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Unusual effects of benzodiazepines and cyclodiene insecticides on an expressed invertebrate $GABA_A$ receptor

Shahid H. Zaman, Robert J. Harvey*, Eric A. Barnard and Mark G. Darlison*

MRC Molecular Neurobiological Unit, MRC Centre, Hills Road, Cambridge, CB2 2QH, UK

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We have previously reported [(1991) EMBO J. 10, 3239–3245] the sequence of an invertebrate γ-aminobutyric acid (GABA) type A ($GABA_A$) receptor polypeptide which forms homo-oligomeric GABA-gated, bicuculline-sensitive, chloride-ion channels upon heterologous expression. We now demonstrate that the benzodiazepines Ro5-4864 (4'-chlorodiazepam) and diazepam, that are active at mammalian peripheral benzodiazepine sites, and not those benzodiazepines specific for central sites, directly activate the homo-oligomeric receptor and evoke larger maximal responses than those elicited by GABA. In addition, members of the cyclodiene class of insecticides block the channel of the receptor in a manner indistinguishable from that of picrotoxin.

Benzodiazepine; Channel blocker; Cyclodiene insecticide; Invertebrate; *Lymnaea stagnalis*; $GABA_A$ receptor; Receptor activation; *Xenopus* oocyte expression

1. INTRODUCTION

Invertebrate GABA receptors have been suggested to be the site(s) of action of several classes of pesticides (reviewed in [1]). In particular, the cyclodienes have been proposed [2–4] to have a similar mode of action to that of picrotoxin (PTX) which is a chloride-channel blocker. In addition, invertebrate GABA receptors appear to have an associated benzodiazepine binding site whose pharmacology resembles that of peripheral benzodiazepine binding sites, rather than those found in the central nervous system, of mammals [5,6]. For example, high-affinity binding sites for RO5-4864, a compound which is selective for the mammalian peripheral benzodiazepine binding site, are present in housefly thorax-abdomen membranes [7]. Furthermore, Ro5-4864 is more effective at displacing tritiated flunitrazepam binding from locust supraoesophageal ganglion membrane preparations than clonazepam [6], which is a centrally-acting benzodiazepine.

We have recently reported [8] the isolation of a complementary DNA (cDNA), from the mollusc *Lymnaea stagnalis*, that encodes a polypeptide that exhibits extensive sequence similarity to vertebrate $GABA_A$ receptor β subunits. When in vitro-transcribed RNA from this clone is injected into *Xenopus* oocytes, GABA-activated chloride-ion channels are formed which can be reversibly blocked by the vertebrate $GABA_A$ receptor antagonist bicuculline. We now report the effects of two cyclodiene insecticides, and several peripheral and central types of benzodiazepines, on the expressed homo-oligomeric molluscan $GABA_A$ receptor.

Abbreviations: cDNA, complementary DNA; DMSO, dimethyl sulphoxide; GABA, γ-aminobutyric acid; PTX, picrotoxin; V_h, holding potential

Present address: Institut für Zellbiochemie und klinische Neurobiologie, Universitäts-Krankenhaus Eppendorf, Universität Hamburg, Martinistrasse 52, 2000 Hamburg 20, Germany.

Correspondence address: S.H. Zaman, MRC Molecular Neurobiology Unit, MRC Centre, Hills Road, Cambridge, CB2 2QH, UK. Fax: (44) (223) 213556.

2. MATERIALS AND METHODS

2.1. *In vitro transcription, oocyte injection and electrophysiology*

In vitro transcription of RNAs encoding the molluscan $GABA_A$ receptor β subunit and the bovine $GABA_A$ receptor α1 subunit, and most electrophysiological methods, were as described previously [8,9]. Capped RNAs, both at a concentration of ~1 ng/nl, were injected (total volume ~40 nl per oocyte) either singly or in combination into manually defolliculated stage 5 or 6 *Xenopus laevis* oocytes. The cells were then incubated at 17°C for a minimum of 48 h prior to recording. Either uninjected oocytes or oocytes that had been injected with an equivalent volume of distilled water were used as controls. All compounds were bath-applied and 'wash-out' times were 5 to 15 min for agonists, depending upon the concentration used, and at least 40 min for PTX. Stock solutions were prepared in the following solvents: GABA, frog Ringer; bicuculline, distilled water; all benzodiazepines, dimethyl sulphoxide (DMSO); PTX and cyclodienes, ethanol. DMSO and ethanol had no effect on oocytes at the dilutions of drugs used, and control oocytes did not respond to either agonists or antagonists. GABA and bicuculline were purchased from Sigma, and Ro5-4864 was from Fluka. PTX, flunitrazepam, diazepam and chlordiazepoxide were a generous gift from Dr. I.L. Martin, and endrin and heptachlor epoxide were kindly provided by Drs. S.C.R. Lummis and D.B. Sattelle.

2.2. *Measurement of PTX and cyclodiene dose-response relationships*

Because the effect of PTX is only very slowly reversible, and those of the cyclodienes tested are essentially irreversible, dose-response curves could not be generated conventionally. However, relative efficacies were obtained by applying increasing concentrations of these compounds (without 'wash-out' between applications) and, at each concentration, measuring the plateau current (see Fig. 1A). Note that the application of a given concentration of each compound was only continued until the current response reached a steady-state.

3. RESULTS

3.1. *Effects of PTX and cyclodiene insecticides*

The application of micromolar concentrations of PTX to *Xenopus* oocytes, which have been injected with in vitro-transcribed RNA that encodes the molluscan $GABA_A$ receptor β subunit [8], produces an outward current at a holding potential (V_h) of −60 mV (Fig. 1B). 'Wash-out' of this compound takes between 40 and 90 min, depending upon the concentration used, thus preventing an investigation of the dose–response relationship by conventional methods. However, a dose–response relationship for PTX has been obtained (Fig. 1C) by applying increasing concentrations of the drug without 'wash-out' between applications (see section 2 and Fig. 1A); the EC_{50} for this compound is 50–60 nM, The reversal potential of the outward current (~−25 mV; data not shown) is similar to that of the GABA-induced inward current [8] and to the chloride-equilibrium potential of the oocyte. A decrease in the membrane conductance in response to PTX (10 μM), which corresponds to the observed outward current, was demonstrated by the application of 10 mV hyperpolarising pulses to oocytes held in the voltage-clamp mode; a conductance decrease was also seen when recording under current-clamp conditions (data not shown). Taken together, these data strongly suggest that the molluscan $GABA_A$ receptor chloride-ion channel closes upon PTX application.

Since cyclodiene insecticides have been suggested [2–4] to act at invertebrate GABA receptors in a similar manner to that of PTX, we tested two members of this class of compound (endrin and heptachlor epoxide) on the expressed molluscan homo-oligomeric receptor. Mi-

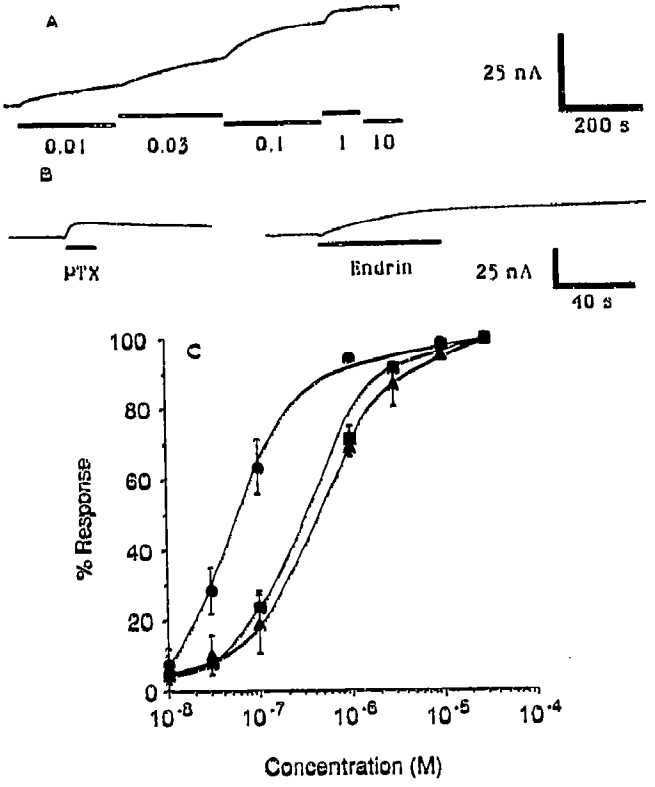

Fig. 1. Effects of PTX and cyclodienes on the expressed molluscan homo-oligomeric $GABA_A$ receptor. (A) Responses of a single oocyte to the application of increasing micromolar concentrations of PTX. (B) Responses of a single oocyte to the application of either 10 μM PTX or 1 μM endrin. The periods of drug application (in A and B) are indicated by horizontal bars; upward deflections indicate outward currents, which were recorded at V_h = −60 mV. (C) Dose–response relationships for PTX (●), endrin (■) and heptachlor epoxide (▲). Responses were normalized to the currents evoked by either 30 μM PTX (I_{max} = 163±46 nA; n = 5 oocytes), 30 μM endrin (I_{max} = 72±9 nA; n=4) or 30 μM heptachlor epoxide (I_{max} = 56±16 nA; n=3). Curves were fitted by eye, and error bars (where greater than the symbol size) indicate the S.E.M. for 3 to 5 oocytes tested for each compound. Note that the outward currents induced by PTX, endrin and heptachlor epoxide appear to be slower in onset at lower concentrations.

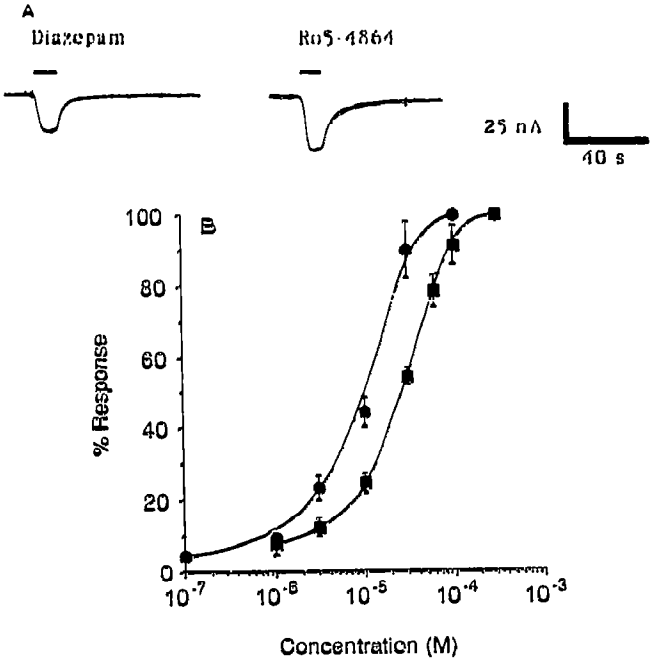

Fig. 2. Effects of benzodiazepines on the expressed molluscan homo-oligomeric $GABA_A$ receptor. (A) Responses of a single oocyte to the application of either 10 μM diazepam or 10 μM Ro5-4864. The periods of drug application are indicated by horizontal bars; downward deflections indicate inward currents, which were recorded at V_h = −60 mV. (B) Dose–response relationships for Ro5-4864 (●) and diazepam (■). Responses were normalized to the currents evoked by either 100 μM Ro5-4864 (I_{max} = 97±29 nA; n=8 oocytes) or 300 μM diazepam (I_{max}=26±8 nA; n=4). Curves were fitted by eye, and error bars (where greater than the symbol size) indicate the S.E.M. for 4 to 8 oocytes tested for each compound. Note that the results presented here were obtained from different oocytes to those used to generate the data shown in Table I.

Table I

Direct activation of the expressed molluscan homo-oligomeric $GABA_A$ receptor.

Agonist	Concentration (μM)	Mean current (nA) ± S.E.M.	Number of oocytes tested
(Data from the same oocytes):			
GABA	3,000	19 ± 5	5
Ro5-4864	100	111 ± 23	5
(Data from the same oocytes):			
GABA	3,000	14 ± 4	10
Diazepam	100	35 ± 8	10

The currents induced by either 100 μM Ro5-4864 or 100 μM diazepam are significantly larger than those evoked by 3 mM GABA; $P \leq 0.01$ (paired Student's t-test). Note that the results presented here were obtained from different oocytes to those used to generate the data shown in Fig. 2A and B.

cromolar concentrations of both compounds resulted in outward currents at V_h=−60 mV (Fig. 1B). The EC_{50} values for endrin and heptachlor epoxide are similar (Fig. 1C; 300–400 nM) but are higher than that for PTX. The reversal potentials of the outward currents induced by the two cyclodienes (~−25 mV) were similar to that of the PTX-induced outward current. In competition experiments, the application of either 10 μM endrin or 10 μM heptachlor epoxide immediately after the application of 10 μM PTX did not result in a significant increase in the outward current (data not shown).

3.2. *Effects of benzodiazepines*

Surprisingly, the application of certain benzodiazepines, such as Ro5-4864 and diazepam, to oocytes injected with the molluscan RNA, resulted in the direct activation of the homo-oligomeric $GABA_A$ receptor (Fig. 2A). Dose-response data (Fig. 2B) reveal EC_{50} values of ~10 μM for Ro5-4864 and ~25 μM for diazepam; these values are much lower than that previously reported [8] for GABA (EC_{50} = 200–300 μM). Furthermore, the maximal responses obtained with these two benzodiazepines were different, but both were significantly larger than that elicited by GABA (Table I). The reversal potential of the inward current induced by Ro5-4864 was −25 ± 1 mV (n = 4 oocytes). Centrally-acting benzodiazepines, such as chlordiazepoxide and flunitrazepam, did not activate the molluscan homo-oligomeric receptor even at concentrations of 50 μM and 100 μM, respectively.

Application of the vertebrate $GABA_A$ receptor antagonist bicuculline (at concentrations up to 100 μM in the presence of 30 μM of either agonist) did not significantly affect the size of the currents induced by either Ro5-4864 or diazepam. This result is consistent with studies on housefly thorax and abdomen membranes in which the binding of Ro5-4864 to GABA receptors was found to be insensitive to bicuculline [7]. Furthermore, Ro5-4864 and diazepam do not appear to potentiate the effect of GABA; the current observed, in any particular oocyte, upon the co-application of GABA and one of the two benzodiazepines is approximately the same as the sum of the currents evoked by the application of the same concentration of each agonist separately.

It has previously been shown [8] that co-injection of *Xenopus* oocytes with the molluscan RNA and RNA for the bovine $GABA_A$ receptor α1 subunit [9] results in the formation of functional hetero-oligomeric (chimeric) receptors. When either Ro5-4864 or diazepam, at concentrations up to 100 μM, were applied to such oocytes, no significant current responses were seen at a V_h of −60 mV (data not shown). However, 3 mM GABA evoked large inward currents (234 ± 40 nA; n = 5 oocytes).

4. DISCUSSION

We have shown here that the effects of two cyclodiene insecticides, on an expressed invertebrate homo-oligomeric $GABA_A$ receptor, are highly reminiscent of that of PTX. The outward current induced by PTX has previously been attributed to the blockade of the integral chloride-selective channel, which is at least partially open even in the absence of agonist [8]. The decrease in conductance, seen in voltage-clamp and current-clamp experiments upon the application of this compound, and the reversal potential of the PTX-evoked outward current, support this suggestion. This effect is probably a consequence of the inefficient assembly, in the oocyte, of single-subunit receptors. Taken together with the finding [2] that cyclodiene-resistant cockroach strains exhibit resistance to PTX, our data lead us to conclude that cyclodiene insecticides bind at, or close to, the PTX site, and that they act similarly as chloride-channel blockers. In this context, the recent report by ffrench-Constant and co-workers [10] of the sequence of a *Drosophila* putative $GABA_A$ receptor subunit, deduced from a cDNA that was isolated using a strategy based on the cloning of a cyclodiene-resistance locus, merits discussion. This polypeptide displays only 38% identity to the molluscan $GABA_A$ receptor subunit whose cDNA was used in the studies reported here, and no more than 41% identity to any vertebrate $GABA_A$ receptor α, β, γ or δ subunit. In fact, the ligand-gated chloride-ion channel subunit to which the *Drosophila* sequence exhibits the highest identity (42%) is the rat glycine receptor α2 subunit [11]. Since, in contrast to the molluscan subunit which forms homo-oligomeric GABA-gated chloride channels, no functional data has yet been presented for the *Drosophila* polypeptide, we believe it is premature to assign this as a $GABA_A$ receptor subunit.

There are three possible explanations of the apparently conflicting data on the site of action of cyclodi-

enes. Firstly, more than one $GABA_A$ receptor subtype may exist in a given invertebrate and the two polypeptides, represented by the *Lymnaea* and *Drosophila* sequences, may be associated with different subtypes, each of which can bind these insecticides. Secondly, two subunits, which are the homologues of the molluscan and *Drosophila* polypeptides, may be required for cyclodiene binding in a single heter-oligomeric receptor. A third possibility is that if the *Drosophila* subunit is not a $GABA_A$ receptor subunit but a component of some other ligand-gated anion-channel complex, then cyclodienes may act as general chloride-ion channel blockers. However, since cyclodiene resistance has been shown, in *Drosophila* and in other insects, to be due to mutation at a single locus [12,13], it would appear that only one receptor/channel gene is mutable.

The finding that Ro5-4864 and diazepam act at the molluscan homo-oligomeric $GABA_A$ receptor is consistent with reports [5–7] of the existence of GABA receptor-associated benzodiazepine binding sites, in several invertebrates, which have a pharmacology that resembles that of the vertebrate peripheral benzodiazepine site. It is surprising, however, that micromolar concentrations of these compounds directly activate the receptor and the apparent affinities for Ro5-4864 and diazepam are at least 10-fold higher than that of the supposedly natural agonist, GABA. Even more remarkable is the observation that the maximal currents induced by these two benzodiazepines are larger than GABA-evoked currents. Since the benzodiazepine-induced currents are not significantly antagonized by bicuculline, and the effects of GABA are not potentiated by either Ro5-4864 or diazepam, we conclude that these benzodiazepines bind to a site on the homo-oligomeric receptor that is distinct from the GABA site, and that binding of agonists to eiher of these sites can result in channel activation. While we do not know of any report on the action of benzodiazepines on 'native' molluscan $GABA_A$ receptors, this clearly now merits investigation.

The most likely interpretation of the lack of direct activation of chimeric receptors by either Ro5-4864 or diazepam is, we believe, that the association of the bovine $\alpha 1$ subunit with the molluscan β subunit masks the binding site for these compounds. Furthermore, the co-assembly of the molluscan and bovine subunits must be extremely efficient, since direct activation is completely abolished upon co-expression. If the cell membrane of co-injected oocytes contained both chimeric receptors and molluscan homo-oligomeric receptors, then some Ro5-4864 and diazepam-induced currents should have been observed.

Our results with benzodiazepines suggest the intriguing possibility that ligands other than GABA can act as agonists at invertebrate $GABA_A$ receptors in vivo. In this context, it is interesting to note that the existence of a second channel-activating site on the *Torpedo* nicotinic acetylcholine receptor, that is separate from the acetylcholine binding site, has recently been reported [14,15]. It is, therefore, tempting to speculate that the occurrence of two (or more) different agonist binding sites may be a common feature of ligand-gated ion-channel receptors.

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