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Insect Ryanodine Receptor: Distinct But Coupled Insecticide Binding Sites for [*N*-C³H₃]Chlorantraniliprole, Flubendiamide, and [³H]Ryanodine

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Abstract

Radiolabeled anthranilic diamide insecticide [*N*-C³H₃]chlorantraniliprole was synthesized at high specific activity and compared with phthalic diamide insecticide flubendiamide and [³H]ryanodine in radioligand binding studies with house fly muscle membranes to provide the first direct evidence with a native insect ryanodine receptor that the major anthranilic and phthalic diamide insecticides bind at different allosterically coupled sites, i.e. there are three distinct Ca²⁺-release channel targets for insecticide action.

The newest major class of insecticides, the diamides, is rapidly replacing major uses of the earlier chlorinated hydrocarbons, organophosphates, methylcarbamates, pyrethroids, and neonicotinoids¹ because they are highly effective on major pests resistant to these earlier chemotypes and appear to be safe for people and the environment.²⁻⁴ There are currently two types of diamide insecticides, i.e., the anthranilic diamides with the important compounds chlorantraniliprole (Chlo)⁵⁻⁸ and cyantraniliprole (Cyan)^{7,8} and the phthalic

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Supporting Information. ¹H and ¹³C NMR spectra for desmethyl-Chlo. Structures of DP-010, DP-033 and compound 44 reported as radioligands without radiosynthesis details.

diamides exemplified by flubendiamide (Flu)⁹⁻¹⁴ (Figure 1). These diamides activate insect ryanodine (Ry)-sensitive intracellular Ca²⁺ release channels (Ry receptors, RyR), an assignment based on electrophysiological and Ca²⁺-release studies⁵⁻¹⁴ and the finding that Flu and the Chlo analog DP-010 stimulate [³H]Ry binding.^{5,9}

Five radioligands have been used in examining the specific binding sites for the diamides. The first is [³H]Ry which defined the functional properties and pharmacological profiles of the insect and mammalian RyRs.¹⁵⁻¹⁷ The second is [³H]Flu^{9, 11, 12} and the third is a ³H-labeled analog (DP-010) of Chlo with multiple structural changes.^{5, 8} These earlier diamide radiosyntheses, without stating the labeling positions or procedures, gave [³H]Flu of 24 Ci/mmol⁹ and [³H]DP-010 of 21 Ci/mmol.⁵ Two other analogs of Chlo designated DP-033 and compound 44 have also been radiolabeled without a detailed report.^{8, 18} Studies with these diamide radioligands did not answer the critical question of whether the anthranilic and phthalic diamides bind at the same or different sites.⁷

The ideal radioligand would be [³H]Chlo because it is highly potent and the most widely-used diamide. The first goal of the present investigation was therefore to prepare [³H]Chlo of high specific activity in a minimum number of labeling steps. Chlo and [³H]Chlo were synthesized from known benzoxazinone 1¹⁶ via desmethyl-Chlo which was reacted with methyl iodide or [³H]methyl iodide, respectively (Figure 2). Desmethyl-Chlo: To a solution of benzoxazinone 1 (46 mg, 0.10 mmol) in tetrahydrofuran (THF) (3 mL) was bubbled ammonia at room temperature. The reaction mixture was stirred for 10 min and concentrated. Recrystallization of the residual solid from acetonitrile afforded desmethyl-Chlo (21 mg, 44%) as a white solid, m. p. 246 - 248 °C. ¹H NMR (500 MHz, dimethylsulfoxide-d₆) δ 10.30 (s, 1H), 8.50 (dd, J = 4.8, 1.3 Hz, 1H), 8.17 (d, J = 8.0, 1.5 Hz, 1H), 7.77 (s, 1H), 7.61 (dd, J = 8.0, 4.5 Hz, 1H), 7.51 (s, 1H), 7.47 (d, J = 2.0 Hz, 1H), 7.40 (d, J = 2.0 Hz, 1H), 7.37 (s, 1H), 2.14 (s, 3H); ¹³C NMR (125 MHz, dimethylsulfoxide-d₆) δ 167.6, 155.7, 148.3, 147.2, 139.5, 139.3, 138.9, 135.6, 131.6, 131.3, 130.9, 127.7, 126.8, 126.6, 125.5, 110.7, 17.7; IR (thin film) ν_{max} 3375, 3249, 1686, 1610, 1578, 1463, 1355 cm⁻¹; HRMS (EI⁺) calc'd for [C₁₇H₁₂BrCl₂N₅O₂+H]⁺: m/z, 467.9630 found 467.9619. Chlo: To a solution of desmethyl-Chlo (5.6 mg, 0.012 mmol) in dimethylformamide (DMF) (0.2 mL) at 0 °C was added NaH (0.6 mg, 0.014 mol). After 10 min, methyl iodide (0.8 μL, 0.012 mmol) was added to the resulting mixture at room temperature. After being stirred for 2 h, the reaction was quenched with H₂O and extracted several times with ethyl acetate. The combined organic layers were washed with H₂O and brine, dried over anhydrous Na₂SO₄, and concentrated in vacuo to yield a yellow solid identical to an authentic sample of Chlo. [*N*-C³H₃]Chlo: The tritium-labeling reaction, product isolation and characterization were carried out by Vitrex Company (Placentia, CA). The procedure above for Chlo was modified to obtain [*N*-C³H₃]Chlo by using [³H]methyl iodide and purification by HPLC (Prevail C18 column, 5 μm, 4.6 × 150 mm) involving mobile phase A 0.05% TFA/H₂O and mobile phase B acetonitrile at a flow rate of 1.0 mL/min and a time program of 0 min 30% B, 10 min 90% B, and 12 min 30% B. A radioflow chromatogram showed a single labeled product (>99% HPLC purity, retention time 9.39 min) with structure identity verified by HPLC co-elution with authentic standard. The specific activity determined by MS was 78 Ci/mmol. It was stored in ethanol at 0.5 mCi/mL at -10°C.

For receptor binding assays, house fly thorax muscle membranes were prepared as previously described¹⁷ with a few modifications. Adults emerged from pupae obtained from Benzon Research (Carlisle, PA) were used to collect the thoraces for membrane preparation. Three buffers were used: (A) 5 mM dithiothreitol and 0.8% (w/v) bovine serum albumin (BSA) in 50 mM Tris-HCl, pH 7.4; (B) 10 μ M phenylmethanesulfonyl fluoride, 0.8% BSA, and 303 mM sucrose in 20 mM Tris-maleate, pH 7.0; (C) 0.8 mM CaCl_2 , 2 mM $\text{ATP}\cdot\text{Mg}^{2+}$ salt, and 1.5 M KCl in 10 mM Hepes, pH 7.4. The frozen tissue was homogenized in ice-cold buffer A with a polytron (Brinkmann Instruments) using 4 15-sec bursts. The homogenate was centrifuged at 500 g for 5 min, and then the supernatant was filtered through eight layers of cheesecloth and centrifuged again at 125,000 g for 1 h. The final pellet was resuspended in buffer B and the protein over and above the BSA component was determined using the standard Bio-Rad Protein Assay method. This membrane preparation was then stored at -80 °C before use. For binding assays, incubation mixtures were prepared by sequential addition to culture tubes of 200 μ L buffer C, next 200 μ L buffer B containing thorax muscle membranes (200 μ g protein) and then ethanol (5 μ L) containing [^3H]Ry (95 Ci/mmol) (Perkin-Elmer Life Sciences, Boston, MA) or [^3H]Chlo to give a final radioligand concentration of 1 nM. Finally test compounds were added in 5 μ L ethanol to give the specified concentrations of Chlo, Cyan, Flu, and Ry. Following incubation for 2 h at 37 °C, the mixtures were filtered through GF/B filters (Whatman, presoaked in ice-cold washing buffer) immediately after dilution with 5 mL ice-cold washing buffer (150 mM KCl, 10 mM Hepes, pH 7.4). Each assay tube was further rinsed twice with 5 mL ice-cold washing buffer and the rinses were passed through the same filter. The filters were then transferred to scintillation vials containing 10 mL Safety-Solve (Research Products International Corporation) and held over-night in the dark before scintillation counting. Non-specific binding was determined with 10 μ M Chlo for [^3H]Chlo and 10 μ M Ry for [^3H]Ry. Specific binding values were as follows for [^3H]Chlo and [^3H]Ry, respectively: percent of total binding 82 ± 1 and 81 ± 2 ; fmol/mg protein 21 ± 2 and 27 ± 3 ; dpm/200 μ g protein 1020 ± 92 and 1000 ± 88 . All data reported are mean $\pm$ standard error for 3 independent experiments.

The results allow a direct comparison of the binding sites for [^3H]Chlo and [^3H]Ry assayed at 1 nM. Specific binding for [^3H]Chlo was enhanced by Ca^{2+} but not ATP (not detailed here) and for [^3H]Ry was Ca^{2+} -dependent, ATP enhanced and saturable.¹⁷ Specific binding for [^3H]Chlo was reduced to 50% by 14 nM unlabeled Chlo and 6 nM Cyan indicating that these anthranilic diamides bind at the same site with a lower apparent affinity for Chlo than for Cyan (Figure 3A). The IC_{50} for [^3H]Ry binding was 3 nM unlabeled Ry (Figure 3B), consistent with its reported K_d (5 nM).¹⁷

The findings from displacement studies with unlabeled compounds of the three chemotypes establish that anthranilic and phthalic diamides and Ry bind at three distinct but coupled sites. The phthalic diamide Flu stimulates rather than inhibits [^3H]Chlo binding establishing different binding sites for Chlo and Flu. Ry also stimulates [^3H]Chlo binding indicating different binding sites for Chlo and Ry. On the other hand, [^3H]Ry binding in our house fly system is stimulated by the anthranilic diamides Chlo and Cyan but not by the phthalic diamide Flu (although Flu does stimulate in other insect systems^{9, 11, 12}). The concentration of Ry to stimulate [^3H]Chlo binding by 2-fold is essentially the same as the IC_{50} value for

Ry (3-5 nM) and correspondingly the concentration of Chlo to stimulate [³H]Ry binding by 2-fold is the same as its IC₅₀ value (14-15 nM) (Figure 3). Thus, there are three distinct but coupled sites in house fly RyR for Chlo/Cyan, Flu, and Ry.

The present study fully supports the proposed mode of action of the insecticidal diamides as RyR modulators and extends the understanding in three ways. First, Chlo and Cyan compete directly with each other and therefore as expected^{8, 18} appear to bind at the same site. Second, Flu and Ry stimulate [³H]Chlo binding by acting at a distinct site or in a different way. Third, the anthranilic diamides Chlo and Cyan stimulate [³H]Ry binding while the phthalic diamide does not in house flies. We interpret these relationships as three distinct binding sites, one for the anthranilic diamides Chlo and Cyan, another for the phthalic diamide Flu and a third for the classical Ry. Flu and Ry are proposed to open the channel for [³H]Chlo binding while Chlo and Cyan and possibly Flu open the channel for [³H]Ry binding.

The finding of different insect RyR binding sites for the anthranilic and phthalic diamides indicates that target site cross-resistance may be minimized or not involved with the two diamide chemotypes. Remarkably, the two diamide binding sites are important in insects but not mammals conferring selectivity²⁻⁴ while the Ry site is similarly sensitive in both insects and mammals.¹⁷ The new [³H]Chlo radioligand allows further characterization of these relationships.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Chlo	chlorantraniliprole
Cyan	cyantraniliprole
Flu	flubendiamide
Ry	ryanodine
RyR	ryanodine receptor

Figure 1.

Anthranilic and phthalic diamides and ryanodine showing tritium-labeling positions.

Figure 2.

Synthesis of desmethyl-Chlo and [N-C³H₃]Chlo. (a) NH₃, THF, 86%. (b) NaH, *MeI, DMF.

Figure 3.
Effects of anthranilic and phthalic diamide and ryanodine insecticides on the specific binding of [³H]Chlo (A) and [³H]Ry (B). S_{2x} designates stimulation of binding by 2-fold