (n = 7; range 15–33 min). Two cell pairs were lost before recovery occurred. For the other five cell pairs, the recordings were maintained long enough that a recovery of electrical coupling to a plateau level was observed. The average recovery, measured at this plateau level (≈90 min after terminating the application of MFA), corresponded to a Gj that was 46 ± 12% (range 18–86%) of the control level. However, the recovery occurred very slowly after terminating the application of MFA. Recovery to 10% of the final plateau occurred after 18.2 ± 2.3 min (range 15–27 min) of washout and recovery to 90% of the final plateau occurred after 80.7 ± 6.2 min (range 68–99 min) of washout.

At the holding potential of −60 mV, the average holding current (Ihold) in the control condition (before application of MFA) was −1.7 ± 3.2 pA (n = 14 cells). After application of MFA, Ihold changed to more positive values and when the electrical coupling was completely blocked, Ihold was 9.6 ± 2.5 pA (P = 0.0004; paired t-test).

FIG. 2. Measuring electrical coupling and junctional conductance (Gj) between AII amacrine cells with dual whole cell recording and discontinuous single-electrode voltage-clamp (DSEVC) amplifiers. A: with a pair of AII amacrine cells in voltage clamp (Vhold = −60 mV), 200-ms voltage pulses (from −40 to +10 mV relative to Vhold; increments of 10 mV) were applied sequentially to the cells (V1, V2). Recording configuration indicated in right diagram. B and C: current responses recorded from the cells (I1, I2) in response to the voltage pulses in A. Hyperpolarizing voltage pulses applied to cell 1 (cell 2) result in inward currents in cell 1 (cell 2) and outward currents in cell 2 (cell 1). Depolarizing voltage pulses applied to cell 1 (cell 2) result in outward currents in cell 1 (cell 2) and inward currents in cell 2 (cell 1). Each trace is the average of 12 trials. Notice that the asymmetry of the voltage pulses relative to Vhold helps to identify the corresponding current responses in the 2 cells. Here, and later, capacitative current transients have been truncated for clarity. D and E: junction current (Ij) vs. junction voltage (Vj) relationships (Ij–Vj) obtained with cell 1 presynaptic–cell 2 postsynaptic (D) and cell 2 presynaptic–cell 1 postsynaptic (E). For each set of measurements, the data points have been fitted with a straight line (slope = Gj).

FIG. 3. Complete and reversible block of electrical coupling between AII amacrine cells by 100 μM meclofenamic acid (MFA). A: Gj as a function of time for a pair of electrically coupled AII amacrine cells in voltage clamp. Gj is calculated as the average of the conductance values measured for each direction of coupling (with voltage pulses applied to either cell 1 or cell 2). MFA was applied in the extracellular perfusing solution during the period indicated by the horizontal bar (duration 40 min). The lower case letters a, b, and c indicate time points during control (a), in the presence of MFA (b), and after washout of MFA (c), where averaged current responses are displayed in B. Recording configuration indicated in inset. B: top: voltage protocol with 200-ms voltage pulses (from −40 to +10 mV relative to Vhold = −60 mV; increments of 10 mV) applied to the cells (V1, V2). Bottom, a, b, and c: current responses recorded from the cells (I1, I2) in response to voltage pulses in the control condition before application of MFA (a); each trace is the average of 7 trials), after complete block of Gj in the presence of MFA (b; each trace is the average of 6 trials), and after partial recovery of electrical coupling after washout of MFA (c; each trace is the average of 8 trials).
Electrical coupling between AII amacrine cells and ON-cone bipolar cells

All amacrine cells are electrically coupled not only to other AII amacrine cells, but to specific types of ON-cone bipolar cells as well. In paired recordings of an AII amacrine cell and an ON-cone bipolar cell, with both cells in the whole cell voltage-clamp configuration, we tested for electrical coupling by applying voltage pulses to the presynaptic cell (Fig. 5A) and recording the current responses in both cells (Fig. 5, B and C; Veruki and Hartveit 2002b). When cells were physiologically coupled, the hyperpolarizing and depolarizing voltage pulses applied to the presynaptic cell evoked outward and inward currents, respectively, in the postsynaptic cell (Fig. 5, B and C).

For each direction of coupling, we plotted the $I_j$ versus the $V_j$ and calculated $G_j$ as the slope of a straight line fitted to the $I_j$–$V_j$ relationship (Fig. 5, D and E). Consistent with previous results (Veruki and Hartveit 2002b), the conductance was very similar for both directions of measurement ($G_j = 711 \pm 50$ pS with ON-cone bipolar cell presynaptic, Fig. 5D; and $G_j = 709 \pm 50$ pS with all amacrine cell presynaptic, Fig. 5E). Accordingly, $G_j$ for a cell pair was calculated as the average of the conductance values measured in each direction (500 ± 100 pS; range 330–760 pS; $n = 4$ cell pairs). In some cases, paired recordings of coupled AII amacrine and ON-cone bipolar cells could be maintained for ≥90 min with stable $G_j$ (unpublished results).

MFA reversibly blocks electrical coupling between AII amacrine cells and ON-cone bipolar cells

We examined the effect of 100 μM MFA on the electrical coupling between AII amacrine cells and ON-cone bipolar cells in the same manner as described earlier. For the cell pair

![Diagram](http://jn.physiology.org/)
illustrated in Fig. 6, the baseline $G_j$ was $\sim 340$ pS (Fig. 6A). When MFA was applied in the bath, $G_j$ was slowly reduced (Fig. 6A). The latency to onset of the reduction was $\sim 4$ min. After $\sim 20$ min of drug application, no electrical coupling could be observed (Fig. 6, A and B). When MFA was washed out by switching to control solution (after 24 min of application), the electrical coupling slowly recovered and reached $\sim 84\%$ of the control level after 90 min of wash (Fig. 6, A and B). Of the four AII amacrine–on-cone bipolar cell pairs examined, two pairs involved a type 7 on-cone bipolar cell and two pairs involved a type 5 or type 6 on-cone bipolar cell.

With 100 µM MFA, the electrical coupling was completely blocked for all four pairs of AII amacrine and on-cone bipolar cells. The latency to onset (10% of maximum block) was 2.4 ± 0.6 min (range 1.6–4 min). The latency to 90% of maximum block was 30.0 ± 8.8 min (range 16–54 min). For the four cell pairs recorded, some recovery of electrical coupling was observed for three pairs. One cell pair was unfortunately lost before washout of MFA could have had any effect on coupling. Another cell pair was lost before recovery reached a plateau, at which time $G_j$ had reached 13% of the control level. For the two remaining cell pairs, the recovery of electrical coupling reached plateau levels corresponding to 36 and 84% of the control level. As for the AII amacrine cells, the recovery occurred very slowly after terminating the application of MFA. Recovery to 10% of the final plateau occurred after 8 and 16 min of washout and recovery to 90% of the final plateau occurred after 57 and 95 min of washout. Because it was more difficult to maintain recordings from on-cone bipolar cells for the same length of time as that for AII amacrine cells, we did not examine the concentration–response properties of the block of electrical coupling between AII amacrine cells and on-cone bipolar cells.

At the holding potential of $-60$ mV, $I_{\text{hold}}$ in the control condition (before application of MFA) was 1.8 ± 7.9 pA for the AII amacrine cells ($n = 4$ cells) and 2.7 ± 3.4 pA for the on-cone bipolar cells ($n = 4$ cells). In the condition of full block of electrical coupling by MFA, $I_{\text{hold}}$ was 7.6 ± 5.7 pA for the AII amacrine and 5.6 ± 10.1 for the on-cone bipolar cells ($P > 0.05$ for both cell types; paired t-test).

**Discussion**

Direct electrical coupling between neurons, independent of chemical synaptic transmission, depends on the presence of gap junction channels between the cells. Simultaneous dual recording is still the only direct and arguably the most convincing way to demonstrate such coupling and to measure the corresponding junctional conductance (Galarreta and Hestrin 2001). In this study we have used dual whole cell DSEVC recording between pairs of AII amacrine cells and between pairs of AII amacrine cells and on-cone bipolar cells to examine the block of electrical coupling evoked by MFA. Using high-resistance pipettes, we have previously demonstrated the feasibility of long-lasting recordings of pairs of electrically coupled AII amacrine cells with stable junctional conductance (Veruki et al. 2008). Our results, based on repeated measurements of junctional conductance, confirm and extend a previous report that MFA reversibly blocks tracer coupling between AII amacrine cells and between AII amacrine and on-cone bipolar cells.

**Fig. 6.** Complete and reversible block of electrical coupling between AII amacrine and on-cone bipolar cells by 100 µM MFA. A: $G_j$ as a function of time for a pair of electrically coupled AII amacrine and on-cone bipolar cells in voltage clamp. $G_j$ is calculated as the average of the conductance values measured for each direction of coupling (with voltage pulses applied to either AII amacrine cell or on-cone bipolar cell). MFA was applied in the extracellular perfusing solution during the period indicated by the horizontal bar (duration $\sim 24$ min). The lowercase letters a, b, and c indicate time points during control (a), in the presence of MFA (b), and after washout of MFA (c), where averaged current responses are displayed in B. Recording configuration indicated in inset. B: top: voltage protocol with 200-ms voltage pulses (from $-40$ to $+10$ mV relative to $V_{\text{hold}} = -60$ mV; increments of 10 mV) applied to the cells ($V_{\text{BC}}$, $V_{\text{AI}}$). Bottom, a, b, and c: current responses recorded from on-cone bipolar cell ($I_{\text{BC}}$) and AII amacrine cell ($I_{\text{AI}}$) in response to voltage pulses in the control condition before application of MFA (a; each trace is the average of 13 trials), after complete block of $G_j$ in the presence of MFA (b; each trace is the average of 13 trials), and after partial recovery of electrical coupling after washout of MFA (c; each trace is the average of 13 trials).
bipolar cells (Pan et al. 2007), but demonstrates that both block and recovery have very slow kinetics.

**MFA as a reversible block of electrical synapses**

The most surprising result from our electrophysiological measurements of block by MFA is the long time from onset of block to complete block and from complete block to maximum recovery. A block of 90% required the application of MFA for 20–30 min on average. In their study of block of gap junction channels between AII amacrine cells or between AII amacrine and on-cone bipolar cells, but for A-type horizontal cells the time course of block with a saturating concentration was <10 min (Pan et al. 2007). A similar time course was reported for reversal of block of tracer coupling. Even if we take into account the uncertainty of such measurements, it seems clear that the kinetics of block and unblock of coupling for AII amacrine and on-cone bipolar cells, as measured by electrophysiological recording, is appreciably slower than that for A-type horizontal cells (Pan et al. 2007). There are at least two possible explanations for this difference. First, it is possible that the time course of block and unblock depends on the identity of the specific connexin(s) mediating the gap junction coupling. Whereas A-type horizontal cells are coupled via Cx50 gap junctions (O’Brien et al. 2006), AII amacrine cells are coupled via Cx36 gap junctions (Feigenspan et al. 2001; Mills et al. 2001) and AII and on-cone bipolar cells seem coupled either by homotypical Cx36 gap junctions or heterotypical Cx36/Cx45 gap junctions (Dedek et al. 2006; Han and Massey 2005; Lin et al. 2005; Maxeiner et al. 2005). We are not aware of published data that are relevant for connexin-dependent kinetics of block by MFA. A second possibility is that the difference in time course primarily reflects a dissociation between junctional conductance, as measured electrophysiologically, and chemical coupling, as measured by tracer coupling. For example, it is possible that elimination of tracer coupling is attained before elimination of electrical coupling. Indeed, there is evidence that chemical tracer coupling and electrical coupling can be independently regulated. In their study of Cx43 gap junction channels, Heyman and Burt (2008) observed gating to a state that is electrically conductive, but chemically dye impermeable. With respect to the action of MFA, one mechanism for such an effect could be that MFA blocks gap junction channels by reducing the single-channel conductance. However, flufenamic acid, an aryaminobenzoate related to MFA, blocks gap junction channels by reducing the channel open probability, without modification of the single-channel conductance (Srinivas and Spray 2003). We are not aware of similar experiments using MFA. Irrespective of the mechanism explaining the different time course of block of electrical and chemical coupling, in the context of investigating signal processing mechanisms it is the time course of block of electrical coupling that is functionally most relevant. This is particularly important when MFA is used to block electrical synapses involving AII amacrine cells in physiological studies of retinal signal transmission (e.g., Manookin et al. 2008).

**Potency of block by MFA of AII amacrine cell electrical synapses**

Because of the slow time course of block by MFA of electrical synapses between AII amacrine cells and between AII amacrine and on-cone bipolar cells, we did not attempt to construct a full concentration–inhibition curve. For the three concentrations examined on pairs of AII amacrine cells, 1 μM resulted in a block of only ~9%, 10 μM resulted in a block of 35%, whereas 100 μM resulted in complete block. These results suggest that 50% block will be reached for a concentration between 10 and 100 μM. We are not aware of other reports where Cx36 gap junctions have been tested quantitatively with MFA. However, in dual whole cell patch-clamp recordings of Cx50-transfected N2A neuroblastoma cells, flufenamic acid (see preceding text) blocked gap junction channel currents with an EC$_{50}$ of 47 μM (Srinivas and Spray 2003). For cells transfected with other connexins (not including Cx36), the half-maximal concentrations were between 20 and 60 μM.

**Kinetics of block of gap junction coupling by MFA**

The long time to complete block and the slow reversal of block of electrical coupling by MFA in our experiments is surprising. It cannot be explained by slow solution exchange. The speed of exchange was followed by the pharmacological block of spontaneous glutamatergic synaptic currents (cf. Veruki et al. 2003) and action currents (cf. Mørkve et al. 2002) and was usually complete within 2–3 min after start of the solution exchange. Furthermore, the time to onset of block (measured as 10% reduction of $G_j$) was relatively fast. The long time to complete block and the slow reversal of block by MFA are in marked contrast, however, to the rapid kinetics observed for similar block of gap junction coupling between cells in culture.

In single-electrode voltage-clamp step response measurements of gap junction coupling in Cx43 expressing NRK cells, 100 μM MFA blocked the electrical coupling within 6 min, and maximum recovery was observed within 7 min (Harks et al. 2001). In dual whole cell patch-clamp recording of transfected N2A neuroblastoma cells, maximal block evoked by flufenamic acid could be observed within 1 to 2 min, depending on the drug concentration and the type of connexin (Srinivas and Spray 2003). Corresponding to this, complete recovery of the junction current was observed after 2 min of washout of the drug. However, an observation that resembles the slow kinetics of block and reversal of the action of MFA on electrical synapses of AII amacrine and on-cone bipolar cells, is the action of mefloquine on Cx36 gap junction channels (Cruikshank et al. 2004). When tested on Cx36 transfected N2A cells, mefloquine rapidly blocked gap junction channel coupling with high potency (EC$_{50}$ = 0.31 μM) and a short time to complete block. In contrast, when tested on pairs of interneurons in neocortical slices, electrically coupled via Cx36 gap junctions, mefloquine dramatically reduced coupling, but maximum blockade occurred more than 70–100 min after application and the concentration required to block coupling in slices was ~25-fold higher (EC$_{50}$ = 15 μM) than for blockade of Cx36 coupling in N2A cells. Recovery from block was not observed for either Cx36-expressing N2A cells or neocortical interneurons.
The reason for the slow block by MFA and mefloquine of electrical synapses between neurons in retinal or neocortical slices is unclear. Cruikshank et al. (2004) suggested that the difference in concentration dependence of block for N2A cells and neocortical interneurons could be related to binding of mefloquine to phospholipid membranes, resulting in slowed diffusion toward the site of action. It is not immediately clear, however, that diffusion in a slice could be so slow as to explain the experimental observations. It is possible that there are other more subtle differences between cells in culture and cells in slices with respect to the local microenvironment of the gap junction channels that could be of importance for the mechanism of block.

Use of MFA as a pharmacological tool to block electrical synapses of AII amacrine cells and on-cone bipolar cells

Meflofenamic acid has a set of properties that makes it attractive as a gap junction blocker. Of all the compounds screened in the study by Pan et al. (2007) of retinal tracer coupling, only MFA was water-soluble and easily reversible. However, the slow block and slow reversal observed for MFA with respect to the junctional conductance of electrical synapses of AII amacrine cells and on-cone bipolar cells are considerable challenges for physiological experiments. At the same time, the fact that reversal can be obtained at all would seem to make MFA preferable over drugs like mefloquine, where reversibility has not been observed for block of Cx36 gap junction channels (Cruikshank et al. 2004). Furthermore, the commonly used gap junction antagonist carbenoxolone seems to have a series of undesirable effects in studies of retinal gap junction coupling. It has a direct inhibitory effect on light responses (Verweij et al. 2003; Xia and Nawy 2003) and Pan et al. (2007) reported that it was difficult to wash out and seems to have a toxic effect on retinal neurons. The reason for the incomplete recovery of $G_j$ after washout of MFA in some of our recordings of AII amacrine and on-cone bipolar cells is unknown.

Despite the desirable properties of MFA, it is clearly not specific for Cx36 gap junction channels (Harks et al. 2001; Pan et al. 2007; Srinivas and Spray 2003) and there is strong evidence that MFA and related fenamates modulate a diversity of ion channels in addition to connexons. For example, MFA inhibits hKv2.1 potassium channels (Lee and Wang 1999) and opens KCNQ2/Q3 potassium channels (Peretz et al. 2005). It is unknown whether MFA has an effect on voltage-gated conductances of AII amacrine or on-cone bipolar cells. The change of $I_{\text{gmax}}$ in AII amacrine cells evoked by MFA could reflect such effects, but they could also be caused by block of electrical synapses. As long as we cannot record from isolated cells, it will be experimentally challenging to discriminate between direct effects on voltage-gated conductances and indirect effects caused by a reduction of $G_j$. However, if MFA has an effect on voltage-gated conductances in AII amacrine or on-cone bipolar cells, it cannot explain the observed reduction of $G_j$. In the paired recordings, we evoked a change in junction voltage by applying voltage pulses to the presynaptic cell, but the junction current was estimated only from the current in the postsynaptic cell, which was voltage clamped at a constant potential. On the other hand, any effect of MFA on voltage-gated conductances in retinal neurons, including AII amacrine and on-cone bipolar cells, might compromise the interpretation of studies where this drug is used specifically to block gap junction channels in investigations of retinal circuit functions. Accordingly, the suitability of MFA as a blocker of gap junction channels in physiological experiments needs to be evaluated separately for each individual study.

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