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Purification and Characterization of β-Leptinotarsin-h, an Activator of Presynaptic Calcium Channels†

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ABSTRACT: A new neuroactive protein, β-leptinotarsin-h, has been purified to near-homogeneity from the hemolymph of the beetle *Leptinotarsa haldemani* by column chromatography. β-Leptinotarsin-h has a molecular weight of 57 000. Rat brain synaptosomes incubated with appropriate radioactive precursors release acetylcholine (ACh), norepinephrine, and 4-aminobutyrate when exposed to β-leptinotarin-h, but do not release lactate dehydrogenase. Release of ACh has been examined in some detail. Release of ACh varies with the concentration of β-leptinotarsin-h in a rectangular hyperbolic fashion. Half-maximal release is stimulated by a concentration of 50 ng/mL. Altering the ionic composition of the bathing solution affects the release in a manner which suggests that neither Na^+ channels nor K^+ channels are affected by β-leptinotarsin-h but that the β-leptinotarsin-h acts to increase permeability to Ca^{2+}. Varying the concentration of Ba^{2+}, Sr^{2+}, Co^{2+}, and Cd^{2+} indicates that β-leptinotarsin-h acts to open the voltage-sensitive presynaptic Ca^{2+} channel. β-Leptinotarsin-h may be a useful tool for studying the Ca^{2+} channel associated with the release of neurotransmitters.

In 1969 Hsiao and Fraenkel reported that the hemolymph of various species of the beetle *Leptinotarsa* was lethal to houseflies and mice. Subsequently, Hsiao (1978) found that partially purified fractions from the hemolymph of seven species of *Leptinotarsa* were also lethal to houseflies and mice. From these seven species, a partially purified fraction from the hemolymph of *Leptinotarsa haldemani* proved to be the most toxic. McClure et al. (1980) examined the effect of the partially purified toxin from *L. haldemani*, designated leptinotarsin-h, on the neuromuscular junction of the rat. Leptinotarsin-h caused a massive, biphasic increase in the frequency of miniature end-plate potentials. Further examination of the effect of leptinotarsin-h revealed that the first phase of release of acetylcholine (ACh)[1] accounted for about 10% of the total release and was abolished by removal of Ca^{2+} from the bathing medium. The second phase of release, however, was little affected by the absence of Ca^{2+}. The data suggest that leptinotarsin-h can induce two modes of quantized release at the neuromuscular junction.

The neurochemical characteristics of both leptinotarsin-h and a partially purified preparation of leptinotarsin from the hemolymph of *L. decemlineata*, designated leptinotarsin-d, were investigated by using synaptosomes from rat brain (McClure et al., 1980; Yoshino et al., 1980). Both preparations caused the preferential release of ACh over that of choline, were inactivated by heat, and were dependent on the presence of Ca^{2+} in the incubation medium. In this paper we report further purification and characterization of the neuroactive component from the hemolymph of *L. haldemani*. These studies were undertaken to clarify the mechanism of action of this toxin in causing the release of neurotransmitters.

Materials and Methods

Hemolymph was extracted from fourth instar larvae of *L. haldemani* and lyophilized. $^{45}CaCl_2$ (18.2 mCi/mg), [*methyl*-^{3}H]choline chloride (80 Ci/mmol), 4-[^{3}H]aminobutyric acid ([^{3}H]GABA) (34.5 Ci/mmol), and [^{3}H]norepinephrine

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[1] Abbreviations: ACh, acetylcholine; GABA, 4-aminobutyric acid; DEAE, diethylaminoethyl; di-O-C_5-(3), 3,3′-dipentyl-2,2′-oxacarbocyanine; LDH, lactate dehydrogenase; NE, norepinephrine; PS, physiological saline solution; Tris, tris(hydroxymethyl)aminomethane; Tris-PS, physiological saline solution with Tris-HCl instead of sodium phosphate; TTX, tetrodotoxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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([^{3}H]NE) (4.5 Ci/mmol) were purchased from New England Nuclear, Boston, MA. Sephadex G-150 was purchased from Pharmacia Fine Chemicals, Piscataway, NJ. Phosphocellulose was bought from Bio-Rad Laboratories, Richmond, CA. Diethylaminoethyl (DEAE)-Sephacel and Reactive Blue 2-agarose were purchased from Sigma Chemical Co., St. Louis, MO. 3,3′-Dipentyl-2,2′-oxacarbocyanine [di-O-C_5-(3)] was the generous gift of Dr. Alan Waggoner of Amherst College, Amherst, MA. Physiological saline solution (PS) consisted of 130 mM NaCl, 4 mM KCl, 10 mM glucose, 2 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM sodium phosphate, pH 7.0. Tris–PS consisted of PS in which sodium phosphate buffer was replaced by 25 mM Tris-HCl, pH 7.0.

Preparation of Synaptosomes. Synaptosomes (crude mitochondrial pellet) were prepared from the brain of a male Sprague-Dawley rat (140–200 g) according to the procedure of De Robertis et al. (1962) and used in all experiments unless otherwise stated. Purified synaptosomes, purified myelin, and purified mitochondria were prepared from a crude mitochondrial pellet according to the procedure of Hajős (1975).

Loading of Synaptosomes with [^{3}H]Choline. Synaptosomes were suspended to a concentration of 1 g of brain/10 mL of PS. A solution of 12.5 μCi of [^{3}H]choline chloride in 12.5 μL of ethanol was dried at 25 °C under a stream of nitrogen and redissolved in 0.7 mL of PS. The resulting solution was added to a vessel containing 2 mL of the synaptosomal suspension, after which the synaptosomes and label were incubated in a shaking water bath for 30 min at 37 °C. The synaptosomes were centrifuged at 5000*g* for 5 min at 4 °C, after which the pellet was resuspended in the initial incubation volume of PS. This washing procedure was repeated twice. The final pellet was resuspended in PS at a concentration of 1 g of brain/13.5 mL of PS. When [^{3}H]choline and [^{3}H]ACh were to be assayed, PS contained 3 mM diisopropyl fluorophosphate to inhibit acetylcholinesterase (EC 3.1.1.7). Synaptosomes previously loaded with [^{3}H]choline were used in all experiments unless otherwise stated.

Loading of Synaptosomes with [^{3}H]GABA or [^{3}H]NE. Synaptosomes were suspended in either PS containing 1 mM β-alanine or PS containing 0.001% pargyline and 0.03% ascorbic acid. β-Alanine causes the preferential loading of GABA into synaptosomes instead of into glial fragments (Schon & Kelly, 1975). Pargyline and ascorbic acid prevent oxidation of NE (Nicklas et al., 1973). Ten microcuries of [^{3}H]GABA in 0.7 mL of PS containing β-alanine was added to 2.0 mL of synaptosomes suspended in PS containing β-alanine. Ten microcuries of [^{3}H]NE in 0.7 mL of PS containing pargyline and ascorbic acid was added to 2.0 mL of synaptosomes in PS containing pargyline and ascorbic acid. Both suspensions were incubated for 15 min at 37 °C in a shaking water bath. Both incubates were pelleted at 5000*g* for 5 min at 4 °C. Both pellets were washed 4 times in their respective solutions of PS and finally resuspended in 2.7 mL of their respective solutions of PS.

Assay for Release of Radioactivity from Synaptosomes. The sample to be tested was diluted to a final volume of 1.0 mL. Isoosmolarity with the cytoplasm of synaptosomes was maintained by adjusting the concentration of NaCl. Synaptosomes (0.2 mL) previously loaded with labeled precursor were added to 1.0 mL of sample solution in a 1.5-mL microfuge tube at 25 °C, and the combined solutions were incubated for 10 min at 37 °C in a shaking water bath. The incubate was centrifuged at 9000*g* for 5 min at 4 °C in a Beckman Model 152 microfuge (Fullerton, CA). The time of incubation was taken as the time between the addition of synaptosomes and the removal of the incubate from the water bath. The supernatant was immediately removed for the determination of released components. After removal of the supernatant, 1.2 mL of water was added to lyse the pelleted synaptosomes. The resulting suspension was centrifuged at 9000*g* for 5 min at 4 °C. The membrane fragments were pelleted, while the released cytosolic components remained in the supernatant. The supernatant to be assayed (0.25 mL) was added to 0.75 mL of water and 5.0 mL of 25% Triton X-114/75% mixed xylenes containing 0.3% 2,5-diphenyloxazole (Anderson & McClure, 1973). The sample was counted in a Beckman Model LS 7500 liquid scintillation counter. Release was calculated as the cpm measured in the experimental supernatant less the cpm measured in the control supernatant.

Determination of [^{3}H]Choline and [^{3}H]ACh. [^{3}H]Choline and [^{3}H]ACh were determined by converting choline to phosphorylcholine with choline kinase (EC 2.7.1.32, ATP: choline phosphotransferase) and ATP (Goldberg & McCaman, 1973). Phosphoryl[^{3}H]choline and [^{3}H]ACh were separated by using a column containing approximately 1 mL of Dowex 50W-X8 (Na^+). The breakthrough and three successive washes with 0.65 mL of water were collected, pooled, and counted as a measure of phosphoryl[^{3}H]choline. The proportions of [^{3}H]choline and [^{3}H]ACh in the original radioactivity were calculated after corrections for recoveries of standards.

Determination of Lactate Dehydrogenase Activity. Lactate dehydrogenase (LDH) (EC 1.1.1.27) activity was determined by monitoring the decrease in absorbance at 340 nm produced by the oxidation of reduced nicotinamide adenine dinucleotide by pyruvate according to the method of Goldberg (1975).

Determination of Protein. Protein was determined by the fluorescamine assay of Bohlen et al. (1973) with bovine serum albumin as a standard.

Polyacrylamide Gel Electrophoresis. Polyacrylamide gel electrophoresis using sodium dodecyl sulfate (SDS–PAGE) was performed according to the procedure of Laemmli (1970) using a Protean 16CM slab gel electrophoresis apparatus (Bio-Rad Laboratories).

Measurement of Depolarization of Synaptosomes. The method of Sen & Cooper (1978) was used to determine the changes in the electrical potential of the plasma membrane of synaptosomes by measuring the change in fluoresence of a dye in the presence of synaptosomes. Five microliters of a solution of di-O-C_5-(3) in methanol (1.5 mg/mL) was added to 2.5 of PS and the basal fluorescence determined. Synaptosomal suspension (0.50 mL) was then added. After 15 min (to allow for stabilization of the fluorescence), the solution to be tested was added, and the fluorescence was measured continuously for 15 min by using a Varian Model SF-330 spectrofluorometer (Palo Alto, CA). The excitation wavelength was 483 nm, and the emission wavelength was 501 nm.

Results

Purification of β-Leptinotarsin-h. Chromatography of *L. haldemani* hemolymph on Sephadex G-150 separated the releasing activity into one major peak (peak A) and one minor peak (Peak B) (Figure 1a). By comparison with standards, the releasing activity in peak A corresponded to a M_r of 57 000 ± 1000 (n = 2 purifications).

The fractions comprising peak A were pooled and chromatographed on DEAE-Sephacel. A portion of the releasing activity in peak A did not bind to the DEAE-Sephacel (peak A_1, Figure 1b). The unbound activity was probably a separate molecular species from the activity which did bind (peak A_2)

Table I: Purification of β-Leptinotarsin-h

procedure	volume (mL)	protein (μg)	release[a] (cpm)	recovery (%)	sp act. (cpm/μg of protein)	purification (times)
hemolymph	2.6	148 000	86 400 000	100	585	1
Sephadex G-150	19.9	13 300	93 300 000	108	7 000	12
DEAE-Sephacel	10.3	1 170	65 700 000	76	56 000	96
phosphocellulose	25.0	300	23 300 000	27	78 200	134
Reactive Blue	9.1	15.5	9 500 000	11	613 000	1050

[a] Units of release are defined under Materials and Methods.

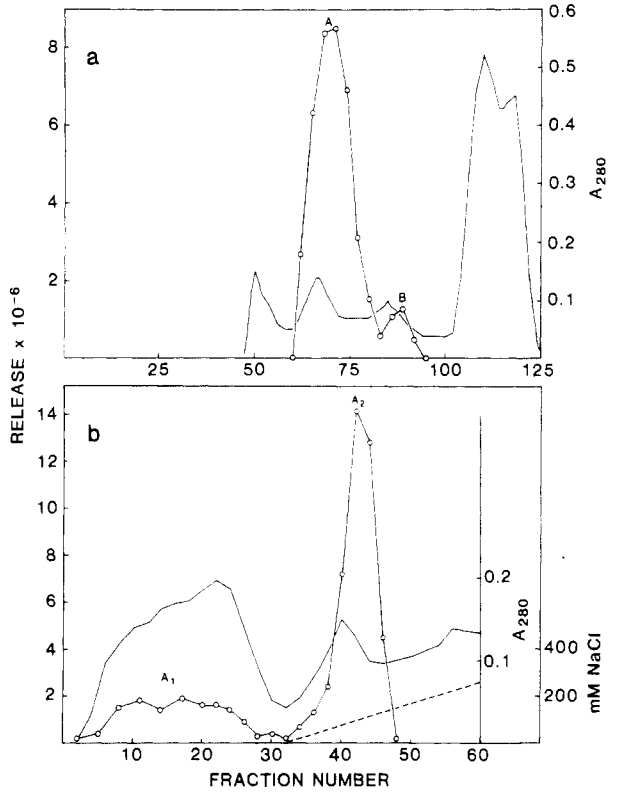

FIGURE 1: Gel filtration of β-leptinotarsin-h on Sephadex G-150 and DEAE-Sephacel. (a) Lyophilized hemolymph (100 mg) from *L. haldemani* was dissolved in 2.6 mL of 50 mM sodium phosphate buffer, pH 7.0, and clarified by centrifugation at 5000*g* for 10 min at 4 °C. The supernatant was applied to a Sephadex G-150 column (1.2 × 108 cm) and eluted with 50 mM sodium phosphate buffer, pH 7.0. Fractions were assayed for releasing activity [(○), cpm per fraction] and absorbance at 280 nm (—). (b) The fractions (60–80) comprising peak A of the Sephadex G-150 column were combined and applied to a DEAE-Sephacel column (3.0 mL) which was subsequently eluted with a linear gradient of 0.0–0.25 M NaCl in 50 mM sodium phosphate buffer, pH 7.0 (40-mL total volume). The eluate was assayed for releasing activity [(○), cpm per fraction], absorbance at 280 nm (—), and NaCl concentration (---) (determined by refractometry).

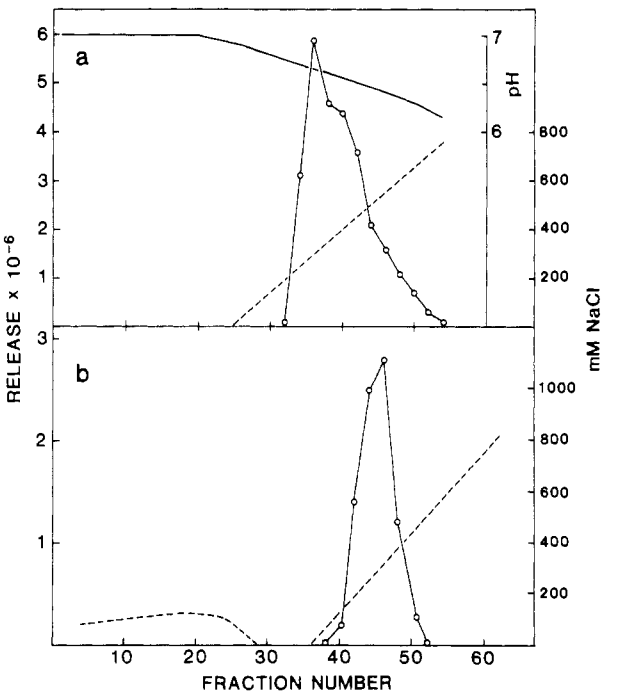

FIGURE 2: Ion-exchange chromatography on β-leptinotarsin-h on phosphocellulose and Reactive Blue 2-agarose. (a) The fractions (38–47) comprising peak A_2 of the DEAE-Sephacel column were combined, diluted in portions 2-fold with water, applied to a phosphocellulose column (6.0 mL), and eluted with a linear gradient from 50 mM sodium phosphate buffer, pH 7.0, to 1.0 M NaCl in 50 mM sodium phosphate buffer, pH 5.5 (40-mL total volume). The eluate was assayed for releasing activity [(○), cpm per fraction], pH (—), and NaCl concentration (---). (b) The pooled active fractions (33–46) from the phosphocellulose column were diluted in portions 2-fold with water, applied to a Reactive Blue 2-agarose column (2.0 mL), and eluted with a linear gradient of 0.0–1.0 M NaCl in 50 mM sodium phosphate buffer, pH 7.0. The eluate was assayed for releasing activity [(○), cpm per fraction] and NaCl concentration (---). The absorbance at 280 nm was too small to be measured.

because when the unbound activity was rechromatographed by using fresh DEAE-Sephacel it still did not bind. The unbound activity was not characterized further.

The active fractions comprising peak A_2 were pooled and chromatographed on phosphocellulose. Releasing activity eluted from phosphocellulose in a single peak (Figure 2a). The activity eluted over a wide salt concentration, and the elution profile tailed. This behavior could indicate that the peak was composed of more than one molecular species possessing releasing activity.

The active fractions from the phosphocellulose column were combined and chromatographed on Reactive Blue 2-agarose. Releasing activity eluted as a single symmetrical peak over a wide concentration range of NaCl (Figure 2b). The fractions containing releasing activity were pooled and characterized. The material possessing releasing activity in these pooled fractions was termed β-leptinotarsin-h.

The pooled active fractions from the various purification steps were assayed for releasing activity and protein (Table I). Although the degree of purification and overall yield varied for different purifications, the values reported in Table I are representative of the results obtained.

Chemical Nature of β-Leptinotarsin-h. Several tests were performed to determine whether β-leptinotarsin-h is a protein. Synaptosomes preloaded with [^{3}H]choline were incubated with β-leptinotarsin-h (54 ng/mL) which had been either (a) heated for 10 min at 75 °C, (b) incubated for 2.5 h at 25 °C with 1.0 mg/mL Pronase, or (c) incubated for 2 h at 25 °C with 1.5 mg/mL trypsin, followed by addition of soybean trypsin inhibitor to a final concentration of 2.0 mg/mL. Most proteins are inactivated by mild heating. Heat-treated β-leptinotarsin-h was ineffective in causing the release of radioactivity from synaptosomes. The protease Pronase completely eliminated

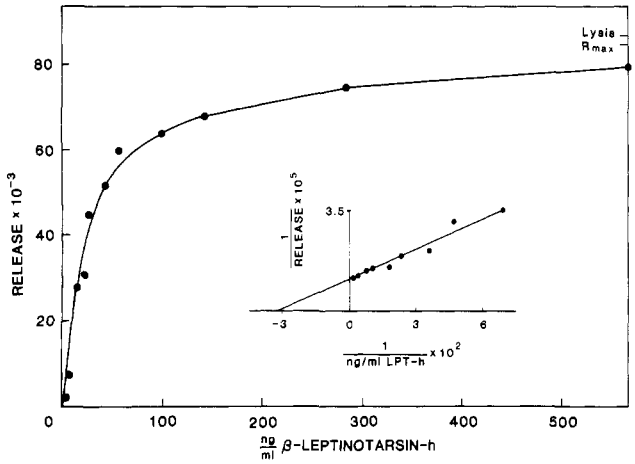

FIGURE 3: Effect of varying concentrations of β-leptinotarsin-h on the release of radioactivity from synaptosomes. Synaptosomes preloaded with [^{3}H]choline were incubated with varying amounts of β-leptinotarsin-h and assayed for release. Lysis represents the radioactivity released from an aliquot of synaptosomes which was incubated without β-leptinotarsin-h and then osmotically shocked with water. Each point is the average of two simultaneous determinations. Inset: Double-reciprocal presentation of the data of the main figure. The maximal release predicted from the inset is indicated as R_{max} on the figure. These data yield a value of 30 ng/mL for the concentration of β-leptinotarsin-h required to stimulated half-maximal release.

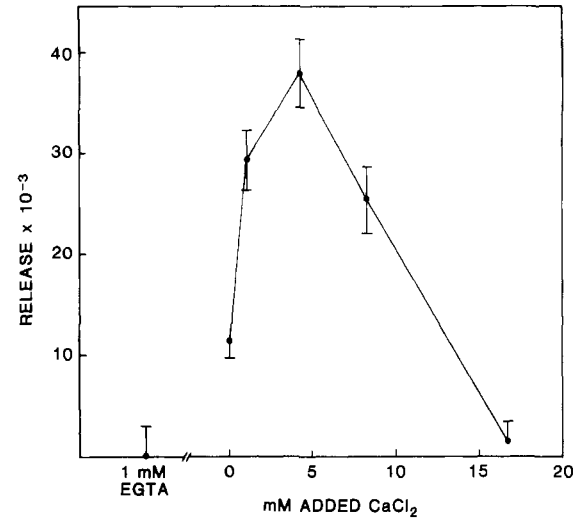

FIGURE 4: Ca^{2+} dependence of release stimulated by β-leptinotarsin-h. Synaptosomes previously loaded with [^{3}H]choline were washed 3 times with Tris–PS lacking $CaCl_2$, resuspended in Tris–PS lacking $CaCl_2$, and incubated in solutions containing β-leptinotarsin-h (90 ng/mL) and various concentrations of $CaCl_2$. The effect of solutions containing no $CaCl_2$ and 1 mM EGTA was also tested (1 mM EGTA). Release was determined in terms of cpm for duplicates ± 1 SD. Values are corrected for release observed in controls lacking β-leptinotarsin-h.

the releasing activity of β-leptinotarsin-h. Trypsin, a more specific protease, had no effect on the releasing activity of β-leptinotarsin-h. The foregoing results suggest that β-leptinotarsin-h is a protein.

The homogeneity and molecular weight of β-leptinotarsin-h were determined by using SDS–PAGE. Electrophoresis of β-leptinotarsin-h resulted in three faint bands of approximately equal intensity. By comparison with standard proteins run on the same gel, the three bands had molecular weights of 57 000, 62 000, and 63 000. Considering the molecular weight (57 000 ± 1000) of β-leptinotarsin-h as determined by chromatography on Sephadex G-150, it is probable that the band at 57 000 is the active species.

Kinetics of Release. The effect of varying the time of incubation on the amount of radioactivity released from synaptosomes stimulated by β-leptinotarsin-h was determined. Synaptosomes were preloaded with [^{3}H]choline, added to solutions containing 42 ng/mL β-leptinotarsin-h, incubated for various times at 37 °C, and assayed for releasing activity. Release reached a plateau after 4 min of incubation. Subsequent experiments were performed with an incubation time of 10 min.

Release increased with increasing concentrations of β-leptinotarsin-h and approached a plateau (Figure 3). The maximal observed release was 92% of the radioactivity available for release, as determined by the amount of radioactivity in lysates of synaptosomes incubated without β-leptinotarsin-h. Release stimulated by β-leptinotarsin-h satisfies a simple rectangular hyperbolic dependence on its concentration (Figure 3, inset). The concentration of β-leptinotarsin-h at half-maximal release of radioactivity is 50 ± 20 ng/mL (n = 3 experiments).

β-Leptinotarsin-h could cause release of radioactivity from synaptosomes by lysis. To evaluate the integrity of the synaptosomes, LDH activity was measured in the same experiments in which the dose–release relationship was determined. The maximal dose of β-leptinotarsin-h tested caused the release of 92% of the available radioactivity while releasing only 2.3% of the available LDH activity.

The proportions of [^{3}H]ACh and [^{3}H]choline released by β-leptinotarsin-h were also determined. [^{3}H]ACh accounted for 74 ± 4% of the total released radioactivity at all doses of β-leptinotarsin-h. Since the radioactivity in the lysates of controls consisted of 65% [^{3}H]ACh, β-leptinotarsin-h releases radioactivity which is somewhat enriched in [^{3}H]ACh, implying that β-leptinotarsin-h exhibits some preference for the release of ACh over that of choline.

Calcium Dependence of Release. The releasing activity of β-leptinotarsin-h was examined in the presence of various concentrations of added $CaCl_2$. In order to perform these experiments, sodium phosphate was replaced with Tris-HCl in the buffer to avoid precipitation of divalent cations. Since Tris-HCl buffer reduces the uptake of [^{3}H]choline into synaptosomes (data not shown), loading of synaptosomes with [^{3}H]choline was performed as usual in PS.

Ca^{2+} is required for the releasing activity of β-leptinotarsin-h (Figure 4). Solutions containing no added $CaCl_2$ and 1 mM EGTA demonstrate no releasing activity. Releasing activity increases with increasing concentrations of added $CaCl_2$ up to about 5 mM $CaCl_2$ and then decreases to zero at 17 mM $CaCl_2$. This behavior is similar to that observed when the hemolymph of *L. haldemani* is applied to the neuromuscular junction of the rat (Dr. Joseph Stimers, unpublished results).

Sodium Dependence of Release. β-Leptinotarsin-h could increase synaptosomal release by opening Na^+ channels in the synaptosomal membrane. A dose of tetrodotoxin (TTX) which substantially reduced veratridine-stimulated release of radioactivity had no effect on the releasing activity of β-leptinotarsin-h (Table II). β-Leptinotarsin-h could also increase synaptosomal permeability to Na^+ if β-leptinotarsin-h were itself a Na^+ ionophore. The releasing activity of β-leptinotarsin-h was not affected by reducing the extrasynaptosomal concentration of Na^+ to a level low enough to eliminate veratridine-stimulated release of radioactivity (Table II).

Effect of β-Leptinotarsin-h on the Synaptosomal Membrane Potential. Although β-leptinotarsin-h does not promote the influx of Na^+ across the synaptosomal membrane, it could depolarize the membrane by some other mechanism, thereby opening the presynaptic Ca^{2+} channels and causing the release

Table II: Effect of Tetrodotoxin and Reduced Extracellular Na^+ on Release Stimulated by β-Leptinotarsin-h[a]

toxin employed	[TTX] (μg/mL)	[Na^+] (mM)	release (cpm)
β-leptinotarsin-h	0.0	130	19 700 ± 900
β-leptinotarsin-h	0.30	130	18 000 ± 4 000
β-leptinotarsin-h	0.0	8	15 000 ± 4 500
veratridine	0.0	130	35 000 ± 2 000
veratridine	0.30	130	10 000 ± 2 000
veratridine	0.0	8	−1 000 ± 2 000

[a] After being loaded with [^{3}H]choline, synaptosomes were washed twice in PS and once in Tris-PS containing no NaCl and resuspended in Tris-PS containing no NaCl. The resuspended synaptosomes were then added to solutions containing β-leptinotarsin-h (54 ng/mL), veratridine (120 μM), TTX, or NaCl (TTX and NaCl concentrations as given above). Isoosmotic conditions were maintained by substituting glucosammonium for Na^+ on an equimolar basis. Values are the means of two measurements of release ± 1 SD. Values of release have been corrected for release observed in controls lacking either toxin.

of radioactivity. As indicated with the aid of di-O-C_5-(3) (Sims et al., 1974), a fluorescent dye which has been used to measure changes in the plasma membrane potential in synaptosomes (Blaustein & Goldring, 1975; Ng & Howard, 1978; Sen & Cooper, 1978), β-leptinotarsin-h caused the depolarization of synaptosomes (Figure 5a). In addition, release of radioactivity correlated well with depolarization, with depolarization somewhat preceding release. Depolarization stimulated by β-leptinotarsin-h achieved a steady state after about 9 min. At t = 15 min, increasing the concentration of KCl in the extrasynaptosomal solution caused a further increase in fluorescence (data not shown). Control experiments showed that β-leptinotarsin-h, either with or without Ca^{2+}, did not alter the fluorescence of the dye.

In experiments to test the ionic dependence of the depolarization induced by β-leptinotarsin-h, removal of Ca^{2+} from the extrasynaptosomal solution caused a 90% reduction in depolarization at 9 min. In a separate experiment performed at 25 °C, releasing activity was also dependent on extracellular Ca^{2+} (Figure 5a).

The synaptosomal suspension used in the depolarization studies is contaminated with myelin and mitochondria (Jones, 1975). To establish the subcellular fraction on which β-leptinotarsin-h acted, sucrose density centrifugation was used to obtain purified synaptosomes, purified myelin, and purified mitochondria. Each of these fractions was assayed with di-O-C_5-(3) and β-leptinotarsin-h. Purified synaptosomes were depolarized with the same time course (Figure 5b) as were unpurified synaptosomes (Figure 5a). Purified myelin was also depolarized, but to a lesser extent than synaptosomes. Purified mitochondria were depolarized to a much lesser extent than myelin, and the depolarization of mitochondria reached a steady state after only 1 min. If the crude synaptosomal suspension were composed of 40% myelin, 40% synaptosomes, and 20% mitochondria (Jones, 1975), then synaptosomes were responsible for approximately 50% of the depolarization indicated in Figure 5a.

Effect of Divalent Cations on Release. Ba^{2+} and Sr^{2+} can substitute for Ca^{2+} in supporting the release of neurotransmitters (Rubin, 1974; Baker & Reuter, 1975; Cotman et al., 1976; Haycock et al., 1978). Synaptosomes previously loaded with [^{3}H]choline were pelleted, washed 3 times with Tris–PS lacking $CaCl_2$, resuspended in Tris–PS lacking $CaCl_2$, and added to solutions containing β-leptinotarsin-h (54 ng/mL) and 1.2 mM either of $CaCl_2$, of $BaCl_2$, or of $SrCl_2$. When Ca^{2+} was replaced by Ba^{2+} or Sr^{2+} in the extrasynaptosomal

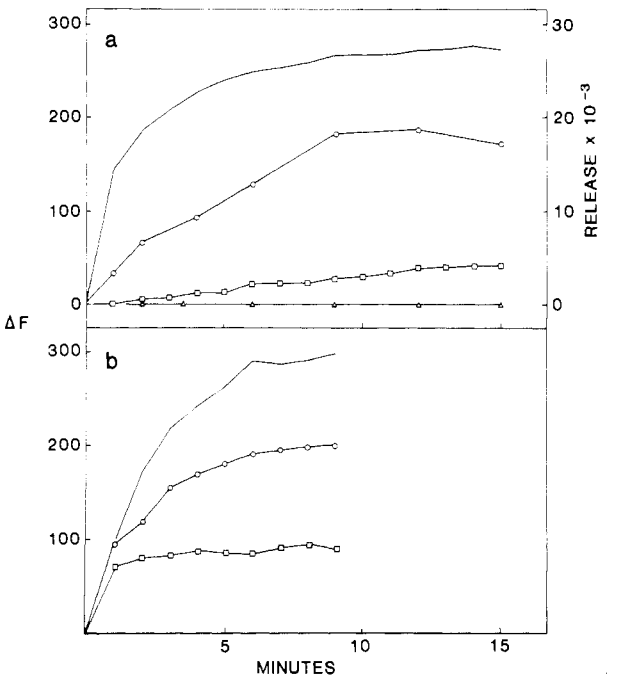

FIGURE 5: Time course of depolarization of synaptosomes stimulated by β-leptinotarsin-h. (a) Unpurified synaptosomes. For depolarization experiments, synaptosomes were washed twice with PS and resuspended in PS. Di-O-C_5-(3) was added and allowed to equilibrate, after which β-leptinotarsin-h (75 ng/mL) was added at time zero. Fluorescence (ΔF, arbitrary units) was calculated as $F_{experimental}$ at time $t - F_{experimental}$ at time 0, corrected for the fluoresence of controls lacking β-leptinotarsin-h. Synaptosomes were also washed once with PS and twice with PS lacking Ca^{2+} and containing 1 mM EGTA and resuspended in the latter solution. The effect of β-leptinotarsin-h on depolarization of these synaptosomes was determined as described above. Releasing activity at 25 °C was determined in a separate experiment. (—) ΔF in the presence of 1 mM $CaCl_2$; (○) release in the presence of 1 mM $CaCl_2$; (□) ΔF in the presence of 1 mM EGTA; (△) release in the presence of 1 mM EGTA. Data are averages of two or three separate experiments. (b) Purified synaptosomes. Each purified fraction (synaptosomes, myelin, or mitochondria) was washed twice in PS and suspended in PS. Di-O-C_5-(3) was added to an aliquot of each suspension and allowed to equilibrate for 15 min. β-Leptinotarsin-h (75 ng/mL) was added at t = 0, and ΔF was determined. (—) Purified synaptosomes; (○) purified myelin; (□) purified mitochondria.

medium, the releasing activity of β-leptinotarsin-h was retained. Release in the presence of Ba^{2+} (17 700 ± 1000 cpm) was somewhat lower than release in the presence of Ca^{2+} (25 700 ± 1700 cpm) or Sr^{2+} (30 000 ± 4500 cpm) (means of duplicate measurements ± 1 SD).

It has been repeatedly demonstrated that Cd^{2+} and Co^{2+} are potent blockers of Ca^{2+} currents in excitable membranes (Hagiwara & Byerly, 1981). Release stimulated by β-leptinotarsin-h was completely blocked by 5 mM Cd^{2+} or Co^{2+} (data not shown). The effect of Cd^{2+} on the inhibition of release stimulated by β-leptinotarsin-h was examined in more detail by varying the concentration of Cd^{2+} (Figure 6). Release was completely blocked by 1.3 mM added Cd^{2+}. Although the concentration of free Cd^{2+} in the incubation mixtures cannot be readily determined due to the complexation of Cd^{2+} by phosphate derived from the aliquot of β-leptinotarsin-h, the kinetic order of inhibition can be determined. Inhibition of release due to Cd^{2+} demonstrated a Hill coefficient of 4.7 (Figure 6).

Calcium Uptake into Synaptosomes. The effect of β-leptinotarsin-h on the uptake of Ca^{2+} into synaptosomes was studied. Purified synaptosomes were used to minimize the effect of mitochondria. β-Leptinotarsin-h stimulated a dose-dependent uptake of $^{45}Ca^{2+}$ into synaptosomes (Figure

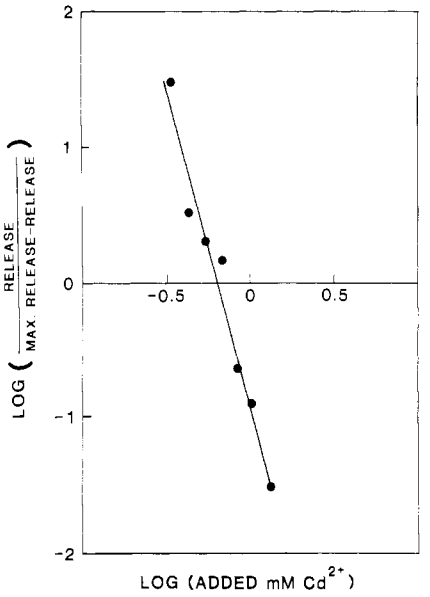

FIGURE 6: Hill plot of the effect of varying concentrations of Cd^{2+} on release from synaptosomes stimulated by β-leptinotarsin-h. Synaptosomes previously incubated with [^{3}H]choline were pelleted, washed 3 times with Tris–PS, resuspended in Tris–PS, and added to solutions containing β-leptinotarsin-h (51 ng/mL) and various concentrations of $CdCl_2$. Maximal release = 58 000 cpm. Data points are averages of two simultaneous determinations.

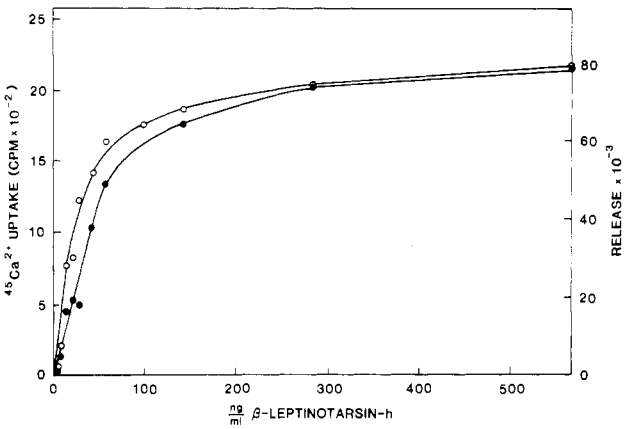

FIGURE 7: Uptake of $^{45}Ca^{2+}$ into synaptosomes stimulated by β-leptinotarsin-h. Purified synaptosomes were suspended in PS containing 1 μM choline chloride and incubated for 30 min at 37 °C in a shaking water bath. The incubate was pelleted and resuspended in PS. Aliquots (0.2 mL) of synaptosomes were added to solutions (1.0 mL) containing 0.2 μCi of $^{45}CaCl_2$ and various amounts of β-leptinotarsin-h and incubated for 10 min at 37 °C. The synaptosomes were pelleted, washed twice in PS, and resuspended in PS. Uptake of $^{45}Ca^{2+}$ (●) was calculated as the difference between the radioactivity in final suspensions originally containing β-leptinotarsin-h and the radioactivity in equivalent suspensions lacking β-leptinotarsin-h. The release data (○) are the same as those in Figure 3. Data points are the averages of two simultaneous determinations.

7). The dose–uptake curve closely paralleled that for release of radioactivity. The concentration of β-leptinotarsin-h which stimulated half-maximal uptake of $^{45}Ca^{2+}$ was 70 ± 10 ng/mL (n = 3 experiments).

Effect of β-Leptinotarsin-h on the Release of Other Neurotransmitters. If the releasing activity of β-leptinotarsin-h be due to the opening of presynaptic Ca^{2+} channels, then β-leptinotarsin-h should also stimulate the release of GABA and NE from synaptosomes. β-Leptinotarsin-h (51 ng/mL) stimulated the release of radioactivity from synaptosomes previously loaded with [^{3}H]GABA or [^{3}H]NE (see Materials and Methods for conditions). When stimulated release is expressed as a percentage of control release, β-leptinotarsin-h is approximately equally effective in promoting the release of GABA (185 ± 6%) and NE (169 ± 1%) (n = 2 experiments).

Discussion

Hemolymph from the beetle *Leptinotarsa haldemani* contains at least three compounds capable of releasing ACh from prelabeled synaptosomes. Of these three, that named A_2 has been examined in detail. The releasing activity in A_2 is probably associated with a protein of M_r 57 000. Although A_2 is demonstrably heterogeneous, analysis of the material by SDS–PAGE indicates that the 57-kD polypeptide is about 30% of the staining material. For purposes of this paper, we have referred to the material in A_2 as β-leptinotarsin-h.

β-Leptinotarsin-h does not appear to be the same protein (leptinotarsin) from *L. haldemani* hemolymph which was isolated by Hsiao & Fraenkel (1969) and found to be lethal to flies. They reported that leptinotarsin has a M_r of 50 000, which is significantly less than the M_r of 57 000 found for β-leptinotarsin-h. In addition, leptinotarsin does not bind to anion-exchange resins, while β-leptinotarsin-h does.

β-Leptinotarsin-h also does not appear to be the same protein as β-leptinotarsin-d, purified by Yoshino (1980) from a closely related insect, *Leptinotarsa decemlineata*. β-Leptinotarsin-d has a M_r of 40 000 compared to a M_r of 57 000 for β-leptinotarsin-h. Trypsin inactivates β-leptinotarsin-d but has no effect on β-leptinotarsin-h. The two proteins behave very differently with respect to ion-exchange resins. β-Leptinotarsin-d binds reversibly to DEAE-Sephadex, carboxymethylcellulose, and sulfoethyl-Sephadex, while β-leptinotarsin-h binds to none of these resins (data not shown). On the other hand, β-leptinotarsin-h binds reversibly to triethylaminoethylcellulose while β-leptinotarsin-d does not. Finally, β-leptinotarsin-d exhibits cooperativity in stimulating release of radioactivity from synaptosomes while β-leptinotarsin-h does not. Even though the two toxins are not identical, they probably act by similar mechanisms.

β-Leptinotarsin-h stimulates release of all the radioactive choline and ACh in synaptosomes over a period of only a few minutes. To compare the time course of action of β-leptinotarsin-h, the time required by *botulinum* toxin for maximum blockade of release of ACh from murine synaptosomes is 60–120 min (Gundersen & Howard, 1978; Wonnacott & Marchbanks, 1976), while black widow spider venom requires 10 min to stimulate maximal release from synaptosomes (Baba & Cooper, 1980).

Release of radioactivity from synaptosomes increased with increasing concentrations of β-leptinotarsin-h, reaching a maximum of 92% of the radioactivity liberated by lysis of control synaptosomes. At the same time, β-leptinotarsin-h released only 2.3% of the LDH activity which was liberated by lysis. It appears that β-leptinotarsin-h can release almost all of the [^{3}H]choline and [^{3}H]ACh contained in loaded synaptosomes without significantly impairing synaptosomal integrity.

β-Leptinotarsin-h has a very potent effect on synaptosomes when compared to other presynaptically active proteins. The concentration of β-leptinotarsin-h which stimulates half-maximal release is 0.8 nM (on the basis of a molecular weight of 57 000). This concentration is probably an overestimate by about 3 times because β-leptinotarsin-h is not homogeneous. Ten nanomolar β-bungarotoxin causes half-maximal release of ACh from synaptosomes of rat (Sen et al., 1976), and 26 nM α-latrotoxin stimulates a moderate release of ACh and NE from murine brain slices (Tzeng et al., 1978). The dose

of β-leptinotarsin-d which stimulates half-maximal release of radioactivity from synaptosomes is 5 nM (Yoshino, 1980).

Although not tested directly, it is unlikely that β-leptinotarsin-h exerts its releasing activity through the action of a phospholipase. Several presynaptic neurotoxins (i.e., β-bungarotoxin, crotoxin, taipoxin) have phospholipase activities which are essential to their neurotoxic activities. These phospholipase activities have a specific requirement for Ca^{2+}. Substitution of Sr^{2+} for Ca^{2+} reduces both the phospholipase activities and neurotoxic activities of these toxins by 90% (Chang et al., 1977; Strong et al., 1976). The releasing activity of β-leptinotarsin-h, however, is unaffected by the substitution of Sr^{2+} for Ca^{2+}, which suggests that a phospholipase activity similar to those found with other presynaptic neurotoxins is not responsible for the releasing activity of β-leptinotarsin-h.

β-Leptinotarsin-h probably does not act as a general Ca^{2+} ionophore since hemolymph from *L. haldemani* failed to stimulate any inward Ca^{2+} current across neuronal membranes of *Limnea stgnalis* (Dr. Joseph Stimers, unpublished results).

The release of neurotransmitters stimulated by β-leptinotarsin-h may involve direct action of the toxin on the presynaptic voltage-sensitive Ca^{2+} channel. Hagiwara & Byerly (1981) have proposed several criteria which must be met in order to show that a Ca^{2+} current exists in a given preparation. Their criteria for Ca^{2+} currents, restated in terms of neurotransmitter release, are the following: (1) Release depends on the external concentration of Ca^{2+}. (2) Replacement of external Na^+ with a large monovalent cation does not affect release. (3) Release is not reduced by 1 μM tetrodotoxin. (4) Uptake of Ca^{2+} must be demonstrated. (5) Release is blocked by less than 10 mM Co^{2+}, La^{3+}, Mn^{2+}, Cd^{2+}, or Ni^{2+}. (6) Replacement of Ca^{2+} with Sr^{2+} or Ba^{2+} maintains release.

Release stimulated by β-leptinotarsin-h satisfies these requirements. Replacing Ca^{2+} in the external medium with EGTA completely eliminated release of radioactivity from synaptosomes. In addition, the magnitude of release increased with increasing concentrations of Ca^{2+} up to 5 mM. The reduction in release at higher concentrations of Ca^{2+} was unexpected, but it may be due to stabilization of the synaptosomal membrane or to a charge barrier to the binding of β-leptinotarsin-h to the presynaptic membrane.

Release of radioactivity stimulated by β-leptinotarsin-h was not affected when the majority of the extracellular Na^+ was replaced by glucosammonium ion. Although the extrasynaptosomal solution contained 8 mM Na^+ derived from the aliquot of β-leptinotarsin-h, this concentration was low enough to eliminate veratridine-stimulated release. β-Leptinotarsin-h does not, therefore, promote the release of radioactivity from synaptosomes by acting as a Na^+ ionophore.

Release of radioactivity promoted by β-leptinotarsin-h was not sensitive to 0.8 μM TTX in the presence of 130 mM Na^+. This concentration of TTX substantially reduced veratridine-stimulated release of radioactivity. Since TTX blocks Na^+ channels (Narahashi, 1964), β-leptinotarsin-h does not act by opening presynaptic Na^+ channels. These data suggest that β-leptinotarsin-h does not cause release of radioactivity from synaptosomes by increasing the permeability of the synaptosomal membrane to Na^+.

β-Leptinotarsin-h promotes the uptake of Ca^{2+} by synaptosomes. When purified synaptosomes were incubated in the presence of β-leptinotarsin-h and $^{45}Ca^{2+}$, a dose-dependent increase in the uptake of $^{45}Ca^{2+}$ into synaptosomes was observed. The concentration of β-leptinotarsin-h which stimulated half-maximal $^{45}Ca^{2+}$ uptake was the same as that which stimulated half-maximal release of radioactivity.

Five millimolar added Cd^{2+} or Co^{2+} was effective in blocking release of radioactivity from synaptosomes stimulated by β-leptinotarsin-h in the presence of 1 mM Ca^{2+}.

Ba^{2+} and Sr^{2+} support synaptosomal release of radioactivity stimulated by β-leptinotarsin-h. Sr^{2+} was as effective as Ca^{2+} in supporting release, while Ba^{2+} was slightly less effective. These results correspond well with those of Cotman et al. (1976) and Haycock et al. (1978), who showed that Ba^{2+} and Sr^{2+} could replace Ca^{2+} in supporting K^+-stimulated release of GABA and NE from synaptosomes.

β-Leptinotarsin-h therefore fulfills all of the rquirements listed previously for a substance which activates presynaptic Ca^{2+} channels. In addition to these criteria, three further observations support an action of β-leptinotarsin-h at the presynaptic voltage-sensitive Ca^{2+} channel. First, the inhibition caused by Cd^{2+} is highly cooperative, with a Hill coefficient of 4.7. The presynaptic Ca^{2+} channel is known to bind Ca^{2+} in a highly cooperative manner (Dodge & Rahamimoff, 1967). If each ion of Cd^{2+} competes for a single Ca^{2+} binding site on the Ca^{2+} channel, an equally high level of cooperativity would be expected for inhibition. Second, β-leptinotarsin-h stimulates release of neurotransmitters other than ACh. GABA and NE are also released, as they should be if β-leptinotarsin-h acts on a presynaptic Ca^{2+} channel which is common to all presynaptic terminals. Third, β-leptinotarsin-h causes a Ca^{2+}-dependent depolarization of synaptosomes with kinetics similar to those of the release of radioactivity. A sufficient influx of Ca^{2+} could both stimulate the release of radioactivity and depolarize the synaptosomal membrane. In the presence of Ca^{2+}, the ionophore A23187 can depolarize synaptosomes (Åkerman & Nicholls, 1981) and cause the release of ACh and choline from synaptosomes (Wonnacott et al., 1978).

Taken together, these data strongly suggest that the mechanism of β-leptinotarsin-h is to activate the Ca^{2+} channels of presynaptic terminals. It is possible that β-leptinotarsin-h can be usefully employed to study the mechanism of activation of the presynaptic Ca^{2+} channel.

Acknowledgments

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Registry No. β-Leptinotarsin-h, 88376-61-8; acetylcholine, 51-84-3; choline, 62-49-7; calcium, 7440-70-2.

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Isolation and Partial Characterization of the Carboxy-Terminal Propeptide of Type II Procollagen from Chick Embryos†

Samantha Curran and Darwin J. Prockop*

ABSTRACT: The carboxy-terminal propeptide from type II procollagen was isolated from organ cultures of sternal cartilages from 17-day-old chick embryos. The procedure provided the first isolation of the carboxy-terminal propeptide in amounts adequate for chemical characterization. The propeptide was isolated as a disulfide-linked trimer with an apparent molecular weight of about 100 000. After reduction, monomers of about M_r 34 000 were obtained. Antibodies were prepared to the propeptide and used to establish its identity. The antibodies precipitated type II procollagen but did not precipitate type II procollagen from which the amino- and carboxy-terminal propeptides were removed with pepsin. No collagen-like domain was found in the propeptide, and the amino acid composition was similar to that of globular proteins. The circular dichroism spectrum of the propeptide suggested the presence of β-structure together with some random-coil structure. The data demonstrated that the type II carboxy-terminal propeptide is similar to the two different carboxy-terminal propeptides of type I procollagen in amino acid composition, molecular size, optical properties, and antigenicity. The homology among the type I and type II carboxy-terminal propeptides is consistent with the current hypothesis that they serve similar functions in vivo. The differences in structure may account for the selection of the appropriate proα chains to form the correct trimers in cells synthesizing several types of proα chains simultaneously.

The three major interstitial collagens, types I, II, and III, are first synthesized as procollagens which contain additional amino acid sequences at both the N- and C-terminal ends of the three proα chains of the molecules (Prockop et al., 1976; Fessler & Fessler, 1978; Bornstein & Traub, 1979; Bornstein & Sage, 1980; Davidson & Berg, 1981; Olsen, 1981a). Both the N- and C-propeptides[1] are cleaved in the conversion of the procollagens to their corresponding collagens. The N- and C-propeptides of several of these type-specific procollagens have been characterized. The primary structure was determined for the N-propeptide of the proα1(I) chain from calf (Hörlein et al., 1979), sheep (Rohde & Timpl, 1979), and chick (Pesciotta et al., 1980) and for the N-propeptide of the proα1(III) chain from calf (Timpl & Glanville, 1981; A. Brandt, D. Hörlein, P. Bruckner, R. Timpl, P. P. Fietzek, and R. W. Glanville, unpubished results). The N-propeptides of the proα2(I) chain from sheep (Becker et al., 1977), chick embryos (Tuderman et al., 1978), and rat (Smith et al., 1977)

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[1] Abbreviations: N-propeptide, amino-terminal propeptide of procollagen; C-propeptide, carboxy-terminal propeptide of procollagen; CD, circular dichroism; $NaDodSO_4$, sodium dodecyl sulfate; CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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