Pesticide Biochemistry and Physiology 95 (2009) 1–5

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Novel mode of action of spinosad: Receptor binding studies demonstrating lack of interaction with known insecticidal target sites

Nailah Orr, Andrew J. Shaffner, Kimberly Richey, Gary D. Crouse *

Dow AgroSciences LLC, Crop-Protection Discovery Group, Bldg. 306/E2/984, 9330 Zionsville Rd., Indianapolis, IN 46268, USA

ARTICLE INFO

Article history:
Received 28 August 2008
Accepted 30 April 2009
Available online 8 May 2009

Keywords:
Spinosad
Spinosyn A
Receptor binding
Insecticide
Periplaneta americana
Musca domestica

ABSTRACT

The ability of spinosyn A to either enhance or displace binding to selected insecticidally-relevant receptors was investigated using a number of radioligands including, [^{3}H]imidacloprid and [^{3}H]ivermectin in tissues from the ventral nerve cord (VNC) membranes of the American cockroach, *Periplaneta americana* and head membranes from the housefly, *Musca domestica*. In these insect neural tissues, spinosyn A does not appear to alter the binding of a number of radioligands suggesting that spinosyn A does not interact directly with a variety of known receptors, including nicotinic or γ-aminobutyric acid (GABA)-based insecticidal target sites. However, available data are consistent with spinosyn A interacting with a site distinct from currently known insecticidal target sites, thus supporting a novel insecticidal mechanism of action for the spinosyns.

1. Introduction

Spinosad is a naturally occurring mixture of two insecticidally active components, spinosyns A (primary component) and spinosyn D (minor component) (Fig. 1), produced by the actinomycete, *Saccharopolyspora spinosa* [1–3]. Since its registration in 1997, spinosad has been used around the world for the control of a variety of insect pests from a wide range of orders including Diptera, Homoptera, Hymenoptera, Isoptera, Orthoptera, Lepidoptera, Siphonaptera, Thysanoptera [3–5], and most recently, for the control of Ctenocephalides felis in dogs [6,7].

The mode of action of the spinosyns has been the focus of several studies which to date suggest that the spinosyns disrupt neural functions, most likely via an alteration of nicotinic receptor function [8–11]. However, it is desirable to have a better understanding of the underlying molecular target site(s) for spinosad and spinosyns since such information could be very useful in the development of resistance management programs and the tools for resistance detection/monitoring, as well as the discovery of future products with this novel mode of action.

The present study summarizes a range of receptor binding studies that have been conducted over several years on spinosyn A and other selected natural and semi-synthetic spinosyn analogs. In general, these studies suggest that spinosyn A does not appear to interact directly with a variety of known insecticidally-relevant target sites, but rather via a novel mechanism. This conclusion is consistent with recent studies that propose a distinct nicotinic receptor subunit as the molecular target site for the spinosyns [11–15].

2. Materials and methods

2.1. Insects

Flies (*Musca domestica*) were maintained at 27 °C, 40% RH for adults and 60% RH for larvae with a 14:10 L:D photoperiod. The flies were fed standard diet and water containing 10% sucrose. Adult flies were collected within 2–3 days of emergence. Adult male cockroaches (*Periplaneta americana*, 1–3 months after the final molt) were taken from a colony maintained at 28 °C with a 12L:12D photoperiod and fed Purina® dog chow and water *ad libitum*. Cockroaches were isolated in Petri dishes, containing a few drops of water and maintained at room-temperature overnight prior to dissection of ventral nerve cord.

2.2. Fly head membrane preparation

Housefly head membranes were prepared as previously described [16]. Briefly, fly heads were homogenized in a chilled Waring™ blender using a 1:10 ratio of grams of fly heads to milliliters of ice-cold homogenization buffer (200 mM sucrose, 1.0 mM EDTA, 10 mM Tris–HCl and 0.1 mM PMSF, pH 7.4). After homogenization, the fly head mixture was filtered through 6–8 layers of cheesecloth and the effluent was centrifuged at 1000*g* for 15 min (4 °C). The supernatant was collected and centrifuged at 35,000*g* for 15–

* Corresponding author. Fax: +1 317 337 3215.
E-mail address: gdcrouse@dow.com (G.D. Crouse).

0048-3575/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.pestbp.2009.04.009

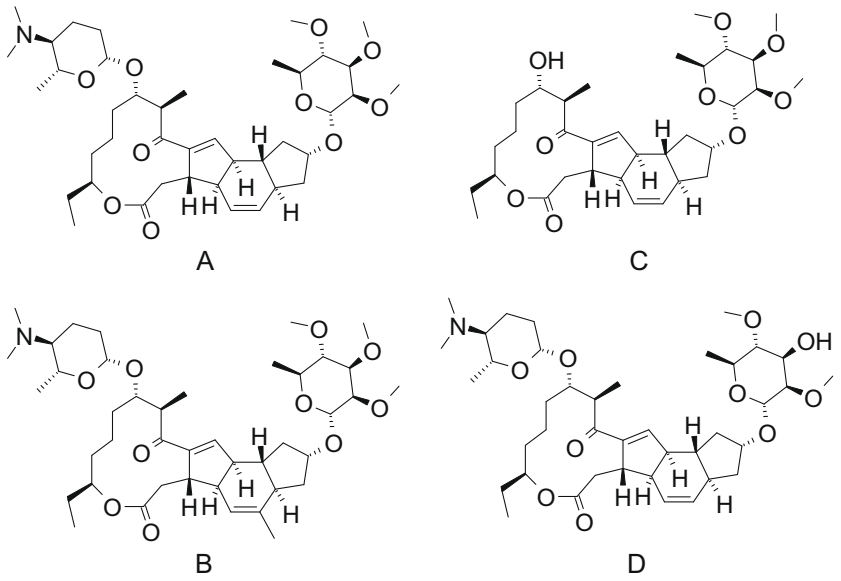

Fig. 1. Structures of spinosyn analogs used in binding studies. (A) spinosyn A; (B) spinosyn D (Spinosad is a mixture of spinosyn A and spinosyn D); (C) C-17-pseudoaglycone of spinosyn A; and (D) Spinosyn J. Analogs C and D were used in [^{3}H]VPM binding studies.

20 min (4 °C). The pellet was collected, resuspended in homogenization buffer and centrifuged at 35,000*g* for another 15–20 min (4 °C). The supernatant from this second high-speed spin was discarded, and the pellet was resuspended in storage buffer (50 mM Tris–HCl, 0.1 mM PMSF, pH 7.4). The protein was stored in aliquots in −80 °C for up to 1 year without loss of activity.

2.3. Cockroach ventral nerve cord membrane preparation

Cockroach ventral nerve cord (VNC) membranes were prepared as previously described [17,18]. Briefly, adult male cockroach ventral nerve cords (without cerebral ganglia) were dissected in ice-cold sucrose buffer (200 mM sucrose, 1 mM EDTA and 50 mM Trizma–HCl, pH 7.4). The isolated nerve cords were homogenized on ice, using a glass–Teflon homogenizer (25 strokes, 700 rpm). This homogenate was centrifuged at 4 °C (36,600*g*, 15 min). The resulting pellet was suspended in sucrose buffer by gentle homogenization and centrifuged as above. The supernatant from this high-speed spin was discarded, and the pellet was resuspended in storage buffer (50 mM Tris–HCl, 0.1 mM PMSF, pH 7.4). The protein was stored in aliquots at −80 °C for up to 4 months without loss of activity For all tissue samples, protein concentration was determined using the Bradford [19] method using bovine serum albumin (BSA) as a standard.

2.4. Chemicals

All chemicals were of analytical grade and were either purchased from Research Biochemicals International (Natick, MA, USA), Sigma Chemical Co. (St. Louis, MO, USA) or synthesized at Dow AgroSciences.

2.5. Radioligands

The following radioligands were used in this study:

1) [^{3}H]α-bungarotoxin ([^{3}H]α-BGT). The binding methods used were adapted from Orr et al. [20]. The radioligand was purchased from GE Healthcare (Piscataway, NJ) with a specific activity (SA) of 61 Ci/mmol (tritiated).
2) [^{3}H]4-*n*-propyl-4′-ethynylbicycloorthobenzoate ([^{3}H]EBOB). The binding method was based on the method of Deng et al. [21]. The radioligand was acquired from PerkinElmer (Waltham, MA) with a SA of 30 Ci/mmol.
3) [^{3}H](±) epibatidine ([^{3}H](±)EPI) binding was based on the procedure described in Orr et al. [18]. The radioligand was purchased from GE Healthcare (Piscataway, NJ) with a SA of 47 Ci/mmol.
4) [^{3}H]imidacloprid ([^{3}H]IMI) binding was based on the previously described method of Liu and Casida [16]. This radioligand was custom synthesized by PerkinElmer (Waltham, MA) with a SA of 37.2 Ci/mmol. All intermediates were synthesized at Dow AgroSciences.
5) [^{3}H]ivermectin ([^{3}H]IVM) binding was based on the method described by Rohrer et al. [22]. This radioligand was synthesized by GE Healthcare (Piscataway, NJ) with a SA of 52 Ci/mmol. All intermediates were synthesized at Dow AgroSciences.
6) [^{3}H]methyllycaconitine ([^{3}H]MLA) binding was based on the method previously described by Davies et al. [23] and Lind et al. [24]. The radioligand was acquired from Tocris Cookson Ltd. (Bristol, UK) with a SA of 43.7 Ci/mmol.
7) [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) binding was based on the method described by Orr et al. [25]. The radioligand was acquired from GE Healthcare (Piscataway, NJ) with a SA of 48 Ci/mmol.
8) [^{3}H]*N*-methyl-verapamil ([^{3}H]VPM) binding was based on the method previously described by Skeer and Sattelle [26]. The radioligand was acquired from PerkinElmer (Waltham, MA) with a SA of 85 Ci/mmol.

2.6. Receptor binding assays

For all binding assays, membranes were either used fresh or frozen. When frozen membrane samples were used, they were thawed on ice and gently suspended by hand-homogenization with a glass–Teflon homogenizer in the appropriate binding buffer. All filtration-based binding experiments were performed either in 96-well polypropylene block-plate (2 ml final volume per well;