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Review Article

Emodepside, a cyclo-octadepsipeptide anthelmintic with a novel mode of action

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Abstract

Due to the development of drug resistance, there is a continual need for new anthelmintics with novel modes of action. Emodepside, a cyclo-octadepsipeptide, was originally thought to modify GABAergic transmission in parasitic nematodes. However, more recent studies using the parasitic nematode *Ascaris suum* and the free-living nematode *Caenorhabditis elegans* indicate that emodepside interacts with a presynaptic latrophilin receptor to release a transmitter or modulator. This presynaptic signaling pathway involves activation of a Gαq protein and phospholipase-β, which mobilizes diacylglycerol (DAG). DAG activates UNC-13, which in turn activates synaptobrevin to increase release of the transmitter or modulator. The released transmitter or modulator acts postsynaptically to modify pharyngeal and somatic body wall muscle activity.

Introduction

Parasitic helminth infection is a worldwide problem with an estimated one-third of the human population affected, resulting in morbidity or even mortality. Most of these infections are preventable. Morbidity can manifest itself in a number of ways, including nutritional imbalance with reduced food intake, reduced growth and development, intestinal obstruction and intestinal bleeding. In addition, parasitic infection of livestock and crops exerts an enormous economic burden on society.

The compounds that have been developed to combat helminth infections can be divided into three major groups: the benzimidazoles/probenzimidazoles, the tetrahydropyrimidines/imidazothiazoles and the macrocyclic lactones. The benzimidazoles and probenzimidazoles, *e.g.*, thiabendazole, fenbendazole and febantel, which is converted to fenbendazole in the host, act by binding free β-tubulin with great selectivity for helminth tissue compared to mammalian tissue (1). However, there is evidence that some benzimidazoles are teratogenic (2). The tetrahydropyrimidines, *e.g.*, pyrantel, and the imidazothiazoles, *e.g.*, levamisole, act as acetylcholine agonists at the nicotinic receptors on nematode body wall muscle (3). These compounds increase the sodium and potassium conductance of the body wall muscle membrane, resulting in spastic paralysis. The macrocyclic lactones, such as the avermectins, *e.g.*, ivermectin, and the milbemycins, *e.g.*, milbemycin D, act selectively on the nematode glutamate-gated chloride channels, including those located on pharyngeal muscle and the motor nervous system, to increase chloride conductance (4-6).

Nematodes possess a wide range of transmitter receptors and ion channels on their tissues which are potential targets for anthelmintics. In addition to nicotinic acetylcholine receptors and glutamate-gated chloride channels, there are transmitter receptor sites in nematodes for 5-hydroxytryptamine (5-HT, or serotonin), γ-aminobutyric acid (GABA), dopamine, octopamine, neuroactive peptides and possibly nitric oxide (7-15). Piperazine, a GABA receptor agonist, has been used as an anthelmintic (16) and acts by opening chloride channels linked to the GABA receptor. This relaxes body wall muscle, resulting in flaccid paralysis (17).

Diethylcarbamazine, a compound related in structure to piperazine, has a very different mechanism of action as an anthelmintic. It is believed to interact with the host-parasite immune system, making the nematode more susceptible to immunological attack (18).

Apart from the major groups of anthelmintics mentioned above, a number of other compounds have been used and these will be briefly reviewed. Certain organophosphates have been used as anthelmintics, *e.g.*, dichlorvos, and act by raising the levels of acetylcholine at the nerve somatic muscle junction, resulting in spastic paralysis due to excessive depolarization of the muscles (18). The problem with these compounds is that they also inhibit host acetylcholinesterases and so can be toxic to the host. The salicylanilides, *e.g.*, closantel, probably act by uncoupling electron transport-associated phosphorylation (18), but they also lower cytoplasmic pH (19). Spinosyns, *e.g.*, spinosyn A and D, are tetracyclic macrolides with two sugars, obtained from the actinomycete *Saccharopolyspora spinosa* (20, 21). These compounds appear to interact with both anion and cation fluxes of insect neurons and may have potential as anthelmintics. Nitazoxanide is another compound which has activity against nematodes but its mechanism of action is not clear (20), although the enzymes of anaerobic electron transport may be potential targets (22). Nodulisporic acid A is a complex natural product from a fungal species of *Nodulisporium* (23) that potentiates the action of glutamate on insect neuronal glutamate-gated chloride channels, which are distinct from those found in nematodes (24). Isothiocyanates, *e.g.*, nitroscanate, alter glucose metabolism in nematodes (18). Paraherquamide, an indole alkaloid closely related to marcfortine A, is produced by *Penicillium paraherqui* and both it and its derivative 2-deoxyparaherquamide have potent *in vivo* activity against a range of nematodes (20, 24, 25). Paraherquamide is equally potent against nematodes that are resistant or sensitive to ivermectin, levamisole and benzimidazoles (26). Paraherquamide induces flaccid paralysis by blocking acetylcholine nicotinic receptors on nematode body wall muscle (27, 28).

Other strategies have been proposed for the development of anthelmintics derived from natural products, *e.g.*, cysteine proteases from the latex or fruit of figs, papaya and pineapples, which digest nematode cuticles (29). Further details on the chemical control of animal parasitic nematodes can be found in a recent excellent review by Conder (2), and an earlier review considers the modes of action of anthelmintics (30). Currently, no anthelmintics have been developed which act on 5-HT, octopamine or dopamine receptors. There are a large number of neuroactive peptides which are specific to nematodes and act on their respective receptors to excite, inhibit or modulate body wall muscle, pharyngeal muscle and other tissues. These include around 60 putative RFamide peptides (flps) and over 130 non-RFamide peptides (nlps) (31-33). These peptide receptors are also potential sites for anthelmintic action. In addition, there are ion channel sites which have been identified, including a small number of calcium channels and around 80 predicted potassium ion channels in *C. elegans*, which could provide target sites for anthelmintics in parasitic nematodes (34-36), although relatively few of these potassium channels are probably normally active physiologically (37).

Isolation of PF-1022A, a member of a new family of anthelmintics

One of the problems with anthelmintics is the development of resistance due to overuse (38, 39). For example, resistance has developed to levamisole due to mutations in nematode nicotinic acetylcholine receptors (40). Resistance is also developing to ivermectin (19, 41), and hence the need to develop anthelmintics which act at novel sites. In 1992, Sasaki *et al.* (42) reported the isolation and structure of PF-1022A (Fig. 1A), a fermentation product of *Mycelia sterilia*, obtained from microflora on a camellia from Japan. PF-1022A is an *N*-methylated 24-membered cyclo-octadepsipeptide consisting of two molecules of D-lactic acid, two molecules of D-3-phenyllactic acid and four molecules of L-*N*-methylleucine, *i.e.*, cyclo(D-lactyl-L-*N*-methylleucyl-D-3-phenyllactyl-L-*N*-methylleucyl-D-lactyl-L-*N*-methylleucyl-D-3-phenyllactyl-L-*N*-methylleucyl). PF-1022A has potent anthelmintic activity against the chick intestinal nematode *Ascaridia galli* and shares a similar structure with bassianolide, an insecticide, and enniatins, which have anthelmintic activity (43). PF-1022A paralyzed a number of parasitic nematodes, including *Angiostrongylus cantonensis* (44), *A. suum* (43), *Haemonchus contortus* (45), *Strongyloides ratti*, *Nippostrongylus brasiliensis, Ancylostoma caninum, Trichostrongylus colubriformis* and *Dictyocaulus viviparus* (46).

The bis-paramorphonyl derivative of PF-1022A, PF-1022-221, was patented in 1993 and is now named emodepside (Fig. 1B). Emodepside is highly effective against a wide range of parasitic nematodes (47) and paralyzes benzimidazole- and ivermectin-resistant nematodes, indicating that it possesses a novel site of action. Recently, a new method has been developed whereby derivatives of PF-1022A with modifications in their benzene ring at the *para*-position can be synthesized (48). This involved introducing the *p*-aminophenylpyruvate biosynthetic pathway from *Streptomyces venezuelae* into *Rosellinia* sp. PF1022 strain deficient in chorismate mutase.

Proposed mechanisms of action of PF-1022A

The first report regarding a possible mechanism of action of PF-1022A was from Terada (44) using *A. cantonensis*. PF-1022A at a concentration of < 1 pM irreversibly inhibited the motility of *A. cantonensis*, while higher concentrations induced paralysis. However, paralysis was partially reversed by the addition of bicuculline (30 μM) and picrotoxin (50 μM). The spasmogenic

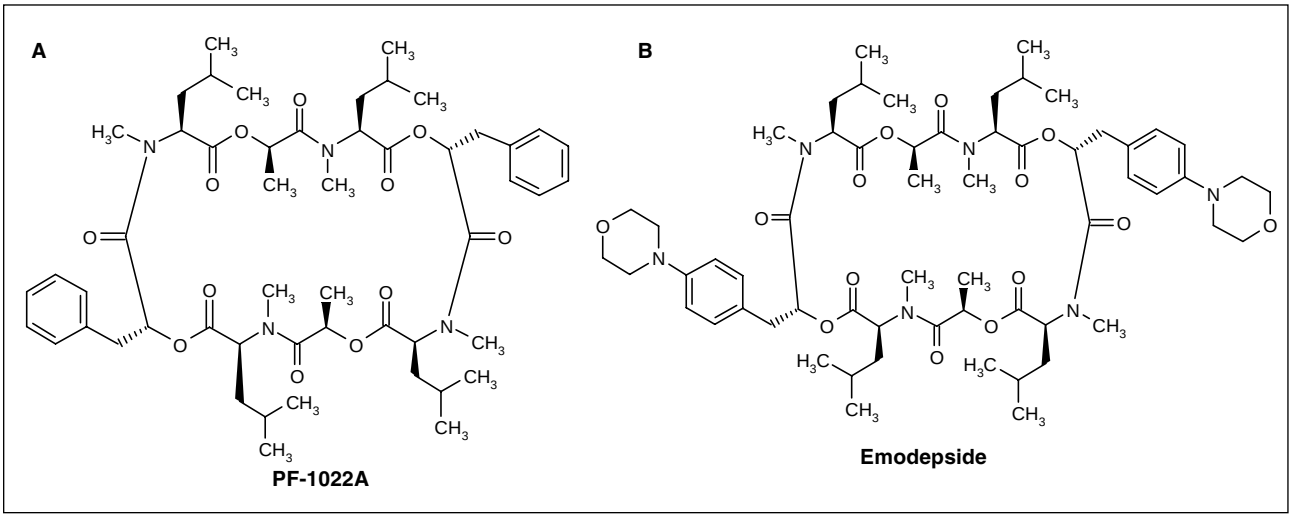

Fig. 1. Structures of PF-1022A and emodepside (PF-1022-221, Bay-44-4400).

effects of *N*-methylcytisine, which releases acetylcholine, were inhibited when given following paralysis induced by 1.05 pM PF-1022A. However, in the presence of PF-1022A and *N*-methylcytisine, picrotoxin did induce contractions. Pyrantel (30 nM) contracted the muscle in the presence of PF-1022A, indicating that PF-1022A did not directly act on nicotinic acetylcholine receptors. The addition of 20 mM calcium contracted the muscle paralyzed with PF-1022A, but this contraction and the accompanying motility could again be blocked with a further application of PF-1022A. PF-1022A (1.05 μM) relaxed frog rectus muscle precontracted with guanidine (2.5 mM), while pyrantel (100 μM) induced a strong contraction of the rectus muscle prerelaxed with PF-1022A. From his results, Terada concluded that PF-1022A stimulated the release of GABA, the body wall muscle-inhibitory transmitter (49), which caused muscle relaxation. In addition, he considered that PF-1022A might affect the release mechanism for acetylcholine.

Subsequent experiments showed that PF-1022A did not antagonize the action of levamisole on *A. suum* body wall muscle (50). These authors found that PF-1022A had no apparent effect on the membrane potential of *A. suum* body wall muscle cells, but did induce a small chloride-dependent increase in input conductance, suggesting a possible role for chloride. This effect did not occur with the optical antipode PF-1022-001.

Cyclodepsipeptides, as with valinomycin, enniatin A and beauvericin, can form ionophores whose ion carrier properties have been investigated using lipid layer membranes. It was proposed that PF-1022A acted through this mechanism (43). Intracellular recordings were made from *A. suum* somatic muscle cells which possessed spontaneous action potentials. When PF-1022A was added to this preparation, all activity ceased within 5 h. In contrast to PF-1022A, its optical antipode PF-1022-001 had no effect on this activity even after 14-h incubation. The ion carrier properties of PF-1022A and PF-1022-001 were also investigated on planar lipid bilayers. Both compounds induced very similar high bilayer conductivity, clearly showing that cation complexation could not be the explanation for the anthelmintic activity of PF-1022A. Thus, while PF-1022A and PF-1022-001 did not differ in their properties as ionophores, they had very different anthelmintic actions. Further evidence for this conclusion came from the use of the linear octadepsipeptide SJB-1822, which is potent in paralyzing *A. suum* and in suppressing muscle cell action potentials, but is completely inactive in terms of modifying bilayer conductivity.

The inhibitory effects of PF-1022A (0.1 nM-10 μM) were investigated on the binding of (2,3-[^{3}H](N))-GABA, (methyl-[^{3}H])-bicuculline and (butyl-4-[^{3}H])-baclofen to isolated muscle membranes of *A. suum* (51). PF-1022A produced a concentration-dependent inhibition of both GABA and bicuculline binding with a K_i of 74.1 ± 8.5 nM and 720 ± 52 nM, respectively. These K_i values were very similar to those obtained for the GABA agonist muscimol, *i.e.*, 78.1 ± 5.8 nM and 790 ± 67 nM, respectively, against GABA and bicuculline binding. The ability of piperazine to displace GABA and bicuculline binding was lower than that of PF-1022A by about a factor of 10. PF-1022A only weakly displaced baclofen binding and its K_i value was over 100 times greater than that for baclofen itself, while piperazine did not displace baclofen binding. These data show that PF-1022A can displace labeled ligands which are selective for the mammalian GABA receptors from *A. suum* somatic muscle membranes. It is not clear what the baclofen was binding to in this preparation, since 5-aminovaleric acid was unable to displace baclofen and baclofen had no effect on *A. suum* muscle membrane potentials (52). In addition, bicuculline does not block nematode GABA receptors, but it does block *A. suum* somatic muscle acetylcholine receptors with an IC_{50} value of 182 μM (53-55).

Another link between GABA and the action of cyclic depsipeptides arises from the interaction between

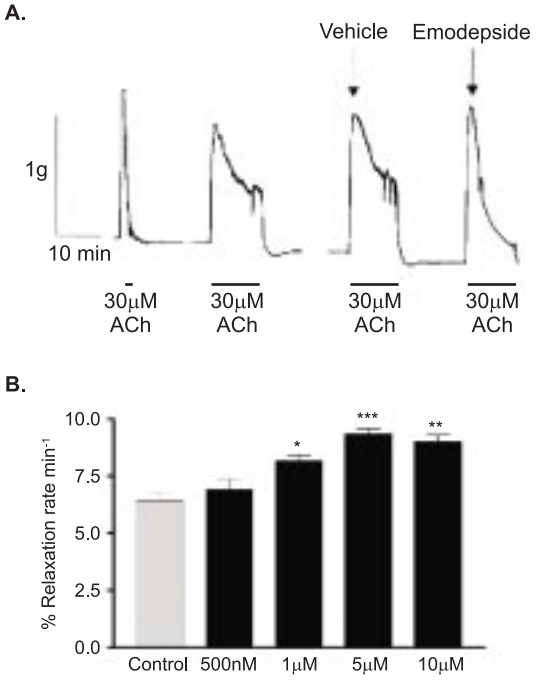

Fig. 2. The effect of varying concentrations of emodepside on prolonged acetylcholine-induced contractions of *A. suum* body wall muscle. **A**. Example of the effect of emodepside on muscle relaxation rate. The horizontal bar indicates the duration of application of acetylcholine. The arrows indicate the addition of either vehicle (0.1% ethanol) or 10 μM emodepside at the peak of the contraction. **B**. Pooled data for different concentrations of emodepside (n=5). The relaxation was measured as the difference in tension at the peak of the response and prior to the wash, expressed as a % of the peak contraction and divided by 10, to yield the % relaxation rate min^{-1}. Control is the relaxation rate with acetylcholine alone. *$p < 0.05$; **$p < 0.01$; *** $p< 0.001$, compared with control (reproduced with permission from Ref. 57, Cambridge University Press).

piperazine and emodepside (56). A combination of piperazine and emodepside produced an expulsion rate of *Heterakis spumosa* from the intestine of mice more than 5 orders of magnitude greater than the sum of the effects of the compounds alone. The combination of these anthelmintics also resulted in a significantly greater degree of degeneration of the intestine and nerve cords of *H. spumosa* compared with the compounds administered on their own.

The experiments described above suggested a possible link between GABA and the mechanism of action of PF-1022A. In the late 1990s, a new study using electrophysiological and pharmacological techniques was undertaken using the body wall muscle of *A. suum* to further explore the mechanism of action of PF-1022A and emodepside (57). For these pharmacological studies, 1-cm strips of dorsal body wall muscle were suspended in an organ bath and recordings made using an isometric transducer. Emodepside, up to 10 μM, produced a small relaxation of basal tension of the muscle strip. Emodepside, 10 μM, also reduced the amplitude of a twitch contraction of the muscle due to application of 30 μM acetylcholine, the body wall muscle excitatory transmitter (58). This effect was maximum following incubation of the muscle with emodepside for 10 min prior to the application of acetylcholine. The threshold for the inhibition of the acetylcholine contraction was around 5 μM. Emodepside also increased the rate of relaxation of the muscle precontracted with acetylcholine, with a threshold of < 1 μM emodepside (Fig. 2A and B).

A comparison was also made between this effect of emodepside and that of GABA on the rate of relaxation of *A. suum* muscle precontracted with acetylcholine (Fig. 3A and B). The effect of GABA was much faster than that of emodepside, with a relaxation rate of 1.1 ± 0.9 g/sec compared with a value of 0.14 ± 0.09 g/sec for emodepside. The control value for muscle relaxation following precontraction with acetylcholine was 0.02 ± 0.01 g/sec. Since the GABA effect on *A. suum* somatic muscle is mediated through an increase in chloride permeability (59), the GABA and emodepside effects on precontracted muscle were investigated using chloride-free saline. While the effect of emodepside was unaffected in chloride-free saline, the effect of GABA was converted to a very slow relaxation (Fig. 3C), or in some cases a slight contraction. These experiments provide evidence that the effect of emodepside is not mediated through a direct GABAergic pathway.

The *A. suum* neuropeptide AF2 (KHEYLRF amide) (60) is also active on nematode body wall muscle, initially relaxing and subsequently inducing a prolonged contraction of *A. suum* body wall muscle (61). During the contraction phase, rhythmic relaxations occur. Application of emodepside at a threshold concentration of 0.5 μM relaxed the AF2-induced muscle contraction, and higher concentrations abolished the AF2-induced rhythmic activity. The direct effect of emodepside on the somatic muscle was reminiscent of the action of the neuropeptides PF1 (SDPNFLRF amide) and PF2 (SADPNFLRF amide) (62) on this muscle (63, 64), and a comparison was therefore made of the effects of emodepside and PF2. When 10 μM emodepside was added first, it reduced the amplitude of the acetylcholine contraction by 29 ± 3%, and subsequent application of 1 μM PF2 reduced the acetylcholine contraction a further 23%, resulting in a reduction to 48 ± 9% of control. When the reverse experiment was performed, *i.e.*, 1 μM PF2 was added first, the acetylcholine contraction was reduced by 43 ± 10%, with no further reduction when 10 μM emodepside was applied. When both compounds were applied together, the acetylcholine-induced contraction was reduced by 56 ± 3% of control. PF2 1 μM also enhanced the rate of relaxation of the acetylcholine-induced contraction in a similar manner to 10 μM emodepside. For example, application of 1 μM PF2 at the point of maximal acetylcholine contraction increased the rate of relaxation from a control value of 7.41 ± 0.25% min^{-1} to 9.2 ± 0.4% min^{-1} ($p < 0.01$). Following recovery from PF2, the addition of 10 μM

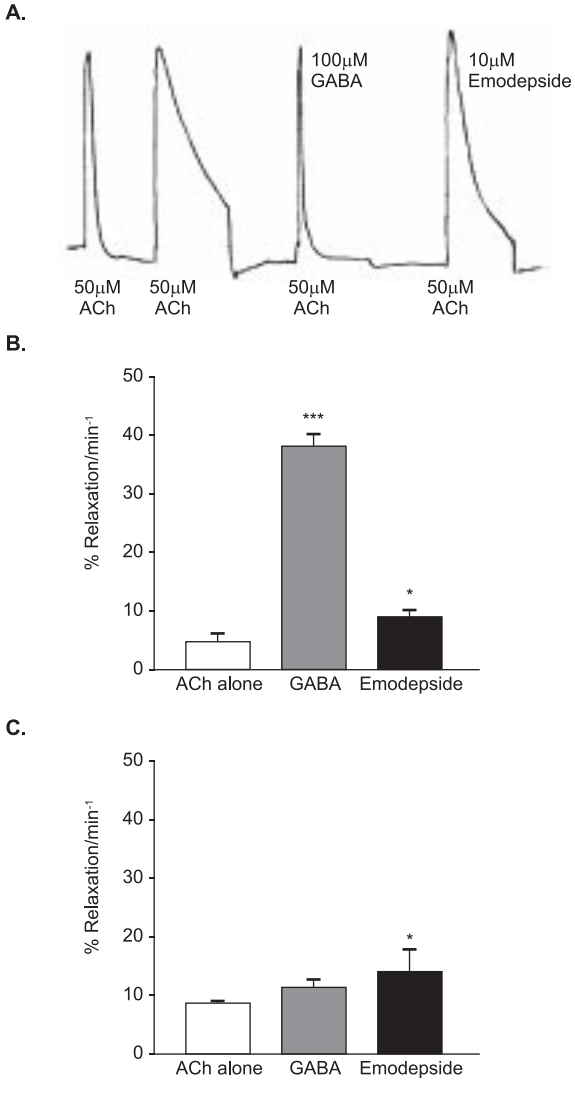

Fig. 3. The effects of GABA and emodepside on acetylcholine-induced contractions of *A. suum* body wall muscle. **A**. The effect of a short 30-sec and a long 10-min application of 50 μM acetylcholine; at the peak of the long response to acetylcholine, 100 μM GABA or 10 μM emodepside was applied. **B**. Pooled data showing the relaxation rates for GABA and emodepside compared with the acetylcholine control. **C**. Pooled data showing the relaxation rates for GABA and emodepside compared with the acetylcholine control, all in chloride-free artificial perienteric fluid. The relaxation was measured as the difference in tension at the peak of the response and prior to the wash, expressed as a percentage of the peak contraction and divided by 10, to yield the % relaxation rate min^{-1}. *$p < 0.05$; ***$p < 0.001$.

emodepside also caused a significant increase in the rate of relaxation of an acetylcholine-induced contraction to 9.0 ± 0.4% min^{-1}. These results demonstrate that emodepside and PF2 appear to act on the muscle in a similar manner.

It is likely that the effects of PF1 and PF2 are at least in part postsynaptic since they act on denervated muscle preparations. In contrast, emodepside had no effect on the acetylcholine-induced contraction in denervated preparations (57). This suggested the possibility that emodepside might act presynaptically to release an inhibitory compound, such as PF1 or PF2. Intracellular recordings from muscle cells revealed that emodepside induced a slow hyperpolarization of the membrane potential without a measurable change in input conductance. Emodepside had no effect on the acetylcholine-induced depolarization of the muscle membrane potential. The slow hyperpolarization induced by emodepside was dependent on external calcium and was enhanced in potassium-free saline, suggesting that both ions are involved in the event. In addition, the potassium channel blockers 4-aminopyridine and tetraethylammonium both reduced the emodepside-induced hyperpolarization, confirming a role for potassium in this event. The emodepside-induced slow hyperpolarization was very different from the fast chloride-mediated response induced by GABA, and it is therefore unlikely to mimic the action of GABA, as previously proposed (50, 51). In contrast, PF1 and PF2 induced slow hyperpolarizations of the *A. suum* membrane potential, which were potassium-mediated events (65, 66). Overall, the data suggested that emodepside acted presynaptically to release an inhibitory neuropeptide which acted postsynaptically to relax the body wall muscle of *A. suum*.

Emodepside as a possible ligand for a latrophilin receptor

A potential receptor for emodepside and related depsipeptides was identified by Saeger *et al*. (67). Using PF-1022A as a ligand, they conducted an immunoscreen against a cDNA expression library constructed from *H. contortus*. A novel orphan heptahelical transmembrane 110-kDa receptor, HC110-R, was isolated. HC110-R had up to 30% amino acid identity with G-protein-coupled receptors of the secretin family and about 31% amino acid identity with latrophilin receptors from humans, cattle and rats. Both HC110-R and mammalian latrophilin receptors share conserved sequences within the transmembrane domains, the lectin-binding domain, a Cys signature, a conserved 4-Cys motif in front of the transmembrane region, and the PEST- and proline-rich regions of the *C*-terminus (Fig. 4). There are also highly conserved Cys-Gly residues in the first extracellular loop preceding the third transmembrane domain and Cys-Trp residues in the second extracellular loop in front of the fifth transmembrane domain. The *N*-terminus of HC110-R does not contain the olfactomedin region or the Pro/Thr region of latrophilin (67). HC110-R and a heptapeptide transmembrane 113-kDa protein, CE B0457.1, from *C. elegans* share 48% identity and 76% similarity (67).

α-Latrotoxin, a neurotoxic protein from the venom of the black widow spider *Latrodectus mactans tredecimguttatus*, is a ligand for the mammalian latrophilin receptor (68). However, the endogenous ligand is

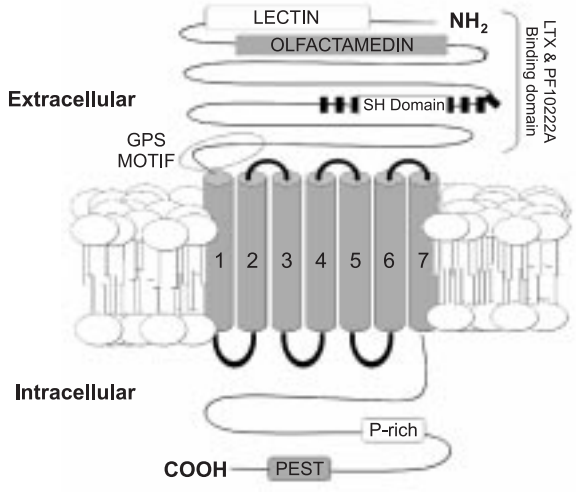

Fig. 4. Schematic view of HC110-R disposition in the plasma membrane (after Ref. 47).

unknown. α-Latrotoxin bound the extracellular *N*-terminal region of HC110-R transfected in HEK-293 cells and induced an influx of calcium through channels which were blocked by both cadmium and nifedipine. Emodepside antagonized α-latrotoxin binding to HEK-293 cells transfected with HC110-R. However, emodepside on its own did not induce calcium signaling in HEK-293 cells transfected with HC110-R. It was concluded that both PF-1022A and α-latrotoxin bound to the *N*-terminal portion of HC110-R (67) and that identification of the natural ligand for HC110-R would reveal which nematode physiological signaling pathways were affected by both PF-1022A and emodepside.

Since *C. elegans* contains a latrophilin-like receptor, CE B0457.1, the effects of emodepside were tested using two *C. elegans* behavioral assays: locomotion (body bends) and pharyngeal pumping (69). Emodepside reduced both locomotion and pharyngeal pumping in *C. elegans* with IC_{50} values of 4.2 nM and 4.6 nM, respectively. PF-1022A also inhibited pharyngeal pumping with an IC_{50} of 25 nM. Experiments using the pharynx were extended to recording electropharyngeograms using extracellular suction electrodes (Fig. 5). Following removal from the animal, the resting pumping rate is around 0.1 Hz, and 5-HT was therefore added to increase the pumping rate (13). The effect of emodepside on pumping rate was determined by comparing the pumping rate following the addition of 5-HT before and after application of emodepside. These experiments comparing the effect of emodepside on the 5-HT response gave an IC_{50} value of 4.1 nM for wild-type *C. elegans*. In addition to numerous *C. elegans* mutant strains lacking genes known to be important in G-protein-mediated vesicular release mechanisms, RNA interference (RNAi) techniques were used to provide further insight into the mediators of emodepside action. The *rrf-3(pk1426)* allele of *C. elegans,* chosen for these RNAi experiments for its hypersensitivity to this technique, was slightly more sensitive to emodepside, with an IC_{50} value of 2.9 nM (Fig. 6C). The significance of this finding is unclear, but it is unlikely that the RNA-directed RNA polymerase (RdRP) encoded by *rrf-3* is involved in the effects of emodepside due to the marginal hypersensitivity observed. Figure 6A shows a control example where a second application of 5-HT (on right of figure) induced a clear response. However, in Figure 6B, following the application of 100 nM emodepside, a second application of 5-HT had no effect. This concentration of emodepside caused a similar inhibition of pharyngeal pumping following stimulation of the pharynx with 100 nM of the *A. suum* peptide AF1 (KNEFIRF amide). PF-1022-001, the optical antipode of PF-1022A, failed to inhibit pumping rate or alter the response to a subsequent application of 5-HT. Intracellular recordings from pharyngeal muscle cells revealed that emodepside 100 nM elicited a slow depolarization (5.8 ± 2.6 mV), with inhibition of pharyngeal action potentials (69). Similar recordings from pharyngeal muscle cells also showed that PF1 and PF2 inhibited the generation of action potentials in this muscle (13).

Identification of the emodepside receptor of *C. elegans*

As mentioned above, a latrophilin-like receptor, B0457.1, LAT-1, was identified in *C. elegans* (67), and these authors suggested that *C. elegans* might be a useful model system for determining the mode of action of PF-1022A and related depsipeptides. A second latrophilin-like receptor, B0286.2, LAT-2, was also identified using a BLASTA search of the *C. elegans* genome data. A latrophilin mutant *lat-1 (ok379)* was resistant to emodepside on the pharyngeal muscle, indicating a role for latrophilin-like receptors in mediating the action of this drug (69). However, emodepside was still effective at inhibiting locomotion in the *lat-1 (ok379)*, suggesting that *lat-2* may be involved in mediatin0g the inhibitory actions of emodepside on locomotion. The expression pattern of *lat-1*, evaluated using a *lat-1*:: DsRed2 reporter construct, demonstrated expression in the pharynx of

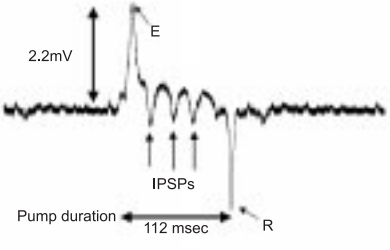

Fig. 5. Example of an electropharyngeogram (EPG) trace of a single pump from *C. elegans*. E and R correspond to excitation and relaxation phases of the pharyngeal muscle contraction. Inhibitory postsynaptic potentials (IPSPs) are thought to arise from synaptic transmission generated from the M3 inhibitory motoneuron.

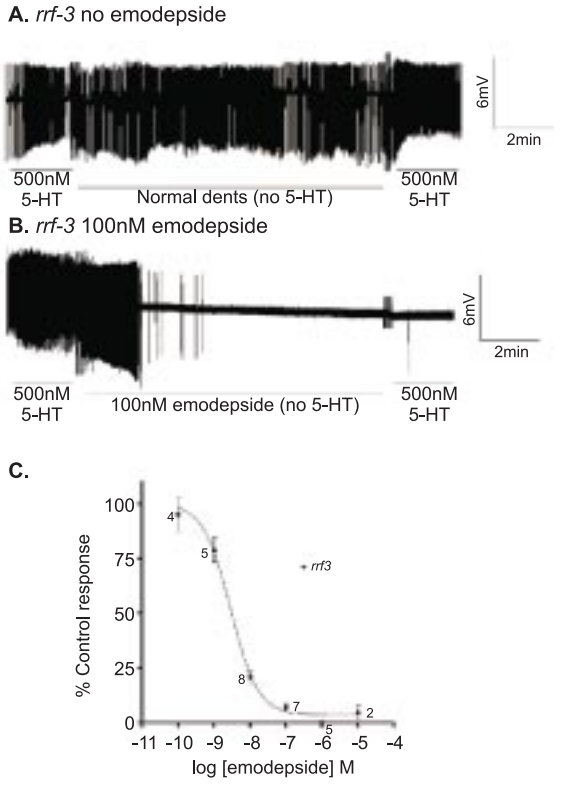

Fig. 6. Effect of emodepside on *rrf-3(pk1426) C. elegans* pharyngeal pumping. **A**. An extracellular recording of the pharyngeal muscle (EPG). Each vertical line represents the electrical activity associated with a single muscle pump, providing a continuous record of muscle activity. 500 nM 5-HT was applied to stimulate pumping. 5-HT was applied for 2-min periods separated by a 10-min application of either vehicle control (0.1% ethanol) or emodepside. **A** is a typical result from a control experiment in which the pharynx continues to pump throughout the entire time course of the experiment and responds to a second application of 5-HT. **B**. The effect of 100 nM emodepside application and the failure of the muscle to respond to a second application of 5-HT. **C**. Concentration-response curve for the effect of varying concentrations of emodepside on pharyngeal pumping rates in *rrf-3(pk1426) C. elegans*. % control response is the rate of pumping in 5-HT following emodepside application compared to the initial pumping rate in 5-HT. Each point is the mean ± S.E. Mean of 'n' determinations as indicated on the graph.

larval stages but not adult animals. However, *lat-1* was expressed in certain pharyngeal and extrapharyngeal neurons, suggesting that emodepside acted presynaptically on a latrophilin receptor to release a transmitter, possibly a neuropeptide, which then inhibited pharyngeal activity and relaxed the muscle. Evidence in support of this came from the use of the rhodamine fluorescent marker FM4-64 to label active synapses (70, 71). Emodepside induced a loss of fluorescence from the synaptic regions compared with the control, where the fluorescence remained stable for up to 30 min. This provided evidence that emodepside induced vesicular release in a manner similar to α-latrotoxin (72, 73).

Role of G-proteins in the action of emodepside

To further investigate the role of a presynaptic signaling pathway in the action of emodepside, the sensitivity to emodepside of a number of mutants for presynaptic proteins was determined (69). Latrophilin receptors couple to Gαq (74) and so the first mutant examined was *egl-30(ad806),* with a loss of function in the Gαq protein (75). Pharyngeal pumping and locomotion were less sensitive to emodepside in these mutants compared with wild-type controls, while in the gain-of-function mutant for *egl-30 (tg26)*, emodepside was more potent on pharyngeal pumping and locomotion compared with wild-type controls. These results suggest that the latrophilin receptor couples to Gαq to mediate the effect of emodepside in *C. elegans*.

Gαq has been shown to stimulate the release of acetylcholine at the *C. elegans* neuromuscular junction through activation of phospholipase C-β (PLC-β) and the production of diacylglycerol (DAG) (76, 77), while Gαo inhibits release through activation of a DAG kinase (78, 79). Mutants were selected to test their sensitivity to emodepside, *e.g.*, *egl-8(md1971)*, *egl-8(n488)* and *goa-1(n1134)*. PLC-β is encoded by *egl-8* (78, 80), while *goa-1(1134)* is a Gαo loss-of-function mutant. These *egl-8* alleles have disruptions to the catalytic Y domain of the gene which encodes PLC-β. Both *egl-8* mutants were less sensitive to emodepside on pharyngeal pumping and locomotion. In contrast, the *goa-1(n1134)* mutants were more sensitive to emodepside on both pharyngeal pumping and locomotion.

PLC-β hydrolyzes phosphatidylinositol-4,5-bisphosphate (PIP2) to produce inositol-1,4,5-triphosphate (IP3) and DAG, and the latter binds to the C1 domain of UNC-13 (81, 82). UNC-13 primes synaptic vesicles for fusion by promoting the open configuration of syntaxin (83), a presynaptic plasma membrane protein involved in synaptic vesicle fusion (84), and therefore, two *unc-13* mutants were tested for their sensitivity to emodepside. Pharyngeal pumping and locomotion in both mutants were less sensitive to emodepside compared with wild-type controls. Emodepside was also less potent on mutants for synaptobrevin, a vesicle-localized protein which mediates vesicle release by forming a complex with syntaxin and SNAP-25 (85).

Conclusions

These results, obtained using *C. elegans* mutants, strongly support the hypothesis that emodepside activates a latrophilin receptor which acts through a PLC-β pathway to hydrolyze PIP2 to produce DAG. This process is negatively regulated through the effect of Gαo. DAG acts on UNC-13 to increase the size of

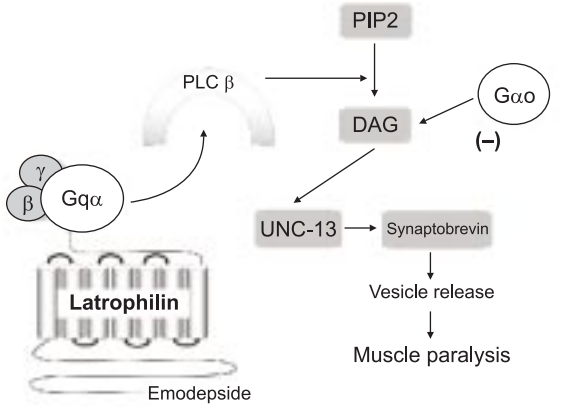

Fig. 7. Proposed mechanism of action following activation of a presynaptic latrophilin receptor by emodepside (after Ref. 69).

the readily releasable pool of transmitter (Fig. 7). This transmitter relaxes both pharyngeal and somatic body wall muscle, resulting in flaccid paralysis of the nematode. Precisely what is released remains to be determined, but intriguingly, the evidence from the *A. suum* studies suggests that emodepside may preferentially release a neuropeptide. The data from the studies using *C. elegans* suggest that emodepside interacts primarily with *lat-1* receptors on pharyngeal muscle and *lat-2* receptors on body wall muscle. The transmitter may also be released at additional sites and alter the activity of other physiological processes in the animal, including egg laying.

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