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Expression of a *Drosophila* GABA receptor in a baculovirus insect cell system

Functional expression of insecticide susceptible and resistant GABA receptors from the cyclodiene resistance gene *Rdl*

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Recombinant baculoviruses containing two alternative splice forms of the *Drosophila Rdl* GABA receptor gene were constructed. *Spodoptera frugiperda* (Sf21) cells infected with either splice form expressed a transcript of expected size (2.5 kb). Western blotting of cell membrane extracts and immunoprecipitation experiments with an anti-*Rdl* antiserum recognized a protein of the expected size of ~65 kDa. Whole cell patch clamp analysis of cells infected with either splice form revealed functional expression of GABA gated chloride ion channels which were blocked by application of 1 μM picrotoxinin. Following replacement of alanine 302 with a serine, a mutation associated with resistance to picrotoxinin and cyclodiene insecticides, mutant channels showed similar levels of insensitivity to picrotoxinin (~100-fold) as those observed in recordings from cultured *Drosophila* neurons. The significance of the expression of an insect GABA receptor in an insect cell line and the similarity of the results from these functional expression studies to recordings from cultured neurons is discussed.

GABA receptor; *Rdl*; Baculovirus; Protein expression; Cyclodiene; Insecticide resistance

1. INTRODUCTION

γ-aminobutyric acid is the major inhibitory neurotransmitter in both vertebrates and invertebrates [1–3]. Vertebrate $GABA_A$ receptors are complex hetero-oligomers that assemble to form GABA gated chloride ion channels [4]. A number of types of $GABA_A$ receptor subunits have been described (α, β, γ, δ and ρ) and multiple isoforms exist for each type (α1–6, β1–4 and γ1–3) [4]. This large potential for receptor diversity is further increased by alternative splicing of small 12- and 24-bp inserts in the presumed intracellular domains of the β4 [5] and γ2 subunits [6]. Although the precise subunit composition of native $GABA_A$ receptors is uncertain, both α and β subunits are required for significant functional expression in heterologous systems [7,8]. In contrast, we have recently identified a novel GABA receptor subunit, isolated from a cyclodiene insecticide-resistant *Drosophila* mutant *Resistance to dieldrin (Rdl)* [9], that is capable of forming highly functional GABA gated chloride ion channels from a single subunit polypeptide [10]. This subunit shows more extensive alternative splicing than vertebrate $GABA_A$ receptors [11]. Further, *Rdl* has equally low sequence identity to any $GABA_A$ receptor type and somewhat higher identity to glycine receptors [12]. *Rdl* therefore appears to belong to a novel class of GABA receptors.

Insensitivity to picrotoxinin and cyclodiene insecticides is conferred by a single amino acid replacement ($Ala^{302} \rightarrow Ser$) in the second membrane spanning domain of *Rdl*, the region thought to line the chloride ion channel pore [10,13]. In this study we describe the functional expression of both cyclodiene susceptible and resistant *Rdl* homomultimers of two alternative splice forms in a baculovirus insect cell system. The pharmacology of these homomultimeric receptors in a heterologous system is comparable to that observed in native receptors examined in cultured neurons. Following expression, resistant recombinant receptors showed similar levels of insensitivity to picrotoxinin as those in cultured neurons, an observation consistent with *Rdl* receptors being composed of single subunit polypeptides in vivo.

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2. MATERIALS AND METHODS

2.1. *Cell culture and virus infection*

Spodoptera frugiperda (Sf21) cells were subcultured as monolayers

at 27°C in TC100 growth medium (Gibco Laboratories) supplemented with tryptose broth (2.6 mg/ml), amphotericin B (0.6 μg/ml), penicillin-G (100 units/ml), streptomycin (207 units/ml), $NaHCO_3$ (0.35 mg/ml) and 10% fetal bovine serum. For infection, monolayers were inoculated with the appropriate virus at an indicated multiplicity of infection (MOI). For the mock-infected control, tissue-culture medium alone was added. The residual inoculum was replaced with tissue-culture medium after a one-hour adsorption period, and infected cells incubated at 27°C.

2.2. *Construction of recombinant transplacement vector and recombinant baculovirus*

Three transplacement vectors containing the susceptible *Rdl* alternative splice forms cDNAs 4.1 and 14.1 [11] and the cyclodiene-resistant mutant *Rdl* cDNA 14.1R ($Ala^{302} \rightarrow Ser$) were constructed. The *Dra*I–*Sac*II fragment of cDNA 4.1 and the *Xho*I–*Sac*II fragment of cDNA 14.1 in pBluescript KS+ (Stratagene) were inserted into the unique *Eco*RV/*Sac*II sites and *Xho*I/*Sac*II sites of the pEV/35K/polybsmcr transfer vector, respectively, downstream of the polyhedrin (*ph*) promoter (Fig. 1). The single base pair substitution (GCG-alanine → TCG-serine) conferring resistance to cyclodienes was introduced into susceptible cDNA 14.1 as previously described [10]. The parent viral DNA, vΔ35K/*lac*Z, was obtained from Dr. Friesen, University of Wisconsin. vΔ35K/*lac*Z is a deletion mutant AcNPV DNA which lacks the native 35K protein gene and contains the *lac*Z gene in place of the structural portion of the *ph* gene. This viral DNA was linearized by digestion of *Bsu*36I site within the *Lac*Z gene. To generate recombinant baculoviruses, 200 ng of linearized parent viral DNA and 2 μg of each transplacement vector DNA were co-transfected into Sf21 cells by using Lipofectin (Gibco BRL).

2.3. *Northern analysis*

Sf21 cells were infected with wild-type or recombinant baculoviruses at a MOI of 10. At 48 h after infection, cells from two dishes were scraped and collected into conical tubes, and centrifuged at 1,000 × *g* for 4 min. Cell pellets were washed three times with 10 ml ice-cold PBS (pH 6.2) and lysed in 0.8 ml lysis buffer containing 0.14 M NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.0), 0.5% Nonidet P40 and 10 mM vanadyl ribonucleoside complex on ice for 5 min, and centrifuged at 2,000 × *g* for 5 min. The supernatant was mixed with an equal volume of 0.2 M Tris-HCl (pH 7.5), 25 mM EDTA, 1.3 M NaCl, 2% sodium dodecyl sulfate (SDS). Proteinase K was added to a final concentration of 0.2 mg/ml, and the sample incubated at 37°C for 30 min. Cytoplasmic RNA was extracted four times with phenol/$CHCl_3$/isoamylalcohol, and precipitated with 2.5 volumes of 100% ethanol. Samples of cytoplasmic RNA were applied to denaturing (formaldehyde)-agarose (1%) gels, separated by electrophoresis and transferred to nylon membranes (Zetabind, CUNO). RNA was cross-linked to the nylon membrane by UV irradiation. Blots were prehybridized for 3 h at 42°C in buffer containing 5 × SSPE (1 × SSPE = 150 mM NaCl, 10 mM NaH_2PO_4, 1 mM EDTA), 5 × Denhardt's, 50% formamide, 0.5% SDS, 10% dextran sulfate and 200 μg/ml of denatured salmon sperm DNA. Hybridization was carried out at 42°C in the same buffer with addition of ^{32}P-labeled probe (1.8 kb *Dra*I/*Sac*II fragment of *Rdl* cDNA 4.1 in pBluescript). Filters were washed and exposed to an X-ray film with an intensifying screen at −80°C.

2.4. *Production of* Rdl *antibodies*

An *Eco*RI/*Sca*I fragment of intracellular domain between the third and the fourth transmembrane regions of *Rdl* was subcloned into *Eco*RI/blunt end (*Hin*dIII and Klenow digestion) sites on the bacterial expression vector pMAL-c2. The fusion protein (378 aa of maltose binding protein and 202 aa of *Rdl*) was expressed in *E. coli* strain DH5α using isopropylthiogalactoside (IPTG) to induce transcription. Purification of the fusion protein was carried out by application of bacterial culture samples to an amylose resin column (New England Biolabs). The column was washed with column buffer (200 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 10 mM β-mercaptoethanol, 0.1 mM PMSF) and eluted with column buffer plus 10 mM maltose in 1-ml fractions. The fraction containing fusion protein was used as immunogen for raising polyclonal antisera. Two New Zealand White rabbits were immunized at the UW antibody facility. Four injections were made, the first intradermally and the following three subcutaneously. Rabbits were bled 6 weeks after each injection. The antiserum was partially purified by ammonium sulfate precipitation, followed by Affi-gel 10 (Bio-Rad) purification, and used in immunoprecipitation and Western blotting.

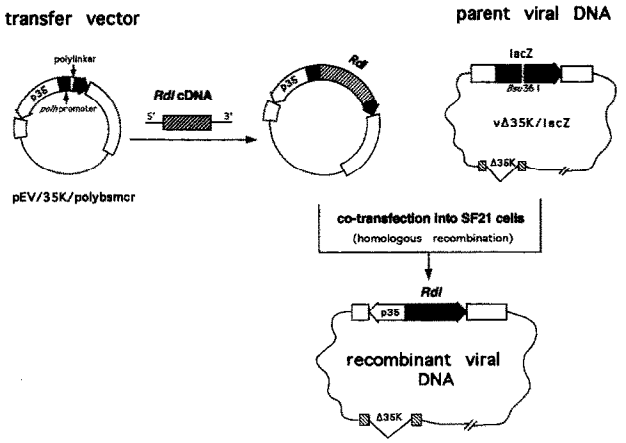

Fig. 1. Schematic representation of the construction of recombinant baculovirus vector (see text for description).

2.5. *Membrane preparation, SDS-PAGE and Western blotting*

Mock and virus-infected Sf21 cells were collected, washed twice and homogenized in a buffer containing 20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml pepstatin A, 10 μg/ml leupeptin, 20 μg/ml trypsin inhibitor. The homogenate was centrifuged at 1,000 × *g* for 5 min to remove nuclei and cell debris. Crude membranes were collected by centrifugation of the supernatant at 13,000 × *g* for 20 min. Membrane pellets were dissolved directly in SDS-PAGE sample buffer for electrophoresis. Western blots were prepared by transferring proteins from SDS-PAGE (10% acrylamide) onto nitrocellulose at 100 V for 1.5 h at 4°C in a Transphor electroblotting apparatus (Hoefer Scientific Instruments). The nitrocellulose membrane was incubated at room temperature for 1 h in a Blocking buffer containing 3% BSA and 2% goat serum in TBS (10 mM Tris-HCl, pH 7.4, 0.9% NaCl). The nitrocellulose membrane was incubated at room temperature for 1 h with the *Rdl* polyclonal antibody (1:400 dilution). Immunoreactivity was detected using goat anti-rabbit second antibody conjugated alkaline phosphatase.

2.6. *Metabolic labeling and immunoprecipitation*

Sf21 cells in 35-mm dishes were infected with wild-type or recombinant baculoviruses at a MOI of 20. At 34 and 46 h p.i., the tissue culture medium was replaced with Grace's methionine-free medium. After 1 h starvation, cells were labeled for 4.5 h with 50 μCi of Tran ^{35}S-label (a mixture of 80% methionine and 20% cysteine; ICN biomedicals) in 0.5 ml of methionine-free medium. Labeled cells were washed twice with ice-cold PBS (pH 6.2) and proteins were extracted in ice- cold lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 5 mM EDTA, protease inhibitors – PMSF, aprotinin, pepstatin, leupeptin, bacitracin). Protein samples were pre-cleared to remove proteins that immunoprecipitate nonspecifically, by incubating the sample with preimmune serum and removing Abs and Ab–Ag complexes with protein A. Following preclearing, specific proteins were immunoprecipitated by incubation with the *Rdl* polyclonal antibody at 4°C overnight, followed by the addition of 30 μl of swollen protein A-Sepharose and incubation for 1 h. Precipitates were washed three times in lysis buffer (containing 0.1% Triton X-100), once with 20 mM Tris-HCl, pH 8.0, and analyzed

in a 10% SDS-polyacrylamide gel. Gels were impregnated with Amplify (Amersham), dried, and exposed at −80°C to X-ray film.

2.7. *Patch clamp analysis of infected cells*

Transfected cells in 35-mm plastic culture dishes were bathed in a solution consisting of 128 mM NaCl, 2 mM KCl, 4 mM $MgCl_2$, 1.8 mM $CaCl_2$, 35.5 mM sucrose, 5 mM HEPES, pH 7.1, and placed on the stage of an inverted phase contrast microscope at room temperature. Whole-cell patch clamp recordings [14] were made with Sylgard-coated patch electrodes filled with 140 mM KCl, 10 mM HEPES, 10 mM EGTA, 4 mM MgATP, pH 7.1. Current was recorded with an EPC-7 patch clamp amplifier interfaced to a personal computer running PCLAMP software (Axon Instruments, Foster City, CA). GABA dissolved in bathing solution (50 μM) was ejected with pressure pulses provided by a Picospritzer (General Valve Corp., Fairfield, NJ) from patch pipettes positioned within 5 μM of the cell under recording. Cells were clamped at various holding potentials as indicated in the figures.

3. RESULTS AND DISCUSSION

3.1. *Detection of* Rdl *message and protein in infected cells*

Two alternative splice forms of *Rdl* and an insecticide resistant mutant were expressed at high levels in the baculovirus insect cell system described here. A message of correct size (2.5 kb) was identified by Northern blotting of cell RNA (Fig. 2) and a highly expressed protein of expected size (65 kDa), alongside many other non-specific bands, was detected in Western blots using an anti-*Rdl* antibody (Fig. 3a). Immunoprecipitation of cell membrane extracts with the antibody reduced the number of non-specific proteins detected and the predominant precipitation product was at 65 kDa (Fig. 3b). The other minor bands detected, which are ~ 10–15 kDa larger, probably represent glycosylated *Rdl* proteins. However, in this study it was not possible to estimate the fraction of protein undergoing proper folding, glycosylation and insertion into the membrane. Large scale production and purification of *Rdl* protein is a prerequisite for a detailed biochemical characterization of this novel GABA receptor subunit and the baculovirus insect cell system described here should facilitate such an analysis.

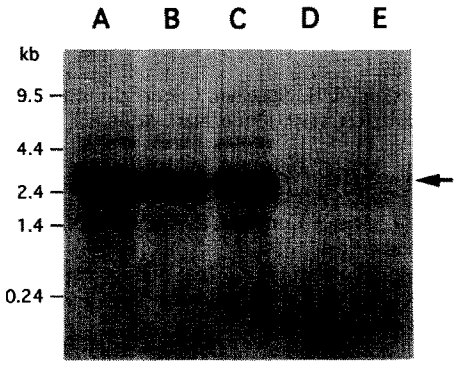

Fig. 2. Northern blot analysis of cytoplasmic RNA isolated from SF21 cells 48 h after infection with recombinant viruses containing cDNA 4.1 (lane A), cDNA 14.1 (lane B) and cDNA14.1R with $Ala^{302} \rightarrow$ Ser (lane C), and infection with mock (lane D) or wild type virus, AcMNPV (lane E). Each lane contains 5 μg of RNA. Molecular weight standards (BRL RNA Ladder) were run in parallel.

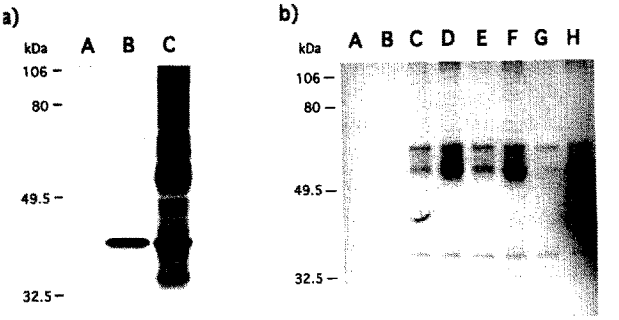

Fig. 3. Expression of *Rdl* protein in Sf21 cells. (a) Western blot analysis of *Rdl* protein in cell membranes. Sf21 cells were infected with mock (lane A), wild type virus (AcMNPV) (lane B), or recombinant baculovirus containing cDNA 14.1 (lane C). Western blots were probed with purified polyclonal antibody against *Rdl* fusion protein and developed with alkaline phosphatase-conjugated goat anti-rabbit second antibody. (b) Immunoprecipitation of *Rdl* protein from SF21 cells infected with mock (lane A), wild type virus at 48 h postinfection (lane B), recombinant virus containing cDNA 4.1 (36 and 48 h p.i.) (lanes C and D); recombinant virus containing cDNA 14.1 (36 and 48 h p.i.) (lanes E and F); recombinant virus containing cDNA 14.1R (36 and 48 h p.i.) (lanes G and H).

3.2. *Patch clamp analysis of infected cells*

GABA application to voltage clamped cells infected with recombinant *Rdl* baculovirus increased the membrane current whereas cells infected with wild type virus showed no response. Responses to brief pulses of GABA were similar in cells infected with either alternative splice form. Current increased linearly with voltage and reversed near 0 mV, as expected for the activation of chloride selective channels in symmetrical chloride solutions (Fig. 4).

Wild type responses could be blocked by 1 μM picrotoxin. Whereas a concentration of 100 μM picrotoxin was necessary to achieve an equivalent level of block in the $Ala^{302} \rightarrow$ Ser mutant (Fig. 5). The mutant channels therefore show ~100-fold resistance to picrotoxinin. This level of reduced sensitivity is similar to that seen both in neurons cultured from susceptible and resistant larvae [15] and following functional expression of mRNA in *Xenopus* oocytes [10]. The similarity of resistance levels shown by this mutant both in vivo and in vitro supports the working hypothesis that *Rdl* receptors may be composed of single subunit polypeptides in the insect nervous system. This hypothesis will be further tested by detailed comparisons of dose–response curves taken from both cultured neurons and infected cells for a number of compounds.

Identification of a functional insect GABA receptor formed from a single subunit polypeptide is important for a number of reasons. Although vertebrate $GABA_A$ receptors have been previously expressed in the baculovirus system [16,17], to our knowledge this is the first report of the expression of an *insect* GABA receptor in

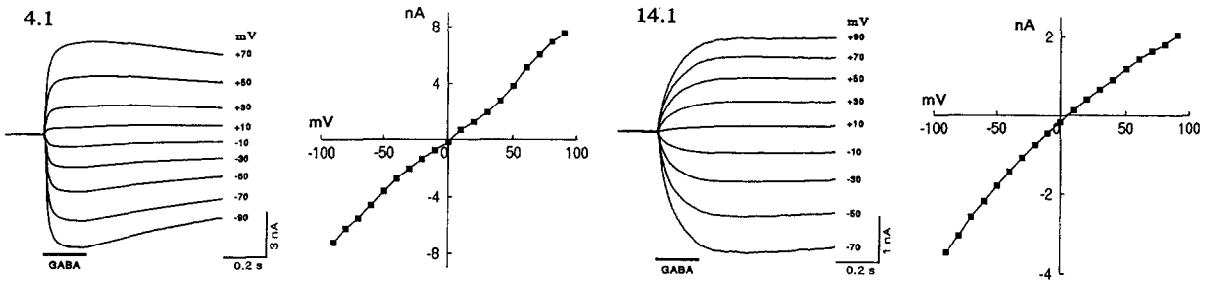

Fig. 4. GABA activated chloride currents in patch-clamped SF21 cells infected with recombinant *Rdl* baculovirus (a) alternative splice form 4.1 and (b) splice form 14.1. Potentials range from −90 mV to 90 mV. Plots of peak current vs. voltage exhibit linear behavior, reversing at or near zero.

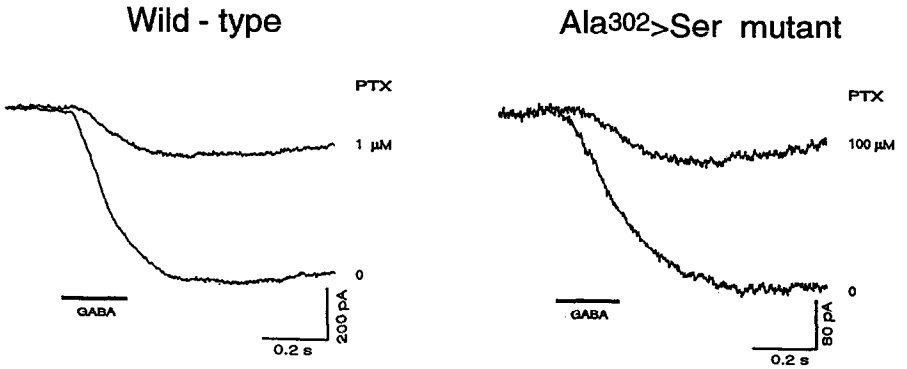

Fig. 5. Responses to GABA from cells infected with susceptible 14.1 (wildtype) and resistant 14.1 ($Ala^{302} \rightarrow$ Ser mutant) recombinant *Rdl* baculovirus differed in the level of block by picrotoxin. Wild-type responses were blocked by 1 μM picrotoxin whereas an equivalent level of block of the mutant was only achieved by 100 μM. Picrotoxin was applied by perfusion of the bathing medium.

an *insect* cell line. Expression of this single polypeptide in a suitable system, such as the insect cell line described here, will allow for detailed site-directed mutagenesis studies of a GABA receptor in the absence of other potentially interfering subunits. Further, the relative ease of deriving dose–response curves in this system in comparison *Xenopus* oocytes, will allow for detailed site directed mutagenesis of ligand binding sites and examination of their interactions with a range of derivatives. Finally, recombinant expression of *Rdl* in cell lines may allow for the design of screens for novel agonists and antagonists of insect GABA receptors which represent attractive but underutilized pesticide targets [18,19].

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