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Effects of [^{3}H]-BIDN, a novel bicyclic dinitrile radioligand for GABA-gated chloride channels of insects and vertebrates

James J. Rauh, Eric Benner, Michael E. Schnee, Daniel Cordova, Caleb W. Holyoke, Michael H. Howard, *Donglin Bai, *Steven D. Buckingham, *Michael L. Hutton, *Alain Hamon, †Richard T. Roush and *,[1]David B. Sattelle

DuPont Agricultural Products, Stine-Haskell Research Center, P.O. Box 30, Newark, DE 19714, U.S.A. †Cornell University, Department of Entomology, Comstock Hall, Ithaca, NY 14853-0999, U.S.A. and *The Babraham Institute Laboratory of Molecular Signalling, University of Cambridge, Department of Zoology, Downing Street, Cambridge CB2 3EJ

1 The radiolabelled bicyclic dinitrile, [^{3}H]-3,3-bis-trifluoromethyl-bicyclo[2.2.1]heptane-2,2-dicarbonitrile ([^{3}H]-BIDN), exhibited, specific binding of high affinity to membranes of the southern corn rootworm (*Diabrotica undecimpunctata howardi*) and other insects. A variety of γ-aminobutyric acid (GABA) receptor convulsants, including the insecticides heptachlor (IC_{50}, 35 ± 3 nM) and dieldrin (IC_{50}, 93 ± 7 nM), displaced [^{3}H]-BIDN from rootworm membranes. When tested at 100 μM, 1-(4-ethynylphenyl)-4-n-propyl-2,6,7-trioxabicyclo[2.2.2]octane(EBOB), 4-t-butyl-2,6,7-trioxa-1-phosphabicyclo[2.2.2]octane-1-thione (TBPS), 1-phenyl-4-t-butyl-2,6,7-trioxabicyclo[2.2.2]octane (TBOB) and picrotoxin failed to displace 50% of [^{3}H]-BIDN binding to rootworm membranes indicating that the bicyclic dinitrile radioligand probes a site distinct from those identified by other convulsant radioligands.

2 Dissociation studies showed that dieldrin, ketoendrin, toxaphene, heptachlor epoxide and α and β endosulphan displace bound [^{3}H]-BIDN from rootworm membranes by a competitive mechanism.

3 Rat brain membranes were also shown to possess a population of saturable, specific [^{3}H]-BIDN binding sites, though of lower affinity than in rootworm and with a different pharmacological profile. Of the insecticidal GABAergic convulsants that displaced [^{3}H]-BIDN from rootworm, cockroach (*Periplaneta americana*) and rat brain membranes, many were more effective in rootworm.

4 Functional GABA-gated chloride channels of rootworm nervous system and of cockroach nerve and muscle were blocked by BIDN, whereas cockroach neuronal $GABA_B$ receptors were unaffected.

5 Expression in *Xenopus* oocytes of either rat brain mRNA, or cDNA-derived RNA encoding a GABA receptor subunit (*Rdl*) that is expressed widely in the nervous system of *Drosophila melanogaster* resulted in functional, homo-oligomeric GABA receptors that were blocked by BIDN. Thus, BIDN probes a novel site on GABA-gated Cl^- channels to which a number of insecticidally-active molecules bind.

Keywords: Ionotropic GABA receptors; *Rdl* gene; [^{3}H]-BIDN; convulsants; dieldrin

Introduction

In mammalian brain, receptors for the neurotransmitter γ-aminobutyric acid (GABA) that gate chloride channels ($GABA_A$ receptors) are important sites of drug action (Seeburg *et al*., 1990). $GABA_A$ receptors possess, in addition to binding sites for the neurotransmitter, sites of action of benzodiazepines, barbiturates, steroids, certain anaesthetics, avermectins and convulsants (Sieghart, 1995). GABA receptors that gate chloride channels are present on insect nerve and muscle cells (Sattelle, 1990) and are targets of insecticides (Rauh *et al*., 1990). Cyclodienes, such as dieldrin, the cyclohexane lindane and the phenylpyrazole fipronil, block insect GABA-gated chloride channels (Matsumura & Ghiasuddin, 1983; Cohen & Casida, 1986; Wafford *et al*., 1989a,b; Bloomquist *et al*., 1992; Buckingham *et al*., 1994; Hosie *et al*., 1995a). Recently, a gene for dieldrin resistance (*Rdl*) has been identified in *Drosophila melanogaster* (ffrench-Constant *et al*., 1991; 1993) and shown to encode a GABA receptor subunit that can be expressed (as a functional homo-oligomer) transiently in *Xenopus laevis* oocytes (ffrench-Constant *et al*., 1993; Buckingham *et al*., 1994), fall army worm (*Spodoptera frugiperda*) Sf9 cells (Lee *et al*., 1993) and stably in a *Drosophila* cell line (Millar *et al*., 1994).

A number of pharmacological differences have been detected between the GABA-gated chloride channels of insects and vertebrates (Rauh *et al*., 1990). The potential exists for exploring such differences in the design of novel, safer pesticides. However, although some information has accumulated on GABA receptors of cockroach (*Periplaneta americana*) (Lummis & Sattelle, 1985; Sattelle *et al*., 1988), housefly (*Musca domestica*) (Abalis *et al*., 1985; Cohen & Casida, 1986) and fruit fly (*Drosophila melanogaster*) (Zhang *et al*., 1984; Rosario *et al*., 1989), to date there have been few studies on GABA receptors of insect pest species. Part of the reason for this is the lack of a suitable radioligand for these species. [^{3}H]-Dihydropicrotoxinin, the first radiolabelled probe of convulsant binding to GABA receptors, exhibits a low ratio of specific to non-specific binding (Matsumura & Ghiasuddin, 1983). This lack of specificity is confirmed by its ability to block vertebrate $GABA_A$ (Sieghart, 1995), $GABA_C$ (Feigenspan *et al*., 1993) and glycine receptors (Pribilla *et al*., 1992) as well as invertebrate GABA (Sattelle *et al*., 1988), L-glutamate (Wafford & Sattelle, 1989) and nicotinic receptors (Sattelle *et al*., 1988; Benson, 1988). [^{3}H]-α-endosulphan shows low specificity of binding and readily partitions into membranes, precluding the determination of standard saturation isotherms and binding kinetics (Cole *et al*., 1994). ([^{35}S]-TBPS) has been applied successfully to the detailed characterization of vertebrate (Casida *et al*., 1985), housefly (Deng *et al*., 1991) and cockroach (Lummis & Sattelle, 1986) GABA receptor convulsant sites, but it has yet to prove useful for major insect pest species. [^{3}H]-1-(4-ethynylphenyl)-4-n-propyl-2,6,7-trioxabicyclo-[2.2.2]octane ([^{3}H]-EBOB) offers a convenient convulsant site probe with a high ratio of specific to non-specific binding and is displaced by several insecticides used in the present study. However, its use to date has been largely confined to studies on

[1] Author for correspondence.

houseflies (Cole & Casida, 1992) and *Drosophila* (Cole *et al*., 1995). Here, we describe the actions of a novel radioligand [^{3}H]-BIDN (3,3-bis-trifluoromethyl-bicyclo[2.2.1]heptane-2,2-dicarbonitrile), a member of a bicyclic dinitrile class of compounds with high insect toxicity (Middleton, 1974; Sattelle *et al*., 1995; Buckingham *et al*., 1995; Hosie *et al*., 1995b). BIDN was found to block GABA-gated chloride channels of invertebrates and vertebrates and [^{3}H]-BIDN binding could be detected in membranes from southern corn rootworm (SCRW), fall army worm, cockroach, boll weevil, Colorado potato beetle and rat brain, thus making it an effective probe of broad utility for GABA receptors.

Methods

Experimental animals

Adult male cockroaches (*Periplaneta americana*) reared at 27°C, ambient humidity, with free access to artificial diet and water, were used throughout this study. Adult southern corn rootworms (*Diabrotica undecimpunctata howardi*) were maintained at 24°C, 75% humidity, with free access to artificial diet supplemented with lettuce leaves. Eggs were collected, and stored for 4–5 days until neonates hatched. The newly hatched larvae were transferred to corn roots, and maintained at 24°C, 75% humidity for 7 days at which point (late first or early second instar) they were either used for fresh tissue preparations, or frozen in liquid N_2 and stored at −80°C for subsequent investigation.

Membrane preparations

Southern corn rootworm (SCRW) and fall army worm membranes Fresh or thawed rootworm larvae were transferred to a Polytron homogenizer containing 9 parts ice-cold Van Harreveld's buffer (mM: NaCl 205, $CaCl_2$ 13.6, KCl 5.4, $MgCl_2$ 2.6, TrisCl 5, pH 7.7, with 1 mM dithiothreitol added fresh). All subsequent procedures were carried out at 4°C. Tissue was homogenized at setting 3 for 2 min and the homogenate centrifuged at 500× *g* for 10 min. The supernatant was filtered through glass wool and centrifuged at 28,000× *g* for 35 min. The pellet (P_2) was suspended in 50 mM TrisCl, 50 mM sodium citrate (pH 7.1) by use of a glass-Teflon homogenizer. The final protein concentration was adjusted to 10 mg ml^{-1} (Lowry *et al*., 1951). The same procedure was used for preparing membranes from larvae of the fall armyworm (*Spodoptera frugiperda*).

Rat brain membranes Frozen rat brains obtained from Pelfreez (Rogers, AR) were thawed and homogenized (with a Polytron) in nine volumes of ice-cold buffer A (50 mM TrisCl, 320 mM sucrose, 1 mM EDTA, pH 7.7, to which 0.1 mM phenanthroline, 0.1 mM PMSF and 0.1 μg ml^{-1} pepstatin A were added immediately before use). All subsequent procedures were carried out at 4°C. The homogenate was further homogenized in a glass-glass homogenizer (10 strokes) and the crude homogenate was centrifuged at 2,250× *g* for 15 min. The supernatant was collected and the resulting pellet resuspended in 3 volumes ice-cold buffer A and subjected to another cycle of homogenization and centrifugation. The resulting supernatants were combined and centrifuged at 22,000× *g* for 30 min. This P_2 pellet was resuspended in 50 mM TrisCl, pH 7.4, and centrifuged again at 22,000×*g* for 30 min. The pellet was resuspended in 50 mM TrisCl, pH 7.4, frozen in a dry ice/ethanol bath for 30 min, thawed at 30°C for 30 min and recentrifuged. The freeze-thaw step was repeated once and the membranes were resuspended to a final protein concentration of 6 mg ml^{-1} (Lowry *et al*., 1951) in 50 mM TrisCl, pH 7.4.

Radioligand binding studies

Solutions of radioligands were prepared in assay buffer containing 5% (v/v) dimethylformamide. All assays were quenched with appropriate assay buffer at 4°C and filtered on GF/C filter sheets with a Brandel Cell Harvester (Cambridge, MA). Filters were rinsed twice with 5 ml 4°C assay buffer, transferred to plastic scintillation vials containing 4 ml Beckman Ready-Safe scintillation fluid (Fullerton, CA) for counting. Triplicate determinations were made for each data point.

Analyses of equilibrium binding and kinetic rate data were carried out by use of the following Biosoft (Cambridge, UK) software: KINETIC, EBDA, LIGAND LOWRY: A Collection of Radioligand Binding Analysis Programs by G.A. McPherson.

[^{3}H]-BIDN binding to insect membranes For saturation binding studies, the assay mixture (1 ml total volume in glass) contained 50 mM TrisCl, 50 mM sodium citrate, pH 7.1 and various concentrations of [^{3}H]-BIDN. BIDN (50 mM) was added for estimation of non-specific binding. The assay was initiated by adding a 1 mg aliquot of membrane protein and was incubated for 45 min at 23°C. For saturation binding studies carried out in the presence of a competing ligand, the ligand was present at a concentration equivalent to its K_i for displacement of [^{3}H]-BIDN from the membranes.

Displacement of [^{3}H]-BIDN by selected compounds was determined by pre-incubation of assay mixture, test compound and 1 mg of membrane protein for 10 min at 23°C. The assay was initiated by addition of [^{3}H]-BIDN (20 nM final concentration) and the mixture was incubated at 23°C for 45 min.

For association rate studies, the final concentration of [^{3}H]-BIDN was 20 nM and unlabelled BIDN was 10 μM. The association assay was initiated by the addition of 1 mg protein and incubation was at 23°C with samples quenched and filtered at intervals of up to 60 min from the start of the assay.

For dissociation rate studies, the final concentrations of [^{3}H]-BIDN and BIDN were as used in the association experiments. Binding was initiated by the addition of 1 mg membrane protein, and continued for 30 min at 23°C. Dissociation was initiated by addition of BIDN (10 μM final concentration). Quenching and filtration took place at intervals during a 60 min period following the start of dissociation. Two sets of assays were employed to determine if a compound displaced [^{3}H]-BIDN by a competitive mechanism. The first set was as described above and served as the control. The second set had 10 μM BIDN, plus the compound at 50× K_i value for displacement of [^{3}H]-BIDN, added to initiate dissociation.

[^{3}H]-BIDN binding to rat brain membranes For saturation binding studies, the assay mixture (1 ml total volume in glass) contained 50 mM TrisCl, 500 mM NaCl, pH 7.4 and various concentrations of [^{3}H]-BIDN. BIDN 50 μM was added for estimation of non-specific binding. The assay was initiated by adding 300 μg of membrane protein and was incubated for 45 min at 37°C.

Displacement of [^{3}H]-BIDN by selected compounds was determined by pre-incubation of assay mixture, test compound and 300 μg membrane protein for 10 min at 23°C. The assay was initiated by addition of [^{3}H]-BIDN (100 nM final concentration) and was incubated for 45 min at 37°C.

Electrophysiology

Identified cockroach motor neurone The cell body of the cockroach fast coxal depressor motor neurone (D_f) was located visually in an isolated, desheathed metathoracic ganglion and impaled with a glass microelectrode filled with 2 M KCl (resistance (R) = 10–20 MΩ). Details of the experimental chamber (total volume 0.5 ml) and the apparatus for membrane potential measurements were as described previously (David & Sattelle, 1984). Unless otherwise indicated the preparation was

bathed in physiological solutions (PS) of the following composition: (mM) NaCl 214, $CaCl_2$ 9, KCl 3.1, sucrose 50 and N-Tris-[hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES) 10, pH adjusted to 7.2 with 1 M NaOH. GABA was applied by syringe-pump injection into the entry port of the experimental chamber as described previously (Bai *et al*., 1991). BIDN in dimethyl sulphoxide was added to the perfusion medium to a final solvent concentration of not more than 0.1%.

Cockroach muscle Coxal muscles 182c and 182d together with attached cuticle were isolated from the metathoracic leg of the cockroach *Periplaneta americana* and mounted in a Perspex chamber (0.5 ml volume) under continuously perfused PS of the following composition (mM): NaCl 180, KCl 3.1, $CaCl_2$ 9, $MgCl_2$ 20, D-glucose 10 and HEPES 10, pH 7.2 adjusted with 1 M NaOH. Intracellular recordings were obtained with two glass microelectrodes filled with 2 M KCl ($R = 18-25$ MΩ). One of the electrodes was used to pass hyperpolarizing current pulses (1–4 nA, 150 ms duration, 0.33 Hz), and subsequent changes in muscle membrane potential and conductance were recorded with an Axon Instruments Axoclamp DC amplifier (Foster City, CA) and displayed on a Gould BS-2772 chart recorder (Cleveland, OH).

SCRW neurones The brain and nerve cord of 3rd instar larval SCRW were isolated, transferred to a Perspex experimental chamber and perfused continuously with PS of the following composition (mM): NaCl 214, KCl 3.1, $CaCl_2$ 9 and TES 10, pH 7.2 adjusted with 1 N NaOH. The isolated preparation was treated with collagenase (Sigma Type X, 10 mg ml^{-1}) and dispase (Sigma Type Y, 2 mg ml^{-1}) in PS for 5 min, after which the softened nerve sheath was removed with finely sharpened needles. Cells on the ventral surface of a thoracic ganglion were impaled with a glass microelectrode filled with 1 M potassium methyl sulphonate ($R = 100-150$ MΩ). GABA was applied from a GABA-filled glass microelectrode located near the impaled neurone by use of a pressure pulse (40 psi) of 20–100 ms duration.

Messenger RNA preparation

Rat brain mRNA Total RNA was extracted from freshly dissected adult rat brains following the procedure of Chomcxyhski and Sacchi (1987). Poly (A^+) mRNA was prepared from total RNA by chromatography on oligo (dT)-cellulose (Sambrook *et al*., 1989) and stored at −70°C).

cDNA-derived mRNA encoding Rdl The *Drosophila Rdl* cDNA was subcloned as an *Eco*RI fragment into the *Eco*RI-site of pBluescript KS+ (Stratagene) in an orientation such that, after linearizing the template DNA with *Hind*III sense RNA *in vitro* transcription with T7 RNA polymerase was produced (Melton *et al*., 1984). Transcripts were capped with m^7 G (5′) G (White, 1985). The synthetic RNA gave a single band of ~2 kb when electrophoresed in a 1.5% (w/v) agarose/formaldehyde gel (Sambrook *et al*., 1989). With the aim of increasing the expression of the *Drosophila* RNA it was polyadenylated (Drummond *et al*, 1985) before injection into oocytes.

Functional expression of rat brain GABA$_A$ receptors in Xenopus *oocytes*

Stage V and VI oocytes of *Xenopus laevis* were defolliculated manually in standard oocyte solution (SOS) of the following composition (mM): NaCl 100, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 1 and HEPES 5, pH 7.6 adjusted with 1 M NaOH. After a minimum recovery period of 3 h, each oocyte was injected with 50 nl mRNA (1 ng nl^{-1}) with a Drummond 500 microdispenser (control oocytes with either uninjected, or injected with 50 nl distilled water). The cells were then transferred into SOS supplemented with 2.5 mM sodium pyruvate and 200 μg ml^{-1} gentamycin sulphate. Before the electrophysiological experiments, oocytes were incubated for at least two days at 18°C to permit translation of the mRNA and incorporation of receptor proteins into the plasma membrane.

For electrophysiological recordings, a single oocyte was retained by fine pins in a 0.5 ml chamber and voltage-clamped at −80 mV with two glass microelectrodes ($R = 2-5$ MΩ) filled with 2 M KCl. The currents induced by GABA were monitored by a Warner Instruments OC725 voltage-clamp amplifier (Hamden, CT) and displayed on a Gould BS-2772 chart recorder (Cleveland, OH). The experimental chamber was perfused continuously at a rate of 2 ml min^{-1} with SOS. The perfusion saline could be switched to one supplemented with 100 μM GABA and/or BIDN at various concentrations.

Chemicals

t-Butylbicyclophosphorothionate (TBPS) was obtained from Research Biochemicals Inc. (Natick, MA). Bicuculline, GABA, picrotin and picrotoxinin were obtained from Sigma Chemical Co. (St. Louis MO). All insecticides were obtained from ChemServ (W. Chester, PA). 3,3-Bis-trifluoromethyl-bicyclo[2.2.1] heptane-2,2-dicarbonitrile (BIDN) was synthesized as previously described (Middleton, 1974).

Radiochemicals

[^{3}H]-BIDN (52.5–54 Ci mmol^{-1}) was custom synthesized by DuPont-NEN (Boston, MA)/ [^{35}S]-TBPS (67.3–139.5 Ci mmol^{-1}) was obtained from DuPont-NEN (Boston, MA). Structures of [^{3}H]-BIDN and other GABA receptor convulsant site ligands used to date in insect GABA receptor studies are shown in Figure 1.

Results

In Figure 1a, the synthetic reaction for incorporation of tritium into BIDN (3,3-bis-trifluoromethyl-bicyclo[2.2.1]heptane-2,2-dicarbonitril) is outlined, and the chemical structure of this novel radioligand is shown. Figure 1b shows the structures of other radioligands previously used for characterization of the convulsant insecticide binding site on GABA receptors of insects.

[^{3}H]-BIDN binding to southern corn rootworm (SCRW) membranes

Membranes prepared from larvae of the southern corn rootworm (*Diabrotica undecimpunctata howardi*), a major pest species, were used to study the binding of [^{3}H]-BIDN. A saturable, specific component of binding was detected (Figure 2a) with 95% of the total binding being specific, when 50 μM unlabelled BIDN was used to estimate non-specific binding. A similarly high ratio of specific to non-specific binding was observed when ketoendrin or dieldrin was substituted for BIDN. Scatchard analysis of this data indicated binding to a single class of sites (Figure 2b). Analysis of all saturation experiments yielded the following binding parameters: $K_d = 26 \pm 6$ nM and $B_{max} = 1{,}748 \pm 250$ fmol mg^{-1} protein (mean ± s.e.mean, $n = 8$). A Hill coefficient of 1.01 was obtained (Figure 2c) indicating non-cooperative binding to a single class of receptor sites.

Examination of the rates of association and dissociation of [^{3}H]-BIDN binding (Figure 3) assumed the simple kinetic model,

$$[L] + [R] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [LR]$$

(where L = ligand and R = receptor). Analysis of these kinetic studies (see Methods) yielded a calculated dissociation constant ($K_d = k_{-1}/k_{+1}$) of 19.1 nM, similar to the value of 26 nM obtained from equilibrium studies.

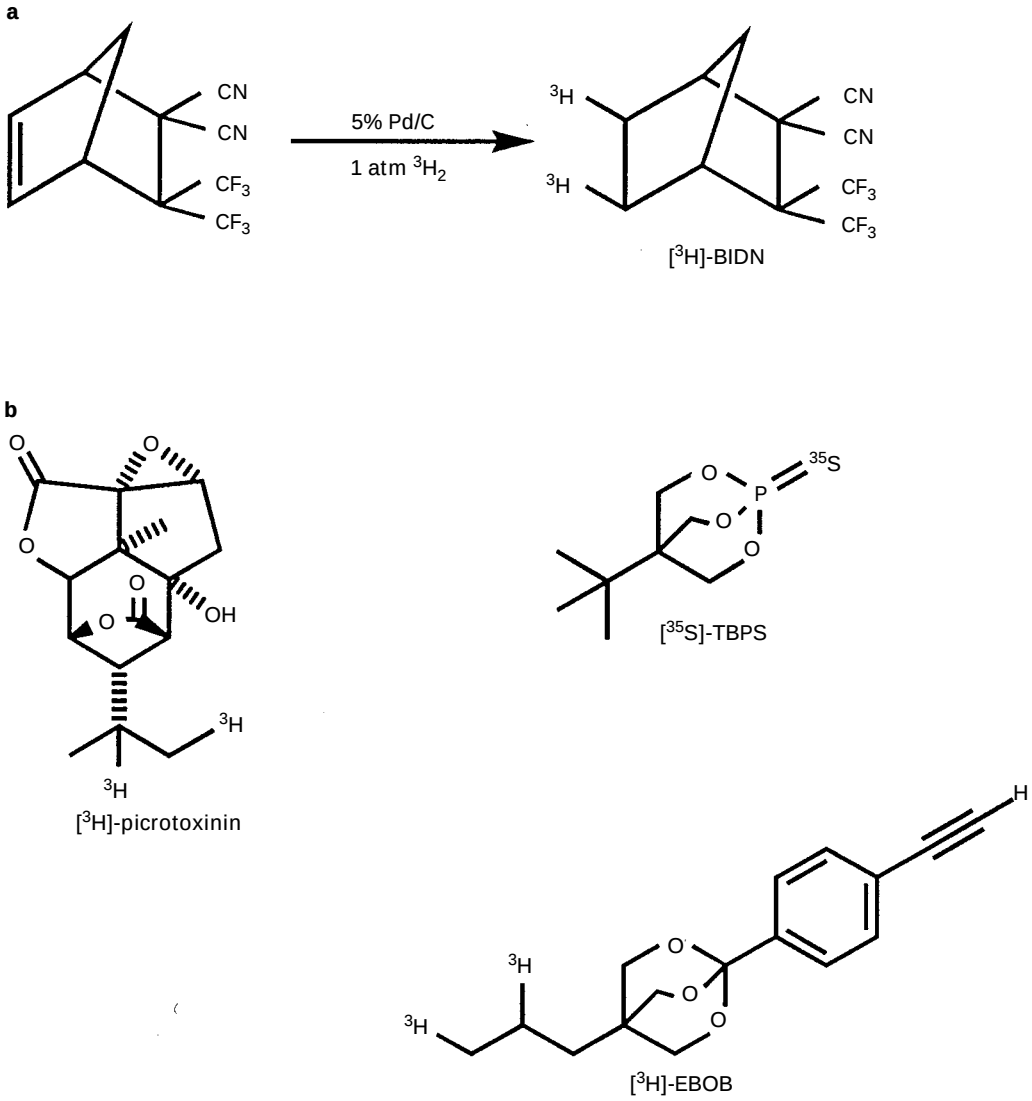

Figure 1 Chemical structure of [^{3}H]-BIDN and other radiolabelled, GABA convulsants. (a) [^{3}H]-BIDN was prepared by incorporation of $^3H_2$. (b) Radiolabelled GABA convulsants previously employed in GABA receptor characterization.

[^{3}H]-BIDN binding to membranes of other insects

Similar studies were performed on other insects. [^{3}H]-BIDN binding to fall armyworm (*Spodoptera frugiperda*) larval membranes yielded the following binding parameters: $K_d = 61 \pm 9$ nM and $B_{max} = 873 \pm 90$ fmol mg^{-1} (mean ± s.e.-mean, $n = 6$). Other insect tissues exhibiting high levels of specific [^{3}H]-BIDN binding include: Colorado potato beetle (*Leptinotarsa decemlineata*) larvae, boll weevil (*Anthonomus grandis*) adults, American cockroach (*Periplaneta americana*) nerve cord and leg muscle, and housefly (*Musca domestica*) head and thorax (Table 1).

Displacement of [^{3}H]-BIDN from SCRW membranes

A variety of GABAergic convulsants displaced [^{3}H]-BIDN from SCRW membranes completely (Figure 4) and with high affinity (Table 2). The most effective compounds, β-endosulphan and heptachlor epoxide, were more effective than BIDN. Of the stereoisomers of endosulphan tested, β was more effective than α. The cage convulsants (EBOB, TBPS, TBOB) and picrotoxinin were inactive at concentrations up to 100 μM. Also inactive were GABA and the potent anthelmintic avermectin B_{1a}.

Competitive/non-competitive displacers of [^{3}H]-BIDN from SCRW membranes

Displacement by dieldrin of [^{3}H]-BIDN binding to SCRW membranes was by a competitive mechanism. This was determined by the inability of dieldrin to alter significantly the B_{max} for [^{3}H]-BIDN binding in saturation binding studies (Figure 5a), and by its failure to increase significantly the dissociation rate for [^{3}H]-BIDN in kinetic studies (Figure 5b). Similar results were obtained for 12-ketoendrin, toxaphene, heptachlor epoxide and the α and β isomers of endosulphan (data not shown).

[^{3}H]-BIDN binding to rat brain membranes

Rat brain membranes exhibited specific [^{3}H]-BIDN binding, but of lower affinity ($K_d = 200 - 300$ nM, $n = 4$) than that detected in insect tissues. Saturable binding was not observed, as solubility problems were encountered at [^{3}H]-BIDN concentrations above 750 nM in these assays. The percentage specific binding observed in rat brain membranes was lower (75% specific binding) than observed for insect membranes. Displacement studies revealed that the GABAergic convulsants effective in displacing [^{3}H]-BIDN from SCRW membranes

were also effective in rat brain membranes (Table 2). In contrast to the findings for SCRW membranes, the cage convulsants and picrotoxin were able to displace [^{3}H]-BIDN from rat brain membranes.

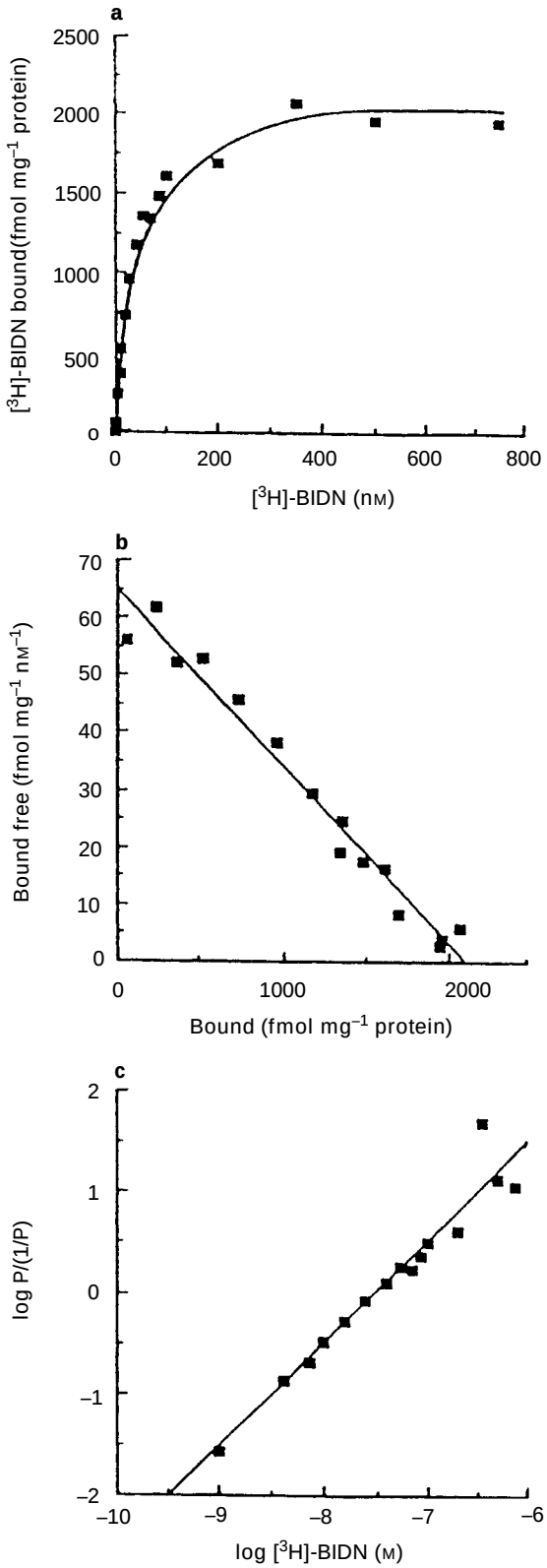

Figure 2 Saturable, specific binding of [^{3}H]-BIDN to membranes from Southern corn rootworm (SCRW). (a) Saturable, specific binding of [^{3}H]-BIDN and SCRW membranes prepared as described in Methods. Each data point represents the mean of triplicate determinations with a s.e. of less than 5%. (b) Scatchard transformation of the saturation binding data. (c) Hill plot of the saturation binding data. Data were analysed by use of the software described in Methods.

BIDN actions on GABA receptors of an identified cockroach motor neurone

GABA, bath applied at a final concentration of 1 mM, evoked depolarizing responses in the cell body membrane of motor neurone D_f of the cockroach (Figure 6a). Responses were in this direction because the recording electrode was filled with KCl to drive the predicted reversal potential for chloride ions away from the resting membrane potential, thereby enhancing the amplitude of the GABA response at resting potential and facilitating pharmacological studies. The response to GABA was reversibly reduced by a 6 min bath-perfusion of 10 μM

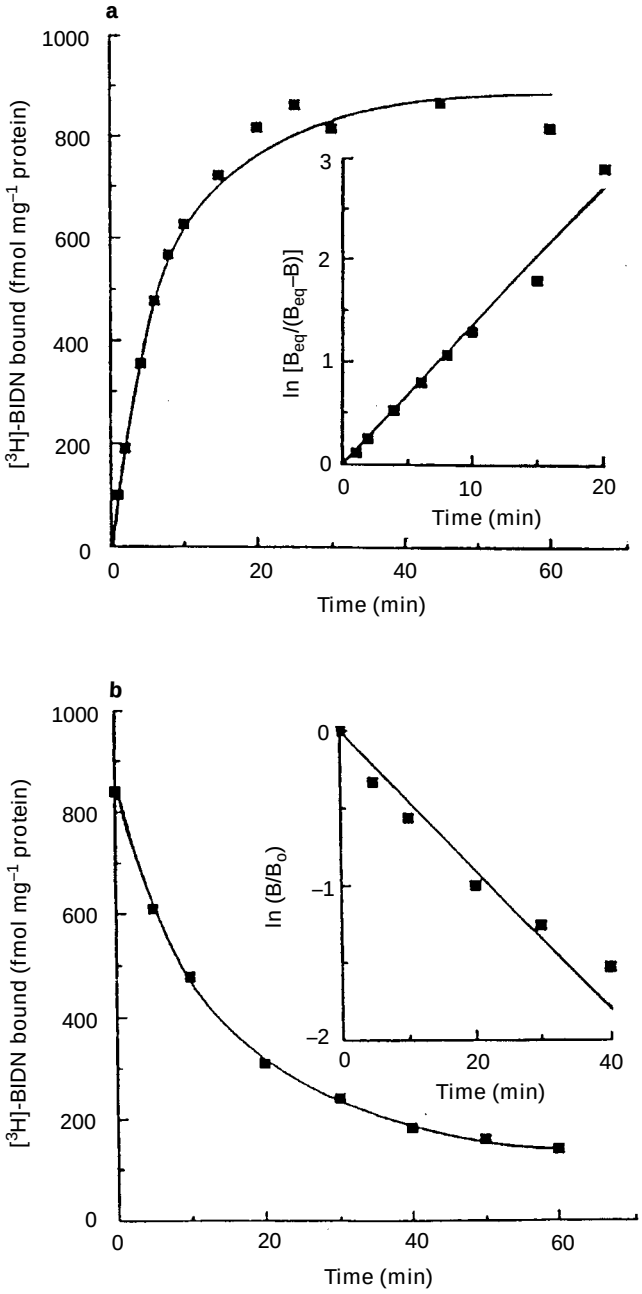

Figure 3 Kinetic analysis of [^{3}H]-BIDN binding to Southern corn rootworm (SCRW) membranes. The time-course [^{3}H]-BIDN association with (a) and dissociation from (b) SCRW membranes is shown. The rate constants were determined after transformation of the binding data (insets) as described in Methods. Each data point represents the mean of triplicate determinations with a s.e. of less than 5%.

Table 1 [^{3}H]-BIDN binding to insect* and rat brain membranes

Tissue source	K_d (*nM*)	B_{max} (*fmol mg*$^{-1}$)	n
Southern corn rootworm (larva)	26±6	1,748±250	8
Fall army worm (larva)	61±9	873±90	6
Rat brain membranes	200–300	>2,000	4

*Other insect tissues with high levels of specific [^{3}H]-BIDN binding include: Colorado potato beetle (larva); Boll weevil (adults); American cockroach (leg muscle); American cockroach (nerve cord); Housefly (head); Housefly (thorax).

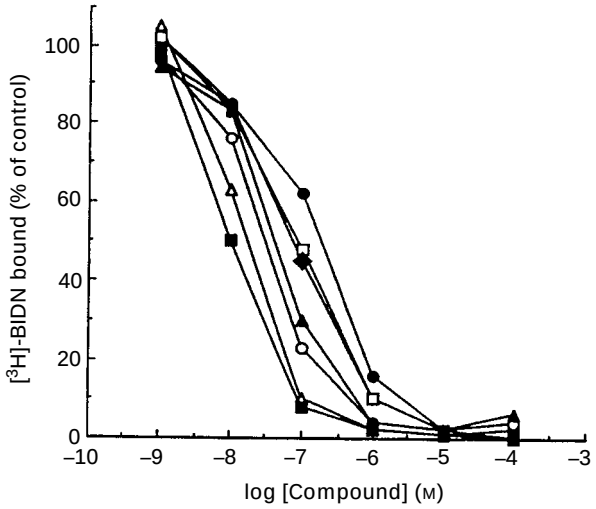

Figure 4 Displacement of [^{3}H]-BIDN from Southern corn rootworm (SCRW) membranes by GABA convulsants. Displacement of bound [^{3}H]-BIDN from SCRW membranes was observed for each of the indicated GABA convulsants. (△, heptachlor epoxide; ◆, α-endosulphan; ■, β-endosulphan; ○, heptachlor, □, dieldrin; ▲, 12-ketoendrin; ●, lindane). Each data point represents the mean of triplicate determinations with a s.e. of less than 5%.

Table 2 Displacement of [^{3}H]-BIDN from southern corn rootworm (SCRW) and rat brain membranes by GABAergic agents

Compound	*Southern corn rootworm* IC_{50} (nM)	*Rat brain* IC_{50} (nM)
β-Endosulphan	9± 1 (3)	225± 65 (4)
Heptachlor epoxide	18± 1 (3)	89± 18 (5)
BIDN	23± 2 (3)	262± 22 (5)
Heptachlor	35± 3 (3)	723±123 (5)
12-Ketoendrin	43± 2 (3)	20± 8 (6)
Dieldrin	93± 7 (3)	35± 13 (4)
α-Endosulphan	93±10 (3)	26± 7 (5)
Toxaphene	186± 4 (3)	32± 9 (4)
Lindane	233± 9 (3)	322± 96 (7)
EBOB	Inactive (3)	32± 5 (4)
TBPS	Inactive (3)	255± 99 (7)
TBOB	Inactive (3)	262± 85 (7)
Picrotoxinin	Inactive (3)	732±136 (7)

Displacement of [^{3}H]-BIDN from SCRW and rat brain membranes by the indicated compounds was determined as described in Methods. IC_{50} values represent the mean±s.e. for the number of independent determinations shown in parentheses. Inactive compounds failed to displace 50% of [^{3}H]-BIDN at a concentration of 100 μM.

BIDN (Figure 6a). The block of BIDN was found to be dose-dependent (Figure 6b) with an estimated IC_{50} of 0.9 μM. Block of greater than 80% of the GABA-induced membrane potential was observed at a BIDN concentration of 10 μM. Higher doses of the drug were not tested. BIDN also blocked smaller amplitude, hyperpolarizing GABA responses with acetate-filled electrodes (data not shown). Responses to 1 mM 3-aminopropylphosphorous acid (3-APPA), a selective mammalian $GABA_B$ receptor agonist, which has recently been shown to activate a $GABA_B$ type receptor on motor neurone D_f (Bai & Sattelle, 1994), were not reduced by a 5 min pre-exposure to

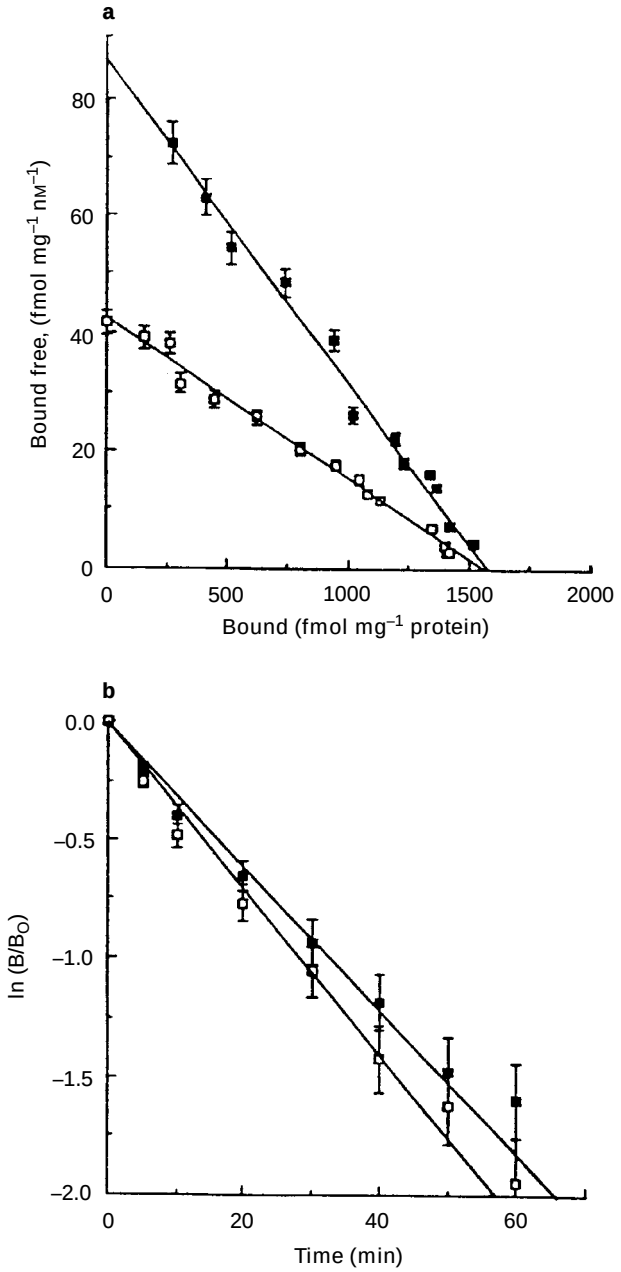

Figure 5 Dieldrin displaced [^{3}H]-BIDN from Southern corn rootworm (SCRW) membranes by a competitive mechanism. (a) Scatchard transformation of saturation binding data obtained for [^{3}H]-BIDN binding in the absence (■) and presence (□) of 54 nM dieldrin was carried out as described in Methods. The presence of dieldrin in the assay increased the K_d for [^{3}H]-BIDN from 18.2 nM to 36.9 nM, but only reduced the B_{max} from 1,575 fmol mg^{-1} to 1,563 fmol mg^{-1}. (b) [^{3}H]-BIDN dissociation rate studies were carried out in the absence (■) and presence (□) of 1.35 μM dieldrin as described in Methods. The presence of dieldrin in the assay slightly reduced the $t_{1/2,dissoc.}$ from 22.8 min to 18.9 min. All data points represent the mean of triplicate determinations; vertical lines show s.e.mean.

10 μM BIDN (data not shown). On the same cell, responses of a nicotinic acetylcholine receptor-gated cation channel (David & Sattelle, 1984) to 10 μM nicotine were unaffected by 10 μM BIDN tested in the same way.

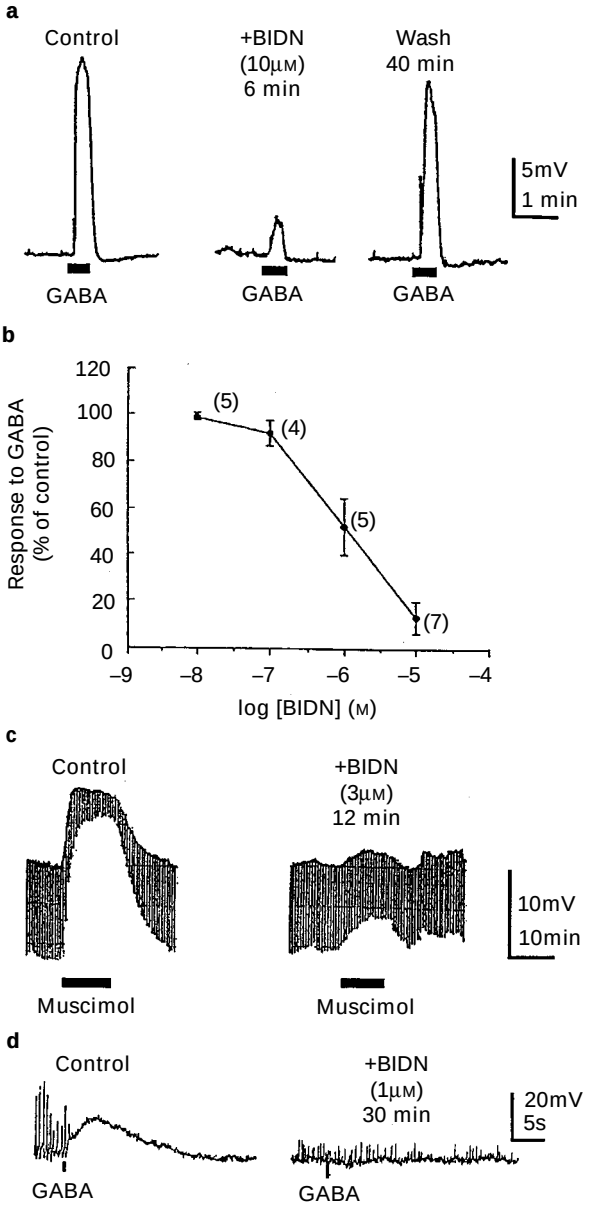

Figure 6 BIDN blocked GABA receptor responses in several insect tissues. (a) A 6 min application of 10 μM BIDN blocked GABA-gated chloride channel responses recorded from the cell body of the cockroach motor neurone D_f. The blocking action was reversed on rebathing the preparation with normal saline. Responses to 1 mM GABA are in the depolarizing direction as they were recorded in chloride-loaded cells (impaled with KCl-filled electrodes), thereby enhancing the amplitude of the GABA response. (b) Dose-response curve for the inhibition by BIDN of GABA-induced responses from motor neurone. D_f. Each data point represents the mean value of at least 4 separate observations as indicated in parentheses. Vertical lines represent twice the s.e.mean. (c) Cockroach coxal muscle cells were impaled with two glass microelectrodes filled with 3 M KCl. One of the electrodes was used to pass hyperpolarizing current pulses, while the second recorded changed in membrane potential. Muscimol (100 μM) induced an increase in the membrane conductance and a depolarization of the membrane potential. A second exposure to muscimol in the presence of 3 μM BIDN failed to elicit a response. (d) Pressure-applied, 50 ms applications of GABA to neurones on the ventral surface of a thoracic ganglion of SCRW larvae, resulted in transient depolarization. This response was blocked following bath application of 1 μM BIDN for 30 min.

BIDN actions on cockroach muscle GABA receptors

A 12 min exposure to 3 μM BIDN suppressed the muscimol-induced depolarization and conductance increase recorded from coxal muscle fibres (Figure 6c). These responses, like those on motor neurone D_f, were chloride-dependent (data not shown). At 0.1–10 μM concentrations, BIDN suppressed in a dose-dependent manner the GABA and muscimol-induced conductance increases with an IC_{50} of 2.5 μM estimated from a dose-inhibition curve. Thus, BIDN was shown to be able to block GABA-gated chloride channels on cockroach nerve and muscle membranes.

BIDN block of GABA receptors on Southern corn rootworm neurones

There have been no previous data obtained of electrophysiological recordings from SCRW neurones. In most thoracic ganglion neurones of larval SCRW examined in this study, action potentials were detected and membrane potentials in the range -35 mV to -45 mV were observed. Pressure applications of GABA (40 psi, 20–100 ms) resulted in transient responses accompanied by temporary cessation of action potentials (Figure 6d). Bath application of 1 μM BIDN for 30 min blocked responses to GABA in SCRW neurones (Figure 6d).

BIDN block of Drosophila *GABA receptor homo-oligomer expressed in* Xenopus *oocytes*

Messenger RNA prepared from *Drosophila Rdl* encoding cDNA resulted in the expression of *Xenopus* oocytes of dose-dependent, picrotoxinin-sensitive, bicuculline-insensitive, GABA-induced inward currents recorded at $E_h = -80$ mV (see also ffrench-Constant *et al*., 1993; Buckingham *et al*., 1994). The amplitudes of these GABA responses were largely suppressed by a 15 min application of 1 μM BIDN (Figure 7a). Block of the expressed *Drosophila* GABA receptor by BIDN, which was partly reversible (Figure 7a), and in a separate study, reduction of the maximal GABA-induced response with a slight rightward shift in the GABA dose-response curve (Hosie *et al*., 1995b), indicated that, as in the case of picrotoxin block (Houamed *et al*., 1984), the action of BIDN was neither completely non-competitive, nor competitive in its blocking action. Picrotoxin (1 μM) was also an effective antagonist of these GABA responses (Figure 7b).

BIDN block of rat brain $GABA_A$ receptors expressed in Xenopus *oocytes*

Messenger RNA prepared from whole rat brains was injected into the cytoplasm of *Xenopus* oocytes. Large (20–150 nA) inward Cl$^-$ currents, observed in response to 100 mM GABA (Figure 7c, see also Houamed *et al*., 1984) were blocked by 30 mM BIDN. The blocking action of BIDN was dose-dependent with an estimated IC_{50} of 5.6 μM. Complete block was obtained at a concentration of 100 μM. The blocking action of BIDN at concentrations above 10 μM was not fully reversible; only a partial recovery was obtained after a 90 min wash. Control, uninjected oocytes showed no consistent responses to either GABA or BIDN at concentrations up to 30 mM.

Discussion

The discovery of saturable, specific [^{3}H]-BIDN binding to insect and vertebrate membranes that is blocked by GABAergic convulsants, together with the block by BIDN of functional GABA receptors in insects and vertebrates indicates its utility as a new probe of GABA-gated chloride channels. Furthermore, the high affinity binding of [^{3}H]-BIDN to membranes of SCRW and other insect pest species, together with the finding that numerous insecticides displaced the binding by a compe-

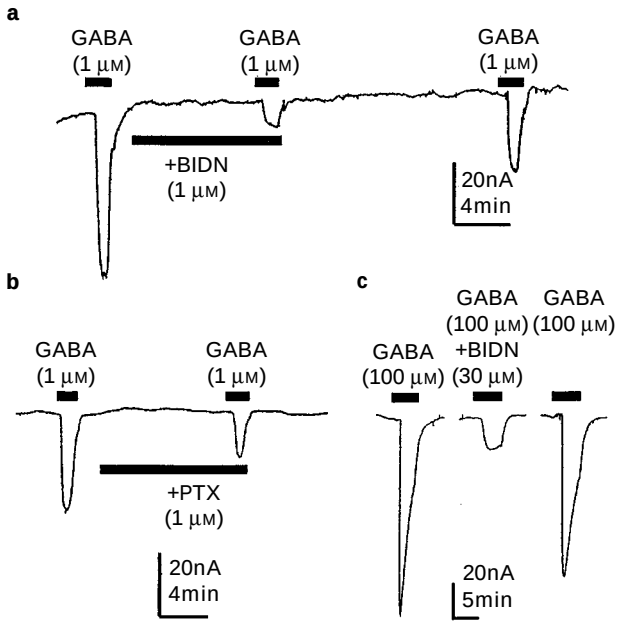

Figure 7 BIDN blocked insect and rat brain GABA receptor subunits expressed in *Xenopus* oocytes. (a) GABA-induced inward currents were recorded from *Xenopus* oocytes following injection into the cytoplasm of messenger RNA prepared from *Rdl*-encoding cDNA of *Drosophila*. Responses to 1 μM GABA were significantly reduced by 1 μM BIDN. This inhibition was partially reversible. (b) Picrotoxin 1 μM (PTX) was also an effective blocker of the response to 1 μM GABA recorded from the expressed *Drosophila Rdl* GABA receptor. (c) Following injection of rat brain mRNA into *Xenopus* oocytes, 100 μM GABA in the perfusing solution resulted in an inward current that was reversibly reduced in the presence of 30 μM BIDN.

titive mechanism, makes it a particularly useful probe in characterizing GABA receptor-insecticide interactions in insect pest species. Several of the caged convulsant compounds used as radioligands for the GABAergic convulsant site in housefly, cockroach, *Drosophila* and vertebrate tissue, principally EBOB, TBPS and TBOB, as well as picrotoxinin, failed to displace [^{3}H]-BIDN from membranes of SCRW and other pest species. This indicates that the convulsant site is likely to be somewhat different in these pest species and possibly explains why these radioligands failed to show high affinity, saturable binding to these tissues.

The discovery that in SCRW neurones BIDN blocks functional GABA receptors provides further evidence that the putative GABAergic convulsant binding site we have characterized is, indeed, a component of a functional insect GABA receptor in this species. This study is the first to obtain electrophysiological data on SCRW GABA receptors. Studies on an identified cockroach motor neurone D_f, the GABA receptors of which have been described in detail (Sattelle *et al*., 1988), showed that BIDN fails to block a nicotinic acetylcholine receptor (a ligand-gated cation channel) and a $GABA_B$-like receptor, indicating selectivity in its actions. Of particular interest is the finding that BIDN blocked a homo-oligomeric, expressed *Drosophila* GABA receptor composed only of the *Rdl* subunit. In this way an insect GABA receptor subunit at which BIDN acts has been identified. BIDN also blocked expressed rat brain $GABA_A$ receptors formed when rat brain RNA is expressed in *Xenopus* oocytes, thereby demonstrating that BIDN can block both insect and vertebrate GABA-gated chloride channels.

Many insecticidally-active molecules displace [^{3}H]-BIDN binding to SCRW membranes, with β-endosulphan and heptachlor epoxide being more potent than BIDN itself. The insecticides heptachlor and dieldrin were also very effective. Competitive block of [^{3}H]-BIDN binding by dieldrin and heptachlor, together with the ineffectiveness of picrotoxinin is of considerable interest in the light of recent findings that a single amino acid substitution ($A^{302}\rightarrow S$) in the second transmembrane (TM2) region of *Rdl* accounts for receptor/channel insensitivity to both dieldrin and picrotoxinin (ffrench-Constant *et al*., 1993). Thus, not all of the actions of picrotoxinin and dieldrin take place at the same site on insect GABA receptors. In this context it is of interest to note that three residues in TM2 of the glycine receptor α subunit (Pribilla *et al*., 1992), another ligand gated ion channel subunit capable of forming homo-oligomers in heterologous expression systems, can influence picrotoxinin sensitivity. Also R^{271} in the extracellular loop linking M2 and M3 of the glycine receptor α-subunit also affects sensitivity of the expressed receptor to picrotoxinin (Lynch *et al*., 1995). Nevertheless, the $A^{302}\rightarrow S$ mutation in *Rdl* also confers resistance to BIDN (Shirai *et al*., 1995) and the novel phenylpyrazole insecticide fipronil (Hosie *et al*., 1995a), indicating some common features of the actions of these convulsants.

Differences in the actions of dieldrin and lindane have been demonstrated at the [^{3}H]-BIDN binding site. Unlike dieldrin which displaced [^{3}H]-BIDN in a purely competitive manner, lindane was neither a purely competitive nor a purely allosteric displacer of [^{3}H]-BIDN, pointing to some differences in the mode of action of the cyclodiene and cyclohexane classes of insecticides. Although dieldrin and lindane (*v*-HCH) blocked GABA-gated chloride channels of native origin (Wafford *et al*., 1989; Zhang *et al*., 1994), and also expressed *Rdl* homo-oligomers (ffrench-Constant *et al*., 1993; Hosie & Sattelle, 1996), the δ-HCH isomer enhances the response to GABA in the expressed *Rdl* splice variant DRC 17-1-2 (Chen *et al*., 1994).

Antibodies to *Rdl* stain intensely in the brain, sub-oesophageal and thoracic ganglia of *Drosophila melanogaster* (Aronstein *et al*., 1995; Harrison *et al*., 1996). Antennal lobe glomeruli, the medulla and lobula of the optic lobes, parts of the central body and the mushroom bodies all stain strongly. Thus it seems likely that BIDN will bind to GABA-gated chloride channels that are widely distributed throughout the nervous system of *Drosophila melanogaster*.

Finally, we have provided direct evidence for an action of BIDN on both a *Drosophila* RDL GABA receptor subunit and on mammalian $GABA_A$ receptors. GABA receptors resulting from the expression of the *Rdl* subunit alone in *Xenopus* oocytes were blocked by BIDN at concentrations lower than those required to block expressed mammalian $GABA_A$ receptors, a finding in broad agreement with the results obtained in [^{3}H]-BIDN binding studies. Compounds active in displacing [^{3}H]-BIDN and which are unaffected by the $A^{302}\rightarrow S$ mutation conferring resistance may conceivably be effective in insect control.

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