Putative ClC-2 Chloride Channel Mediates Inward Rectification in *Drosophila* Retinal Photoreceptors

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Abstract. We report that *Drosophila* retinal photoreceptors express inwardly rectifying chloride channels that seem to be orthologous to mammalian ClC-2 inward rectifier channels. We measured inwardly rectifying Cl^- currents in photoreceptor plasma membranes: Hyperpolarization under whole-cell tight-seal voltage clamp induced inward Cl^- currents; and hyperpolarization of voltage-clamped inside-out patches excised from plasma membrane induced Cl^- currents that have a unitary channel conductance of $\sim$3.7 pS. The channel was inhibited by 1 mM Zn^{2+} and by 1 mM 9-anthracene, but was insensitive to DIDS. Its anion permeability sequence is $Cl^- = SCN^- > Br^- >> I^-$, characteristic of ClC-2 channels. Exogenous polyunsaturated fatty acid, linolenic acid, enhanced or activated the inward rectifier Cl^- currents in both whole-cell and excised patch-clamp recordings. Using RT-PCR, we found expression in *Drosophila* retina of a ClC-2 gene orthologous to mammalian ClC-2 channels. Antibodies to rat ClC-2 channels labeled *Drosophila* photoreceptor plasma membranes and synaptic regions. Our results provide evidence that the inward rectification in *Drosophila* retinal photoreceptors is mediated by ClC-2-like channels in the non-transducing (extra-rhabdomeral) plasma membrane, and that this inward rectification can be modulated by polyunsaturated fatty acid.

Key words: ClC-2 channel — *Drosophila* — Photoreceptors — Patch clamp — Linolenic acid

Introduction

Inward rectifiers are important in retinal function, in photoreceptor cells, bipolar cells, horizontal cells, amacrine cells, and ganglion cells (Tachibana, 1983; Bader & Bertrand, 1984; Kaneko & Tachibana, 1985; Hestrin, 1987; Maricq & Karenbrot, 1990; Yagi & Macleish, 1994; Han, Jacoby & Wu, 2000; Kawai et al., 2002). Inward rectification has also been described in depolarizing invertebrate photoreceptors (Phillips, Bacigalupo & O'Day, 1992; Wang et al., 2005). In many cases these inward currents are carried by chloride channels (Jentsch et al., 2002).

The ClC superfamily of voltage-dependent chloride channels is the largest known family of anion channels. The various ClC isoforms exhibit a wide range of gating, permeation properties and selectivity sequences (Fahlke, 2001; Jentsch et al., 2002). ClC-2 chloride channels are activated by hyperpolarization, modulated by pH (Thiemann et al., 1992; Gründer et al., 1992; Staley et al., 1996; Jordt & Jentsch, 1997; Schwiebert et al., 1998; Rutledge et al., 2001; Nehrke et al., 2002) and possess a characteristic anion permeability sequence. Recent evidence indicates that ClC-2 channels are gated by intracellular Cl^- (Pusch et al., 1999; Haug et al., 2003; Niemeyer et al., 2003; Zuñiga et al., 2004), and polyunsaturated fatty acids, such as arachidonic acid, reportedly can activate ClC-2 channels (Tewari et al., 2000; Cuppoletti et al., 2001, 2004a). In addition, human ClC-2 channels are activated by protein kinase A and low extracellular pH (Sherry et al., 1997; Tewari et al., 2000; Cuppoletti et al., 2004b).

ClC-2 is very widely expressed, and although functional roles have not been clearly established, evidence suggests that the ClC-2 channel is important in several cellular processes. ClC-2 channels may be

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involved in regulating synaptic transmission in central neurons (Staley et al., 1996; Mladinic et al., 1999). Roles for ClC-2 channels in retinal function have also been suggested. Expression of ClC-2 channels in retinal bipolar cells has been reported (Enz, Ross & Cutting, 1999), and a ClC-2 knockout affects retinal development in mice (Bösl et al., 2001).

Here we report the first evidence that *Drosophila* expresses a functional inward rectifier Cl$^-$ channel that appears to be orthologous to mammalian ClC-2. This channel exhibits the characteristic anion selectivity and pharmacology that distinguishes ClC-2 currents from other inward rectifier anion currents. Our results suggest that this ClC-2 channel is expressed in retinal photoreceptors and mediates inward rectification. The inward rectifier Cl$^-$ channel can be activated by the polyunsaturated fatty acid, linolenic acid (Chyb, Raghu & Hardie, 1999), which has also been proposed as an endogenous agonist of the light-dependent channels in *Drosophila* photoreceptors.

Materials and Methods

ELECTROPHYSIOLOGY

Wild-type *Drosophila* (Oregon-R) and trpl^{302};trp^{343} mutants (courtesy of Dr. Charles Zuker) in late pupal stage were used in the experiments. Ommatidia were prepared as described previously (Hardie, 1991a). Briefly, retinae were dissected under dim red light illumination in Ca^{2+}/Mg^{2+}-free external solution and gently triturated to separate surrounding pigment cells from the ommatidia. Ommatidia were allowed to settle onto a coverslip that was mounted on the stage of an inverted microscope, and they were viewed under infrared illumination using a CCD camera and monitor. The experiments were carried out at room temperature ($\sim$21°C). Single-channel and whole-cell voltage-clamp recordings from cells in ommatidia were made using an Axopatch-1D (Axon Instruments, Union City, CA) patch-clamp amplifier. Data were acquired digitally with a LabMaster interface (Scientific Solutions, Mentor, OH) and sampled and analyzed with pCLAMP software (Axon Instruments, Inc.). Patch pipette resistances were $\sim$4 MΩ for whole-cell and $\sim$10 MΩ for single-channel recordings.

Normal extracellular solution contained (in mM): 120 NaCl, 5 KCl, 8 MgSO$_4$, 1.5 CaCl$_2$, 10 HEPES, 2.5 L-proline, and 25 sucrose, pH 7.15. The Mg^{2+}- and Ca^{2+}-free external solution for ommatidial dissociation contained (in mM): 120 NaCl, 4 KCl, 10 HEPES and 32 sucrose, pH 7.15. The control pipette solution for excised patch recordings contained (in mM): 130 NaCl, 2 MgCl$_2$, 1.5 CaCl$_2$ and 10 HEPES, pH 7.15. The normal internal solution for whole-cell recordings contained (in mM): 120 CsCl, 10 HEPES, 2 MgSO$_4$, 1.1 EGTA, 0.1 CaCl$_2$, 0.5 NaGTP, 2 NaATP, and 15 TEA-Cl, pCa 8.0, pH 7.15. CsCl and TEA were used to block voltage-dependent K$^+$-currents. Whole-cell recordings were performed in the dark to eliminate light-sensitive currents. For selectivity studies, the excised patches were perfused with solutions containing (in mM): 121 NaX (where X signifies an anion), 1.5 CaCl$_2$ and 10 HEPES, pH 7.15. Linolenic acid (LNA) was purchased from Sigma-Aldrich (St. Louis, MO) and maintained as 20 mM stock solution in ethanol. Solutions were prepared daily from a stock stored at −20°C.

The relative anion permeability sequence of the channel was established by extrapolation of the linear regression function fitted to the experimental values of the *I-V* relations. The assumption that the *I-V* curve obtained from single ClC-2 channel currents is linear is validated by the fact that the *I-V* relation of macroscopic tail currents does not rectify (Niemeyer et al., 2003). Shifts of E_{rev} were used to estimate the relative permeabilities for the different anions according to the equation,

$$\Delta E_{rev,X^-} = E_{rev,Cl^-} - E_{rev,X^-} \frac{RT}{zF} \ln \frac{P_{X^-}[X^-]}{P_{Cl^-}[Cl^-]}$$

where E_{rev,Cl^-} is the apparent reversal potential in normal bath chloride and E_{rev,X^-} is that measured when bath chloride was substituted by test anion X$^-$, and where R is the universal gas constant, T is the absolute temperature, z is valence, and F is the Faraday constant.

For the analysis of the voltage dependence of the open probability (P_o) of unitary transitions, traces showing a single current level were selected. Voltage-dependence parameters for channel gating were obtained by fitting a Boltzmann distribution to single-channel data using the equation,

$$P_o = P_o^{max} \cdot \{1 + \exp[-(V - V_{0.5})/A]\}^{-1}$$

where A corresponds to slope factor containing the voltage dependence of channel activation and $V_{0.5}$ is the voltage at which $P_o = 0.5 \cdot P_o^{max}$. In a two-state model for channel activation, A is equivalent to $RT/z'F$, where z' corresponds to the product of z, the number of charges or activation particles, and δ, the fractional distance the particles move within the transmembrane electric field. Similarly, the conductance (G) was fitted using the equation

$$G = G_{max} \cdot \{1 + \exp[-(V - V_{0.5})/A]\}^{-1}$$

RT-PCR AND RESTRICTION ANALYSIS

Retinae were dissected under a microscope with microforceps and a fire-polished glass micropipette was used to remove as much of the non-retinal tissue attached to the eye as possible. Total RNA from *Drosophila* wild-type (Oregon-R) retinas was isolated using Trizol (Gibco BRL, Carlsbad, CA); the concentration and purity were estimated by UV spectrophotometry and the integrity was assessed by agarose gel electrophoresis. The cDNA was prepared using 3 μg of total RNA using the oligo dT and random primers in the presence of RNAse inhibitor and SuperScript II reverse transcriptase (Gibco BRL). The PCR amplification was carried out using a Perkin Elmer GeneAmp 2400 thermal cycler (Boston MA). Primers used in RT-PCR were 5′-gatagttcaaagcaatcacc-3′ for sense and 5′-agaaaacggaaatcatgcttga-3′ for antisense (*see* location in Fig. 5*A*). Standard reaction mixture contained aliquots of cDNA, 0.2 mM of each primer, 2.5 units Taq DNA polymerase (Fermentas, Hanover, MD), 100 mM dNTPs, and 1.5 mM MgCl$_2$ in a total volume of 50 μl. Conditions were as follows: initial denaturation at 95°C for 2 min, 30 cycles at 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min, and final extension at 72°C during 5 min.

IMMUNOLABELING

For Western blot analysis, standard procedures were used. Briefly, a total extract was prepared from approximately 70 *Drosophila* retinae prepared in a glass-glass homogenizer in homogenization buffer (in mM: 50 TrisHCl, 50 NaCl, 1 EGTA, pH 7.5) and protease inhibitors (in μg/ml, 10 aprotinin, 10 leupeptin, 10 pepstatin and, in μM, 100 benzamidine, 100 PMSF), and then centrifuged at 4,000 × *g* for 5 min at 4°C. Proteins in the supernatant were separated by

SDS-PAGE, transferred to nitrocellulose, and probed with anti-rat ClC-2 antibody (1:200, Alomone Labs. Ltd., Jerusalem, Israel). The binding of secondary antibody to reactive proteins was detected by chemiluminescence (ECL, Amersham-Pharmacia, Piscactaway, NJ).

For immunohistochemical analysis of *Drosophila* retinae, whole heads were fixed in 4% paraformaldehyde, incubated in PBS 30% sucrose overnight and embedded in Tissue Freezing Medium™. A CRYO-CUT II cryostat (American Optical, New York, NY) was used to obtain 10 μm sections at −20°C. The sections were adhered to silane-covered microscope slides, blocked with BSA, washed, and incubated with primary antibody overnight at 4°C. After wash, the sections were incubated with a secondary antibody coupled to FITC for 1 h at room temperature. Control sections were incubated only with the secondary antibody. Optical sections of 1 μm thickness were obtained with a confocal microscope (Zeiss, model Axiovert 135M, Germany).

SEQUENCE ANALYSIS

Sequence alignments and analysis were aided by CLUSTAL W and PROSITE software online (Thompson, Higgins & Gibson, 1994; Bairochetal, 1997).

Results

INWARD RECTIFIER CHANNELS IN *DROSOPHILA* PHOTOECEPTORS

We found inwardly rectifying currents in *Drosophila* retinal photoreceptors in voltage-clamp recordings from excised plasma membrane patches and from whole cells. In patches excised from extrarhabdomeral (non-transducing) plasma membrane, we measured single-channel activity at hyperpolarizing voltages (Fig. 1). Shown by each trace is the amplitude histogram of the total number of points along the recorded current. Channel activity increased as the membrane patch was further hyperpolarized (Fig. 1A). The current-voltage relation for unitary currents was obtained by linear regression of the measurements from 3 separate patches (Fig. 1B). Linear extrapolation gave an apparent reversal potential close to zero (6.7 mV ± 5.6; mean ± SD, $n = 3$), as expected for symmetrical NaCl solutions. The average single-channel conductance was 3.7 ± 0.8 pS ($n = 3$). The fit of the open probability (P_o) *vs.* voltage data to a Boltzmann distribution yielded $V_{0.5} = -82.8 \pm 27.5$ mV and $z' = 4.5 \pm 2.1$ ($n = 8$) (Fig. 1C).

In our records we observed channel transitions that resembled the double-barreled behavior previously described for ClC-0 and ClC-1 channels (Hanke & Miller, 1983; Middleton, Pheasant & Miller, 1996). This characteristic was observed in our recordings with a low probability. The most frequently visited state of the channel, by far, that of smaller conductance. We calculated the dwell time considering only the most visited open state. The inset shows three examples where this characteristic behavior is evident.

To characterize the hyperpolarizing Cl⁻ current described above, we conducted whole-cell recording experiments. Figure 1D shows representative whole-cell currents induced by hyperpolarizing voltage steps from a holding potential of −70 mV. The inward currents exhibited slow activation, with a time course that varied considerably among cells ($\tau = 310 \pm 184$ ms, mean ± SD, at −130 mV, $n = 21$), and did not inactivate for voltage steps of up to 2 s (*not shown*). The conductance values were fitted to a Boltzmann distribution, with $V_{0.5} = -98.6 \pm 6$ mV and $z' = 5.6 \pm 0.4$ ($n = 3$) (Fig. 1E).

CHANNEL ANION SELECTIVITY AND PHARMACOLOGY

To evaluate the ion selectivity, we compared the reversal potentials of the hyperpolarization-activated currents in single-channel recordings obtained after several ion substitutions. When the impermeant cation, N-methyl-D-glucamine (NMDG⁺), was substituted for Na⁺ in the pipette, we observed no effect on the current-voltage relation or on the single-channel conductance (*data not shown*), indicating that Na⁺ does not permeate the channel in any significant way.

To determine the permeability sequence for this conductance, we recorded and analyzed single-channel currents under bi-ionic conditions. Figure 2 shows current records from inside-out patches exhibiting channel activity obtained after Cl⁻ was replaced by thiocyanate, bromide or iodine ions in the bath solution. We estimated E_{rev} values under the different biionic conditions. The E_{rev} for SCN⁻ was -6.79 ± 7.1 mV and 29.6 ± 3.51 mV for Br⁻ (Fig. 2E). These values determined an anion permeability sequence for the *Drosophila* photoreceptor chloride channel of Cl⁻ = SCN⁻ > Br⁻ >> I⁻, as for ClC-2 channels (Fahlke, 2001).

The Cl⁻ channel blocker, Zn^{2+} (1 mM), suppressed the unitary transitions almost completely when applied to the internal side of excised patches (Fig. 3A). Internal application of 1 mM anthracene-9-carboxylic acid (9-AC) also inhibited the single-channel currents (Fig. 3B). Recovery was complete after removing the blockers and restoring normal Cl⁻ solution, being rather fast (seconds) for Zn^{2+} and somewhat slower (over 1 minute) for 9-AC. Chloride currents were insensitive to internal 1 mM DIDS (*data not shown*). This profile is a distinguishing characteristic of ClC-2 channels.

MODULATION OF THE INWARD RECTIFIER BY LINOLENIC ACID

Polyunsaturated fatty acids, including arachidonic acid and linolenic acid (LNA), have been reported to activate or modulate several channel types including those involved in photo-transduction. Evidence indicates that ClC-2 channels can be activated by
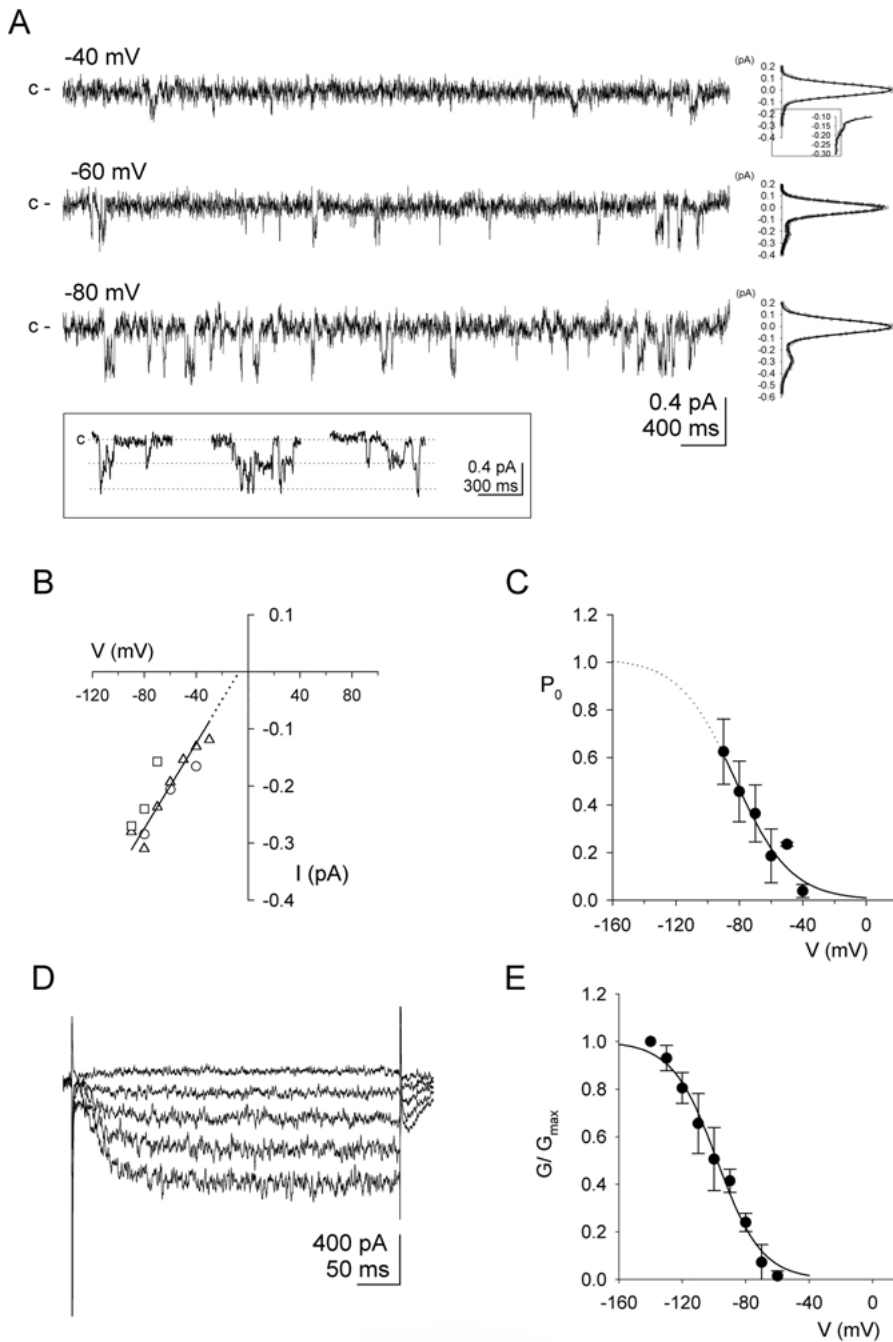
Fig. 1. Hyperpolarization-activated single-channel currents in *Drosophila* retinal photoreceptors. (*A*) Single-channel recordings from an inside-out excised patch from extrarhabdomeral plasma membrane, in symmetrical NaCl solutions. Command voltages are indicated. Closed-current level is indicated for each trace. The amplitude histogram for all points is shown by each trace. The light lines correspond to the experimental values and the dark lines were determined by fitting the experimental data to a double Gaussian function. The inset presents three selected traces filtered at 0.5 KHz that clearly show the double-barrel behavior of ClC channel. (*B*) Current-voltage plot constructed from the unitary currents from three patches. Slope conductance $= 3.7 \pm 0.8$ pS (mean $\pm$ SD), apparent reversal potential, $E_{rev} = 6.7 \pm 5.6$ mV. (*C*) Plot of open probability (P_o) *vs.* voltage for single-channel recordings from 8 different patches. The data were fit by a Boltzmann distribution (*see* Materials and Methods), with $V_{0.5} = -82.8 \pm 27.5$ mV and $z' = 4.5 \pm 2.1$. (*D*) Family of whole-cell currents induced by a series of hyperpolarizing voltage steps from a holding potential of -70 mV. (*E*) Conductance *vs.* voltage plot for 3 different experiments. The fitting determined a $V_{0.5} = -98.6 \pm 6.0$ mV and $z' = 5.6 \pm 0.4$.

arachidonic acid (Tewari et al., 2000; Cuppoletti et al., 2001, 2004a). We thus examined whether the *Drosophila* photoreceptor Cl$^-$ conductance might be also modulated by LNA. Indeed, we found that 50 μM linolenic acid (LNA, 18:3 cis) applied to the internal side of inside-out patches increased the Cl$^-$ channel open probably (Fig. 4*A*). An analysis of P_o in a steady-state recording before and after the addition of LNA is shown in Fig. 4*B*. Although P_o varied during time, activation by LNA is evident and can reach values up to 0.8, much higher than those observed in its absence. This LNA effect was dose-dependent: 5 μM increased the basal open probability

two-fold (1.96 ± 0.13; mean $\pm$ SD, $n = 3$) and 50 μM increased it three-fold (3 ± 0.28, $n = 3$) (*data not shown*). LNA did not alter the unitary conductances. Similarly, we found that LNA enhanced the inward rectifier Cl$^-$ currents in intact cells. To analyze the effect of LNA on macroscopic Cl$^-$ currents in intact cells, we sought to eliminate any transduction currents that might be influenced by LNA. Thus, we recorded from cells from trpl^{302};trp^{343} mutants, which lack functional TRP and TRPL channels (Niemeyer et al., 1996). External perfusion with 20 μM LNA enhanced the hyperpolarization-activated current, measured using a voltage-clamp ramp protocol; this
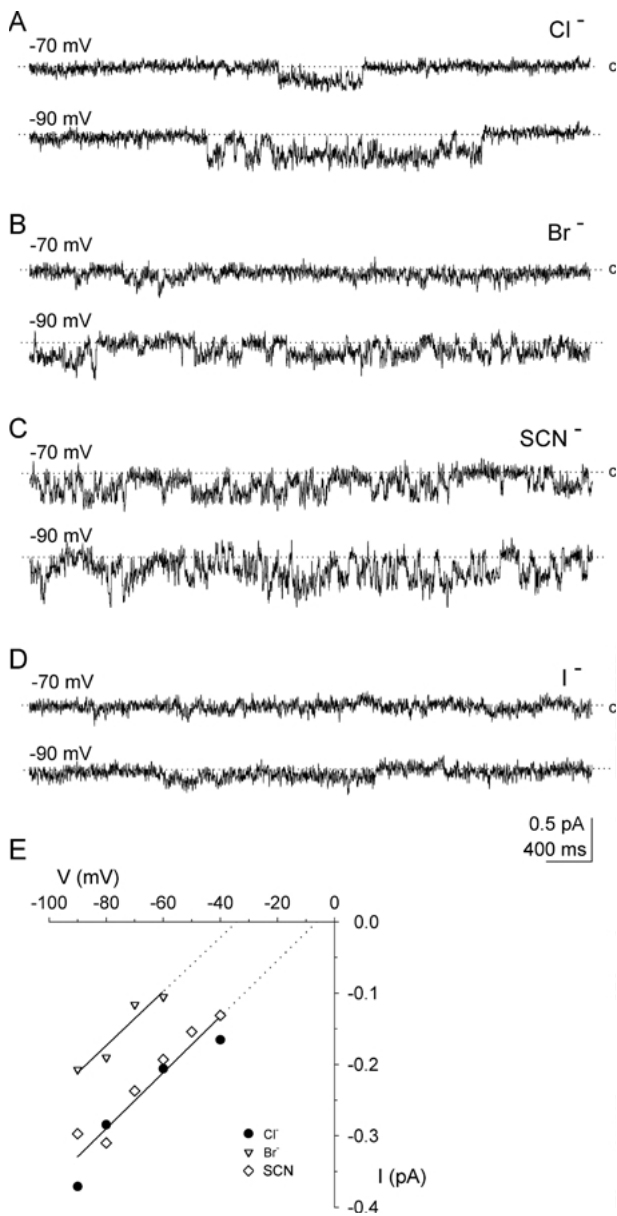
Fig. 2. Anion selectivity of the hyperpolarization-activated channel. Voltage-clamp-current traces were recorded from inside-out patches under biionic conditions. (*A*) Unitary transitions recorded under symmetrical conditions for Cl^-. (*B–D*) Records obtained when Cl^- was replaced by Br^-, SCN^-, and I^-, as noted. Closed-current level is indicated for each trace. (*E*) *I-V* relations under Cl^-/Cl^- (*circles*), Cl^-/Br^- (*triangles*) and Cl^-/SNC (*diamonds*) solutions for the recordings in (*A*).

current was blocked by 1 mM 9-AC (Fig. 4*C*). These results indicate that the inward rectifier Cl^- conductance can be activated both in intact cells and in patches by LNA.

CLC-2 INWARD RECTIFIER CHANNEL EXPRESSION IN THE *DROSOPHILA* RETINA

The results described above strongly suggest that the *Drosophila* photoreceptor Cl^- channel belongs to the

ClC-2 family. In order to determine whether a ClC-2 homologue was present in the *Drosophila* genome (Adams et al., 2000), we conducted BLAST searches with mouse ClC-2. Orthology was suggested by the fact that the mouse and *Drosophila* proteins were mutual best hits in BLAST searches against one another's genomes. A cDNA sequence from this *Drosophila* gene (accession number AY119539) contains the ORF and 5′ and 3′ untranslated regions; it encodes a protein of 1176 residues with 32% global identity to rat ClC-2, with 62% identity in the region containing the putative transmembrane segment. RT-PCR with gene-specific primers (Fig. 5*A*) amplified a product of the expected size from retinal RNA. Restriction analysis confirmed the identity of the amplicon (Fig. 5*B*). We conclude that a *Drosophila* ClC-2 ortholog is expressed in the retina.

IMMUNOREACTIVITY OF *DROSOPHILA* RETINA TO AN ANTI-RAT CLC-2 ANTIBODY

We found that polyclonal anti-rat ClC-2 antibody bound to an $\sim$84–90 kDa polypeptide in Western blots of a total retinal extract (Fig. 6*A*). By immunohistochemistry (Fig. 6*B*), we found that the same ClC-2 antibody labeled principally the extrarhabdomeral plasma membrane of photoreceptors (Fig. 6*Bb*, cross-section at retina level), and in the distal portion of the photoreceptor axons in the lamina (Fig. 6*Be*), the first optic ganglion where terminals of R1–R6 photoreceptors synapse with monopolar neurons. Reactivity was not detected in tissue sections that were incubated only with secondary antibody (Figs. 6*Bc* and 6*Bf*)

Discussion

In this work we describe a novel inwardly rectifying chloride conductance of *Drosophila* retinal photoreceptors. Its unitary conductance value ($\sim$3.7 pS) is comparable to a ClC-2-like channel recorded in excised parches from cultured rat cortical astrocytes (Nobile et al, 2000). It is also similar to the value extracted by noise analysis from rat ClC-2 macroscopic currents expressed heterologously in *Xenopus* oocytes (2–3 pS; Lorenz, Pusch & Jentsch, 1996) and to that of concatemers ClC-2/ClC-2 in the same expression system (2.6 pS; Weinreich & Jentsch, 2001).

As discussed by Jentsch et al. (2002), the activation kinetics from either heterologously expressed ClC-2 channels or from native systems is quite variable. This variability can be considered as reflecting modulation of ClC-2 channels by several different mechanisms. The kinetics of activation we observed was similar to ClC-2 chloride currents in other preparations (Jentsch et al., 2002). Our esti-
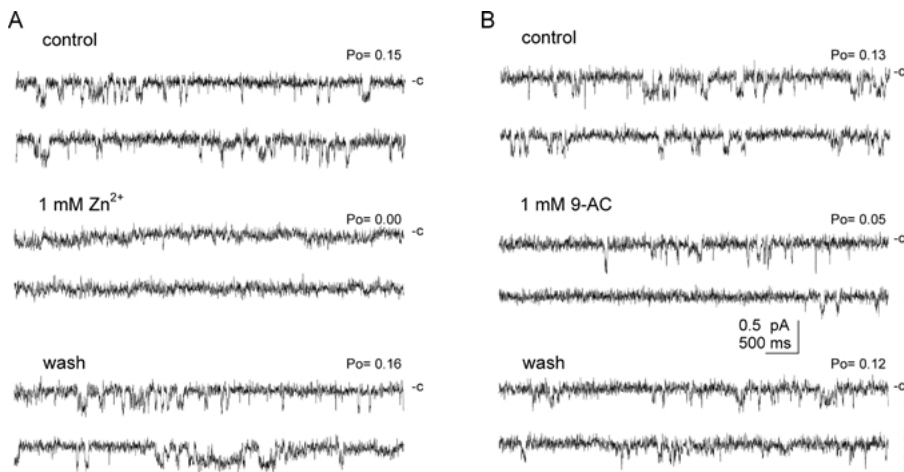
Fig. 3. Pharmacological blockage of the hyperpolarization-activated chloride channel. (*A*) Single-channel currents were inhibited by 1 mM Zn^{2+} at the intracellular face of the excised patch. (*B*) 1 mM anthracene-9-carboxylic acid (9-AC) abolished the single-channel currents. Both effects were fully reversible after washout. Closed-current level is indicated for each trace. Command potential = −80 mV.
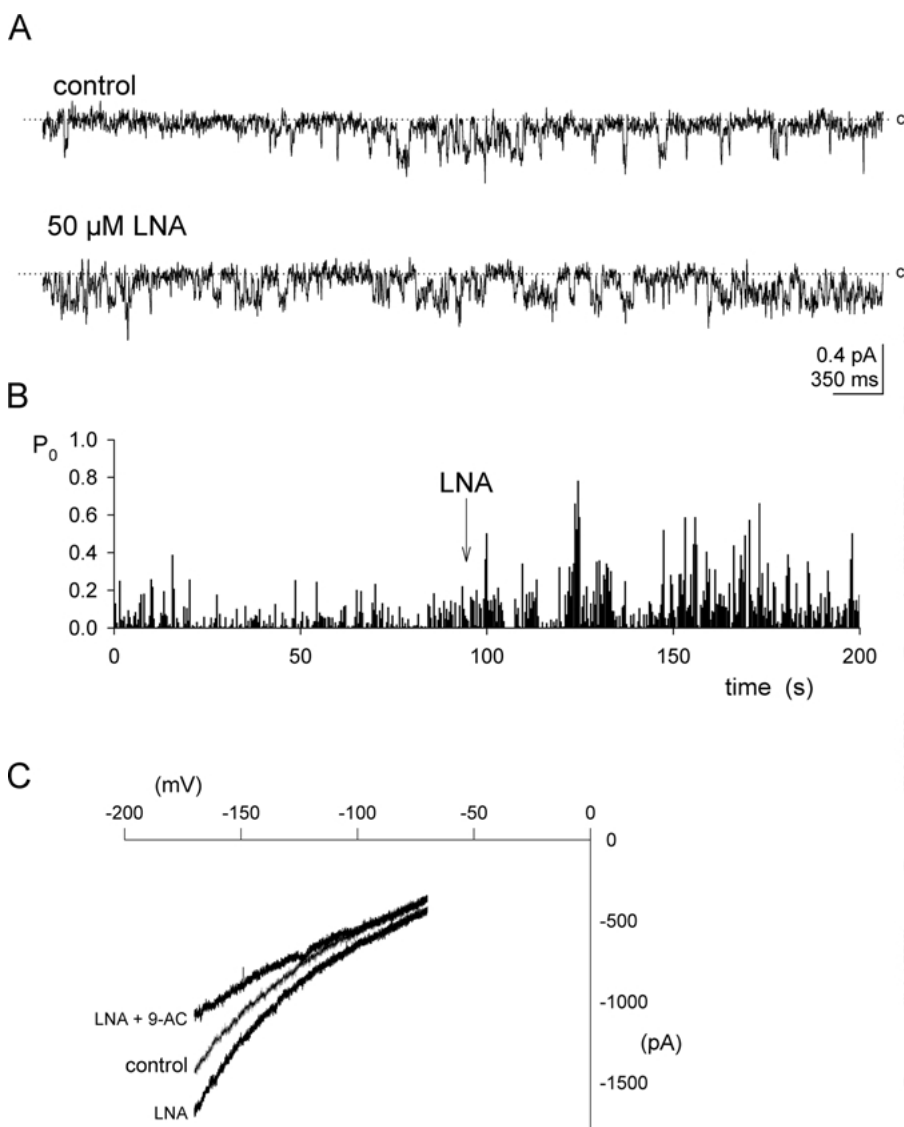

Fig. 4. Linolenic acid (LNA, 18:3 cis) activation of the hyperpolarization-activated Cl^- conductance. (*A*) Recordings from an inside-out excised patch containing a channel held at −80 mV in symmetric Cl^-. Representative traces before and after application of 50 μM LNA to the internal side of the channel are shown. (*B*) Open probability plot *vs.* time for a 200 s continuous recording; time of LNA application is indicated. (*C*) Whole-cell current-voltage traces obtained with 750 ms voltage-ramps from −70 to −170 mV in a trpl[302];trp[343] mutant photoreceptor, under control, 20 μM LNA and LNA + 1 mM 9-AC added to the external solution.

mates of $V_{0.5}$ from single-channel data (−82.8 ± 27.5 mV) varied considerably. The higher variability of the $V_{0.5}$ values from single-channel data compared to the variability of the macroscopic data (−98 ± 6 mV) might be due to a regulatory mechanism that was affected after excision of the membrane patches.
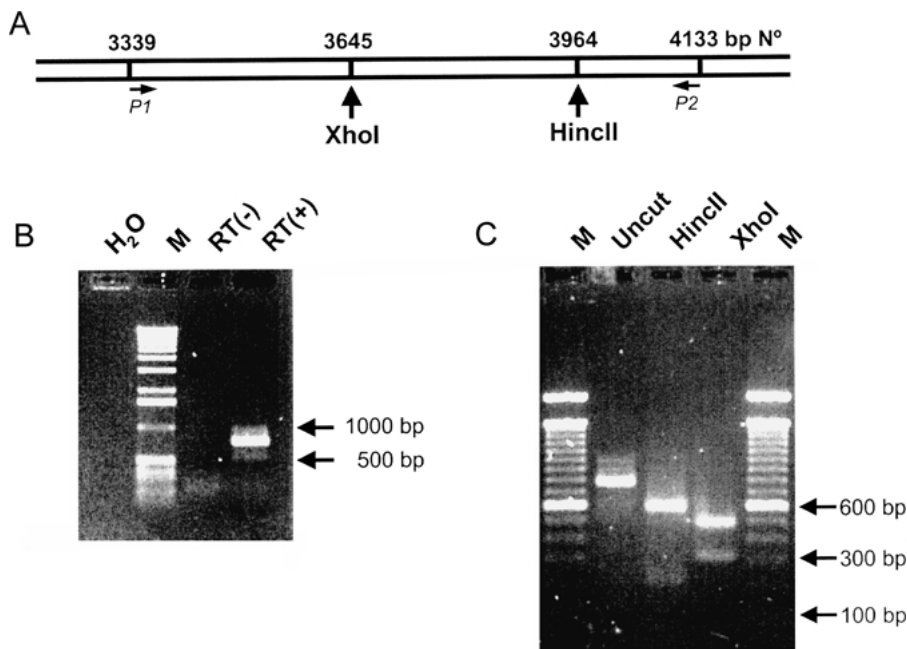
Fig. 5. RT-PCR amplification of a ClC-2 orthologue in *Drosophila* retina. (*A*) Scheme illustrating part of the *Drosophila* ClC-2 transcript, showing bp numbers for positioning primers and restriction sites used in subsequent analysis. (*B*) Agarose gel electrophoresis of PCR products amplified with the P1 (sense) and P2 (antisense) primers using cDNA from an eye preparation. H_2O corresponds to amplification without DNA template, and $RT(-)$ corresponds to a reaction without reverse transcriptase. A lane with the 1000 and 500 bp molecular weight markers (*arrows*) is also shown (*M*). $RT(+)$ shows an amplified fragment of the gene of the expected size ($\sim$800 bp). (*C*) Agarose gel electrophoresis showing the restriction analysis of the PCR product. Lanes show the uncut product and the restriction fragments obtained after treatment with HincII and XhoI. The fragments obtained after digestion are as expected. Two lanes with the 100 bp molecular weight markers are also shown (*M*, *arrows* at 600, 300, and 100 bp).

Nevertheless, these values are similar to other ClC channels regarding $V_{0.5}$. For torpedo ClC-0 incorporated into planar bilayers, $V_{0.5}$ is -85 mV (Hanke & Miller, 1983). $V_{0.5}$ from macroscopic currents of splice variants of guinea pig ClC-2 channels is -101 mV (Cid et al., 2000). The values of z' determined here ($\sim$5) are higher than for heterologously expressed mammalian ClC-2 channels ($\sim$1) (Cid et al., 2000; Varela et al., 2002; Niemeyer et al., 2004). Thus, the *Drosophila* Cl$^-$ channel has a voltage dependence similar to the squid axon Na$^+$ ($z' = 6$) and K$^+$ channels ($z' = 4.5$) (Hille, 2001).

Estimates from ClC-2 channels expressed heterologously indicate that the rectification properties of this channel are due to gating and not to channel conduction (Fahlke, 2001). Therefore, it was reasonable to estimate the reversal potentials by extrapolating the *I-V* curves of the single-channel currents. These values defined a permeability sequence as that of other ClC-2 channels (Furukawa et al., 1998; Clark et al., 1998).

The pharmacology of the *Drosophila* photoreceptor Cl$^-$ channels corresponds to that of human, rabbit and rat ClC-2 channels (Thiemann et al., 1992; Clark et al., 1998; Furukawa et al., 1998; Enz et al., 1999). It has been suggested that 9-AC, an aromatic compound formed by three benzenic rings, binds to a conserved hydrophobic region in ClC channels

(Gelband, Greco & Martens, 1996; Estévez et al., 2004). Although external Zn^{2+} has been generally used to block ClC-2 currents (Staley et al., 1996, Clark et al., 1998), it has been reported that this divalent can also block inward rectifying chloride currents when applied internally (Staley, 1994), as is the case for these channels (Fig. 3). Several reports indicate that ClC-2 channels are gated by intracellular Cl$^-$ (Pusch et al., 1999; Haug et al., 2003, Niemeyer et al., 2003; Estévez et al., 2004; Zuñiga et al., 2004) and that a specific glutamate residue confers this feature to the channel. A comparison of ClC-2 sequences illustrates that this glutamate and several surrounding residues are conserved in mammalian and *Drosophila* ClC-2.

Our PCR analysis supports the possibility that this channel is an ortholog of mammalian ClC-2 channels. Since the gene has not been functionally expressed, we refer to the gene product as a putative *Drosophila* ClC-2 channel.

Immunolabeling of non-rhabdomeral plasma membrane with anti-ClC-2 antibody (Fig. 6*Bb* and 6*Be*) and the patch-clamp recordings of Cl$^-$ channel currents from the same membrane (Fig. 1) suggest that the Cl$^-$ channels reside in the non-transducing regions of the photoreceptors, like the other voltage-gated channels (Hardie, 1991b). We cannot rule out the possibility that a few channels localize to the
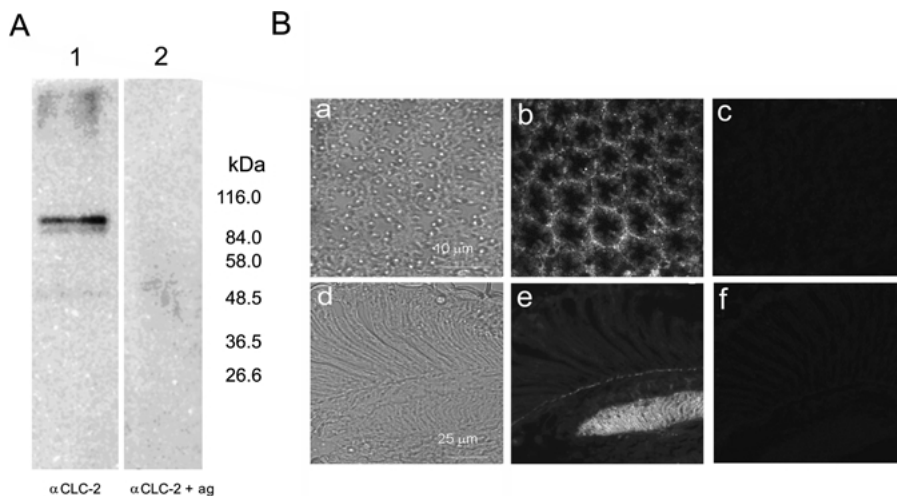
Fig. 6. Immunoreactivity of *Drosophila* retina to ClC-2 antibody. (*A*) Lane *1*: Western blot analysis of a total extract of *Drosophila* retinas shows an ∼84–90 kDa band labeled with an anti-rat ClC-2 antibody (1:200). Lane *2*: Immunolabeling was blocked when the antibody was preincubated with the antigen peptide. (*B*) ClC-2 antibody labeled photoreceptor extrarhabdomeral plasma membranes and lamina. *Ba* and *Bd*, transmission micrographs of 10 μM cryostat sections of a *Drosophila* retina. Image *a* corresponds to a transversal section through the photoreceptor layer and *c* to an orthogonal section to the cornea of retina. *Bb* and *Be*, 1 μM confocal fluorescence micrographs of corresponding sections, showing rat ClC-2 immunoreactivity. Reactivity is apparent in the photoreceptor borders (extrarhabdomeral plasma membrane) (cf. panel *Ba*) and in the distal portion of photoreceptor axons in the lamina (cf. panel *Bc*). *Bc* and *Bf*, confocal fluorescence micrographs (1 μm optical sections) of corresponding sections, that were incubated only with secondary antibody.

rhabdomeral membrane and could have been undetected by the antibody. The strong ClC-2 immunolabeling of distal portions of axons from photoreceptors in the lamina may be due either to a higher density of reactive protein in this compartment or to a highly ordered arrangement of axons extending to the lamina where they synapse with monopolar neurons. ClC-2 antibodies also label mammalian retinal synapses (Enz et al., 1999). Interestingly, there are several reports of inward rectifiers at synapses that may have significant physiological impact (Fletcher & Chiappinelli, 1992; Ponce et al., 1996; Beaumont & Zucker, 2000; Leonoudakis et al., 2001). The activity of ClC-2 channels is thought to regulate intracellular Cl⁻, shifting the Cl⁻ equilibrium potential and modifying the GABA response in some GABAergic neurons (Staley et al., 1996).

Our finding that the polyunsaturated fatty acid linolenic acid (18:3 cis) can enhance or activate inward rectifier currents (Fig. 4) agrees with other studies. Indeed, several lines of evidence show that ion channels can be modulated directly by fatty acids (Ordway, Singer & Walsh, 1991; Cuppoletti et al., 2004a) and that this modulation may be physiologically significant. Arachidonic acid (20:4, cis) reportedly activates human ClC-2 currents (Tewari et al., 2000; Cuppoletti et al., 2001). Polyunsaturated fatty acids may serve as endogenous agonists of the light-dependent channels in *Drosophila* photoreceptors (Chyb et al., 1999; Raghu et al., 2000; Hardie, 2003). These messengers would be generated from diacylglycerol, a product of IP$_3$ hydrolysis by phospholi-

pase C, activated in the excitation cascades. Supporting this idea is the detection of LNA in the phosphatidyl inositol molecule from *Drosophila* heads (Stark et al., 1993). Thus, if free fatty acids are generated in the phototransduction cascade, they might diffuse from the rhabdomere, where the excitation cascades take place, to other cellular compartments. These fatty acids may modulate conductances present in the extrarhabdomeral plasma membrane, where the voltage-gated channels reside. In this case, the Cl⁻ conductance could be increased by the excitation cascades and, in turn, modulate subsequent light-induced conductance changes, influencing the overall electrical response of photoreceptors to light stimuli.

Physiological relevance of ClC-2 channel function has been suggested for vertebrate photoreceptors, based on progressive retinal degeneration of ClC-2 knock-out mice (Bösl et al., 2001). Although the relevance of a ClC-2 channel in the *Drosophila* photoreceptor physiology is unknown as yet, gene cloning and mutational analysis will provide clues to its function. In other invertebrate photoreceptors and other cell types, evidence suggests that the inward rectifier conductance prevents excessive hyperpolarization (e.g., by electrogenic Na/K-exchange), which could be damaging to cells (Phillips et al., 1992; Wang et al., 2005). In such cases the inward rectifier confines dark resting potential to a range near E_K. This may also be the case in *Drosophila* photoreceptors. The fact that, under some conditions, a fraction of the Cl⁻ channels are open at rest (−70 mV) suggests that these channels may serve to enhance temporal

contrast in vision. In this case, the input resistance at rest would be low, limiting the size of responses to dim lights. Subsequent light-induced depolarization would close the inward rectifier channels, reducing a Cl^- shunt, thus providing a higher input resistance and yielding larger light responses than if the inward rectifier channels were open.

The putative *Drosophila* ClC-2 ortholog contains six consensus sequence sites for phosphorylation by cyclic-AMP-dependent protein kinase (PKA sites), all of which share homology with human ClC-2. Two of these sites in human ClC-2 have recently been identified (Cuppoletti et al., 2004b) as PKA phosphorylation sites near the C-terminus that are involved in channel activation by PKA. It is therefore interesting to speculate that these sites might underlie modulation of the *Drosophila* inward rectifier channel. Functional properties of other inward rectifiers appear to be modulated by PKA (Wischmeyer & Karschin, 1996; Enz et al., 1999; Beaumont & Zucker, 2000) or other kinases (Henry, Pearson & Nichols, 1996; Shin et al., 1997). Similar modulation may contribute to the variability we have observed in functional properties we report here, e.g., voltage dependence and kinetics of chloride-channel gating. Interestingly, modulation of the light response in *Drosophila* by PKA has been suggested based on the effects of cAMP on electrophysiological light responses (Chyb et al., 1999); however, no mechanism for PKA-dependent modulation has been found.

Although our evidence supports the functional expression of a chloride conductance with ClC-2 properties in *Drosophila* photoreceptors, the definitive proof will be provided by cloning studies and heterologous expression of the putative ClC-2 *Drosophila* gene.

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