A Novel Chloride Channel in Drosophila melanogaster
Is Inhibited by Protons*

Katrin Schnizler‡§, Beate Saeger‡§, Carsten Pfeffer‡, Alexander Gerbaulet‡,
Ulrich Ebbinghaus-Kintscher‡, Christoph Methfessel‡, Eva-Maria Franken‡, Klaus Raming‡,
Christian H. Wetzell, Arunesh Saras§, Hermann Pusch§, Hanns Hatt§, and Günter Gisselmann***

From ‡Bayer AG, Bayer Technology Services GmbH, 51368 Leverkusen, §Bayer AG, Bayer CropScience,
40789 Monheim, and the ¶Fakultät für Biologie, Lehrstuhl für Zellphysiologie ND4, Ruhr-Universität-Bochum,
Universitätsstrasse150, D-44780 Bochum, Germany

A systematic analysis of the Drosophila genome data reveals the existence of pHCl, a novel member of li-
gand-gated ion channel subunits. pHCl shows nearly identical similarity to glutamate-, glycine-, and hist-
aamine-gated ion channels, does however not belong to any of these ion channel types. We identified three
different sites, where splicing generates multiple tran-
scripts of the pHCl mRNA. The pHCl is expressed in
Drosophila embryo, larvae, pupae, and the adult fly. In
embryos, in situ hybridization detected pHCl in the
neural cord and the hindgut. Functional expression of
the three different splice variants of pHCl in oocytes of
Xenopus laevis and SF9 cells induces a chloride current
with a linear current-voltage relationship that is in-
hhibited by extracellular protons and activated by aver-
nectins in a pH-dependent manner. Further, currents
through pHCl channels were induced by a raise in
temperature. Our data give genetic and electrophysi-
ological evidence that pHCl is a member of a new
branch of ligand-gated ion channels in invertebrates
with, however, a hitherto unique combination of phar-
macological and biophysical properties.

Ligand-gated ion channels (LGICs)† mediate the fast
inhibitory and excitatory responses of neuronal and muscle
cells to neurotransmitters. A universal feature of the type
of “Cys-loop” class of LGIC is a common topology of four
membrane-spanning segments (M1–M4) and a huge N-terminal
extracellular domain with a hyperconserved cysteine-
bridge motif (1). In vertebrates this “Cys-bridge” family of
phylogenetically related genes codes for cation channels ac-
tivated by acetylcholine and serotonin or for anion channels
activated by GABA and glycine (1). In addition, glutamate-
and serotonin-gated anion channel genes are known in inver-
tebrates (2, 3). Recently, genes for histamine-gated chloride
channels and GABA-gated cation channels were identified in
invertebrates (4–7). The molecular basis of further channel
types like acetycholine-gated chloride channels in inverte-
brates is, however, still unknown (8). Information from the
Drosophila melanogaster genome sequencing project allows
identifying all members of the superfamily of ligand-gated
ion channels occurring in this species by bioinformatic anal-
ysis of new homologous genes. The summarized data ob-
tained from several published bioinformatic analyses (5, 6, 9,
10) show that the group of ligand-gated “chloride” channels
consists of 12 genes that are coding for GABA, histamine, and
glutamate receptors or new, homologous ion channel types.
Four members of this group cannot be directly assigned to the
GABA, glutamate, or histamine branches and thus code for
putative new types of ligand-gated chloride channels with yet
unknown function. In a systematic expression approach of
these predicted novel types of ion channels in Xenopus oo-
cytes, it was found that none of the typical neurotransmitters
activated these novel types of channels (6). Therefore, we
extended the molecular biological analysis of the mRNA and
found that the gene CG6112 encodes for transcripts that
undergo extensive splicing. The functional expressions of
these splice variants in Xenopus oocytes and SF9 cells re-
vealed a unique combination of pharmacological and biophys-
ical properties.

EXPERIMENTAL PROCEDURES

Computer Analysis—The pHCl clones were sequenced using the LI-
COR 4200 laser fluorescent sequencing system (MWG Biotech, Ebers-
berg, Germany), Fluorescence Labeled Cycle Sequencing Kit (Amer-
sham Biosciences), and infrared fluorescence-labeled primers (MWG) as
described before (11). The sequences were analyzed with SAPS (12),
ScanPROSITE, Prot-Param, and Predict Protein (13). The programs
FASTA (14), BLITZ (15), BLASTN (16), and TBLASTN (17) were used
to search EMBL and Swiss-Prot data bases. pHCl splice variants and
similar sequences identified by FASTA were aligned by ClustalW (18).
Detection of pHCl Splice Variants in Different Drosophila Stages by
RT-PCR—Total RNA of D. melanogaster imagos, pupae, larvae III, and
eggs was isolated from the RNeasy Mini Kit (Qiagen, Hilden, Germany),
according to the manufacturer’s protocol for animal tissues. DNA was
removed by on-column digestion during RNA purification with the
Qiagen RNase-Free DNase Set, whereas the RNA was bound to the
giga-gel membrane. The OneStep RT-PCR System (Qiagen) was used
to transcribe 1 μg of total RNA at 30 min, 50 °C and 15 min, 95 °C;
subsequently, PCR amplification was performed for 35 cycles of 1 min,
94 °C; 1 min, 60 °C; and 2 min, 72 °C with final extension at 72 °C for
10 min employing splice variant-specific primer pairs (0.6 μM). As a
negative control RT-PCR and PCR amplification was performed with-
out template, and as a second control 1 μg of total RNA was used in a
PCR amplification with gene-specific primers but without prior RT-
PCR step. Detection of variants A–C were as follows: variant A, a 296-bp
fragment with HT-F1 plus HT-R2 or 550 bp with HT-F2 plus HT-R2,
variant B: 499 bp with HT-F2 plus HT-R2, variant C: 620 bp with
HT-F1 plus HT-R2. (The locations of the primers are in Fig. 1A: ▶.

* The costs of publication of this article were defrayed in part by the
payment of page charges. This article must therefore be hereby marked
“advertisement” in accordance with 18 U.S.C. Section 1734 solely to
indicate this fact.
† The nucleotide sequence(s) reported in this paper has been submitted
to the GenBank™/EBI Data Bank with accession number(s) AY880248,
AY880249, and AY880250.
‡ Both authors contributed equally to this work.
§© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.
" in accordance with 18 U.S.C. Section 1734 solely to

Received for publication, October 15, 2004, and in revised form, January 27, 2005
Published, JBC Papers in Press, February 15, 2005, DOI 10.1074/jbc.M411759200

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
The plasmids pCT19189A–C, which contain PCR products of the different pHCl splice variants generated with the primer pair AATT-
GATGAGTCAGTTGAGTTAAGG and GCTTAATTTGACGAAAATCC, originated from the systematic expression screening approach described previously (6). The cDNAs were cloned into the blunt-ended XbaI site of the expression vector pSMyc (19). This vector construct facilitates expression of a fusion protein consisting of the N-terminal membrane import sequence of the guinea pig serotonin receptor (20) followed by a myc tag and then by the pHCl channel beginning at amino acid 39. Such constructs have been proven useful for the functional expression of ligand gated ion channels in heterologous systems and can substitute for the missing endogenous membrane import sequence (21). Plasmid DNA used for microinjection was prepared using an endotoxin-
free Qiagen Maxiprep kit (Qiagen, Hilden, Germany) dissolved in water to yield 1 μg/ml and frozen in aliquots until use for injection.

Whole Mount in Situ Hybridization with D. melanogaster Embryos— Antisense and sense RNA probes were labeled with digoxigenin-UTP (DIG-RNA-labeling kit SP6/T7, Roche Applied Science) by in vitro tran-
scription in the presence of α-32PUTP with 4 μl of TC4 RNA-polymerase. A TOPO (Invitrogen) containing the complete ORF of pHCl-A was linear-
ized with HindIII (antisense) or EcoRV (sense) and served as the tem-
plate. RNA probes were hydrolyzed at 60 °C under alkaline conditions (0.2 m sodium carbonate, pH 10.2) to yield probes with a length under 500 nucleotides. In situ hybridization on embryos was performed ac-
cording to the method of Tautz and Pfeifle (22). Briefly, embryos were collected 12–15 h after egg laying (5). Eggs were fixed with 50% sodium hypochloride bleach (Sigma) and washed several times with 1× phosphate-buffered saline, fixed with a solution contain-
ing equal parts F-PBT (4% paraformaldehyde in 1× phosphate-buffered saline, filtered), and heated for 20 min with frequent shaking. Embryos were devitellinized with equal parts heptane and methanol for 2 min with vigorous shaking and allowing embryos to settle for 3 min. Five times washes with 100% methanol were given in 100% methanol at 4 °C. After rehydration for several times with 1× PBT (pH 7.4, 130 mm NaCl, 7 mm NaH2PO4, 3 mm Na2HPO4, 0.1% Tween 20 (v/v)) and first fixation with 1× F-PBT (4% paraformaldehyde in 1× PBT) for 20 min while shaking embryos were washed and shaken 3 × 5 min in 1× PBT. First fixation was followed by digestion with proteinase K (25 μg/ml in 1× PBT) for 1 min. Reaction was stopped by incubating embryos with 1× F-PBT while shaking. Embryos were incubated therefor 1 h at 50 °C in enzyme buffer (60 mm Tris, 1 mm EDTA, 50 mm NaCl, 10 mm MgCl2, 100 mm Tris, pH 7.4, 200 mosmol/kg) (23). The pH of the solutions was altered by addition of either NaOH or HCl and routinely checked before and during experiments. Functionally expressing oocytes were identified with the Roboocyte by clamping the oocytes to −80 mV and superfusion with a frog Ringer’s solution of pH 9. Further electrophysiological and pharmacological experiments were carried out on a manual set up. Cells were penetrated with two micro-

tip electrodes and usually clamped to −80 mV with a voltage clamp amplifier (TEC01/02, npi, Tamm, Germany), and the membrane currents were recorded. If not stated differently, recordings were performed at a holding potential of −80 mV and a sampling rate of 20 Hz. Substances were delivered from two reservoirs reaching the cell 7 s after valve opening and exchanging the solution in the recording chamber within 2 s. Data acquisition and analysis were performed with the software program Clampex (Axon Instruments, Foster City, CA and CytoSoft, Bioelectronics, Germany). All measurements were carried out at room temperature (23–28 °C) except those investigating temperature-dependent effects. For those, the glass-enclosed temperature sensor of a digital ther-
mosimeter (Mawitherm, Germany) was positioned near the oocyte into the flowing stream of the extracellular solution.

Cell Culture and Transfection of Sf9 Cells—Patch-clamping Experi-
ments—9–12 h old Sf9 cells were transfected with 1 μg of pHCl splice variants were recorded in the whole cell configuration of the patch clamp technique (28). Application of test substances and bath solutions of various pH were applied using the U-tube-reversed-flow technique (29) with an application time of 1–2 s at intervals of 1 min. The perfusion chamber had a volume of ~0.5 ml and was continuously perfused (flow rate 3 ml/min) with external bath solution driven by gravity. The standard external bath solution contained (in mM): 150 NaCl, 4 KCl, 2 MgCl2, 20 Hepes (pH 7.3 adjusted with 1 N NaOH, 320 mosmol/kg). The pipette solution contained (in mM): 150 KCl, 10 K-EGTA, 10 Hepes (pH 7.3 adjusted with 1 N KOH, 320 mosmol/kg). For pH-variation experiments, the external bath solution was adjusted to pH 6.1 with 1 N HCl. In the case of pH 8.6 Tris buffer was used instead of Hepes buffer. For chloride exchange experiments, the pipette solution contained (in mM): 120 KF, 30 KCl, 10 K-EGTA, 10 Hepes (pH 7.5 adjusted with 1 N KOH, 320 mosmol/kg). Microelectrodes were pulled from borosilicate glass capillaries (external diameter 1.6

mm, Hilgenberg, Malsfeld, Germany) on a Zeitz Puller. The resistance of the pipette medium was normal frog Ringer’s solution containing (in mM): NaCl 115, KCl 2.5, CaCl2 1.8, HEPES 10 (pH 7.2, 240 mosmol/kg). Where stated the

Electrophysiological ex-
periments—Electrophysiological ex-
periments on oocytes were carried out using the two-electrode voltage clamp method (27). The standard extracellular superfusion solution was normal frog Ringer’s solution containing (in mM): NaCl 115, KCl 2.5, CaCl2 1.8, HEPES 10 (pH 7.2, 240 mosmol/kg). Where stated the

Electrophysiological ex-
periments—Electrophysiological ex-
periments on oocytes were carried out using the two-electrode voltage clamp method (27). The standard extracellular superfusion solution was normal frog Ringer’s solution containing (in mM): NaCl 115, KCl 2.5, CaCl2 1.8, HEPES 10 (pH 7.2, 240 mosmol/kg). Where stated the

Electrophysiological ex-
periments—Electrophysiological ex-
periments on oocytes were carried out using the two-electrode voltage clamp method (27). The standard extracellular superfusion solution was normal frog Ringer’s solution containing (in mM): NaCl 115, KCl 2.5, CaCl2 1.8, HEPES 10 (pH 7.2, 240 mosmol/kg). Where stated the

Electrophysiological ex-
periments—Electrophysiological ex-
periments on oocytes were carried out using the two-electrode voltage clamp method (27). The standard extracellular superfusion solution was normal frog Ringer’s solution containing (in mM): NaCl 115, KCl 2.5, CaCl2 1.8, HEPES 10 (pH 7.2, 240 mosmol/kg). Where stated the

Electrophysiological ex-
periments—Electrophysiological ex-
periments on oocytes were carried out using the two-electrode voltage clamp method (27). The standard extracellular superfusion solution was normal frog Ringer’s solution containing (in mM): NaCl 115, KCl 2.5, CaCl2 1.8, HEPES 10 (pH 7.2, 240 mosmol/kg). Where stated the

Electrophysiological ex-
periments—Electrophysiological ex-
periments on oocytes were carried out using the two-electrode voltage clamp method (27). The standard extracellular superfusion solution was normal frog Ringer’s solution containing (in mM): NaCl 115, KCl 2.5, CaCl2 1.8, HEPES 10 (pH 7.2, 240 mosmol/kg). Where stated the
The analog signals were low-pass (Bessel) filtered at 3.15 kHz (whole cell measurements) and digitized at 1 kHz. For recording and analysis the PClamp software (Axon Instruments, version 6.03) was used.

A Novel Chloride Channel Subunit in Drosophila

The genomic region around the gene CG6612 that encodes a putative novel type of invertebrate ligand-gated ion channel was examined for coding regions and deduced transcripts homologous to known Drosophila ligand-gated ion channel subunit sequences. This analysis led to a postulated mRNA sequence that was experimentally proved to exist by RT-PCR and sequencing. The longest transcript identified experimentally in this way encompasses nearly the complete open reading frame of the postulated transcript except for a few nucleotides at the 5′ end.

The originally found cDNA was named pHCl according to the later identified features of the expressed channel (pH-sensitive chloride channel) has an open reading frame of 1464 nucleotides that predicts a protein of 487 amino acids (56 kDa). The extracellular N terminus consists of 277 amino acids in toto, starts with a signal peptide of 18 amino acids (30) followed by the conserved Cys-bridge (positions 195 and 209) and the four predicted transmembrane regions (M1-M4) conserved in ligand-gated-chloride channels (Fig. 1A). A hydrophobicity plot detects three hydrophobic regions in the central part and one at the C-terminal part that fit to the location of M1–M4 in other LGICs (data not shown). The putative pore forming M2 region of pHCl is similar to the M2 region of other ligand-gated chloride channels, suggesting that the pHCl pore is chloride-selective also (Fig. 1B). As in other LGICs consensus sequences for putative N-glycosylation sites (positions 135, 180, 250, 263, and 336) and a protein kinase C-phosphorylation site (position 383) can also be detected (Fig. 1A). A putative orthologous gene exists in Anopheles gambiae; in addition to that, pHCl shows the greatest homology to invertebrate glutamate, teleosts, and mammalian glycine receptor subunits and exhibits a considerable amino acid identity with the D. melanogaster glutamate-gated (28%), the histamine-gated (23%), and the Rattus norvegicus α3-glycine-gated chloride channels (24%), respectively (Fig. 1A). However, a tree constructed of the known and postulated amino acid sequences of Drosophila LGICs shows that the pHCl protein does not fit into the GABA, glutamate, or histamine groups of LGIC and forms a sub-branch of its own (Fig. 2).

Sequencing of the cloned cDNAs revealed the existence of several splice variants (pHCl-A, pHCl-B, and pHCl-C). We identified three sites of different splicing that can theoretically generate a variety of eight different splice variant combinations. In the N-terminal region (positions 68–92, Fig. 1A), a stretch of 25 amino acids is present (Variant 1, pHCl-A and pHCl-B) or lacking (pHCl-C) due to the presence or absence of an exon in the mRNA. In the region located at M1–M2, at the splicing site 2, pHCl-C differs at five positions due to the alternative use of an exon in the mRNA (Variant 2, Fig. 1A). In the cytoplasmic loop between M3 and M4, pHCl-A differs at a stretch of 17 amino acids at the splicing site 3. In the variants pHCl-B and pHCl-C, the stretch is absent due to the usage of different splice sites (positions 385–401, Fig. 1A).

Electrophysiological Characterization of pHCl Homomers—

Oocytes injected with cDNA of one of the pHCl splice variants (pHCl-A, pHCl-B, or pHCl-C) exhibit pH-sensitive currents that are not found in non-injected controls. Changing the pH of the extracellular solution from pH 7.2 to 5.8 strongly reduced the membrane current, whereas changing it to a more basic pH of 9.0 evoked a non-desensitizing membrane current in pHCl-A-expressing oocytes (Fig. 5A). The splice variants pHCl-A and pHCl-C showed the same qualitative dependence of the current on the extracellular pH when expressed in oocytes (data not shown). The pHCl-A splice variant expressed most reliably in Xenopus oocytes and the electrophysiological characterization was therefore concentrated on this splice variant. All three pHCl-splice variants could also be functionally expressed in SF9 cells, respectively, and were activated by basic and inhibited by acidic extracellular pH (Fig. 5B). The membrane current of non-transfected SF9 cells showed no sensitivity to the pH of the extracellular solution. As Fig. 6 shows, the membrane current in pHCl-A-expressing oocytes was half-maximal at pH 7.33 ± 0.16. In normal frog Ringer’s solution, the membrane current of pHCl-A-expressing oocytes was significantly higher than that of non-injected controls indicating that an additional conductance exists at pH 7.2 due to expression of pHCl-A (−777 ± −594 nA (n = 58) versus −136 ± −133 nA (n = 28)). We also observed that oocytes kept in Barth’s solution of pH 6.0 remained longer viable than those kept at pH 7.2.

The current-voltage relationship of the additional membrane current in pHCl-A-expressing oocytes activated by enhancing the pH of the extracellular solution is slightly rectifying and has a reversal potential of −41 ± 5 mV (n = 16) in normal frog Ringer’s solution (Fig. 7). This is in the range of the reversal potential of −53 mV for chloride ions calculated by the Nernst equation assuming an intracellular chloride concentration of 15 mM. Reducing the extracellular chloride concentration to 36.3 and 12.1 mM shifts the reversal potential of the pH-induced current to more positive potentials (−28 ± 14 mV, n = 9 and −12 ± 17 mV, n = 9). To maintain a constant offset potential at the bath electrode we used agar bridges for measurements with low extracellular chloride concentrations.

The deviation of the measured from the calculated reversal potential for chloride ions (−22 mV for 36.3 mM and +5 mV for 12.1 mM chloride extracellularly) may be attributed to the fact that the intracellular chloride concentration of the oocyte is not constant. Lowering the extracellular chloride concentration could induce leaking of chloride ions from the cytoplasm into the extracellular solution and result in a less positive reversal potential for chloride ions. The reversal potential of the pH-induced current did not depend on the extracellular pH, showing that no proton- or hydroxide-ion currents are involved (data not shown). To further support the finding, that chloride ions permeated through pHCl channels, we performed similar ion exchange experiments in SF9 cells. Under nearly symmetrical...
In chloride conditions, the current carried by the spontaneously open pHCl-A channels reversed at 6.2 mV, a reversal potential near zero, as expected for these ionic conditions ([Cl]i = 150 mM, [Cl]o = 162 mM; Fig. 8). Lowering of the intracellular chloride concentration shifted the reversal potential to 45.3 mV, in good agreement with the prediction by the Nernst equation for a chloride-selective ion channel ([Cl]i = 30 mM, [Cl]o = 162 mM = > ECli = −43.3 mV at 25 °C; Fig. 8).

**Pharmacological Properties of the Currents through pHCl Channels**—The extracellular application of 10 μM ivermectin on pHCl-A-expressing oocytes activates a membrane current that desensitizes slowly in the presence of ivermectin. However, we mostly observed that after ivermectin application the membrane current did not return to its original value nor did it reach a stable plateau after 10-min superfusion with frog Ringer’s solution. The amplitude of the additional current stimul...
A Novel Chloride Channel Subunit in Drosophila

of normal frog Ringer’s solution at the respective pH.

The chloride channel blockers niflumic acid, flufenamic acid, and phenylalantraine acid did not block currents through pHCl-A channels at a concentration of 1 mM, respectively. Neither did the insecticide fipronil (10 μM) induce any change in the membrane current of pHCl-A-expressing oocytes at pH 7.2 or 9. 1 mM of picrotoxin, a plant-derived non-selective blocker of ligand-gated anion channels, led to a half-maximal inhibition of currents through pHCl-A channels induced by extracellular pH of 9.0. Compared with channels that are considered to be blocked by picrotoxin (31, 32), the pHCl-A channel is not sensitive to picrotoxin.

**pHCl-A Channels Are Temperature-sensitive and Inhibited by Capsaicin**—The membrane currents of pHCl-A-expressing cells are strongly modulated by the extracellular temperature. In contrast to non-injected oocytes where membrane currents exhibited only little temperature sensitivity, we found that reducing the temperature decreased and increasing the temperature stimulated currents through pHCl-A channels (Fig.

![Fig. 2](Image 119x359 to 246x557)

**Fig. 2.** A dendrogram showing the relationship of pHCl to the different groups of LGICs. The dendrogram was constructed by MegAlign (Lasergene) on the basis of the ClustalW algorithm (18). pHCl clearly forms a separate branch in the group of LGICs that further contains the families of GABA-, glutamate-, and histamine-gated channels.

![Fig. 3](Image 336x568 to 543x738)

**Fig. 3.** Detection of pHCl splice variants in different Drosophila stages by RT-PCR. RT-PCR with splice variant-specific primer pairs detected expression of pHCl in all tested stages of Drosophila (adult, pupae, larvae III, and eggs). pHCl-A: a 296-bp fragment with primers HT-F1 plus HT-R2; pHCl-A/B: 550 bp (for pHCl-A, 499 bp for pHCl-B), with HT-F2 plus HT-R2; pHCl-C: 620 bp with HT-F1 plus HT-R2. (+: cDNA; −: mRNA prior reverse transcription).

![Fig. 4](Image 489x557)

**Fig. 4.** Whole mount in situ hybridization with D. melanogaster embryos and larvae I. Antisense pHCl-A RNA probes labeled with digoxigenin-UTP detected high expression levels in the central nervous system/neural cord (A–D) together with a particular hybridization signal in the hindgut (B and C). The negative controls, sense pHCl-A RNA probes, showed no hybridization signals at all (E and F). Antisense probes: A, embryo lateral view; B, embryo dorsal; C, larvae I lateral; D, embryo ventral. Sense probes: E, embryo ventral; F, embryo lateral. Scale bar: 100 μm; nt: neural tube; hg: hindgut.

![Fig. 5](Image 506x557)

**Fig. 5.** Influence of the external pH on pHCl mediated currents. A, membrane currents of a pHCl-A-expressing oocyte and a non-injected oocyte controlled by pH upon superfusion with normal frog Ringer’s solution of acidic (5.8) and basic (9.0) pH. B, membrane currents of a pHCl-C-expressing Sf9 cell and a non-transfected cell controlled by pH. Short application of pH 6.1 completely blocked the membrane current of a pHCl-C-expressing Sf9 cell under control conditions (pH = 7.3), whereas changing the pH from 7.3 to pH 8.6 doubled the membrane current.
A Novel Chloride Channel Subunit in Drosophila

FIG. 6. Dependence of the membrane current of pHCl-A-expressing oocytes on the pH of the extracellular solution. Current amplitudes were referred to the amplitude induced by changing the pH from 7.2 (normal frog Ringer's) to pH 8 after subtraction of the current measured at pH 5.83. Data points were fitted by \( I/I_{\text{max}} = 1/(1 + (EC_{50}/x^a)) \) yielding a half-maximal activation at a \( H^+ \) concentration of \( 4.71 \times 10^{-4} \) ± \( 7.58 \times 10^{-4} \) mol, which corresponds to a pH of 7.33 ± 0.16 and a Hill coefficient of 1.07 ± 0.18 (\( n = 3-5 \) per data point).

FIG. 7. Current-voltage relationship of pHCl-A mediated current in oocytes. Current-voltage relationship of the additional current activated by changing the extracellular pH from 7.2 to 9.0 in a pHCl-A-expressing oocyte and non-injected oocyte. From a holding potential of −80 mV the membrane potential was clamped for 400 ms from −100 mV to 60 mV in 20-mV steps, total pulse duration was 500 ms. Current values are mean amplitudes of the last 100 ms of the voltage step.

FIG. 8. Dependence of the reversal potential of the pHCl-A carried current on the intracellular chloride concentration. Under physiological ([Cl]o = 30 mM, [Cl]i = 162 mM) or almost symmetrical ([Cl]o = 150 mM, [Cl]i = 162 mM) chloride concentrations the current through pHCl-A channels expressed in Sf9 cells reverses at −45 mV and 6 mV respectively, which is in good agreement with the Nernst equation that yields −43 mV and 0 mV. At a given holding potential extracellular solution of pH 6.1 was applied for 2 s. Current values are the blockable membrane current by application of the pH 6.1 solution for 2 s.

A systematic analysis of the Drosophila genome data reveals the existence of a novel branch of ligand-gated ion channel (LGIC) subunits. It was named pHCl according to the proper-

10A). Starting from a room temperature between 23 and 28 °C, oocytes were superfused first with cold frog Ringer’s solution that lowered temperature in the bath chamber to 13–20 °C as measured with a sensor right besides the oocyte. On average, membrane currents were reduced by 16 ± 10 nA per degree Celsius (\( n = 10 \)) in pHCl-A-expressing oocytes compared with 1.8 ± 4 nA per degree Celsius (\( n = 8 \)) in non-injected controls. Raising the temperature of the extracellular solution yielding 31–44 °C near the oocyte stimulated the membrane currents in pHCl-A-expressing oocytes by 46 ± 26 nA per degree Celsius (\( n = 10 \)) and by 7 ± 4 nA per degree Celsius (\( n = 9 \)) in non-injected oocytes. To further characterize the temperature dependence of the membrane currents, the temperature coefficients \( Q_10 \) were determined in an Arrhenius plot in which the amplitude of the common logarithm of the current was plotted against the reciprocal of the absolute temperature (33, 34). The factor by which the peak membrane current decreased upon a 10 °C drop or increased upon a 10 °C rise in temperature were \( 1.7 ± 0.24 (n = 9) \) and \( 1.7 ± 0.43 (n = 13) \), respectively. The reversal potential of the additional membrane current evoked by the rise in the temperature of the extracellular solution was −44 ± 9 mV (\( n = 7 \)) in pHCl-A-expressing oocytes compared with −20 ± 9 mV (\( n = 3 \)) in non-injected controls.

Capsaicin did not activate a membrane current in pHCl-A-expressing oocytes but slightly reduced the membrane current at 7.2 where approximately half of the pHCl-A channels are open. Further quantification of this effect showed that capsaicin blocked the additional current evoked by basic extracellular pH with an IC50 of 51 ± 13 \( \mu \)M (\( n = 5-7 \)) per data point, Hill coefficient of 1.4 ± 0.26 (Fig. 10B).

DISCUSSION

A systematic analysis of the Drosophila genome data reveals the existence of a novel branch of ligand-gated ion channel (LGIC) subunits. It was named pHCl according to the proper-
ties of the expressed ion channel that is sensitive to pH and permeable for chloride ions. The overall structure clearly classifies pHCl unequivocally as a member of the superfamily of the cys-bridge type of LGICs. pHCl shows nearly identical similarity to glutamate-, glycine-, and histamine-gated ion channels; however, it does not belong to any of these ion channel types. It seems possible that pHCl shares the same common ancestral gene as postulated for the ligand-gated chloride channels (35) but separated early and evolved independently from the later ion channel subunits. This new branch of ion channels is possibly unique for insects (or maybe arthropods). A putative orthologue gene is present in *Anopheles*, but in the genomic data of nematodes, teleosts and mammals, no such gene can be found. pHCl encodes for a variety of ion channel subunits due to splicing of the mRNA at, at least, three different positions. The three different splicing sites can generate a variety of eight different ion channel subunits. An RT-PCR analysis suggests that all combinations are expressed in all developmental stages of *Drosophila*. To elucidate, if the splice variants are responsible ion channel subunits with different properties, we chose the pHCl-A, pHCl-B, and pHCl-C subunits that are different at all three positions for a detailed functional characterization.

We found that all characterized splice variants of pHCl are strongly sensitive to external pH, activated by the insecticidal compounds ivermectin and avermectin B1a, respectively, in a pH-dependent manner and modulated by extracellular temperature. Thus, the described chloride channels most likely encode pH-sensitive ion channels, and compounds affecting these channels have the potential to provide novel strategies in agriculture and public health.

Various LGICs are sensitive to protons. In invertebrates, a modulation by external pH has been reported for a GABA-gated Cl− conductance in the crayfish leg opener muscle fiber (36). However, in contrast to the pHCl channels, this conductance was inhibited by raising the extracellular pH. In vertebrates, protons were found to differentially regulate neuronal GABA<sub>A</sub> receptors, resulting in potentiation, inhibition, or no effect (37–41). An inhibition of single channel currents of GABA<sub>A</sub> receptors by H<sup>+</sup> in outside-out granular cell patches of early developmental stages was shown to result from an increase in the long shut times (39). In primary hippocampal neurons the major effect of protons on GABA<sub>A</sub> receptors was revealed to be an enhancement of the desensitization and binding rates by decreasing proton concentration (42). Specifically, it was shown that even variations of a few tenths of a pH unit can have major effects on the amplitudes and kinetics of GABA<sub>A</sub> receptors. Also, modulation of GABA receptors by external pH was shown to be dependent on the receptor subunit composition (39, 43). Lately, a single histidine residue in the ion channel domain of the β-subunit was identified to be solely responsible for proton regulation of α<sub>1</sub>β<sub>3</sub> heteromers (44). The pHCl-A channel shows moderate similarity to the vertebrate ρ1 GABA receptors that were also shown to be sensitive for protons (45, 46). GABA-mediated currents through receptors composed of the rat ρ1 subunit show a similar sensitivity to protons over a wide range of acidic and alkaline pH like the pHCl-A. However, on ρ1-oligomers, protons act as a modulator of GABA-evoked currents but do not activate channels by themselves as they do on the pHCl-A channel.

From our data, we cannot decide if the ion channel is gated by hydroxyl ions and protonation/deprotonation of amino acids in a putative ligand binding site (model 1) or if the protons modulate the open probability of the channel by acting on an amino acid in the pore region (model 2). The fact that LGICs have a considerably open probability in the unliganded state has been reported for several type of ion channels (5, 47). According to model 2 the current evoked in pHCl-A at an elevated pH would then represent the unliganded form of the receptor that exhibits a considerable open probability in this state. Ivermectin or an as yet unidentified endogenous ligand would then gate this channel. The open ligand-bound state is then equally modulated by the pH, resulting in a small activation at an acidic pH and an enhanced activation at a basic pH. So, in model 2, the pH modulation of the pore and the gating of the channel (by ivermectin) would then be two separable processes and ivermectin would act as an agonist. In model 1, the hydroxyl ion would really “gate” this channel; e.g. by deprotonating an amino acid side chain in a potential ligand binding region and ivermectin would act as an allosteric modulator as described for glutamate gated channels in *Caenorhabditis elegans* and *Drosophila* (48, 49) and the nicotinic acetylcholine receptor (50).

It is tempting to speculate whether the pHCl-channel is a modulatory subunit that can confer pH sensitivity to a heteromultimeric ligand-gated anion channel. The expression of pHCl in the neural cord implicates a role of pHCl in synaptic transmission. Transient changes in the extracellular pH are produced by excitatory and inhibitory neuronal activity (51–53). Variations of the interstitial pH are also induced by the release of vesicles with acidic contents into the synaptic cleft (54) as well as the reuptake of neurotransmitter (55). The local proton concentration near the plasma membrane is influenced by the passive and active ion transport across the membrane. Specifically, for the GABA<sub>A</sub> receptor, the permeation of HCO<sub>3</sub> ions influences the pH in the immediate environment of the ion channel (37, 56). If located postsynaptically, pHCl could modulate the strength of GABA-mediated inhibitory synaptic transmission. At an extrasynaptically located receptor, acidifi-
cation or alkalization of the extracellular space would strongly modulate inhibitory neurotransmission in general, and this would have physiological implications on the neuronal activity of the total Drosophila.

The temperature sensitivity of the pHCl-A splice variant was quantified by dependence of the membrane currents of pHCl-A-expressing oocytes upon a 10 °C decrease or increase in extracellular temperature. The Arrhenius plot was linear over the whole temperature range yielding a Q10 value of 1.7 for both hot and cold temperatures. This relatively low Q10 value together with the linearity of the Arrhenius plot indicates that the pHCl channel is not gated by temperature but merely modulated as many ligand- or voltage-gated ion channels. Similar temperature sensitivities of current amplitudes have been reported for GABA-induced chloride currents in sensory frog neurons (57) and for the mean open time of nicotinic acetylcholine receptor channels in BC3H-1 cells (58).

Some members of the family of transient receptor potential channels that are activated by temperature are also activated by capsaicin, the main active component of red hot chili peppers. We therefore tested whether pHCl-A is sensitive to capsaicin and found that pHCl-A-mediated currents in oocytes are inhibited by capsaicin with an IC50 of 51 μM. This IC50 for the inhibitory effect of capsaicin is 100-fold higher than that for the activation of the vanilloid receptor (59) but is of the same order of magnitude reported for the inhibition of various voltage-gated cation channels (60–66). In a more recent report (67), the inhibitory effect of capsaicin is 100-fold higher than that for the cAMP-stimulated CFTR currents with an apparent EC50 of 48 μM. This nonspecific block of voltage-gated ion channels in the nodose ganglion, the primary sensory ganglion of the vagus, would have physiological implications on the neuronal activity modulate inhibitory neurotransmission in general, and this might also be possible that the pHCl channel is expressed in the enervating neurons rather than in the epithelium of the hindgut which cannot be decided by in situ hybridization.

Acknowledgments—We acknowledge the excellent technical assistance of A. Stocke, S. Seil, and M. Kuester.

REFERENCES

A Novel Chloride Channel in *Drosophila melanogaster* Is Inhibited by Protons

Katrin Schnizler, Beate Saeger, Carsten Pfeffer, Alexander Gerbaulet, Ulrich Ebbinghaus-Kintscher, Christoph Methfessel, Eva-Maria Franken, Klaus Raming, Christian H. Wetzel, Arunesh Saras, Hermann Pusch, Hanns Hatt and Günter Gisselmann

doi: 10.1074/jbc.M411759200 originally published online February 15, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M411759200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 70 references, 19 of which can be accessed free at [http://www.jbc.org/content/280/16/16254.full.html#ref-list-1](http://www.jbc.org/content/280/16/16254.full.html#ref-list-1)