SPECIAL REPORT

Drosophila melanogaster GRD and LCCH3 subunits form heteromultimeric GABA-gated cation channels

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In addition to its action as a fast inhibitory neurotransmitter, γ-aminobutyric acid (GABA) is thought to mediate excitatory action by activating cation currents in some cell types in invertebrates. However, to date no GABA receptor capable of mediating such action has been identified at the molecular level in insects. Using a systematic expression screening approach, we found that the *Drosophila* ligand-gated ion channel subunits GRD and LCCH3 combine to form cation-selective GABA-gated ion channels when coexpressed in *Xenopus laevis* oocytes. The heteromultimeric receptor is activated by GABA ($EC_{50} = 4.5 \mu M$), muscimol ($EC_{50} = 4.8 \mu M$) and *trans*-4-aminocrotonic acid ($EC_{50} = 104.5 \mu M$), and partially by *cis*-4-aminocrotonic acid ($EC_{50} = 106.3 \mu M$). Picrotoxin effectively blocked the GABA-gated channel ($IC_{50} = 0.25 \mu M$), but bicuculline, TPMTA, dieldrin and lindane did not. The benzodiazepines flunitrazepam and diazepam did not potentiate the GABA-evoked current. Our data suggest that heteromultimeric channels composed of GRD and LCCH3 subunits form GABA-gated cation channels in insects.

Introduction Ligand-gated ion channels (LGICs) mediate the fast responses of neuronal and muscle cells to neurotransmitters. They form a large family of anion and cation channels activated by various neurotransmitters (Ortells & Lunt, 1995). The widespread anion-selective γ-aminobutyric acid (GABA) receptors mediate rapid inhibitory neurotransmission in the nervous systems of both vertebrates and invertebrates (Lee *et al.*, 2003). In addition, GABA can also act as an excitatory neurotransmitter (Beg & Jorgensen, 2003; Stein & Nicoll, 2003). One of several suggested mechanisms for this action is that in invertebrates GABA might mediate excitation *via* cation currents (Zhang *et al.*, 1997; Swensen *et al.*, 2000; Stein & Nicoll, 2003), and in *Ceanorhabditis elegans* a GABA-gated cation channel, named EXP-1, has been cloned (Beg & Jorgensen, 2003). Our knowledge about the structure and function of ionotropic GABA receptors in *Drosophila* is fragmentary; two GABA receptor subunits from *Drosophila* have been characterized by recombinant expression of cloned cDNA: the exhaustively investigated RDL receptor (resistance to dieldrin locus), which is an important target for insecticides (Ffrench-Constant *et al.*, 1991), and the ß-subunit LCCH3 (LCCH3: ligand-gated chloride channel homolog 3) (Henderson *et al.*, 1993). LCCH3 alters the pharmacological properties of the RDL receptor, when the subunits are coexpressed (Zhang *et al.*, 1995), but it is not thought to function as a ß-subunit for RDL *in vivo* because the two subunits exhibit different spatial patterns (Hosie *et al.*, 1997). Multiple types of pharmacologically distinct GABA receptors in invertebrates have been characterized *in vivo* (Sattelle *et al.*, 1988), particularly in the arthropods, where they have been studied in more detail (Dudel & Hatt, 1976; Zhainazarov *et al.*, 1997). It is probable that this variety of GABA receptors is coded by more than two subunit types. Apart from RDL and LCCH3, one additional subunit has been cloned in *Drosophila*, named GRD (GABA and glycine-like receptor of *Drosophila*), which exhibits considerable homology to vertebrate ionotropic GABA receptors (Harvey *et al.*, 1994). Analysis of the *Drosophila* gene sequences predicts one additional candidate, AAF48539, which clearly fits into the GABA group (Witte *et al.*, 2002). To address the question of whether ion channel proteins coded by GRD and AAF48539 may form functional GABA receptors, possibly in combination with the ß-subunit LCCH3, the *in vitro*-transcribed cRNAs of these subunits were expressed using the *Xenopus laevis* oocyte expression system. Here we report the functional expression and pharmacological characterization of a new type of heteromultimeric GABA-gated channel formed by GRD and LCCH3.

Methods *Construction of expression vectors* mRNA was isolated from adult *Drosophila melanogaster* using the Pharmacia QuickPrep Micro mRNA Purification Kit. cDNA was constructed using an oligo(dT)17 primer and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.). PCR products containing the coding region of LGICs were obtained in a PCR buffer containing $1.5 mM$ $MgCl_2$, $0.2 mM$ of each dNTP, ~10 ng *Drosophila* cDNA, $0.5 mM$ of the corresponding primer and 2.5 U *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.). PCR amplification was performed according to the following schedule: 94°C for 1 min, 55°C for 1 min, 72°C

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for 2 min, for 40 cycles. The resulting fragments were subcloned blunt end into pSGEM (courtesy of M. Hollmann) previously digested with *Eco*RV and *Sma*I, resulting in the plasmids pGEM-LCCH3 (P1–P2), pGEM-GRD (P3–P4) and pGEM-AAF48539 (P6–P7):

P1: gccaccatgacatgttttacgcgcgtcggag
P2: tcattccagaatataaaacagccagtagc
P3: gccaccatgtgcacaatgccagcaacaagag
P4: tcaggtgctcgcatccggcgtgttgg
P5: accatgggaccctcggcgattttaac
P6: catgactatgacttcatattggacc

Expression of receptor cRNA in Xenopus *oocytes* RNAs were synthesized using the AmpliCap T7 high-yield message maker kit (Epicentre, Madison, WI, U.S.A.), according to the manufacturer's protocol, with *Pac*I-linearized pSGEM plasmids as templates. *X. laevis* oocytes were prepared by standard methods. After 24 h, stage V–VI oocytes were injected with cRNA (about 50 ng per oocyte) and incubated at $18°C$ in Barth's solution (88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.33 mM $Ca(NO3)_2$, 0.41 mM $CaCl_2$, 2.4 mM $NaHCO_3$, 5 mM Tris-HCl, pH 7.4) supplemented with 100 U ml^{-1} penicillin and 50 μg ml^{-1} streptomycin. Oocytes were tested for functional expression of LGICs after 3–7 days.

Electrophysiological recording Two-electrode voltage-clamp recording was used to obtain current responses to applied substances. Agonists and antagonists were diluted to the concentrations indicated with *Drosophila*-Ringer solution (115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES, pH 7.2) and were applied by means of a multibarrel, single-tip superfusion device. Electrodes were pulled from borosilicate glass using a Kopf vertical pipette puller and were backfilled with 3 M KCl. The membrane potential was controlled and current signals were recorded with a two-electrode voltage-clamp amplifier (TURBO TEC-03, npi, Tamm, Germany) and pCLAMP software (Axon Instruments, Union City, CA, U.S.A.). The I/V curve and the ion selectivity experiments were performed essentially as described for the EXP-1 receptor (Beg & Jorgensen, 2003) and, to get comparable results, similar experimental conditions were used. Briefly, all points were obtained by a 10 s application of 5 μM GABA. The reversal potentials were determined in the following solution: 115 mM NaCl, 1.8 mM $BaCl_2$, 10 mM HEPES, pH 7.2. For sodium exchange experiments, 50 or 75% of NaCl was replaced by Tris-HCl, and for chloride exchange, sodium gluconate was used. In the sodium-free potassium Ringer, NaCl was completely replaced by KCl. SigmaPlot 7.0 (SPSS Inc.) was used to analyze concentration–response data. A four-parameter logistic equation was used to fit curves to the averaged, normalized data. All data are ± s.e.m.

Results In order to investigate the function of the cloned cDNAs, RNA was *in vitro* transcribed from the linearized plasmids pGEM-LCCH3, pGEM-GRD and pGEM-AAF48539 and microinjected individually or in combination into the cytoplasm of *Xenopus* oocytes. After 3–7 days, oocytes were recorded in the two-electrode voltage-clamp configuration and tested for responses to agonists. To test the functional expression of the GABA receptors, we applied GABA and several other neurotransmitters including glycine, acetylcholine, histamine and serotonin (1 mM, $n > 30$ for each combina-

tion). Only oocytes co-injected with cRNAs for LCCH3 and GRD exhibited a detectable current evoked by GABA (Figure 1a). Oocytes coexpressing LCCH3 and GRD responded to GABA with a concentration-dependent inward current, when clamped at −80 mV (Figure 1a). The rapidly developing current had amplitudes typically in the range 0.5–4 μA at a holding potential (V_h) of −80 mV. From analysis of concentration–response data, an EC_{50} value of $4.3 ± 0.4$ μM ($n = 7$) was estimated for the heteromultimeric receptor (Figure 1b and d). The Hill coefficient was $1.73 ± 0.26$.

Next we characterized the pharmacological properties of the heteromultimeric GRD/LCCH3 receptors. The agonists muscimol and *trans*-4-aminocrotonic acid (TACA) activated about 120% of the maximal GABA-induced current (Figure 1c and d) (EC_{50} for muscimol $= 4.8 ± 1.1$ μM, $n = 4$; EC_{50} for TACA $= 104.5 ± 5.3$ μM, $n = 4$), whereas *cis*-4-aminocrotonic acid (CACA) was a weak partial agonist (EC_{50} for CACA $= 106.3 ± 17$ μM, $n = 4$) (Figure 1d). Bicuculline, an antagonist for $GABA_A$ receptors, and for some populations of GABA receptors in insects (Hosie *et al.*, 1997), did not antagonize the current evoked by 6 μM GABA at a concentra-
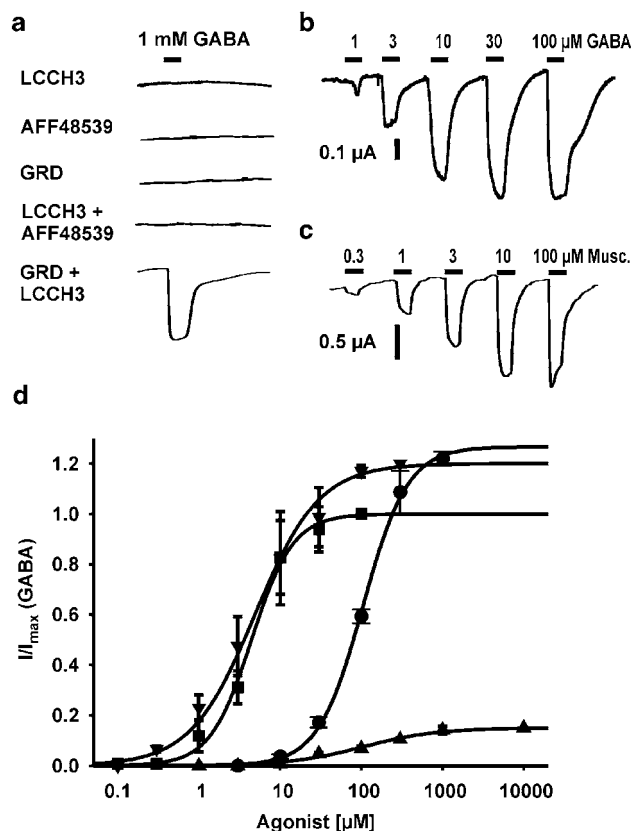

Figure 1 (a) Oocytes were voltage clamped ($V_h = −80$ mV) 3–7 days after injection of 25 ng of various cRNA mixes. GABA applied at 1 mM for 10 s produced inward currents only in oocytes coexpressing GRD and LCCH3. The duration of application of agonists is indicated by bars. (b, c) Currents evoked by GABA and muscimol in oocytes co-injected with cRNA encoding GRD and LCCH3 subunits voltage clamped at –60 mV. (d) Agonist profile of oocytes coexpressing GRD and LCCH3. Recordings were performed as in (b, c). Each point represents the average value of four to seven oocytes. Data were normalized to the maximum GABA response (100 μM) seen in each oocyte. The concentration–response curve was fitted to the logistic equation.

tion as high as $100 \mu M$. The same was found for the $GABA_C$ antagonist (Bormann, 2000) 1,2,5,6-tetrahydropyridine-4-yl (TPMPA, 100 mM; data not shown). γ-Hexachlorohexane (γ-HCH, lindane), a ligand related to cyclodienes that interacts with the picrotoxin (PTX)/cyclodiene binding site and blocks the RDL receptor (Zhang *et al.*, 1994), had no effect at $10 \mu M$ concentrations (Figure 2a). Dieldrin too was not an effective blocker at concentrations up to $100 \mu M$. The antagonist PTX, known to be an effective noncompetitive blocker for both vertebrate and invertebrate GABA receptors, virtually completely blocked the GABA-induced current at concentrations above $10 \mu M$ (Figure 2a and b). The heteromultimeric channels were highly sensitive to PTX; the IC_{50} for PTX was 250 ± 24 nM. The low Hill coefficient of 0.84 ± 0.06 is in good agreement with a simple single-site blocking mechanism (Figure 2b). Numerous allosteric modulators, including benzodiazepines, are known to regulate $GABA_A$ receptor function. To test if benzodiazepines potentiated the GABA-evoked current, we co-applied a near-EC_{10} concentration of GABA ($1 \mu M$) with $30 \mu M$ flunitrazepam or $30 \mu M$ diazepam and found no enhancement of the evoked current (data not shown).

The current exhibited a linear current–voltage relationship (Figure 3a). The GABA-dependent current reversed at -11.2 ± 1.6 mV ($n = 9$). Under the ionic conditions of the experiments, the reversal potentials are roughly -22 mV for a chloride and ~ 0 mV for mixed K^+–Na^+ currents in *Xenopus* oocytes, provided typical intracellular concentrations of 108 mM K^+, 10.8 mM Na^+ and 40 mM Cl^- are present. These data are consistent with the hypothesis that the channel could conduct chloride, but the shift in the reversal potential to more positive values suggests that other ions may also permeate the channel. To identify the permeating ions, we determined the reversal potential in different extracellular solutions. The
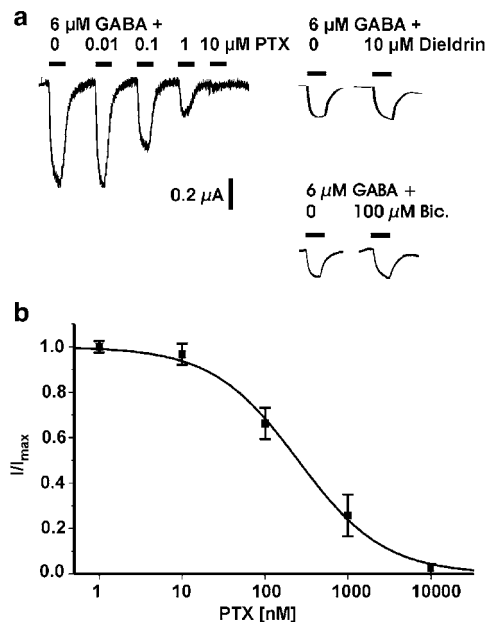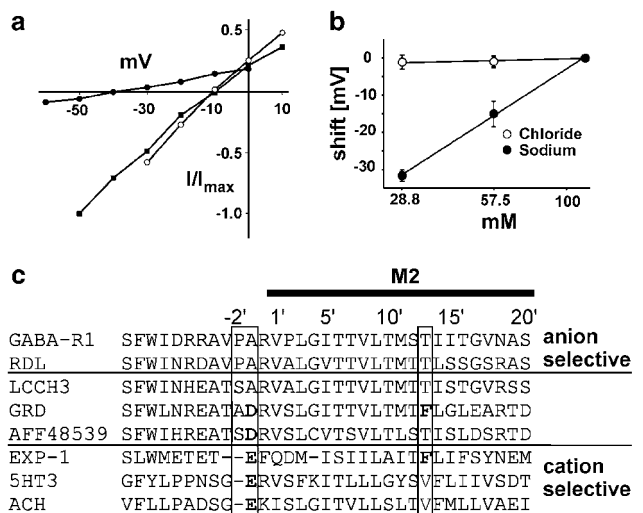

Figure 3 (a) Current–voltage relationship. The averaged peak currents of the I/V curves were determined under varying external ionic conditions (normalized to the response in 100% Na^+, 100% Cl^- Ringer): 100% Na^+, 100% Cl^- Ringer ($n = 9$); 25% Na^+, 100% Cl^- Ringer ($n = 4$); 100% Na^+, 25% Cl^- Ringer ($n = 4$). (b) Reversal potential shifts for ionic substitution experiments. Reversal potentials were measured in solutions with varying amounts of sodium ($n = 4$) or chloride ions ($n = 4$). (c) The pore-forming M2 domain of GRD (CAA55144) contains the molecular determinants for cation selectivity and differs in the three critical positions $-2'$, $-1'$ and $13'$ (Wotring *et al.*, 2003) from the ligand-gated chloride channel RDL (AAA28556) and the rat $\rho1$-GABA receptor (P50572) and resembles ligand-gated cation channels such as EXP-1 (AY383563), the rat $5HT_3$ receptor (NP_038589) and the rat nicotinic $\alpha7$ ACh receptor (NP_036964) at these positions. AFF48539 and LCCH3 (AAB27090) show these differences to a lesser extent.

reversal potential was virtually independent of the external chloride concentration ($n = 4$), an indication that chloride ions make no significant contribution to the current (Figure 3b). In contrast to this, variation of the external sodium concentration shifted the reversal potential to more negative values. The shift of -31.6 ± 1.6 mV ($n = 4$) in 25% sodium Ringer is comparable to the theoretical shift of -34.3 mV predicted by the Nernst equation for the given ionic conditions. This proves that sodium permeates through the channels. To test if the channel also conducted potassium, we replaced sodium Ringer with potassium Ringer and found a shift in the reversal potential of 7.1 ± 0.7 mV ($n = 5$) to more positive values (data not shown), an indication that potassium permeated the ion channel pore even more effectively; the estimated selectivity of sodium over potassium was 0.75, assuming no other ions were involved. A possible reason for the cation selectivity of this channel was discovered by analyzing the amino-acid sequence of the pore-forming M2 region, which determines the ion selectivity of LGICs. Three residues at positions $-2'$, $-1'$ and $13'$ have been identified by mutation studies as being critical for ion selectivity (Wotring *et al.*, 2003). The M2 region of the GRD subunit differs from a typical chloride-selective pore in all three critical positions: a proline is absent at position $-2'$, a phenylalanine is located at position $13'$ and , importantly, a charged aspartic acid is found at position $-1'$ (Figure 2c). In LCCH3 subunits, the proline at position $-2'$ is absent.

Discussion Pharmacologically distinct classes of invertebrate GABA-gated ion channels existing *in vivo* have been reported

Figure 2 (a) Antagonist profile of oocytes coexpressing GRD and LCCH3. Oocytes were voltage clamped ($V_h = -80$ mV) and $6 \mu M$ GABA was co-applied with various concentrations of PTX, dieldrin or bicuculline. (b) Concentration–block curve for PTX. Each point represents the average value of three to five oocytes. Concentration–response curves were fitted to the logistic equation.

by many groups (Dudel & Hatt, 1976; Sattelle *et al.*, 1988; Zhainazarov *et al.*, 1997), but nearly all investigations of recombinantly expressed channels have focused, in contrast, on chloride channels such as the RDL receptor and its homologs. There are indications from physiological studies in invertebrates that GABA mediates excitation by activating cation currents (Zhang *et al.*, 1997; Swensen *et al.*, 2000). Recently, a homomultimeric GABA-gated cation channel in *C. elegans*, named EXP-1 (Beg & Jorgensen, 2003), was cloned that belongs to the superfamily of LGICs. Our data show the existence of a second, distinct type of heteromultimeric GABA receptor occurring in *Drosophila*, made up of LCCH3 and GRD subunits, which also form cation-selective channels when coexpressed in *Xenopus* oocytes. LCCH3 and GRD are not receptors of the EXP-1 type. Their homology to EXP-1 is weak (17–21% identical amino acids). LCCH3 has been described as a clear homologue of vertebrate GABA$_A$ ß-subunits (Hosie *et al.*, 1997) and GRD is similar to the vertebrate GABA and glycine α-subunits (Harvey *et al.*, 1994). The high homology of AAF48539 with GRD suggests that it could be an additional potential α-subunit. However, AAF48539 alone or in combination with GRD or LCCH3 did not form any detectable GABA receptors when expressed in oocytes. It should be noted that the cDNA used for expression of AAF48539 was generated by RT–PCR based on the genome sequence data only. Hence we cannot rule out the possibility that the cDNA was incomplete and thus coded for a nonfunctional protein.

What causes the cation selectivity of the ion channel formed by GRD/LCCH3? GRD differs from GABA-gated chloride channels at three positions, $-2'$, $-1'$ and $13'$, that are critical for the ion selectivity of the pore (Wotring *et al.*, 2003). At these positions, GRD shows more similarity to ligand-gated channels with cation selectivity. Changes at these positions are sufficient to change the ion selectivity of the pore of LGICs from chloride to cation selective (Wotring *et al.*, 2003). GRD and EXP-1 share more or less the same differences to chloride-selective channels in the pore region. The importance of these amino-acid exchanges is discussed for EXP-1 in detail by Beg and Jorgensen (2003). LCCH3, which can function as a subunit with RDL in chloride channels (Zhang *et al.*, 1995) and with GRD in cation channels, lacks proline at position $-2'$ but displays no charged amino acid at position $-1'$. Therefore, the GRD subunit seems to be the molecular determinant of the ion selectivity, analogous to findings that, in GABA$_A$ receptors, the M2 region of the GABA$_A$ ß-subunit alone can determine ion selectivity in a heteromultimeric channel (Zhang *et al.*, 1997).

The pharmacological characteristics of the *Drosophila* receptor are similar to the excitatory muscimol II type GABA receptor found in the v-LCDN giant neurons of the African giant snail *Achatina fulica* (Zhang *et al.*, 1997). At both receptors, GABA, muscimol, TACA and CACA are agonists,

TACA activating the receptor with greater efficacy and CACA with lesser efficacy than GABA. Furthermore, both receptors are insensitive to diazepam. In some neurons of the stomatogastric ganglion of the crab *Cancer borealis*, a GABA-evoked excitatory response occurs (Swensen *et al.*, 2000) that shares the muscimol and PTX sensitivity of the *Drosophila* receptor. Therefore, it seems possible that the GRD/LCCH3 receptor is the prototype of this excitatory type of muscimol-activated GABA receptor. The nematode EXP-1 channel (Beg & Jorgensen, 2003) differs from the *Drosophila* receptor in several properties: for example, the EXP-1 receptor is homomultimeric and not antagonized by PTX, whereas the heteromultimeric *Drosophila* receptor is highly sensitive to PTX.

Recently, it has been shown that LGICs with a complex subunit composition exist in *Drosophila*, such that glutamate and GABA receptors are both found in the same heteromultimeric ion channel (Ludmerer *et al.*, 2002). This fits with the finding that the LCCH3 subunit can co-assemble with both RDL and GRD subunits. The ß-subunit LCCH3 alters the pharmacological properties of the RDL receptor when they are coexpressed. In RDL/LCCH3 heteromultimers, PTX sensitivity is drastically reduced, by nearly three orders of magnitude, from 1 to 500 μM, compared to RDL homo-oligomeres (Zhang *et al.*, 1995). In contrast, heteromultimeric GRD/LCCH3 channels are approximately as sensitive to PTX as are RDL receptors, so that the possibility that LCCH3 alone is sufficient to obstruct PTX binding can be excluded. The fact that γ-HCH does not block the GRD/LCCH3 receptor is surprising because PTX and γ-HCH are thought to bind to the same site in insecticide-resistant insect strains; a mutation in the RDL-GABA receptor influences sensitivity to the antagonists PTX and γ-HCH in the same manner. γ-HCH and PTX also have similar blocking properties at GABA receptors in other invertebrates (Zufall *et al.*, 1989). In contrast, GRD/LCCH3 receptors are highly sensitive to block by PTX but not by γ-HCH. This difference may be caused by the altered pore structure.

The identification of heteromultimeric GABA receptors with cation selectivity broadens our view of GABA receptors in insects. It can be speculated that heteromultimeric receptors composed of proteins with homology to GRD and LCCH3 form the molecular basis of the excitatory action of GABA in several invertebrates. Functional expression studies will enable us to characterize the pharmacological properties of this new class of GABA receptor and their potential use as targets for new kinds of GABA-receptor insecticides.

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