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Characterization of β-leptinotarsin-h and the effects of calcium flux antagonists on its activity

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Abstract

β-Leptinotarsin-h, purified from the hemolymph of the beetle *Leptinotarsa haldemani*, is a potent (~1 nM) neuroactive protein that rapidly (few seconds) stimulates Ca^{2+} influx and neurotransmitter release. Our goals were to further characterize β-leptinotarsin-h and to test the hypothesis that it stimulates Ca^{2+} influx through presynaptic Ca^{2+} channels. Analysis of partial amino acid sequences revealed that β-leptinotarsin-h is a unique protein with significant similarity to only one other protein, the juvenile hormone esterase of *Leptinotarsa decemlineata*, commonly known as the Colorado potato beetle. We have examined the effect of β-leptinotarsin-h on Ca^{2+} current, Ca^{2+} uptake, Ca^{2+} levels, and neurotransmitter release in synaptosomes, cell lines, and neuronal systems. We found that its preferred site of action appears to be mammalian presynaptic nerve terminals. We tested antagonists of Ca^{2+} flux for their effects on β-leptinotarsin-h-stimulated Ca^{2+} uptake in rat brain synaptosomes. The non-selective Ca^{2+} channel blockers flunarizine, Ni^{2+}, ruthenium red, high-concentration thapsigargin, and SKF 96365 inhibited β-leptinotarsin-h's activity, but none of the tested selective blockers of voltage-operated Ca^{2+} channels (ω-agatoxin IVA, ω-conotoxin GVIA, ω-conotoxin MVIIC, nicardipine, nifedipine, SNX-482) was inhibitory. Selective inhibitors of ligand-operated, store-operated, and transduction-operated channels were also not inhibitory. β-Leptinotarsin-h did not stimulate Na^{+} uptake, ruling out Na^{+} channels and many non-selective cation channels as targets. We conclude that β-leptinotarsin-h stimulated Ca^{2+} uptake through presynaptic Ca^{2+} channels; which channel is yet to be determined. β-Leptinotarsin-h may prove to be a useful tool with which to investigate calcium channels and calcium flux.
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Keywords: Ca^{2+} channel; Ca^{2+} uptake; Synaptosomes; *Leptinotarsa haldemani*

1. Introduction

The level of intracellular Ca^{2+} regulates many processes in neurons, including neurotransmitter release (Bennett, 1997). Release of neurotransmitters is normally triggered by a rapid influx of Ca^{2+} through voltage-operated Ca^{2+} channels that are opened by depolarization of the presynaptic membrane. Any agent, however, that promotes Ca^{2+} influx and a sufficient increase in the intraterminal level of Ca^{2+} can cause neurotransmitter release.

β-Leptinotarsin-h is a potent, rapidly acting protein that promotes the influx of Ca^{2+} and release of neurotransmitters from presynaptic terminals (McClure et al., 1980; Crosland et al., 1984; Madeddu et al., 1985a,b). In a seminal

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14. ABSTRACT

ß-Leptinotarsin-h, purifed from the hemolymph of the beetle Leptinotarsa haldemani, is a potent neuroactive protein that stimulates Ca2+ influx and neurotransmitter release. Our goals were to further characterize ß-leptinotarsin-h and to test the hypthesis that it stimulates Ca2+ influx through presynaptic Ca2+ channels. Analysis of partial amino acid sequences revealed that ß-leptinotarsin-h is a unique protein with significant similarity to only one other protein, the juvenile hormone esterase of Leptinotarsa decemlineata, commonly known as the Colorado potato beetle. We examined the effect of ß-leptinotarsin-h on neurotransmitter release, Ca2+ current, Ca2+ uptake, and Ca2+ levels in several cell lines and neuronal systems. We found that its preferred site of action appears to be mammalian presynaptic nerve terminals. We tested antagonists of Ca2+ flux for their effects on ß-leptinotarsin-h-stimulated Ca2+ uptake in rat brain synaptosomes. We found that the nonselective Ca2+ channel blockers flunarizine, Ni2+, ruthenium red, high-concentration thapsigargin, and SKF 96365 inhibited ß-leptinotarsin-h's activity, but that non of the selective blockers of voltage-operated Ca2+ channels (w-agatoxin, IVA, w-conotoxin GVIA, w-conotoxin MVIIC, nicardipine, SNX 482) that we tested was inhibitory. Selective inhibitors of ligand-operated, store-operated, and transduction-operated channels were also not inhibitory. ß-leptinotarsin-h did not stimulate Na+ uptake, ruling out Na+ channels and nonselective cation channels as targets. We conclude that ß-leptinotarsin-h stimulated Ca2+ channels, which channel is yet to be determined.

15. SUBJECT TERMS
leptinotarsin, neurotransmitter release, calcium channel, pharmacology, modulation

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study (McClure et al., 1980) β-leptinotarsin-h caused a large increase in the frequency of miniature endplate potentials at the rat neuromuscular junction, followed by a decrease in frequency to zero. In the same study β-leptinotarsin-h stimulated the release of acetylcholine from rat brain synaptosomes. Both effects of β-leptinotarsin-h were reduced when Ca^{2+} was omitted from the bathing solutions. Crosland et al. (1984) confirmed the extracellular Ca^{2+} dependency of β-leptinotarsin-h-stimulated neurotransmitter release and discovered that β-leptinotarsin-h promoted the uptake of Ca^{2+} into synaptosomes, suggesting that β-leptinotarsin-h stimulated neurotransmitter release by promoting Ca^{2+} influx and increasing the intrasynaptosomal level of Ca^{2+}.

Further investigation (Crosland et al., 1984) demonstrated that β-leptinotarsin-h fulfilled the classical requirements of a substance that stimulates Ca^{2+} current (Hagiwara and Byerly, 1981), as indirectly measured by neurotransmitter release from synaptosomes, and it was hypothesized that β-leptinotarsin-h promoted Ca^{2+} uptake through presynaptic Ca^{2+} channels. This interpretation was called into question, however, when verapamil failed to inhibit β-leptinotarsin-h-stimulated Ca^{2+} uptake in guinea pig cortex synaptosomes and PC12 cells (Madeddu et al., 1985a), and verapamil and nitrendipine failed to inhibit β-leptinotarsin-h-stimulated release of dopamine from synaptosomes and PC12 cells (Madeddu et al., 1985b). At that time it was believed that verapamil and nitrendipine inhibited 'the' voltage-operated Ca^{2+} channel. It is now known that there are six pharmacologically defined voltage-operated Ca^{2+} channels based on 10 pore-forming subunits. In addition, there are many other types of Ca^{2+} channels on which β-leptinotarsin-h could act (Meir et al., 1999). Some Ca^{2+} channels have quite selective inhibitors that can be used to identify a particular channel (Uchitel, 1997). We investigated the effects of these inhibitors and other antagonists of Ca^{2+} flux on the activity of β-leptinotarsin-h, with the aim of determining on which Ca^{2+} channel it acts.

2. Materials and methods

2.1. Reagents

We purchased 2-APB from Calbiochem (San Diego, CA), KB-R7943 from Tocris Cookson (Ellisville, MO), and SNX-482 from Alomone Labs (Jerusalem, Israel). All other reagents not otherwise noted were from Sigma Chemical Co. (St Louis, MO). We referred to compounds by their catalog names. Water-insoluble reagents were dissolved in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide was no more than 1% by volume, which had no effect on Ca^{2+} uptake (data not shown). We dissolved all water-soluble reagents except fluorescent dyes in balanced saline solution (140 mM NaCl, 4.5 mM KCl, 10 mM glucose, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM HEPES, and 0.25 mg/ml BSA, pH 7.4).

A calcium ion fluorescent dye kit (catalog number R-8041) was purchased from Molecular Devices, Sunnyvale, CA. The kit contains a proprietary no-wash dye (Krahn et al., 2002). Calcium ion dye solution was prepared by dissolving the contents of one bottle of the dry reagent in Hank's Balanced Salt Solution/HEPES to a volume of 15 ml. In our hands, this was the maximal useful dilution for the cells studied here. The dye solution was kept in the dark and was stable at least 8 h at room temperature and 2 weeks in a −20 °C freezer. The response of the dye was sensitive to dye concentration and loading time, as are other intensity dyes. The efficiency of dye loading was quite good in all lines reported here, allowing a reduction in dye usage of 5- to 12-fold relative to recommended loading described in package literature, without substantial degradation of response characteristics.

2.2. Animals

BALB/C, 20–25 g mice and Sprague–Dawley, 100–150 g rats (Harlan Sprague–Dawley, Frederick, MD) were maintained on a 12 h light–dark (18:00–06:00) cycle and allowed free access to food and water. Their care and use complied with the Animal Welfare Act (USA) and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (USA).

2.3. Purification of β-leptinotarsin-h

We prepared β-leptinotarsin-h according to Madeddu et al. (1985a) after Crosland et al. (1984) except that size exclusion chromatography was performed using a Waters TSK 300SW stainless steel column on a Waters isocratic high pressure liquid chromatography system (Waters Corporation, Milford, MA). Due to the limited supply of hemolymph we used the active fractions (termed 'Peak A') from the size exclusion column for many experiments in order to maximize the amount and stability of the Ca^{2+} uptake activity. 'β-Leptinotarsin-h' refers to the highly purified preparation eluted from the Cibacron Blue 3GA Sepharose column (Supelco, St Louis, MO). Peak A is approximately 1% β-leptinotarsin-h by mass (Crosland et al., 1984; Madeddu et al., 1985a) and has the same characteristics as β-leptinotarsin-h with respect to Ca^{2+} uptake activity, making it a satisfactory preparation for activity measurements (Crosland et al., 1984; Madeddu et al., 1985a).

A characteristic of β-leptinotarsin-h that hindered its study was the very low recovery of activity after it had been in contact with artificial membranes (e.g. dialysis tubing), suggesting that β-leptinotarsin-h has some hydrophobic character, which was also observed by Madeddu et al. (1985a). This characteristic made concentrating β-leptinotarsin-h or exchanging its solution difficult. Recovery of

activity could be enhanced by adding another protein (e.g. bovine serum albumin (BSA), aprotinin) at concentrations >0.25 mg/ml, but this was not always desirable.

Protein was assayed according to the procedure of Lowry et al. (1951) or Bradford (1976) using BSA as a standard.

2.4. Ca^{2+} uptake

We prepared rat brain synaptosomes according to Hajós (1975). The final synaptosomal pellet was suspended in 6 ml of oxygenated, balanced saline per gram of brain.

Depending on the experiment, we used one of two assay protocols. In experiments not requiring preincubation of synaptosomes with agent (e.g. assaying column fractions), the agent of interest (typically β-leptinotarsin-h) (in 0.9 ml of balanced saline containing 0.5 μCi of $^{45}Ca^{2+}$ per sample (ICN Pharmaceuticals, Inc., Costa Mesa, CA)) and 0.1 ml of synaptosomes (~0.1 mg protein) were preincubated separately for 15 min at 30 °C, and then mixed (Protocol 1).

In experiments that required preincubation of synaptosomes with agent, synaptosomes and the agent of interest (typically an antagonist of Ca^{2+} flux) were preincubated together for 15 min at 30 °C, and then the Ca^{2+} uptake-stimulating agent (usually β-leptinotarsin-h) and $^{45}Ca^{2+}$ were added (Protocol 2). For some experiments, the temperature and length of time of incubation were varied.

Typically after 75 s of incubation at 30 °C with both protocols, we added 4 ml of ice-cold stopping solution (140 mM NaCl, 4.5 mM KCl, 1 mM $MgCl_2$, 1 mM $NiCl_2$, and 10 mM HEPES, pH 7.4). The synaptosomes were immobilized on prewashed Whatmann GF/B filters (Brandel, Gaithersburg, MD) on a Brandel M-30R cell harvester or on prewashed Millipore AAWP filters on a Millipore Sampling Manifold (Billerica, MA) and washed three times with 4 ml of cold stopping solution. Radioactivity associated with the washed synaptosomes was counted in 5 ml of Ready-Protein+ (Beckman Corporation, Fullerton, CA) and considered a measure of Ca^{2+} uptake. β-Leptinotarsin-h-stimulated Ca^{2+} uptake was calculated as nmol of Ca^{2+} uptake in the presence of β-leptinotarsin-h less nmol of Ca^{2+} uptake in its absence (control).

2.5. Na^+ uptake

We measured Na^+ uptake using modifications of the procedure of Tamkun and Catterall (1981). Synaptosomes were preincubated at 22 °C in 0.75 ml of incubation solution (containing 0.25 mg/ml BSA instead of 1 mg/ml). After 15 min we added 0.25 ml of incubation solution containing 10 mM ouabain, 10.6 mM NaCl, 1 μCi of $^{22}NaCl$ (Amersham Biosciences, Piscataway, NJ), and the agent of interest, and continued incubation for 2.5 min. We stopped uptake with 4 ml of cold incubation solution containing 1 mM $NiCl_2$. The synaptosomes were processed as described above for Ca^{2+} uptake, and the radioactivity associated with the washed synaptosomes was considered a measure of Na^+ uptake.

2.6. Production and selection of monoclonal antibodies

We immunized mice with approximately 10 μg of β-leptinotarsin-h. Monoclonal antibodies were generated by cell fusions with SP2/0 parent myeloma cells following the procedure of Rener et al. (1985) (Crosland, 1996). We identified antibodies of interest by their ability, when combined with Protein G-Sepharose (Sigma Chemical Co., St Louis, MO), to immunoprecipitate Ca^{2+} uptake activity from solutions containing Peak A. The supernatants of four wells contained antibodies that presumably bound β-leptinotarsin-h, the most robust of which (MAb 2C3; IgG_1 subclass) we chose for further study.

2.7. Immunopurification of β-leptinotarsin-h

We cleared lyophilized *Leptinotarsa haldemani* hemolymph by first dissolving 25 mg of it in 1.0 ml of lysis buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, pH 7.8). After 20 min at room temperature, the solution was microfuged, and the supernatant was transferred to a fresh microfuge tube. Twenty micrograms of goat anti-mouse IgG antibody (Kirkegaard & Perry, Gaithersburg, MD) and 75 μl of diluted Protein G-Sepharose were added to the supernatant and rotated for 2 h at 4 °C. The tube was microfuged, and the supernatant was transferred to a fresh microfuge tube containing 75 μl of fresh, diluted, Protein G-Sepharose. After 1 h at 4 °C the tube was microfuged, and the supernatant (cleared hemolymph) was removed and stored at 4 °C overnight.

We added 0.50 mg of MAb 2C3 to the cleared hemolymph and rotated the solution for 2 h at 4 °C. We then added 450 μl of diluted Protein G-Sepharose and rotated the solution for an additional 2 h at 4 °C. The solution was microfuged, and the supernatant was discarded. The beads of Protein G-Sepharose were washed with 1.0 ml of lysis buffer until the supernatant was no longer yellow. The beads were then washed twice in 1 M NaCl and twice more in lysis buffer. We then added 25 μl of 2× Tricine SDS sample buffer (without reducing agents) (Novex, San Diego, CA) and stored the solution overnight at 4 °C.

We heated the solution for 10 min at 90 °C, applied it to a 10% Tricine sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) in a Novex apparatus, and electrophoresed it for 90 min at a constant 100 V. The gel was stained in 0.1% Coomassie blue/1% acetic acid/20% methanol for 5 min and then destained in the same solution sans Coomassie blue. We excised bands of the gel and stored them at −20 °C.

2.8. Internal sequence analysis

We followed the procedure of Jenö et al. (1995) to hydrolyze β-leptinotarsin-h in the excised PAGE bands

and extract the resultant peptides. We used endoproteinase lysine-C (Boehringer Mannheim Corp., Indianapolis, IN) without prior reduction or alkylation of β-leptinotarsin-h.

The extracted peptides were filtered through either 0.25 or 0.45 μm microcentrifuge filters (PGC Scientific, Gaithersburg, MD). Filtered extracts (5 μl) were injected onto a C4 (7 μm, 1000 Å) capillary column (180 μm × 15 cm) (LC Packings, San Francisco, CA). Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile. We eluted the peptides using the following solvent gradient and total flow rates: 0–20% solvent B for 20 min at 150 μl/min, 20–60% solvent B for 20 min at 150 μl/min, and 60–100% solvent B for 20 min at 250 μl/min. The capillary column was attached to an electrospray ionization source fitted to a Finnigan TSQ 700 mass spectrometer (San Jose, CA). The electrospray sheath liquid consisted of 80% isopropanol in 0.1% formic acid and flowed at 4 μl/min.

We acquired electrospray ionization mass spectra by scanning the instrument from *m/z* 400–2000. Product ion spectra were acquired using collision-induced dissociation of doubly charged ions (argon gas at 3.5 mTorr nominal pressure and 10 eV) and scanning from *m/z* 40–1800.

To assist with amino acid composition and sequence determinations, separate aliquots of peptides were methylated, acetylated, or subjected to manual Edman degradation. Masses and product ion spectra were acquired after each treatment. To confirm a putative peptide sequence, experimental sequences were used to synthesize peptides, which were subjected to the same treatment(s) as liberated peptides. We compared the mass spectra of these treated, synthetic peptides to the spectra obtained for the liberated peptides and found them to be identical.

2.9. Intracellular Ca^{2+}

We measured intracellular Ca^{2+} according to the protocol of Fitch et al. (2003) without modification, including reagents, cells, and cell culture conditions (see Section 2.1). Cells were preincubated with the proprietary composition fluorescent dye, a baseline obtained, and Peak A added. We added a cocktail of 5 μM ionomycin + 20 μM *p*-(trifluoromethoxy)phenylhydrazone (FCCP) + 100 μM carbachol (final concentrations) at the termination of the experiment to maximize intracellular Ca^{2+} and provide a reference value with which to normalize measurements to correct for dye loading and cell count. Intracellular Ca^{2+} level [Ca(*i*)] was expressed as $(F_t - F_0)/(F_{max} - F_0)$. Absolute Ca^{2+} concentrations were not measured.

2.10. Data analysis

All data are presented as means ± SEM. We performed one-way analyses of variance, and in the case of a significant difference ($p < 0.05$) among the means, we used the Dunnett post hoc test to ascertain significant differences of individual means from control. Statistics and concentration-effect parameters were calculated using Prism (GraphPad Software, San Diego, CA). We performed all experiments at least twice, and the data were combined.

3. Results

3.1. Monoclonal antibody 2C3 and β-leptinotarsin-h

Titration experiments showed that 10 μg of MAb 2C3 bound the maximal amount of Ca^{2+} uptake activity (data not shown), but that the maximum bound was only 85% of the activity in β-leptinotarsin-h. This revealed the presence of at least two immunologically distinct substances with Ca^{2+}-uptake activity. Immune serum from the mouse that produced MAb 2C3 bound all of the uptake activity at all stages of purification (data not shown), demonstrating that the immunizing β-leptinotarsin-h preparation and Peak A contained all of the Ca^{2+}-uptake activity present in *L. haldemani* hemolymph and that the mouse produced antibodies against the activity.

Although MAb 2C3 bound β-leptinotarsin-h, it did not inhibit its Ca^{2+}-uptake activity (Table 1) or, by inference, β-leptinotarsin-h's binding to synaptosomes. This suggests that MAb 2C3 may be a useful reagent for labeling β-leptinotarsin-h and locating its binding site(s) and target molecule(s).

Preimmune mouse serum had no effect on β-leptinotarsin-h's activity (Table 1), demonstrating that naïve mouse serum contained no substances that inhibited activity. Immune mouse serum, on the other hand, essentially completely inhibited β-leptinotarsin-h's activity. This suggests that immune mouse serum contained antibodies other than 2C3 that inhibited activity, which is consistent with immune

Table 1
Effect of MAb 2C3 on β-leptinotarsin-h-stimulated Ca^{2+} uptake activity

Condition	% of β-Leptinotarsin-h activity[a]
β-Leptinotarsin-h only	100 ± 10
β-Leptinotarsin-h + 10 μg of MAb 2C3	116 ± 9
β-Leptinotarsin-h + 0.75 μl of preimmune mouse serum	103 ± 2
β-Leptinotarsin-h + 0.75 μl of immune mouse serum	6[b] ± 5

β-Leptinotarsin-h and MAb 2C3 or mouse serum were preincubated together for 15 min at 22 °C, then 30 min at 4 °C before determining Ca^{2+} uptake using Protocol 1.

[a] β-Leptinotarsin-h activity = 1.2 ± 0.1 nmol of Ca^{2+} uptake per 0.1 mg synaptosomal protein above basal uptake of 0.9 ± 0.1 nmol.

[b] Significantly different from β-Leptinotarsin-h only.

mouse serum's ability to remove all Ca^{2+} uptake activity from solution.

3.2. Immunopurification and partial sequencing of β-leptinotarsin-h

In order to obtain homogeneous β-leptinotarsin-h for sequencing, we immunoprecipitated it with MAb 2C3 and Protein G-Sepharose. SDS-PAGE resulted in a single prominent band unique to β-leptinotarsin-h at 56 ± 1 kDa (Fig. 1). This value agreed with previous estimates of the molecular weight of β-leptinotarsin-h as determined by size exclusion chromatography (57 kDa) and SDS-PAGE (57 kDa) (Crosland et al., 1984) and size exclusion chromatography (50–60 kDa) and two-dimensional SDS-PAGE (55 kDa) (Madeddu et al., 1985a). We sometimes observed a second, very faint band at 22 ± 1 kDa.

After excising β-leptinotarsin-h from a SDS-PAGE gel and hydrolyzing it with endoproteinase lysine-C, we isolated and sequenced three peptides (Table 2). Comparison of the amino acid sequences of these peptides with sequences in public protein and DNA databases revealed only one protein with significant similarity: the juvenile hormone esterase of *Leptinotarsa decemlineata*, commonly known as the Colorado potato beetle.

3.3. Effects on cell lines

Previous studies (Madeddu et al., 1985a,b) demonstrated that β-leptinotarsin-h was active with PC12 cells, albeit with less potency and efficacy than with synaptosomes. We extended these studies to several other cell lines. Our objective was to find a cell line that is as responsive to β-leptinotarsin-h as synaptosomes are, since cell lines have advantages as an experimental system with which to study neuroactive substances.

As a comparison to cell lines we initially measured the effect of increasing concentrations of Peak A on Ca^{2+} uptake in synaptosomes (Fig. 2). The EC_{50} of Peak A was 6 μg/ml. Assuming that Peak A contains 1% β-leptinotarsin-h, whose molecular weight is 56 kDa, then the EC_{50} of β-leptinotarsin-h with respect to Ca^{2+} uptake was 1 nM, and the apparent Hill coefficient (n_{app}) was 0.9. These values agree well with previous studies (Crosland et al., 1984; Madeddu et al., 1985a).

Table 2
Amino acid sequences of peptides from β-leptinotarsin-h

1.	YGTAVQWIGNSSK[a]
	YGTAVQWIGTVSE (AA 45–57 of juvenile hormone esterase)[b]
2.	YFPEFIIDIAK
3.	RIQVIEEDIDK
	RIQVLEEELDR (AA 189–199 of juvenile hormone esterase)[b]

[a] The mass spectrometer cannot distinguish between leucine and isoleucine.
[b] Vermunt et al. (1997a).

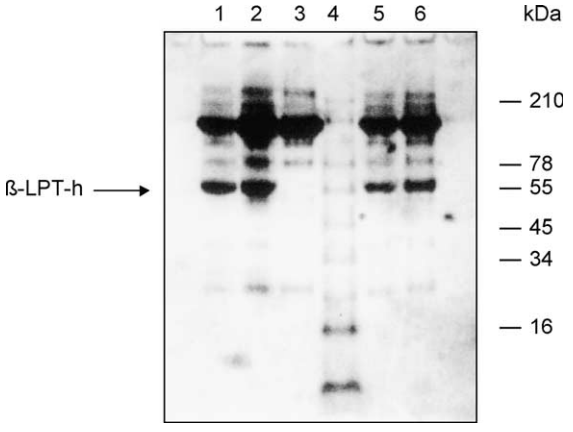

Fig. 1. Immunoprecipitation of β-leptinotarsin-h. β-Leptinotarsin-h (β-LPT-h) was immunoprecipitated and separated on a SDS-PAGE gel as described in Methods. Lanes 1, 2, 5, and 6 contained β-leptinotarsin-h + MAb 2C3. Lane 3 contained MAb 2C3 only. Lane 4 contained molecular weight standards. The molecular weight standard closest to β-leptinotarsin-h is glutamic dehydrogenase, 55 kDa.

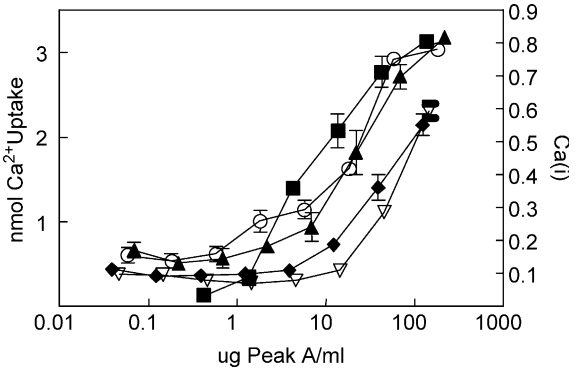

Fig. 2. Concentration–effect of Peak A on intracellular Ca^{2+} levels. Ca^{2+} uptake in synaptosomes (Protocol 1) and intracellular Ca^{2+} levels [Ca(*i*)] in cell lines (Fitch et al., 2003) were measured at 22° after 3 min of incubation with Peak A. Values are expressed per 0.1 mg of synaptosomal/cellular protein. Error bars are SEM. Synaptosomes (filled squares) (Ca^{2+} uptake), SH-SY5Y (open circles), HEK (filled triangles), IMR-32 (filled diamonds), TE-671 (open triangles).

Our measurement of β-leptinotarsin-h's effect in cell lines was change caused by Peak A in intracellular Ca^{2+} (Fitch et al., 2003). Peak A stimulated increases in intracellular Ca^{2+} in all four tested cell lines, but with reduced potency compared to its effect on Ca^{2+} uptake in synaptosomes (Fig. 2). Peak A was approximately one-fourth as potent with HEK ($EC_{50}=21$ μg/ml) and SH-SY5Y ($EC_{50}=24$ μg/ml) cells compared to synaptosomes and even less potent with IMR-32 and TE-671 cells. We could not measure full concentration response curves for IMR32 and TE-671 cells due to concentration limitations of our purification protocol. Of the cell lines tested, SH-SY5Y is the most promising for experiments with β-leptinotarsin-h because it is the more responsive of the neuronal cell lines. We therefore examined Peak A's effect on SH-SY5Y cells in more detail.

Peak A stimulated a continual increase in intracellular Ca^{2+} in SH-SY5Y cells (Fig. 3) similar to that observed by Madeddu et al. (1985a) in PC12 cells. Peak A's effect at all doses was eliminated in the presence of ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) (Fig. 3), which was consistent with previous observations (Crosland et al., 1984; Madeddu et al., 1985a) and strongly suggestive that the observed increases in intracellular Ca^{2+} were due to influx of extracellular Ca^{2+}, as observed with synaptosomes, and not due to release of Ca^{2+} from internal stores. Peak A's effect had a very rapid onset and was possibly delayed only by the time required for mixing.

3.4. Na^+ uptake

We explored the possibility that β-leptinotarsin-h stimulates Ca^{2+} uptake through a non-selective cation channel by investigating its ability to promote Na^+ uptake in synaptosomes. β-Leptinotarsin-h failed to stimulate Na^+ uptake under conditions in which veratridine promoted a robust increase in Na^+ uptake (Table 3). Since Ca^{2+} could be required for β-leptinotarsin-h's binding to synaptosomes, we included 1 mM Ca^{2+} in some of the tests and found that the inclusion of Ca^{2+} did not alter β-leptinotarsin-h's ability to promote Na^+ uptake. Finally, with the addition of 1 mM Ca^{2+} and $^{45}Ca^{2+}$, we found that β-leptinotarsin-h stimulated Ca^{2+} uptake under the conditions used to measure Na^+ uptake. This Ca^{2+} uptake was undiminished compared to that measured with our standard Ca^{2+} uptake protocols. The results of these experiments suggest that β-leptinotarsin-h did not promote Ca^{2+} uptake through a non-selective cation channel and that any channel that passes Na^+ is not required for β-leptinotarsin-h's action.

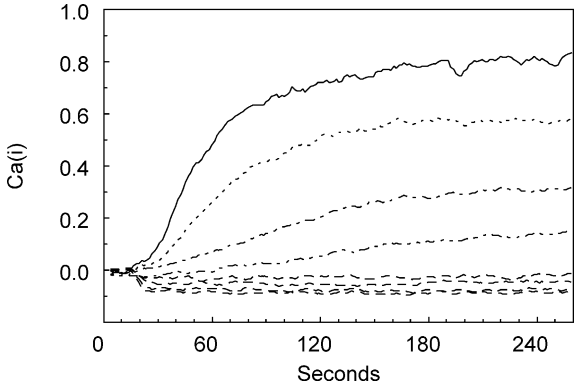

Fig. 3. Time course of Peak A-stimulated changes in intracellular Ca^{2+} [Ca(*i*)] in SH-SY5Y cells. Peak A was added at 16 s. μg Peak A (per 0.1 mg cellular protein): 1.8 (●●-), 18 (●-), 58 (●●●), 180 (—). The effect of Peak A (all four amounts) was also tested with 10 mM EGTA (- - -) added to the incubation medium. SEM of Ca(*i*) is 5–10% of the mean.

Table 3
Effect of β-Leptinotarsin-h on Na^+ uptake

Condition	% of Control[a]
Na^+ uptake	
Control[a]	100±5
β-Leptinotarsin-h	96±5
β-Leptinotarsin-h +1 mM Ca^{2+}	84±5
25 μM Veratridine	171*±7
Ca^{2+} uptake	
Control[b]	100±8
β-Leptinotarsin-h	188*±3

*Significantly different from control.
[a] Control activity = 0.99±0.07 nmol of Na^+ uptake per 0.1 mg synaptosomal protein.
[b] Control activity = 2.4±0.2 nmol of Ca^{2+} uptake per 0.1 mg synaptosomal protein.

3.5. Ca^{2+} flux antagonists

Our principal hypothesis is that β-leptinotarsin-h stimulates Ca^{2+} uptake through Ca^{2+} channels in the presynaptic membrane. As a test of this hypothesis, we examined the effects of a number of antagonists of Ca^{2+} flux on β-leptinotarsin-h-stimulated Ca^{2+} uptake in synaptosomes using Protocol 2 (Table 4). The antagonists were initially tested using Peak A, and we found five to be inhibitory (flunarizine, Ni^{2+}, ruthenium red, SKF 96365, and thapsigargin). Flunarizine, Ni^{2+}, and ruthenium red were also tested using β-leptinotarsin-h, with results similar to those using Peak A, bolstering our confidence that Peak A is a suitable surrogate for β-leptinotarsin-h with respect to Ca^{2+} uptake activity.

The antagonists that inhibited β-leptinotarsin-h-stimulated Ca^{2+} uptake were less selective antagonists, although all of them act on voltage-operated Ca^{2+} channels in addition to other Ca^{2+} channels. None of the selective

Table 4
Effects of Ca^{2+} flux antagonists on Peak A-stimulated Ca^{2+} uptake

Antagonist	Channel[a]	% of Peak A activity[b]	IC_{50}	n_{app}[c]
1 μM ω-agatoxin IVA	P, Q (VO)	95±7		
20 μM 2-APB	IP_3R,SO	140*±10		
10 μM anandamide	T,VR-1[d] (TO)	117*±2		
1 μM atropine	Muscarinic (LO)	119*±3		
10 μM capsazepine	VR-1 (TO)	113±7		
10 μM CNQX	AMPA/kainate (LO)	105±5		
1 μM ω-conotoxin GVIA	N (VO)	100±5		
5 μM ω-conotoxin MVIIC	N,P,Q (VO)	125±10		
33 μM D-AP5[e]	NMDA (LO)	118*±6		
10 μM flunarizine	VO, Na	34*±8	8 μM	−4.0
10 μM flunarizine[f]		41*±4		
33 μM GYKI 52466	AMPA/kainate (LO)	120*±4		
10 μM KB-R7943	Na/Ca exchange, SO	119*±5		
10 μM mecamylamine	Nicotinic (LO)	127*±2		
10 μM MK-801	NMDA (LO)	115±3		
1 mM Ni^{2+}	All Ca	24*±2	0.3 mM	−2.0
1 mM Ni^{2+}[f]		−6*±6		
10 μM nicardipine	L, T (VO)	110±4		
10 μM nicotine[d]	Nicotinic (LO)	111±3		
10 μM nifedipine	L,T (VO)	102±7		
1 μM ruthenium red	RyR, VO, TO	31*±3	0.5 μM	−1.5
1 μM ruthenium red[f]		16*±3		
10 μM SKF 96365	VO, SO, other	88±5	30 μM	−2.0
320 nM SNX-482	R (VO) (some)	117±4		
144 mM tetraethyl ammonium[g]	K	113±4		
1 μM thapsigargin	SERCA	93±9		
10 μM thapsigargin	SERCA, VO, other	11*±6	9 μM	−10.0
1 μM tetrodotoxin	Na	104±5		

*Significantly different from Peak A alone.
[a] IP_3R, IP_3 receptor; LO, ligand-operated; RyR, ryanodine receptor; SERCA, sarcoplasmic reticulum endoplasmic reticulum Ca^{2+} ATPase; SO, store-operated; TO, transduction-operated; VO, voltage-operated.
[b] Peak A activity averaged over all experiments = 3.24±0.04 nmol Ca^{2+} uptake per 0.1 mg synaptosomal protein above basal uptake of 2.43±0.04.
[c] n_{app} Apparent Hill coefficient reported to nearest 0.5 unit.
[d] Agonist.
[e] D-(−)-2-amino-5-phosphonovaleric acid.
[f] Tested against β-leptinotarsin-h.
[g] 22° and 180 s.

antagonists of voltage-operated Ca^{2+} channels (ω-agatoxin IVA, ω-conotoxin GVIA, ω-conotoxin MVIIC, nicardipine, nifedipine, SNX-482) was inhibitory. Some of the antagonists (2-APB, anandamide, atropine, D-AP5, GYKI 52466, KB-R7943, and mecamylamine) actually enhanced Ca^{2+} uptake somewhat.

We further characterized the effects of the inhibitory antagonists on Peak A activity with concentration-effect experiments (Fig. 4), which were the source of the IC_{50} and n_{app} values in Table 4. With the exception of thapsigargin, the values were within the expected ranges. Noteworthy is the large negative n_{app} (−10) of thapsigargin. We found a similar effect of thapsigargin on 40 mM K^+-stimulated Ca^{2+} uptake ($n_{app} = -5$, data not shown). Others have observed a steep concentration–effect response with respect to thapsigargin's inhibition of K^+-stimulated increases in internal Ca^{2+} in adrenal glomerulosa cells ($n_{app} = -14$, our calculations from the authors' data) (Rossier et al., 1993).

4. Discussion

β-Leptinotarsin-h promotes the influx of Ca^{2+} and release of neurotransmitters from presynaptic terminals (McClure et al., 1980; Crosland et al., 1984; Madeddu et al., 1985a,b). The primary aims of this study were to further characterize β-leptinotarsin-h and to test the hypothesis that β-leptinotarsin-h promotes Ca^{2+} influx through presynaptic calcium channels.

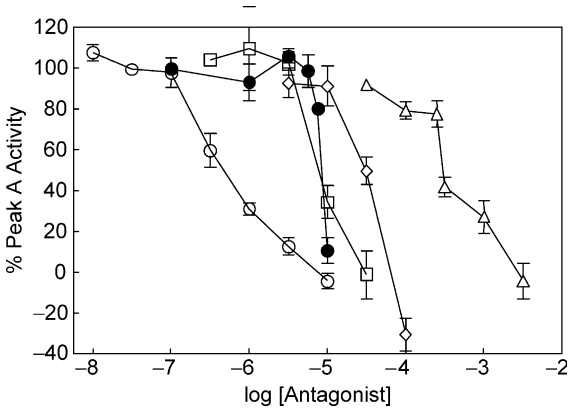

Fig. 4. Concentration–effect of selected antagonists of Ca^{2+} flux on Peak A-stimulated Ca^{2+} uptake. We tested varying concentrations of inhibitors using Protocol 2. Results are expressed as a percentage of Peak A-stimulated Ca^{2+} uptake without inhibitor. Flunarizine (open squares), Ni^{2+} (open triangles), ruthenium red (open circles), SKF 96365 (open diamonds), thapsigargin (closed circles).

4.1. Amino acid sequence comparison to other proteins

In order to investigate the possible similarity of β-leptinotarsin-h to previously reported proteins, we purified it by immunoprecipitation followed by hydrolysis with endoproteinase lysine C. Sequence analysis of selected resulting peptides revealed significant partial similarity with only one protein in public databases. That protein is the juvenile hormone esterase of *L. decemlineata*. Juvenile hormone esterase hydrolyzes juvenile hormone in insects. Juvenile hormone maintains the larval stage, and its hydrolysis is necessary for the initiation of metamorphosis.

In addition to partial amino acid sequence similarity, β-leptinotarsin-h shares other characteristics with the juvenile hormone esterase of *L. decemlineata* (Crosland et al., 1984; Madeddu et al., 1985a; Vermunt et al., 1997a,b). Both β-leptinotarsin-h and juvenile hormone esterase are present in the hemolymph of their respective *Leptinotarsa* species and they have similar isoelectric points, 5.5–5.6. β-Leptinotarsin-h has a molecular weight of 56 kDa, while juvenile hormone esterase is a dimer of two 57 kDa subunits.

Although β-leptinotarsin-h and juvenile hormone esterase share some similarities, there are also differences between them. Juvenile hormone esterase is approximately five times more abundant in hemolymph than is β-leptinoleptinotarsin-h. Under non-denaturing conditions juvenile hormone esterase migrates as a 120 kDa dimer, while β-leptinotarsin-h migrates as a 56 kDa monomer, with no indication of dimerization. Finally, there is a partial amino acid sequence (No. 2 in Table 2) in β-leptinotarsin-h that is not present in juvenile hormone esterase, and partial sequences 1 and 3 of β-leptinotarsin-h that are similar to sequences in juvenile hormone esterase are not completely contained in the latter's sequences. Interestingly, partial sequence 1 of β-leptinotarsin-h contains a potential *N*-glycosylation site (NSS) that is absent in the similar sequence of juvenile hormone esterase.

4.2. β-Leptinotarsin-h and Na^+ uptake

We investigated the possibility that β-leptinotarsin-h promoted Ca^{2+} influx through a non-selective cation channel by testing its ability to stimulate Na^+ uptake. β-Leptinotarsin-h failed to stimulate Na^+ uptake in synaptosomes (Table 3), suggesting that no channel that passes Na^+ was required for β-leptinotarsin-h's action. This observation eliminated non-selective cation channels such as nicotinic, glutamatergic, purinergic P2X, and serotoninergic 5-HT_3 ligand-operated Ca^{2+} channels, and transduction channels as sites of action of β-leptinotarsin-h, as well as Na^+ channels. It was also consistent with the failure of mecamylamine, CNQX, D-AP5, GYKI 52466, MK-801, anandamide, and tetrodotoxin to inhibit β-leptinotarsin-h's activity (Table 4). The results suggest that β-leptinotarsin-h stimulated Ca^{2+} uptake through a selective cation channel.

It is noteworthy that under the experimental conditions of the Na^+ uptake protocol there is little or no depolarization of the synaptosomal membrane when Na^+ uptake is stimulated (Tamkun and Catterall, 1981). β-Leptinotarsin-h-stimulated Ca^{2+} uptake was undiminished using a modification of this protocol, lending support to the suggestion that membrane depolarization is a consequence of, rather than a cause of, Ca^{2+} uptake. This suggestion is further bolstered by the lack of effect of replacing Na^+ with the K^+ channel blocker tetraethylammonium on β-leptinotarsin-h-stimulated Ca^{2+} uptake (Table 4), which, in addition, confirmed that K^+ channels were not involved in β-leptinotarsin-h's activity.

4.3. β-Leptinotarsin-h and Ca^{2+} uptake

Early studies (Yoshino et al., 1980; Crosland et al., 1984) showed that β-leptinotarsin-h stimulated Ca^{2+} uptake and neurotransmitter release from rat brain synaptosomes. β-Leptinotarsin-h-stimulated neurotransmitter release was dependent on external Ca^{2+}, supported by Ba^{2+} and Sr^{2+}, blocked by Cd^{2+} (with high cooperativity) and Co^{2+}, and not reduced by tetrodotoxin or replacement of Na^+ with glucosamine—all characteristics of neurotransmitter release stimulated by Ca^{2+} influx through Ca^{2+} channels. In subsequent studies Madeddu et al. (1985a,b) substantially confirmed and also extended the characterization of β-leptinotarsin-h's action. Based largely, but not exclusively, on the observation that verapamil and nitrendipine failed to reduce the effects of β-leptinotarsin-h, Madeddu et al. (1985a) concluded that β-leptinotarsin-h did not act by opening presynaptic Ca^{2+} channels. At that time it was

believed that verapamil and nitrendipine inhibited 'the' (L) voltage-operated Ca^{2+} channel. Since then, five additional voltage-operated Ca^{2+} channels (N, P, Q, R, T) based on 10 pore-forming subunits have been discovered, in addition to other classes of Ca^{2+} channels. Sufficient Ca^{2+} influx through any of these channels could stimulate neurotransmitter release. There are now inhibitors of some of these channels, and we employed them with the aim of determining which Ca^{2+} channel is stimulated by β-leptinotarsin-h.

We found that β-leptinotarsin-h-stimulated Ca^{2+} uptake was inhibited by the general Ca^{2+} uptake blockers Ni^{2+} and ruthenium red (Table 4), the latter of which demonstrates La^{3+}-like properties with respect to blocking Ca^{2+} uptake in synaptosomes (Tapia and Velasco, 1996). Ca^{2+} uptake was also inhibited by the general voltage-operated Ca^{2+} channel antagonist flunarizine, which implicated voltage-operated Ca^{2+} channels in the action of β-leptinotarsin-h, since voltage-operated Na^{+} channels (also antagonized by flunarizine) are not involved in β-leptinotarsin-h's action (see above and Tables 3 and 4) and (Crosland et al., 1984; Madeddu et al., 1985a). Thapsigargin inhibited β-leptinotarsin-h's activity, but at doses (>1 μM) above those normally associated with inhibition of the sarcoplasmic-endoplasmic Ca^{2+} ATPase. At these higher doses, thapsigargin also inhibits voltage-operated Ca^{2+} channels (Shmigol et al., 1995). Similarly, SKF 96365 inhibited β-leptinotarsin-h's activity at doses that inhibit voltage-operated Ca^{2+} channels and store-operated Ca^{2+} channels (Arakawa et al., 2000).

Inhibition by Ni^{2+}, flunarizine, ruthenium red, thapsigargin and SKF 96365 is suggestive but not probative of the involvement of voltage-operated Ca^{2+} channels in β-leptinotarsin-h's action. We therefore tested the effects of selective inhibitors of voltage-operated Ca^{2+} channels on β-leptinotarsin-h's activity. None of the inhibitors that we tested (ω-agatoxin IVA, ω-conotoxin GVIA, ω-conotoxin MVIIC, nicardipine, nifedipine, SNX-482), however, had any effect on β-leptinotarsin-h-stimulated Ca^{2+} uptake. This result made it unlikely that L, N, P, or Q channels were involved in the activity of β-leptinotarsin-h, although it is possible that β-leptinotarsin-h stimulated uptake through one of these channels in a novel way that overcomes the effect of these inhibitors.

T channels are also probably not involved because nicardipine and nifedipine were ineffective, and they can antagonize T channels as well as L channels (Davila, 1999). In addition, the T channel antagonist anandamide (Davila, 1999) did not inhibit β-leptinotarsin-h's activity. Finally, Ni^{2+} antagonized β-leptinotarsin-h activity at concentrations above those that generally block T channels in neurons (Todorovic and Lingle, 1998), although Lee et al. (1999) reported that with cloned T channels only α1H current was blocked by a low concentration of Ni^{2+} ($IC_{50}=13$ μM), while much higher concentrations of Ni^{2+} were required to block α1I and α1G currents ($IC_{50}=216$ and 250 μM, respectively, similar to the IC_{50} of Ni^{2+} vis à vis β-leptinotarsin-h's activity).

The R voltage-operated Ca^{2+} channel can be defined as the channel that subserves any high-voltage activated Ca^{2+} current that remains in the presence of the selective inhibitors of the other (L, N, P, Q) high-voltage activated Ca^{2+} channels (Randall, 1998). The R channel is blocked by low (<100 μM) concentrations of Ni^{2+} in many systems (Sochivko et al., 2003). There is evidence, however, that the R channel may comprise two channels, one that is sensitive to low concentrations of Ni^{2+} and one that is only sensitive to higher concentrations of Ni^{2+} (100–1 mM) (Dietrich et al., 2003; Tottene et al., 1996; Wu et al., 1998) that are in the range of nickel's inhibitory effect on β-leptinotarsin-h's activity. The Ca^{2+} flux antagonist SNX-482 inhibits the R channel in some, but not all, systems (Sochivko et al., 2003). Thus, its failure to antagonize β-leptinotarsin-h's activity does not necessarily exclude the R channel as a possible site of action of β-leptinotarsin-h.

Studies have been less than consistent in concluding which voltage-operated Ca^{2+} channels in what proportions are responsible for depolarization-stimulated Ca^{2+} uptake in rat brain synaptosomes (Maubecin et al., 1995; Meder et al., 1997; Yu-an et al., 1999). The reported inconsistencies could be due to different brain regions and/or experimental conditions that were investigated. The inconsistencies notwithstanding, one can reasonably draw the general conclusion from the studies that N, P, and/or Q channels in varying proportions subserve Ca^{2+} uptake in rat brain synaptosomes. Furthermore, all of the studies do agree that selective antagonists of voltage-operated Ca^{2+} channels do not completely block Ca^{2+} uptake, suggesting that the R, T and/or some unknown Ca^{2+} channel may be responsible for the remaining uptake. Perhaps β-leptinotarsin-h acted on one of these channels.

There are other possible routes of β-leptinotarsin-h-stimulated Ca^{2+} entry into synaptosomes besides the voltage-operated Ca^{2+} channels. These include reversal of the Na^{+}/Ca^{2+} exchanger and the plasma membrane Ca^{2+} ATPase, ligand-operated Ca^{2+} channels, store-operated Ca^{2+} channels, and transduction-operated channels.

Reversal of the Na^{+}/Ca^{2+} exchanger is unlikely to be involved because the inhibitor KB-R7943 did not inhibit the activity of β-leptinotarsin-h, and KB-R7943 preferentially inhibits the reverse (Ca^{2+} entry) mode of the exchanger (Iwamoto et al., 1996). The plasma membrane Ca^{2+} ATPase has no selective inhibitor, and thus its involvement could not be tested. We tested inhibitors (CNQX, D-AP5, GYKI 52466, mecamylamine, MK-801) of two prominent ligand-operated Ca^{2+} channels, the glutamate- and nicotine-operated channels, and none of them inhibited β-leptinotarsin-h's activity. In addition, the nicotinic agonist nicotine did not affect activity. Finally, ligand-operated Ca^{2+} channels are generally non-selective cation channels, and β-leptinotarsin-h did not stimulate Na^{+} uptake. We can

therefore tentatively look to another class of Ca^{2+} channels as the target of β-leptinotarsin-h.

At standard concentrations none of the inhibitors of store-operated Ca^{2+} channels that we tested inhibited Ca^{2+} uptake, suggesting that this channel is not a site of action of β-leptinotarsin-h. SKF 96365 was inhibitory at higher concentrations ($EC_{50}=30$ μM), however, where it exhibits non-selective effects, including inhibition of store-operated Ca^{2+} channels. KB-R7943 actually increased β-leptinotarsin-h-stimulated uptake somewhat, and 2-APB increased it substantially, while having no effect on basal uptake (data not shown).

At least one transduction-operated Ca^{2+} channel, the VR-1 channel, is not a likely candidate for β-leptinotarsin-h's action because the VR-1 inhibitor capsazepine was without effect. In addition, transduction-operated Ca^{2+} channels are generally non-selective cation channels, and β-leptinotarsin-h did not stimulate Na^{+} uptake.

4.4. Where does β-leptinotarsin-h act?

Table 5 summarizes the effects of β-leptinotarsin-h on various systems. It was most effective at the rat neuromuscular junction and rat/guinea pig brain synaptosomes, while being ineffective at the frog and Drosophila neuromuscular junctions and at snail neuron and rat dorsal root ganglion neuron cell bodies. It did not alter the postsynaptic muscle cell membrane potential in rat or Drosophila. It was active, but less effective than with synaptosomes, in several mammalian cell lines of both neuronal and non-neuronal origin. Considered in toto, the data suggest that β-leptinotarsin-h's preferred site of action is the mammalian presynaptic terminal.

Although our hypothesis is that β-leptinotarsin-h's action can be explained by its activation of a presynaptic Ca^{2+} channel, there is the possibility that it is or forms an ionophore to permit the influx of Ca^{2+}. Given the specificity of its action with respect to neuronal systems, it could not be a general ionophore, but would require some aspect of specificity (e.g. binding site) with respect to the cell membrane. It would also need to have the classical characteristics of Ca^{2+} channels and not pass Na^{+}.

Others and we have shown that β-leptinotarsin-h has some hydrophobic nature, as would be expected of an ionophore. We have commented that we could not recover activity after β-leptinotarsin-h had been in contact with artificial membranes. Madeddu et al.

Table 5
Effect of β-leptinotarsin-h on divers systems

System	Effect[a]	Reference
Rat or guinea pig brain synaptosomes	Ca^{2+} influx	This study
		Crosland et al. (1984)
		Madeddu et al. (1985a)
	Membrane depolarization	Crosland et al. (1984)
		Madeddu et al. (1985a)
	Increased internal Ca^{2+}	Madeddu et al. (1985a)
	ACh, GABA, NE release	Crosland et al. (1984)
	DA, NE release	Madeddu et al. (1985b)
Rat neuromuscular junction	Increased mepp frequency[b]	McClure et al. (1980)
		O.D. Uchitel, p.c.
PC-12 rat pheochromocytoma cells[c]	Ca^{2+} influx	Madeddu et al. (1985a)
	Increased internal Ca^{2+}	Madeddu et al. (1985a)
	Membrane depolarization	Madeddu et al. (1985a)
	DA release	Madeddu et al. (1985b)
HEK, IMR-32, SH-SY5Y, TE-671 cells[c]	Increased internal Ca^{2+}	This study
Drosophila neuromuscular junction	None[b,d]	J.A. Umbach, p.c.
Frog neuromuscular junction	None	J.R. Stimers, p.c.
		Madeddu et al. (1985b)
Rat dorsal root ganglion cell body	None[e]	E.W. McClesky, p.c.
Snail neurons	None[e]	J.R. Stimers in
		Crosland et al. (1984)

p.c. = personal communication. Previously unpublished effects are reported here for the first time.

[a] ACh, acetylcholine; DA, dopamine; GABA, gamma-amino butyric acid; NE, norepinephrine.

[b] No effect on muscle cell resting potential.

[c] β-Leptinotarsin-h was one-fourth to one-tenth as potent with cell lines as it was with synaptosomes.

[d] Spontaneous and evoked release of neurotransmitter.

[e] Whole cell current.

Table 6
Characteristics of β-leptinotarsin-h, α-latrotoxin and glycerotoxin

Characteristic[a]	β-Leptinotarsin-h	α-Latrotoxin	Glycerotoxin
kDa	56	130	320
Minimal effective concentration	High pM[b]	High pM	High pM[b]
Species specificity	Rat, not Frog	Frog & Rat	Frog, not Rat
Ion specificity	Ba^{2+}, Ca^{2+}, not Na^{+}	Ba^{2+}, Ca^{2+}, Na^{+}	Ba^{2+}, Ca^{2+}, not Na^{+}
Spontaneous NT release	Stimulates	Stimulates	Stimulates
Onset of action	Few seconds	1+ min	Few seconds
Duration of increased Ca^{2+} level	Sustained	Sustained	Transient

[a] Only characteristics that have been documented for all three proteins are presented.
[b] Estimate.

(1985a) demonstrated that β-leptinotarsin-h probably has the capacity to bind to lipid bilayers, but not to insert across them unless a transpositive voltage is applied. This suggests that β-leptinotarsin-h would not insert into the presynaptic membrane at its negative resting potential. In support of this speculation, MAb 2C3 did not inhibit the Ca^{2+} uptake activity of β-leptinotarsin-h (Table 1), implying that MAb 2C3 also did not interfere with β-leptinotarsin-h's binding to synaptosomes. Since MAb 2C3 is three times the molecular weight of β-leptinotarsin-h, it is unlikely that β-leptinotarsin-h bound to MAb 2C3 would insert itself into the plasma membrane, thus casting doubt on β-leptinotarsin-h's possible role as an ionophore. It is more likely that β-leptinotarsin-h bound to MAb 2C3 acts at the extracellular surface to promote Ca^{2+} uptake.

Table 6 compares characteristics of β-leptinotarsin-h, α-latrotoxin, and glycerotoxin. All three proteins stimulate Ca^{2+}-dependent neurotransmitter release. α-Latrotoxin is the venom component of *Lactrodectus mactans* (black widow spider) that targets vertebrates (Ushkaryov et al., 2004). It forms pores that permit the influx of cations, including Ca^{2+}, into presynaptic terminals, resulting in the massive release of neurotransmitter. Glycerotoxin is a component of the venom of the sea worm *Glycera convoluta*. It has recently been shown to promote neurotransmitter release by acting extracellularly on N-type Ca^{2+} channels to stimulate Ca^{2+} influx (Meunier et al., 2002). β-Leptinotarsin-h shares some characteristics with both toxins and some characteristics with one toxin, but not the other. All three proteins stimulate the spontaneous release of neurotransmitter and are effective in the high pM range. β-Leptinotarsin-h and α-latrotoxin, but not glycerotoxin, cause a prolonged increase in intracellular Ca^{2+}. β-Leptinotarsin-h and glycerotoxin, but not α-latrotoxin, act almost immediately and do not stimulate Na^{+} influx. These last two characteristics are indicative of an extracellular activator of Ca^{2+} channels.

5. Conclusion

β-Leptinotarsin-h is a potent (~1 nM), rapidly acting (few seconds) protein that promotes Ca^{2+} influx and neurotransmitter release. We found that it is a unique protein, with sequence similarity to only one other protein. We also found that it acted preferentially at mammalian presynaptic nerve terminals, probably at the extracellular surface. In addition, we demonstrated that β-leptinotarsin-h likely promoted Ca^{2+} influx through selective cation channels. The results of our investigation of the effects of Ca^{2+} flux antagonists, coupled with previous studies, support the hypothesis that β-leptinotarsin-h stimulates Ca^{2+} influx through Ca^{2+} channels, although which channel is yet to be determined.

Neuronal voltage-operated Ca^{2+} channels are composed of one of 10 known α_1 subunits, one of at least four β subunits, and one of at least three $\alpha_2\delta$ subunits, creating a large number of possible voltage-operated Ca^{2+} channel complexes (Fisher and Bourque, 2001). Add these to the other classes of Ca^{2+} channels and their variants (Meir et al., 1999), and we have conceivable targets of β-leptinotarsin-h that easily number in the hundreds. β-Leptinotarsin-h may prove to be a useful tool with which to investigate calcium channels and their variants.

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References

Arakawa, N., Sakaue, M., Yokoyama, I., Hashimoto, H., Koyama, Y., Baba, A., Matsuda, T., 2000. KB-R7943 inhibits store-operated Ca2+ entry in cultured neurons and astrocytes. Biochem. Biophys. Res. Commun. 279, 354–357.

Bennett, M.K., 1997. Ca^{2+} and the regulation of neurotransmitter secretion. Curr. Opin. Neurobiol. 7, 316–322.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the priniciple of protein-dye binding. Anal. Biochem. 72, 248–254.

Crosland, R.D., 1996. Technical note on production of 2C3, anti-β-leptinotarsin-h (β-LPT-h, CAS [88376-61-8]). Hybridoma 15, 314.

Crosland, R.D., Hsiao, T.H., McClure, W.O., 1984. Purification and characterization of β-leptinotarsin-h, an activator of presynaptic calcium channels. Biochemistry 23, 734–740.

Davila, H.M., 1999. Molecular and functional diversity of voltage gated calcium channels. Annals NY Acad. Sci. 868, 102–117.

Dietrich, D., Kirschstein, T., Kukley, M., Pereverzev, A., von der Brelie, C., Schneider, T., Beck, H., 2003. Functional specialization of presynaptic $Ca_v2.3$ Ca^{2+} channels. Neuron 39, 483–496.

Fisher, T.E., Bourque, C.W., 2001. The function of Ca^{2+} channel subtypes in exocytotic secretion: new perspectives from synaptic and non-synaptic release. Prog. Biophys. Molec. Biol. 77, 269–303.

Fitch, R.W., Xiao, Y.X., Kellar, K.J., Daly, J.W., 2003. Membrane potential fluorescence: a rapid and highly sensitive assay for nicotinic receptor channel function. Proc. Natl Acad. Sci. USA 100, 4909–4914.

Hagiwara, S., Byerly, L., 1981. Calcium channel. Ann. Rev. Neurosci. 4, 69–125.

Hajós, F., 1975. An improved method for the preparation of synaptosomal fractions in high purity. Brain Research 93, 485–489.

Iwamoto, T., Watano, T., Shigekawa, M., 1996. A novel isothiourea derivative selectively inhibits the reverse mode of Na^+/Ca^{2+} exchange in cells expressing NCX1. J. Biol. Chem. 271, 22391–22397.

Jenö, P., Mini, T., Moes, S., Hintermann, E., Horst, M., 1995. Internal sequences from proteins digested in polyacrylamide gels. Anal. Biochem. 269, 75–82.

Krahn, T., Paffhausen, W., Schade, A., Bechem, M., Schmidt D., 2002. Patent US:6, 4.1.B.AG.; Patent US:6,420,183, Bayer AG.

Lee, J.-H., Gomora, J.C., Cribbs, L.L., Perez-Reyes, E., 1999. Nickel block of three cloned T-type calcium channels: low concentrations selectively block α1H. Biophys. J. 77, 3034–3042.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Madeddu, L., Pozzan, T., Robello, M., Rolandi, R., Hsiao, T.H., Meldolesi, J., 1985a. Leptinotoxin-h action in synaptosomes, neurosecretory cells, and artificial membranes: stimulation of ion fluxes. J. Neurochem. 45, 1708–1718.

Madeddu, L., Saito, I., Hsiao, T.H., Meldolesi, J., 1985b. Leptinotoxin-h action in synaptosomes and neurosecretory cells: stimulation of neurotransmitter release. J. Neurochem. 45, 1719–1730.

Maubecin, V.A., Sanchez, V.N., Rosato Siri, M.D., Chersky, B.D., Sugimori, M., Llinás, R., Uchitel, O.D., 1995. Pharmacological characterization of the voltage-dependent Ca2+ channels present in synaptosomes from rat and chicken central nervous system. J. Neurochem. 64, 2544–2551.

McClure, W.O., Abbott, B.C., Baxter, D.E., Hsiao, T.H., Satin, L.S., Siger, A., Yoshino, J.E., 1980. Leptinotarsin: a presynaptic neurotoxin that stimulates release of acetylcholine. Proc. Natl Acad. Sci. USA 77, 1219–1223.

Meder, W., Fink, K., Gothert, M., 1997. Involvement of different calcium channels in K+- and veratridine-induced increases of cytosolic calcium concentration in rat cerebral cortical synaptosomes. Nauyn-Schmiedeberg's Arch. Pharmacol. 356, 797–805.

Meir, A., Ginsburg, S., Butkevich, A., Kachalsky, S.G., Kaiserman, I., Ahdut, R., Demirgoren, S., Rahamimoff, R., 1999. Ion channels in presynaptic nerve terminals and control of transmitter release. Physiol. Rev. 79, 1019–1088.

Meunier, F.A., Feng, Z.-P., Molgó, J., Zamponi, G.W., Schiavo, G., 2002. Glycerotoxin from *Glycera convoluta* stimulates neurosecretion by up-regulating N-type Ca^{2+} channel activity. EMBO J. 21, 6733–6743.

Randall, A.D., 1998. The molecular basis of voltage-gated Ca2+ channel diversity: is it time for T? J. Membr. Biol. 161, 207–213.

Rener, J.C., Brown, B.L., Nardone, R.M., 1985. Cloning hybridoma cells by limiting dilution. J. Tissue Culture Meth. 9, 175–177.

Rossier, M.F., Python, C.P., Burnay, M.M., Schlegel, W., Vallotton, M.B., Capponi, A.M., 1993. Thapsigargin inhibits voltage-activated calcium channels in adrenal glomerulosa cells. Biochem. J. 296, 309–312.

Shmigol, A., Kostyuk, P., Verkhratsky, A., 1995. Dual action of thapsigargin on calcium mobilization in sensory neurons: inhibition of Ca2+ uptake by caffeine-sensitive pools and blockade of plasmalemmal Ca2+ channels. Neuroscience 65, 1109–1118.

Sochivko, D., Chen, J., Becker, A., Beck, H., 2003. Blocker-resistant Ca^{2+} currents in rat CA1 hippocampal pyramidal neurons. Neuroscience 116, 629–638.

Tamkun, M.M., Catterall, W.A., 1981. Ion flux studies of voltage-sensitive sodium channels in synaptic nerve-ending particles. Molec. Pharmacol. 19, 78–86.

Tapia, R., Velasco, I., 1996. Ruthenium red as a tool to study calcium channels, neuronal death and the function of neural pathways. Neurochem. Int. 30, 137–147.

Todorovic, S.M., Lingle, C.J., 1998. Pharmacological properties of T-Type Ca2+ current in adult rat sensory neurons: effects of anticonvulsant and anesthetic agents. J. Neurophysiol. 79, 240–252.

Tottene, A., Moretti, A., Pietrobon, D., 1996. Functional diversity of P-type and R-type calcium channels in rat cerebellar neurons. J. Neurosci. 16, 6353–6363.

Uchitel, O.D., 1997. Toxins affecting calcium channels in neurons. Toxicon 35, 1161–1191.

Ushkaryov, Y.A., Volynski, K.E., Ashton, A.C., 2004. The multiple actions of black widow spider toxins and their selective use in neurosecretion studies. Toxicon 43, 527–542.

Vermunt, A.M.W., Koopmanschap, A.B., Vlak, J.M., de Kort, C.A.D., 1997a. Cloning and sequence analysis of cDNA encoding a putative juvenile hormone esterase from the Colorado potato beetle. Insect Biochem. Molec. Biol. 27, 919–928.

Vermunt, A.M.W., Vermeesch, A.M.G., de Kort, C.A.D., 1997b. Purification and characterization of juvenile hormone esterase from hemolymph of the Colorado potato beetle. Arch. Insect Biochem. Physiol. 35, 261–277.

Wu, L.-G., Borst, J.G.G., Sakmann, B., 1998. R-type Ca2+ currents evoke transmitter release at a rat central synapse. Proc. Natl Acad. Sci. USA 95, 4720–4725.

Yoshino, J.E., Baxter, D.E., Hsiao, T.H., McClure, W.O., 1980. Release of acetylcholine from rat brain synaptosomes stimulated with leptinotarsin, a new neurotoxin. J. Neurochem. 34, 635–642.

Yu-an, Z., Imanishi, T., Wada, T., Ichida, S., 1999. Proportions of Ca2+ channel types in chick or rat P2 fraction and NG108-15 cells using various Ca2+ blockers. Neurochem. Res. 24, 1059–1066.